Horses as Sentinels of Emerging Infectious Disease

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STATEMENT OF AUTHENTIFICATION

The research presented in this thesis is, to the best of my knowledge, original except as acknowledged in the text.

I hereby declare that this material, either in full or in part has not been submitted for a degree at this or any other institution.

I certify that the intellectual content of this thesis is the product of my own work and that all the assistance received in preparing this thesis and sources have been acknowledged.

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ABSTRACT

Horses suffering illness consistent with Hendra virus (HeV) are routinely sampled and submitted along with case descriptions to government laboratories for timely testing. Extensive investigations for further infectious agents are rare, and yet <1% of >1000 horses test HeV positive annually. Most that test HeV negative feature infectious-like signs such as acute, severe neurological or respiratory illness and pyrexia, yet do not receive causative diagnosis.

HeV in horses and testing of suspect cases has highlighted challenges and gaps in significant zoonotic disease investigation. Yet horses investigated for HeV-like disease also present unique opportunities for improvements of broad and profound biosecurity benefit. Horses are maintained in close association with other animals and humans, monitored thoroughly for disease; susceptible to agents transmitted by insects and wildlife such as bats (HeV/ ABLV).

This research identified significant pathogens amongst Horses with severe HeV-like illness, beyond those currently recognised and included in priority disease investigations and progressed interpretation of their disease significance.

Fore-front diagnostic techniques were integrated with information theoretical, epidemiological, and virological analyses. Systematic pathologic-basis-guided interpretation of disease descriptions, sensitively informed case likelihood of infectious cause, and case diagnostic responsibility. Three innovative pillars were developed:

1. purpose-built SQL database integrating bio-banked laboratory sample information, de-identified sample event, subject and clinical details (incl. syndromic classification) with parallel research testing results;
2. extensive multiplex microbead immunoassay explorative serological testing approach screening both IgG and IgM for a wide range of EID pathogens;
3. advanced molecular methods testing for novel and emerging infectious agents, including high-throughput pan-PCR, metatranscriptomic sequencing and bioinformatical pipelines.

Integrated application to suitably bio-banked clinical samples served proof-of-concept for proactive convergence research detection and interpretation of emerging infectious disease agents that could affect One health, livestock, trade and industry security and public health.

EXECUTIVE SUMMARY

This thesis addresses the **hypothesis that:**

> **some cases of severe Hendra virus-like disease in Australian horses are due to spillover infection of related but divergent bat-borne viruses – particularly *Pararubulaviruses* and *Henipaviruses* (*Paramyxoviruses*).**

Our awareness of these viruses is emerging.

Horses are susceptible to infection with Hendra virus (HeV) via natural spillover pathways. Spillover transmission is thought to occur via infected bat urine, inhaled as microscopic droplets by horses during their inquisitive grazing behaviour, gaining entry via cells of their extensive upper and lower respiratory surfaces. The detection and management of HeV in horses relies on recognition of suspect cases by veterinarians in private practice. Their appropriate sampling and submission for priority state laboratory investigation enables necessarily timely diagnosis via quantitative polymerase chain reaction (qPCR) testing, targeting the HeV matrix (M) gene. Serology testing may also be performed, using the enzyme linked immunosorbent assay (ELISA) detecting anti-HeV receptor binding protein (RBP) IgG antibodies.

Veterinarians have been challenged and burdened with this crucial role for many reasons, including implications for workplace health and safety (WPHS), with professional and financial liability. Their understanding that HeV disease cases manifest with non-specific signs, common to many other conditions in the horse, has been particularly troubling.

Both to aid practising veterinarians in recognising suspect cases, and in selecting the most suitable cases for further infectious diagnostic investigation, this research began with analysis of HeV disease manifestations to develop an understanding of the clinical syndrome resulting from infection and its pathogenic basis (at the subcellular, cellular, tissue/organ and systemic levels) over time. Relevant literature was reviewed and coupled with qualitative (collating and interpreting first-hand veterinary assessment) and quantitative (analyses of all reported signs from HeV infected horses) analyses of HeV disease.

The approach consolidated the understanding that HeV causes disease, in horses and humans, by diffuse endothelial vasculitis supported by multiple host immune evasive mechanisms and formation of giant cells, with secondary cellular damage and compromised organ function. Signs of disease in the horse may be mild initially, with viral shedding enabling transmission, to in-contact animals and humans, up to two days prior to the onset of overt clinical disease. The majority of recognised infections in horses, and all experimental infections, have featured acute severe disease with pronounced respiratory compromise and encephalitic manifestations, usually justifying euthanasia on humane grounds (fatality rate is over 80%). Chronic and relapsing encephalitis features among horses that survive acute infection. A minority of natural infections survive (<20%), following acute severe disease, having experienced mild, or no recognised disease, with infection confirmed by consistent epidemiology and seroconversion. The review of the clinical manifestations of HeV in horses, revealed that gingival mucous membrane changes consistently feature, yet have been relatively lacking in published literature and messaging.

A relational database (SQL) was constructed to best serve this research-extended surveillance activity. Case details were determined from attending veterinarian descriptions reported via pdf submission forms (de-identified). Each sampled case-subjects' disease event, was categorised based on pathologic syndromic and epidemiologic consistency of reported disease manifestations and context, with HeV, or related bat-borne paramyxovirus of similar pathogenicity, and linking with available bio-banked samples (appropriately handled, processed, and catalogued). The database and biobanking approach further supported systematic plating of samples for parallel serology and molecular research testing based on assigned case categorisation and available sample types.

The approach prioritised safe and efficient (high throughput) sample handling, supported interpretation and analysis of extensive test results, while leaving information sensitive to primary stakeholders with the state laboratory. Reported disease manifestations were systematically interpreted in terms of their pathogenic basis and categorised by likelihood of infectious cause.

Serology testing for antibodies against HeV, as part of routine priority disease investigations, has been limited to ELISAs targeting IgG antibodies against the HeV RBP. In acute horse

infections, the first antibodies produced will be IgM, thus serological assays targeting these antibodies might be expected to be of great diagnostic value in acute infection. Yet, developing IgM assays, and interpreting their individual case results is challenging, as they are cross-reactive and have poor specificity.

The HeV subunit vaccine that has been available as a One Health risk management tool since 2012, elicits targeted immunity reliably preventing infection, utilising a recombinant version of the RBP (G glycoprotein). IgG antibodies against this protein are detected by serological assay similarly in vaccinated horses making interpretation of positivity requiring reliable knowledge of the horse-subject's vaccine history.

Interpretation of serology as part of investigations for suspected HeV cases (ELISA targeting IgG) and in sero-epidemiological surveillance has been limited because of these challenges. This is due to the low positive predictive value of the approved ELISA assay (for IgG targeting the G glycoprotein) in detecting acute cases and the inadequacy of the testing approach in differentiating between viral infection/exposure and immune response to vaccination. An assay is needed for differentiating infected and vaccinated animals (DIVA). The use of paired sera (as in human infectious disease testing) would improve the diagnostic interpretation of infections. This is inherently unavailable in cases that die or are euthanased in their initial veterinary consultation, and not commonly collected in those horses that survive.

This research developed and applied an extensive panel of relevant recombinant viral protein antigens as a multiplex microbead fluorescent immunoassay utilising the Luminex/ Bioplex platform, targeting both IgG and IgM antibodies in parallel. The panel included both RBPs (G glycoprotein for Henipaviruses and sHN for Rubulaviruses) considered most specific for their specific viral species, and nucleocapsid (N) proteins considered sufficiently cross reactive so as their elicited sero-reactivity indicates exposure to related divergent related viruses.

Routine priority diagnosis of HeV in suspect diseased horses relies on application of a quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) assay targeting the HeV M gene performed on minimally invasive swab samples (oral, nasal and rectal) and blood. This approach necessarily facilitates rapid conclusive detection in acute infections.

RNA viruses are transmitted readily among flying foxes due to their communal roosting and relative immunocompetence relating to their high metabolism yet relative longevity, and other specific immune adaptions arising from their having co-evolved with these viruses. Given their consistent wide host species and tissue (incl. endothelial) tropism, and mechanisms of immune evasion, many divergent paramyxoviruses are expected to pose similar potential for domestic mammalian spillover and zoonotic infection and to be similarly pathogenic in these hosts.

Spillover infection of Menangle virus (MenPV) (*Pararubulavirus, Paramyxoviridae*) in New South Wales (NSW), Australia, in 1997 from flying foxes, resulted in porcine foetal loss and respiratory disease with influenza-like illness amongst in-contact humans serves an example of this potential. Internationally, multiple strains of Nipah virus (NiV) (Henipavirus, *Paramyxoviridae*) have been observed to cause severe disease and spread amongst a range of domestic mammalian hosts and humans. NiV is the closest known relative of HeV - sufficiently similar to have equivalent pathogenicity and immunogenicity.

In this context, reliance on the highly specific single qRT-PCR assay approach for priority screening is inadequate to detect divergent and emerging viruses, of which many are understood to circulate amongst flying foxes. This exposes all relevant stakeholders and our nation to intolerable One Health and biosecurity risk.

This research trialled and developed molecular testing approaches beyond those routinely available for government-based priority disease investigation (passive surveillance), including nested conventional PCR assays targeting the most-conserved regions of the viral N protein genome (targeted), and metatranscriptomic next generation sequencing (NGS) capable of detecting widely divergent and novel viruses (unbiased/ general). To optimise the integrity of the RNA required for the success of these approaches, attention was applied to sample handling, cold-chain storage, and nucleic acid extraction methods.

Developing and applying each of the above approaches, this research aimed to significantly enhance Australia's biosecurity and emerging infectious disease preparedness. Their integrated application, to suitably processed bio-banked clinical samples from HeV suspect equine disease cases, as a **three-pillared 'Horses as Sentinels' convergence research process** targeting the detection and characterisation of emerging bat-borne

paramyxoviruses, **demonstrated convincing proof of concept for this and other many emerging infectious agent and One Health disease contexts.** Facets of the innovative approach include:

1. a purpose-built relational database (SQL), capturing bio-banked sample details, with their de-identified sample event, case subject and clinical details, allowing systematic categorisation of case-likelihood of infectious cause and linking for sample processing and numerous parallel testing outcomes
2. an extensive serological multiplex microbead immunoassay utilising novel recombinant viral proteins enabling screening for exposure (IgG and IgM) to emerging viruses
3. parallel high-throughput application of next-generation (metatranscriptomic sequencing) and traditional (pan-PCR) molecular testing and specialised bioinformatical methods to identify divergent and novel viruses.

This research identified a novel prototypic variant of HeV (HeVg2) in multiple samples from a horse that was humanely destroyed following rapid severe disease. This horse's clinical disease manifestations were indistinguishable from HeV, yet it had tested negative by priority routine government-based testing. The 'Horses as Sentinels' research group developed and optimised a novel duplex qPCR assay to detect both the discovered variant of HeV (HeVg2) and the formerly known lineage (HeV). This was shared and integrated into existing state biosecurity diagnostic approaches. Nine months later, the HeV-g2 virus was detected as part of routine operations in a horse at Wallsend, near Newcastle, NSW, within this project's timeline.

Furthermore, the research identified several novel paramyxoviruses beyond the HeV variant described above via molecular pathogen discovery approaches from samples collected from significant HeV-like equine disease events. These included a novel Pararubula-like virus − a highly divergent paramyxovirus most closely related to Rubulaviruses and Henipaviruses in mammals, and Canine Morbilliviruses (vaccine strain and novel wild virus). The serology findings from this research further supported the consistency of spillover of bat borne Rubulaviruses from flying foxes to Australian horses across a wide geography. It also consolidated disease association with seroconversion of IgG and IgM antibodies in three outbreaks of severe HeV-like disease in horses in NSW.

Beyond paramyxoviruses, this 'Horses as Sentinels' research has identified many other potentially significant viruses via molecular means. These viruses included Equine Rhinitis virus, Equine Torovirus, equine Hepaci viruses, Equine Pegiviruses, a novel Norovirus, novel Astrovirus, novel Nidovirus and a coronavirus that was most like bovine coronaviruses. Furthermore the 'Horses as sentinels' research serological approach, extended to a 33-plex assay, showed evidence of exposure to filoviruses, borna-like virus, Hepatitis-E virus and arboviruses such as flaviviruses, alphaviruses, and bunyaviruses, as well as bacterial zoonoses - Brucella spp. and Leptospira spp.. Interpretations of such extensive findings are ongoing and considered beyond the scope of this PhD thesis.

This research gained an understanding of necessarily complicated network of sectors, stakeholders, expertise and disciplines that require strategic proactive problem focused integration in order to detect HeV and emerging infectious disease (EID) agents in Australian horses and manage their potential zoonotic disease risks. By combining these three foundational pillars, extending from the routine surveillance system, this project aimed to demonstrate the utility of an innovative, integrated research approach for risk-based targeted and general syndromic sentinel surveillance. This approach would extend, support and guide existing systems and operations for routine biosecurity at national, state, clinical veterinary, and farm/stable levels, and holds strong analogous relevance to many other domestic animal and One Health disease contexts.

This research detecting significant paramyxoviruses spilling over from bat hosts, and causing disease in Australian horses, resolves the above stated hypothesis categorically.

Furthermore, the thesis highlights both:

- the reality that significant EID cases will have been missed across a broad geography, resulting in unmanaged zoonotic disease risk in each sporadic spillover event and
- that critical scientific knowledge and capacity may be offered by such research approaches in sentinel pathogen discovery that combine strengths of multiple disciplines to detect emerging pathogens of both Human and animal health significance. This will guide effective surveillance and biosecurity.

AUTHORSHIP ATTRIBUTION

AUTHORSHIP ATTRIBUTION FOR PUBLISHED MATERIAL

I, Dr Edward J Annand, was first author and corresponding author for the following published manuscripts contained within the body of the thesis:

Chapter 8 of this thesis is published as one manuscript:

A novel Hendra virus variant detected by sentinel surveillance of horses in Australia. Emerging Infectious Diseases, 2022, 28: 693-704. doi: 10.3201/eid2803.211245.

AUTHORSHIP ATTRIBUTION FOR MATERIAL INTENDED FOR, OR SUBMITTED TO, PEER-REVIEWED JOURNALS

Chapter 3 is intended for peer-reviewed journal submission, but it has not yet been submitted. I drafted the first complete manuscript (2018) and received feedback from PhD supervisors and wider project team member Dr Peter Reid. Dr Anne Jackson provided editorial feedback and guidance, especially where the original draft was excessive in length (2019); I undertook revisions of the manuscript to meet the PhD chapter format expectations (2021).

A concise preliminary version of this chapter was published by the Northern territory (NT) CDC newsletter (June 2017) and republished by the NSW DPI (2018), and was circulated similarly to practising veterinarians. The NT CDC version is appended to this thesis as Appendix 6: An update on Hendra virus (HeV), HeV-like illnesses and horses as sentinels for emerging infectious disease, *The Northern Territory Disease Control Bulletin Vol 24, No. 2, June 2017.*

Chapter 2, 4, 5, 6 and 7 have been compiled specifically for this thesis with anticipation of subsequent further consideration for inclusion in peer review publications.

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I acknowledge both the traditional custodians of the lands upon which this research was carried out, and the diverse communities that maintain, live and work with horses in Australia. Horses are kept for a wide range of purposes across Australia, enriching lives of those to whom they matter most – owners, breeders, carers, riders, drivers, trainers, and families. Particularly thanks to my wonderful clinical clients and friends with horses, that share my passion for the health, well-being and performance of the horse. There was a moment when managing the diverse challenges that the burden of this prolonged research effort and my professional career had placed on my personal well-being, when it was you who made it possible to carry on.

Finally, I would like to acknowledge all the equine veterinarians of Australia, their families and their representative organisation, Equine Veterinarians Australia. I dedicate this thesis to them. Managing the challenges around HeV in horses has weighed heavily on our already often over-burdened shoulders since discovery in 1994. I hope this research, the changes and further research it might inspire, somewhat lessen these burdens by increasing both core knowledge to guide proactive management, prompt further supportive One Health and biosecurity governance frameworks and assisting their proactive recognition of suspect cases and promote their continued sharing of experiences and supportive friendships.

Contents

PUBLICATIONS ARISING MOST DIRECTLY FROM THIS THESIS *(CHRONOLOGICAL ORDER)*

1. **An update on Hendra virus (HeV) and HeV – like illnesses and horses as sentinels for emerging infectious disease.** The Northern Territory Disease Control Bulletin, Vol 24, No. 2, June 2017. Annand, E.J., Reid, R., Smith, I., Diallo, I., Lynch, S., Weir, R., Melville, L., Secombe, C., Britton, P.N., Gilkerson, J., Jones, C., Dhand, N. http://digitallibrary.health.nt.gov.au/prodjspui/bitstream/10137/506/550/Vol.%2024%20no%202%20June%202017.pdf

2. **Citizens' juries give verdict on whether private practice veterinarians should attend unvaccinated Hendra virus suspect horses.** *Australian Veterinary Journal*, 98 no.7:273-279, 2020. Annand, E., Reid, P.A., Johnson, J., Gilbert, G., Taylor, M., Walsh, M., Ward, M., Wilson, A., Degeling, C.

3. **Spillover of bat borne Rubulavirus in Australian horses - Horses as sentinels for emerging infectious diseases.** Annand, E., Barr, J., Balasubramanian, N.S., Reid, P.A., Boyd, V., Burneikienė-Petraitytė, R., Žvirblienė, A., Grewar, J., E. Laing, Eden, J.S., Lynch, S.E., Diallo, I., and Yan, L., Secombe, C., Britton, P., Jones, C., Broder, C., Dhand, N.K., Smith I. International Journal of Infectious Diseases. 2020 Dec 1;101:401–2.

4. **Novel Hendra Virus Variant Detected by Sentinel Surveillance of Horses in Australia.** Emerging Infectious Diseases, 28, 3. March 2022. 10.3201/eid2803.211245. Annand, E.J., Horsburgh, B.A., Reid, P.A., Poole B., Brown N., de Kantzow, M.C., Xu, K., Tweedie, A., Michie, M., Grewar, J.D., Tachedjian, M., Jackson, A. E., Singanallur, N., Plain, K., Diallo, I., Jackson, L., Secombe, C., Laing, E.D., S Sterling, S., Yan, L., Jones, C., Peel, A.J., Breed, A.C., Broder, C.C., Britton, P.N. Dhand, N.K., Smith, I.L., Eden, J.S.

   Associated Scientifc abstract: **Severe Hendra virus disease in a horse in 2015 due to a previously-unrecognised Hendra virus variant matching that detected in a grey-headed flying fox.** EVA Bain Fallon/ ANZCVS abstracts, The Australain Equine Veterinarian, Vol XX, No X, 2022. (Authorship as above)
   Awarded the Equine Veterinarians Australia Peter Irwin Literary prize 2022.

5. **Hendra virus - Two viruses, Same Fatal Disease.** Annand, E.J., Reid, P.A., Jackson, A. E., Eden, J.S., Smith, I.L. The Australian Equine Veterinarian, vol. 41 No. 2, 2021. https://www.sciquest.org.nz/browse/publications/article/166341

6. **Novel Hendra Virus Variant Circulating in Black Flying Foxes and Grey-Headed Flying Foxes, Australia.** Emerging Infectious Diseases, 28, 5. May 2022. 10.3201/eid2805.212338. Peel, A.J., Yinda, C.K., Annand, E.J., Dale, A.S., Eby, P., Eden, J.S., Jones, D.N., Kessler, M.K., Lunn, T.J., Pearson, T., Schultz, J.E., Smith, I.L., Bat One Health, Munster, V.J., Plowright, R.K.

7. **Potent monoclonal antibody-mediated neutralization of a divergent Hendra virus variant.** Proceedings of the National Academy of Sciences. 31.05.2022. 10.1073/pnas.2122769119 Wang, Z., Dang, H.V., Amaya, M., Xu, Y., Yin, R., Yan, L., Annand, E.J., Horsburgh, B.A., Reid, P.A., Smith, I., Eden, J.S., Xu, K, Broder, C.C., Veesler, D.

8. **Novel Variant Hendra Virus Genotype 2 Infection in a Horse in the Greater Newcastle Region, New South Wales, Australia.** Taylor J, Thompson K, Annand E.J., Massey P, Bennett J, Eden JS, et al. One Health, Vol. 15, Dec 2022. https://doi.org/10.1016/j.onehlt.2022.100423

PRESENTATIONS ARISING FROM THIS THESIS *(CHRONOLOGICAL ORDER)*

INTERNATIONAL

2022 **Novel Hendra Virus Variant Detected by Sentinel Surveillance of Horses, Australia.** Centres for Disease Control and Prevention, Emerging Infectious Diseases journal podcast https://tools.cdc.gov/medialibrary/index.aspx#/media/id/671420

2021 **The research discovery of a variant genotype of Hendra virus (HeVg2) affording prospective spillover detection and revision of geographical spillover risk.** Invited presentation. One Health & Outbreak Surveillance Online Symposium | One Integrative Surveillance System for Pandemic Preparedness, Hosted by: Centre for

Infectious Disease Epidemiology & Research, Saw Swee Hock School of Public Health, National University of Singapore, 29 November 2021.

NATIONAL

2022 **Novel variant Hendra virus infection in a horse in Newcastle, New South Wales.** Hodgson, E. Annand, E.J., Poster Bain Fallon National Equine Veterinary Conference, Melbourne July 2022. (Appendix 3) **Awarded EVA Clinical Case-Based Poster Prize**

2022 **Taking the reins on equine health – three emerging biosecurity risks for Australian horses – Dr Ed Annand - Horses as sentinels for emerging infectious disease research Integrating disciplines and perspectives to tackle One Health disease threats early & the Hendra virus variant discovery.** Aust. Government, Department of Agriculture Water and the Environment Biosecurity Webinar Series, May 2022.

2022 **Interdisciplinary *Horses as Sentinels for* emerging infectious disease research; detection & interpretation of a *variant genotype of Hendra virus* (HeVg2); causing fatal HeV disease of equivalent One Health significance; enabling contemporary spillover detection (Newcastle 2021); and revision of geographical risk of HeV spillover**. Veterinary Epidemiology and Surveillance Throughout Australia (VESTA) Government Biosecurity and Surveillance webinar series. 7/3/2022, Canberra, ACT.

2021 **A novel Hendra virus variant detected by sentinel surveillance of Australian horses.** ASID Annual Scientific Meeting: Zoonoses, Brisbane (virtually hosted 13 Oct 2021).

2021 **Two viruses, Same Fatal Disease.** Opening plenary keynote presentation: Equine Veterinarians Australia 50TH Annual 'Bain Fallon' Conference, Sydney (postponed 2022 Melbourne) – published in AEVJ spring edition 2021. (Appendix 2)

2021 **The discovery of a variant prototypic Hendra virus 'Horses as Sentinels' for emerging zoonotic viruses and potential public health risk.** Presented separately to: Wildlife Health Australia Bat Health Focus Group annual scientific meeting. 19th May 2021, virtually hosted, Sydney; and Regional One Health Partnership: Hunter New England and North Coast 10th May 2021, virtually hosted, Newcastle.

2018 **Serological evidence of bat borne *Rubulavirus* in Australian horses.** EJ Annand, PA Reid, J Barr, V Boyd, R Burneikienė-Petraitytė, A Žvirblienė, C Broder, CA Jones, N Dhand and I Smith. EVA Bain Fallon / ANZCVS absracts, The Australian Equine Veterinarian, 38, Vol 38, No 2, 2018.

2017 **Horses as sentinels in a one health syndromic approach for detecting emerging infectious diseases.** EJ Annand, P Reid, I Smith, JS Eden, C Jones, P Britton, N Dhand. CSIRO AAHL Recent Advances in Emergency Animal Diseases Annual Symp. 2017**.**

LOCAL

2020 **Hendra virus, other Zoonotic Emerging Infectious Diseases and Australian horses.** Presentation University of Illinois/ University of Sydney Veterinary Public Health and research international elective. EJ Annand. January 2020.

Living with Flying foxes in a changing climate and managing the risk Hendra virus in horses. Wollondilly Shire Council, Council's Community Forum, 11 November 2019.

Horses as sentinels for emerging zoonotic viruses and potential public health risk. EJ Annand, PA Reid, I Smith, JS Eden and NK Dhand. CVO and International research Workshop & Update on Hendra virus and other Australian bat-borne viruses, and spillover Brisbane 18 March 2019.

2018 **Serological evidence of bat borne *Rubulavirus* in Australian horses.** MBI Zoonoses Node Workshop 2018, November 2018, The University of Sydney. EJ Annand, PA Reid, J Barr, V Boyd, R Burneikienė-Petraitytė, A Žvirblienė, C Broder, CA Jones, N Dhand and I Smith.

Update on the clinical signs of Hendra virus (HeV) - the challenge of forming DDx list for Australian horses with HeV – like illness. EJ Annand, PA Reid, I Smith, NK Dhand. 100th NSW District Veterinarians Annual, Orange, March 20-22.

Zoonoses: Horses as sentinels in a one health syndromic approach for detecting emerging infectious diseases. EJ Annand, PA Reid, I Smith, NK Dhand. 100th NSW District Veterinarians Annual Conference, Orange, March 20-22.

2017 **Horses as sentinels in a one health syndromic approach for detecting emerging**

infectious diseases. EJ Annand, PA Reid, I Smith, CA Jones, PN Britton, NK Dhand. Invited presentation Grand Rounds, Westmead Children's Hospital, Westmead, Sydney, NSW. 9th November.

Hendra Virus in horses and related issues - Perspective of equine veterinarians and insight from viral research. EJ Annand, PA Reid, I Smith, C Broder. Expert-witness representation as part of NHMRC funded Jury based study Rockhampton (21st October) & Lismore (11th November).

2017 **Surveillance for emerging infectious diseases to protect horse and human health.** EJ Annand, PA Reid, I Smith, CA Jones, PN Britton, NK Dhand. Marie Bashir Inst. for Biosecurity & Infectious Disease - Annual colloquium November 2017.

Australian bat lyssavirus in two horses (2013) Horses as sentinels in a one health syndromic approach for detecting emerging infectious diseases. EJ Annand, PA Reid, I Smith, NK Dhand. Agriculture Victoria state lab DEDJTR, Agribio.

Horses as sentinels in a one health syndromic approach for detecting emerging infectious diseases. EJ Annand, PA Reid, I Smith, NK Dhand. Training Workshops for Veterinary Practitioners in Emergency Animal Disease Recognition, Investigation. The Queensland Department of Agriculture and Fisheries (Biosecurity Queensland), Townsville (30th July 17) and Rockhampton (13th August 17).

Syndromic Surveillance and Australian Horses as Sentinels for Emerging Infectious Diseases. EJ Annand, PA Reid, I Smith, CA Jones, PN Britton, NK Dhand. Invited presentation Grand Rounds, Vanderbilt University Medical Center, Nashville TN USA.

2016 **Can We Use Australian Horses as Sentinels for Emerging Infectious Disease?** EJ Annand, PA Reid, I Smith, NK Dhand. Training Workshops for Veterinary Practitioners in Emergency Animal Disease Recognition, Investigation. The Northern Territory Department of Primary Industry and Resources (Animal Biosecurity Branch).

PUBLICATIONS MORE BROADLY RELATED TO THIS THESIS *(CHRONOLOGICAL ORDER)*

2021 **Cooperativity mediated by rationally selected combinations of human monoclonal antibodies targeting the henipavirus receptor binding protein.** *Cell reports*, 36 no. 9 2021. Doyle, M.P., Kose, N., Borisevich, V., Binshtein, E., Amaya, M., Nagel, M., Annand, E.J., Armstrong, E., Bombardi, R., Dong, J., Schey, K.L., Broder, C.C., Zeitlin, L., Kuang, E.A., Bornholdt, Z.A., West, B.R., Geisbert, T.W., Cross, R.W., Crowe, J.E. https://doi.org/10.1016/j.celrep.2021.109628

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6. **Horses as sentinels for emerging zoonotic viruses and potential public health risk.** EJ Annand, PA Reid, I Smith, JS Eden and NK Dhand. CVO and International research Workshop & Update on Hendra virus and other Australian bat-borne viruses, and spillover Brisbane 18 March 2019.

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1. **'Expert witness affidavit' towards the Class Action relating to Equivac HeV vaccine** – Federal Court of Australia proceedings no NSD 406 of 2018, Rachael Abbott v Zoetis Australia Pty Ltd. consigned by 'Piper Alderman' lawyers acting for the defendant. **Sworn in relation to the discovery of a Hendra virus variant by Horses as Sentinels research, outlining implications on perceived risk of HeV spillover geographically and the content of the national update relating to One Health disease management and Biosecurity via the Chief Veterinary Officers and Chief Health Officers of Australia.** March 2021.

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LIST OF TABLES (NOT CONTAINED IN PUBLISHED MATERIALS)

LIST OF FIGURES (NOT CONTAINED IN PUBLISHED MATERIALS)

Horses as Sentinels of Emerging Infectious Disease

Chapter 1

INTRODUCTION: STRENGTHS AND GAPS IN HENDRA VIRUS (HeV) SURVEILLANCE AND BIOSECURITY AND JUSTIFICATION FOR 'HORSES AS SENTINELS' RESEARCH

1.1 Emergence of Zoonotic RNA Viral Pathogens in the Context of Australian Horses

Infectious diseases pose an increasing threat to Australia's human and animal health, agriculture industry and trade. Close and complex human and animal interaction, intensifying international mobility, and progressing climatic and anthropogenic pressures on wildlife populations, contribute to a scenario in which disease spillover is both a persistent considerable risk, yet difficult to detect and manage.

1.1.1 Hendra virus (HeV)

HeV causes severe acute respiratory and encephalitic disease mediated by endothelial vasculitis, with high fatality and chronic encephalitis amongst survivors (3–5). Discounting the retrospective and prospective detections enabled by this research, there have been 63 known natural spillovers of HeV in Australian horses. These resulted in 105 horse deaths, with four of the seven confirmed human cases infected via exposure to an infected horse having been fatal (6–8). Table 1.1 summarised all cases of human and horse HeV infection detected since 1994 discovery (1994 – 2022).

Initial discovery of HeV resulted from investigation of an outbreak of highly fatal zoonotic disease occurring in racehorses and humans with close contact in 1994. The outbreak resulted in the death of 13 horses, with seven dying in a single 12-hour period − described by the attending clinician as drowning in bloody respiratory fluid. Two in-contact humans were infected, resulting in one fatality. The outbreak, understandably, received extensive multi-agency and -disciplinary priority diagnostic investigation.

HeV infection in horses usually results in an acute fulminating disease course with rapid progression over two to three days and a case mortality rate of approximately 80%. In acute cases, the most common signs observed in all infected horses from 1994 to 2018 have been a rapid onset of illness, anorexia, tachycardia, pyrexia, depression, and mucous membrane injection/congestion. There is usually rapid deterioration to moribund condition with either overt respiratory and/or neurological signs (8,9).

Table 1.1 Recognised HeV spillover to horses (1994−2022)

YEAR/S	QLD	NSW	TOTAL	Horse Fatalities	Human	
					Exposures/illnesses requiring intensive treatment	Fatalities
1994−2010	13	1	14	48	10 (7+3)	4 (3+1)
2011	10	8	18	24	0	0
2012	8	0	8	10	1	0
2013	4	4	8	8	(1*)	0
2014	3	1	4	4	6	0
2015	2#	2	4	4	(1*)	0
2016	0	1	1	1	0	0
2017	1	3	4	4	3	0
2018	0	1	1	1	0	0
2019	0	1	1	1	0	0
2020	0	1	1	1	0	0
2021	0	1#	1	1	0	0
2022	1	0	1	1	2	0
TOTALS	42	24	66	108	24 (15)+2*	4

Note that Equine Hendra virus vaccination (Equivac HeV®) began in 2012.
Underlined Human cases indicate received monoclonal antibody m102.4. Cases marked with an * represent human laboratory exposures (2013 US NiV 2015 CSIRO HeV) and thus are not included in the tally.
The two known Hendra virus variant genotype (HeV-g2) cases occurring in 2015 in QLD (detected as part of this research – see chapter 8) and in 2021 in NSW (detection enabled by communication of these research findings and sharing of updated PCR testing capacity) are included and marked with #.

1.1.2 Australian Bat Lyssavirus (ABLV)

A second example of a similarly high consequence, yet low incidence, bat-borne RNA viral zoonotic pathogen with wide host tropism and disease significance infecting Australian horses is Australian bat lyssavirus (ABLV). ABLV was detected following the death of two horses in South-east Queensland in 2013 (10–12). Both horses were initially sampled and tested for HeV infection and other significant known causes of infectious equine encephalitis including flaviviruses and Equine herpesvirus (EHV). Exceptional circumstances included multiple highly valued animals affected, with many more at risk, numerous significant human exposures, and a medical practitioner owner who supported a thorough causative investigation. The circumstances prompted collaborative further investigation between me as the attending clinician and the state biosecurity duty pathologist and virologist. This example highlighted the potential for bat-borne zoonotic viral pathogens of high consequence yet infrequent and sporadic incidence to be missed by routine priority disease investigations in Australian horses with severe HeV-like illness.

1.1.3 Emerging RNA Viruses of Potential Spillover from Wildlife Reservoirs to Australian Horses and Subsequent Zoonotic Transmission Risk

Advances in disease detection mechanisms and diagnostic technology are rapidly increasing our understanding of infectious disease dynamics and helping us identify previously unknown pathogens. It is increasingly clear that we have previously underestimated many infectious causes of disease. Zoonotic agents comprise many of these newly identified pathogens. The human and animal health impacts and resultant socio-economic burden of emerging infectious diseases (EIDs) is considerable.

The emergence and management of Hendra virus (HeV) in Australia demonstrates the challenges of One Health disease risk management yet has resulted in opportunities via an increased awareness of zoonotic diseases.

1.1.3.1 Pteropid flying foxes as a reservoir of emerging zoonotic paramyxoviruses and the potential of spillover to Australian horses

Pteropodid flying foxes are the natural reservoir for highly-pathogenic Hendra virus (HeV) (13). Anti-HeV antibodies were detected in all Australian mainland flying fox ‘species’, with seroprevalence between ~20% and ~65%.(14,15) In the grey-headed flying fox (GHFF),

seroprevalence was approximately 40% from roosts sampled in South Australia (SA) and Victoria (VIC),(16) and >50% from one roost in southeast QLD. The spatial distribution of previously detected spillovers to horses, as well as molecular HeV testing of flying fox urine, suggested that transmission was predominantly from black flying foxes (BFF, *P. alecto*) and spectacled flying foxes (SFF, *P. conspicillatus*).(17,18)

Horses are the predominant species known to be infected with HeV by natural spillover from flying foxes in Australia. Two dogs(19) and all known human infections resulted from close contact with infected horses. HeV transmission from flying foxes to horses occurs primarily via urine(17) with mainly black (BFF) and spectacled flying foxes thought to play active roles in disease transmission.(17,18) Horses are likely to be vulnerable to respiratory droplet HeV transmission because of their grazing behaviour, large respiratory tidal volume, and large surface area ratio of their highly vascular upper respiratory epithelium.(20–22)

1.1.3.2 Related bat borne paramyxoviruses

The majority of emerging infectious diseases are caused by RNA viruses.(23) The differing processes and biology of RNA viruses allow for distinctly different evolutionary features to double stranded DNA organisms – these include high mutation rates, small genome size (<45,000 nt) and very large population sizes.(23,24) These features have resulted in the ability to infect multiple host species (spillover) but RNA viruses are challenged in establishing sustained transmission cycles in emergent hosts.(23) Bats (order *Chiroptera*) are one of the most diverse, abundant and geographically dispersed vertebrates, representing around 20% of mammalian species living on all continents except Antarctica.(25,26) Bats, specifically flying foxes (family *Pteropodidae*) provide an optimal niche for RNA virus persistence and evolution due to their relative immunity to infection, relative longevity for their body size, large communal roosting habits and inter-colony migratory interactions.(25,27–30) As such bats are identified as the reservoir origin of the majority of significant emerging infectious diseases that are capable of spillover to domestic animals and humans, as well as those capable of ongoing transmission cycles in emergent hosts.(27,28,30,31) Flying foxes in particular are considered to have co-evolved with many viruses. They have been identified as the reservoir for many recently identified RNA viruses with potential for interspecies transmission and high

likelihood of featuring the adaptive mutations required for cross-species transmission.(27,32,33)

In Australia, paramyxoviruses shed from flying foxes(33,34) are likely to have the potential to follow the same proven spillover pathway to horses as HeV.(35) The opportunity that flying foxes provide RNA viruses to persist, vary and evolve could be likened to a 'training ground' for viruses of spillover potential. This increases the likelihood that variants capable of persistence in spillover hosts will occur and interspecies transmission will take place.

Research isolating viruses in cell culture from Australian flying fox urine samples (collected via plastic sheets laid out underneath roosting colonies) has not only detected HeV but an additional member of the Henipavirus group, Cedar virus (CedPV), as well as multiple novel Rubula viruses – Menangle (MenPV), Teviot (TevPV), Hervey (HerPV), Yeppoon YepPV and Grove (GroPV) viruses.(34,36) Further recent research has identified that Rubulavirus spillovers occur in parallel to the shedding of HeV, with periods and locations of increased virus shedding coinciding.(35) All of these recently identified Rubulaviruses are of unknown clinical significance but feature equivalent immune-evading variable P gene translation to MenPV, and thus have the potential of causing similar disease.(37–40) Similarly obtained bat urine samples analysed by molecular methods have demonstrated evidence for as many as 30 novel paramyxoviruses in Australian bat urine.(33)

1.1.4 Veterinary disease detection and testing

Less than 1% of more-than 1,000 horses tested for Hendra virus (HeV) annually are found to have the virus. Many of these horses have displayed signs consistent with HeV (acute, severe neurological and or respiratory illness, often with pyrexia), test negative and do not receive a causative diagnosis. Many veterinarians experience frustration in handling cases of severe equine illness that demonstrate a clinical course and presentation consistent with HeV and are negative for HeV by PCR but receive no alternative diagnosis. The 2011 Queensland Government Ombudsman's investigation highlighted the importance of conducting further testing 'where the clinical signs of the horse were suggestive of Hendra virus or where the cause of the horse's illness remained unknown after other investigations'.(41)

Passive disease surveillance and biosecurity risk management for HeV and EIDs in Australian animals largely relies on clinical syndromic recognition and management of suspect disease cases by private veterinarians. These veterinarians play crucial roles, relevant to animal and human health.(42) The management of HeV in Australian horses is an example of a challenge of zoonotic disease risk met with great success through the pooling of expertise, data, knowledge, functional networks, operational systems, and stakeholders. This success has resulted in increased zoonotic disease awareness, risk management and preparedness in Australia.

The detection of HeV in the horse relies on veterinary attendance and consideration of the disease as a differential diagnosis based on clinical findings, sampling of the horse and timely laboratory testing. Many challenges face the private practitioner veterinarians that attend and sample the potential cases.(42) These include considerations of professional liability, workplace health and safety risks, personal, staff, owner and public safety, financial and time pressures, and practical challenges associated with the application of personal protective equipment (PPE) in Australian conditions. In this context, considering that any unwell horse could be infected with HeV, and that clinical assessment will not provide increased predictive value has added to the difficulty experienced by clinicians. It has been established that HeV infected horses may shed virus for many days prior to demonstrating clinical signs(1) and as with many viruses, a minority of horses may become infected, shed virus, and recover without demonstrating clinical disease.

Veterinarians often have limited windows of time for observation and can be restricted in diagnostic procedure options. Restricting factors include availability, experience, owner financial constraints and limitations associated with the management of the risk for zoonotic disease exposure (prior to testing for HeV and Australian bat lyssavirus, ABLV), including workplace health and safety restrictions (WPHS) and guidelines. Horses vary greatly in their clinical response to pain generally, severe inflammation, abdominal pain, and toxaemia. They often demonstrate signs easily confused with and/or overlapping neurological dysfunction such as, but not restricted to ataxia, uncontrolled thrashing and collapse or secondary respiratory changes relating to pain rather than respiratory dysfunction. Vaccination of horses remains the single most effective risk management strategy for HeV. With many horses remaining unvaccinated, cases are certain to continue

intermittently. Accurate recognition of HeV as a differential diagnosis in horses by attending clinicians is fundamental to the success of One Health HeV disease management.

Surveillance via suspect disease testing is affected by a strong regional bias for areas where HeV has previously been detected, and where domestic horse populations overlap with the distribution of BFFs. The range is from eastern coastal QLD to northern NSW, area often referred to as the 'Hendra Belt' (Figure 1.1). Testing for HeV is less commonly performed on horses suffering similar disease further south in Australia due to a perception that the likelihood of disease occurrence is lower in regions without this flying fox species. Historically, less-than 7% of testing of suspect horse disease cases has been outside NSW and QLD.

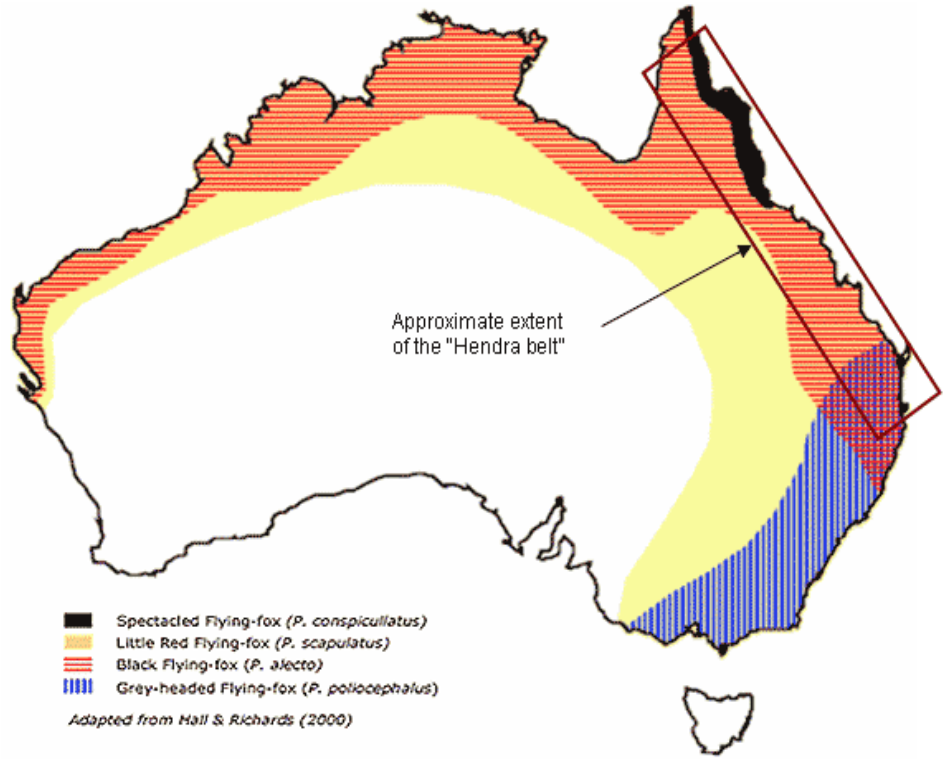

Figure 1.1: Map of Australia with the previously proclaimed 'Hendra Belt', a misnomer

Around 1,000 horses are tested for HeV annually. Many of these are suffering disease that is clinically consistent with HeV, such as acute severe respiratory and/or neurological disease, often featuring pyrexia and other signs of disease mediated via endothelial vasculitis. However, less than 1% of horses tested annually are found to give a positive result (Table 1.2, Figure 1.2).

Table 1.2: HeV testing as part of Emergency Animal Disease Investigations 2016 – 2018

National Animal Health Information System (NAHIS) Data Summary

Year	ACT	NSW	NT	QLD	SA	TAS	VIC	WA	Total
2016	0	256	11	685	3	1	14	6	976
2017	0	274	8	631	9	3	93	12	1030
2018	0	157	9	442	1		6	15	630
Total	0	687	28	1758	13	4	113	33	2636

(Equine testing only; date range 1/01/2016 - 27/12/2018)

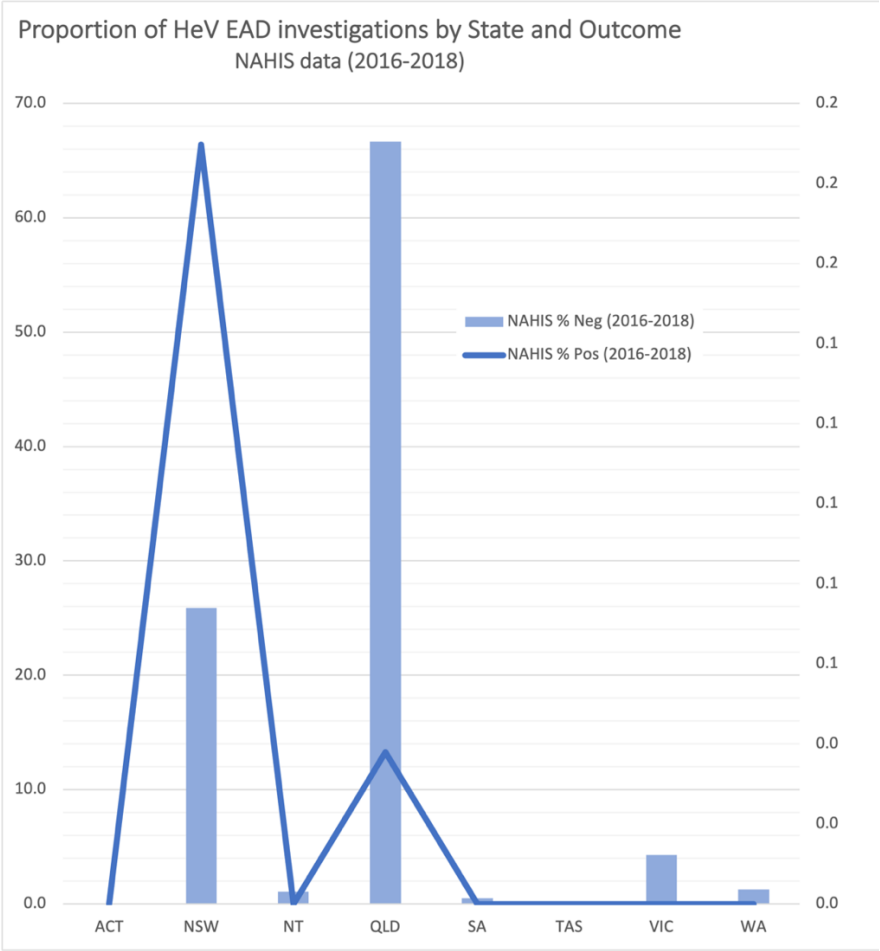

Figure 1.2: Proportion of HeV disease investigations by state and outcome

Proportion of Horse disease investigations conducted between 2016 and 2018 (during the time range of the study population) tested for HeV by state and by positive and negative test outcome. Strong case submission bias favors those regions in which HeV cases have been detected previously. In Qld, highest awareness sees high case numbers investigated for very few detections (<1%). Of particular note is that horses in regions of highest awareness for HeV (Qld and Northern coastal NSW) have highest uptake of HeV vaccine which has been available for use in horses since 2012. Missed cases are inevitable, yet every missed case matter for the veterinarians and owners involved. It is thus crucial to emphasise that while likelihood of any individual horse becoming infected may be low (especially if not near watercourses and flying fox roosting or feeding habitats), the consequences for infected horses or humans are usually catastrophic.

1.2 Emerging Infectious Disease (EID) Sentinel Opportunity

The prioritised management of HeV in Australian horses and the testing of suspect cases has highlighted the challenges, limitations, and gaps in diagnostic investigation beyond a few well-understood pathogens. This challenging context represented an opportunity for strengthening Australia's biosecurity.

Australian horses:

- are kept in close association with other domestic animals – both companion and agricultural production species. This includes in peri-urban regions that pose particular risk to biosecurity for production species such as pigs, sheep, and poultry
- are kept in close association with human communities – rural, semirural, and peri-urban
- are monitored and investigated more closely for disease with far greater frequency and sensitivity than production species. Disease is investigated by veterinarians even when subtle, in isolated cases or when affecting only small groups
- more overtly demonstrate signs of infectious disease compared to production species such as cattle and sheep
- are susceptible to biting insects and often kept both close to water sources and human dwellings/communities, thus acting as sentinels for arthropod-borne diseases of human, animal health and national biosecurity significance
- are comparatively susceptible to emerging infectious disease spillover from wildlife such as flying foxes (HeV) and other bats (ABLV).

Horses are of lower potential influence on international trade and gross domestic product (GDP) compared to other production livestock species. Thus, detection of emerging disease threats in them is lower risk to market and food security while allowing further scientific interpretation of their infection dynamics and significance.

Australian horses investigated for HeV-like disease offer a unique opportunity to take a closer look at the agents causing these disease syndromes in horses. This would improve disease diagnostic capacity and the wellbeing of horses and may detect infections that are spilling over from wildlife reservoirs that could affect Australia's livestock, trade and

industry security and public health. For these reasons we considered horses a particularly suitable species as a sentinel for infectious diseases to improve biosecurity.

Taking a closer look at the agents causing HeV-like disease syndromes in horses served as proof-of-concept in a highly suitable sentinel species for piloting our innovative transdisciplinary and inter sectoral approach to detecting, characterising, and interpreting infections that are spilling over from wildlife reservoirs. These infections could affect Australia's livestock, trade and industry security and public health.

This project aimed to demonstrate how three highly innovative pillars may be combined and applied to existing resources to deliver significant enhancement of Australia's biosecurity and emerging infectious disease preparedness.

The three pillars of this research comprised the following activities:

1. The operation of an innovative purpose-built SQL database that combined the bio-banked laboratory samples with their de-identified sample event, subject, and clinical details (including syndromic classification) and their diagnostic testing results. This approach allowed for optimised epidemiological assessment of disease causality while leaving unnecessary sensitive information with the primary submitting laboratory
2. The application of a novel serological microbead immunoassay enabling screening for exposure to a wide range of relevant pathogens in bio-banked clinical horse samples
3. The parallel application of advanced molecular testing methods to identify novel and emerging infectious agents in the same cohort of horses. Testing methods include high-throughput pan-PCR, metagenomic next-generation sequencing (NGS) and specialised bioinformatical pipelines.

A purpose-built, de-identified SQL database was built to capture and compare subjects, geography, clinical features, samples, and results, while appropriately leaving sensitive horse and owner details with state biosecurity departments and local stakeholders. Advanced molecular and serological testing was applied in parallel to samples from horses collected during infectious-like clinical syndromes consistent with but found negative for HeV when tested as part of routine biosecurity. The same testing was applied to samples of equivalent type. These were stored and processed from horses that were free of infectious-

like disease at the time of sampling and thus acted as comparative controls supporting both assay development and interpretations. This comparative analysis allowed for insights into the significance of novel and emerging infectious agents and their likelihood of causal association with disease.

This research approach aimed to:

- deliver crucial knowledge of and preparedness for emerging infectious disease threats
- develop novel diagnostic tools based on new knowledge with priority, in collaboration with state and national biosecurity stakeholders
- develop testing algorithms suitable for integration into government laboratories
- demonstrate 'proof of concept' of an optimised syndromic surveillance system that combines leading research technology and innovative biobanking with routine biosecurity, complementing existing systems to suggest improvements.

Thus, this project aimed to demonstrate the utility of an innovative, integrated research approach to syndromic surveillance. The approach should extend, support and guide existing systems and operations for biosecurity at routine national, state, clinical veterinary, and farm/stable levels.

Trust between sectors was facilitated establishing collaborative relationships with co-operative stakeholder engagement that aimed to understand differing perspectives of relevance. The project fostered holistic, respectful, and open-minded understanding of the priorities, operations, systems, and perceptions of tolerable vs intolerable risk unique to each technical operation, sector, and stakeholder. Significant results were communicated with government departments to allow for relevant biosecurity response and preparedness.

This approach was expected to significantly improve Australia's biosecurity by overcoming technical and cultural barriers currently limiting routine disease surveillance and biosecurity management of Australia's horses. The approaches and successes of the project also hold relevance for Australia's biosecurity in other animal species, disease, and pest contexts, in agriculture, human health and similarly to regional and global biosecurity internationally.

1.3 Overview of Thesis Structure

Following this thesis introduction (***Chapter 1***), the remainder of the thesis is divided into three Parts (see Figure 1.3). **Part 1** gives a critical review of clinical features and

pathophysiological basis of Hendra virus infection and disease in horses. This Part is broken down into two chapters: ***Chapter 2***, which reviews literature that converge on the study of Henipavirus (and related/ emerging pathogen) infection in horses (virological, immunological, pathological, epidemiological, and clinical) to form a holistic basis for recognising suspect cases that warrant further investigation; and ***Chapter 3***, which reviews the literature relevant to the consideration of differential causes of HeV-like disease in horses in Australia, and relevant epidemiology of emerging infectious diseases of potential One Health relevance in this context.

Part 2 introduces the key concepts of the research strategy. ***Chapter 4*** discusses and demonstrates the clinically-focused approach (qualitative and ethnographic): a series of case studies of horses are closely described integrating first-hand veterinary accounts with expert panel critical review. These include both case series confirmed to have been infected with HeV, and some suspected to have been infected with HeV or closely related pathogen - but in which diagnostic confirmation was not realised. ***Chapter 5*** describes the complex One Health setting surrounding the sporadic occurrence of HeV and EIDs in Australian horses, using a systems processes and associated systems thinking causality framework, to identify current knowledge gaps and process/ system pain-points, inspired by the ethnographic approach of understanding real world cases, and the chain of critical processes required for their proactive recognition and appropriate One Health/ Biosecurity risk management, as always occurring within a wider context. The chapter concludes that risk-based and targeted active surveillance of horses with HeV-like illness is justified: horses can be sentinels of emerging zoonotic viral diseases – and introduces the strategic three-pillared approach with which the empirical research proceeded. ***Chapter 6*** provides a guide for how this can be done, providing specific guidelines (syndromic and pathological analyses) to inform the selection of cases for further analysis. Quantitative analysis is presented of all publicly and scientifically reported signs of equine HeV disease cases. The analysis uses indexing to the Medical Subject Heading descriptor term database (MeSH) to understand their pathologic basis and syndromic co-occurrence. Syndromic analysis was extended via plotting of signs, as they occurred in combination among individual cases, utilising UPsetR.

Part 3 reports on the most-significant virus discovery (HeVg2 – including characterisation, interpretation, and communication/ system operational responses) and an overview of

additional findings that resulted from this research. ***Chapter 7*** describes the significant, confirmed paramyxovirus and filovirus serology findings in Australian horses along with preliminary interpretation of seropositivity to other emerging zoonotic RNA viral agents and select bacterial zoonoses. A strategically selected panel of recombinant viral protein antigens was developed (33 plex) and applied as a multiplex microbead fluorescent immunoassay targeting both IgG and IgM antibodies for Emerging Infectious Disease agents relevant to the Australian and HeV-like horse disease contexts (informed by the critical review findings of Part 1), with Bayesian information theory approaches for assessment of test performance and continuous bio-marker cut-off (Mean florescent Intensity MFI) binary test outcome classification. Findings included novel evidence of bat-borne Pararubulavirus spillover to horses and associated clinical HeV-like disease. A selection of most-notable findings beyond Paramyxoviruses are also discussed in this Chapter. Some findings have been published and presented nationally and internationally in scientific abstracts.(43,44) ***Chapter 8*** is a published manuscript (*Emerging Infectious Diseases*) that reports the discovery of a novel HeV variant, with interpretation of equivalent pathogenicity and immunogenicity. The publication also covers implications for extended geographic spillover risk. ***Chapter 9***, the discussion and conclusion, offers a reflective overview of this Horses as Sentinels research, with synthesis of insights, conclusions, and future directions**.** This research developed an integrated transdisciplinary three pillared approach, bolstering veterinary-initiated government-based syndromic priority disease surveillance, suitable for detection of EID agents of zoonotic significance. **This thesis demonstrates how significant enhancements can be made to Australia's biosecurity and emerging infectious disease preparedness. Each component of the 'three pillars' offers significant value individually, but greatest value is in their problem-focused example, combined application and integration with routine biosecurity processes - across settings of clinical veterinary practice, state biosecurity, national biosecurity and epidemiology, and national reference laboratories.** This can be understood as a remarkable proof-of concept for the potential for such agent/host scenario-focused, research-based extensions of state government-based biosecurity investigations, to improve significant pathogen characterisation, biosecurity and One Health management. This research identified significant novel infectious agents that were going undetected at the time of the research, yet which were causing severe disease with relevance for both animal and human health.

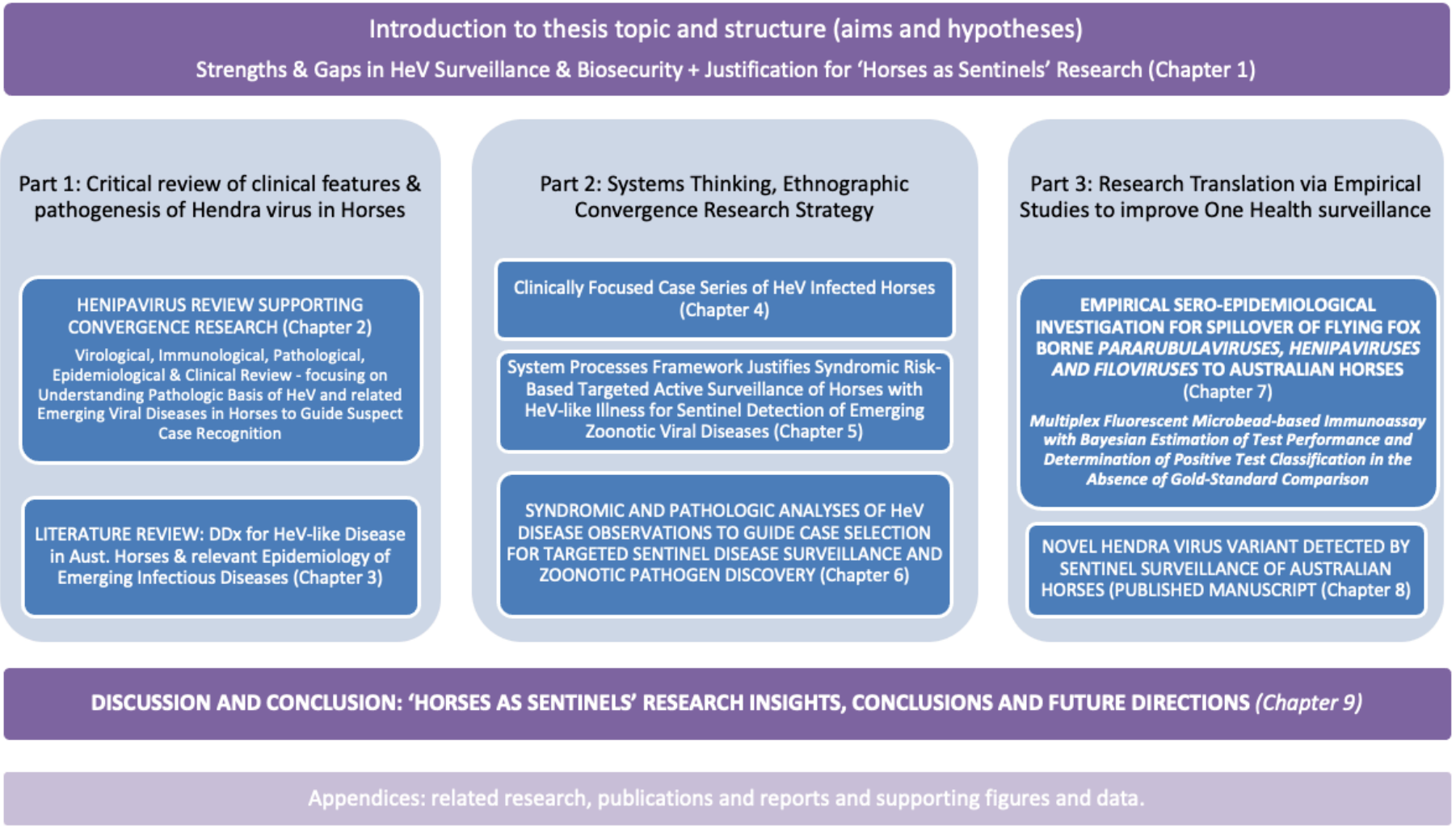

Figure 1.3: Schematic outlining structure of the thesis – Horses as Sentinels of Emerging Zoonotic Diseases in Australia.

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Horses as Sentinels of Emerging Infectious Disease

Chapter 2: HENIPAVIRUS REVIEW SUPPORTING CONVERGENCE RESEARCH - Virological, Immunological, Pathological, Epidemiological, & Clinical Review - focusing on Understanding Pathologic Basis of HeV and related Emerging Viral Diseases in Horses to Guide Suspect Case Recognition

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Statement of personal contribution: I drafted the first complete manuscript (2018) and received feedback from PhD supervisors and wider project team members Dr Peter Reid and Dr Anne Jackson. I acknowledge additional supportive contribution from Dr Nicole brown, Ms Nicole Popovic, Dr Ruth Zadoks, Dr Caroline Spelta and Dr Carly Garling.

Format: This chapter has been prepared with a view to peer-reviewed journal submission however has not yet been submitted to a journal.

A concise manuscript drawn from this text was published in 2021 as a priority following recognition of HeV-g2. The paper supports proactive recognition and management of suspect HeV infected horses amongst consulting veterinarians as: **Hendra virus − Two viruses, Same Fatal Disease.** Annand, E.J. The Australian Equine Veterinarian, vol. 41 No. 2, 2021. https://www.sciquest.org.nz/browse/publications/article/166341

2.1 Introduction

HeV and Nipah virus (NiV) are closely related viruses in the *Henipavirus* genus of the *Paramyxoviridae* family.[1–3] Both cause highly lethal zoonotic disease. They were discovered in the mid to late 1990s with disease spillover events from their natural reservoir hosts, several species of flying foxes (*Pteropus* spp). They are classified as biosecurity level 4 (BSL-4) pathogens and listed in the World Health Organisation's Blueprint for Research and Development as *Priority Diseases* given their wide host tropism, high pathogenicity and case fatality, and lack of registered available vaccine or therapy in humans.[4,5]

Hendra virus (HeV) occurs as a low incidence but high consequence infection in horses, usually resulting in acute severe disease. The disease is predominantly mediated through systemic endotheliotropic infection, resultant vasculature compromise and vasculitis, with rapid progression to death or moribund condition justifying veterinary euthanasia.[6,7] Infected horses usually show respiratory (broncho-pneumopathologic) and/or neurological (neuropathologic) disease manifestations. As for most viruses, clinical manifestations vary over the course of infection and may be discreet in some time-point examinations of infected horses (especially in early stages of infection course), or as for many RNA viruses particularly, may be non-specific or diffuse (reflecting pathology of any organ or body system).[8]

A rapid, accurate and specific real time PCR assay[9] was developed for the detection of HeV in clinical samples. This has been used extensively as part of priority equine disease investigations (optimised passive syndromic surveillance), almost always initiated by attending private practising veterinarians.[10]

Recognition of potentially fatal Hendra virus disease in humans is greatly dependent on confirmation of HeV infection in the horses to which they have been exposed. While no immune therapy for HeV infection is licensed for human use yet, monoclonal antibody (m102.4) has been administered on 16 occasions to people with high perceived viral exposure or confirmed infection (provisional use on compassionate grounds).[11] This treatment has demonstrated safety and efficacy in phase one human trails.[12] A human vaccine is currently being evaluated in phase-one clinical trails[13] based on the same HeV G glycoprotein ectodomain (sG) as the licensed horse subunit vaccine (Equivac ® HeV) that has been used highly-effectively in horses since 2012.[14] Other monoclonal antibody therapies are in

development.[11,15,16] Efficacy of monoclonal therapies greatly depends on their being administered promptly following viral exposure. Thus, private veterinarians carrying out suspect significant disease investigations on horses serve a critical and unique One Health role in their recognition of suspect cases of HeV and other emerging infectious diseases.[10]

Since the first recognised outbreak, HeV spillover infections have occurred sporadically, resulting in the natural death or euthanasia of 107 horses and severe disease in seven humans, four of whom have died. One human death occurred 13 months following apparent recovery from initial illness. Death in this case was due to a relapse featuring severe meningoencephalitis.[17] Three humans recovered from initial illness, although one is reported to have persistent neurological deficits.[18] As of June 2022, there have been 41 outbreaks in Qld and 24 in the central coast to northern New South Wales (NSW) regions. Included in these tallies are two cases caused by a novel HeV-variant genotype (HeV-g2) detected and interpreted as part of this research. Cases include the retrospectively diagnosed occurrence near Gympie in Queensland in September 2015 from this research subject cohort and a contemporary case near Newcastle in NSW in October 2021. This case was diagnosed by the routine public biosecurity sector, enabled by proactive suspect disease consideration by the attending clinician and diagnostic support and scientific insight afforded by this research.[19,20]

These numerous spillover detections and their timely biosecurity and One Health management show how systematic HeV diagnosis in Australian horses is an example of how lethal zoonotic disease risk can be effectively managed. Management can be achieved through a collaborative interdisciplinary and intersectoral surveillance networks that draw together disease recognition expertise from several sources: owners and consulting clinical veterinarians with government biosecurity duty pathologists, laboratory scientists and epidemiologists for significant One Health diagnostic responsibility.

Secondary benefits of HeV disease management have included increased zoonotic disease awareness, risk management strategies including knowledge and application of staged personal protective equipment (PPE),[21] and appreciation of operative One Health perspectives.[10] A survey of Australian veterinarians in 2011 found a 70% majority were unaware of industry guidelines or standards relating to zoonotic disease, but 70% of those

who reported awareness, indicated familiarity with equine standards related primarily to Hendra virus.[22]

Unfortunately, significant burdens for veterinarians and other primary stakeholders have persisted alongside these successes. Management of One Health HeV disease risks associated with horses potentially infected with HeV challenge private veterinarians (on which suspect case recognition so heavily relies) across professional, financial, and personal liability realms.[10] Challenges can relate to delay or limitation in treatment available and case management options in suspect cases, until negative priority government biosecurity diagnostic testing for HeV infection is available, often resultant compromise of horse welfare and/or health outcomes, along with potentially compromised owner experience, confidence in veterinary services generally or in specific service providers.[10] Additional challenges arise for veterinarians attending suspect HeV cases in meeting Work Health and Safety Act and Biosecurity Act obligations in the context of overseeing often wide ranging and case context-specific biosecurity risks.[10] Such challenges often confound already demanding field conditions and stressors associated with commercial veterinary practice. These pressures are thought to contribute to some veterinarians restricting their consultation to vaccinated horses and even abandoning equine practice altogether in HeV endemic regions.[23–25]

Veterinarians have also been challenged in their recognition of suspect HeV infected horses and thus management of the associated zoonotic risks by the apparent excessive variability of clinical signs. There is an associated lack of confidence in recognising HeV clinical syndrome/s.

Holistic proactive consideration of the manifestations of diseased horse cases by consulting veterinarians as part of their clinical assessments, as consistent with the pathologic basis of Henipavirus (HNV) infection, critically influences timely recognition of suspect HeV infection cases. Case diagnostic assessment and therapeutic management more broadly (including considered differential causes/disease influences) are also supported by this approach, as are appropriate timely One Health and biosecurity management. Clinical and pathological considerations specific to the individual disease case may be further proactively interpreted in light of attending practitioner consideration of epidemiological case context. Such epidemiological considerations may include microbiological, environmental, individual horse

and herd background health as well as husbandry, socio-economic and cultural (such as horse purpose). Wider considerations may relate to welfare, insurance and referral treatment options including in relation to vaccination. Such holistic information best guides proactive suspect case recognition and biosecurity management in such a way as to most benefit and least burden primary stakeholders - those to whom the condition of the horse matter most such as owners, trainers, breeders, and carers.

Given that appropriate human disease risk management depends on timely laboratory diagnosis of HeV infection in the horse/s, it is of great public health benefit for veterinarians to be most familiar with the clinical disease course expected with HeV infection, variable single time-point clinical manifestations/syndrome/s, and their pathologic bases. This manuscript reviews clinical disease in HeV infected horses. It highlights the importance of pathogenesis-orientated consideration of disease presentation for proactive recognition of suspect cases and clearest interpretation of case-specific HeV and EID diagnostic responsibility.

2.1.1 Evolving understanding on clinical recognition of HeV disease spillover to Australian horses

HeV was first identified in 1994 in two neighbouring thoroughbred racing stables in Hendra, Queensland (Qld). Of 20 infected horses, 13 either died or were euthanased in moribund condition on humane grounds, and one of two infected humans also died.[26,27] The time between the onset of overt clinical disease and death in horses was typically two to three days.[5] Previously no highly lethal zoonotic diseases affecting humans and horses were known to exist in Australia,[28] and appropriate personal protective equipment was not routinely used by veterinarians and carers when managing horses with severe disease of suspect infectious cause.

Although clinical signs indicating central nervous system (CNS) dysfunction were observed and recorded by the attending veterinarian in Hendra in 1994 (P Reid, Chapter 2 of this thesis – *Clinically focused case series of HeV disease*), early publications concentrated on the disease's pronounced respiratory signs and pathological features. Pleural haemorrhages, interstitial pneumonia and signs of neurological damage were reported at necropsy.[26] The causative organism was initially referred to as equine morbillivirus. Descriptions (including

those conveyed as part of post- and under-graduate veterinary education) emphasised acute equine respiratory syndrome featuring marked pyrexia, rapid progression to death with terminal frothy nasal discharge in some horses. Awareness among veterinarians focused on acute severe disease or 'sudden death' featuring terminal frothy nasal discharge.

Broader and less overtly respiratory signs of disease were not well recognised in veterinary education reports and messaging on recognition of HeV disease cases. This had the effect of limiting consideration of HeV in diseased horse cases attended by most practising veterinarians to those with overtly consistent disease featuring terminal frothy nasal discharge and most clearly observable exposure to flying foxes. Even fatal cases considered most consistent with HeV infection, in light of current understanding of expected pathologic and clinical disease course, were often left without causative diagnosis or were presumed likely attributable to alternative non-infectious causes. A common alternative presumptive diagnosis has been snake envenomation in cases of acute progressive fatal disease featuring neurological dysfunction and coagulopathy.[29] This has applied even when disease manifestations and epidemiological context were highly consistent with HeV infection (Author (E Annand) experience Bowen Qld. 2008), and coagulopathy may similarly result from disseminated intravascular coagulation (DIC) in advanced HeV infection and disease.[30,31]

Through the 15 years following its discovery (between 1994 and 2009), HeV spillover infection cases were diagnosed sporadically.[6] Veterinarians who recognised HeV infection in north Queensland in this time were prompted to suspect HeV in 'unusual disease presentations', heeding the advice of an experienced clinician, B. Pott. This veterinarian, having attended HeV infected horse cases, offered colleagues advice to consider HeV infection 'If the case is unusual' (T. Annand – personal communication). Such advice prompted sampling and investigation of two horses near Bowen, Qld in 2008, found to have HeV infection. Diagnosis was based on their demonstrating severe acute systemic, respiratory, and neurological disease without context consistent with alternate cause such as recent travel (that can prompt travel-associated pleuropneumonia) or exposure to toxic plants (such as *Crotilaria* spp. or *Indigophera* sp.). (T. Annand – personal communication) (See Chapter 4 of this thesis – *Clinically focused case series of HeV disease*)

The second largest recognised HeV spillover event occurred in an equine hospital in Thornlands (formerly Redlands), south-east Queensland in 2008, involving eight cases that died or were euthanased with acute severe disease. HeV infection was confirmed by PCR in five, with diagnosis of the other three by fluorescent antigen test performed on blood smears where samples for PCR were unavailable. Sudden onset illness, fever, rapid deterioration, respiratory and neurological disease manifestations were described, with one horse displaying terminal frothy nasal discharge.[32]

The five PCR confirmed cases, reported as predominantly neurological manifestations, were considered potentially novel clinical disease presentations compared with previously reported HeV cases for which respiratory manifestations had been emphasised (in published literature).[18,33] Media reports and equine online forums conjectured that the cases were caused by the neurological form of equine herpes virus-1, rabies or that a novel strain of HeV had given rise to significantly different disease.

Responding to consideration of such potential varied HeV disease molecular, phylogeny, in-vitro and in-vivo studies were undertaken comparing the virus isolated from this outbreak (Hendra Virus/Australia/Horse/2008/Redlands) with prototypic HeV. The isolate was found to be 98% similar at the nucleotide level and 99.6% similar at the amino acid level to the 1994 HeV isolate.[1] Clinical disease and pathological features described from experimental infections of three horses using the Redlands 2008 isolate were consistent with those recorded in earlier studies using the original HeV isolate.[1] The data indicated conserved infectivity and tissue tropism of all strains of HeV identified in horses at the time. The prominent neurological manifestations described as 'novel clinical features' in field cases[33] most-likely reflected:

1. underreporting of neurological features in published recounts of previous cases
2. the spectrum of HeV disease in horses, as influenced by the route of exposure (viral entry portal), the infectious dose, individual host immune-susceptibility and the anatomical course of infection and pathology
3. the relative prominence of outwardly observable clinical signs of neurological dysfunction compared to that of other organs and body system and
4. understandable bias limiting recollection of overt pneumotropic disease signs given delayed recognition of suspect HeV disease and retrospective reporting in the context of zoonotic human fatality.

These considerations of potentially extended signs of clinical disease, and high publicity of the 2008 Redlands outbreak, increased awareness of a broader range of clinical manifestations consistent with HeV infection, and thus the expected HeV disease syndrome, amongst horse carers, veterinarians, and government biosecurity duty pathologists. As a result, there have been greatly increased numbers of suspect case investigations in subsequent years – especially in regions where HeV has been previously detected.[34] This has had the benefit of increasing the sensitivity of surveillance for HeV spillover infection that relies on passive suspect case reporting by consulting clinical veterinarians. However, recognition that horses infected with HeV may present with wide ranging (non-specific) clinical manifestations (including mild illness especially early in infection course) has challenged many veterinarians tasked with recognising suspect HeV infection. Veterinarians must decide when priority government biosecurity laboratory submission and investigation is justified/indicated while best managing clinical therapy, animal welfare and One Health and biosecurity risks. Their case management must also align with professional indemnity, OHS and biosecurity legislation.[10,35] Indeed such challenges, along with concerns for personal and family bio-safety, financial and other practical challenges, have influenced some veterinarians to cease equine practice or relocate to regions of perceived lower risk for HeV.[23,24]

State biosecurity departments have similarly been challenged by greatly increased HeV PCR testing requirements. Asymptomatic, unvaccinated horses requiring biosecurity clearance for invasive or intensive medical veterinary management and/or admission to equine hospitals must also be tested. These challenges highlight that funding and infrastructure are currently grossly inadequate to support operational and governance specifically for the management of zoonotic diseases in non-production domestic animal species. Adequate support might most suitably and sustainably be achieved via integrated investment and engagement from the government human health sector to which such disease contexts of highest priority including support for all necessary operations and processes that link the diseased animal in the field to confirmed laboratory diagnosis and prompt interagency response. This issue is widely recognised among those managing zoonotic disease in the animal health and biosecurity sector globally and has been discussed in interdisciplinary One Health scientific literature in the Australian context.[36] Inadequate bespoke resourcing

integrated with human health has meant that resources to meet this increasing demand have drawn variably, with less accountability and contingency, from the common limited pool available for managing animal health and biosecurity. Similarly, such zoonotic disease investigations are prone to barriers relating to conflicting or competing priorities between those of animal owners, private practicing veterinarians, Animal health biosecurity, freedom of disease and trade, and human health. Many diseases of high impact to animal production industries, food security and wildlife health are affected by such barriers relating to these conflicting priorities. Improvements in recognition of clinical disease most consistent with HeV infection, and that of similar emerging RNA viruses of One health significance, by responsible clinicians, duty pathologists, laboratory scientists, and epidemiologists, should improve the sensitivity and efficacy of operative surveillance, and support proactive interpretation and management of emerging One Health disease risks.

Naturally acquired equine HeV infections usually result in severe disease, with fever and increased heart rate (tachycardia) and rapid deterioration over one to three days, featuring signs of respiratory and/or neurological dysfunction and a very high case fatality (over 80%[6]). Incubation periods of four to 16 days have been observed in naturally infected horses.[7,28,37,38] Actual case mortality is expected to be somewhat lower given some cases of infection go undetected (especially those with mild or unrecognised disease) meaning true spillover prevalence is expected to be higher than known. Also, previous biosecurity protocol instructed for euthanasia of all horses deemed to have been infected with HeV, including even those that recovered, to manage then-uncertain ongoing transmission risks. Biosecurity advice has been revised more recently in light of evidence that supports no transmission risk from horses that survive infection and mount adequate adaptive immune response. Decisions about management of surviving infected horses are currently made on a case-by-case basis by each state's Chief Veterinary Officer with owner and veterinary consultation.[7,39]

2.1.2 Challenges with clinical recognition of HeV disease

Recognition and diagnosis of HeV infection relies critically on veterinary attendance, consideration of the disease as a differential diagnosis based on clinical findings in light of

relevant epidemiological context, appropriate sampling of the horse, and timely laboratory testing.

The potential variability of signs observable with HeV infection, at any single clinical presentation, challenges veterinarians to recognise suspect cases and to manage biosecurity of their associated zoonotic disease risk.

Such perceived low clinical diagnostic confidence, yet high diagnostic responsibility, confounded by potentially diverse and significant primary stakeholder burdens, limits the sensitivity of passive surveillance for this priority-listed and notifiable disease and related emerging diseases by directly limiting sampling and laboratory submission.

In principle, clinical signs observed by veterinarians are expected to vary, reflecting the 'time window' of the examination during the course of disease. Additionally, disease severity is influenced by the infectious dose of virus inoculum, route of infection and individual animal variation in immune and inflammatory responses. The thoroughness of the examination and reporting accuracy is also likely to contribute to the observed findings that have been previously recorded.

A broad range of individual clinical signs are observed in HeV infected horse cases.[6] Early signs can be mild and non-specific, and clinical disease manifestations observed at any one time-window vary significantly. Stage of infection, anatomical progression through different body systems, and resultant progressive course and their pathologic basis at the subcellular, cellular, tissue, body system and systemic levels all affect the clinical disease presentation. For example, many individual respiratory disease clinical signs attributable to HeV infection are consistent with alternative aetiologies, such as respiratory irritants, immune mediators and other infectious disease agents. Similarly, widely variable clinical manifestations of non-infectious 'acute abdomen' disease (colic), can overlap with some typical signs of HeV infection such as respiratory distress, injected mucous membranes and tachycardia. Furthermore, manifestations of pain and circulatory dysfunction, such as posture and gait changes, common in severe non-infectious 'acute abdomen' disease, may be misinterpreted as indications of neurological dysfunction resulting from HeV or other CNS viral infection.

A small minority of HeV infections have been asymptomatic or mildly symptomatic in horses, without pyrexia.[40] Many factors influence severity of HeV disease − particularly horse age, acquired and innate immune response and infectious viral load. Approximately 10% of horses infected survive acute infection aided by their development of neutralising antibodies.[6,26,41,42] Some horses that survive acute infection develop persistent (chronic) neurological disease manifestations, attributed to persistent non-suppurative meningoencephalitis, such as myoclonic gluteal or triceps muscular spasms and persistent jaw champing (co-author observation − P Reid) − See thesis Chapter 4: Clinically Focused Case Series of HeV Disease).[3,7,37]

Cases of horse illness fitting the case description for HeV disease, prior and after the initially recognised spillover event in Hendra September 1994, and the retrospectively diagnosed Mackey spillover August 1994, are likely to remain undetected. They may be attributed to unknown cause or suspected alternative but unconfirmed causes. Differential diagnoses in cases of HeV-like illness in Australian horses may include the following: snake bite, toxic plant poisoning (Crofton weed, *Crotalaria* spp., avocado, Brassicaceae, *Indigophera* spp.), mouldy grain aflatoxin poisoning, ionophore toxicity (monensin, salinomycin), botulism, anthrax, chemical poisonings (paraquat, phostoxin, zinc phosphide, fluoroacetate, cyanide), equine herpes virus-1, myeloencephalopathy, purpura haemorrhagica, pleuropneumonia, pneumonia, and sepsis from unconfirmed or untested bacterial or viral pathogens caused by progression of undiagnosed pre-existing disease (See Chapter 3 - Table 3.1).

2.1.3 Laboratory diagnosis of HNVs and related EIDs in animals and humans

Laboratory diagnosis in animals of infection caused by both known HeV genotypes (prototype HeV *HeV-g1*, and novel variant genotype *HeV-g2*) is made by detecting RNA in clinical and/or post-mortem samples. Reverse transcriptase real-time PCR (RT-qPCR) is used, targeting the matrix (M) gene of both genotypes. This testing approach allows rapid screening of suspect cases as part of priority disease investigations by government biosecurity departments to guide timely emergency biosecurity management. These investigations constitute risk-based surveillance requiring suspect disease recognition by consulting clinical veterinarians, with appropriate sampling, submission and diagnostic request (passive reporting).

Confirmatory testing is performed at the Australian Centre for Disease Preparedness (ACDP, formerly AAHL) by real-time PCR testing targeting M and nucleocapsid (N) genes. Virus isolation is also attempted on retained samples. A serological test such as a virus neutralisation test or an sG iELISA test may also be performed.[43] This is considered of limited diagnostic value, however, when interpreted without a paired convalescent sample, in acutely affected, rapidly progressing fatal cases yet to mount an antibody response, and/or in horses of unknown HeV vaccine and/or past exposure status.[9,34]

Molecular methods able to detect related and emerging agents from clinical samples are increasingly feasible such as those customised and applied in parallel for this research: high-throughput application of Targeted 'Pan-viral-family' nested conventional PCR [141]; and Metatranscriptomic total RNA sequencing coupled with bespoke viral discovery bioinformatical analyses (informed by host genome, available microbiological genetic diversity including expected incidental, contaminant and context relevant known viral diversity).[142] The challenge currently is in practical integration of such methods with routine exclusion testing and known disease case definition based priority animal disease surveillance activities. Especially given their required expertise and infrastructure, cost and operational time, justifying suitability/ feasibility only in sample/ case cohort batches. Similarly, interpretation in situations of divergent and emerging infectious agents, require diverse extensive innovative transdisciplinary epistemological and scientific inference are necessary to guide response for which policy and guidelines, and the core scientific, pathologic, diagnostic and epidemiology knowledge, that underpins them, will inherently be lacking. Nevertheless, meeting this demand is increasingly possible with fore-front probabilistic and machine learning modelling (max-entropy; agent, DAG and scenario tree based), bioinformatical, emerging epidemiological, as well as system thinking and analytical approaches that may incorporate broader influences including cultural, legal, industry, WPHS and system contexts.

Similarly, serology testing platforms suitable for determining antibody reactions against a wider viral diversity of and numerous target significant disease agents including multiplex florescent microsphere immunoassay (MIA) platforms utilising appropriately selected recombinant antigenic virus proteins for either IgG and IgM and with options to utilise for sera of diverse mammalian species.[144-145] Again such testing is best suited to application in

batches of suitably selected animal sera and ideally with paired (disease case sample event with subsequent convalescent sampling to allow determination of rising or falling IgG or IgM antibodies). JEV in humans has highlighted the need for multiple, paired and extensive serological testing results required for confidence in diagnosis.[146] In the horse setting increased opportunity exists for increased utilisation of paired sampling serology and for that of in contact horses to bolster potential infectious viral disease diagnosis. This would require communication advice and protocols for private practitioner and other field veterinary submitters. While many cases of suspect HeV diseased horses will be euthanased prior to the required interval for convalescent sera sampling. In such cases potential improved inference from serology testing may be achieved via concurrent and convalescent sampling of other horses on the case property (in direct and not in direct contact) - with signalment, health and exposure status recorded as available. Availability of bespoke quality mammalian expressed recombinant protein targeting antibodies to emerging local viruses is another limitation for best implementation of these approaches. Chapter 7 of this research showcases the benefit of such sample group serology testing supporting interpretation of putative significant disease spillover infection diagnoses and disease events in which diagnosis via molecular means was not achieved (in some cases due to sampling limitations relative to target virus abundance, tissue/ sample type, contamination, cold-chain and sample quality etc.).

2.1.4 Improved clinical recognition of HeV cases considering pathologic basis of disease

HeV infected Australian horses pose a risk of fatal human disease and for high biosecurity consequence yet occur sporadically in low frequency. Thus, detection of cases is challenging, and given such sporadic and low incidence missed cases are inevitable, posing significant unmanaged human health risk that is difficult to measure. Missed HeV infections in horses due to failure to sample and test likely occur consistently with similar low and sporadic incidence. Opportunity can be missed for appropriate timely management of fatal disease risks in exposed/infected humans, where diagnostic investigation and emergency health care in humans, depends largely on laboratory confirmed animal case exposure. Thus, recognition by consulting veterinarians of each suspect equine case greatly influences HeV One Health disease management and biosecurity outcomes.[10]

The lynchpin of effective veterinary disease management is thorough clinical examination, repeated at various time-points if possible. The examiner should consider aberrations from the horse's expected condition in light of case-specific underlying health and immune status, epidemiology, and type, husbandry and purpose/use of the horse. Unfortunately, vets are challenged in determining priority suspect cases of HeV infection, where clinical manifestations in any single time-window of observation may be poorly specific and highly variable. Consulting veterinarians who may find this understanding challenging in the context of suspecting HeV infection, might relate more readily by analogy, to examples of tendon injury or dental abnormality. For these conditions, recognition and appropriate management is similarly best guided by considering the pathologic basis of the cases clinical disease manifestations drawn through such systematic, detailed and often repeated general and focused clinical examinations, supported by understanding broader influencing contexts (such as exercise history, age, diet, dentition, parasitology, farriery, and underlying health).

Pathology is the study of disease (most literally translated as 'suffering').[44] Pathology understands disease as resulting from cellular and molecular aberrations that influence survival of cells and function of tissues and body systems.[44] Understanding the pathologic basis of clinical disease as resulting from structural, biochemical and functional changes in cells, tissues, organs and body systems[45] guides case management and diagnosis very well in most clinical contexts. The diagnostic value of understanding and considering the pathologic basis of presenting disease is especially beneficial in challenging diagnostic and management settings. Examples are cases presenting early in their infection/disease chronology, with non-specific, mild and/or covert manifestations or cases with multiple potential and often unknown (idiopathic) cause/s.

Clinical consideration of viral infection in the horse is underpinned by understanding associated disease manifesting as chronological disruption to molecular, cellular, tissue, organ and systemic homeostasis and function. Mechanisms of disease include direct viral effects and host responses to viral infection, with associated cell, tissue and organ injury. Clinical observations, case history and field epidemiological context all contribute to understanding pathologic basis of cases of infectious disease, and their progression anatomically and over time. Improved awareness of how pathogenesis of HeV gives rise to

the range of observable disease in horses may potentially greatly improve veterinary recognition of suspect cases and associated One Health biosecurity risk management.

This manuscript reviews the pathologic basis of HeV disease, focusing on relevance to recognition of suspect horse-case infection based on observable clinical manifestations. The review will serve to inform this sentinel active disease surveillance research activity, in selection of priority subject-cases for targeted and open-ended serological and molecular testing pathways. It will also inform routine surveillance processes, in proactively determining diagnostic responsibility, and assist consulting veterinarians by bolstering their recognition of suspect cases, affording appropriate timely management of One Health and biosecurity risks.

2.2 Review of HeV and NiV epidemiology, and diagnostic considerations relevant to suspect horse HeV case recognition and management of One Health and biosecurity risk

2.2.1 Reservoirs of HeV and other henipaviruses (HNVs)

HNVs maintain infection reservoirs among pteropod flying foxes across their extensive distribution globally; this includes Africa, Asia and Australia.[46] Neutralising antibodies against HeV are evident in *Pteropus* spp. flying foxes in all Australian states and territories, with detection in samples that pre-date the earliest reported equine cases.[47] It is inherently difficult to appreciate disease manifestations in wildlife compared with domestic animals, nevertheless HeV infected flying foxes demonstrate less pathology and clinical disease. Hypothesised bases for this discrepancy include superior flying fox host adaptive and innate immune responses to HNVs and related paramyxoviruses in the *Pararubulavirus* genus, as for many other RNA viruses of emerging One Health significance with which they are understood to have co-evolved. This is supported by high host metabolism and viral transmission, and dense communal living.[48] Flying foxes roost in dense colonies with great opportunity for exposure when young. They have potential advantages in immunity and resilience against disease associated with their high metabolism, consistent with their having adapted to flight uniquely amongst mammals.[42] Nevertheless pathologic changes consistent with HeV infection in horses, including endothelial vasculitis and neuronal perivascular cuffing have been observed in HeV infected bat tissue from Australian flying foxes. These were sampled due to suspicion of ABLV and thus were more likely to have

shown overt disease manifestations that might include any of inability to fly, weakness, altered mentation and/or behaviour, and general signs of immunocompromise (such as alopecia).[49]

Early genomic sequence analysis of all prototype HeV (HeV-g1) isolates known prior to the discovery of HeV-g2, including from horses, a human case and pteropodid bats, have shown ~99% RNA nucleotide sequence identity. Conversely wide genomic diversity of paramyxoviruses in the henipaviruses and the pararubulavirus families were known to infect and circulate within flying fox populations. Although previously developed molecular assays were regarded as sensitive (when performed on suitable sample types that undergo optimal RNA preservation and extraction) and highly specific, agent-targeted real-time assays may produce false negatives due to wider target agent (similarly pathogenic) genomic diversity.[50,51] This was confirmed by this research identifying a novel HeV variant genotype (HeV-g2) as causing equivalent disease in horses, posing equivalent zoonotic transmission and disease risk.[52] There is, however, sufficient genotypic similarity between the two HeV variants, HeV, HeV-g1 and HeV-g2, and NiV-B and NiV-M to show that they transmit, infect and cause disease via the same overall mechanisms. Evidence is based on their phenotypic innate immune evasion attributes associated with their variable transcription of the P gene, tissue tropism and equivalent host cellular entry receptor binding affinities.[11]

NiV, also arising from spillover transmission from flying fox reservoirs, was identified in 1998 when it caused outbreaks predominantly of acute encephalitis with associated respiratory symptoms in pig handlers in Malaysia.[53] There, the intermediate amplifying hosts were identified as pigs. In the initial spillover events, approximately 40% of 265 human patients died from encephalitis, and more than one million pigs were subsequently destroyed. Late onset and relapsed encephalitis cases were also identified in Malaysia and Singapore, where the genotype was labelled NiV-M. Outbreaks with a new variant (NiV-B) was first recognised in Bangladesh in 2001. Since then, outbreaks have occurred nearly annually in Bangladesh and India, with case fatality rates of over 90%.[54,55] Significant research on HeV and NiV has ensued, while international focus has predominantly been on NiV, given its higher recognised numbers of human infections that have featured human-to-human transmission, and its consequent further recognised pandemic potential.

A third HNV, the non-pathogenic Cedar virus (CedV), was identified following isolation of virus from flying fox urine as part of HeV surveillance activities in Queensland.[56] Distinct from NiV and HeV, the genome of CedV does not encode for V and W proteins, which suppress host interferons and are critical for evasion of the host innate immune response, via its P gene.

Mojiang henipavirus (MojV) was identified in swab samples from rodents (*Rattus flavipectus*) in a cave in Yunnan province, China. It was believed to be the source of fatal infectious pneumonia in three miners in 2012, and not in samples from the insectivorous greater horseshoe bats (*Rhinolophus ferrumequinum*) or shrews that were also tested.[57] The detection highlights the potential for infection and transmission of henipaviruses amongst and between wide-ranging mammalian species, often going undetected by routine timely health and animal health diagnostic testing. It is important to note that no published scientific findings support rodent species acting as reservoir hosts for HNVs in Australia. Subsequently it has been noted that the closest known related virus to SARS-CoV-2 (strain RaTG13/ CoV4991) was identified from greater horseshoe bats sampled in the same mine shaft cave. This complicated the diagnostic inference on the cause/s of the associated fatal human disease.[58] The similarities of SARS and HNV disease in humans create a challenge in determining causation in these human illnesses in the absence of sufficient contemporary molecular diagnostic testing. This highlights the suitability of the acute viral pneumotropic disease syndrome for active emerging infectious disease surveillance activities. Such surveillance activities can extend and support routine animal and human health government diagnostic and passive surveillance operations for infective disease.

Given the consistency of disease resulting from all known strains of HeV in the horse and similarly that arising from NiV infection internationally (for which more published scientific literature on pathogenesis is available), this review focuses on HeV pathogenesis with reference to the more abundant literature on NiV when useful.

2.2.2 Transmission of HeV to horses

Horses are likely to become infected with HeV via the nasal and oro-pharyngeal routes. Their inquisitive grazing behaviour, large respiratory tidal volume, and high surface area of

densely vascularised upper airway mucosa are thought to potentiate transmission via virus-laden droplets or aerosols from flying fox fruit spats or excreta − especially from urine-contaminated vegetation.[1,3,20]

Virus particles may be inhaled accompanying fomites. Infected body fluid such as urine, dust particles or soil of 1 µm diameter or less are examples, capable of entering the lower airway bronchioles, alveolar ducts and alveoli precipitating infection establishing via the respiratory route.[1,3,59]

Horses' oro-naso-pharyngeal mucosae are highly vascularised and closely associated with the central nervous system via the ethmoid and the cribriform plate. In association with their flehmen behaviour, horses sniff unusual odours such as urine, accumulating small molecules that concentrate over the vomeronasal organ on nasal mucosa associated with sensory nerve endings of the olfactory nerve. NiV has been shown to reach the central nervous system via infection ascending along olfactory nerves similarly to the respiratory route in pigs[60] and hamsters.[61] For HeV, anterograde neuro-invasion along sensory olfactory neurones has similarly been demonstrated in laboratory infection in mice. This is a highly plausible simultaneous route of HeV infection to the lower respiratory route in horses,[42] especially considering the anatomical and behavioural features of the species. Indeed, typical neuro-histopathological changes have been observed in experimentally infected horses that have undergone post-mortem and histopathological examination of these relevant structures.[1,7]

Transmission via ingestion of contaminated pasture or water is also considered possible. HeV transmission can occur by sporadic spillover from flying foxes to horses, by horse-to-horse transmission through direct contact with infectious body fluid or through indirect contact via equipment contaminated with infectious body fluids (fomites).

2.2.3 Laboratory diagnosis considerations relevant to suspect horse HeV case recognition and management of One Health and biosecurity risk

Laboratory diagnosis of infection in animals caused by both known HeV genotypes (prototypic HeV-g1 and novel variant HeV-g2) is made by detecting RNA using reverse

transcriptase real-time PCR (RT-qPCR) targeting the matrix (M) gene of both genotypes specifically. This testing approach allows rapid screening diagnosis of suspect cases as part of priority disease investigations by government biosecurity departments, allowing for prompt conclusive results to guide emergency management of biosecurity and One Health transmission risks. These investigations constitute passive surveillance and require initiation by clinical veterinary suspect case recognition, appropriate sampling, laboratory submission and diagnostic request.

Confirmatory testing is performed at the Australian Centre for Disease Preparedness (ACDP) by PCR testing targeting the M and nucleocapsid (N) genes. Unfortunately, in the past this has only been undertaken for those cases testing positive by highly specific real-time PCR. Further testing was not carried out on cases found negative, regardless of diagnostic responsibility attributable to case disease manifestation and epidemiology contexts.[20] Virus isolation is also attempted on retained confirmed HeV infected horse samples. A serological test such as a virus neutralisation test or an sG iELISA test may also be performed.[43] This testing, however, is perceived of limited diagnostic value in acutely affected, rapidly progressing fatal cases yet to mount an antibody response, in the absence of a paired convalescent sample (often unavailable given the high incidence of acute fatality) and when case-subject vaccine status is unclear. That is, when status is not adequately reported to the veterinarian by the horse carer and/or when submitting veterinarians inadequately specify details via submission forms.[9,34]

2.2.4 Virus shedding from infected horses and transmission risks

All cases of HeV infection in humans have arisen from being exposed during close contact with infected horses, either during necropsy of infected horses or from close contact with virus-laden respiratory secretions or blood from infected horses. Transmission may also occur from infected horses to humans via droplet and/or aerosolised partial inhalation, via fomite contact via mucous membrane contact. In one case, human HeV infection is thought to have occurred through contact with an asymptomatic infected horse in the 72 hours before the horse displayed signs.[18] Department of Health and Biosecurity guidelines include 72 hours before overt disease manifestations occur in a horse in the transmission risk period.[63]

A Philippines HNV outbreak involving horses as primary spillover hosts featured transmission pathways to humans via their exposure during butchering and/or ingestion of infected horse meat, as well as transmission from infected horses to multiple other in-contact domestic animals.[64] While direct transmission from flying foxes has not been reported for HeV (or other HNVs) in Australia, humans have served as primary spillover hosts of NiV. Indirect transmission without intermediate domestic animal host was via human exposure and consumption of date palm contaminated with NiV by infected pteropid flying foxes.[54]

There is little specific data on the variable risks of transmission of HeV at different times in the source host infection course.[1,18] However, by the time infected horses show overt disease, the virus has spread systemically and is present in sufficient abundance to support onward transmission in multiple body fluids.[1,7] Significant load of viral genetic material has been detected in nasal/nasopharyngeal secretion samples from unvaccinated HeV infected horses obtained as early as two days after exposure and including all periods when demonstrating acute disease manifestations, potentially signifying excretion of infectious virus. Therefore, there is a risk of onward transmission to other susceptible species prior to infected horses developing clinically detectable disease.

Studies of experimentally infected horses have shown that HeV, and/or its molecular components, may be detected in lung, kidney, brain, lymphoid tissues, brain and spinal cord, meninges, upper respiratory tract, heart, adrenal gland, respiratory secretions, saliva, urine and faeces.[1,65,66] The risk of transmission of the virus from an infected horse is likely to be greater when carers and veterinarians perform longer, and/or relatively more invasive, health-care procedures such as nasal intubation or routine dental work. Post-mortem examination of a horse that died of acute HeV infection is considered to be associated with highest transmission risk.[18,67] Infectious virus may persist on surfaces contaminated by body fluids at necropsy for a variably prolonged period.

Reliable detection of viral genetic material in nasal swabs from infected horses, on which routine, government facilities, priority significant disease diagnostic testing relies, is consistent with viral replication in the upper respiratory tract and infectious HeV shedding into the nasal cavity.[50] Research using infected animals as models suggests that NiV appears

to replicate in high abundance in tracheal epithelium, whereas HeV does less so. It has been suggested that the increased person-to-person transmission may be due to the higher virus load in lower respiratory tract secretions produced by NiV compared with HeV.[21] Nonetheless it is important to note that HeV has been isolated from a nasopharyngeal aspirate (NPA) from a human case, indicating that there is likely to be some human to human transmission risk for HeV similarly as for NiV.[18]

Infected horses may be asymptomatic or only mildly symptomatic, shed virus and recover and PCR testing has identified low levels of viral RNA components in a small number of recovered horses. Importantly, however, shedding of infectious virus from recovered seropositive horses, or recovery of the infectious virus from tissue of any organ including brain, has not been identified in recovered horse subjects.[7,17,68] Similarly, shedding or recovery of the infectious virus has not been reported in human patients who have recovered from HeV infection or those with late-onset or relapsed NiV encephalitis.[17,68–70]

One of the features of HeV and NiV in human patients is late-onset and relapsing encephalitis, which can follow recovery from acute infection.[40,59,71] A Mackay farmer who became infected in August 1994 by assisting in a veterinary post-mortem examination on an infected horse, recovered, subsequently relapsed 13 months afterwards and died with neurological symptoms in October 1995.[17,70] A relapsed case of HeV appeared to be the cause of a horse death in Casino in 2016.[72] Experimental observation and study of such chronic disease cases in horses is not possible because of the high occupational bio-safety risk for operators, the lower incidence of this form of disease among horses, and associated animal-subject welfare considerations and cost-prohibitive laboratory requirements.[72,73]

Chronic HeV disease in horses and humans is understood to result from viral recrudescence, where latent virus is maintained in tissue, possibly in the smooth muscle cells of the vascular system, and associated delayed relapse virus replication and/or pathologic alterations.[74] The smooth muscle cells of the tunica media of blood vessels contain the virus and are sites of virus replication, yet these cells do not show pathology.[75] This is thought to be how infections reappear in bat colonies over time; the smooth muscle cells act as a reservoir for susceptible animals.[76]

2.3 Clinically focused review of the pathologic basis of HeV disease in the horse

2.3.1 The pathogenic basis of viral disease at the cellular and subcellular level

Viral infections cause disease by altering cellular homeostasis and causing cellular injury. The clinical disease manifestations resulting from viral infection reflect effects of the virus on homeostatic biological function of the host at the subcellular, cellular, tissue, organ and systemic levels. As infection courses through various anatomical tissues and body systems the host immune response produces subsequent effects.[77,78]

Disruption of cell homeostasis is caused by: viral induced disruptions in host cell genes, proteins and metabolites that influence cell function and survival; resultant compromised tissue and organ function, and pathological effects of host immune responses to cellular, tissue and organ injury.[44,78] Cellular injury includes several biological mechanisms. adenosine triphosphate is depleted, cell membranes become permeable, biochemical pathways are disrupted, the host cell genome is damaged, prompting disruption of cell homeostasis, and cells undergo degeneration or death by lysis or apoptosis.[78]

Genomic, phenotypic, and functional aspects of an infectious agent all exert pathologic influence on host cell and system function. Additionally, host immune defences operate largely at the subcellular level and are influential determinants of the clinical disease associated with infection.

Viruses have evolved to specifically utilise ‘target cells’ as ‘portals of entry’ in susceptible animals suitable for hosting their replication. This is based on specific ligand-receptor interactions between viral surface proteins (envelope or capsid attachment proteins) and receptors presented on the membrane of host target cells. There are many potential cell membrane surface receptors suitable for viral attachment on any mammalian cell (10^4 to 10^6 per cell) and the pattern in which they are presented in addition to their individual phenotype may be recognised by targeted viral attachment mechanisms mediated by surface proteins.

Cells that allow viral replication are termed permissive cells. They are destroyed by the effect of viral replication, whereas infected non permissive cells will survive, often playing a

role in disseminating virus throughout the body and onward transmission from infected hosts. Outcomes for infected cells in response to viral replication include: death by apoptosis, necroptosis and pyroptosis[79], persistent infection, proliferation and malignant transformation.

All stages of viral infection − cellular entry, viral replication and dissemination − may disrupt cellular homeostasis and trigger signalling pathways that often result in cell death.[79] In some instances and stages of infection, cellular death can partially benefit the viruses by assisting in viral proliferation. However, viruses have also necessarily evolved to develop strategies to evade triggering host immune responses and signalling, typically anti-viral cell-death pathways. This allows for their successful infection and replication.[79]

Nucleocapsid containing viral genome is released into the target cell cytosol, where viral RNA-dependent RNA polymerase transcribes mRNAs from a negative sense RNA genome, beginning at a single promoter located at the 3' end of the viral RNA and terminating downstream at specified regions for each viral gene. This transcription mechanism results in gene product polarity. Those closest to the 3' end are expressed in greater abundance than those downstream, facilitating preferential gene translation in initial stages of infection that evades host immune signalling.[80,81] Once sufficient concentration of nucleocapsid protein has been expressed in the cytoplasm, RNA-dependent RNA polymerase switches from transcribing HNV genes to full viral genome replication. This involves synthesis of full-length positive-strand RNAs, which are then transcribed into the progeny genomic negative-sense RNA strands. The maturing virions finally gain their envelopes, with incorporated membrane-trapped viral glycoproteins, by budding through the host cell outer membrane.

Paramyxoviruses broadly also induce host cellular syncytium formation when surface proteins used in viral cellular entry induce fusion of infected host cell membranes with neighbouring cell plasma membranes.[82] The approach allows for further extensive viral replication within the polykaryonic (merged cell) cytoplasm. It delays exposure to host neutralising antibodies and extrinsic innate immune signalling that trigger cellular lysis, prior to mass dissemination following eventual infected polykaryonic cell death. Infection and subsequent death of dozens of cells is potentiated in this way via infection of a single target cell, theoretically with even a single virion. This mechanism is especially adventitious when

occurring among target cells at portals of viral entry. There it supports establishment of infection and local intercellular spread, evasive of innate host immune defences, prior to systemic dissemination as occurs in respiratory epithelial cells. These cells demonstrate some of the highest rates of syncytia of any cell type in hosts infected with paramyxoviruses utilising this entry-portal route for transmission to susceptible hosts.[83–85]

Disease of all tissues and organs begins with the disruption of homeostatic cellular biochemical conditions and function, resulting in cell injury[78]. Understanding the underlying pathologic basis of HeV disease over its typical chronological course of infection, in terms of the response of cells, tissue and organs to injury, is essential for interpreting varied clinical manifestations of HeV infection and recognising suspect cases.

The relative severity of disease associated with infection is referred to as viral pathogenicity. It is determined largely by the biological activity and interactions that result from the proteins expressed by viral genes. Actions of viral proteins considered as influential virulence factors fall predominantly into two groups: those involved with attachment to, replication in and shedding from target cells, and those that modulate and/or evade host defence mechanisms against infection.

Pathogenicity of viral infection relates directly to the survival and function of infected target cells. These in turn depend on both how the virus utilises and alters the function of cell organelles, genome transcription and protein translation; and how replicated virus escapes from the cell (via dissemination by cell lysis or viral particle budding from cell membrane).

Five stages of viral replication are recognised: cellular attachment, cellular entry, spread, replication and shedding. Three categories of host cells are targeted by viruses. These are cells acting as portals of entry, such as mucosae and epithelial cells; cells that facilitate spread of infectious agent locally, regionally, or systemically, such as lymphocytes, macrophages and dendritic cells; and cells located systemically within other organ systems.[86] Mucosal epithelial cells and mucosa-associated lymphoid tissues are targeted by many viruses, both as suitable portals of entry and as part of mechanisms that facilitate systemic spread respectively.

Experimental studies involving HeV-infected horses have been limited in case subject number and duration of observed disease course due to the practical, biosafety and welfare limitations of ethically maintaining horses in the required BSL-4 high-biocontainment laboratory conditions. Studies are also limited by the resources required to meet the extensive biosafety measures, including that all personnel be extensively trained and wear fully enclosed positive-pressure suits that must be decontaminated by chemical shower on each occasion the animal enclosure is entered.[1] Additionally, to preserve their welfare, research animal ethical standards require all experimentally infected horses to be euthanased at humane endpoints, relatively early in the infection course.

The pathological mechanisms and manifestations of HeV and NiV are generally similar in human and animal infections. Therefore, this review draws on scientific description of both the more numerous NiV infected human cases, and published reports of horses experimentally infected with HeV, experimental animal infections as models of HeV infection and disease, and the limited number of observed human HeV infections.[18,59,74,87–89]

2.3.2 Review of virological (biological) and immuno-pathophysiological features of HeV infection in horses relative to clinical disease course

HeV infection in horses usually results in an acute fulminating course of disease with rapid progression over two to three days and a case mortality rate of approximately 80%. The most frequently observed clinical signs of disease in acute cases have been a rapid onset of illness, with anorexia, tachycardia, pyrexia, depression, and mucous membrane injection/congestion. There is then typically progressive deterioration to moribund condition with either overt respiratory and/or neurologic signs.[6,20,39]

Respiratory disease manifestations have included tachypnoea, hyperpnoea (with deep inspiratory or expiratory effort), dyspnoea and nasal discharge shortly ante-mortem and/or post-mortem. Reduced respiratory sounds due to pulmonary consolidation can feature on auscultation in the later stages of disease. Clinical signs of neurological dysfunction have predominantly been encephalitic signs.

Mucous membrane changes feature hyperaemia initially around the gingival margins of the incisors, progressing to hyperaemic petechial/ecchymotic areas across gingivae. These

coalesce, then become more deeply reddened and eventually cyanosed.[20] (additionally see first-hand clinical observations by Dr P Reid described as part of Chapter 4 of this thesis: *Case series: HeV disease revisited First-hand clinical veterinary findings*)

A minority of infections have been asymptomatic (covert) or only mildly symptomatic. Some horses have developed persistent neurological signs consistent with non-suppurative meningoencephalitis subsequent to surviving acute infection.[6,18,39]

2.3.3 Henipavirus (HNV) structure and function

Henipaviruses (HNVs) are non-segmented enveloped viruses with a 6-gene 18.2K, negative-sense, single-stranded RNA genome; it is the largest of known paramyxoviruses. The genome encodes nine proteins: a nucleoprotein (N), phosphoprotein (P), interferon antagonists W, V and C proteins, a matrix protein (M), transmembrane anchored viral fusion (F) and attachment (receptor binding protein RBP − formerly G) glycoproteins and a large polymerase (L).[90] The virus particles are pleomorphic, with spherical or filamentous forms observed by electron microscopy.

Henipaviruses enter susceptible host cells via fusion of the viral and cell membranes, mediated by the concerted action of the two viral surface glycoproteins.[11] The binding of the RBP to the cell surface ephrin B2 and B3 receptors triggers a conformational change in the HNV F protein, facilitating viral and cell membrane fusion.[91] Highly conserved across invertebrate and vertebrate species, ephrin B2 and B3 are ubiquitously expressed cell-surface protein-tyrosine kinases that enable angiogenesis and axonal guidance.[92] Utility of these receptors for cellular entry allows for the wide host-species and -tissue tropism that enables HNVs to infect and transmit between broadly diverse mammalian species and cause diffuse host tissue pathogenesis. Neutralising host antibodies targeting the HeV and NiV RBPs and disrupting binding of ephrin B2/B3 have been the principal target of antiviral strategies.[11,12,14–16,40]

HNV transcription and replication takes place in the cytoplasm, with many of the proteins (P,V,W, C and M) inhibiting host innate immune responses including proapoptotic signalling.[79,90] HNV M and W proteins enter the cell nucleus, including the nucleolus. They inhibit cellular genome maintenance, and transcription[90] of cellular defence antiviral genes, and other manipulations of the host cell status and response. This supports the construction

of viral particles, and their cell membrane engagement and budding, which is orchestrated by the M protein and promoted by the C protein.[93]

2.3.4 Cellular entry

Henipaviruses infect cells through fusion of the their viral envelope with host cell membranes, mediated by concerted action of their two surface glycoproteins (F and G or RBP).[94] The first point of viral access occurs via the RBP protein, embedded in the viral envelope, attaching to ephrin B2 ligands on the pulmonary epithelial cell membrane. A conformational change of the F protein then enables fusion of viral and host cell membranes, translocating the viral genome to the cytoplasm, where replication occurs.[91,92] Henipavirus host infection specificity is based on these ligand-receptor interactions between the viral surface proteins and Ephrin B2 and/or B3 receptors on the membrane of host target cells.

The HNV surface glycoprotein (G), also known as the receptor-binding protein (RBP), is homotetrameric, featuring an N-terminal transmembrane domain, a stalk domain and a C-terminal head domain. The latter binds host cell ephrin B2 and ephrin B3 host entry receptors[11,95–99] in what has been termed 'a diabolically elegant mechanism'.[100] Ephrin B2 and B3, two members of a large family of receptor tyrosine kinases expressed in cellular membranes across wide ranging mammalian tissues, are phenotypically conserved amongst broad ranging mammalian species. This supports the wide host species and tissue tropism of HNVs.[95,101] Indeed in-vitro experimental comparison of prototypic HeV (HeV-g1) and HeV-g2 RBPs binding to human and *P. alecto* flying fox Ephrin B2 and B3 receptor ligands has demonstrated remarkably high consistency.[11]

In normal cells, interactions between the ephrin receptors and their ligands form part of complex signalling that mediates changes in cellular shape, motility and proliferation.[92,95,102,103] These interactions are involved in immunomodulation, trafficking of immune cells to sites of inflammation, chemotaxis, adhesion and transmigration of cells through the vascular endothelium. They also regulate neurogenesis and bone formation.[89,100,103] The location of these receptors within the host explains the tissue tropism of many viral diseases.[3,104] Ephrin B2 ligand receptors are expressed on endothelial and smooth muscle cells in small arteries, pulmonary alveolar and bronchial epithelial cells,

at sites of angiogenesis, in the brain and on cardiomyocytes.[102,105] They play an essential role in immune regulation and are also expressed on macrophages, monocytes and T-lymphocytes.[102,105] Ephrin B3 is found throughout the brain and brainstem, and on endothelial cells.[105]

HNV F proteins are homotrimeric, surface expressed class I viral fusion glycoproteins.[106] They are processed by host cathepsin L in the cell's endosomal compartment as part of a cellular protein recycling process.[94,107,108]

HNV infection is dependent on host-viral interactions by both RBP(G) and F proteins. Both are targeted by neutralising host humoral immune responses,[94,109–112] with serum antibodies against F and G correlating with protection from both HeV and NiV infection in animals experimentally.[94,113–115]

2.3.5 Henipaviruses (HNVs) and the immune system

Host immune responses to viral infection are broadly categorised into innate and adaptive (or specific). Adaptive immunity mechanisms are antigen-specific. They include cell-mediated T lymphocytic responses to intracellular pathogens and humoral immunity via B lymphocytes targeting extracellular pathogens and toxins.[116] Memory B cell and immunoglobulin-mediated adaptive immune responses require previous exposure of the host to the antigenic target of the specific viral agent or equivalently for closely related viruses and/or antigenic priming by immunisation. Innate immune responses, particularly molecular signal expression by innate lymphoid cells (ILCs), initiate and sustain the adaptive immune responses.[116]

Innate immune responses of the host are non-pathogen-specific first-line defence mechanisms that engage promptly following initial infection. They comprise anatomical barrier systems such as skin or mucosae, host physiologic properties such as pH, mucus layer and body temperature, and inflammatory and phagocytic responses. The epithelial cells in, for example intact and healthy respiratory mucosae, act as initial barriers against viral infection.[116] After a pathogen penetrates the epithelial cell barrier, it enters the highly vascularised extracellular matrix of endothelial cells in the lamina propria or submucosae.[86] Phagocytic cells then play roles in the innate response. Neutrophils, monocytes and tissue

macrophages, innate lymphoid cells and dendritic cells play a part, as well as plasma proteins such as those comprising the complement system.

Henipavirus tropism for Ephrin B2 and B3 that are expressed on the surface of upper and lower airway respiratory epithelial cells and associated endothelial cells, afford affinity with target cells. This allows HNVs to utilise the respiratory system as a predominant portal of entry.

Thus, the respiratory epithelium, serving the main portal of entry for HNV, is also a key first-line barrier for the host innate response. Any compromise of respiratory epithelial integrity may increase the risk of HNV infection establishing in horses that are subsequently exposed. Such compromise may include the muco-ciliary mucous clearance function, such as may occur in prolonged transport conditions (when horses are unable to lower their heads for a prolonged period), concurrent viral infection, inflammatory airway disease and allergic airway disease.

Experimental animal model HNV infection studies have demonstrated that disease morbidity and mortality may also be influenced by the infectious dose of the virus (demonstrated in Hamsters while not in Ferrets) and route of infection (Neurotropic versus pneumotropic and lymphocyte-associated/ systemic spread).[117,118] Age, general health, immunocompetency, and any co-morbidities and/or co-infection in horses are also expected to greatly influence their immune response to HeV infection. Older horses are over-represented among recognised natural spillover cases.[6]

Another key component of innate immune defence of mammalian cells against viral infection is the alpha/beta interferon (IFN-α/β) response.[90] These type 1 interferons are produced in response to detection of virus in the host cell. Activating signal transducers and activators of transcription (STAT) proteins are released by infected and neighbouring cells, which in turn translocate to the nucleus. Here they upregulate the expression of antiviral genes, establishing an antiviral state.[92]

Henipaviruses downregulate host innate antiviral responses by disrupting the interferon-mediated signal pathways and inhibiting the activation of the interferon α/β gene.[84,85,87,119] The P, V, W and C viral proteins variably encoded by the P gene, via the unique

Paramyxovirus trait of 'nucleotide slippage' during this gene's transcription - all contribute to HNV host immune evasion. Production of these proteins has been demonstrated to be a critical potentiator for infection and disease experimentally.[120,121]

Once HNVs infect lower respiratory tract epithelial cells, adjacent resident alveolar macrophages respond to the presence of viral nucleic acids by expressing inflammatory mediators to attract neutrophils, leucocytes and macrophages aiming to inactivate viral particles.[84,92] Cytokines, including interleukin (IL)-2, IL-6, MCP-1 and G-CSF, may then engage, potentiating pulmonary cellular immune response. Furthermore, activated macrophages may generate a cascade of tumour necrosis factor α, interleukin-1 β and interleukin-6 to recruit additional macrophages.

In addition to these innate immunity mechanisms, to which the virus has evolved defences, infected host defence draws on adaptive immunity involving dendritic cells that couple innate immune signals like IFN and activate immune T cell response. Henipaviruses limit this T cell signalling and recruitment in infected endothelial cells; this critically contributes to their capacity for viral replication in these tissues and resultant systemic viral distribution via the host circulatory systems.[123] There is some evidence that T cell-mediated immune response may vary between infected individuals. This is one factor influencing variable host susceptibility to infection and similarly influencing disease severity (morbidity and mortality). Recent immunological studies stress the influence on disease severity and outcome of variable host cell-mediated and humoral immune responses to HNV infection.[124] When hosts mount and maintain antiviral T cell responses, this is understood to be a critical defence against severe disease resulting from infection with many RNA viruses, with variation between individuals considered influential on differences in disease severity. Prasad et al. showed that HNV disease was associated with prolonged signalling of innate immune response pathways, dysregulation of complement and suppression of T cell responses.[125]

2.3.6 Endotheliotropic (circulatory vessel associated) HeV infection mechanisms of disease

Healthy endothelial cells regulate blood flow, inhibit coagulation, control vascular permeability, and respond to inflammatory stimuli by rapidly recruiting leucocytes.[123] Microvascular endothelial cells regulate the extravasation of fluids, solutes, and plasma

proteins. Activation of the inflammatory and antiviral pathways in response to viral infection of endotheliocytes increases blood flow and vascular leakage, shifting these cells into a prothrombotic state.[123] HNV infection also has a direct cytopathic effect on endotheliocytes, causing them to clump together to form syncytial cells. This effect increases immune evasive virus replication (as described in section 3.3.1 above).[123]

The exact mechanism by which viruses cross the epithelial/endothelial barrier into the bloodstream has not been conclusively established. It is likely, however, to include direct leakage via compromised endothelial-blood barrier, transcytosis and traffic of carrier leucocytes, and infected and prokaryotic cell death.[123] HeV is disseminated via the vasculature systemically throughout host anatomy, infecting any cells that express the Ephrin B2 and B3 receptors, including cells of the cardiovascular and respiratory systems.

Multinucleated syncytial endothelial cells (giant cells) are formed by fusion of infected cells with adjacent non-infected cells. This contributes to local viral tissue spread via direct cell to cell transfer of viral particles, prolonged immune evasion and increased systemic and tissue dissemination upon eventual infected polykaryonic cell death.[126] Giant cells also form in other viral diseases, particularly in epithelial cells, smooth muscle cells and macrophages. Not all is known about their function, beyond these discussed features of immune evading viral proliferation and their association with host cell apoptosis. Endothelial cells damaged by viral infection are unable to contain macromolecules and fluids within vascular circulation, thus their dysregulation ultimately leads to local oedema and bleeding. This is a predominant feature of HeV infection in most tissue types and organs.[123]

Syncytia are also observed in neuronal and endothelial tissue of the CNS, albeit in lower frequency. It is unclear whether this difference represents a different neural pathogenesis or study bias associated with the limitations of early infection end points in experimentally infected horses. Brain tissue has been very rarely available for study in natural spillover cases given risks of zoonotic exposure associated with invasive post-mortem sampling. Nevertheless, it is important to note that syncytia have been observed in neuronal tissue from experimentally infected animals sampled at pre-symptomatic and early disease stages.[126]

The combined activation of immunological pathways promoting cell death in defence of viral infection, plus direct effects of infected virus compromising functionality and integrity of blood vessel walls, results in severe vascular leakage, which itself induces a cytokine response. This amplifies the cascade of additional cytokine release, cellular, tissue and systemic inflammation, infiltration of macrophages and lymphocytes, increased coagulability, thrombosis in the small vessels and local necrosis. Widespread infection of the vasculature itself results in blood vessel damage, contributing to extravascular leakage and circulatory compromise. Ultimately DIC can follow, with multi-organ failure.

The disease manifestations that these pathologic mechanisms generate depend on the anatomical location, extent of the cellular injury and functional tissue compromise.

As HNVs illicit diffuse pathology, manifested via endotheliotropic infection, observed clinical signs are also influenced by which body systems and abnormalities appear most readily via clinical physical examination. For example, respiratory and neurological dysfunction and changes to mucous membrane perfusion are far more readily evident on clinical examination than disturbance of the gastrointestinal, renal or hepatic systems, while disease in these systems will also manifest over the course of HeV infection. Respiratory and neurologic entry portals of infection may further increase the relative clinical observation of dysfunction of these body systems.

2.3.7 How pathogenesis explains clinically observed disease in HeV infected horses

Disease resulting from HeV infection across a range of tissues, organs and body systems has given rise to a broad range of clinical signs described in horses. Necrotising lymphadenitis and widespread necrotising pulmonary alveolitis with marked fibrinous alveolar exudates are common. Parenchymal cells containing viral inclusions have been observed in both the lungs and central nervous system. Syncytial vascular and lymphatic endothelial cells are seen histologically within alveolar walls, renal glomeruli and lymphoid tissues.[1,37,89] Tissues demonstrating pathology evident on histology have included the lungs, central nervous system, lymphoid tissues, kidney (glomeruli), female reproductive tract, nasal mucosa, adrenal gland, liver, heart, stomach and intestine.

The most common signs observed in all infected horses suffering acute severe disease have been rapid onset of illness, anorexia, tachycardia, pyrexia, depression, mucous membrane

congestion, and subsequent progressive deterioration with a predominance of respiratory and/or neurological disease signs. These signs have affected 80–90% of cases infected naturally and 100% of those infected experimentally.

Early manifestations are somewhat equivalent to influenza-like symptoms, as feature in the early stages of human henipavirus disease, with incubation periods in horses estimated as ranging from few days to two weeks.[18,28,74,127,128] Illness in all seven recognised cases of human HeV infection has begun with influenza-like symptoms, including fever, headache, myalgia, and drowsiness. Redness, swelling, heat and pain are generated from increased local blood flow and plasma leakage, which causes lymphocyte recruitment; these produce the mediators that activate C-type sensory nerve cells.[123] Thus disease arises both because of direct viral influences on cellular and tissue function as well as secondary host immune responses.

2.3.7.1 Respiratory system (pneumotropic) disease (pathophysiological) manifestations

Respiratory disease signs in HeV infected horses include tachypnoea, hyperpnoea (deep inspiratory or expiratory effort), dyspnoea and nasal discharge. Typically, nasal discharge is initially clear, progressing to a stable white, yellow or blood-stained froth (shortly antemortem or post-mortem). This likely reflects oedema, (leakage of fluid both into the pulmonary interstitium and the alveolar and airway lumen) and resultant functional pulmonary failure, exacerbating proliferative discharge of the characteristic stable foam through the consequent respiratory distress and tachypnoea/dyspnoea. On auscultation of the lung fields, it can be difficult to hear normal lung sounds due to pulmonary consolidation in the later stages of the disease.[129] Swollen, oedematous submandibular, sternal and bronchial lymph nodes are also feature.[1]

In vitro studies show differences in viral replication and induction of the immune response between small airway and bronchial cells, tissues of various species, and minimally between HeV and the two variants of NiV.[85,122] Notably, greater inflammatory response, with higher activation of IFN α and β signalling pathways and higher levels of cytokines (particularly interleukin-6 and interleukin-8), has been observed in lower airway cells infected by HeV compared to NiV. It has been hypothesised that such difference may contribute to the more

overt lower respiratory disease manifestations in horse and human cases of HeV compared with NiV.

2.3.7.2 Neurological (neurotropic/neuropathological) effects

In the central nervous system (CNS) of HeV infected horses, direct cytopathic effects and blood-brain barrier disruption associated with syncytia formation give rise to encephalitic disease manifestations, including motor and sensory neurological deficits.[102] HeV may initially enter the brain facilitated by increased permeability of the blood-brain barrier. Change in permeability is mediated by pro-inflammatory cytokines and/or ante-retrograde neuro-invasion infection along sensory olfactory neurones (as discussed in section 3.2.2 above). NiV has been identified in the olfactory nerve and olfactory bulb of experimentally infected pigs sampled shortly after oro-nasal inoculation.[130] This highlights the plausibility of this relatively direct CNS infiltration via ante-retrograde neurotropic pathways. This is particularly relevant in horses given their anatomy and behaviours, and potentially explains the predominance of neurological signs reported in some naturally exposed cases. There may be multiple foci of necrosis, and necrotising vasculitis with associated focal haemorrhages differing in severity and extent. The virus may also infiltrate the CNS within infected lymphocytes[5,118] constituting a 'trojan horse' mechanism.

The clinical features of neurological dysfunction in horses infected by neurotropic viruses of the order Mononegavirales (including henipaviruses, bornaviruses and lyssaviruses) vary according with the severity and extent of pathological lesions and their location.[77,131,132] Multiple foci of necrosis and necrotising vasculitis with associated focal haemorrhages can manifest with neurotropic HeV infection.[1,7,37,41,85]

Henipaviruses are thought to have a direct cytopathic effect on infected neurons. Non-suppurative meningitis with lymphocytic infiltration is predominantly reported in horses with HeV, and in animal models for which CNS histopathology has been undertaken. As evidence, viral antigen is detected in the meninges, ependyma, choroid plexus, neurons and glial cells of the trigeminal ganglion, thalamus, medulla, and the cerebellum.

Pathology evident in the brain and CNS in animal experimental infections (horses, mice, pigs, hamsters, ferrets and African green monkeys) have variably included: meningeal haemorrhaging and oedema, nonsuppurative meningitis, multifocal encephalitis,

perivascular cuffing, neurons with eosinophilic inclusion bodies, neuronal enlargement and/or degradation (featuring vacuoles) or necrosis (fragmentation with karyolysis), neuronal microglial activation and glial reaction.[1,62,113,118,130,133]

Clinical signs of neurological dysfunction observed amongst HeV infected horses have included: muscle twitching, fasciculations, head tilt, aimless walking, circling or pacing in an apparent dazed state, ataxia, apparent blindness, terminal muscular spasms or convulsions, nystagmus, opisthotonus, maniacal and uncontrollable behaviour with difficulty or inability to stand, and collapse.[7,129] There may also be clinical signs with a neurological basis affecting other body systems. These signs include: absence of gut borborygmi on auscultation, stranguria (may be interpreted as incontinence) and weight shifting. The latter is seen particularly in the early stages of disease; this might also be due to laminitis resulting from endotheliotropic infection and vasculitis within the lamellae and corium plexus – particularly in horses and ponies with underlying chronic subclinical and/or intermittent laminitic disease.[7,129] HeV can also cause relapsing encephalitis months after acute infection in horses that survive.[134]

Figure 3.1 and Table 3.1 illustrate the reported clinical manifestations of HeV with description of their currently understood underlying pathologic processes. Chapter 6 of this thesis analyses all reported clinical signs of HeV cases as syndromes. The aim is to further aid recognition of suspect cases by clinicians and diagnostic case selection as part of biosecurity surveillance systems. The analyses support the understanding conveyed in this review of the consistency of HeV disease understood as manifestation of the expected pathologic processes of viral infection and host responses.

Figure 2.1. Pathologic processes induced by HeV infection over the course of disease and their associated clinically observable signs

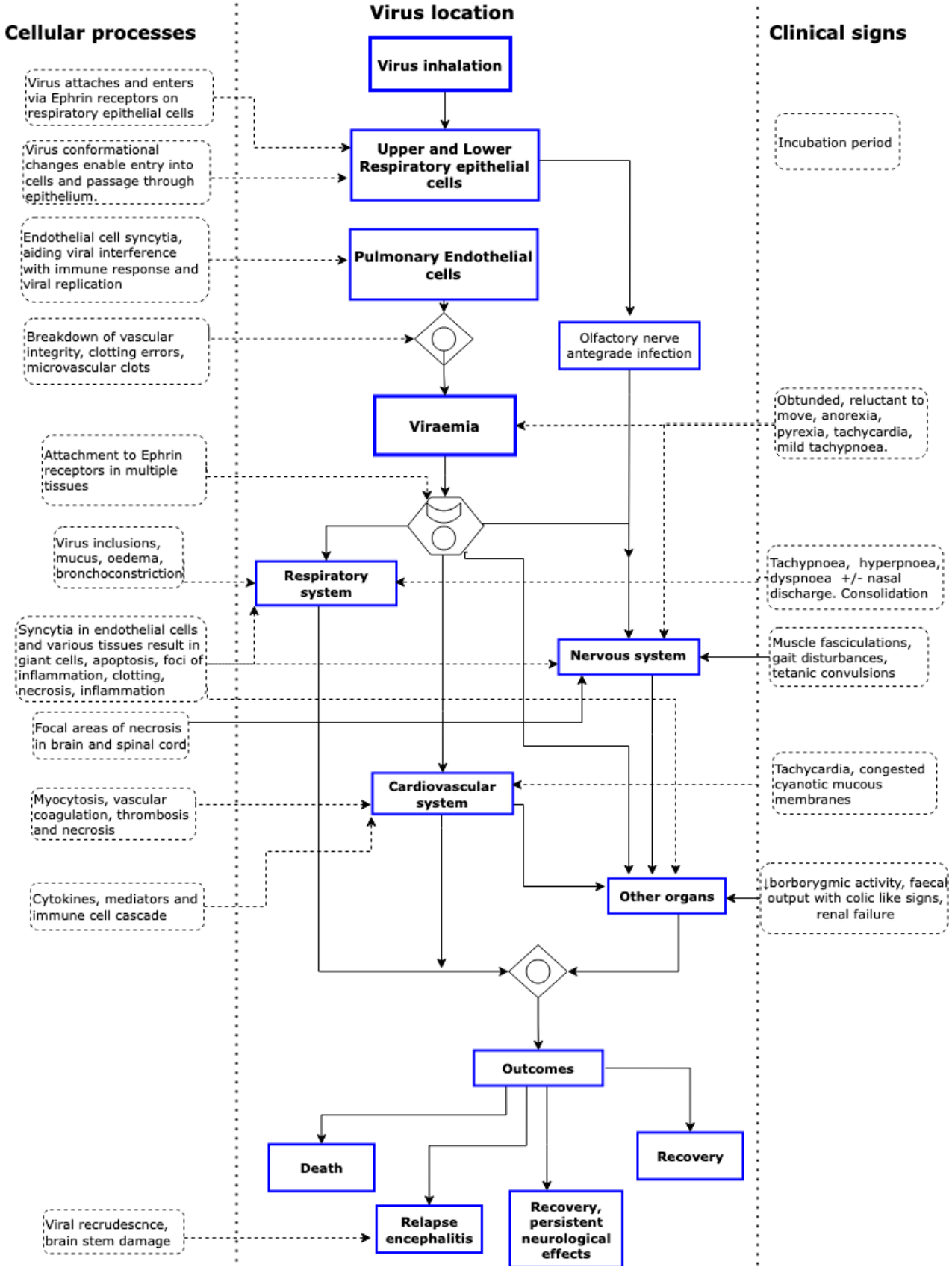

Table 2.1 Clinical signs and their currently understood pathogenesis

Location	Signs	Suspected underlying cause
Generalised acute infection/'influenza-like'	Obtunded demeanor, anorexia, pyrexia, tachycardia, mild tachypnoea, depression, muscular weakness, intermittent pyrexia, some feed intake	Degrees of endothelial, cellular and tissue damage. Immune responses. Systemic effects of vasculitis
Mucous membrane changes	Petechial haemorrhages coalesced to progressing ecchymoses	Abnormal bleeding from defects in vessel walls
	Oral mucosae injected to lightly cyanosed	Restricted blood flow, respiratory alveolar damage
	Palpebral conjunctivae oedematous and hyperaemic	Defects in vessel walls, leaky vessels, increased blood flow
	Mucous membrane hyperaemia (bright red) at the gingival margins	Increased blood flow, inflammation
	Mucous membrane generalised hyperaemia, jaundice	Increased blood flow, inflammation, increased bilirubin, liver function impairment e.g. from internal haemorrhage
	Hyperaemia incisor gingival margins, progressing to hyperaemic petechial/ecchymotic areas across gingivae	Microvascular inflammation, endothelial cell damage and leakage. As the hyperaemic areas coalesce, the membranes become more deeply reddened, with increased capillary refill time
	Mucous membranes cyanosed (dark red/purplish tint), associated with decreased peripheral perfusion and hypoxia	Fluid leakage into alveolar spaces because of the extensive vasculitis associated with the small blood vessel endothelial cells and poor gas exchange
Cardiovascular	Prolonged capillary refill time (2-3 seconds)	Compromised peripheral perfusion
Respiratory/ Cardiovascular	Congested cyanotic mucous membranes	Poor lung aeration, compromised cardiac function, tissue perfusion
Respiratory system	Mildly reduced lung sounds, adventitious bronchiovesicular sounds	Lung consolidation, oedema
	Tachypnoea progressing to hyperpnoea and dyspnoea	Lung consolidation, oedema, myocarditis
	Blood stained, frothy nasal discharge	Excess pulmonary fibrinous oedema, congestion
	Difficulty hearing normal lung sounds	Pulmonary consolidation (oedema)

Table 2.1 (Continued)

Location	Signs	Suspected underlying cause
Neurological	Muscle twitching, fasciculations, head tilt, aimless walking, circling, altered mentation/dazed state, ataxia (often progressive), apparent blindness, muscular spasms or convulsions, nystagmus, opisthotonus, maniacal uncontrollable behaviour, difficulty or inability to stand, collapse, decreased or absent borborygmic activity, stranguria	Vascular perfusion abnormalities in muscles, extracellular and intracellular electrolyte abnormalities Focal areas of histological damage throughout the brain. Severity of signs depends on the extent and location of the damage
	Muscle fasciculations predominantly affecting the quadriceps and triceps muscle groups	Focal areas of histological damage throughout the brain. Vascular perfusion abnormalities in muscles, extracellular and intracellular electrolyte abnormalities Severity of signs depends on the extent and location of the damage
	Limb paddling, tetanic convulsions, intermittent opisthotonus	Focal areas of histological damage throughout the brain. Vascular perfusion abnormalities in muscles, extracellular and intracellular electrolyte abnormalities Severity of signs depends on the extent and location of the damage
	Progressive myoclonic muscle spasms	Focal areas of histological damage throughout the brain. Severity of signs depends on the extent and location of the damage.
	Stranguria	Sensory and motor neurone damage, CNS and spinal cord damage
Gastrointestinal Neurological	Decreased normal borborygmic activity and faecal output	Compromised cellular and tissue perfusion. Damage to autonomic nerves
Gastrointestinal signs	Colic like signs	Pain, bowel tissue infarctions, compromised abdominal vasculature. Pain due to tissue damage caused by thrombosis and infarction in GI tract
Kidney	Renal failure	Giant cells in the glomerulus. Areas of necrosis in renal cortex Compromised cardiovascular function, cellular damage Glomerular and tubular necrosis
General	Tachycardia	Pain, stressful stimuli Damage to autonomic nerves Compromised lung aeration, compromised cellular and tissue perfusion

2.4 Discussion

Appropriate case management of equine disease is underpinned by astute veterinary clinical appraisal of horses in the field. Ideally this combines unbiased and responsive consideration of clinical, pathological, and epidemiological features of disease including microbiological, environmental, husbandry, socio-economic and cultural details. Comprehensive review of the pathogenic basis of infectious diseases relevant to their patient population and geography, holds great benefit for clinically practising veterinarians of all experience and specialties.

Private veterinarians carrying out clinical disease investigations are uniquely positioned to recognise suspected cases of HeV and other emerging infectious diseases in Australian horses.[10] Critically, timely risk assessment of human exposure depends firstly on veterinarians recognising HeV as a differential diagnosis. Appropriate sampling and early state laboratory testing should follow.

For many practising veterinarians, considering the potential for HeV infection in horses based on their clinical presentation is challenged by reported association with extensive broad ranging and non-specific clinical signs. Contextual relation to underlying pathogenesis and expected disease course is often lacking.

Clinical veterinary appreciation of the pathologic basis of disease in the horse follows holistic and contextualised understanding of disease as 'a chronological sequence of events from both the morphologic and mechanistic perspectives with an emphasis on the response of cells, tissue and organs to injury'.[77] Among wide-ranging clinical disease contexts observed in Australian horses, recognition of suspect HeV disease can be greatly assisted by appreciating the established pathological chronology of HeV infection in horses. This is coupled with consideration of case consistency with expected resultant clinical disease manifestations.

This review collates pathologic understanding of HeV disease in horses key to clinical case recognition. The review draws, where relevant, from the broader scientific literature describing human HNV disease pathology and experimental animal models. Beyond pneumotropic and neurotropic pathology, HeV causes endotheliotropic pathology, manifesting as systemic vasculitis and infecting cells of wide-ranging tissue type; thus a

broad range of clinical signs may be seen in horses. These depend on the window in time in which clinical observations are made, the infectious dose of virus and route of infection, individual animal variation in immune and inflammation response, the extent of the examination afforded, and reporting accuracy.

This review highlights that all HeV infected horse cases observed and reported have demonstrated clinical disease consistent with such pathologic understanding, and that the expected range of disease manifestations over time correspond well to the progression of infection, host cell injury and immune responses. Suspect case recognition and clinical decision making, including the important decision to sample and submit for priority laboratory testing, can thus be guided by such consideration of expected clinical disease in the context of disease pathogenesis.

The emergence of HeV (and emerging recognition of HeV-g2) requires veterinarians to increase familiarity and experience with biosecurity issues related to zoonotic diseases. There are many inherent challenges and occupational stresses related to these issues.[10,22] These include personal stress, staff and owner workplace health and safety risk stresses, horse welfare[35] and mortality insurance issues. Further stressors include professional liability, financial and time pressure, loss of income due to possible quarantine restrictions being imposed on veterinary premises, and practical challenges associated with using personal protective equipment in Australia's high-temperature climates.[10,23,24]

Comprehensive guidelines are available for veterinarians investigating suspect cases.[39] Advice to veterinarians by all relevant government agencies and the Australian Veterinary Association is that HeV infection should be considered as a differential diagnosis in any diseased horse when the cause of illness is not readily apparent, particularly when signs progress quickly with rapid deterioration.[129]

For an exposed person to receive timely assessment, and an infected person to receive potentially life-saving treatment quickly, the infected horse to which they have been exposed must be diagnosed through government laboratory testing, with minimal delay to their infective exposure. According to the Australian Government Department of Health guidelines for responding to HeV, a response is required to either a confirmed human or equine case or ‘where heightened suspicion of infection in a horse exists as advised by the

relevant animal health agency'. The response includes a detailed analysis of the nature and magnitude of risk, because post-exposure prophylaxis with monoclonal antibody m102.4 needs to be administered early to highly exposed individuals to be effective.[12]

Therefore, accurate and timely recognition of HeV as a differential diagnosis by consulting clinical veterinarians, in horses that demonstrate consistent disease manifestations, is fundamental to prompting streamlined sampling and laboratory submission. Biosecurity and successful One Health HeV disease management, which may save human and horse lives, depends on these actions. This review highlights that the numerous challenges posed to field veterinarians in responding to sick horses with respiratory or neurological signs may be somewhat alleviated by their proactive interpretation of clinical manifestations. Interpretation should be informed by an understanding of the chronological molecular, cellular, tissue, organ and systemic pathogenesis expected with HeV infection, facilitating timely testing to manage biosecurity and One Health risks.

Veterinarians can be restricted in diagnostic options by several factors. These include geographical distance to government laboratories, their clinical experience and confidence, owner hesitancy relating to potential imposition of quarantine restrictions, owner financial limitations, and concerns associated with management of risk for zoonotic disease exposure. Compliance with workplace health and safety regulations is another factor of heightened perceived weight following the prosecution of three veterinarians despite their subsequent revision supporting interpretation of shared responsibility for veterinarians, animal owners/ carers and infected property stakeholders.[135,136]

The risk of zoonotic disease restricts tissue sampling and necropsies in horses with suspected HeV and/or ABLV infection. Coupled with the logistic and technical challenges in collecting brain/CNS samples, the proportion of horses that undergo detailed histology is limited. Although the high sensitivity of PCR diagnosis specifically for known HeV strains is well-established, false negatives do occur due to genome divergence.[52] Deteriorated sample condition can also contribute to false negative results as the RNA targeted by the assay rapidly degrades. Brain and CNS tissue sampling (ideally via minimally invasive techniques) affording diagnostic testing of tissue, including histology, could reduce missed diagnosis of emerging infectious encephalitic disease. This was highlighted by the 2013 novel detections of Australian bat lyssavirus having caused fatal encephalitic infection in two horses.[137,138]

In any particular diagnostic window, the range of presenting disease manifestations in horses acutely infected with HeV result from disruption of normal function. This is caused by systemic multi-organ vasculitis affecting meninges, brain, mucosa, trachea, lung, diverse lymph nodes, spleen, liver, kidney, heart, reproductive organs, stomach and intestine coupled with progressive cellular immune responses. Disease arising from damage to respiratory and neurological system tissue may predominate because of several factors: specificity of the virus attachment to the local ephrin B2 and B3 receptors; the role of these systems as entry portals of infection; and/or sensitivity of the horse's clinical condition to functional disruption of these body system.

It is of significant benefit to horses, their owners and carers and the veterinarians themselves to have optimised veterinary interpretation of potential HeV infection. Clinical appraisal should be informed by an understanding of the pathologic basis of disease at the subcellular, cellular and tissue level. It also benefits passive-syndromic disease surveillance by informing diagnostic responsibility to guide proactive government diagnostic investigative activities. In the context of HeV-like diseased Australian horses, this is furthermore vital given that the pathological manifestations of many viral infections of emerging One Health significance similarly include features of hypoperfusion, oedema, bleeding and thrombosis because of vascular dysregulation.[123] Achieving animal-case infection diagnosis is critical to identifying emerging agents and management of their One health disease and biosecurity risks.

The consistent clinical disease course resulting from HeV infection in horses may be best understood when informed by its expected chronological pathogenesis. An understanding of the pathologic basis of disease [78] is taught in undergraduate veterinary training. However, increasingly specialised internal medicine and surgery disciplines of therapeutic emphasis predominate in clinical training and postgraduate professional development in modern veterinary practice. HeV disease can be understood to result from dual pathologic mechanisms. A breakdown of vascular function occurs, causing severe vascular compromise, hypoperfusion, oedema, haemorrhage and thrombosis; and the effects of host immune responses mounted against infection. Such immune responses are similar to that for other emerging RNA Mononegavirales such as SARS-CoVs, filoviruses and bornaviruses.[84] Thus optimising understanding of subcellular, cellular and tissue level pathogeneses offers great

benefit for the identification of possible HeV cases by attending clinicians and proactive surveillance for similar emerging infectious diseases.

Improving veterinary appraisal and interpretation of the pathologic basis of clinical equine disease offers significant and appreciable benefits to the patient and their carers, where it may most suitably guide treatment and further diagnostic investigative options. This may help to minimise owner expenditure and improve patient condition, welfare and overall case management and disease outcome. Such proactive veterinary interpretation of the pathologic bases of equine diseases may further support primary stakeholder (including the attending veterinarian and the horse owner, carer, breeder or trainer) engagement with government facilitated surveillance and management of risks to biosecurity and One Health.

Horses infected with HeV demonstrate predictable clinical disease syndromes that are best understood by appreciating the disease pathogenesis. The underlying mechanism of HeV disease is understood to be an overwhelming inflammatory response syndrome with a breakdown of vascular function, causing severe vascular compromise. The dual pathogenic mechanism for causes of tissue injury are vasculopathy-associated microinfarctions coupled with direct parenchymal cell damage.[66]

Recent commentary has suggested a new descriptive term, ‘acute immune dysrhythmia syndrome’ for a group of diseases that cause similar disruption of the immune system. These include COVID-19, Ebola virus, NiV, SARS and MERS.[139] This infectious disease syndrome classification is proposed to improve management of disease through concerted development of immunomodulatory drugs. These drugs may potentially be commonly therapeutic to the group of RNA pathogens, given similarities in their pathogeneses.

Interpreting HeV epidemiology, clinical presentation and disease progression from a disease pathogenesis perspective offers an opportunity to improve suspect case recognition. This allows for appropriate timely testing and One Health biosecurity measures. This review supports the most accurate and predictable clinical description of Hendra virus infection in the horse to be **'Acute severe and often rapidly progressing fatal illness usually featuring pyrexia and mucous membrane injection, congestion and/or petechial haemorrhage, with respiratory and/or neurological signs’**. Understanding the well-established disease pathogenesis and expected chronologic progression strongly justifies this definition.

Vaccination has been advocated as the single most effective way of reducing risk of HeV in horses. A subunit adjuvanted vaccine against HeV, utilising a soluble form of the virus attachment glycoprotein (sG), was developed for horses with high priority given the associated fatal human disease risks.[14] It was approved in November 2012 by the APVMA on a minor use basis, with subsequent full registration (2015) and registration for use in pregnant mares (2016), with more than one hundred and fifty thousand horses immunised by 2019. The vaccine provides public health and work health and safety benefits by reducing the risk of HeV transmission to humans and other susceptible animals.[40] Nevertheless, vaccination uptake in regions of lower perceived spillover risk has been low, and even many horses living in regions of well perceived spillover risk remain unvaccinated.[140,141] Monoclonal antibody m102.4, specific for the same G glycoprotein antigen, has been administered to 16 individuals as emergency post-exposure therapy on compassionate basis and demonstrated safety, tolerability and intended immunogenicity in a phase-one clinical trial.[11,12] Furthermore a human vaccine, based on the same G glycoprotein, is currently being evaluated in phase 1 trails, intended to protect against both HeV and NiV infections.[13]

We expect assessment of potential HeV infection in horses based on their clinical disease manifestations, informed by understanding of the expected range and chronology of expected pathogenesis, will optimise HeV case recognition. This understanding will also aid recognition and interpretation of cases of emerging and novel infectious agent disease and their One Health significance. It will also support proactive interpretations of diagnostic responsibility to guide most appropriate government biosecurity testing pathways for emerging infectious differential diagnoses. It may lessen inherent yet poorly understood barriers to efficacy of surveillance relying on passive disease reporting, such as minimal (sometimes insufficient) clinical and epidemiological description on laboratory submission.

Every year, many horses still present with HeV-like disease without receiving a causative diagnosis. If we can improve the understanding of HeV disease pathogenesis as it relates to the presentation of clinical signs and the progression of disease, we will greatly assist veterinarians attending Australian horses in recognising suspect HeV infection and emerging Infectious diseases.

2.5 References

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Horses as Sentinels of Emerging Infectious Disease

Chapter 3

LITERATURE REVIEW: DIFFERENTIAL DIAGNOSES FOR HENDRA VIRUS LIKE DISEASE IN AUSTRALIAN HORSES AND RELEVANT EPIDEMIOLOGY OF EMERGING INFECTIOUS DISEASES

Statement of personal contribution: I drafted the first complete manuscript (2018) and received feedback from PhD supervisors and wider project team member Dr Peter Reid as well as Equine Medicine expert A/Prof Cristy Secombe and arboviral expert Dr Stacey Lynch. Dr Anne Jackson provided editorial feedback and guidance especially where the original draft was excessive in length (2019) and I undertook revisions of the manuscript to meet the PhD chapter format expectations (2021).

Format: This chapter has been prepared with a view to peer-reviewed journal submission but has not been submitted to a journal as yet.

A concise preliminary version of this chapter was published by the Northern territory (NT) CDC newsletter (June 2017) and republished by the NSW DPI (2018) and circulated similarly to practicing veterinarians.

The NT CDC version is appended to this thesis as Appendix 6: An update on Hendra virus (HeV), HeV-like illnesses and horses as sentinels for emerging infectious disease, *The Northern Territory Disease Control Bulletin Vol 24, No. 2, June 2017.*

3.1 Introduction

Emerging infectious diseases (EIDs) are of critical global concern due to their impacts on animal and human health. Recent examples include Ebola virus, severe acute respiratory syndrome coronavirus (SARS) and pandemic influenza virus, all of which have caused enormous socioeconomic burdens. It has been estimated that 60−75% of EIDs are zoonotic, most having wildlife reservoirs and commonly amplifying or adapting in domestic animal populations before spilling over to humans.[1-3] Estimated overall costs of emerging pandemics range between at-least US$ 3 billion for Ebola virus disease in sub-Saharan Africa[4], US$ 10 billion for SARS[5,6] and up to US$ 7 trillion for a severe influenza pandemic representing a significant economic burden to societies worldwide.[7] At the time of this thesis submission (subsequent to the original drafting of this chapter), global recognition of the impact of emerging infectious zoonotic disease has surged beyond recent history with the experience of COVID-19. The estimated total global financial costs will dwarf such previous figures.

Similarly, the impacts of EIDs are often devastating on animal populations. For example, the outbreak of Nipah virus (NiV) in Malaysia 1998/99 saw approximately one million pigs slaughtered, causing the near collapse of the country's one billion-dollar pig farming industry.[8] In the USA, the 2002 West Nile virus (WNV) outbreak saw over 15,257 horses affected across 43 states.[9] In North Dakota alone, 569 horses were affected with a case fatality rate of 22%, the losses estimated to be at least US $1.9 Million.[6] The 1994 outbreak of Hendra virus (HeV) suspended racing for nine days and is reported to have cost the state betting agency $7 million in lost revenue.[10] A recent economic study showed that mitigation of pandemic threats would be more cost-effective in the long term than relying on outbreak responses and policies.[7]

Our ever-expanding society poses a significant impact on our environment and contributes to shifts in the distribution and interaction of animal species, leading to new health challenges for humans and animals. For example, dramatically reduced coastal forest habitat is considered likely to have influenced the feeding and migration patterns of bat colonies. These changes intensify their interactions with domesticated animals and humans

and increases the potential for disease spillover.[11-13] In this context, proactive identification of novel and emerging pathogens is preferable.

The emergence of HeV disease in Australian horses led to an unprecedented appreciation of the importance of the management of lethal zoonotic disease risk in a modern One Health perspective. This awareness and associated improved disease investigation pathways also influenced the novel detection of Australian bat lyssavirus (ABLV) in two horses in 2013.[14,15] As for all Lyssaviruses, ABLV poses the challenge of often cryptic exposure, variable and potentially very long incubation period and 100% fatality once symptomatic. Yet when exposure is recognised, timely post-exposure prophylaxis is highly reliable if administered prior to the onset of disease. Extensive diagnostic investigations applied in the 2013 horse cases of ABLV, including post-mortem retrieval of brain tissue, and testing beyond what is routinely undertaken targeting alternative viral causes of encephalitis, were prompted by three factors: the author's consideration of HeV and other emerging viral encephalitides as differential diagnoses; rapidly progressive fatal neurological disease in multiple horses with mild pyrexia; and high human handler exposure. Prior to these unprecedented diagnoses and following negative results for HeV, Flavivirus encephalitis had been considered the most likely diagnosis in these cases. It was for this reason that brain tissue was obtained following extensive consultation with state biosecurity duty pathology and diagnostic scientist staff. Unfortunately, such sampling and testing is rarely available. This is partially due to the practical challenges in the horse of obtaining central nervous system samples, the occupational risks given the potential of HeV and other diagnoses, and difficulty knowing and communicating indication of highest diagnostic responsibility to justify pursuing such extensive investigations for infectious cause.

This chapter reviews current knowledge of differential diagnosis for HeV-like disease in Australian horses as a basis for the research presented in this thesis. This research explores the investigation of equine illness as a component of surveillance for known and emerging diseases in Australia.

3.2 Investigations for HeV infection in horses

When HeV is recognised as a differential diagnosis in Australian horses presenting with illness to consulting veterinarians, samples are collected and sent to the respective

government state laboratory for timely testing. Rapid and conclusive determination of HeV infection status is needed to direct clinical and biosecurity case management by the attending veterinarian.

In the 14 years following the identification of HeV (1994−2008) just 84 samples were submitted to the Queensland (Qld) state laboratory for HeV testing.[16] In this same period just 67 suspect disease investigations for HeV infection had been undertaken nationally across all species (Sup Table s3.1). A significant increase in practitioner initiated suspect disease investigations followed the 2008 Redlands outbreak. General awareness was heightened due to the public attention and loss of yet another veterinary surgeon's life, and recognition of wider disease manifestations than had been previously widely understood by veterinary practitioners. In the following six years (2008−2014) 2,467 cases were submitted and tested at the Queensland (Qld) state laboratory alone. [16] This dramatic increase in testing of suspect cases, driven by increased veterinary awareness, is thought to mostly explain the increase in disease detection.[16] Despite the high and increasing number of submissions, less than 1% have been positive for HeV in all years since 2008 other than 2011 in which 2.4% of submissions were positive.[16] The number of equine submissions for HeV testing nationally between July 2017 and July 2018 was 867 with three positive cases being identified (National Animal Health System Database NAHIS). The state with the largest number of submissions in this single-year-period was Qld with 586 submissions (68%), followed by NSW with 247 (28.5%), VIC with 13 (1.6%), WA with nine (1.0%), the NT with six (0.7%) and Tasmania with two (0.2%) (NAHIS). The difference in submissions reflects the degree of perceived risk of spillover based on the location of previous cases. Many more investigations are undertaken in regions from where HeV cases have been previously identified, due to strong regional bias − See Table 1.2. The regional sampling bias has applied to some extent since the discovery of HeV, however this has been most significantly realised since the increases of testing for HeV in Qld and north-eastern NSW since 2009 (Sup Table S3.1).

The recognition of HeV, ABLV and WNV in Australian horses has increased investigation of significant diseases in this species, well above that usually applied to domestic animals more broadly for such infectious diseases of emerging One Health relevance. Of a total of 10,605 HeV disease investigations carried out since 1994 in Australian animals, the vast majority

have been in horses (10,455) followed by 94 dogs, 25 cats, 12 pigs, 6 bats and 5 cattle (Sup Table S3.2). There is great sentinel potential for investigating infectious-like disease for agents of emerging significance in horses.

In the period in which horses have been tested for HeV as part of state government biosecurity significant disease investigations, despite the very low test-positive rate (<1% - see Sup Table S3.3), there has been comparably minimal testing of alternate known significant infectious causes. In comparison to 2,636 investigations for HeV in horses nationally, over the period of HeV tested sample acquisition for this study (2016−2018), just 60 horses were investigated for ABLV and 90 for WNV (NAHIS).

It is of great value to all stakeholders to consider the potential that undiagnosed causes of significant infectious agents are accounting for some of the horses, presenting with signs consistent with HeV infection, that test negative by priority PCR testing. Veterinarians, horse owners, animal, human health care professionals and government animal biosecurity agencies are all stakeholders most directly engaged in this One Health context. More broadly workplace health and safety, insurance, animal- and wildlife-health entities and equine industries are also relevant.

Many veterinarians experience frustration in handling cases of severe equine illness that demonstrate a clinical course and presentation consistent with HeV, test negative for HeV by PCR but for which no alternative diagnosis is reached. The 2011 Queensland Government Ombudsman's investigation highlighted the importance for further testing to be conducted 'where the clinical signs of the horse were suggestive of Hendra virus or where the cause of the horse's illness remained unknown after other investigations'.[17]

In the equine HeV disease syndrome, horses present with acute, rapidly progressive disease usually featuring an obtunded demeanour and 'influenza-like' signs initially (while with less serous nasal discharge). Further signs often feature pyrexia and injected/ congested mucous membrane changes, progressing to severe respiratory and or neurological manifestations and moribund condition.

Differential diagnoses for an Australian horse presenting with similar acute severe illness can be classified into infectious causes, acute abdominal cause (colic), toxicity, neoplasia, iatrogenic causes, and miscellaneous categories (Table 3.1). Many features of the equine

HeV disease syndrome are suggestive of an infectious cause (particularly when concurrent). Examples are pyrexia, mucous membrane changes, acute respiratory illness and/or neurological signs, and in some cases incidence in horses. Equivalent aetiologically underdiagnosed human disease syndromes have been recognised as suitable for enhanced infectious disease surveillance. Similarly, the HeV-like syndrome is suited to investigation for other infectious causes of acute illness in the horse, when a horse is negative to HeV. Toward this aim, it is timely to review plausible differential diagnoses for acute severe illness featuring respiratory and/or neurological signs in the Australian horse in addition to HeV disease.

Table 3.1 Differential diagnoses that could be considered in an Australian horse presenting with acute severe illness categorised into infectious causes, acute abdominal cause (colic), toxicity, neoplasia, iatrogenic causes and miscellaneous

Predominant causal category		Differential diagnosis
Infectious	**Bacterial**	Bacterial meningitis/abscessation, bacterial pneumonia; bacterial systemic toxaemia; anthrax
	Viral	Viral infection (encephalitis/meningitis, vasculitis, severe respiratory)##
	Mycotic	Mycotic infection - particularly *Cryptococcus* spp. (pneumonia/encephalitis)
	Protozoal	Equine protozoal myeloencephalitis*/amoebic encephalitis*, Trypanosomiasis* #
Acute abdomen (colic)		Colic due to acute abdominal conditions (examples include strangulating intestinal or infarctive lesions)
Toxicity	Plant toxicities	Avocado, pyrrolizidine alkaloids (in the NT *Crotolaria* spp. especially *C. crispate*), annual ryegrass toxicity, cardiac glycosides e.g. Indigofera
	Snake envenomation	Brown, tiger, taipan
	Tick paralysis	*Ixodes holocyclus*
	Poisons	1080, paraquat, monensin, lead
	other	Tetanus, botulism, metaldehyde, ergot alkaloidosis
Trauma		Traumatic encephalopathy: severe pain due to trauma such as associated with lacerations and/or bone fracture
Iatrogenic		Hypersensitivity reactions and immune mediated disease of unknown cause

* Not known to occur in Australia. # Surra (*Trypanosoma evansi*) is not found in Australia but is endemic in our close neighbours. Native trypanosome species are of unknown presence or clinical significance in horses.

See table 3.2 detailing viruses that might be considered differential diagnoses for HeV-like illness in Australian horses.

Table 3.2 Viruses present in Australia as potential viral causes of HeV-like illness in Australian Horses

Virus Species	Genus/Family	Reservoir (recognized)	Arthropod vector	Confirmed infection in:		Confirmed antibodies in:		Horse references
				Humans	Horses	Humans	Horses	
Australian bat lyssavirus (ABLV)	*Lyssavirus/Rhabdoviridae*	All bats	None	Yes	Yes			Annand et al. 2014[14]
Cedar henipavirus (CedV)	*Henipavirus/ Paramyxoviridae*	Flying foxes	None			No	No	-
Menangle pararubulavirus (MenPV)	*Pararubulavirus/ Paramyxoviridae*	Flying foxes	None	Yes	Yes			Annand et al. 2018[18] & 2019[19]
Yeppoon pararubulavirus (YepPV)						No	Yes	Annand et al. 2018[18] & 2019[19]
Grove pararubulavirus (GroPV)						No	Yes	Annand et al. 2018[18]& 2019[19]
Hervey pararubulavirus (HerPV)						No	No	-
Teviot pararubulavirus (TevPV)						No	No	-
Elsey virus (ELSV)/ *Peruvian Horse Sickness virus* (PHSV)	*Orbivirus/Reoviridae*	Flying foxes and macropods	Mosquitoes, *Culicoides*	No	Yes			Attoui et al. 2009[20] Agnithori et al. 2016[21]
Murray valley encephalitis virus (MVEV)	*Flavivirus/Flaviviridae*	Birds / small mammals	Mosquitoes	Yes	Yes			Gordan et al. 2012[22]
Alfuy virus (ALFV) (close genomically to MVEV)				No	Yes			Prow et al. 2013[20]
West Nile virus (WNV) *formerly Kunjin virus or strain*				Yes	Yes			Prow et al. 2013[20]
Kokobera virus (KOKV)								
Japanese Encephalitis virus (JEV)								Lian et al. 2002[24]
Ross River virus (RRV)	*Alphavirus/ Togaviridae*	Macropods birds, many other species	Mosquitoes	Yes	Yes			Azuolas et al. 2003[25]
Barmah Forest virus (BFV)			Mosquitoes	Yes	Yes			Gummow et al. 2018[26]
Trubanaman virus (TRUV)	*Orthobunyavirus/ Bunyaviridae*	Macropods birds, many other species	Mosquitoes, *Culicoides and ticks*	Yes	No			Gauci et al. 2016[27]
Gan Gan virus (GGV)				Yes	No			
Equine Herpes virus -1 (EHV – 1)	*Varicellovirus/ Alphaherpesvirus*	Horse	None	No	Yes			Ma et al. 2013[28]
Equine arteritis virus (EAV)	*Arterivirus/Arteriviridae*	Horse	Mechanical only	No	Yes			Huntington et al. 1990[29]
Equine infectious anaemia virus (EIAV)	*Lentivirus*, *Retrovirus*	Horse/wild horses	Mosquitoes	No	Yes			Lepherd 1981[30]
Borna disease virus (BoDV)#	*Bornavirus/Bornaviriadae*	Shrews and squirrels + divergent lineages in birds	Unknown	Yes	Yes	Yes	Yes	Caplazi et al. 1998[31] Flower et al. 2008[32] Kamhieh et al.[33]

Antibodies only detected in Australia – see Table 3.3 and section 3.5.3.2 for further description

3.3 Other viruses as potential causes of acute, severe respiratory and/or neurological illness in Australian horses

Table 3.2 lists viruses known to be present in Australia that might appropriately be considered differential causative agents in Australian horses suffering acute, severe respiratory and/or neurological signs, yet returning negative priority molecular HeV testing. Especially in cases with features further suggestive of infectious cause – pyrexia, mucous membrane changes or outbreak or clustering case incidence. Many of these viruses may also transmit directly or indirectly (via fomite or arthropod vector) to humans, posing One Health considerations, as does HeV.

3.3.1 Australian viruses that may spill over to domestic species from bat reservoir hosts

Bats, (order Chiroptera) serve as highly suitable reservoir hosts for RNA viruses. Their communal roosting behaviours and high metabolism (being flying mammals) have allowed them to co-evolve with a wide range of RNA viruses. As such many of the most significant emerging infectious zoonotic viruses are considered to have their origin in bats. These include coronaviruses, lyssaviruses, filoviruses and henipaviruses. Mostly, while these viruses cause severe disease when they spill over to terrestrial mammals, they cause minimal or far less evident disease in their bat reservoir hosts.

Australian bat lyssavirus (ABLV), detected in two Australian horses following testing for HeV, is considered clinically indistinguishable from rabies virus in the horse.[14] The incubation period for lyssaviruses may be highly variable. It ranges from days to years, thought to be affected by the virus strain, host species, inoculum dose and proximity of the inoculation site to the central nervous system (CNS). Early signs can be non-specific, subtle, and inconsistent, ranging from lameness, colic, behaviour change, mild pyrexia, hyperaesthesia, ataxia, anorexia, frequent whinnying, apparent thirst but inability to swallow, and paralysis or paresis to sudden death. Overt neurological signs may not appear until late in the disease course but typically will be rapidly progressing and reflect diffuse CNS dysfunction.[14] Since its spillover to horses was confirmed in 2013, Australian state laboratories have been performing ABLV testing on some equine cases when the horse presents with neurological disease features, or at the specific request of submitting veterinarians, at the time of HeV

testing. Between June 2016 and June 2018, 62 suspect HeV-disease equine-case submissions were also tested for ABLV without detection (NAHIS). Marked regional bias to Queensland was again evident in these disease investigations. Of the 62 cases, 55 were tested in Queensland, but just seven in other states (two in New South Wales, two in Victoria, one in South Australia, one in Western Australia and one in Tasmania). This bias most likely reflects increased practitioner and laboratory scientist/duty pathologist familiarity with the differential diagnosis, following the only recognised cases having occurred in Queensland in 2013 (NAHIS).[14] Importantly this geographic sampling bias is at odds with the scientific consideration of risk, as pertaining similarly across the country, wherever horses live with Australian flying foxes and any of the approximately 64 species of insectivorous bats.

ABLV spillover infection in horses is expected to occur rarely, sporadically, across broad geography, making case detection particularly challenging. Further barriers to detection in horses are challenges associated with submitting central nervous system tissue, due to practicality (animal size), and occupational risks in obtaining samples for such a deadly zoonosis given HeV is often yet to have been ruled out by test results at the time of sampling. This research engaged in the development of minimally invasive sampling techniques (supplementary scientific abstract) to improve the potential for ABLV infection investigations. CNS tissue was utilised when available, as well as PCR being performed on oral swabs. This sample type has proven diagnostic potential, where both 2013 cases were found positive by testing oral swabs. Consulting veterinarians are reminded to consider ABLV as a differential diagnosis in cases of progressive neurological disease in horses and other domestic species with conceivable bat contact. [14]

Menangle virus (MenPV), a Paramyxovirus in the genus *Pararubulavirus*, was initially identified as the cause of an outbreak of severe reproductive failure in pigs and severe febrile illness with a macular rash in two in-contact humans in 1997 near Sydney.[34,35] Transplacental infection was evident in pigs, resulting in mummified foetuses, stillborn piglets and malformations.[36] The origin of the virus has been found to be flying foxes and there is evidence that Australian flying foxes harbour this virus accross a wide distribution, with 46% of black flying foxes (*P. alecto*) demonstrating neutralising antibodies (VNT titers >

or = 8).[37] MenPV is very similar to *Tioman pararubulavirus* (TioPV) – identified in Malaysia also in flying foxes as part of research into the source of NiV.[38]

Recent research performing viral culture in mammalian cells from Australian flying fox urine samples (collected via plastic sheets laid out underneath roosting colonies) has detected HeV and MenPV, and an additional member of the Henipavirus family, Cedar virus (CedPV), as well as multiple novel Pararubulaviruses – Teviot (TevPV), Hervey (HerPV), Yeppoon YepPV and Grove (GroPV) − all closely related to MenPV and TioPV pararubulaviruses (Table 3.2).[39,40] All these recently identified viruses are of unknown clinical significance but could potentially cause similar disease.[41-44] Similarly obtained Australian bat urine samples, analysed by molecular methods, have demonstrated evidence for as many as 30 novel paramyxoviruses.[45]

The only Henipavirus isolated from Australian samples, beyond HeV, CedPV has not been shown to cause clinical signs in experimental animal infections including ferrets (the animal model utilised for HeV and NiV pathogenesis studies) and guinea pigs.[39] The major genetic difference between CedPV and prototypic Henipaviruses, HeV or NiV, is the coding of the P gene, which is known to play an important role in evading the host innate immune system. Unlike almost all known paramyxoviruses, the CedPV P gene lacks both RNA editing and coding capacity for the highly conserved V protein.[39] As a result, the virus demonstrates a comparably compromised ability to counteract the host IFN/STAT response.[46] Molecular evidence of more Henipavirus variants in Australian flying fox urine highlight the possibility that potentially pathogenic novel Henipaviruses circulate amongst Australian flying foxes.[39,46]

Paramyxoviruses shed from Australian flying foxes could follow the same spillover pathway as HeV, and cause HeV-like illnesses in Australian horses. This research presents multiple findings that confirm this potential, including the recognition of the HeV variant (HeV-g2 (Chapter 8), additional molecular detections (Chapter 7) and extensive serology findings (Chapter 7). Seroconversion of IgG (one horse) and IgM targeting (3 horses) recently identified Australian Pararubulaviruses was demonstrated in association with HeV-like acute severe respiratory horse disease, featuring pyrexia disease, in a small outbreak of in NSW (Chapter 7 and published as scientific abstracts Annand et al. 2018[18] & 2019[19]).

3.3.2 Arboviruses present in Australia

Australia is host to a diverse biting arthropod fauna, many of which are established as vectors of significant viral disease agents. Many mosquito species, midges (*Culicoides* spp.) and hard ticks (*Ixodes* spp.), as well as larger biting flies are capable of transmitting viruses to domestic species. They are abundant both broadly (for many species) and in geographic niches across wide climatic and ecological Australian landscape. Recently, Japanese encephalitis virus was detected causing disease in pigs, horses, and humans. It was found across unprecedented geography and numerous detections have been made of hitherto unrecognised potential disease agents in the Bunyavirus and Orbivirus families via next generation sequencing molecular research testing. These findings have highlighted the likelihood that more arboviruses of One health significance will be recognised in Australia in years to come.

Horses are relatively 'thin-skinned' with light coat cover, live near water bodies and courses, and in close association with human settlement. Furthermore, horses readily demonstrate manifestations of arboviral disease, are often closely monitored by their carers with veterinary consultation sought promptly and readily (even for mild conditions). These features of horses, make them highly suitable sentinel species for arboviral disease, supporting early detection of novel agents of significance, prior to infection and disease being otherwise recognised in humans and other domestic species. Indeed, horses have served as highly suitable sentinels for West Nile virus (WNV) and other emerging arboviruses internationally (South Africa and USA).

Arboviruses within the viral families *Flaviviridae*, *Togaviridae*, *Reoviridae*, *Rhabdoviridae* and *Bunyaviridae* pose significant burden on human and animal health and are among the major emerging and re-emerging zoonotic pathogens of the last decade.[47] Viruses that utilise arthropod vector transmission pathways exhibit considerable host plasticity, resulting in zoonotic transmission from wild and domestic animals to humans without direct contact.[48]

More than 75 arboviruses have been identified in Australia, some of which are associated with human and equine disease. Arbovirus infection has been considered a likely differential diagnosis for in as many as 60% of people suffering 'undifferentiated febrile illness' in Australia.[49] Infection with some Australian arboviruses can cause acute illness in horses,

with many similar clinical manifestations as HeV infection − particularly pyrexia, oedema, signs of systemic inflammation, and progressive neurological signs.[22,50,51]

Northern Australia represents a hotspot for arbovirus activity including incursions via regular cyclonic weather, which can carry insects over large distances.[52] Arbovirus surveillance has been carried out since 1980 following the isolation of blue tongue virus (BTV) in 1975. Surveillance was optimised through the National Arbovirus Monitoring Program (since 1993) using approaches that include insect trapping and maintenance of sentinel cattle for virus isolation and serology.[53] Viruses from *Togaviridae* (genus *Alphavirus*), *Flaviviridae*, *Bunyaviridae*, *Rhabdoviridae* and *Reoviridae* (genus *Orbivirus*) families are regularly isolated, and a large collection of unidentified isolates have accumulated despite extensive diagnostic testing.[54] Horses have demonstrated serologically to be a reservoir host of recently identified and prolific Australian Stretch Lagoon orbivirus (SLOV), which shares species classification with Umatilla virus (UMAV) from the USA.[54,55] Another newly identified Australian orbivirus Middle point orbivirus (MPOV), is closely related to Yunnan orbivirus (YUOV), isolated in China and its Peruvian strain Rioja virus (RIOV) and associated with neurological signs in donkeys, cattle, sheep and dogs.[20,56] The spread and transmission of orbiviruses (as for bunyaviruses) involves both mosquitoes and *Culicoides* spp*.* vectors, so the potential geographical coverage is greater than for Australian alphaviruses and flaviviruses which are thought to predominantly utilise mosquito spp.. ELSV/PHSV (described below under 3.3.2.3 Reoviruses) and other Orbiviruses should be considered as differential diagnoses in horses presenting with infectious-like neurological disease, including many HeV-suspect cases, especially in the Northern Territory, Northern Western Australia and Queensland where *Culicoides* are abundant.

Recent research utilising metagenomic approaches has investigated Australian mosquitoes and other arthropods for virus sequences, focusing on those that cause cytopathic effects in inoculated mammalian cell cultures. The research has revealed strains of Liao ning virus (LNV) (Reovirus, Seadornavirus previously detected only in China), SLOV, two novel dimarhabdoviruses (a supergroup within Rhadovirodae, Beaumont (BEAUV) and North Creek viruses are similar to viruses detected in European mosquitoes),[57] and two novel orthobunyaviruses (MURBV and SASHV).[58,59]

3.3.2.1 Flaviviruses

Viruses within the family *Flaviviridae,* genus *Flavivirus* cause significant disease in several mammalian species, with occasional human and equine mortalities reported in Australia.[22,51,60] Several viral species have been described in Australia and the most significant zoonotic arboviruses are WNV and Murray valley encephalitis virus (MVEV), within the Japanese encephalitis serogroup.

Horses are considered dead-end hosts for WNV group flaviviruses. These flaviviruses pose significant human health burden in the same way (causing similar neurological disease in humans via mosquito transmission). The incidence of human epidemics correlates well with those recognised in horses relating to bird reservoirs and biting arthropod vector migration/abundance in suitable climatic conditions. Horses have thus already been utilised and proven optimal as sentinels for human infection in multiple countries.[64,65] Between July 2017 to July 2018 there were 42 investigations of horses for WNV by government biosecurity in Australia, with no positives detected (NHAIS). Other closely related Australian flaviviruses considered part of the KOKV complex include Stratford (STRV) and New Mapoon (NMV) viruses; these are similarly likely to infect horses.[62] Edge Hill virus (EHV), a member of the yellow fever group and identified in Australia, is considered a significant public health threat, causing mild disease in humans. Association with equine disease for these additional flaviviruses, however, remains unknown.[63]

West Nile virus (WNV) has a worldwide geographical distribution with infection in humans, horses and birds ranging from sub-clinical manifestations to fatal disease including encephalitis.[22,23,60] The Kunjin strain of WNV (WNV_{KUN}) is enzootic to northern Australia, but the incidence of clinical disease in all species in this region is low, with most infections being asymptomatic.[23,66] Murray Valley encephalitis virus (MVEV) is geographically restricted to Australia, Indonesia and Papua New Guinea, and like WNV is considered enzootic in northern Australia.[60] Infection with MVEV commonly produces a subclinical infection in humans, however, large outbreaks of encephalitis resulting in deaths have been reported in 1951, 1954 and 1974.[60] Alfuy virus (ALFV) is considered to be a strain of to MVEV (https://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?id=44017), that as yet has not been associated with human disease.[67] WNV and MVEV are thought to be maintained in

a transmission cycle involving water birds, with humans and horses considered dead-end hosts.[68] Rare epizootic disease activity is detected on occasions in southern Australia, often following a significant weather event and increased avian reservoir and vector abundance.[23]

In 2011, following higher than usual rainfall, an outbreak of approximately 900 cases of equine encephalitis occurred in south-eastern Australia involving 231 properties. [23] Clinical equine disease, including fatalities, was reported for both MVEV and WNV_{KUN} infections.[69] Initially the high incidence of clinical disease was attributed to a newly emerged WNV_{KUN} strain.[51] However, similar virulence was exhibited in a mouse virulence assay between the strain from the 2011 outbreak and 13 WNV_{KUN} isolates collected from different regions of Australia during 1960 – 2012.[70] Further investigations found that the 2011 strain replicated to higher levels in *Culex annulirostris* mosquitoes in comparison with another strain of the virus assayed,[66] suggesting that along with relative geographic immunonaïvity amongst humans and horses (following the dry conditions of preceding years), the increased efficiency of viral transmission contributed to the large outbreak.

In 2022, Japanese encephalitis virus (JEV), notably closely related to WNV and MVEV, was recognised to have caused disease in domestic pigs (predominantly reproductive loss) and humans (sometimes fatal encephalitis) across a broad Australian geography.[177] JEV had previously been detected only intermittently in far-north Australian regions, but as of June 1st 2022, 40 human cases have been found, comprising 13 in each of NSW and Victoria, nine in South Australia and five in Qld (https://www.health.gov.au/health-alerts/japanese-encephalitis-virus-jev/about). Infection and disease were confirmed in more than 70 piggeries across Victoria, NSW, South Australia, and Qld. At the time of this thesis submission at least 30 probable cases of equine JEV infection have been recognised in NSW (https://www.dpi.nsw.gov.au/__data/assets/pdf_file/0006/1405176/CVO-Bulletin-for-Industry-Probable-cases-of-Japanese-Encephalitis-in-NSW-horses-23-May-2022.pdf). Clinical disease manifestations of JEV in horses are consistent with those attributed to infection with other flaviviruses (WNV and MVEV). Most cases are asymptomatic, or demonstrate mild disease only, recognised by serology as having had infection. However, a consistent minority of cases suffer severe disease that can feature fever, jaundice, lethargy, anorexia, and encephalitic manifestations (hyperaesthesia, ataxia, difficulty swallowing, blindness). This can be fatal or justify euthanasia due to moribund condition.

Viral reservoir and transmission cycles remain insufficiently understood currently for both WNV and JEV in Australia. It seems most likely, however, that water birds play an important reservoir host role. It is also likely that the endemic multispecies transmission persists in the north of Australia with variable southern spread in association with favourable seasonal climatic conditions. Weather patterns such as La Niña likely promote disease epidemics in regions that are usually relatively free – especially following years of dry/drought conditions that may allow susceptible host species immunity to wane (including cross-reactive immunity due to exposure to related agents).

Several other flaviviruses have been detected in Australia in humans and horses, including members from the Kokobera group: Kokobera (KOKV), New Mapoon and Stratford viruses, and yellow fever group viruses (Edge Hill and Fitzroy Creek viruses).[23,71-73] While their contributions to equine disease remain unknown, and some (KOKV) have been shown to cause no disease in laboratory infected mice, their public or wider animal health impact has not been well defined other than the association with occasional human disease including fever, rash and/or polyarthritis.[23, 71,72] Recent positive serology has been reported for KOKV and Stratford virus in South Eastern Queensland[23] and Fitzroy Creek virus (12.6% of 87 sentinel horses) in Northern Territory.[74] They are now understood to be endemic in horses in northern Australia, likely spreading intermittently, with favourable seasonal climatic conditions promoting southern spread of mosquito vectors and avian reservoir species, to horse populations of relative immuno-naivety in southern regions.[23,69]

Laboratory diagnosis of equine infection with JEV-group Flavivirus is challenged by transient and low viraemia (in comparison to other viral agents), meaning that molecular diagnosis of mammalian infection is mostly limited to testing of CNS samples (tissue and/or CSF fluid) in highly suspect cases. As a result, serology (IgG and IgM) often relies on paired (acute and convalescent) serum samples. The high degree of cross-reactivity of antibodies deployed in response to a target virus, to other related viruses in the group further complicates clear interpretation. Assays capable of greater specification often require higher laboratory biocontainment. Nevertheless horses, while serving as dead-end hosts for these viruses (posing no significant onward transmission risk), have been shown to be highly effective sentinel species in facilitating early detection of WNV in South Africa and the USA prior to subsequent human infections. [64,65,75] Indeed a human case of $WNV_{(kun)}$ and seven MVEV

were recognised in the second quarter of 2011 in Australia (http://www.health.gov.au/internet/main/publishing.nsf/Content/cda-cdi3504i.htm) following recognition of these agents circulating and causing disease in horses.[69]

An inactivated vaccine (Equivac® WNV) containing West Nile virus, strain VM-2, was registered and approved for use in Australia in 2016 (https://portal.apvma.gov.au/pubcris) for the active immunisation of horses six months of age or older against WNV disease. However, it has not been used to date.[76] Consideration for use of this vaccine in protecting horses against different Australian flaviviruses may support strategic use in identified high risk regions, climatic conditions, and husbandry scenarios. It could also be used for industry and at-risk horse populations such as foals, horses with equine metabolic syndrome (EMS) and in older horses residing in higher risk habitats (in proximity to wetlands and paddocks with heavy tree coverage) and geography.

3.3.2.2 Alphaviruses

Mosquito borne alphaviruses (family *Togaviridae*, genus *Alphavirus*) represent a globally distributed group of pathogens that cause rheumatic disease (including Chikungunya virus, and Ross River and Barmah Forest viruses) and encephalitis (EEV, WEE, VEE). Ross River (RRV) and Barmah Forest viruses are both indigenous to Australia and considered zoonotic pathogens, with RRV disease reported in both horses and humans.[77,78] Clinical disease manifestations in horses infected with RRV and related alphaviruses have included pyrexia, exercise intolerance, synovial effusion, laminitis, multi-limb lameness, muscle stiffness, increased respiratory rate, ventral and or distal limb oedema, behavioural changes, reduced appetite, colic, lethargy and neurological signs of ataxia or incoordination.[25,50,77,78] Equine RRV disease contributed, alongside MVEV and KUNV, to the large equine arbovirus outbreak in 2011.[69] A wide range of mammals (including horses), marsupials and birds have been considered likely reservoirs of RRV in Australia.[79]

Several other alphaviruses have been detected in Australia, for which animal and human health significance has been poorly defined or considered minimal due to asymptomatic infections predominating. These include Whataroa virus (WHAV), Bebaru virus (BEBV) and Sindbis virus (SINV).[26]

There is the potential for significant cross reactivity between these alphaviruses in diagnostic assays. Seroprevalence studies conducted in Queensland in the 1960s demonstrated widespread antibodies to group A arboviruses reacting with higher titres to RRV than to GETV, Bebaru virus (BEBV) or Sindbis virus (SINV) in man, horses, cattle, kangaroos and wallabies.[55,80] More recent equine serology research that sampled horses from 134 north Queensland properties identified horses with seropositivity to BFV on 22 and to WHAV on 2 properties.[26] Further study of these viruses and their potential significance to equine and One Health disease is warranted.

The closely phylogenetically related Getah virus (GETV) causes acute and sometimes severe disease in horses internationally.[25,26,50,77-79] First identified in Malaysia in 1955, GETV has been responsible for epidemics of mild to severe disease in horses in India and Japan. Signs have included depression, anorexia, pyrexia, serous nasal discharge, urticaria, submandibular lymphadenopathy, lymphocytopaenia and distal limb oedema.[77,81-83] Outbreaks in horses and pigs have resulted in significant economic losses.[82] Seropositive horses have been identified in South Korea, Malaysia, Hong Kong, Borneo, Cambodia, China, Indonesia, Japan, Mongolia, the Philippines, Russia, Thailand, Sri Lanka and Vietnam (Calisher and Walton 1996; Fukunaga et al. 2000; Timoney 2004).[77,81-83] In the Queensland serology studies mentioned above, positivity to GETV and BEBV were considered likely due to cross-reacting antibody to RRV in most cases; however, serum from one Brisbane based child neutralised GETV and showed no evidence of antibody RRV.[55,80] While yet to be conclusively identified in Australia, the consistency with which GETV has been recognised as causing clinical disease in horses highlights the potential for related viruses to contribute to disease in Australian horses (likely currently under-recognised). Its circulation in regional international geography, along with numerous viable local mosquito vectors, suggests there may be value in screening suitable equine disease cases for this virus.

3.3.2.3 Reoviruses

Viral species within the family *Reoviridae*, genus *Orbivirus* represent a third group of arboviruses recognised as having caused equine disease in Australia. Elsey virus (ELSV), considered an Australian isolate of the species Peruvian horse sickness virus (PHSV) (>99% homology), was originally isolated from two horses that demonstrated severe neurological

signs in the Northern Territory.[20,84] A third case of ELSV was identified following testing, which ruled out HeV and WNV by viral isolation performed on samples from a horse in QLD that had shown neurological disease signs.[21] internationally PHSV causes clinical disease featuring encephalitic manifestations similar to that recognised for EEV.[20] The genus *Orbivirus* (family *Reoviridae*) consists of 22 known species that are genetically distinct based on vector species. Viruses in this genus are transmitted to mammals via *Culicoides* spp.. Diseases in livestock include bluetongue virus (BTV) in cattle and sheep are considered of great economic impact, along with epizootic haemorrhagic disease virus (EHDV) in wild ungulates or cattle and in equines, African horse sickness virus (AHSV), equine encephalosis virus (EEV) and PHSV.[85]

At least seven serogroups of the known orbiviruses are endemic to Australia, causing disease ranging from minimal to severe, in a wide range of host species.[86] A retrospective serological survey of stored equine sera available from 411 Northern Territory based horses demonstrated 10% seropositivity to ELSV (1:5 dilution neutralisation assay). Antibodies were detected in sera from horses in the Katherine region collected as early as 1981.[84] Seropositive horses were subsequently detected in 12 locations within the Northern Territory's recognised arbovirus endemic area, with seroconversion demonstrated in three horses that had displayed mild colic and behavioural changes.[84] Further prospective testing of horses demonstrated 13/107 positive for ELSV in 2001, and 14/71 in 2002/2003. [84] Cross species serological investigations have demonstrated very high titres and a prevalence of 46% (36/78) in flying foxes (*Pteropus alecto* and *P. scapulatus*), four percent prevalence in macropods (11/253), minimal prevalence in cattle and pigs and no detections in rats.[84] Antibodies were detected in horses to other orbiviruses including the mosquito-borne Stretch Lagoon orbivirus (species *Umatilla* virus)[54] and Corripata virus and the tick-borne orbivirus Nugget virus.[49]

3.3.2.4 Bunyaviruses

Trubanaman (TRUV), Gan Gan (GGV) and Mapputta (MAPV) viruses (genus *Orthobunyavirus*, family *Bunyaviridae*) originally isolated from Australian mosquitoes in 1966, 1970 and 1960 respectively, are members of the Mapputta group, which includes Maprik virus (MPKV) from Papua New Guinea.[27] Buffalo Creek (BUCV), Murrumbidgee (MURBV) and Salt Ash (SASHV)

viruses are recently identified Australian additions to this group. MURBV and BUCV are considered strains of the TRUV species and SASHV a strain of the GGV species.[27] An extensive NSW based human sero-epidemiological study found prevalence for GGV to be 4.7% and 1.4% for TRUV, concluding that these two viruses have caused human infection in all health regions of NSW.[87] Syndromic surveillance utilising paired sera from clinical patients in NSW showed seropositivity for GGV. Detections included a small epidemic of self-limiting acute febrile illness in the Murrumbidgee irrigation region featuring malaise, myalgia, polyarthralgia or polyarthritis, with or without sore eyes or rash. TRUV was associated with very similar illness in two people from Bourke and Griffith. [87] Diagnostic testing for this group of viruses has been very limited, yet it is suggested that GGV and TRUV may be significant causes of RRV-like illness in Australians, [27,87] and thus we consider them likely unrealised causes of RRV-like illness in horses.

Recent research using modern molecular approaches (PCR and Next generation sequencing) has identified widespread prevalence of these bunyaviruses, including TRUV in Australian mosquitoes in WA, NSW, Victoria, and southern Australia. TRUV and others related to GGV are widely distributed throughout Australia.[88] Investigations of the natural host range of TRUV undertaken on mammals sampled in the Northern Territory between 1960 and 1966 by Doherty et al. detected antibodies in highest proportion in horses (25/55) and kangaroos (21/53) with lower antibody seroprevalence in wallabies (34/70), humans (9/227), cattle (7/197), pigs (2/34), sheep (1/38), and poultry (1/59)[89]. These investigations followed isolation of TRUV from an *Anopheles annulipes* mosquito, prevalent in both coastal and inland regions Australia wide. (Summary accessed CDC Arbovirus Catalog – Trubanaman virus: https://wwwn.cdc.gov/arbocat/VirusDetails.aspx?ID=492)

3.3.3 Equine viruses present in Australia causing disease manifestations most like HeV

The following viruses occur in Australian horses without potential for zoonotic transmission yet are associated with sufficiently consistent disease to be considered leading differential diagnoses of HeV infection. Clinical signs include acute and/or progressive respiratory and/or neurological manifestations, often with pyrexia.

3.3.3.1 Equine Alphaherpesviruses

The order *Herpesvirales* consists of three families: *Herpesviridae, Alloherpesviridae and Malacoherpesviridae.*[90] Members of the family *Herpesviridae* infect mammals, birds and reptiles and comprise three subfamilies: *Alpha-, Beta- and Gammaherpesvirinae*.[90] Nine herpesviruses have been identified infecting equines.[28] Equine abortion virus (EHV-1), equine coital exanthema virus (EHV-3), equine rhinopneumonitis virus (EHV-4), asinine herpesvirus 1 (EHV-6), asinine herpesvirus 3 (EHV-8) and gazelle herpesvirus 1 (EHV-9) all belong to the genus *Varicellovirus*, subfamily *Alphaherpesvirinae*. The other three viruses, equine cytomegalovirus or equid herpesvirus 2 (EHV-2), equid herpesvirus 5 (EHV-5) and asinine herpesvirus 2 (EHV-7) belong to the *Gammaherpesvirinae* subfamily. EHV-5 has been associated both naturally and experimentally with equine multinodular pulmonary fibrosis (EMPF) similar to Epstein-Barr virus (EBV) associated with lung fibrosis in humans.[91] The horse is host to EHV-1, -2, -3, -4 and -5, while the donkey is host to EHV-6, -7 and -8. A neurotropic virus, EHV-9, has been identified in a range of species, and was originally isolated from a gazelle.[28]

EHV-1 and EHV-4 are ubiquitous in horse populations on all continents with more than 80% of horses estimated to be latently infected.[28,92] EHV-4 causes relatively mild upper respiratory infection whereas EHV-1 causes systemic infection affecting multiple organ systems. It causes a range of disease including rhinopneumonitis, pneumonia in foals, neonatal death, chorioretinopathy and most significantly abortion and potentially lethal equine herpesvirus myeloencephalopathy (EHM). EHV-1 and -4 are endemic in Australia, with highest incidence considered likely to be in thoroughbred (TB) breeding populations, given the necessity for live breeding in this breed and their typically higher density breeding and youngstock populations. EHV-1 is of greatest economic significance due to propensity to cause foetal loss (including outbreaks). It is also considered a differential diagnosis of HeV infection in Australian horses, given its clinical manifestations can similarly include both respiratory and neurological disease. It is highly probable that EHV-1 associated illness is underdiagnosed, because the testing for this virus has traditionally been sporadic amongst horses showing consistent disease from non-breeding populations.

Key epidemiological features of EHV-1 include high incidence of respiratory infection in foals and young stock. Most infected horses establish latent infections, and when reactivated, there is frequent shedding of virus, resulting in transmission to naïve horses.[93] Importantly, EHV-1 latency often gives rise to abortion, and neurological disease in closed horse populations. They mostly occur as single EHV-1 abortion case events only rarely leading to abortion storms, highlighting widespread endemicity in some equine breeding populations.[94] Sero-epidemiological investigation of a large TB horse stud farm in NSW showed a prevalence of antibodies to EHV-1 of 26.2% in mares and 11.4% in foals and more than 99% of mares and foals seropositive to EHV-4.[95] Infected stallions can shed virus in their semen but the role of seminal shedding in venereal transmission remains undetermined.[94] Mare and foal populations act as the reservoir of EHV-1 with transmission demonstrated amongst young stock typically around weaning and in foals from 30 days of age.[96]

Both EHV-1 and -4 are known to circulate in vaccinated mares and their unweaned foals, with many exposed in the first five weeks of life, despite use of combined EHV-1 and EHV-4 inactivated whole virus vaccines.[97] The respiratory tract is primarily infected, and transmission of infectious virus is mostly via respiratory secretions, contact with aborted foetuses or placentas, and via fomites. Initial infection is followed by a cell-associated viraemia and transition from viraemic-infected cells to latently infected cells several weeks later.[92] Experimental infections in horses have demonstrated viral shedding as early as Day 1 post challenge, ceasing within approximately one to two weeks in most cases. [94] Latent EHV-1 infection, often lifelong, is understood to establish in lymphoid tissues, circulating lymphocytes and trigeminal ganglia. Reactivation occurs from these sites, often without clinically overt disease.[28,94]

It is estimated that about 10−40% of horses in North America that develop EHV-1 viraemia go on to develop EHM. EHM is unlikely to develop without viraemia, and it is considered more likely to manifest in cases that feature longer and more severe viraemia.[92] EHV-1 respiratory illness is most severe in immunologically naïve younger horses. It often lasts two to three weeks, featuring a biphasic fever, depression, anorexia, and coughing, with initially serous progressing to mucopurulent nasal and ocular discharge. Clinical signs of EHV-1

induced EHM are associated with dysfunction and inflammation of the CNS, particularly the caudal spine. Signs include ataxia ranging from mild transient cases with weakness of hind limbs, and variable paresis to severe cases of recumbency, paralysis and even tetraplegia, sensory deficits in the perineal area and bladder dysfunction with urinary incontinence. Cases featuring cortical, brainstem or vestibular involvement, characterised by obtunded demeanour, recumbency, head tilt, ataxia and cranial nerve deficits, present less frequently. EHM typically develops suddenly after a secondary pyrexia without respiratory disease between six and 10 days after initial signs. Disease often becomes most severe over two to three days, resulting in euthanasia. Many of these clinical features are also seen in horses infected with either HeV or ABLV (RABV). Outbreaks feature typical EHM disease in multiple horses and a history of fever, abortion or respiratory disease in affected horses.[92] Chorioretinopathy featuring permanent 'bullet or shotgun lesions' of the retina appear between four weeks and three months after EHV-1 infection in approximately 80% of horses, resulting from viral infection affecting the choroidal vasculature.[92] Such lesions are a very common incidental finding on ophthalmic examinations of Australian horses of many breeds such as those undertaken on behalf of prospective purchasers pre-sale. (Pers Comm)

There has been minimal detection of the specific strain/s of EHV-1 causing EHM internationally in Australia and confirmed cases of EHM have remained sporadic. A study published in 2011 typed the genomes of 66 EHV-1 isolates that had been collected and archived from Australian cases of abortion, perinatal foal disease and EHM since 1977. The study found that 97% (64/6) were of the less-neuropathogenic genotype offering potential explanation for the consistently lower incidence in Australia compared to the EU and USA.[99] Only one of four viruses isolated prospectively in the same study from cases of EHM in Australia contained the genome sequence with greatest EHM disease association. [99] This low incidence of viruses most-associated with EHM in Australia is consistent with the low incidence of EHM disease. In recent years, the increased number of outbreaks in the US and Europe directly correlates with a similar increase in isolations of viruses with the genome type associated with EHM. This suggests that the higher incident EHM viral strains have established high and persistent rates of endemic infection in these horse populations. Each year many TB racehorses and breeding stallions travel to Australia from these regions, posing a risk of further introductions of higher-incident EHM strains. Increased passive and

active surveillance testing and promotion of strategic biosecurity measures for high-risk populations may prevent similar increases in EHM outbreaks in Australia.

Antemortem diagnosis of EHV-1 associated EHM is supported by ruling out other neurologic conditions, demonstration of xanthochromia and an elevated cerebrospinal fluid protein concentration and identifying or isolating EHV-1 from the respiratory tract, buffy coat, or CSF.[92] As mentioned above in the context of Flavivirus diagnosis, the requirement of extensive testing of optimal tissues can be additionally challenging in Australia. Occupational biosafety hazard commonly restricts sampling to minimally invasive approaches prior to priority molecular screening is negative for HeV and ABLV. Optimal biosecurity on breeding farms is underpinned by early recognition of suspected cases, enabling quarantine and monitoring of high-risk horses to prevent EHV-1 EHM and abortion outbreaks.[92]

Two Australian studies published in 2001 detail the development of sensitive PCR assays for the diagnosis EHV-1 And EHV-4, as well as PCR for EHV-3, a semi-nested PCR for EHV-2 and single round PCRs for EHV-5, EAdV-1, EAdV-2 and RT-PCRs for EAV and ERAV.[100,101] These are highly specific and sensitive, providing a major advance for rapid diagnosis or exclusion of these endemic equine virus diseases in Australia.

Currently EHV-1 PCR and serology assays are undertaken by both government state laboratories and by commercial pathology upon specific request by attending veterinarians. The cost of testing is often attributed to the horse owner, unless testing occurs as part of a government facilitated significant disease investigation. This limits the number of investigations.

3.3.3.2 Equine viral arteritis virus

Equine arteritis virus (EAV) (genus *Arterivirus*, family *Arteriviridae*) also causes acute severe disease in equids with infection termed equine viral arteritis (EVA). Endothelial pathology (like HeV) results in widespread tissue and organ oedema and haemorrhage. Other members of this viral family include simian haemorrhagic fever virus (SHFV) which infects many wild primate species, porcine reproductive and respiratory syndrome virus (PRRSV) – a serious infectious disease of pigs costing the US pork industry over USD $560 million

annually, and wobbly possum disease virus (WPDV) which causes neuropathology in wild Australian brushtail possums (*Trichosurus vulpecula*) in NZ and Australia.[102,103]

EAV Infection causes disease with a wide variety and severity of clinical manifestations in horses, yet many infected remain asymptomatic while still capable of shedding and transmitting virus. Viral shedding occurs via respiratory droplets, fluids associated with parturition (including aborted foetuses) and significantly via semen.[104] Infected stallions (10−70%) can become 'shedders', releasing virus for months, years or even life, due to persistent virus replication in fibrocytes of the ampullae.[105] Clinical signs in overtly diseased cases may include fever, depression, respiratory symptoms including nasal discharge, oedema (especially distal limb), conjunctivitis, abortion and severe, sometimes fatal neonatal disease.[106]

EVA is considered a notifiable disease in all states of Australia. While serology has consistently supported that Australian horses are infected at low prevalence, EVA disease has not been recognised, prompting interpretation that the strains circulating in Australia are less virulent.[106] The first Australian isolation of EVA was from Standardbred (SB) stallions imported from North America, reported in 1990.[29] This prompted serological investigation of the Australian horse population (including archived sera) that determined that Australian horses had been infected with EAV at least since 1975. Breeding stallions (SB and TB) were prospectively tested, and out of 170 stallions, 73% of SB and 8% of TB were seropositive. Seropositivity was also detected in 71% of SB mares, 6% of SB racehorses and 1% of TB mares and racehorses.[29] An inactivated vaccine is available − Equip Artervac (Zoetis),[107] but is only used for import and export purposes in Australia. The United Kingdom (UK) and other countries recommend routine vaccination of all breeding stallions and pre-breeding testing certification of stallions (live cover) and semen (artificial insemination).[104]

Testing for EAV in Australia is mainly limited to investigation of pregnancy loss by submitting veterinary requests. The last serological surveys for EIAV in Australia was published in 1981 and 1990.[30,108] Contemporary knowledge on the incidence, prevalence, and effects of EAV on the Australian equine breeding population is lacking. Significance for horse and industry health and biosecurity, and potential for EVA to cause HeV-like illnesses in Australian

horses, strongly justify both increased passive surveillance testing for EVA and active surveillance activities.[109]

The role of widespread feral horses, numbering approximately 400,000 (https://www.ecolsoc.org.au/hot-topics/feral-horses-australia) in Australia as reservoirs of EIAV has been considered but empirical data is lacking.[110] EVA was diagnosed in three horses June 2004, on the central Qld coast near Mackay.[111]

Indeed, surveillance for EVA of horses is challenged globally. Responding to positive cases is problematic, given the substantial value of many breeding horses; lifelong carrier status and potential to act as a source of infection to naïve horses, justify owners to consider ceasing breeding activity, and even humane destruction. Most countries limit testing of domestic horses to owner/veterinarian elective testing, freedom from disease status before movement to events, investigation of consistent disease (initiated by veterinarian with owner consent), pre-breeding freedom of disease testing for stallions and export and import purposes. Recommended responses to positive status vary by jurisdiction in Australia, mostly recommending but not enforcing euthenasia.[109,112] Biosecurity legislation in the UK maintains that any horse testing positive will be subject to compulsory slaughter without compensation.[113]

Surveillance testing in Canada between 2009 and 2012 was analysed retrospectively. The analysis was prompted by recognition of these barriers to owner initiated passive surveillance for EIA, and resultant compromise of accurate estimation of EIAV prevalence and EAV disease, which varied greatly by juristiction.[114] Biosecurity for EIAV began in 1975 in Canada. The latest of multiple reforms operated shared responsibility between the equine industry, encouraging voluntary testing in the domestic population, with the Canadian Food Inspection Agency (CFIA) managing responses to positive cases.[114] Carrier animals are removed, reducing population prevalence and proportional incidence over time, providing the detection rate exceeds the rate of new cases. [114] Annually, 14% of horses are tested (owner-requested), succeeding in reducing the Canadian annual EIAV incidence to 0.04 cases for every one thousand horses. [114] While the estimated Canadian horse population (~400,000) is less than half of Australia's, the Canadian program highlights the potential for successful EIAV/EVA control, driven by industry and supported by government.

Like Canada, Australia follows the standards for the safe international trade and movement of equines set by the World Organisation for Animal Health (WOAH) Terrestrial Animal Health Code.[115] The code outlines requirements for the importation of equines: veterinary certification attesting absence from consistent clinical signs; and that all premises where an imported horse has resided in the prior three months be free of EIAV with sero-negativity throughout the quarantine periods (30 or 90 days for temporary and permanent migration respectively). Frozen semen imported into Australia similarly must be prepared by approved international facilities adhering to similar quarantine requirements and must be accompanied by certification.

Chilled semen is imported to Australia from NZ exclusively (mostly SB lacking accompanied certification of freedom of EIA/EVA), as distance prohibits viability from more remote locations (USA/EU/UK). Great emphasis is placed on domestic and international chilled semen being accompanied by stallion freedom of EIA and EVA certification in the UK. This is partly due to perceived incursion threat from EU stallions, and liability that might accompany recognition of iatrogenic transmission through artificial insemination. Veterinarians are instructed to inseminate mares only with semen accompanied by EIA/EVA freedom certification. Domestic UK stallions are strongly encouraged to undergo preseason swabbing and screening for these pathogens as outlined in the annually published codes of practice.[113]

Traditionally few Australian horses (other than thoroughbreds) have a passport or are identified by microchip and registration. Microchip identification has increased, as all horses receiving HeV (administered since 2012), or equine influenza vaccines (as part of the 2007/2008 outbreak response and eradication), have been required to be microchipped. Improving equine identification and traceability would benefit national biosecurity, equine health, and industry bio- and economic-security.

3.3.4 Equine viruses present in Australia causing some disease manifestations like HeV

The following viruses are known to occur intermittently in, and/or circulate amongst, Australian horses, manifesting in disease features similar to HeV. Signs such as pyrexia, injected mucous membranes, upper respiratory abnormality, and malaise (influenza-like)

may be consistent with HeV infected horses, especially milder cases of HeV, and even severe cases of HeV examined relatively early in the disease course.

3.3.4.1 Equine adenoviruses

Equine adenovirus (EAdV) infection is associated with generally mild or self-limiting respiratory and/or gastrointestinal infection in horses and foals with virus detected in both healthy and sick animals. [116] Two serotypes are described − equine adenovirus type 1 (EAdV1) associated mostly with upper respiratory tract infection and equine adenovirus type 2 (EAdV2) associated mostly with gastrointestinal infection.[117] The significance of these viruses is highest in foals, particularly those with immunosuppression, concurrent infection or immunodeficiency in which particularly EAdV1 has caused fatal bronchopneumonia.[117] A 2008/09 serosurvey of 122 NSW based TB horses for EAdV found moderate titres to EAdV1 and/or EAdV2 in 96 horses. Eighteen of these were positive to both serotypes when tested by a standard serum neutralisation assay, of higher sensitivity than a comparison ELISA.[117] EAdVs are not considered a significant differential for HeV-like illness in adult horses.

3.3.4.2 Equine rhinoviruses

Equine rhinitis viruses are single stranded RNA viruses (family *Picornaviridae*) that are widespread in horses globally. They cause mild respiratory disease featuring serous discharge, pyrexia and compromised athletic performance. Four serotypes are recognised: equine rhinitis A virus (ERAV) (genus *Apthovirus*) and equine rhinitis B viruses − ERBV1, ERBV2 and ERBV3 (genus *Erbovirus*).[118] To further understand ERAV disease, experimental infection was performed with a Canadian isolate obtained from an outbreak of pyrexia and respiratory disease.[119] Mild respiratory tract disease was observed featuring mild pyrexia, nasal discharge, adventitious lung sounds and enlarged mandibular lymph nodes.[119] Lower airway inflammation with increased mucous/mucopurulent material was observed endoscopically for at least 21 days after inoculation.[119] Ponies with neutralising antibodies against ERAV did not develop clinical signs of disease when reinoculated one year later.[119] An effective RT-PCR is available and would be suitable for investigation of equine disease featuring respiratory signs and mild pyrexia in Australia.[120] While ERAV and ERBVs are likely significant causes of outbreaks of respiratory disease in young horses and likely contribute

to inflammatory airway disease (IAD), reduced performance and exercise intolerance, they are not considered a likely differential for HeV-like illness.

3.4 Viruses causing HeV-like disease in horses internationally as perspective on unrecognised, emerging or future infectious causes in Australia

Viruses reviewed here cause sufficiently similar disease to HeV in horses internationally that, while currently considered exotic, have potential to occur in Australian horses via incursion or emergence of hitherto unrecognised endemic variants. Considering established and recently identified viral causes of acute severe equine illness with respiratory and/or neurological signs internationally, offers additional perspectives potentially supporting identifying novel and emerging infectious causes of HeV-like disease in Australian horses. Several international and exotic viruses have been discussed above in relation to related viruses present in Australia. Here we will discuss a selection of others of potential significant to Australian HeV-like diseased horses (Table 3.3).

Many parts of the world share climates, reservoirs (particularly flying foxes and migratory birds) and arthropod vector types and species with Australia. Variant strains and similar species of many RNA viruses, with wide host tropism and One Health relevance, are recognised globally. The frequency of emerging infectious diseases is highest in countries with tropical and subtropical climates. Higher abundance of arthropod vectors and wildlife reservoirs, and greater biodiversity enhance frequency. Australia hosts broad ecological and climatic agricultural, peri-urban and wild habitats, including extensive regions of similar tropical and subtropical climate to many nations in which a wider (and ever increasing) range of infectious agents of animal and/or One Health significance are recognised. A tradition of world-leading animal infectious disease investigation, significant agent detection, scientific research and epidemiology operates in Australia. Our national government biosecurity agencies and laboratories serve as a regional diagnostic reference for Southeast Asia and the Pacific islands for many animal diseases via World Organisation for Animal Health (WOAH formally OIE) and Food and Agriculture Organization of the United Nations (FAO).

Table 3.3 Viruses causing HeV-like disease in horses internationally

Virus	Genus /family	Reservoir/vector	Confirmed infection in:		Geography recognised	Equine disease syndrome
			Horses	Humans		
Nipah virus (NiV)	*Henipavirus/Paramyxoviridae*	Flying foxes	Yes*	Yes	Southern Asia	Hemorrhagic endothelial vasculitis, Encephalomyelitis & SARS#
Rabies virus (RABV)	*Lyssavirus/ Rhabdoviridae*	Terrestrial carnivores and bats	Yes	Yes	Globally	Encephalomyelitis
African horse sickness virus (AHSV)	*Orbivirus/ Reoviridae*	Culicoides, mosquitoes and ticks	Yes	No	Africa, Europe, South Asia	Hemorrhagic endothelial vasculitis & SARS
Equine Encephalosis virus (EEV)		Culicoides	Yes	No	South Africa	Encephalomyelitis, - less freq. abortion & SARS
Peruvian horse sickness (PHSV)**		cattle & sheep/ culicoides & mosquitoes	Yes	Yes	South America (Australia)	Encephalomyelitis
Eastern equine encephalitis virus (EEEV)	*Alphavirus/ Togaviridae*	Birds, small mammals, & reptiles	Yes	Yes	North/ South America & Caribbean	Encephalomyelitis
Venezuelan equine encephalitis virus (VEEV)		Small mammals & mosquitoes (Direct mammalian transmission poss. incl. aerosol)				
Getah virus (GETV)		mosquitoes	Yes	Yes	South-East & East Asia	Fever, oedema & lymphadenopathy
Shuni virus (SHUV)	*Orthobunyavirus/ Bunyaviridae*	Culicoides, mosquitoes, ticks & flies	Yes	Yes	South Africa	Encephalomyelitis
California encephalitis virus (CEV) Serogroup incl. Jamestown Canyon virus (JCV); La Crosse virus (LACV) & Snowshoe hare virus (SSHV)		Rodents and Lagomorphs	Yes	Yes	North America	
Borna disease virus (BoDV)	*Bornavirus/ Bornaviriadae*	Shrews and Squirrels + divergent lineages in birds	Yes	Yes	Central Europe (potential global distribution)	Encephalomyelitis
Tick-borne encephalitis virus (TBEV)	*Flavivirus/ Flaviviridae*	Small mammals, bats, domestic mammals/ Ixodes Spp. ticks	Yes	Yes	Europe and Asia	Encephalomyelitis
Louping ill virus (LIV)		Sheep, small mammals & Grouse/ Ixodes ticks.	Yes	Yes	UK, Divergent lineages in Europe and Asia	
Powassan virus (POWV)		Small mammals, Deer/ Ixodes spp. ticks	Yes	Yes	North America & Russia	
St. Louis encephalitis virus (SLEV)		Birds / Mosquitoes	Yes	Yes	North/ Central America, Caribbean & Canada	
Equine Influenza (EI) (H3N8 & H7N7)	*Alphainfluenzavirus/ Orthomyxovirus*	Potentially Birds	Yes	No	Global	Influenza, incl. vasculitis, lymphadenopathy & respiratory dis.

*Confirmed in 1998/1999 Malaysia / Singapore.outbreak. and in a henipavirus outbreak in 2014 in the Philippines
**The Australian ELSV is considered an isolate of this virus. # Severe Acute Respiratory Syndrome (SARS)

3.4.1 Equine Influenza

Equine influenza (EI) is one of the most highly contagious and common respiratory infectious diseases of horses globally. It is caused by infection with Equine influenza virus A type 2 (H3N8) and Equine influenza virus A type 1 (H7N7), family Orthomyxovirus. Disease manifestations in horses include inappetence, pyrexia, lethargy, reduced athletic performance, a harsh dry cough, hyperaemia of nasal and conjunctival mucosae, tachycardia, dyspnoea, stiffness, distal limb oedema, myalgia, lymphadomegaly, serous nasal discharge (often progressing to mucopurulent), abortion, and a low incidence (mainly in foals) of pneumonia and death.[121]

Australia is currently considered free from EI. Virus incursion via an infected visiting TB breeding stallion, and escape from the quarantine facility, resulted in an unprecedented outbreak in 2007/2008 in Australia. The outbreak involved infection in over 70,000 horses, spread across 9,000 premises in two states (NSW and Qld).[122] Remarkably, eradication was successfully achieved with prompt national, and subsequent regionally zoned, horse movement restrictions, biosecurity protocol, risk classification zoning and ring vaccination. Costs to the economy associated with the outbreak and requirements for regaining WOAH freedom of disease status are estimated as having approached A$1Billion.[123]

Internationally EI is considered a leading differential diagnosis for horses presenting with acute pyrexia and respiratory disease. Since 1980, there has been minimal identification of H7N7 in horses and H3N8 has been established as the predominant cause of EI. Distinct Eurasian and American H3N8 lineages are recognised, with the American lineage responsible for most recent outbreaks worldwide. H3N8 is considered endemic globally other than in NZ, Iceland, and Australia, which regained EI-free status after the extensive outbreak of 2007.[124]

H3N8 is not known to circulate asymptomatically and is eliminated rapidly by the equine immune response. Outbreaks in immunonaïve populations may be avoided by quarantine of newly arriving horses for a minimum of 14 days, and by vaccination. Horses with partial immunity may be infected, often sub-clinically, yet shedding virus. Immunity following natural infection is thought protective for approximately one year, whereas immunity following currently available (inactivated) vaccination is short-lived, justifying six-monthly

booster immunisation intervals. Recently vaccinated horses can thus become infected and shed virus. Despite vaccination, outbreaks occur every few years in endemic regions, due to waning immunity and viral antigenic drift.

Maximum-likelihood phylogenic analysis of H3 influenza genomes, including recently isolated strains, suggest that equine influenza (H3) viruses are of avian lineage and may have originated in wild birds. Six distinct H3 viruses were isolated from wild waterbirds in Chile with high nucleotide homology to the 1963 lineage of H3N8 equine influenza. While these viruses were antigenically characterised as more like avian influenzas, having adapted and co-evolved with their avian hosts, these findings suggest equine influenza-like H3 viruses maintain wild bird reservoirs.[125]

3.4.2 Bat borne viruses in horses internationally

A 2014 outbreak of Henipavirus in the Philippines highlighted the wide host tropism and potential for HeV and NiV to cause highly fatal infection, capable of transmission between humans and domestic animal species following spillover from flying foxes. The outbreak featured horse to human as well as human to human transmission with 17 human cases and a case fatality rate, in those who developed acute encephalitis syndrome, of 82%. There were 15 known animal cases, all fatal, comprising ten horses, four cats and one dog.[126] Prior to this, horses detected as seropositive as part of investigations surrounding the 1998/99 NiV outbreak in Malaysia, were thought to have been exposed from infected pigs.[127-129]

Internationally, Rabies virus (RABV) has long been infrequently, yet consistently, detected in equids with at least three reports of human contraction from horses in modern literature.[130,131] Many equine cases result from bites from infected carnivores such as dogs and foxes in countries with terrestrial mammalian reservoirs, but spillover from bats is also recognised and not limited to the vampire bats of South America.[132] Young horses are noted to be at particular risk of transmission interactions (being bitten or scratched), given their heightened curiosity and tendency to approach sick or injured bats.[14,132] Incidentally the two ABLV cases in 2013 occurred in young paddock-mates, who, based on bat ecological and epidemiological findings were considered likely to have contracted the virus via such inquisitive contact with a single infected micro-bat.[13]

Borna disease virus (BoDV), BoDV-1 has been identified as the cause of severe neurological disease in horses, humans and sheep in central Europe (Germany, Switzerland and Austria.[133-135] The virus is known to infect cats and shrews in Europe and Western China. There it exhibits broad tissue tropism while causing less overt disease with histopathology sometimes not evident, yet characteristic neuropathology in some.[136] Antibodies to BoDVs have been detected across a range of mammalian and avian species, including in regions beyond where domestic mammalian and human disease have been recognised. However, epidemiology interpretation should caution expected propensity for sero-crossreactivity of antibodies generated in response to relatively diverse viral genomic lineages.[137] Relatively genetically diverse lineages of BoDVs have been identified in avian species in which they cause often fatal proventricular dilatation disease. [137]

BoDV-1 and a closely genomic lineage BoDV-2 have been recognised as causes of fatal encephalitis in a humans,[134,138-140] subsequently suspected to have spilled over from shrew and squirrel host reservoirs respectively.[135,141-144] An outbreak of fatal encephalitis in four horses in upper Austria in the winter of 2015/16 demonstrated the potential for high fatality rate in horses. The viral reservoir host and putative spillover source in this outbreak was considered two shrew species, *Crocidura leucodon* and *Sorex araneus*. virus was detected, with histological neuropathology, in one of each species, and seroprevalence of IgG was 67% and 8% of 9 and 13 sampled subjects respectively.[136] BoDV-1 infection in horses is selectively neurotropic, causing non-purulent encephalitis featuring substantial CNS T-cell infiltration, giving rise to progressive encephalitic manifestations. Signs include depression and ataxia, with an outcome that is usually fatal.[33]

Molecular detection or isolation of BoDV or related viruses are currently lacking in any Australian mammalian or marsupial species, but limited testing has occurred. A 2006 human sero-epidemiological study demonstrated serological evidence of BDV infection in Australia (26 antibody positive and 6 antigen positive of 705 subjects tested) with highest seroprevalence among subjects that had received blood transfusions (14/168) and in those suffering depression (5/104) compared to no cases of seropositivity amongst 42 appropriately comparable control subjects.[32] The same research team was prompted by

their findings on human infection to undertake further investigation. They conducted a serological survey of 553 horses sampled in NSW Australia. They screened for BoDV antigen, circulating immune complexes (CICs) and antibodies by monoclonal antibody based ELISAs.[33] The study found a low frequency (0.7%) of serological markers considered plausibly associated with BoVD infection and interpreted the findings as inconsistent with endemic infection. [33]) Rather, intermittent spillover from an unknown wild reservoir host is supported by such low frequency seropositivity. Prompted by these additional findings the same researchers then undertook a further serology study on Australian cats using monoclonal antibody based ELISA with confirmatory IFA and western blot assays.[147] They identified seroprevalence of 3.2% and 0.8% in cats sampled in NSW and SA respectively, indicating that there may be a low prevalence of BoDV infection amongst Australian cats.[147]These findings, taken together with international knowledge on BoDVs, justify further active surveillance activities and research to inform of potential One Health disease and biosecurity risks posed by BoDVs in Australia.

Several international studies have failed to conclusively determine a causal association between human infection with BoDV and mental illness.[145,146] Considering the high relative incidence of mental illness in Australian veterinarians and their heightened occupational exposure risks, further studies into zoonotic transmission of BoDVs should be prioritised.

3.4.3 Arthropod-borne viruses causing HeV-like disease in horses internationally

Ticks are a well-established vector of viruses that can cause acute pyrexia and neurological disease, particularly those of Orthobunyavirus and Flavivirus families. Louping ill virus (LIV) (family *Flaviviridae*) causes encephalomyelitis in horses in the British Isles and the Iberian Peninsula.[148-150] In 2006, seven cases of equine neurological disease were attributed to LIV, in a region of the UK in which cases had been detected in farmed ruminants. Two cases were seropositive of 68 sampled within two miles of the infected premises. Signs included pyrexia (up to 40.5 °C), inappetence/anorexia, depression, obtunded demeanour, muscle tremors of the neck and facial area, altered head carriage, mild to severe ataxia, avoidance of bright daylight, and constant or exaggerated chewing.[151] Two other tick-borne Flaviviruses causing encephalomyelitis in horses are Powassan virus (POWV) in the USA

and Russia and Tick-borne encephalitis virus (TBEV) in Asia, the EU, Finland and Russia.[148]

Recent research detecting emerging viruses in Australian ticks (contemporary and archived samples) using advanced molecular techniques, support that ticks may play a role in transmission of viruses in the families *Bunyaviridae*, *Flaviviridae*, *Nairoviridae*, *Phenuiviridae* and *Reoviridae*. These viruses closely align with viruses that have demonstrated disease significance in domestic animals, and while are yet to be detected, may currently circulate and cause disease in Australian mammals and/or pose unrecognised biosecurity risk.[152,153]

The Californian serogroup of bunyaviruses (CSGV) (genus *Orthobunyavirus*, family *Peribunyaviridae*) are transmitted by mosquitoes. They include Jamestown canyon (JCV) La Crosse (LACV) and snowshoe hare (SSHV) viruses, all established causes of encephalomyelitis in horses and humans in the USA and Canada, with serology in humans and horses correlating geographically.[148,154-156] Serology has shown that orthobunyaviruses commonly infect livestock including horses and wild deer in the Yucatan Peninsula of Mexico with unknown disease significance.[157] Bunyamwera virus (BUNV) was established as the cause of encephalitis and abortion in horses in Argentina in 2015.[151] BUNV serogroup viruses (family *Bunyaviridae*, genus *Bunyavirus*) include Maguari, Cache Valley, Main Drain, Kairi and Santa Cruz virus strains. They are known to circulate in Africa and the Americas and have been established causes of pyrexia and/or encephalomyelitis in horses for many years.[151,158]

Both horses and humans are considered 'dead-end' hosts for lineages 1 and 2 WNVs (Flaviviruses, 7 lineages in the WNV group), given viral load is relatively low in these species (in comparison to infected pigs and bird species). Infections in horses are not thought to result in onward mosquito vector transmission.[137] A large outbreak of WNV occurred in the US in 2012 resulting in infection of 5,674 humans and 690 horses with infections in horses serving valuable sentinel indication for detections in humans.[159]

Endemic transmission of WNV lineage 2 has long been recognised in Africa. Infection in humans is associated with flu-like illness, and recently both increased virulence and geography have been recognised with neuroinvasive disease in African horses and humans, and detection across Europe in Hungary, Austria, Italy and Greece. Neurological disease

cases of WNV infection have been recognised in Greece and Serbia, Southeast Asian countries, New Guinea, and India.[160] At least four epidemic cycles involving neurological disease in humans and horses have been reported in Greece. [161] When infected by JEV or WNV both older people and older horses appear more susceptible to neuroinvasive disease.[160] Age-associated risk factors included hypertension and diabetes mellitus in humans, and while investigation into risk factors in older horses is lacking, underlying cardiac disease, equine metabolic syndrome, and pituitary adenoma are proposed.[160]

Relatively low viral load in incidental human and horse hosts of WNV results in unreliability of molecular detection other than in optimal CNS samples. This places importance on serology, both paired IgG and IgM and the detection of IgM antibody response in antemortem diagnosis.[148] Extensive PCR testing of equine WNV cases in the US has determined that due to the limited viral load, accurate testing of appropriate tissues consisting of several locations (thalamus, hypothalamus, rostral colliculus, pons, medulla and anatomically identified spinal cord) is necessary to optimise diagnosis when relying on real-time PCR.[148] Nested PCR is more sensitive and has the added benefit of detecting variant strains and viral isolation remains important in determining pathogenesis.[148] Immunohistochemistry (IHC) and In situ hybridisation (ISH) are diagnostic techniques applied to fixed tissue; these have proven highly effective in detection of WNV and EEEV in horse neurological tissue.[162]

France has utilised horses very successfully as sentinels for human WNV infection since 2002, along with call ducks and domestic poultry.[163] WNV was detected in both horses and humans for the first time in Cuba.[160] Recent research in South Africa has identified West Nile Virus Lineage 2 to be causing significant neurological disease in horses and other animals with infection in horses correlated with infection in veterinarians.[65]

Eastern, Western, and Venezuelan equine encephalitis viruses (EEE, WEE and VEE) (family Alphavirus, family *Togaviridae*) are well recognised causes of equine and human neurological disease in North and South America and the Caribbean. [148] Many horses are thought to become infected without overt disease. However, those that develop neurological disease (most frequently young animals, as for humans) suffer encephalitic

manifestations and rapid disease progression similar to that caused by HeV infection. Neuropathology includes necrotising encephalitis and neuronal dysfunction, considered a direct result of viral replication. Post-mortem examinations reveal significant meningeal congestion and often focal areas of darkened discoloration in slices of cerebellum. Pleocytosis and sometimes IgM are evident in CSF, predominantly neutrophilic for EEV in contrast to monocytophylic, which is typical for WNV. Histological findings include neuronal necrosis, neurophagia, marked perivascular cuffing, mononuclear and polymorphonuclear leukocyte infiltration, with focal and diffuse microglial proliferation.

Horses are considered 'dead-end' hosts for EEEV and WEEV as for flaviviruses in the WNV group. Case-fatality rate is very high in encephalitis cases of EEEV, while lower (between 20% to 30%) for WEEV. [148]

In addition to similar enzootic vector borne infection, having further adapted to mammalian hosts, VEEV is recognised to be transmitted in epizootic infection cycles. The virus causes outbreaks of disease in horses and humans, potentially involving transmission without need for reservoir hosts.[164] Case fatality rate in diseased horses has been estimated as high as 90%. Such epizootic infection cycles may feature transmission between horses without mosquito vectors, given recent evidence of aerosol transmission experimentally in mice. [165]

Equine disease control and biosecurity risk management for these viruses in the Americas relies on vaccination. This is considered 100% effective in horses receiving three doses annually when young. [148]

Semliki Forest virus (SFV), (family *Alphaviridae*), the original member of the Semliki Forest complex to which RRV and GETV belong, is found in both East and West Africa, where it is known to cause human and equine disease including encephalomyelitis.[148, 166]

3.4.4 African horse sickness and equine encephalosis viruses

African horse sickness (AHS and AHSV) (family *Reoviridae*, genus *Orbivirus*) is one of the most significant viruses of horses worldwide that is transmitted primarily by biting midge *Culicoides imicola*. It is endemic in regions of Africa where it infects all Equidae, with horses most prone to disease, followed by mules, donkeys and zebras. The range of *Culicoides*

imicola extends north each year into Europe, potentially influenced by global warming, where other competent vectors reside. Middle eastern countries such as Spain, Portugal, and Morocco experience frequent epidemics.[85] Initial outbreak response aims to eradicate disease through quarantine, vector control and euthanasia of exposed horses. An inactivated vaccine has been shown to be protective against all experimental AHS challenges. Vaccination is used in endemic areas to prevent losses and during epidemics, when ring vaccination is utilised to limit spread.[85] Successful eradication entailing vaccination of all equines (170,000), was achieved following the 1989 outbreak in Portugal, costing $1,955,514. A review of cases in Namibia, however, reported many vaccinated horses succumbing to disease.[167]

Initial viral replication of AHSV takes place in regional lymph nodes (incubation period 3−15 days), followed by spread via the bloodstream involving erythrocyte bound virus and severe viraemia.[168] Secondary replication occurs in endothelial and mononuclear cells prior to infection of target organs (lung, heart, spleen and lymphoid tissue). A second viraemic phase then involves endothelial damage and macrophage activation with associated release of inflammatory cytokines.[168]

Four forms of AHS disease are recognised, described here in decreasing order of severity. The pulmonary form is peracute; many horses are found dead without observed disease. Features are marked pyrexia (39−41° C) and severely obtunded demeanour followed by severe dyspnoea (respiratory distress), severe sweating, periods of recumbency and frothy nasal discharge, with mortality rates exceeding 95%.[168] Disease involving a combination of cardiac and pulmonary manifestations (mixed form) is the most common form, with approximately 70% mortality. Deaths usually occur within three to six days of first signs (pyrexia). The cardiac or subacute form features prolonged pyrexia (39−40°C for several weeks) and prominent subcutaneous oedema, particularly of the head (including the supraorbital fossae), neck and chest with mortality rate exceeding 50%. Other signs include colic, conjunctivital congestion, petechiae of the mucous membranes and ecchymotic haemorrhages on the ventral surface of the tongue. The fourth form, horse sickness fever, features mild to moderate pyrexia and subcutaneous oedema, particularly of the supraorbital fossae, and is usually not fatal. It typically is seen with less virulent strains of AHSV and/or in donkeys, zebras and vaccinated mules and horses.[168]

Subclinical infections can occur in donkeys and zebras, with these animals acting as a reservoir host. [167] Potential reservoir Infection has long been appreciated in zebras and donkeys, but subclinical infection of horses is also now known to occur.[168] For practicality AHS has also been categorised into peracute/acute (sudden clinical onset and fatal outcome within two days), sub-acute (slower progression of the disease and fatal outcome) and AHS fever (milder symptoms with recovery). [167] As with HeV infection, typical marked oedema and effusions are thought to result from viral induced endothelial injury and consequent increased vascular permeability, with potential contribution from inflammatory cells and mediators. Viral tropism for cardiac or pulmonary endothelial cells is observed variably between strains, and virulence is associated with a strain's ability to cause endothelial injury resulting in intravascular coagulation.[168] Such strain characteristics, as well as host immune status, and the species of infected equid, determine the form and severity of disease. Differential diagnosis of AHS include EIA, EVA, EEV, HeV, anaplasmosis, babesiosis or theileriosis, anthrax, and purpura haemorrhagica.[168]

EEV is also endemic to east and west Africa and the cause of equine encephalosis (EE) in horses. The virus is also transmitted by *Culicoides* spp., supporting a very similar distribution pattern to AHSV.[169] Infection is most commonly mild or subclinical, but can be severe and even fatal in rare cases. As for many arboviruses, diseases is mostly recognised in young horses and associated with spread to immunonaïve populations, often due to altered weather patterns. A widespread outbreak of severe but not fatal EE disease was identified in Israeli in 2009. Infected horses exhibited pyrexia, oedema of the neck, legs, eyelids, and lips.[170] Similar disease was observed recently in an outbreak of EE in TB weanlings in the Western Cape Province of South Africa. [169] An outbreak of EE occurred in Israel in 2008/09, affecting 60 properties with no fatalities recorded and incidence ranging from 2% to 100%. Additional clinical manifestations to those listed above include pyrexia, unrest, inappetence, tachycardia, tachypnoea, congested mucous membranes, and oedema of the head (periorbital and lips), neck and legs.[171]

3.5 Suitability of Syndromic Surveillance of Horses as Sentinels for One Health and EIDs

This review of the broad range of RNA viruses infecting horses in Australia and internationally, causing disease of variable consistency with HeV, has highlighted the suitability of horses to act as sentinels for emerging infectious diseases. Infectious agent epidemiology involves complex animal species, often posing One Health disease and biosecurity risks. The consistency of their associated clinical syndromes (disease manifestations) further highlights the suitability of risk based active surveillance activities (general and targeted). These activities may extend and inform routine syndromic passive disease surveillance facilitated by government biosecurity, enabling proactive interpretation of emerging One Health disease threats.

Broad ranging discipline perspectives must be effectively combined to undertake such disease and biosecurity surveillance improvements. Necessary disciplines include clinical veterinary, biosecurity laboratory diagnostic pathology, serology and molecular science, virology, epidemiology, ecology and social sciences. Multiplex and active surveillance activities coupled with syndromic disease investigations (passive reporting) for HeV-like disease in horses may realise such potential use of horses as sentinels for EIDs.

Several members of the families Flavivirdae and Togavirdae, considered endemic pathogens of significance to human health in Australia, are particularly well suited to such approaches, including nationally notifiable human diseases such as Ross River virus, Barmah Forest virus, Murray Valley encephalitis virus, West Nile virus and Flavivirus disease (unspecified).[58] Traditionally testing of Australian horses for known flaviviruses and RRV has not been routinely included in state laboratory investigations despite their confirmed association with disease. Rather, their inclusion in testing varies greatly by state and case-specific perceived diagnostic responsibility such as when prompted by practitioner request, as part of research initiatives or epidemic responses. Disease notification status (justified by human health significance rather than animal health) for these viruses has traditionally varied, more so than ABLV and HeV, between Australian jurisdictions. This affects laboratory testing priorities and diagnostic cost attribution.

There has been some increase in state laboratory testing for these viruses in recent years, likely due to practitioner awareness following the 2011 outbreak and due to increased availability of RT-PCR for the WNV group flaviviruses.[172] Indeed, increased awareness for zoonotic viral causes of equine neurological disease in Australia contributed to the detection of this outbreak. Awareness has arisen following the emphasis on neurological symptoms in the 2008 Redlands HeV outbreak and the resultant increase in optimised syndromic surveillance for HeV-like illness. WNV group flaviviruses pose a significant human health burden. They cause epidemics of neurological disease in humans similarly (via mosquito transmission), related to vector geographical expansion beyond endemic reservoirs, and as such equine infection is appreciated to serve sentinel indication for human disease.[64,65] Recent human medical publications and public health advice have identified ALFV, KOKV, STRV and EHV viruses (flaviviruses), SINV and GGV as neglected Australian arboviruses. These viruses likely contribute to infectious-like, yet idiopathic, undifferentiated febrile illnesses (UFI) in humans justifying increased surveillance mechanisms and pan-viral diagnostic options.[49]

A subsequent significant weather event in Victoria in 2016 prompted the Victorian State Government Health Department to fund investigations of horses presenting with neurological disease for these viruses. Risk-based surveillance targeting detection of mosquito-borne viruses of human health significance, including Murray Valley encephalitis, virus was undertaken in 2018. Sixty-four cases were investigated in the one-year period, with neither HeV (prior to testing for HeV-g2), MVEV or KUNV identified as causes of neurological disease. Although Victoria was not then considered an endemic region for HeV, the virus was considered a differential diagnosis for all horses presenting with acute neurological disease. Thus, many arbovirus infections in horses pose a parallel diagnostic challenge to that appreciated in human medicine, with UFI in Australia often presumed to be arboviral in cause yet without diagnosis in as many as 60% of cases.[49]

One Health virology research recently conducted in South Africa has included investigation into previously undiagnosed causes of neurological syndrome in horses.[47,65,173,174] Two novel viruses have been detected and considered causative agents – Shuni virus (SHUV) and Middleburg virus (MIDV) (considered to have zoonotic potential).[173,174] The same research team identified old world alphaviruses in 52 of 623 horses with febrile or neurologic disease,

SINV in eight (five with mild signs and three severe and fatal cases – two of which involved co-infections), and MIDV in 44 horses (28 with neurologic disease and 12 fatal).[155] Subsequently, SHUV has recently been confirmed as a cause of previous unrecognised neurological infection and disease in humans in Africa.[176]

Similarly, research investigating infectious causes of equine neurological disease utilising metagenomic methods in the USA has identified a novel parvovirus, a previously described hepacivirus closely related to human hepatitis C virus (HCV), and EHV-2 in clinical cerebrospinal fluid samples.[175]

3.6 Conclusion

Managing Hendra virus (HeV) in Australian horses has met One Health disease risk challenges with great success. Biosecurity and One health management has integrated expertise, data, knowledge, functional networks, operational systems, and stakeholders, resulting in increased zoonotic disease awareness. HeV causes a usually fatal acute disease in horses mediated by an endothelial vasculitis. While clinical manifestations observed by consulting veterinarians vary, especially when constrained to a limited window of observation, consideration of the pathologic basis of disease aids recognition of HeV as a differential diagnosis. This review of differential diagnosis for HeV-like disease in Australia supports understanding that consideration of the pathologic basis of HeV-like disease in Australian horses also holds potential to increase diagnosis of a range of other RNA viruses of emerging One Health significance.

Although there is a diversity of potential viral pathogens circulating in Australian horses, wild animal species including bats, and biting arthropods, routinely available diagnostic testing targets a comparatively minimal range of agents. It is thus highly likely that alternative infectious causes of One Health and biosecurity significance, unknowingly contribute to HeV-like illness in a significant number of horses annually. International and human health perspectives support this hypothesis.

Advances in scientific knowledge and diagnostic techniques afford unprecedented diagnostic opportunity, including detection of novel pathogenic variants and species. Such diagnostic

techniques include highly sensitive multiplexed serology screening assays (Microsphere immunoassays utilising recombinant antigenic proteins) and advanced molecular techniques such as RNA-seq and other advancing NGS platforms such as Nanopore. Combining these systemically with existing traditional clinical, pathological, microbiological, and epidemiological diagnostic approaches, as well as with human and animal health biosecurity systems, mechanisms and policy, is fundamental to successful operation and interpretation. The application of advanced diagnostic testing beyond that routinely performed in investigation of Australian equine neurological disease has afforded recent diagnoses of WNV and MVEV (2011 epidemic), Elsey virus in 2011 and ABLV in 2013.

There is concern among veterinarians that infectious agents causing similar signs to HeV are going undiagnosed. Although many equine neurological and acute severe respiratory presentations in Australian horses feature characteristics of infectious causes, in most cases, no causative pathogen is identified. Regular and timely sampling of clinically affected horses for HeV testing offers a unique opportunity to increase understanding of possible under-detected equine diseases of high significance to industry and biosecurity, and novel or emerging pathogens. Horses are monitored closely for individual illness. They are often heavily exposed to biting arthropods and subject to spill-over infection with bat borne viruses (HeV and ABLV). These factors make them a highly suitable sentinel species for early detection of emerging infectious diseases of potential human and livestock significance.

Horses have already served valuable One Health sentinels internationally. Recent studies in America and South Africa investigating cases of equine neurological disease have identified numerous novel viruses of emerging zoonotic potential. Further research and optimisation of syndromic disease investigations in Australia will provide much needed additional knowledge and preparedness. The horse industry, veterinarians, human health professionals, horse owners and the public all stand to benefit, including with clarification of potential human health risk associated with direct contact with diseased horses. Diagnostic improvements should also benefit biosecurity, treatment, and outcomes for horses, sometimes compromised by a lack of causative diagnosis. Thus, great One Health benefit may be realised through further infectious disease investigation extending existing syndromic surveillance of acute severe equine illness.

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3.8 Supplementary Material for Chapter 3

Table S3.1 Hendra virus investigations by state and year (all species) #

Year	ACT	NSW	NT	QLD	SA	TAS	VIC	WA	Total
1995						2			2
1996		10		8		1	1	2	22
1997		7		6	2			2	17
1998		3		3	3	1		3	13
1999		11		6		1			18
2000		4		14		1			19
2001		4		3	1		1	1	10
2002		2		6				1	9
2003		2							2
2004		1		3					4
2005		2	1	3	1		1		8
2006		2	2	9			3	1	17
2007		16	1	6				1	24
2008		22	3	29	2	1	8	2	67
2009	2	77	5	400		1	5	5	495
2010	4	93	1	472	4		10	4	588
2011	1	304	4	931	19		23	30	1312
2012	1	79	14	518	10		12	13	647
2013		104	17	520	11		16	2	670
2014		195	8	543	11	3	25	9	794
2015		351	6	852	6	1	35	20	1271
2016		257	11	690	4	1	14	7	984
2017		280	8	632	9	3	98	12	1042
2018		209	9	586	1	2	24	17	848
2019		285	13	675	5	2	47	16	1043
2020	1	187	4	455	3	1	16	12	679
Total	9	2507	107	7370	92	21	339	160	10605

Table S3.2: Species tested as part of Hendra virus investigation, 1995 – 30/09/2020 #

Species	ACT	NSW	NT	QLD	SA	TAS	VIC	WA	Total
Alpaca		1						1	2
Bat			1			1		4	6
Camel				1				1	2
Camelid		1							1
Cat		3	1	16	1		3	1	25
Cattle		1	2		1	1			5
Dog		22	2	68			1	1	94
Eastern grey kangaroo				1					1
Horse (or Donkey)	9	2477	101	7279	87	19	334	149	10455
Goat								1	1
Pig		2		5	3		1	1	12
Sheep								1	1
Total	**9**	**2507**	**107**	**7370**	**92**	**21**	**339**	**160**	**10605**

Table S3.3: outcome of Hendra virus investigations in Australia, 1/7/2009 - 30/9/2020 #

Jurisdiction	Total	Aust. Total (%)	No. positive	Aust. prop. Positive (%)	State prop. Positive (%)
ACT	9	0.1	0	0.0	0.0
NSW	2411	23	26	0.3	1.1
NT	97	0.9	0	0	0.0
QLD	7205	70	52	0.5	0.7
SA	83	0.8	0	0	0.0
TAS	14	0.1	0	0	0.0
VIC	325	3.2	0	0	0.0
WA	147	1.4	0	0	0.0
Total	**10291**	**100**	**78**	**0.8**	0.8

Data presented in Tables S3.1, S3.2 and S3.3 were extracted from the National Notifiable Animal Disease Investigation (NNDI) program in NAHIS on 2 March 2021 using the date range 1/1/1995 to 31/12/2020. Data with the disease field 'Infection with Hendra virus' were then extracted into a separate file from which these table summaries reported. A small number of investigations (4) were unvalidated at the time of the query and were thus excluded. Each investigation may involve more than one animal; however, animal level data was not available. Most investigations (10434/10605; 98.4%) were in horses; investigations in alpacas, bats, camels, camelids, cats, cattle, dogs, donkeys, kangaroos, goats, pigs, and sheep are also in included in these summary figures with species break down specified in Table S3.2. Data afforded by the Department of Agriculture, Water and the Environment (DAWE) as part of their support of this research via Biosecurity Innovation Project 2020–21 scheme, Project ID 202043, Metagenomic Investigation of Horses as Sentinels.

Horses as Sentinels of Emerging Infectious Disease

Chapter 4

CLINICALLY FOCUSED CASE SERIES OF HEV INFECTED HORSES

Initially prepared with intention for peer-reviewed journal submission – adapted for thesis context and format.

Contributions: I drafted the first version of this manuscript in 2018, drawing on extensive first-hand clinical perspectives from veterinarians Dr Peter Reid, Dr Tim Annand, Dr Doug English, and Dr Greg Baldwin. I received manuscript editing support on various version from Dr Anne Jackson, feedback, and guidance from PhD supervisors, particularly A/Prof Jenny-Anne Toribio. I further acknowledge supportive feedback on earlier drafts of this manuscript from Prof Ruth Zadoks, Dr Nicole Brown and Dr Carly Garling.

4.1 Introduction

Emerging awareness of Hendra virus (HeV) spillover to Australian horses has seen the veterinary profession build strengths and realise challenges in managing One Health zoonotic disease risks. Outbreaks of HeV disease in horses arise from spillover from the virus reservoir in flying foxes (*Pteropus* spp.), posing risk of zoonotic infection for humans with close contact with infected-horses. (1)

Most HeV infected horses suffer pneumotropic and neurotropic while diffuse disease, which is rapidly progressive (acute) and severe, usually fatal, mediated by systemic endothelial vasculitis. Disease in infected humans follows the same path. Most infected horses demonstrate rapidly progressive severe disease featuring respiratory and/or neurological signs with variable pyrexia and injected/congested mucous membrane changes. Any single veterinary examination allows for clinical observations that represent a limited moment in time during the course of infection, with a wide range of signs thus possible including mild and non-specific manifestations − especially during early infection.

Transmission from flying foxes to susceptible horses is thought to predominantly occur via the oro-nasal route, during exposure to contaminated flying fox urine on paddock pasture and/or in association with flying fox feeding or water sources. Exposure to flying foxes is often perceived in confirmed spillover cases based on direct veterinary and/or owner-reported observations of flying foxes frequenting flowering trees and/or water bodies such as dams, creeks, or rivers, flying directly overhead and/or roosting in trees in the vicinity. Nevertheless, numerous spillover detections have featured no perceived exposure; thus HeV should be considered a potential cause of consistent disease in any unvaccinated horse living in a region within the range of flying fox species. (2)

Horses are inquisitive in their grazing behaviour, investigating and smelling the pasture they graze as well as any incidental animal excreta and miscellaneous objects such as spat (chewed fruit foods) that flying foxes may drop when flying overhead. These behaviours, together with their highly vascularised mucosal lined upper respiratory anatomy of large surface area, prominent olfactory neurology associated with their flehmen apparatus, and their high tidal volume may predispose their species to HeV spillover infection by both pneumotropic and neurotropic infection entry routes. (See Chapter 2)

Diagnosis of HeV infection in horses occurs as part of a priority disease investigation by quantitative real-time RT-PCR. Submission is reliant on suspect case recognition by the attending private practising veterinarians and their voluntary significant disease reporting and appropriate sample submission to government biosecurity laboratories (passive surveillance). Zoonotic infection risk management for in-contact humans, in-turn, relies on laboratory diagnosis of the infected horse to which they have been exposed. (3,4)

This case series of HeV infected horses extends from review of the pathologic basis of HeV disease in horses (Chapter 2). The series integrates first-hand veterinary perspectives with focused expert review highlighting the consistency of clinically observed disease when understood as manifestations of pathologic processes expected over the course of infection.

Clinical veterinarians and those engaged with surveillance and management of HeV One Health disease and biosecurity risks, should benefit in their recognition of suspect HeV disease cases for priority diagnostic investigation from the presented perspectives. Beyond this fundamental benefit, suspect case descriptions by clinicians and interpretations by duty pathologists as part of priority disease investigations are likely to be improved by consideration of this case series and associated pathologic review and syndrome analyses.

HeV spillover to horses is recognised across a wide geography with sporadic low frequency, yet each spillover event poses risk for high-consequence zoonotic disease. Increases in sensitivity of case recognition as part of passive surveillance activities should benefit disease preparedness, save human and animal lives and bolster Australia's biosecurity both directly (in relation to HeV) and for emerging zoonotic disease threats. Such potential has already been realised by this 'Horses as Sentinels' research, which enabled both retrospective discovery and subsequent prospective detection of a previously unrecognised HeV variant (HeVg2) (Chapter 8). (4)

Beyond linking the known pathologies of HeV disease to expected clinical presentation, this manuscript highlights that consideration of the horse-patient and location/farm contexts (field epidemiology) as well as priorities and perspectives of primary-stakeholders (those to whom the horse matter most – e.g. owners, trainers, and breeders) can also improve the

case detection and assist attending veterinarians in promoting informed stakeholder support for testing and compliance with biosecurity procedures.

Suspect case recognition and diagnostic submission has several challenges:

- early and non-specific disease manifestations coupled with variable veterinary examinations – this has led veterinary and/or primary stakeholder to lack confidence in recognising the disease
- cryptic spillover host exposure, in which flying foxes have not been seen in the area
- anticipated burdens and costs associated with significant disease investigation and biosecurity management requirements. These may include delay or restriction of veterinary treatment options when considering HeV as differential diagnosis; enterprise reputation damage and/or operation disruption; and professional or workplace health and safety liability.

The presented case series promotes veterinary consideration of cases in a cultural context (such as horse husbandry, purpose, sentimental and monetary value, and insurance) and describes epidemiological features that may be appreciated by consulting veterinarians to bolster their consideration of suspect HeV infection. These include:

- horse population, immune susceptibility, health and disease history and condition
- potential for flying fox exposure regionally and locally (on farm) including consideration of flowering and fruiting trees, flying fox flight paths and proximity to known colony roosts
- environmental features relevant to pathogen transmission opportunity including of pasture and trees and water source conditions.

We present retrospective veterinary descriptions of clinical and epidemiological observations and case events for nine laboratory-confirmed spillover events of naturally HeV infected horses (section 4.2), and two incidents of suspect HeV-infected horses in which diagnosis was not achieved (section 4.3). The descriptions are then considered within the context of the evolved veterinary knowledge of clinical HeV manifestations, identifying features, perspectives and approaches that have supported veterinary detection or acted as barriers to laboratory diagnosis respectively.

4.2 Chronological Case Series of HeV Infection in horses

4.2.1 Hendra, Queensland – September 1994

The first recognised and largest outbreak of HeV infected horses to date occurred in September 1994 at Brisbane racing stables. Publications associated with this outbreak focused on the predominance of respiratory disease manifestations of HeV as an acute equine respiratory syndrome featuring marked pyrexia, rapid progression to death and terminal frothy nasal discharge. (4) Dr Peter Reid was the consulting veterinarian for these cases. Dr Reid has contributed intellectual support to many aspects of this research, scientific and professional mentorship to the author, and acted as project partner and representative on behalf of the Australian national professional veterinary organisations (Equine Veterinarians Australia and the Australian Veterinary Association). This description is based on his previously unpublished first-hand clinical and contextual observations of the disease outbreak.

Of the 20 horses involved, 13 died or were euthanased in moribund condition on humane grounds. Of the seven horses that seroconverted and were later euthanased under government legislation orders of destruction, four were clinically unwell, one had demonstrated mild transient pyrexia of less than a day accompanied with dullness and two were not observed to develop clinical signs.

The index case (case 1) was transported to the stables from a peri-urban paddock. The paddock had dry, poor pasture and the resident horses received little supplementary feed. Grazing was underneath a Moreton Bay fig tree, and dried flying fox excreta was evident. The mare had been thought (but not confirmed) to be in foal but was not pregnant, had lost body condition and reportedly demonstrated mild ataxia at the time of transport.

On day 1, the mare was observed to be in very poor body condition, was obtunded with low head carriage and reluctance to move, and had anorexia, pyrexia (39.4°C), tachycardia (60 bpm), mild tachypnoea (24 bpm) and halitosis. She had oedematous swellings around the head. Mildly reduced lung sounds and abdominal borborygmi were evident by thoracic and abdominal auscultation respectively. No significant cough, ventral oedema, nasal or ocular discharge or abdominal pain (colic) were observed. Oral mucosae were uniformly injected to lightly cyanosed with a prolonged capillary refill time (2-3 seconds). The palpebral

conjunctivae were oedematous and hyperaemic. Un-masticated and un-swallowed food was evident on oral examination, remaining after attempts to manually introduce feed by the mare's carers.

The mare received parenteral broad-spectrum antibiotic and non-steroidal anti-inflammatory injections, resulting in no observable clinical improvement. Ataxia worsened and 20 hours after intial veterinary presentation, the mare collapsed several times and died without assistance. A small amount of clear, stable frothy nasal discharge was evident immediately before and after death.

Racehorses within the stable continued to exercise as usual in the following week. On day 12 following exposure to the index case, some horses demonstrated exercise intolerance and signs of exertional rhabdomyolysis. Fatal cases 2 to 13 developed per acute illness between day 12 and day 19. Within this period, nine horses died or were euthanased in less than 36 hours and seven of these in less than 12 hours. Cases 3 and 4 both raced on day 10 to expectation before developing acute severe progressive disease resulting in euthanasia on days 13 and 14.

Clinical signs and disease progression of the affected horses were consistent. Early signs were sudden onset of anorexia, depression, muscular weakness and marked pyrexia (39−41° C). Other signs as the illness progressed were worsening tachycardia (50 bpm acutely to exceeding 100 bpm terminally) and tachypnoea, progressing to hyperpnoea and dyspnoea.

Thoracic auscultation showed rapidly developing adventitious bronchiovesicular sounds. Terminally, air movement was difficult to detect, most likely due to pulmonary consolidation or marked oedema. Coughing was not a feature of the acute disease process. Nasal discharge was not apparent until close to death and varied from a serous (clear liquid) to white or bloody froth.

Mucous membranes were initially hyperaemic at the gingival margins. Changes progressed to generalised hyperaemia (bright red), hyperaemia and jaundice, then to a dark red appearance and finally a cyanotic appearance terminally, indicating hypoxia and anoxia. Capillary refill time was delayed, which suggested compromised peripheral perfusion. Some horses displayed petechial haemorrhages, which coalesced to progressing ecchymoses.

Other clinical signs included stranguria, reduction or loss of normal borborygmic activity evident by abdominal auscultation, signs of colic and reduced faecal output. Muscle fasciculations predominantly affecting the quadriceps and triceps muscle groups were observed in many fatal cases, and horses became progressively ataxic until they collapsed. Terminal horses leaned against walls or rested their heads and necks on stable doors or other supporting objects. After collapse, horses would generally struggle and seizure, often resulting in self harm and making veterinary intervention and euthanasia challenging because of human safety concerns. For example, case 3, euthanased on day 13, frantically washed and rinsed his head and mouth in water for hours before developing progressive ataxia, collapse, terminal violent agonal struggles and a bloody nasal discharge.

The time between the onset of clinical signs and death was typically two to three days. Spread was evident outside the index stable, as case 7 originated from a neighbouring stable on day 15 after it had routine castration performed nine days previously. The gelding was noticed by staff to have an obtunded demeanor on day 14 and veterinary attention was sought when he collapsed the following day. Tachycardia, congested cyanotic mucous membranes, limb paddling, tetanic convulsions, intermittent opisthotonus, and blood-stained frothy nasal discharge were evident. He was euthanased, and necropsy with laboratory testing confirmed HeV infection.

Of the seven horses that survived, having seroconverted to HeV and being euthanased subsequently, three survived acute illness at the primarily affected stable. One survived an acute illness described (incompletely) as showing ‘colic-like’ signs while at a spelling property. One horse sharing the same stable complex as case 7 displayed mild inappetence and transient pyrexia for less than 24 hours.

Surviving horses demonstrated some of the clinical signs seen in the fatal cases; however, they were generally less severe. Intermittent pyrexia, some feed intake and mild mucous membrane changes without progression were considered reasonable prognostic indicators for survival.

Two of the clinically affected surviving horses developed myoclonic muscle spasms, possibly neurological in origin. Case 19 developed twitching of the triceps muscles, which lasted three to four days before resolving. Case 16 developed myoclonic spasms of the lower lip

and mandible on day 18, progressing to involve the ventral neck musculature before resolving on day 20. This stallion exhibited convoluted tongue rolling movements while the mandibular and lip spasms were present, but he could prehend and masticate food, drink and swallow. Discreet bilateral erosions on the lateral borders of the tongue resulted from accidental self-trauma during involuntary 'jaw champing' motions. He developed myoclonic spasms in the right gluteal musculature on day 26, progressing to involve the quadriceps group, inner thigh and anterior tibial muscle and persisting until euthanasia on day 59. He occasionally rested the right hind leg on the toe but had no obvious abnormalities in voluntary limb movements, sensation, or proprioception.

Cases 17 and 20 seroconverted without clinical signs. A dry, non-productive cough was observed in two recovered cases.

4.2.2 Mackay Queensland 1994

Two 1994 HeV infected horse cases in Mackay, Queensland were retrospectively diagnosed late in 1995 following the testing of bio-banked samples. Testing was prompted by diagnosis of HeV infection as the causative agent of a fatal illness in a person that undertook their post-mortem examination. The pregnant mare showed ‘severe respiratory distress, ataxia and marked swelling of the head, particularly of the infraorbital fossa and cheeks’. The stallion displayed ‘aimless pacing, muscle trembling and a haemorrhagic nasal discharge’. In both cases death occurred within 24 hours.(5)

Between January 1999 and June 2008, six additional cases were confirmed in Queensland. In addition, there was one epidemiologically suspected but not laboratory confirmed case.

4.2.3 Trinity Beach, Cairns, Queensland January 1999

The third recognised HeV spillover was in a nine-year-old thoroughbred mare near Cairns, in north Queensland in 1999.(6) Initially, disease manifestations included inappetence, depression, and oedema of the face, lips and neck. Dry mucous membranes, prolonged mucous membrane capillary refill, lung auscultation consistent with pleural effusion, yellow frothy nasal discharge, and jaundice of the ocular sclera were observed on closer clinical examination. Differential diagnoses initially included bacterial pleuropneumonia, and colic signs were also recognised. Generally supportive treatment was administered, including

fluids and paraffin oil by nasogastric tube, involving significant exposure of the veterinarian to blood and bodily fluids. Veterinary support staff were also exposed to contaminated equipment and bodily fluids during cleaning. The mare died overnight, prompting those involved to consider the potential of Hendra infection. Government biosecurity laboratory testing was then associated with an approximate three-day turn-around due to distance. The diagnosis caused great personal health concern amongst the veterinary hospital staff given their considerable exposures. All staff were tested without any detections of human infection or seroconversion.

4.2.4 Redlands, Queensland - June (May) 2008

The second largest outbreak of HeV occurred in a veterinary referral hospital at Thornlands in the Redlands shire, southeast of Brisbane in 2008.

In the month before the first confirmed hospitalised case, three cases in contact with the hospital index case, all died with clinical signs consistent with probable HeV infection. Diagnosis was unconfirmed due to a lack of suitable samples to allow conclusive testing retrospectively. The cases experienced a sudden onset of clinical signs, fever, rapid deterioration, and respiratory or neurological clinical signs, including one horse displaying terminal frothy nasal discharge.(7,8) The first of the three cases developed acute onset of illness while residing in a paddock in a peri-urban region of southeast Queensland close to a flying fox roost before being transported, with pyrexia and ataxia, to the referral hospital and dying the following day (June 7). The CSIRO scientist who had overseen all horses experimentally infected with HeV in the laboratory believed that there was a high chance that the three horses were HeV cases. This opinion was based on their temporaneous disease, reported clinical manifestations, and their case proximities to the confirmed HeV-infected hospitalised index case.(7) Necropsies were not carried out, and conclusive retrospective HeV testing was not possible on archived fixed and stained blood smears.

The five clinical case presentations confirmed positive on laboratory testing and were described as predominantly neurological in origin compared with all previous case presentations.(9) Clinical signs reported from the five horses included sudden onset of illness, with a rapid disease progression resulting in death or euthanasia on humane grounds in four horses. Other signs reported were pyrexia, depression, anorexia, pawing of

the ground, and neurological signs of circling, ataxia, stranguria, head tilt, disorientation, apparent facial paralysis, leaning against walls, periods of recumbency, maniacal behavior and violent thrashing. One recovered horse that survived acute illness was described as presenting with sudden onset of anorexia, pyrexia, ataxia, recumbency and then gradual improvement until euthanasia (having seroconverted), 46 days after first presenting with clinical signs.

4.2.5 Bowen (Qld) September 2009

Two quarter horse geldings of advanced age (20 and 19 years) were kept in an extensive paddock on a family-run beef-cattle farm near Bowen. Both horses were retired and maintained, with limited owner involvement, for sentimental value given past family riding purpose.

Case 1 presented with a history of muscle fasciculations and reluctance to move – the owner noticed the horse standing in the same position since the previous day. A different ambulatory equine veterinarian had attended the horse the evening prior but had not suspected HeV infection.

Clinical examination determined: good body condition yet obtunded demeanor and weakness; muscular twitching (fasciculations) at approximately one second intervals; reluctance to move (ataxia and/or weakness); moderate tachycardia (55 bpm); mild to moderate congestion of the gingival mucous membranes; shallow rapid respiration and apparent diaphragmatic twitch (thumps). Teeth grinding was also observed without perceived pain and abdominal borborygmus was within the expected normal on auscultation.

The combination of abdominal and CNS signs and the presence of toxic crotalaria in the dry, sparse paddocks suggested the most likely differential diagnosis of acute liver failure. However, because the clinical signs were unusual and there was more awareness of the risks posed by HeV, further investigation using a belly tap was not performed. All equipment was washed in a bucket of Betadine scrub before leaving the premises.

On the afternoon of day 2, the horse had deteriorated significantly. The horse was administered broad spectrum antibiotics (procaine penicillin) and non-steroidal anti-inflammatories (flunixin, phenylbutazone), along with 8 litres of water via a nasogastric tube. By 10 pm, the horse had deteriorated further with tachycardia (62 bpm) and a muscle twitch every two seconds. The owner mentioned that the horse had been slightly 'off colour' for 10-14 days, and because he was deteriorating despite significant intervention, the horse was euthanased. A necropsy was performed in the field 18 hours after death. Upon initial opening of the abdomen, the distended intestine was lacerated accidentally, resulting in heavy contamination of clothes with intestinal contents.

Post-mortem examination showed that the liver appeared normal, and so crotalaria was an unlikely cause. The pelvic flexure region appeared abnormal, but no other significant intestinal abnormalities were detected. Samples were taken of liver and pelvic flexure, and fixed in formalin. The thorax was not opened because of the perceived risk of HeV or other zoonoses. The owner buried the remains.

Two days later, HeV was suspected in another horse on the same property. The owner was warned of the risk and was advised to quarantine the animal from other horses and human contacts. The horse had pyrexia (39.0° C), tachycardia (65 bpm), exaggerated chewing movements, teeth grinding, increased capillary refill time, and dry and mildly congested mucous membranes. Gastrointestinal borborygmi were minimal to absent on auscultation. Nasal swabs and blood samples were collected for submission to the state government biosecurity laboratory for prioritised HeV testing. The horse was administered epsom salts ($MgSO_4$) in water and paraffin oil via nasogastric tube, penicillin and phenylbutazone.

Biosecurity precautions included having a second veterinarian present, who remained in a defined decontaminated zone to provide equipment and materials from the vehicle. The attending veterinarian applied PPE intended to constitute Stage 5 PPE as limited by materials available. All procedures were performed at a distance and downwind from the vehicle. Any potentially contaminated materials required to leave the farm with the veterinarian were washed with Betadine or sealed in double plastic bags.

No clinical improvement was evident on examination of the horse the following morning when an absence of gastrointestinal borborygmi was noted on abdominal auscultation. The same medications were administered by a nasogastric tube to stimulate intestinal activity. There was no improvement in the evening, and he was euthanased. Necropsy was not performed and samples from both cases were forwarded to DPI.

Initial HeV positive laboratory results were given on day 8, and HeV confirmed by fluorescent antibody test at Australian Animal Health Laboratory (AAHL) on the intestine on day 9. On day 10, the other in-contact but asymptomatic mare (the only other paddock mate of the two diseased horses) was euthanased in compliance with state government biosecurity recommendations, because the property had limited options for sufficient quarantining from neighbouring (and wild) horses.

The horses had been held in flat riverine country adjacent to the river mouth, on flood plains that were nearly bare of grass. Horses chose to graze in the 2−3 m deep drainage washouts, which contained large Melaleuca trees and green grass. Bats were noted in the trees.

4.2.6 *Kuranda (Koah) (Qld) 2011*

A pony mare presented with neurological disease featuring circling and disorientation, raising suspicion of Hendra virus and prompt case attendance with stage PPE deployed. Immediate high concern for the potential of HeV infection prompted the attending veterinarian to advise all persons that had had contact with the pony to shower, change clothes and observe strict quarantine. A thorough physical exam was not undertaken given the zoonotic exposure risk, but distant examination revealed apparent blindness, neck muscle fasciculations and an absence of overt nasal discharge. The mare's condition progressively deteriorated throughout the day, with coma and agonal struggles followed by death on dusk. HeV was detected by PCR in rectal swabs and blood samples, but not in nasal swabs. A video of the affected mare is available online (https://youtu.be/o0EopcE2Lcc). The mare was one of 38 at a tourism riding facility that had more than 100 (mostly foreign) customers scheduled for rides the following day (a Monday). The attending veterinarian made the difficult decision to advise the facility to close, based on the high suspicion of HeV

infection and associated zoonotic risk. Diagnostic confirmation took three days, and the affected business is estimated to have lost approximately $40,000 in earnings when they were closed as part of biosecurity response for three months.

4.2.7 Murwillumbah (NSW) 2015

A 15-year-old stock horse gelding was found deceased in a paddock close to the owner's house (late June 2015). The farm where the gelding lived was a beef grazing property on the outskirts of the coastal township and bordered by the Tweed River. The property was lightly covered with Australian native trees, including rainforest species, and mature specimens of the introduced camphor laurel. Flying foxes had been observed visiting trees near the house and within the paddock where the gelding was kept. The owner reported having noticed that the gelding was listless, lethargic, and depressed, and had segregated himself from his three paddock mates, two days prior to being found dead. One of the paddock mates had been removed and taken to a nearby property in Chillingham, NSW at that time. The owner recounted having had minimal contact with the subsequently deceased horse on this day, limited to patting on the forehead (without PPE). The owner reported no subsequent contact with the gelding until discovery of his death.

The following morning, the consulting veterinarian attended to sample the horse, while wearing stage 5 PPE considering the relatively high probability of HeV infection, given the location, acute fatality without overt alternative cause and the season (winter). Jugular blood clot, urethral, rectal, oral, and nasal swab samples were obtained and submitted for priority government biosecurity laboratory testing. Manifestations consistent with HeV pathology were recognised by the attending veterinarian during their minimally invasive post-mortem examination and sampling, including blood-tinged stable foamy nasal discharge. The gelding was buried on site, near where it was found. The two other horse that remained on the property were examined from a distance, appearing of usual health and behaviour. HeV was detected in all submitted samples by TaqMan PCR. All horses residing on the infected premises, and those on the property to which the one horse had been relocated, were sampled, vaccinated against Hendra, quarantined, and monitored closely. A subsequent paddock assessment by the district veterinarian identified the likely source of the virus as a water trough close to a 'foaming bark tree' that flying foxes had been known to visit.

4.2.8 Casino (Qld) December 2016

In December 2016, a horse at Casino presented with inappetence, nasal discharge, ataxia, disorientation, weight loss and oral discomfort. The horse was reported to initially improve before dying approximately 18 days later. Samples including swabs and blood, collected on day 1, tested negative for HeV by PCR. Subsequently, high levels of immunoglobulin G (IgG) antibodies to HeV were detected by Luminex assay in sera collected on day 1. The positive test was confirmed by viral neutralisation assay, which featured a high neutralising antibody titre consistent with an immune response to natural infection. The virus or viral genome was probably not detected by PCR initially due to infection in the horse having already passed the acute stage. Infection could also have been with a variant genotypic lineage such as HeV-g2, known to have sufficient M gene nucleotide mismatches in the region targeted by the then available real-time HeV TaqMan PCR to fail detection. Nasal samples collected from the carcass approximately one week following death showed one weak PCR detection of HeV and two negative results. The circumstances, in this case, are consistent with a relapse or late-onset fatal encephalitis following an earlier sublethal infection in the horse, probably involving a low viral infectious dose. It is most likely that the PCR detection of HeV in one of the three post-mortem samples indicated the presence of a small amount of viral genome, possibly from autolysed brain material, rather than shedding of infectious virus. Alternatively, the weak detection may have related to this case having been due to a variant of the then known HeV strains (such as HeV-g2 identified by this research).

4.2.9 Murwillumbah (NSW) August 2017

In August 2017, an unvaccinated 13-year-old thoroughbred part-bred gelding presented to the same clinician that had attended the 2015 Murwillumbah case, with a three-day history of inappetence and lethargy. The horse was kept on a small acreage farm in Terranora, NSW. The gelding was a retired pony club horse, usually of good general health. The paddock was lined by trees along the boundary fence, consisting mainly of Australian natives, including *Melaluca* spp. Occasionally, flying foxes were known to have visited these and other trees on the property. The owners had been trying to force feed and water the horse during the three days prior to veterinary attendance.

Stage 5 PPE and highest biosecurity precautions were adopted by the consulting veterinarian, based on their perception of a relatively high probability of HeV infection. More specifically,

this assessment was based on: immune susceptibility (unvaccinated); the calendar timing of the presentation; and perceived consistency of clinical presentation with HeV infection and pathology (with which the attending veterinarian had first-hand experience – 2015 described above).

The gelding was tachycardic (HR 64), pyrexic (39.0° C), and had markedly reduced gut sounds in all abdominal quadrants with no ileo-caecal sounds. The mucous membranes were observed as 'markedly injected' featuring a 'toxic line' at the gingival margin. Small petechial haemorrhages were also noted on the oral mucosa above the incisors. The gelding presented in moribund condition, with low head carriage, ocular blepharospasm (squinting) and was markedly ataxic to the extent of being reluctant to move. He had the appearance of a horse that had been sedated.

The gelding was administered broad spectrum antibiotics (procaine penicillin) and non-steroidal anti-inflammatory (phenylbutazone) medications. Blood samples and swabs (oral, nasal and rectal) were collected and submitted to NSW DPI laboratory for priority disease instigation including HeV PCR.

The attending veterinarian communicated to the owners regarding the high probability that the gelding was infected with Hendra virus, and of the likelihood that the gelding would die overnight, given the severity of presenting signs. Euthanasia was considered but the veterinarian opted against this, given the associated human exposure risk. The gelding died of HeV disease overnight and was buried under the supervision of the district veterinarian.

4.2.10 Cobaki, Tweed Shire (NSW) September – October 2018

An unvaccinated four-year-old Arabian part-bred mare presented in moribund condition with nystagmus, severe pyrexia (42.0° C), tachycardia (130 bpm) and respiratory distress (dyspnoea, resp. rate 60 bpm). The owner had noticed lethargy and inappetence the day before. The mare's condition deteriorated rapidly, with severe disease manifestations including ataxia and marked proprioceptive deficits noted by the following morning, progressing to inability to stand and recumbency persisting some hours until veterinary consultation.

The attending veterinarian noted moribund and hypotonic posture on their arrival, progressing to flaccid lateral recumbency (unable to lift head or correct head posture). Intermittent 'paddling' of the forelimbs and distressed breathing were also remarkable, increasing the attending veterinarian's suspicion of HeV infection. The veterinarian wore PPE for the examination, which determined marked pyrexia (42° C) and 'maroon' mucous membrane discolouration (heavily congested), overt nystagmus and rapid distressed breathing, prompting euthanasia by lethal injection with owner consent.

Blood (serum clot and EDTA), nasal, oral, ocular, vaginal and rectal swabs were obtained, and instructions were communicated on the use of PPE and appropriate carcass disposal (burial). The attending veterinarian couriered the samples to the Queensland government biosecurity laboratory (QDAF BSL) personally to expedite results, in view of the high perceived owner exposure.

The only two horses in direct contact (paddock mates) were sampled by a NSW department of biosecurity field veterinarian on 10th October and sent to the laboratory at Elizabeth Macarthur Agricultural Institute (NSW) on the same day − blood (serum, EDTA and lithium heparin tubes) with nasal, oral, and rectal swabs in PBGS. Negative qPCR results were reported for all (interim) the following day, 11th Oct.

4.2.11 Murwillumbah (Stokers Siding) (NSW) 2020

Having attended numerous HeV infected cases, the attending veterinarian considered this case, a 17-year-old thoroughbred in current ownership for two years and maintained on a single property, to be somewhat atypical, given that pyrexia did not feature. Nevertheless, they held high suspicion of HeV infection based on the case details related by the owner by phone, which included unusual behaviour and disorientation. The normal rectal temperature (37.6° C) made the veterinarian question their suspicion of HeV infection. However, other manifestations including severe lethargy, marked tachycardia (approx. 80–90 bpm), teeth grinding, cyanotic mucous membranes and abnormal behaviour were sufficient to maintain their suspicion. Treatment including broad spectrum antibiotics (oxytetracycline) were administered, rather than advising to immediately euthanase, based on the diagnostic doubt cast by the lack of pyrexia.

Differential causes considered by the attending veterinarian included Crofton weed toxicity, however, none was found on a paddock walk. The owner had not heard of Hendra virus.

The following morning, a Saturday, the horse's condition had severely deteriorated. Repeat examination revealed hypothermia (36° C), and moribund condition, justifying euthanasia by lethal injection with owners' consent. The owners dug a hole by hand with help from their neighbours to bury the horse, which they had done before the positive test results became available. This potentially high exposure justified two in-contact persons to receive emergency post-exposure therapy with monoclonal antibody m102.4 on compassionate grounds.

Of note, beyond extensive experience with numerous confirmed HeV cases, the attending veterinarian had heightened awareness for HeV at the time, due to their having attended another highly suspect case just one week earlier. This was a young, well-fed pony that was unable to rise, dyspnoeic and hypothermic (36.2° C), but which tested negative for HeV by PCR. This pony was treated for three days with antibiotics prior to receiving the negative test result and died of its HeV-like disease only hours after the results became available, without causative diagnosis.

A summary of the clinical signs of HeV cases are summarised below (Table 4.1).

Table 4.1. Hendra virus cases and signs of disease (adapted from https://www.business.qld.gov.au/industries/service-industries-professionals/service-industries/veterinary-surgeons/guidelines-hendra/incident-summary)

Date	Location	Confirmed cases (Unconfirmed cases)	Clinical signs and observations	Notification of the cases
August 1994 #	Mackay/ Qld	2	**Horse 1:** Severe respiratory distress, ataxia, marked swelling of the head (infraorbital fossa and cheeks). Death within 24 hours **Horse 2**: Pacing, muscle trembling, haemorrhagic nasal discharge. Death within 24 hours	https://pubmed.ncbi.nlm.nih.gov/8894044/
Sept. 1994 #*	Hendra, Brisbane/ Qld	7 (13)	Acute equine respiratory syndrome, pyrexia, rapid progression to death and terminal frothy nasal discharge (see section 4.2.1 for further details)	Reid P, personal communication, Selvey LA, Wells RM, McCormack JG (1995). 'Infection of humans and horses by a newly described morbillivirus'. *Medical Journal of Australia*. **162** (12): 642–5.
January 1999 #*	Trinity Beach, Cairns/ Qld	1	Depressed and inappetant, oedema of the face, lips and neck. Dry mucous membranes, capillary refill sluggish, excess pleural fluid, a yellow frothy nasal discharge, and jaundice of the ocular sclera	https://onlinelibrary.wiley.com/doi/epdf/10.1111/j.1751-0813.2000.tb11758.x
October 2004	Cairns/ Qld	0 (1)	Restlessness, elevated heart rate, increased respiratory effort and profuse sweating, fever, blood-stained frothy secretions issuing from the nose. A veterinarian was confirmed serological positive for HeV after performing a necropsy on a horse that died suddenly with signs consistent with HeV.	Hanna, JN; McBride, WJ; Brookes, DL (2006). "Hendra virus infection in a veterinarian". *Medical Journal of Australia*. **185** (10): 562–4
December 2004	Townsville/ Qld	1	Depressed, fever, elevated heart rate and respiratory rate, brownish nasal discharge	As above
June 2006	Peachester/ Qld	1	Opisthotonus, restlessness, vocalising (terminal), elevated heart rate, elevated respiratory rate, fever, markedly swollen lips, purple gums, died suddenly	As above
October 2006	Murwillumbah/ NSW	1	Lethargic, ataxic, penile erection, disorientation, mandibular swelling, dyspnoea, fever, wide-based stance, high stepping gait, occasional tongue protrusion, coughing	Notifiable Diseases: Hendra Virus" (PDF). *Animal Health Surveillance*. **4**: 4–5. 2006
June 2007	Peachester/ Qld	1	Wide-based stance, rocking, relaxed penis, colic signs, inappetant, lethargic, depressed demeanour, elevated heart rate, recumbent and unable to rise	
July 2007	Clifton Beach, Cairns/ Qld	1	Nasal discharge, wet lung sounds, fever, elevated heart rate, elevated respiratory rate, terminal neurological signs	ProMED Archive Number 20070903.2896
June 2008 #	Redlands/ Qld	5 (3)	**Horses 1–3:** Unknown **Horse 4**: Inappetant, depressed, maniacal/erratic behaviour **Horse 5**: Head tilt, ataxic, circling, inappetant, depressed, fever, recumbent periods **Horse 6**: Central neurological signs, severely ataxic, inappetant, depressed, recumbent, thrashing violently **Horse 7**: Depressed, deteriorated rapidly **Horse 8**: Febrile, depressed	ProMED Archive Number: 20080708.2076
July 2008	Proserpine/ Qld	3 (1)	**Horse 1**: Unknown **Horse 2**: Some respiratory manifestation, head down, non-responsive, elevated heart rate, swollen muzzle, recumbent, red fluids from mouth, died **Horse 3**: Short neurological illness, ataxic, recumbent, horse appeared very stressed, trouble standing and walking **Horse 4**: High stepping, dull	ProMED Archive Number: 20080725.2260
July 2009	Cawarral/ Qld	3 (1)	**Horse 1**: Unknown **Horse 2**: Heavy breathing, nasal froth, fever, elevated heart rate, elevated respiratory rate, blood slow to clot, difficulty walking, collapsed, died **Horse 3**: Progressive neuromuscular spasms, incoordination, died **Horse 4**: Possible mild neurological signs − weaving, head pressing, ataxic on day of euthanasia	ProMED Archive Number: 20090826.2998

Table 4.1. (Continued)

Sept.r 2009 #*	Bowen/ Qld	2	**Horse 1**: Shallow respiration, diaphragm twitching, muscle twitching, short stepping, reluctant to move, elevated heart rate, teeth grinding, head down, congested oral mucous membranes **Horse 2**: Foam from mouth and nose, laterally recumbent with extensor rigidity, no gut sounds, fever, elevated heart rate, oral mucous membranes dry and congested, weak, jaw champing, teeth grinding, rapid deterioration	ProMED Archive Number: 20090910.3189
May 2010	Tewantin/ Qld	1	Twitching of mouth muscles, blindness, ataxic, seizures, inappetant, lethargic	ProMED Archive Number: 20100520.1673
June 2011	Beaudesert/ Qld	1	Increased respiratory effort, hind limb incoordination, depressed, fever, congested oral mucous membranes with petechial haemorrhage, died	ProMED Archive Number: 20110629.1984
June 2011	Boonah/ Qld	3	**Horse 1**: Colic, recumbent, thrashing **Horse 2**: Twitching of muscles and eyes **Horse 3**: Slight nasal discharge, dull, depressed, fever, elevated heart rate	ProMED Archive Number: 20110629.1984
June 2011	Logan/ Qld	1	Ataxic, mild colic signs, fever, elevated heart rate, bloody ocular discharge	ProMED Archive Number: 20110725.2243
June 2011	Wollongbar/ NSW	2	**Horse 1**: Fever, congested mucous membranes, ataxic with wide-based stance, asymmetrical facial paralysis, blindness, euthanased **Horse 2**: Depressed, fever, slightly ataxic, dyspnoea, copious amounts of nasal foam bilaterally after euthanasia	ProMED Archive Number: 20110702.2012
July 2011	Park Ridge/ Qld	1	Incoordination, fever, very weak, progression to death overnight	ProMED Archive Number: 20110706.2045
July 2011	Macksville/ NSW	1	Sudden onset depression, blindness, head pressing, died 36 hours after first signs	ProMED Archive Number: 20110710.2084
July 2011 #*	Kuranda (Koah)/ Qld	1	Ataxic, disoriented, neck muscle fasciculation, circling, inappetant, recumbent, depressed	ProMED Archive Number: 20110715.2149
July 2011	Lismore/ NSW	1	Found dead. Observed normal by owners 24 hours previously	ProMED Archive Number: 20110717.2167
July 2011	Hervey Bay/ Qld	1	Attempting to stand but stumbling, dry faeces, fever, elevated heart rate, oral mucous membranes injected, capillary refill time less than 4 seconds	ProMED Archive Number: 20110716.2158
July 2011	Boondall/ Qld	1	Mild clear nasal discharge, acute onset ataxia, intermittent inappetence, lethargic, fever	ProMED Archive Number: 20110716.2158
July 2011	Chinchilla/ Qld	1	Terminal nasal discharge, ataxic, recumbent, dull demeanour, respiratory signs	ProMED Archive Number: 20110725.2243
July 2011	Mullumbimby/ NSW	1	Found dead. Observed normal by owners 18 hours previously	ProMED Archive Number: 20110729.2274
August 2011	Ballina/ NSW	1	Slightly ataxic then recumbent 12 hours later, muscle twitching, unable to stand and euthanased 3 hours later	ProMED Archive Number: 20110818.2512
August 2011	South Ballina/ NSW	2	Both horses (mare and foal) found dead. Absentee owner	ProMED Archive Number: 20110818.2512
August 2011	Mullumbimby/ NSW	1	Found dead. Observed normal by owners 15 hours previously	ProMED Archive Number: 20110818.2512
August 2011	Gold Coast Hinterland/ Qld	1	Gait problem, lethargic, fever	ProMED Archive Number: 20110823.2570
August 2011	North Ballina/ NSW	1	Depressed, ataxic, wide-based stance. Found dead approximately 12 hours after onset of first signs	ProMED Archive Number: 20110830.2666
October 2011	Beachmere/ Qld	2 (1)	**Horse 1**: Unknown **Horse 2**: Ataxic, large distended bladder, decreased gut sounds, lethargic **Horse 3**: No clinical signs observed	ProMED Archive Number: 20111012.3057

Table 4.1. (Continued)

January 2012	Townsville/ Qld	1	Bilateral serous nasal discharge, blind, ataxic, sudden brief irritation, dull, fever, elevated heart rate, elevated respiratory rate, oral mucous membranes injected, capillary refill time greater than 4 seconds, clotting time 2 minutes, ileus, facial swelling	ProMED Archive Number: 20120106.1001359
May 2012	Rockhampton/ Qld	1	Elevated respiratory rate, fever, bilateral frothy nasal discharge, muffled heart sounds, bilateral epistaxis at death	ProMED Archive Number: 20120531.1151213
May 2012	Ingham/ Qld	1	Fever, ataxic, circling, hanging head, dull demeanour	ProMED Archive Number: 20120531.1151213
June 2012	Mackay/ Qld	1	Horse found moribund	ProMED Archive Number: 20120728.1218748
July 2012	Rockhampton/ Qld	3	**Horse 1**: Ataxic, apparent blindness, droopy bottom lip and salivation **Horse 2**: Off food, reluctant to move, extended neck, muscle fasciculation, increased respiratory effort **Horse 3**: Off food, dull demeanour, reluctant to move, hanging head, droopy bottom lip, ataxic, mild increase in respiratory effort, pawing at ground	ProMED Archive Number: 20120728.1218748
July 2012	Cairns/ Qld	1	Inappetant, tremors, staggering, neurological signs worse on handling, slight nasal discharge, elevated heart rate, elevated respiratory rate, fever, delayed capillary refill time, congested conjunctival and oral mucous membranes, grinding teeth, penis protruding, muscle fasciculation, blood from nose at death	ProMED Archive Number: 20120728.1218748
September 2012	Port Douglas/ Qld	1	Ataxic, high stepping (left side), death	ProMED Archive Number: 20120907.1284588
October 2012	Ingham/ Qld	1	Anorexia, slight bilateral nasal discharge, laboured respiration (respiratory rate 20), heart rate 60 bpm, lowered head, unsteady on feet, progression to recumbency	'Horse dead after contracting Hendra virus'. Australian Broadcasting Corporation. 3 November 2012
January 2013	Mackay/ Qld	1	Slow moving, ataxic, absent blink reflex, found dead approximately 24 hours after clinical signs were first noticed	ProMED Archive Number: 20130124.1512182
February 2013	Atherton Tablelands/ Qld	1	Slow moving, off food, ataxic. Found dead several days after the onset of clinical signs	ProMED Archive Number: 20130224.1557005
June 2013	Macksville/ NSW	1	Observed normal in the early morning and found dead in the late afternoon	ProMED Archive Number: 20130612.1768141
June 2013	Brisbane Valley/ Qld	1	Depressed, unsteady on feet, reluctant to move, elevated heart rate and muddy mucous membranes, deteriorated and euthanased	ProMED Archive Number: 20130626.1792190
July 2013	Gold Coast Hinterland/ Qld	1	Off feed, dull, lethargic, elevated respiratory rate and mild bilateral serous nasal discharge, deteriorated to collapse, intermittent convulsions and unresponsive state, euthanased	ProMED Archive Number: 20130712.1820724
July 2013	Macksville/ NSW	1	Ataxic, weak, stumbling and rolling, euthanased	ProMED Archive Number: 20130721.1837123
July 2013	Kempsey/ NSW	1	Ataxic, elevated heart rate, mild fever; neurological signs include loss of balance, staggering and laterally recumbent	ProMED Archive Number: 20130712.1820724
July 2013	Kempsey/ NSW	1	Observed seriously ill and failed to respond to antibiotics. The horse died the following day	ProMED Archive Number: 20130712.1820724
March 2014	Bundaberg/ Qld	1	Off food, elevated respiratory rate, injected gums, frothy nasal discharge after death	ProMED Archive Number: 20140320.2343538
June 2014	Beenleigh/ Qld	1	Off food, small amount of green nasal discharge turned bloody at later stage, ataxic, mild fever and toxic mucous membranes	ProMED Archive Number: 20150729.3543733
June 2014	Murwillumbah/ NSW	1	Found in swamp unable to stand. Low body temperature; died overnight	ProMED Archive Number: 20140621.2557020

Table 4.1. (Continued)

July 2014	Gladstone/ Qld	1	Altered gait, off food, depressed, mild mucoid nasal discharge, died overnight	ProMED Archive Number: 20140721.2626012
June 2015 #*	Murwillumbah/ NSW	1	Lethargy for 2-3 days before death	ProMED Archive Number: 20150625.3463123
July 2015	Atherton Tableland/ Qld	1	Observed acutely ill 3 days before death. Ataxia, discharge, no respiratory distress	'Hendra virus case confirmed after horse dies in North Queensland'. Australian Broadcasting Corporation. 24 July 2015.
September 2015	Lismore/ NSW	1	Observed to be unwell, off food, before it collapsed. Horse was euthanased	ProMED Archive Number: 20150905.3625268
September 2015	Gympie/ Qld	1	Acute disease onset with rapid deterioration over 24 hours. Depressed (obtunded) demeanour, injected/congested gingival mucous membranes (darkened red/purple change with darker periapical line and prolonged capillary refill time), tachycardia (75 beats/min), tachypnoea (60 breaths/min), normal rectal temperature (38.0° C), muscle fasciculations, head pressing and collapse, euthanased	Annand et al., 2022
Dec. 2016 #*	Casino/ NSW	1	The horse had been through a period of illness. Initial clinical signs: failure to graze, nasal discharge, some ataxia, mild disorientation, weight loss and oral discomfort. Further behavioural abnormalities were seen prior to death	ProMED Archive Number: 20161223.4720721
May 2017	Gold Coast Hinterland/ Qld	1	Depressed, inappetant, not walking, mild fever, elevated heart rate and respiratory ate, euthanased after rapid deterioration	ProMED Archive Number: 20170526.5065372
July 2017	Lismore/ NSW	1	Off feed, wobbly on feet, lethargic; euthanased after health condition deteriorated	ProMED Archive Number: 20170709.5162048
August 2017 #*	Murwillumbah/ NSW	1	Lethargic and not eating properly, unsteady on its feet and unwilling to move, decreased gut sounds, elevated temperature and poor blood circulatory function	ProMED Archive Number: 20170805.5229510
August 2017	Lismore/ NSW	1	Observed unusually quiet and disorientated one day prior to showing clinical signs – fever, increased respiration, poor circulation, teeth grinding; euthanased after rapid health deterioration	'Hendra virus confirmed near Lismore - By NSW DPI'. NSW Department of Primary Industries. 9 July 2017.
Sept. 2018 #*	Tweed Heads/ NSW	1	Depressed and not eating. Worse by the next morning, fever and staggering; euthanased	ProMED Archive Number: 20180915.6030923
June 2019	Scone, Upper Hunter Valley/ NSW	1	Sudden onset of neurological signs and unresponsive; euthanased. Of significance this diagnosis prompted hitherto unrecognised risk for HeV in this prominent horse breeding region. Diagnosis was challenged by delays in sourcing veterinarian with experience and willingness to sample and submit the case for priority government laboratory testing, due to widespread perception amongst vets in region of no risk of HeV in the region. Persistence by attending general practitioner, based on perceived consistency of disease and of possible human exposure, and sampling by Local Land Services veterinarian based in neighbouring region 3 hours away with experience working in HeV infected regions previously.	ProMED Archive Number: 20190621.6531307 Personal comm.
June 2020 #*	Murwillumbah (Stokers Siding)/ NSW	1	Depressed and having difficulty breathing, euthanased after rapid health deterioration	ProMED Archive Number: 20200602.7420665
October 2021	West Wallsend, Newcastle/ NSW	1	Neurological signs. Unvaccinated against HeV, euthanased after rapid health deterioration	ProMED Archive Number: 20211016.8699079

case description/s included in this case series, * including first-hand attending veterinarian description.

4.3 Clinical case reports of severe equine disease events for which HeV or related bat-borne paramyxovirus was considered most likely, yet without laboratory diagnosis

The following two suspect HeV or related bat-borne virus spillover events were attended by the author of this thesis at varying stages of clinical experience. The first case was attended one year after graduation and prior to greater awareness of wide-ranging clinical manifestations that followed the second recognised larger natural HeV infection spillover (occurring almost simultaneously in Redlands). The second outbreak event was attended the year following the experience of the first known cases of ABLV in two horses originally tested for HeV. Epidemiological, pathological and clinical features in both cases strongly support probability of infection with HeV or a related bat-borne virus, and yet in both cases causative agent diagnosis was not achieved.

4.3.1 HeV suspect disease, Proserpine, July 2008

The horse was seen for acute progressive infectious-like fatal hemorrhagic disease involving severe quadriparesis and respiratory distress with prolific hemorrhagic respiratory failure. The attending veterinarian was consulted earlier the same day when mild illness including lethargy and mildly increased respiratory rate was noted by the owner in one of a small group of horses maintained extensively (seen only every two weeks or so), in a paddock where mango trees were growing and frequently visited by flying foxes. The horse was lethargic, perceived to have lost weight/condition, anorexic, with mild dehydration. A unilateral firm painless mass was palpable near the larynx. The horse was normothermic and had normal heart rate. Lung field auscultation revealed broadly increased respiratory sounds and respiratory rate and a mild mucoid nasal discharge. Dental/oral examination revealed no significant abnormality and rectal examination findings were normal. Nasal swabs were taken.

Parotid lymph node abscess due to *Streptococcus equi* or neoplasia were considered as leading differential diagnoses by the veterinarian, and the horse was started on a course of procaine penicillin. A fine needle aspirate performed on the mass produced blood-stained fluid; in house cytology showed blood and many leucocytes and was potentially consistent with an abscess.

The same evening the horse unexpectedly collapsed, was in severe respiratory distress (laboured and rapid breathing) and was not able to rise. On injection of flunixin, the venipuncture site haemorrhaged subcutaneously. Generalised flaccid paralysis was noted, with no resistance to manual manipulation of the hind limbs through extension and flexion, and absence of patellar reflex in both hind limbs (assessed in lateral recumbency with unlocked stay apparatus). A nasogastric tube was passed while the horse was in lateral recumbency, unusually producing more than 1 L of blood mixed with gastric fluid via the tube lumen, suggesting internal haemorrhage. There was marked hypothermia (36.2° C), and blood collected into serum tubes appeared to have longer than normal coagulation time.

The horse was euthanased. Necropsy showed two small pale liver masses that were a similar consistency to the laryngeal mass and were thought to be unrelated to death. Free blood was present in the lung parenchyma, trachea, and stomach, suggesting coagulopathy. There was no evidence of gastric ulceration or gastric neoplasia.

The veterinarian concluded that the respiratory abnormality might not have been related to the acute signs. The combination of haemorrhage, lack of patellar reflex[1], hind limb paralysis and the presence of numerous snakes on the property supported the high likelihood of snake envenomation. No further testing was performed. There was concern about the unusual clinical presentation, the knowledge of the flying foxes frequenting mango trees on the property, and the veterinarian's exposure to blood, because all procedures were performed with only latex gloves as PPE.

One month later, a case of HeV was confirmed 10 km away at Proserpine. A senior clinician from the practice of the attending vet became concerned that this case may have been infected with HeV. The extensive property was in close proximity and the horses may conceivably have shared fence-line contact. The consistency of the clinical presentation as recounted and reported in the practice system was also suggestive. His discussion with a Department of Primary Industries (DPI) veterinary biosecurity officer confirmed that the

[1] Assessment of patella reflex is rarely used in equine practice – the clinician (EJ Annand) performed the same technique on horses and foals when opportunity arose (lateral recumbency) confirming the abnormal finding in years that followed.

level of suspicion indicated further investigation, including testing of the stored samples. However, when consulted by phone the attending veterinarian (the author of this thesis in their first year following graduation) considered the risk excessively low based on their perception that the signs and presentation were so conclusively consistent with snake envenomation, no consistent frothy discharge had been observed (as had been described in undergraduate their training). No further action was taken on this basis.

4.3.2 HeV or related bat borne Paramyxovirus suspect outbreak, Dayboro, August 2014

A miniature pony (case 1) developed neurological symptoms (aimless walking reported by the carer) that had rapidly worsened over 24 hours on the 4th of August 2014. Seven horses (two of which were partially vaccinated for HeV only) were maintained in a paddock in Dayboro, Qld, that backed directly onto a water source heavily frequented by a large flying fox population. Night-time flying fox activity on the property was noted to have been particularly high over the preceding weeks.

Additional case history reported to the attending veterinarian included that the mare (Case 1) had eaten as usual the evening before overt illness, but 'was not her usual bossy self'. Close observation over the following hours did not reveal further overt signs of illness to the carer. The following morning the mare was clearly demonstrating neurological disease including frequent whinnying, pacing aimlessly, hypermetric gain all four limbs, ataxia, ocular discharge (clear) and 'dazed look in the eyes'. No toxic plants were known to occur in the paddock and the only ration supplementing grazing was lucerne hay; this was examined and found to be normal.

By midday the mare's condition had deteriorated severely, prompting urgent veterinary consultation. Clinical examination findings included injected/congested and mildly cyanotic mucous membranes, tachycardia (60 bpm), diffusely increased lung sounds on auscultation, respiratory distress (resp. rate 120), absent borborygmi on auscultation over all four GIT abdominal quadrants, marked pyrexia (40.8° C), frothy oral excretions and a range of neurological disease manifestations. These signs included vertical head shaking, profound ataxia, altered proprioception, unusual behaviour (attracted to dam in paddock from which it was repeatedly retrieved by carer) and posture ('dog sitting' and falling into fence), reduced vision/awareness, hyperaesthesia and muscle fasciculations. An abdominocentesis

sample was obtained of normal consistency and colour. Blood and swab (oral mucosal, rectal mucosal and nasal) samples were obtained before euthanasia was performed by lethal injection, justified by moribund condition with owner consent. Two skin biopsies that included facial sensory hair follicles (palpebral and muzzle) were collected immediately post-mortem.

The six paddock mates were then examined clinically. A 12-year-old unvaccinated thoroughbred gelding (case 2) demonstrated inappetence, mildly obtunded demeanour (lethargic with low head position), increased digital pulse amplitudes and extended forelimb stance (history of laminitic disease), mild tachypnoea (20bpm) with broadly elevated sounds on auscultation of the lung fields (most evident on deep breathing prompted by a modified 're-breathing examination'), and pyrexia (40.3° C). Treatment administered included broad spectrum antibiotics (procaine penicillin and gentamicin), nonsteroidal anti-inflammatories (phenylbutasone), along with electrolyte fluid (8 L), charcoal and paraffin oil by nasogastric tube. Blood and swab samples were collected as for Case 1 for government laboratory testing and quarantine instructions were communicated.

A third horse demonstrated pyrexia (39.7° C), mild dehydration and mildly increased lung-field auscultation (on modified rebreathing) with no other signs of illness. This horse had received a primary dose of HeV vaccine in the preceding fortnight and only blood samples were collected. The other four horses were clinically normal.

All samples from Case 1 and 2 were submitted for priority HeV testing as part of the government biosecurity significant disease investigation. Biosecurity instructions were provided, including on the use of PPE, and the carcass of Case 1 was covered and quarantined from horse access and to protect against predation. Given the high perceived diagnostic responsibility, including for zoonotic disease agents, post-mortem was planned for the following day, providing HeV and ABLV test results supported relative procedural biosafety.

Real time TaqMan PCR assays targeting HeV and ABLV, performed on the oral swabs of Case 1 and 2, were negative allowing for post-mortem examination on Case 1 approximately 24 hours after euthanasia. Abnormal findings (see Figure S4.1, Panels A – H) included: marked

haemorrhagic congestion of both lung lobes with mottled haemorrhagic consolidations apparent on the serosal surface and bloody tissue consolidation evident on section, and bloody pleural effusion on the non-gravitationally dependant side; marked pericardial haemorrhage; intra-cranial haemorrhage with petechiae of the meninges adjacent to the cerebellum; and sub-serosal bleeding associated with small intestinal lymph nodes. Samples collected included brain and cervical spinal cord tissue portions submitted both fresh chilled and fixed, lung (also fresh chilled and fixed), along with kidney, liver, and spleen (fixed).

The remaining six horses were re-examined on this following day. Case 2 demonstrated persistent pyrexia (40° C despite further nonsteroidal anti-inflammatories) and reduced GIT borborygmi. Case 3 demonstrated persistent pyrexia (39.4° C), mildly increased lung sounds, and a prolonged skin tent consistent with 5% dehydration. A 34-year-old miniature pony gelding (Case 4), that had presented normally the previous day, demonstrated diffusely increased bronchovesicular sounds (not wheezes or crackles) only on re-breathing test.

Subsequent government laboratory results included no detection of EHV-1 and -4 by qPCR on nasal swabs and lung samples, or ABLV by multiplex qPCR on oral swab, brain and spinal cord. Fluorescent antibody assay performed on impression smears of the brain and spinal cord also tested negative. Aerobic bacterial culture of the cerebrum and lung resulted in no significant growth. Histopathology interpretation was greatly limited due to sample degeneration and autolysis. There was marked congestion of lung parenchyma, and congestion of the meninges with moderate numbers of red blood vessels in extravascular connective tissue. Samples were not retained for further investigation as part of routine operations as there was reduced laboratory cold storage capacity at the time.

4.4 Specific case contexts as barriers and facilitators of HeV and EID diagnostic processes

Consideration of case-specific field clinical, epidemiological and cultural case contexts can highlight context-variable barriers and facilitators for diagnostic processes for HeV and related significant emerging viral infections. By way of explanation, preliminary considerations of clinical, epidemiological, procedural and cultural features of cases described in this manuscript, identified to have likely influenced diagnostic processes, have been synthesised in as potential facilitators (Table 4.2) and barriers (Table 4.3). Such intelligence could support strategic biosecurity risk management.

Table 4.2 Clinical, epidemiological, procedural, and cultural case features that likely acted as promotors of suspect case recognition and diagnosis

			Case features or context promoting or facilitating suspect case recognition and laboratory diagnosis									
			Epidemiological			Clinical			Procedural		Cultural	
	Perceived Diagnostic responsibility	Diagnostic outcome	Numerous cases and/or perceived case transmission by proximity or contact	Perceived flying fox exposure	Perceived or potential significant human exposure or synchronous disease	Attending veterinarian familiarity with HeV or other EID (ABLV) in horses	Attending veterinary experience generally	Proactive interpretation of suspect pathologic bases of clinical disease	Veterinarian familiarity with diagnostic options available	Veterinarian familiarity with sample submission protocol and commitment to overcome practical barriers	Veterinary consideration of primary stakeholder experiences to promote investigation yet avoid burdens	Owner/carer preferences or horse purpose promoting diagnostic responsibility
HeV diagnosed events												
Hendra Qld Sept 1994,	High increasing to very high with human illness	20 Confirmed	Yes	No	Yes	No	Yes	Yes	No	Yes	Yes	Yes
Redlands Qld May/June 2008,	Low increased to very high with human illness	3 Suspect only 5 confirmed	Yes	No	Yes	No	Yes	No	No	No	No	No
Bowen Qld Sept 2009,	Low increased to moderate with increased HeV awareness between cases	2 Confirmed	Yes	Yes	No	No	Yes	Yes	No initially then Yes after local workshop	No initially then Yes after local workshop	Yes	No
Koah, Tweed Shire NSW July 2011	High given human exposure prior to PPE and public riding facility context	1 confirmed	No	Yes geographic and seasonal	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No
Murwullimba NSW June 2015	Moderate	1 confirmed	No			No	Yes	Yes	Yes	Yes	Yes	No
Murwullimba NSW July 2017	High	1 confirmed	No			Yes	Yes	Yes	Yes	Yes	Yes	
Piggabeen NSW Sept – Oct 2018	High	1 confirmed	No			Yes	Yes	Yes	Yes	Yes	Yes	
Stokers Siding 2020	High	1 confirmed	Yes *Heightened suspicion given contemporaneous HeV like disease*			Yes	Yes	Yes	Yes	Yes	Yes	
HeV or EID suspected events without diagnosis												
Proserpine Qld July 2008	Low by vet despite govt. perception of high	1 Suspect, untested	Yes	Yes	No	No	No	No	No	No	No	Yes
Dayboro Qld Aug 2014	High by vet, moderate by govt. biosecurity	4 suspect, testing without diagnosis	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes

Table 4.3 Clinical, epidemiological, procedural, and cultural case features that likely acted as barriers for suspect case recognition and diagnosis

			Case features or context acting as barriers to suspect case recognition and laboratory diagnosis									
			Epidemiological			Clinical			Procedural		Cultural	
	Perceived Diagnostic responsibility	Diagnostic outcome	Isolated case and/or lack of perceived case transmission by proximity or contact	Lack of perceived flying fox exposure	Perceived lack of human exposure or disease risk	Attending veterinarian lack of familiarity with HeV or other EID (ABLV) in horses	Attending veterinary inexperience generally	Limited interpretation of pathologic bases of clinical viral disease	Vet lacking familiarity with diagnostic options available	Sample submission practicalities e.g. Distance to laboratory and sample storage	Concern for disruption of primary stakeholder or veterinary business (e.g. burdens relating to biosecurity or negative publicity)	Owner/carer preferences or horse purpose limiting diagnostic responsibility
HeV diagnosed events												
Hendra Qld Sept 1994,	High increasing to very high with human illness	20 Confirmed	No	Yes	No	No	No	No	No	No	Yes	No
Redlands Qld May/June 2008,	Low increased to very high with human illness	3 Suspect only 5 confirmed	No	Yes	No	Yes	No	Yes	Yes	No	Yes	No
Bowen Qld Sept 2009,	Low increased to moderate with increased HeV awareness between cases	2 Confirmed	Yes then No	No	Yes	Yes initially, then No after local workshop	No	Yes initially, then No	Yes initially, then No after local workshop	Yes	Yes	Yes
Koah, Tweed Shire NSW July 2011							No	Yes		?		
Murwullimba NSW June 2015						Yes	No	Yes		Yes		
Murwullimba NSW July 2017						No	No	Yes		Yes		
Piggabeen NSW Sept – Oct 2018						No	No	Yes		Yes		
Stokers Siding 2020						No	No	Yes		No		
HeV or EID suspected events without diagnosis												
Proserpine Qld July 2008	Low by vet despite govt. perception of high	1 Suspect, untested	Yes	No	Yes	Yes	Yes	No	No	No	No	No
Dayboro Qld Aug 2014	High by vet, moderate by govt. biosecurity	4 suspect, testing without diagnosis	No	No	No	No	No	Yes	Yes	Yes	Yes	No

4.5 Discussion

The circumstances in these cases highlight the challenges posed to field veterinarians in responding to sick horses with respiratory or neurological signs, and the range of considerations and factors that may influence their consideration of HeV infection, appropriate sampling, and submission of government laboratory investigation. Furthermore, the highlight factors and contexts on which appropriate timely diagnostic investigation depends, sampling and submission limitations, molecular assay specificity and performance and the importance of serological testing to determine previous exposure to HeV, including with a suitable DIVA assay, and multiplexed antigens for related paramyxoviruses (as presented in Chapter 7) once available. Both IgG and IgM and paired sera should be tested when available for suspect cases and a selection of in-contact horses. The cases also demonstrate the importance of familiarisation amongst veterinary practitioners and horse carers and their implementation of staged PPE and other precautionary biosecurity practices in areas where horses are maintained that are also frequented by flying foxes (regardless of species) and of vaccination of horses to minimise the risk of infection to horses and humans.

The two suspect spillover events (Proserpine 2008 and Dayboro 2014) described in section 4.3 highlight challenges differentiating HeV infection from other conditions based on clinical and field examinations. Conclusive timely diagnostic processes, critical to One Health and biosecurity management, associated with HeV and related bat-borne paramyxovirus infection in Australian horses, may fail due to a wide variety of factors specific to each case. In Proserpine Qld (2008), suitable retrospective samples were available, and the government biosecurity agency wished to explore potential diagnosis further; it was the relatively inexperienced consulting veterinarian, informed by limited knowledge of clinical manifestations who precluded further investigation. Such barrier may have been averted by emphasis on the need for shared interpretation of diagnostic responsibility in protocols guiding handling of suspect cases and increased familiarity of the expected HeV clinical syndrome underpinned by its pathologic basis – both subsequently improved markedly following the Redlands outbreak occurring that same winter season.(2)

In Dayboro Qld 2008, extensive case investigations including post-mortem tissue sampling were undertaken. However, laboratory sample processing and storage limitations, along

with limited available diagnostic options to test for divergent/ emerging viruses, beyond specific known lineages of HeV, served as barriers to conclusive diagnosis. Furthermore, this spillover event featured paramyxoviral-like disease manifestations in two persons within conceivable time window. These people had direct animal case contact prior to veterinary attendance when PPE and biosecurity measures were instigated. The affected horse carers followed consulting veterinarian advice and sought general practitioner medical consultation. However, testing or significant diagnostic processes were unavailable, relating to the lack of diagnostic confirmation in the diseased horses to which they had exposure.

We need to reduce such barriers and support proactive responsive diagnostic surveillance for HeV and related emerging infectious diseases of One Health significance. This requires support for extensive systems and collaborative networks as part of a truly transdisciplinary, multi-sectoral and multi-agency One Health approach.

4.6 References

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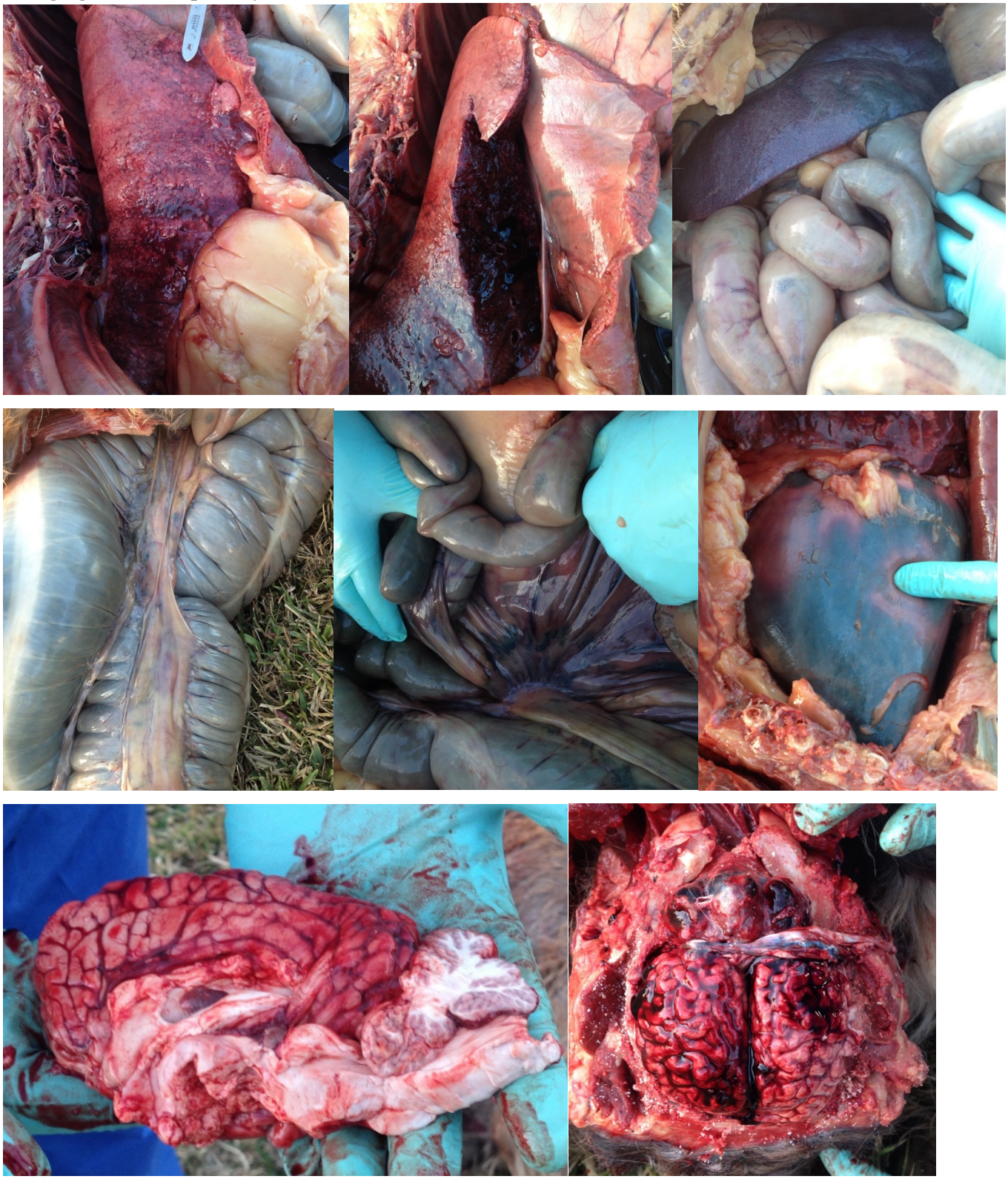
4.7 Supplementary materials for Chapter 4

Figure S4.1 (Panels A – H) Images of gross findings on post-mortem of undiagnosed HeV-like or related emerging bat-borne paramyxovirus.



Descriptions: Panel A: Serosal surface of positionally non-dependent lung lobe showing interstitial haemorrhagic consolidation. **Panel B:** cut transection of same lung lobe. **Panel C:** diffuse microvasculature haemorrhage evident throughout small intestinal serosa. **Panel D**: petechial haemorrhage associated with ascending colon lymph and mesentery. **Panel E:** Vasculature leakage evident at the small intestinal mesenteric route. **Panel F:** pericardial haemorrhage. **Panel G:** cross section of the brain showing diffuse microvasculature haemorrhage. **Panel H:** Marked meningeal and CNS microvasculature haemorrhage evident on opening cranium.

Chapter 5

SYSTEM PROCESSES FRAMEWORK JUSTIFIES SYNDROMIC RISK-BASED TARGETED ACTIVE SURVEILLANCE OF HORSES WITH HEV-LIKE ILLNESS FOR SENTINEL DETECTION OF EMERGING ZOONOTIC VIRAL DISEASES

Statement of personal contribution: I drafted the first complete manuscript (2019−21) and received feedback from PhD supervisors. I received support in the system process analyses from epistemology scholar Tim Jackson and technical support from project research assistants Nicole Popovic and Anna Gonzalez.

Format: This chapter has been prepared primarily for this purpose (rather than for peer review journal submission).

Much of the material in this chapter describes the research approach similarly to that reported as part of the Biosecurity Innovation Program Project 2020-21 Project ID 202043*: Metagenomic Investigation of Horses as Sentinels: Innovative metagenomic, multiplex serology and custom database combined in testing cases of priority infectious clinical syndromes to greatly improve diagnosis of infectious diseases and Australia's biosecurity***,** Examples are in milestone documents and project reports. To my knowledge these are not anticipated to be published publicly but rather used to guide internal procedures in Australia's government departments.

Thus, some of the findings and perspectives included in this thesis chapter may be further communicated and incorporated into grey literature regarding biosecurity and surveillance for EID in Australia. Where suitable and over time, these may be presented as part of peer-reviewed scholarly research outputs.

5.1 Introduction

5.1.1 Context for EID surveillance and biosecurity

Infectious diseases pose an increasing threat to Australian and global human, animal and ecological health, agricultural industry, trade and food security. Extensive and complex human and animal interaction, intensifying international mobility and trade, anthropogenic and climatic pressure on wildlife populations coupled with progressive diagnostic technology, all contribute to a scenario where disease spillover and biosecurity risk are increasingly evident. They also remain difficult to detect, characterise and manage.

The prioritised management of HeV in Australian horses and the testing of suspect cases has highlighted the challenges, limitations, and gaps in diagnostic investigation for emerging infectious diseases beyond a select few well-understood pathogens (See Chapters 1 – 4). There is an opportunity for strengthening Australia's biosecurity with improved proactive surveillance for emerging disease beyond known diseases to augment biosecurity preparedness. Recognition of reductions in socio-economic burdens by early detection of pathogen spillover[1–3] has increased global interest in surveillance approaches that are proactive and biosecurity risk-focused, and interdisciplinary and intersectoral. A recent economic study showed that mitigation of pandemic threats would be *more cost effective in the long-term* than continuing with outbreak responses and policies.[1]

By definition, animal health surveillance involves systematic ongoing collection, collation, analysis and interpretation of information of diseases occurring in animal populations to support timely communication that guides policy and strategic decision making.[4,5] Sergeant and Perkins (2015)[4] offer a basic schematic (Figure 5.1) of the broadly-accepted concept of how effective animal health surveillance operates to guide decisions and processes among stakeholders concerned. It comprises surveillance activities that draw on information generated in the first instance for other purposes (passive surveillance) and activities that collect data to obtain information on specific disease status of an animal population (active surveillance). Furthermore, active surveillance activities that aim to detect specific pathogens are termed 'targeted active' surveillance. Those that undertake preferential sampling of animals with the highest likelihood of being infected are termed 'syndromic' and/or 'risk-based' surveillance.[4] Active surveillance activities may extend from passive

surveillance operations, be conducted in parallel or be applied separately, and the distinction between the forms may not always be clear.

An adequate health surveillance program will include active and passive surveillance activities that in combination provide the required information on distribution of known pathogens and enable detection of exotic and new pathogens to guide priority One Health and biosecurity management. Figure 5.2 illustrates the relationships between the components of an animal health surveillance program. It emphasises that the foundation of an effective program is the application of surveillance infrastructure and functional resources within the context of knowledge of susceptible host populations and of their environments.[4] This requires extensive consideration and planning to optimise supportive governance, bureaucracy and communication as well as allocation of the necessary substantial infrastructure and resources where they are needed across multiple sectors and operational stakeholders. Resources may include personnel with appropriate training and diverse expertise, equipped laboratories, timely protected data sharing capacity, testing submission and reporting pathways, supporting legal, policy and procedural frameworks, and established transport, communication, and reporting networks. Many of these inherent requirements are further challenged by zoonotic diseases on One Health relevance.

Surveillance based on the outcome of veterinarian-initiated priority disease reporting is generally considered ‘passive surveillance’ as the cases investigated arise sporadically from the general population of animals that veterinarians routinely attend across a range of consultation contexts. Prioritised diagnostic consideration of voluntarily reported cases of diseased animals for a select few infectious agents of established concern by government sector laboratories is a highly efficient risk-based targeted disease surveillance activity underpinned by passive disease reporting. However, this approach is inherently limited in its potential to detect emerging and novel disease agents (Figure 5.2) and to provide epidemiological estimation of the true incidence of pathogen spillover and disease prevalence.[6]

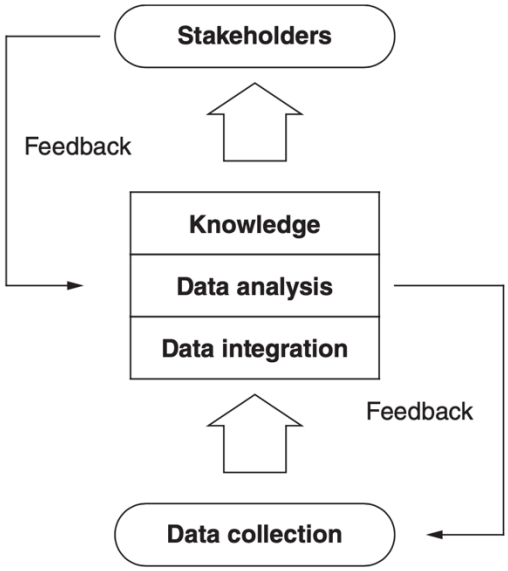


Figure 5.1. Broad concepts of disease surveillance as presented by Sergeant and Perkins 2015[4]

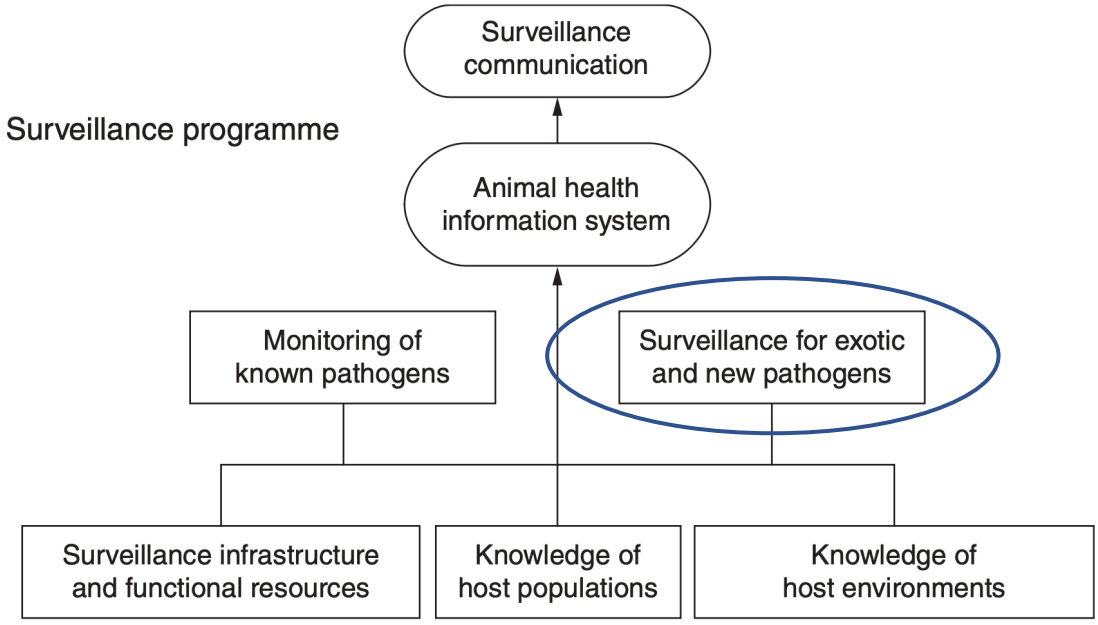


Figure 5.2. Relationships among different components of a population health surveillance program as presented by Sergeant and Perkins 2015[4] incorporating *World Organisation for Animal Health Code* concepts[5]. Capacity of routinely operating passive surveillance investigating reported disease cases for priority agents such as HeV in horses is limited for detecting exotic and novel pathogens (blue circle)

5.1.2 Barriers and challenges limiting effective EID surveillance of HeV-like disease in Australian horses

For many diseases, such as HeV in horses, the highest priority is management of human (zoonotic) infection risk. The success of passive disease reporting systems can be significantly compromised in such diseases by diverse barriers for proactive engagement of stakeholders in biosecurity investigation and management. Investigations for HeV and related EIDs, regardless of diagnostic outcome, can result in significant disruption and burden for primary stakeholders[2]. Another limitation is the fact that funding and governance for the management of zoonotic diseases is variable, and often inadequate to sufficiently support extensive operation of processes within the animal health system that ultimately are to serve a human health priority in the zoonotic disease contexts.

Sensitivities for primary stakeholders, including veterinarians and horse owners, trainers, and breeders, can include financial liability relating to direct disruption to enterprise operations (including from quarantine requirements), and/or perceived risks associated with the investigated premises. This can lead to a loss of business due to reductions in customer confidence. In addition, there may be a compromise in the range and timeliness of veterinary care and/or animal welfare[7], liability under the WPHS and biosecurity act, professional indemnity to the veterinarian, and compromise of animal insurance policy compliance or eligibility.[8] Since 2014, underwriters of Australian equine mortality insurance have included exclusion clauses relating to insured animal losses that receive compromised veterinary treatment due to unvaccinated status and delays relating to HeV exclusion testing. This restriction applies regardless of the ultimate cause of death.

There are also barriers to proactive engagement with HeV and EID surveillance for higher level stakeholders,[3] such as those operating in industry and government. Barriers may relate to potential for changes in disease free status, freedom of international horse trade, and biosecurity or financial liability, when new agents of significance are discovered prior to

[2] Primary stakeholders are those to whom the condition of the animal/s matter most. When considering the health and diseases of horses these include owners, breeders, carers, and trainers, as well as consulting veterinarians.

[3] Higher level stakeholders relevant to disease surveillance include relevant industries along with government agencies and others responsible for maintaining biosecurity, the economy, natural resources, and ecological health.

sufficient knowledge being available to interpret and manage biosecurity risks most sensitively.

Other challenges to the effectiveness of surveillance for HeV and EIDs in Australian horses are outlined in Box 5.1.

Box 5.1. Challenges to surveillance of HeV and EIDs in Australian horses

Related to ecological context:

- Expected sporadic and low incidence of spillover infection of HeV and other bat-borne paramyxoviruses that have been detected in flying foxes with potential to follow the same spillover pathway to horses, in which they may cause similar disease
- Difficulty obtaining funding for discovery research, with inherent high risk of failing to detect significant target pathogens, due to expected rarity
- Potential for positive agent identification to adversely impact the public perception of threatened wildlife species risking legal and illegal population disruption.[15]

Related to processes:

- Limitations to available sample types and volume imposed by requirement of minimally invasive sampling techniques to manage zoonotic exposure risks. Usually swabs (nasal, oral and vaginal mucosal and rectal) and blood are all that is obtained in HeV suspect horses due to occupational risk involved in acquiring tissue samples that have traditionally offered greater diagnostic potential for emerging infectious diseases
- Sole sampling events rather than paired (initial and subsequent) sampling, as is a usual requirement for confirmation of infection by serological assays of individual diseased animals; lack of sampling of animals that share the paddock and farm with varying disease and exposure statuses
- Varied adequacy of cold chain to preserve sample integrity by minimising degradation of the viral RNA. This is difficult to ensure due to large distances between many sampling locations and the state animal health laboratory
- Biosafety concerns given biohazard rating of target pathogens
- Lack of developed and/or validated assays targeting emerging and divergent bat-borne paramyxoviruses
- Difficulties interpreting equine and/or zoonotic disease associations for divergent and emerging viruses when discovered as single case detections
- Barriers to developing and applying updated routine diagnostic capacity for significant agent discoveries made using novel research approaches.

5.1.3 Approach

Clearly there are a wide range of stakeholders involved in routine systems for HeV surveillance and biosecurity management. These stakeholders experience diverse challenges spanning core scientific, diagnostic, epidemiological, ecological, and cultural contexts. It is apparent that many factors may influence the capacity of these stakeholders to undertake critical process steps required for timely case detection, diagnosis, and biosecurity risk management. However, these influencing factors are poorly understood and inadequately described, as is their impact on actioning critical procedural steps and decisions on which successful timely diagnosis depend. Further, the ability of any single stakeholder or sector to understand the factors influencing critical processes with which they are not involved is likely to be constrained or limited by their discipline and role focused perspective.

Given this situation, to progress understanding, a system processes approach was applied as a means to establish a framework for understanding the complex context that shapes the surveillance of HeV and EIDs for diverse actors engaged with Australian horses. The system processes framework was developed through an analysis of procedures, engagement with stakeholders, and a review of relevant literature. Schematic flow diagrams were useful in clarifying relationships and perspectives, and a directional acyclic graph mapped influencing factors. This was undertaken with a view to thinking through how the recommendations developed in this research could be applied effectively in the real-world scenarios considered here by identifying and addressing ‘pain points’.

5.2 System processes framework of routine surveillance for HeV and EIDs in Australian horses

5.2.1 Stakeholder engagement with HeV surveillance and management influencing critical processes

Many stakeholders are affected by and related to each process in the sequence of events by which a sick animal is diagnosed with a significant disease and then the biosecurity and case management implemented.

Effective surveillance and appropriate biosecurity management for HeV and EIDs in Australian horses relies on engagement by stakeholders of diverse discipline and sector with various expertise, experience, influenced in turn by even wider ranging perspectives and priorities (Box 5.2).

Box 5.2. Stakeholders engaged with surveillance for HeV spillover infection in Australian horses and appropriate One Health biosecurity management

- Private practice mixed species veterinarians* (R)
- Private practice equine focused veterinarians* (R)
- Public sector field veterinarians (R & OHR)
- State and national government sector biosecurity laboratory scientists (L)
- State and national biosecurity and epidemiology as part of routine surveillance (OHR)
- Biology and epidemiology as part of scientific research (L & OHR)
- Human health communicable diseases governance, infectious disease clinical and diagnostic departments (OHR)
- Wildlife health, ecology, and science multi-discipline representation (OHR)
- Government sector managing freedom of disease, trade agreement, international animal and biological sample movements (OHR)
- Equine industry representation (R) − e.g: racing (galloping and harness), sport horse disciplines (jumping, eventing, and dressage), team sporting disciplines (polo crosse and polo), western and stock associated disciplines (roping, cutting, camp-drafting and western saddle)
- Horse owners, breeders, carers and trainers.* (R)

Stage of critical processes primarily influenced by stakeholder: Suspect case recognition and laboratory submission (R), Laboratory diagnosis and reporting (L), and One Health responses to positive case diagnosis (OHR)

**Primary stakeholders to whom the condition of the horse matters most, and who have the most direct exposure prior to realisation of zoonotic disease risks and involvement with subsequent PPE and biosecurity measures. These stakeholders face the greatest disruption and inconvenience from both investigation of suspect cases found to be negative and in cases of detection of HeV and EID infection.*

Four key stakeholder groups were identified as being involved in the critical processes for recognition of suspect disease cases in the field, laboratory submission and biosecurity management. These were:

1. horse owners, trainers, carers, and breeders
2. private veterinary clinicians in equine practice comprising a mix of:
    - first opinion practitioners
    - referral centre practitioners

3. private veterinary clinicians in mixed-species practice
4. public sector field veterinarians:
    - district vets (government funded animal health roles) relevant to case recognition and management post detection
    - state biosecurity officers, field epidemiologists and duty pathologists.
      This group becomes involved in managing biosecurity following significant disease detection. They play an important role in communicating with private practising and district veterinarians both to raise awareness for case recognition, sampling, submission processes and appropriate biosecurity risk management and in timely responses to suspect and confirmed cases.

Three further specifically defined stakeholder groups were included. These were identified as engaging with laboratory diagnostic investigation and reporting processes:

5. state and national government sector biosecurity laboratory scientists
6. state and national biosecurity and epidemiology as part of routine surveillance
7. biology and epidemiology scientists as part of research partnering with or extending routine operations.

Many stakeholders engage with the system more broadly, acting in ancillary roles to the operation of the system for detecting HeV infected horses. Those from the human health sector include general practitioners and infectious disease specialists, in regions where flying foxes and horses cohabit. In addition, there are stakeholders involved in human health governance including those relevant to funding for zoonoses. Government sector stakeholders managing freedom of disease and trade, and those overseeing live animal international movements and the international movement of biological samples are also important stakeholders following notifications of outbreaks of EIDs.

5.2.2 Developing an understanding of crucial processes for HeV and One Health EID surveillance of Australian horses

This research's convergence approach drew inspiration from ethnographic research methods[11,23,24,25] to harness the holistic (broad) and specific (focused) perspectives required in systems thinking[9] framework mapping and analytical approaches.[26] The aim was to

develop a holistic understanding of the various roles, perspectives and priorities of stakeholders concerned with surveillance of HeV and EIDs in Australian horses.

Accounts of each role and perspective were recorded and transcribed. These informed the construction of the mapping of influencing factors relevant to each stakeholder and their decisions and actions critical to the critical system processes required for optimal proactive case recognition and biosecurity/ one health management.

Once system framework mapping and analyses had been undertaken for the example this research context of HeV and EID, workshops were held to engage key stakeholders (Box 5.2) and to validate or improve our understanding and perspectives. Through this process, we identified the critical gaps in knowledge and procedural pain-points in the current surveillance system and seek agreement on plausible/ feasible potential system improvements extending from this research.

These workshops were held for the purpose of collective consideration of the significant findings and perspectives drawn from this research by stakeholders of most direct relevance as part of the Australian Government Department of Agriculture, Water and the Environment Biosecurity Innovation Program research project. Focus group meetings were held in two stages, firstly as discipline/ sector specific groups and secondarily as groups of mixed stakeholder and discipline representation. In both cases participants considered the HeV g2 research finding and a summary of the system process frameworks presented in this chapter and deliberated and contributed by way of anonymous interactive virtual (online) white board (Mural). A report on the full findings of the workshop are beyond the scope of this thesis, but were presented in an internal project government report. The workshops are mentioned here to the extent that they give and indication of the methods and thinking that went into the system processes analyses.

Through these processes, we identified three broad points that are critical to effective surveillance for emerging pathogens in the context of horses investigated for HeV-like disease in Australia. These are:

- intersectoral collaboration in diagnostic discipline;

- diagnostic sampling (case selection, sample types, transport, cold chain and laboratory submission);
- diagnostic testing (testing approaches and algorithms applied routinely and in research or surveillance supporting routine testing, interpretation of results and of disease and biosecurity significance, sample intellectual property (IP) and data management, reporting and testing procedures).

5.2.3 Processes and decisions in HeV and One Health EID surveillance of Australian horses and biosecurity depicted as a current ('as is') system framework to highlight strengths, gaps and pain points

By considering the critical processes and decisions that enable a horse infected with HeV in the paddock to receive timely laboratory diagnosis, we can highlight the diverse stakeholder engagement and integrated supportive national and state biosecurity systems that are required. Appropriate biosecurity and zoonotic disease risk management rely on such timely diagnosis.

In this research, we collated and analysed first-hand empirical experience in the specific Australian equine disease context[8,10] and other relevant EID One Health contexts.[11] We also included the experience, discipline expertise and engagement of research project collaborators and external sector partners in the broader stakeholder group.

5.2.3.1 Outline of critical processes

We defined critical processes as those that must necessarily and effectively take place to allow timely positive case diagnosis, and for which failure will result in the system missing detection, with unmanaged disease risks. Failure of any single critical process means the system overall will fail to detect a disease event, with potentially catastrophic animal or human health consequences. Missed animal case detections are inevitable given the low and sporadic incidence of viral spillover events. However, ensuring these are kept to a minimum is nevertheless a key goal of the surveillance and biosecurity management for zoonotic viruses.

These necessary critical processes may logically be considered in three chronological stages, outlined in Figure 5.3: 1) disease recognition in the field and appropriate submission of samples and case details, 2) state laboratory testing and reporting and 3) responses to

positive diagnosis including confirmatory testing and One Health responses. Any one stakeholder role is variably limited to engage and influence processes in one or more of these stages. Stakeholder familiarity is often similarly limited to the processes in which they are most directly engaged.

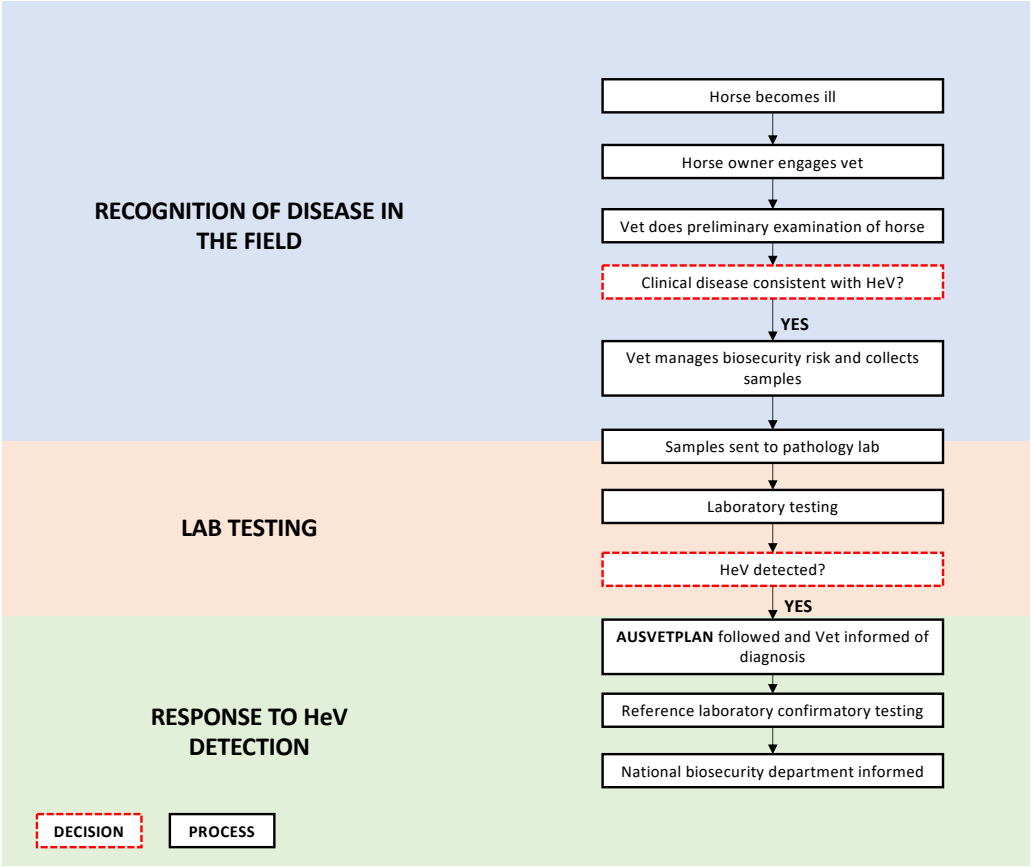


Figure 5.3. High-level 'as is' flow-chart of critical processes required to take place as a chain of events in order to identify and appropriately respond to HeV disease in horses

5.2.3.2 Flow-diagram representing system processes framework currently operating ('as is') as surveillance for HeV spillover infection in Australian horses

A schematic flow diagram representing the system processes and variable stakeholder engagement as it currently operates ('as is') was constructed to better understand roles through which each stakeholder engages with system decisions and processes. The diagram also shows the extent to which effective case detection and timely and appropriate One Health biosecurity management depend on these roles (Figure 5.4). The model was adapted to incorporate human health system engagement (Supplementary Figure 5.1) both in response to confirmed (well established) and to suspect, but not laboratory confirmed, cases (less established). It is important to note that suspect human case recognition has always relied heavily on known (laboratory confirmed) animal disease exposure. This limits

potential for human cases to be recognised where diagnosis has not occurred in an infected horse.

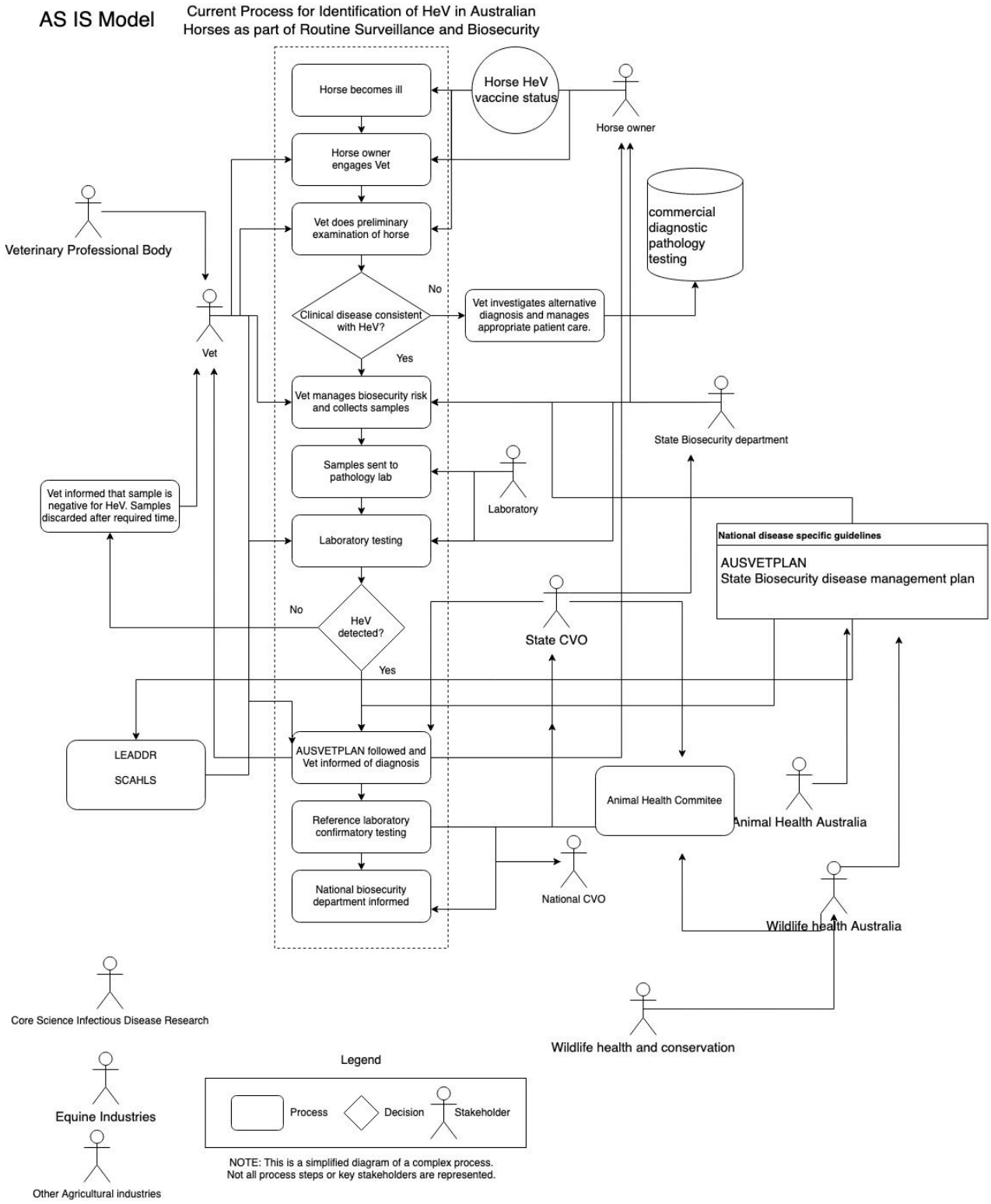


Figure 5.4. Schematic flow diagram representing the critical processes, decisions, stakeholders that are involved in the current detection and surveillance of HeV ('as is'). *Note current system*

framework lacks reliable accountable, most effective integration of research and industry actors and stakeholders

5.2.4 Directional acyclic graph (DAG) guided mapping of influences on stakeholder decisions and processes critical to system performance

Bayesian structural network models[12] such as the directional acyclic graph[13] (DAG) have been increasingly employed to improve causal inference in guiding epidemiological study design and informing multiple regression analyses that aim to overcome challenges of diverse confounders and interactions relevant to multiple and complex causation.[14] DAGs comprise factors as nodes with connections representative of qualitative causal relationships (direct or indirect). The factors are considered in their most directed influence toward an outcome of interest (without feedback loops). The Daggity package in R may be used directly via command-line instruction and/or via a web browser hosted (SHINY) interface. This package has greatly reduced the technical barrier to use of DAGs, which are now a readily available tool for wide application.

To improve understanding of this complex system with multiple influential factors, a structured DAG approach was employed to consider the network of influences as diverse (and mostly unmeasurable) qualitative predictor variables on chronological critical processes (as outcomes), to inform qualitative estimation of strengths (enablers) and pain points (bottle necks subject to strongly inhibiting or numerous influencing factors) of the current HeV surveillance system processes (Supplementary Figure 5.2).

The decision process for clinical veterinarians were mapped out − the recognition of a possible HeV infection in a horse (Supplementary Box S5.1) is followed by the procedures and decision processes that occur with upon receipt of submissions at the state biosecurity laboratory (Supplementary Box S5.2), and then diagnosis (Supplementary Box S5.3). The key stakeholder decisions and processes were mapped via the DAG onto the ‘as is’ system processes flow diagram (Figure 5.5).

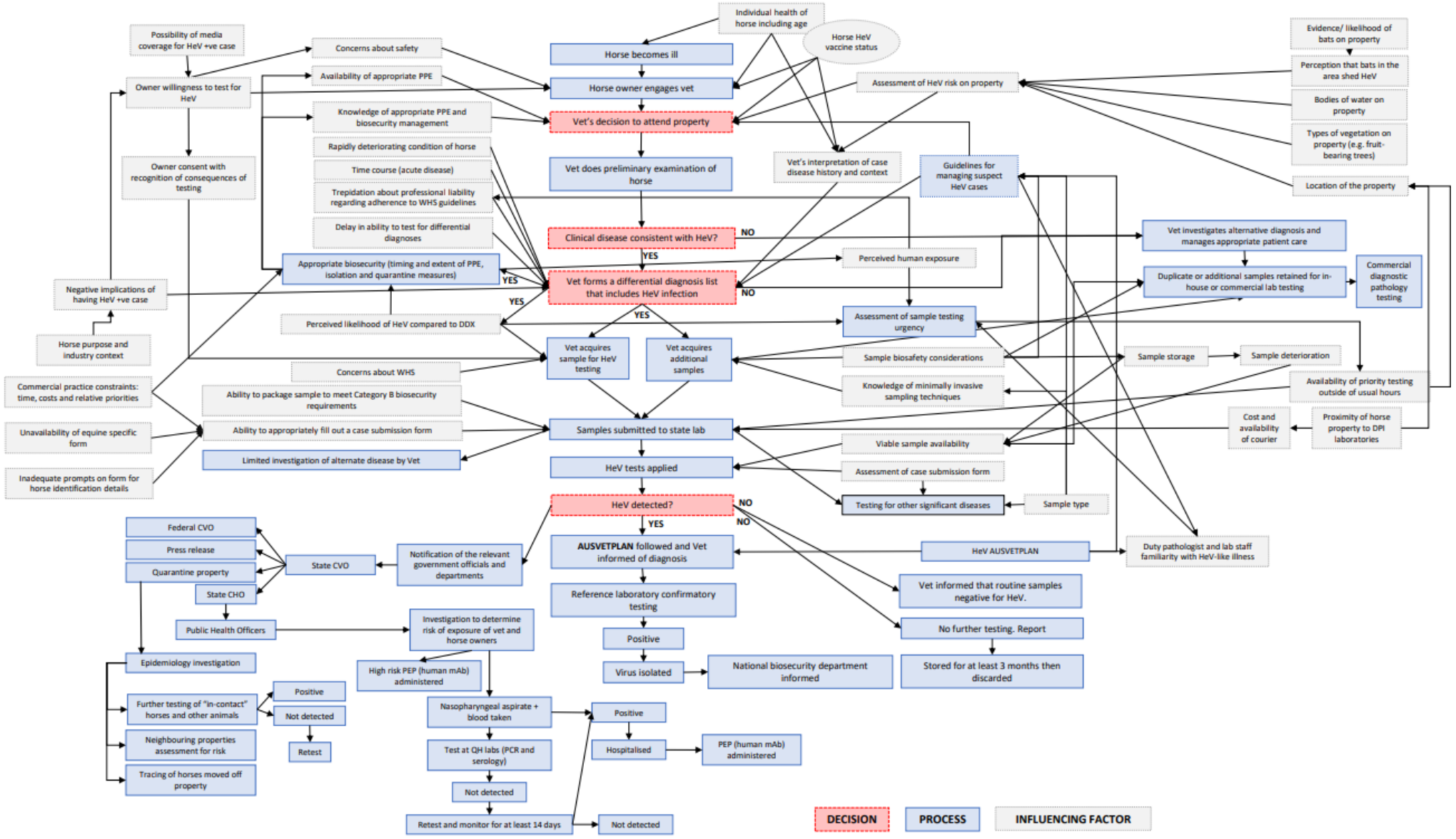


Figure 5.5: Major influential factors for the steps in the systems process informed by partial DAGs built to consider each stage of critical system processes

5.3. Current critical system processes as strengths and pain points especially in detection of novel and emerging zoonotic pathogens

'Pain points' represent opportunities for significant improvement, and with improvement should translate into better overall outcomes.[15] Critical decisions and processes have many influencing factors acting as barriers (recognised or potential). Key pain points identified in the system process included:

- clinical recognition of suspect cases
- sampling and laboratory submission for priority testing
- laboratory diagnostic testing beyond specific HeV detection by qPCR
- determination of the significance of an agent.

5.3.1 Clinical recognition of suspect HeV disease cases as both a strength and pain point

Effective surveillance and biosecurity management of HeV spillover infections in horses relies on accurate clinical recognition of suspect cases by private veterinarians.

Awareness and recognition of HeV in horses by private veterinarians is clearly a strength of the current significant disease detection system, especially in areas where HeV has been previously detected (i.e. Queensland and northern NSW). However, some inherent challenges are evident, including the breadth of potential disease signs and potential for viral shedding in early mildly symptomatic or even pre-symptomatic cases. Therefore, clarifying and improving understanding of clinical and epidemiological indications of suspect cases will be of benefit to improving private practitioner confidence for detecting suspect cases and managing the biosecurity and zoonotic risks of HeV including WPHS.

There is clearly room for improvement in sample submission processes, including case descriptions, to assist in the interpretation of illnesses by laboratory staff. Duty pathologists and laboratory staff may mis-interpret the intended understanding of submitting veterinarians regarding disease characteristics and level of suspicion for priority disease if they are not clarified either by appropriately detailed submission forms or via responsive consultation, for example by phone.

Furthermore, the current criteria for suspecting HeV disease limits consideration to horses residing in (or having travelled from) certain geographic locations where equine HeV disease

has been previously detected. This results in profound sampling bias based on geographic location, rather than appropriate sampling and testing in all cases of consistent equine disease and in all regions frequented by flying foxes.

5.3.2 Diagnostic sampling of suspect HeV disease horse cases and laboratory submission as strengths and pain points for EID surveillance

Several practical issues limit diagnostic sample collection from horses with suspect infectious disease and laboratory submission. For routine priority HeV testing the use of minimally invasive sampling options that minimise the risk of zoonotic disease transmission has been thoroughly outlined in government communicated guidelines for veterinarians. This is well established in regions in which the risk of HeV spillover has been demonstrated (eastern Qld and North-eastern NSW). Regional lymph node biopsy (fresh chilled) tissue samples would offer increased suitability for diagnosis of divergent or novel HeV-like agents. These samples might be obtained ante- or post-mortem via relatively minimally invasive technique. Post-mortem tissues of greatest diagnostic value would include central nervous system (CNS) tissue, lung lobe biopsy, lymphoid tissue associated with internal organs such as intestine, and hepatic tissue. The opportunity for relatively invasive post-mortem tissue sampling is rarely provided in regions where HeV is considered a differential diagnosis. This is due to necessary delays in initial screening of minimally invasive samples for HeV, resulting in delayed access to safe carcasses; most are buried for biosecurity risk management and/or practical stakeholder reasons. Additionally, many clients are less supportive of veterinary consultation occurring after a fatal outcome due to their assessment of poor cost versus benefit.

Access to out of hours testing is based on suspicion of human exposure rather than strength of suspicion of HeV in the horse. Clear guidelines are required surrounding when out-of-hours testing is justified. Improved sample receival over weekends and outside business hours would also be beneficial, even in cases that do not meet the criteria for priority weekend testing. This would help to maintain sample quality and ensure reliable timely receival.

5.3.3 Current laboratory diagnostic processes as strengths and pain points for detection of bat-borne emerging infectious zoonotic diseases

Advanced laboratory diagnostic capability was identified as a strength of the current system. Rapid real-time PCR analysis of suspect HeV cases offers timely agent-specific conclusive laboratory screening to guide zoonotic risk management, and national reference laboratory capacity offers positive case confirmation and further infectious agent characterisation.

However, a fundamental limitation in the current system is that further characterisation of infectious agent is presently only applied to samples that have already tested positive at the state laboratories. Additional testing of samples where HeV has not been detected for other potential significant infectious zoonotic diseases could enable the system to move beyond exclusion testing towards causative diagnostics. The development of an emerging infectious disease likelihood algorithm that interprets animal-case details to prompt further testing could assist in this process. Challenges to further testing are listed in Box 5.3.

Box 5.3. Challenges identified with regards to testing capacity:

- Highly specific and single assay/molecular-target qPCR testing, which rules out the possibility to detect HeV variants possibly causing HeV-like disease
- Limited funding with reduced capacity for broad-based diagnostic work-up beyond exclusion testing at state laboratories
- Limited time allocation at state laboratories for further exploratory testing and screening of samples beyond exclusion tests
- Difficulties in thorough validation of new diagnostic tests and evaluation of these tests in the context of low-occurrence diseases
- Challenges in the serological detection of HeV and inability to differentiate between natural exposure vs vaccination.

5.3.4 Determination of the significance of disease as a current strength for HeV specifically but a pain point for divergent and novel viruses of emerging significance

The determination of the disease significance of a novel agent when detected can be very difficult due to wide ranging challenges predisposing to high risk of disruption to stakeholders. This is due to the need for a conservative interpretation of risks in the absence of scientific knowledge on features of disease and transmission (Box 5.4).

Box 5.4. Challenges interpreting the significance of a novel infectious agent when initially detected

- Determining disease causality and pathogenesis in the absence of multiple cases and sufficient epidemiology without targeted sentinel research supporting routine surveillance
- Consistency or otherwise with infectious conditions defined by AUSVETPLAN and other relevant existing guidelines
- Frequency and distribution of spillover transmission risks from wildlife reservoirs maybe unknown
- Zoonotic and ongoing domestic animal transmission risks
- Management of surviving infected animals including consideration of compulsory humane destruction
- Determining expected efficacy of available vaccines and post-exposure therapies and protocols
- Primary stakeholder communication, appropriate biosecurity and quarantine management
- Implications to trade and freedom of disease status
- Understanding of biosafety relating to laboratory and biological samples
- Limited capacity for appropriate biobanking of samples and adapted submission and reporting pathways to allow for research to support routine surveillance while maintaining chain of diagnostic custody.

Specific approaches considered suitable to support timely interpretation of novel detected disease significance by this research system thinking analyses are listed in Box 5.5.

Box 5.5. Suggested approaches to support timely interpretation of novel detected disease significance

- Improved communication of the known pathogens as well as the unknowns. This ensures transparency and active communication between those at the forefront of research involving pathogen discovery and those most at risk in the field
- Extended disease investigations beyond HeV negative results in cases featuring disease signs and syndromes consistent with potentially significant and/or zoonotic infectious cause
- Integration or extension of routine diagnostic pathways with Next Generation Sequencing (NGS) platforms for investigation of disease beyond the known few for which testing is available
- Formation of a nationally co-ordinated restricted access database for timely sharing of molecular sequences. This enables Australian researchers and government sector laboratories tasked with testing samples and/or agents of potential biosecurity significance to access data appropriately that is not in the public domain. *Entry of sequences could be a requirement of any diagnostic or research testing benefiting from state and federal funding and/or utilising samples of potential national biosecurity significance. This is particularly important as sequences of relevance to routine biosecurity currently may be delayed awaiting scientific publication or never reach public databases such as GenBank*
- Adaption of modern testing technologies and approaches to evaluating diagnostic test performance. Adaptions should optimise the routine and extended analysis of submitted samples; currently options are restricted by availability of suitable samples and sample types. For example, positive control samples are lacking for rare or novel diseases, and there is a need for minimally invasive or ante-mortem sampling due to significant zoonotic disease risk
- Investigation of other diagnostic testing approaches such as for inflammatory biomarkers suitable for use in the absence of pathology since tissues are not collected
- Description and communication of minimally invasive approaches to optimal tissue sample collection for EID investigations beyond limited routine qPCR on swabs and blood, for example lymph nodes, CSF and CNS biopsy. (Approach for equine context has been evaluated in Horses as Sentinels collaboration with Dr Caroline Spelta)
- Increased options for paired sera in individual or low case number potential significant events of infectious disease (as for human infectious disease diagnostic testing) and use of IgM assays in addition to IgG
- Ensuring that virus detection is linked to pathology and clinical data, and consideration by multiple relevant discipline expertise to assess relevance
- Similar targeted sentinel approaches supporting routine biosecurity could be adopted for production species for the identification of diseases that present with a likely infectious cause
- Research and targeted population testing approaches and guidelines supporting routine biosecurity.

5.4 Horses as sentinels for EID

There are clear biological, cultural and socio-ecological reasons to support the suitability and benefits of utilising horses as sentinels for emerging infectious diseases.

Firstly, horses are maintained in rural, peri urban and even urban settings, near other significant domestic animal species such as commercial cattle, sheep, poultry and pigs, as well as in close proximity to humans and backyard poultry and pigs. Secondly horses are monitored closely for disease and receive veterinary consultation both routinely for optimised performance and in response to even the mildest of health concerns and disease signs. Many owners of horses will engage veterinary consultation more readily, and for less significant conditions, than they would seek medical attention for themselves (personal observation). Thirdly, horses are susceptible hosts to a very wide range of disease agents (with and without significant clinical disease) including many of multi-species and human health significance (see Chapter 3). Of pertinence to this research, horses are susceptible to RNA viruses relevant to Australia's One Health, including those spread by biting arthropods (ticks, mosquitoes and flies), flying foxes and insectivorous bats. Also horses serve as appropriate sentinel species nationally and internationally − especially for mosquito-borne flaviviruses in the Japanese encephalitis group (WNV, SLEV, MVEV, JEV).[16–18]

Finally, horses are relevant for international trade agreements including evidence for freedom of disease and industry impacts can arise from recognition of novel, emerging and/or hitherto unrecognised infectious agents in horses. In many instances, however, the trade consequences may be greatly less substantial than for other industries. There is greater opportunity for investigation as an individual disease event to gain understanding of pathogenic significance that can act as an early warning signal. This can prompt further investigations into broader implications, including those relating to production animal trade agreements and food security.

Despite these clear benefits and justifications, this research has highlighted several critical barriers for using Australian horses as sentinels for emerging infectious diseases along with potential solutions. Potential solutions in the context of investigation of HeV-like horse disease cases in Australian horses for EIDs, for broader sentinel benefits for One Health are summarised in Box 5.6.

Box 5.6. Potential solutions to overcome current barriers for EID detection in HeV-like horse disease cases

- Increase disease investigations beyond known-agent-specific (exclusion) testing as extension of routine significant disease investigations – esp. in cases of defined context/s (diagnostic responsibility/ies) of increased One Health or biosecurity relevance through augmented risk-based syndromic surveillance. Including via open and targeted research diagnostics such as this example and via suitably informed multiplex EID molecular and serological testing diagnostic algorithms.
- Develop minimally invasive sampling techniques, especially of brain tissue, which is seen as the gold standard to diagnosis of many viral diseases. Development and validation of technologies could present safer options for sampling for veterinarians but also expand the scope of current diagnostics (e.g. testing for more viruses and a greater range of tissue from which to sample). Veterinarians would also then need to be trained in these minimally invasive sampling techniques.
- Extend the current repertoire of tests at state laboratories to screen for divergent pathogens in samples from horses that are displaying severe disease and are Hendra negative.
- In highly suspicious cases, encourage serial sampling to obtain paired samples to confirm a diagnosis.
- Develop capabilities at all state laboratories for NGS to enable discovery of pathogens by partnering with research institutions.
- Obtain increased funding to facilitate:
    - extra staff allocated to processing samples
    - extra scientists focusing on investigation of causes of animal disease
    - development of an information system to couple information between laboratories to allow sharing of relevant data while protecting personal information
    - biobanking facility and suitable sample testing and products processing (RNA

5.5 Conclusion

Considerations of system processes framework assisted our research and the stakeholders engaged in workshops to understand how each process, stakeholder and factor influencing engagement related to the operation of the system (as a whole) in effectively detecting significant disease and managing biosecurity risks.

All individual components of the current system are required to function effectively to prevent failure of the entire system in its core aim. That aim is to detect and appropriately manage animal cases of significant zoonotic and emerging diseases as they occur sporadically in nature. An understanding of the context and framework in which each required surveillance act, diagnostic step and response process operates, and of the level of

individual stakeholder engagement is thus essential to the success of the surveillance system.

Adequate surveillance for emerging bat-borne paramyxoviruses specifically,[19] and emerging zoonotic diseases in other similar complex multi-species contexts, requires a truly integrated One Health approach.[20–22] In order to minimise the impacts of zoonotic disease and biosecurity risks, a wide range of expertise is required, including local and international scientists, ecologists, veterinarians, health professionals, social scientists and government policy agents.

The pain points identified as part of the analyses allowed me to highlight areas where improvements could be made within the system to benefit surveillance for EIDs. Improved clinical recognition and sampling of cases of significant disease by clinical veterinarians is the crucial first step in improving surveillance (See Chapter 2, 3 and 4). Improvement of the system for recording clinical signs to allow interpretation of cases where an infectious cause is highly likely beyond HeV diagnosis will help to identify new EIDs (Chapter 6). Strategic implementation of new technologies as part of the routine suite of techniques in a diagnostic laboratory or by integration with research laboratory-based analyses (Chapters 7 and 8), present opportunities to improve and strengthen the system by actively searching for emerging infectious diseases of significance to humans and animals.

Three innovative pillars were developed, which may be combined and applied to existing resources to deliver significant enhancement of Australia's biosecurity and emerging infectious disease preparedness (represented figure S5.3)

The three pillars were:

1. Operation of an innovative purpose-built SQL database that combines bio-banked laboratory samples with their de-identified sample event, subject and clinical details (including syndromic classification), and their advanced diagnostic testing results. This approach allowed for optimised epidemiological assessment of disease causality while leaving unnecessary sensitive information with the primary submitting laboratory (Chapter 6)

2. Application of a novel serological microbead immunoassay enabling screening in bio-banked clinical samples for exposure to a wide range of relevant pathogens (Chapter 7)

3. Parallel application of advanced molecular testing methods to identify novel and emerging infectious agents in the same cohort of horses. Testing methods include high-throughput pan-PCR, metagenomic next-generation sequencing (NGS) and specialised bioinformatical pipelines (Chapter 8).

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5.6 Supplementary materials for Chapter 5

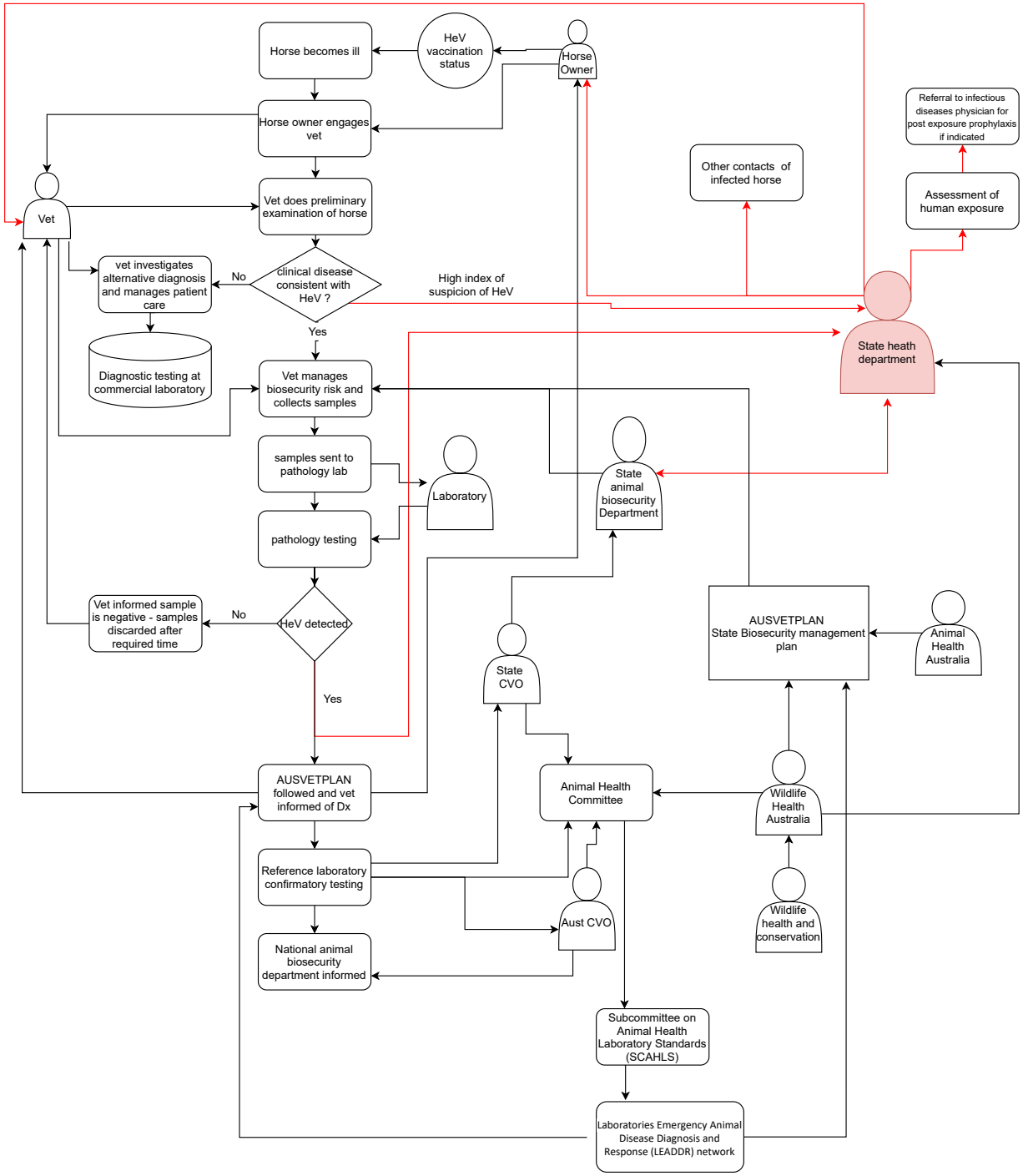

Figure S5.1. Schematic flow diagram representing the core processes, decisions, stakeholders and influencing factors that are involved in the current detection and surveillance of HeV ('as is') including Human Health. *Acknowledgment for contribution in incorporating the human health links to Dr Sandra Steel who drew on her own One Health Zoonoses focused PhD research investigating connections from animal to human health in the context of zoonotic disease in Australia.*

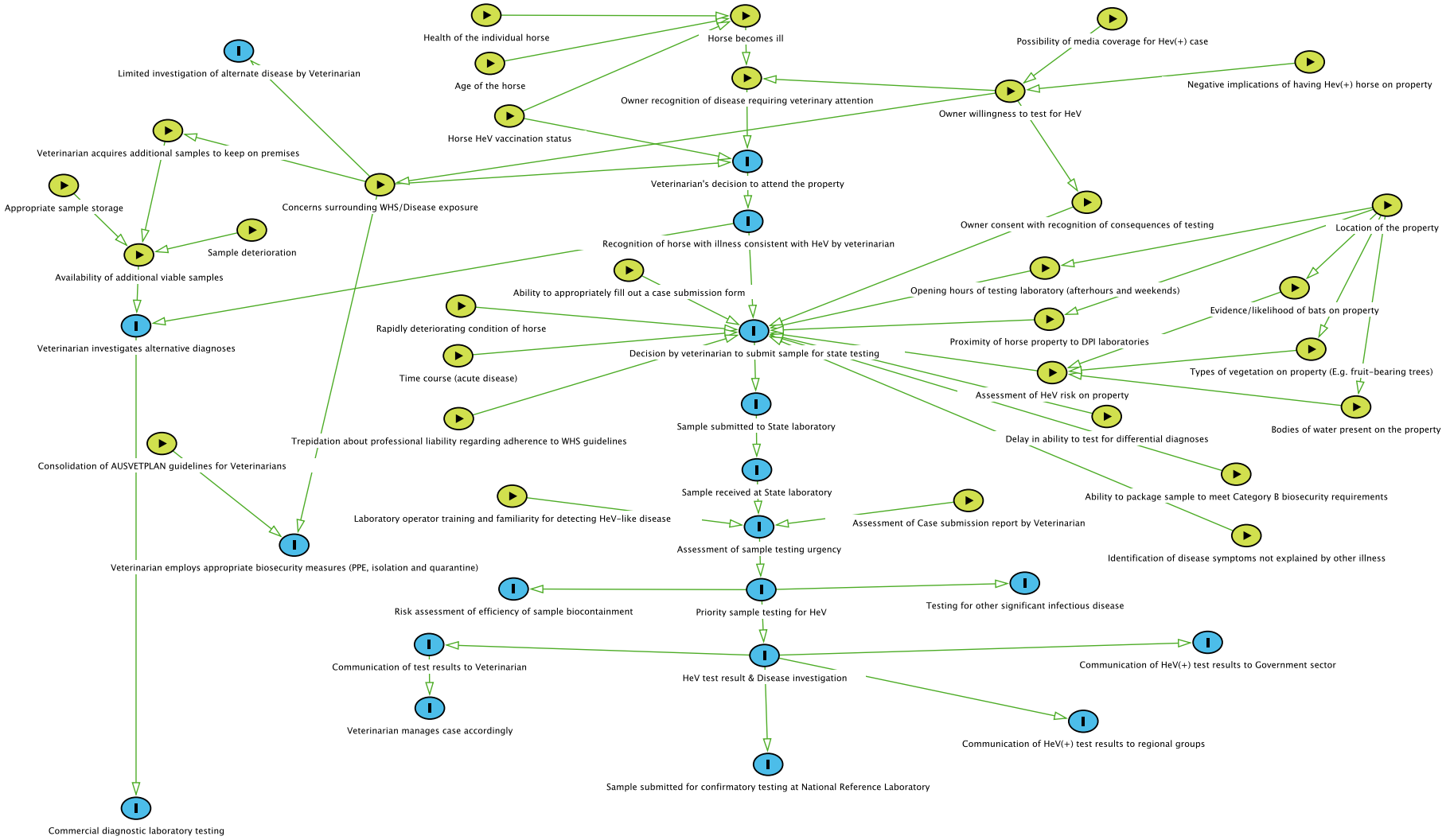

Figure S5.2: Wider influencing factors mapped using Directional Acyclic Graph (R Daggity) in their interactions to core processes to guide their incorporation into the system process framework understanding of HeV surveillance operations

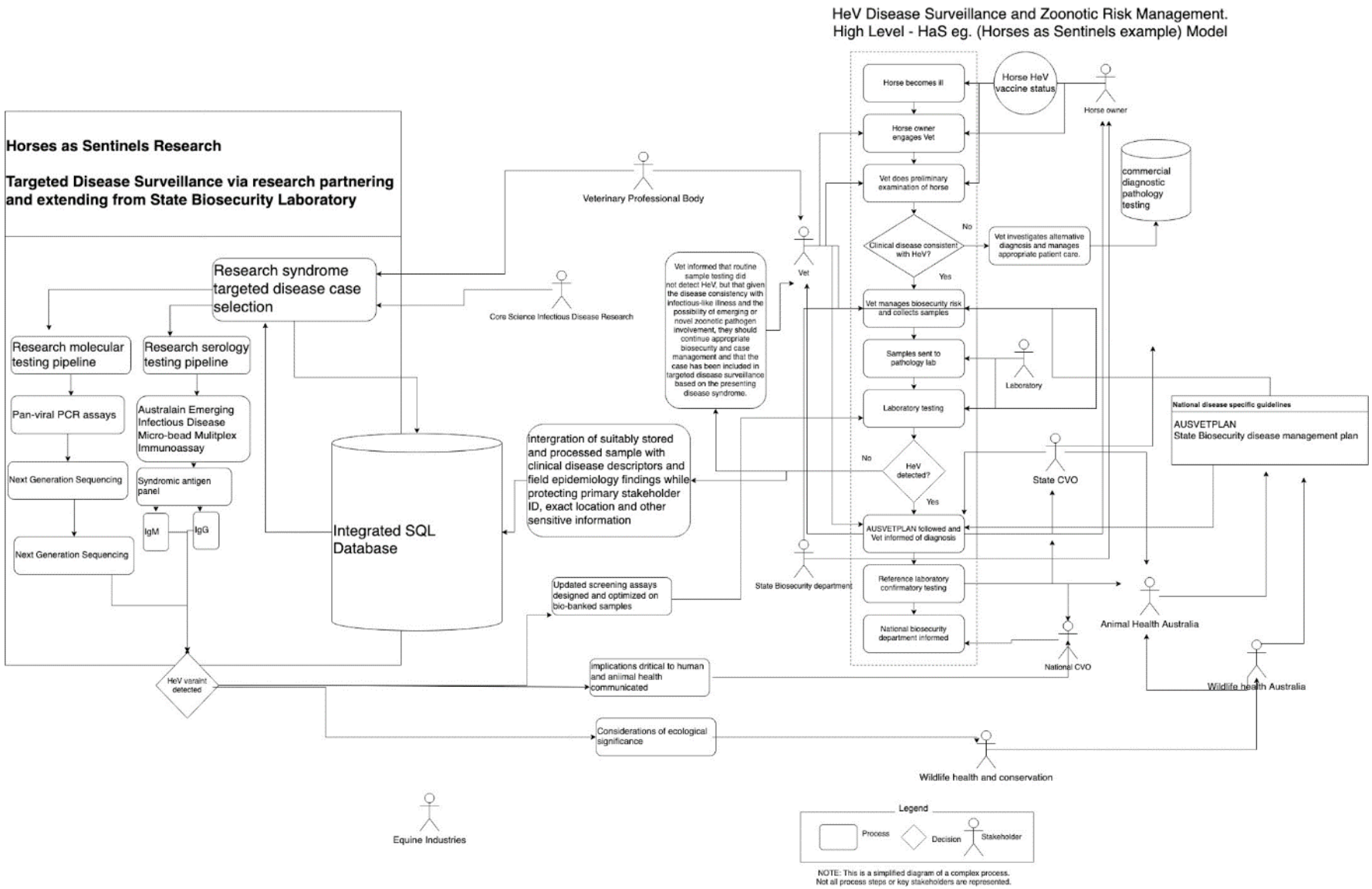

Figure S5.3 Extensions of the routine systems processes enabled via the Horses as Sentinels research approach

Box S5.1. Decision process for the veterinarian

The decision for a veterinarian to attend a property with a potential HeV case is primarily instigated by a horse becoming ill and the owner recognising the requirement for veterinary attention. The likelihood of the horse becoming ill depends upon its general health, the location of the property where it resides (as specific disease risks and prevalence may be influenced by geographical location) and its HeV vaccination status. The owner's decision to contact a veterinarian may be influenced by their willingness to test for HeV, which may be adversely influenced by the possibility of negative outcomes associated with having an HeV (+) horse on their property and the implications of media coverage associated with a confirmed case of Hendra. Alternatively, a concern for the possible exposure of both humans and horses to HeV may influence the owner to contact a veterinarian for attention and subsequent testing for HeV.

The Veterinarian's decision to attend the property and conduct sampling of the horse may be similarly influenced by concerns regarding work health and safety (WHS) and disease exposure risk (for either circumstance, a veterinarian may wish to avoid the potential risk of HeV exposure). The owner's willingness to test for HeV may contribute to the concern for WHS and disease exposure risk for the veterinarian, particularly in circumstances in which the owner does not wish to test for HeV but requests for the veterinarian to investigate alternative diagnoses (under which circumstances, a veterinarian's examination and approach to diagnosis of the horse may be limited by their concern for personal HeV exposure). Upon recognition of a horse with illness consistent with HeV, a veterinarian may instigate precautionary biosecurity measures, including isolation and quarantine of the identified horse and the use of PPE appropriate for HeV management.

The decision for a veterinarian to submit a sample for state testing may be facilitated by a consolidation of the AUSVETPLAN Guidelines (providing guidelines pertaining to disease investigation including requirements for isolation of the identified horse and requirement for appropriate PPE measures). This decision may also be influenced by the veterinarian's assessment of the HeV risk on the property − factors that contribute here would be the property's location, the possibility of bats on the property, a consideration of the vegetation on the property (particularly, the identification of tree species that may attract bats, such as fruit-bearing trees) and the presence of bodies of water − and the proximity of the horse property to DPI laboratories. The latter may have implications for the logistics of sending away a sample. Furthermore, a veterinarian's experience with encountering previous suspect HeV cases that have resulted in a negative HeV result without an alternative diagnosis provided by the state laboratory may influence their likelihood of submitting a sample.

A veterinarian's suspicion and decision to sample for HeV infection may be supported by the identification of disease symptoms during physical examination of the horse that cannot be explained by another known or differential diagnosis. Also, the nature of disease including its time course and the rate of deterioration of the horse's condition would be considered. A consideration of the owners' circumstances may influence the decision made by a veterinarian, such as the cost of HeV testing to the owner (particularly, the indirect costs associated with the possibility of requiring subsequent disease investigation if the horse is HeV negative) and the ability to obtain owner consent with confidence that the owner sufficiently understands the potential consequences of testing. Consequences include the potential burdens for the horse owner relating to compromises in most appropriate treatment, and in the extent or the timelines of appropriate treatment due to biosecurity risk until negative results of Hendra testing are returned. As a result, veterinarians may be less inclined toward submitted samples in suspect cases. Veterinarians may face significant mental distress while awaiting the results of HeV testing, especially when considering that most vets will choose to isolate themselves from others to alleviate the potential risk of HeV transmission (despite there being no instances showing human-to-human HeV transmission). Furthermore, in circumstances where the perceived risk of exposure is low the samples may not be given priority testing, in which case veterinarians and owners may be required to wait for a longer time before receiving results. This may lead to rapid deterioration of the horse in question and significant distress for the individuals involved. As it currently stands, a veterinarian is not able to update the laboratory if the index of suspicion becomes more urgent (for example, the horse begins to demonstrate clinical signs that are more convincing of HeV infection or significant disease progression). In these circumstances, the vet may need to euthanase the horse and collect and store samples for future analysis, which may put them at a higher risk of exposure.

Box S5.2. Procedures that occur following laboratory receival of a sample and diagnostic testing

Once the sample arrives at the laboratory, an assessment of the urgency of testing is made, and the suitability of biocontainment of the sample will additionally be assessed. Any sample requiring HeV testing must be contained according to Category B biocontainment requirements, and the sample needs to be clearly marked as suspecting HeV as a differential so that the sample can be unpackaged and tested by appropriately trained staff within the confines of a physical containment 3 (PC3) biocontainment hood. If the laboratory staff suspect that a breach has occurred in the biocontainment of the sample, then immediate risk assessment and tracing of the transport company and any other potentially exposed points in the submission pathway are undertaken.

Following the submission of a suitable sample for state laboratory testing, the veterinarian will be informed of a potential HeV diagnosis. The veterinarian will await the result of priority testing for HeV and other infectious causes of significant diseases for which state test is available and relevant. The veterinarian may investigate alternative diagnoses through commercial diagnostic laboratory testing only following notification by the State laboratory that a horse has tested negative for HeV infection, as samples acquired for HeV testing are recognized as a biohazard until a negative diagnosis is confirmed. This results in a time delay before alternative diagnoses can be explored.

Often, Veterinarians fail to acquire additional samples at the time of initial sampling, and the risk of possible repeated HeV exposure required to obtain additional samples on a subsequent occasion (including risk to veterinary practice staff and the veterinarian's family) may breach their efforts to remain biosecure. These complications may result in the only suitable samples being sent to the State Laboratory; as such, the opportunity to conduct commercial or inhouse diagnostic testing that the Veterinarian would otherwise rely on to guide best treatment and management of the case (including haematology, biochemistry and inflammatory marker serology testing) would be lost.

Other significant factors influencing poor sample retention include a failure to provide adequate sample storage conditions, including appropriate refrigeration of the sample due to a lack of in-field resources (such as a dedicated biohazard storage refrigerator) or inappropriate packaging of the sample for storage (additionally potentiating a biohazard risk of possible HeV exposure). Suggestions for improvement include (1) improved/reduced turnaround for Veterinarians to receive HeV test results, (2) improved guidelines for how Veterinarians can suitably store samples whilst they are awaiting laboratory results (both in terms of maintaining sample viability and reducing biosecurity risks associated with biohazard sample storage) or (3) the state laboratory could provide improved incentive for Veterinarians to send in samples for HeV testing by offering to provide concurrent routine commercial/inhouse type sample analysis at a reduced cost to competitor companies (offering, for example, fibrinogen measurement and biochemical profiling). This may include improved guidelines for collecting samples from suspected HeV case horses (including additional guidelines for providing extra tubes/samples required for alternative disease testing) that may ultimately reduce the risk of Veterinarians losing the entire sample acquired at initial case presentation to HeV testing alone and may reduce the time delay between receiving a negative test result and exploring alternative differential diagnoses.

Box S5.2. (Continued)

Providing greater support and incentive for veterinarians to critically engage with laboratory testing and recommended testing guidelines (for example, by improving/creating additional guidelines, offering concurrent diagnostic testing opportunities within the state laboratories) may be imperative for improving HeV management within the field. From a professional liability standpoint, veterinarians may be vulnerable when approaching HeV management, particularly when considering the previous prosecution of three veterinarians for WHS biosecurity breaches observed during active HeV cases. This event prompted the amendment of WHS guidelines leading to the provision of greater context for the interpretation of guidelines to ensure that they are specific to the context and interpreted as a shared responsibility for the animal owner, the vet and the property owner.

Submission of the samples to the state animal health laboratory is accompanied by a case submission form that may be completed on line or in hard copy. This form follows the structure and requirements for the biosecurity department to undertake a significant disease investigation. It asks for description of the property and the clinical signs of disease in sampled individuals as well as in the context of the farm animal population. It also provides opportunity for the submitting veterinarian to specify differential diagnoses including any notifiable diseases for which testing will be covered by the state.

However, there are missed opportunities to best interpret the disease presentation and clinical manifestation of the disease if there is insufficient detail entered on the form by the veterinarian. This may lead to increased difficulty for analysis and interpretation by the duty pathologist and laboratory staff, who are faced with the challenge of diagnostic analysis in the face of insufficient case history and clinical details. This situation may arise due to a lack of specific prompting for increased detail on the submission form for the most appropriate information in their species and disease context. This is fuelled by the historic use of these forms for use in production species, without sufficient modification to include information that may relate to domestic or performance species such as the horse. In particular, the requirement for listing a property identification code within these forms is not satisfied by vets on these properties, as such a code is not routinely available for non-primary production properties, such as the peri-urban properties that commonly keep horses. Secondly, these forms offer very little opportunity to identify an individual animal by name or, even more appropriately by a microchip identification number (or defining animal characteristics such as markings, brandings and scars). Due to the spatial restrictions for listing leading differentials on these forms (which may prove relevant in circumstances of livestock production where only a few leading differentials may be considered), there is less opportunity to describe the considerations for differential diagnoses that would otherwise be relevant to individual equine cases that have undergone animal-specific extensive clinical investigation.

Another major constraint on the ability for the veterinarian to provide extensive clinical details on the case submission form is that the vet is often limited by time constraints and/or the appropriate opportunity in which to readily fill out the form. Time constraints are fuelled by the addition of time-consuming sampling and submission processes that are often unplanned for and fall within a veterinarian's busy daily schedule. Furthermore, vets who complete these case submission forms often do so while they are still within full PPE at the sampling site and may include the paperwork into the pathology sample packaging. To improve these conditions in the future, specific guidance and instruction on how best to fill out the case submission form under the difficult field and biosecurity circumstances surrounding a suspected HeV case could be provided as part of training sessions that additionally address the most suitable sampling, case identification and biosecurity management procedures.

A particular challenge that presents around receiving priority assessment of a suspected HeV case occurs when a case identification falls outside of normal laboratory hours (for example, over a weekend). This problem is contributed to by an inability to submit a sample in regions where remote location and regional labs are no longer available. Submitting an out-of-hours sample may require specific case approval from the CVO (only once assessment and emergency animal disease hotline have been consolidated) and a high likelihood of Hendra based on location, clinical signs of the presenting horse and your medical perception as a veterinarian (often with a need for significant exposure of humans to the suspected case before priority assessment is received). Since this approach is not reliable, extra resources have been allocated toward hiring an on-call employee to provide Hendra exclusion analyses on weekends; however, the difficulty of contacting federal and state department members at these times to inform them of a potential case remains a significant dilemma.

Box S5.3. Procedures that occur following the receival of a positive or negative testing outcome

Suspected HeV samples undergo HeV exclusion testing at the state veterinary laboratory. During this time, testing for all other possible differentials is prohibited (to attenuate the risk of potential exposure to HeV cases).

In circumstances where HeV is not detected, the samples are stored for three months prior to being discarded (as required by NATA). No further testing is conducted on these samples, and a report outlining the findings will be provided.

In circumstances where the horse tests positive for HeV, the state laboratory will immediately notify the relevant government officials of the case, and the sample will be prepared to be sent to the Australian Centre for Disease Preparedness (ACDP) for confirmatory testing and isolation of the virus. The state Chief Veterinary Officer (CVO) will be alerted to the case and this information will be passed onto the federal CVO as HeV is a notifiable disease that must be reported to the OIE. The state CVO will enforce a quarantine of the affected property, and all movement of horses in and out of the property will be prevented. An epidemiological investigation (managed by the Department of Agriculture) will follow to assess the risk posed to neighbouring properties, to trace any horses that may have moved off the property and to conduct HeV exclusion testing on all horses that have been suspected of having contact with the index case. Horses that appear HeV negative will undergo serial sampling and retesting for the duration of the expected incubation period (five to 16 days).

The state Chief Health Officer (CHO) will additionally be notified, and Public Health Officers will be recruited to identify the risk of HeV exposure (assessed via interviews) of the attending veterinarian, horse owners and any other individuals that have come into contact with the affected horse. Individuals at a high risk of HeV exposure − high risk potentially exposed persons (PEPs) − will be tested for HeV antibodies and undergo a comprehensive medical examination, and may be offered human mAb therapy on compassionate grounds. Individuals that are less heavily exposed can also request HeV testing.

For all potentially exposed persons, nasal-pharyngeal aspirates and blood samples will be acquired for testing at QH laboratories. Baseline serology will be acquired and repeated at 21 days and 42 days post-exposure. Following testing, if HeV antibodies are not detected individuals will be required to undergo serial retesting and monitoring (for development of HeV-related symptoms) over a 14-day period (incubation period in humans: five to 21 days). In circumstances where HeV antibodies are detected (either at initial testing or within the 14-day monitoring period), individuals will be hospitalised and PEP human mAb therapy will be administered.

Horses as Sentinels of Emerging Infectious Disease

Chapter 6

SYNDROMIC AND PATHOLOGIC ANALYSES OF HeV DISEASE OBSERVATIONS TO GUIDE CASE SELECTION FOR TARGETED SENTINEL DISEASE SURVEILLANCE AND ZOONOTIC PATHOGEN DISCOVERY

Statement of personal contribution: I drafted the first complete manuscript (2019−21) and received feedback from PhD supervisors. I received support in the approach to SQL design and construction and Upset plot in R from project collaborator Dr John Grewar who had specifically relevant expertise process and assistance in Clinical descriptor term classification and case data entry and infectious disease category priority assessment from project collaborators Dr Anne Jackson and Dr Nicole Brown respectively.

Format: This chapter has been prepared primarily for this purpose (rather than for peer review journal submission).

Much of the material in this chapter describes the research approach similarly to that reported as part of the Biosecurity Innovation Program Project 2020-21 Project ID 202043*: Metagenomic Investigation of Horses as Sentinels: Innovative metagenomic, multiplex serology and custom database combined in testing cases of priority infectious clinical syndromes to greatly improve diagnosis of infectious diseases and Australia's biosecurity*, Examples are in milestone documents and project reports. To my knowledge these are not anticipated to be published publicly but rather used to guide internal procedures in Australia's government departments.

Thus, some of the findings and perspectives included in this thesis chapter may be further communicated and incorporated into grey literature regarding biosecurity and surveillance for EID in Australia. Where suitable and over time, these may be presented as part of peer-reviewed scholarly research outputs.

6.1 Introduction

The emergence and management of Hendra virus (HeV) in Australia has highlighted a great many challenges to proactive management of highly fatal zoonotic disease risk for the veterinary profession.

HeV disease outbreaks in horses result from spillover from the reservoir of the virus in flying foxes (*Pteropus* spp.) and occasionally result in zoonotic infection in close-contact humans. HeV causes acute, usually fatal, pneumotropic and neurotropic while diffuse disease in horses and humans, mediated by systemic endothelial vasculitis. Horses infected with HeV usually demonstrate rapidly progressive respiratory and/or neurological disease with while the combination and variety of clinical signs evident in any single time-point observation are numerous. Each time point represents a moment in the course of infection, often featuring non-specific and wide-ranging clinical signs, especially during early infection. Virus may be shed by infected horses up to two days prior to clinical illness.[1]

HeV infection in horses is diagnosed as part of a priority disease investigation by quantitative real-time RT-PCR. Importantly, this is reliant on suspect case notification and sample submission by attending private practising veterinarians. Managing the risk of in-contact human disease relies on timely conclusive laboratory diagnosis in the horse.

This chapter extends from review of the pathologic basis of HeV disease in horses, focusing on how disruption of subcellular, cellular, tissue and systemic homeostasis arising from infection of the virus give rise to the expected manifestations of clinical disease (Chapter 2), and description (qualitative) of practitioner perspectives from confirmed and highly suspect cases (Chapter 4). A pathologically guided quantitative syndromic assessment of HeV disease observations is presented consolidating the previously argued understanding and demonstrating an approach that holds operational benefit for proactive research and/or surveillance system recognition of suspect cases and assignment of appropriate case-specific diagnostic responsibility. Individual clinical signs were condensed by equivalence to most representative terms and categorised in their pathologic basis including indexing by Medical Subject Descriptor database (MeSH). Detailed signs as well as their pathologic category as they occurred in individual cases were plotted as frequency of observed combinations (syndromes) using 'UpSetR'.

As a rare and fatal disease in horses, our understanding of the natural history of infection with HeV and its catalogue of clinical signs is hampered by biosecurity measures to limit exposure to infected animals. Indeed, several factors have limited efforts to establish consistent clinical presentation, which would support timely recognition of HeV as a differential diagnosis. These are:

- the wide range of clinical features that may be observed in any single examination
- the subtlety and non-specificity of early disease
- variable and often limited opportunities in equine veterinary practice for extensive and repeated clinical observation.

This recognition is a critical step towards prompting suspect horse-case sampling and laboratory submission, biosecurity and case management including appropriate timely recognition of human exposure and infection to enable time dependent potentially life-saving treatment. Therefore, we undertook a systematic analysis of all reported clinical signs drawn from published scientific and grey literature, to further develop a systematic approach to optimise the sensitivity of suspect case selection, informed by expected clinical-pathogenic disease progression of HeV infection in horses (Chapter 2), and to guide assignment of diagnostic responsibility on which laboratory investigations depend, regardless of limitations relating to the duration or detail of examination and extent of communicated descriptions via laboratory submission forms.

The primary objectives were to:

- fully describe the variety of clinical observations reported in HeV-infected horses
- consider any limitations of relying on a restricted list of clinical parameters as indicators of possible HeV infection
- improve the understanding of the clinical natural history and pathogenesis of HeV disease in horses
- develop an enhanced description of the clinical syndrome of HeV in horses to aid field veterinarians in suspect case recognition and to inform the design and interpretation of laboratory submission forms
- prioritise cases that had the highest likelihood of an infectious cause to undergo a comprehensive molecular and serological laboratory analysis.

Initially an analysis of all reported clinical descriptions of past HeV cases is undertaken. Individual clinical signs are categorised by their pathogenic basis informed clinical equine veterinary experience and pathologic basis of HeV disease (Chapter 2) and standardised by indexing to most representative Medical Subject Headings database (MeSH). Descriptive analyses of observed signs categorised in this way was then undertaken highlighting the predominance of vascular inflammatory disease processes in addition to respiratory and neurological manifestations. Syndromic plotting (UpSetR) of signs as they occurred in combination in individual cases, both as detailed signs and collapsed into pathogenic categories was then undertaken using a bespoke package in R (UpSetR) offering further consolidated understanding of how broad ranging signs may be most sensitively interpreted in their combined manifestations as consistent with HeV infection. These combined approaches are relevant for clinical, biosecurity and epidemiology veterinary interpretation of suspect case descriptions to guide assigning diagnostic responsibility. The findings serve to assist practising veterinarians in recognising suspect cases and managing biosecurity risks to humans in the clinical setting. In this sentinel risk-based active surveillance research activity, the analysis informed prioritisation of cases from a large sample biobank to focus resources for greatest likelihood of detection of both target emerging (known unknown) and novel (unknown unknown) pathogens. In government biosecurity laboratories, the analysis will also serve to assist duty pathologists in interpreting case descriptions as part of prioritising limited testing resources.

These analyses were coupled with the development of a purpose-built SQL database that combined laboratory and sample details with de-identified sample event, subject and clinical details. This allowed determination and systematic processing of samples that had the highest likelihood of having an infectious cause of target or emerging significance and that therefore warranted further laboratory investigations. Importantly, this approach serves as a model that may be applied more broadly to inform future passive and targeted surveillance programs to identify cases of priority emerging and novel infectious diseases.

6.2 Quantitative and qualitative analyses of syndromic consistency amongst all reported clinical manifestations of HeV infection in horses by systematic reference to pathologic bases of disease in clinical disease terms.

6.2.1 Description of Clinical Signs

A comprehensive list of all reported clinical signs associated with HeV diseased horses was compiled systematically for a previous purpose unrelated to this manuscript in 2017 by veterinarian Dr Richard L'Estrange, and then extended here to include the cases of 2018 and 2019. Briefly collation of data involved systematically reviewing all publicly available data sources for content relating to clinical signs or observations attributed to individual horses assessed as having been infected with HeV. The original purpose had been to establish the fullest detail and variety of clinical descriptor terms associated with HeV infection to establish the widest range of clinical representations in which HeV infection might be pose zoonotic risk highlighting the benefit of vaccination as the single most effective measure to reduce zoonotic HeV infection risk in the clinical setting. Thus, this approach afforded data of a very alternate potentially biasing priority to that of this research activity which conversely prioritised maximising the coherence and consistency of descriptions to guide most sensitive suspect case recognition for surveillance purposes.

The accessed sources included the commissioned expert report into the original HeV outbreak,[2] published peer-reviewed literature, HeV outbreak notifications issued by state departments of agriculture or primary industries, and the HeV summary documents that are updated on the Biosecurity Queensland (now Business Queensland) website.[3] We limited the use of personal communication data to a minimum to avoid recall bias. Practice records were not accessed due to time and labour limitations. Finally, it should be noted that some sources described clinical progression of cases (longitudinal records), while others simply listed clinical signs attributed to a case (cross-sectional records).

Initial processing of these data for this research activity involved consolidating listed terms for which terms of duplicate meaning were included into their most representative detailed medical descriptor term from the perspective of clinical examination. (Table 6.1 & 6.2). For example, listed term 'depression lethargy or dullness' was used to represent listed terms 'somnolent' and 'head held low'. Combining equivalent listed terms in this way refined the number of terms from 125 to 77 (Table 6.2).

Notably a single clinical descriptor term was found to have been incorrectly associated with HeV disease due to reporting bias. This was 'hypopnea' (red shading and # in table 6.2) which refers to reduced respiration rate. This term was included in published literature description of the initial Hendra 1994 spillover cases without inclusion of the attending clinician (R. Reid) in the authorship. In this instance as in the only other instance (Bowen 2009) this research cross checked firsthand clinical descriptions (Chapter 4) and found this to be misinterpretation of 'rapid shallow breathing' through the process of government agency recording of clinical signs including by phone interview following HeV infection diagnosis had been confirmed. This highlights the importance of consultation with attending clinicians of all reports including description of clinical findings to avoid misinterpretation (reporting bias) that may confound challenges for practicing clinicians in recognising suspect cases.

Individual clinical descriptor terms listed in association with HeV infection were tabulated and indexed to their most representative equivalents in the MeSH medical descriptor term database. The table was then organised by body system and syndrome association and columns populated considering each individual term in relation to their potential meanings when used to describe the clinical condition of horses (See Table 6.1 and Appendix 7 Spread sheet: Section 2); terms defined clinical and patho-physiological syndromic assessment (Table 6.1/ Appendix 7 Spread sheet: Section 3); Their potential association with conditions and causes of disease in horses (Table 6.1/ Appendix 7 Spread sheet: Section 4); Seasonal and geographical influences of these clinical manifestations or their underlying causes in Australian horses (Table 6.1/ Appendix 7 Spread sheet: Section 5); and the frequency with which they are observed in domestic Australian horses (Table 6.1/ Appendix 7 Spread sheet: Section 6).

Table 6.1. Descriptions of columns by Section of 'Appendix 7 - *Spread sheet example of systematic consideration of clinical descriptor terms listed in association with HeV infection and amongst a comparative cohort of severe equine disease events of lower likelihood of infection cause (adverse events following immunization AEFI)'*

Section Ref.	Column heading	Column description
Description of columns in **Section 1 - Descriptor term sources**		
1.0	**Clinical term standardised**	
1.1	**Clinical descriptor term observed with HeV disease in horses (most representative and detailed)**	
1.2	**Listed equivalent descriptor term as listed in association with HeV infection**	
1.3	**Clinical descriptor term as listed in association with AEFI**	
1.4	**Listed equivalent descriptor term as listed in association with AEFI**	
1.5	**Listed collective terms**	
1.6	**Pathologic syndromic term**	
1.7	**Collective disease or body system terms**	
Description of columns in **Section 2 - Descriptor terms defined**		
2.0	**Term defined**	
2.1	**Descriptor term category/ies**	Order listed: Most-common to least-common when used in equine practice towards description of clinical condition. (Physiological phenomena/ Physiological parameter/ Altered physiological parameter partially specified in magnitude, direction or location/ Altered physiological parameter insufficiently specified in direction, magnitude or location/ Patho-physiological phenomena/ Patho-physiological parameter specified in magnitude, direction or location/ Patho-physiological parameter insufficiently specified in magnitude, direction or location/ Pathological Syndrome/ Broad pathological syndrome/ General pathological descriptive term/ Clinical sign/ Clinical syndrome/ Non-scientific general term/ Non-specific scientific term, relatively specific non-scientific term)
2.2	**Specificity in clinical description of disease and causal consideration**	(very general and common/ low/ moderate/ high/ very specific or pathognomonic)
Description of columns in **Section 3 – Clinical and patho-physiological syndromic assessment**		
3.0	**MeSH hierarchical indexing in 'Pathological conditions, signs and symptoms' schema**	Indexing and hierarchy of listed descriptor term under 'Pathological conditions, signs and symptoms' schema in the MeSH descriptor database 2020
3.1	**Pathological conditions, signs and symptoms MeSH index number**	'Pathological conditions, signs and symptoms' schema index-reference under in MeSH descriptor database 2020
3.2	**Associated syndrome**	Clinical syndrome most equivalent to the listed descriptor term and most appropriate for syndromic interpretation
3.3	**Associated organ and body systems**	Organs and body systems of highest relevance to the listed descriptor term reflecting the diseases with which it might most likely be observed
3.4	**MeSH hierarchy body system disease schema**	Indexing and hierarchy of listed descriptor term to 'Disease descriptor headings' in MeSH descriptor database 2020 relating to Disease processes indexed by body system
3.5	**Diseases by body system MeSH index**	Index-reference of most 'Diseases by body system' in MeSH descriptor database 2020
3.6	**Additional MeSH hierarchy**	Additional appropriate indexing in MeSH descriptor database 2020. (Selection of most relevant included only.)
3.7	**Associated MeSH disease categories ordered by frequency observed**	Disease categories of highest relevance to the listed descriptor term as listed in MeSH data base 2020 ordered by frequency observed in clinical equine practice Comprising: bacterial infections and mycoses/ virus diseases/ parasitic diseases/ neoplasia/ musculoskeletal diseases/ digestive system diseases/ stomatognathic diseases/ respiratory tract diseases/ otorhinolaryngologic diseases/ nervous system diseases/ eye diseases/ urological and male genital diseases/ female genital diseases and pregnancy complications/ cardiovascular diseases/ hemic and lymphatic diseases/ congenital, hereditary, and neonatal diseases and abnormalities/ skin and connective tissue diseases/ nutritional and metabolic diseases/ endocrine system diseases/ immune system diseases/ disorders of environmental origin"
3.8	**Lay term equivalents of associated MeSH disease categories ordered by frequency observed**	Lay term equivalents of Disease categories listed in 3.7 Comprising: Bacterial and fungal infections/ Viral infections/ Parasite burden/ Cancers/ Musculoskeletal diseases/ Digestive system diseases/ Oral and dental diseases/ Respiratory diseases/ Ear, nose and throat diseases/ Nervous system diseases/ Eye diseases/ Urinary system diseases/ Stallion genital diseases/ Mare genital diseases and pregnancy complications/ Heart and blood vessel diseases/ Blood and body fluid diseases/ Hereditary and congenital diseases/ Skin, coat, hoof wall and sole diseases / Nutritional and metabolic diseases / Hormonal system diseases/ Immune system diseases/ Trauma, injury, inflammation, toxicities and other diseases of environmental cause.

Table 6.1 (continued)

Section Ref.	Column heading	Column description
Description of columns in **Section 3 – Clinical and patho-physiological syndromic assessment**		
3.0	**MeSH hierarchical indexing in 'Pathological conditions, signs and symptoms' schema**	Indexing and hierarchy of listed descriptor term under 'Pathological conditions, signs and symptoms' schema in the MeSH descriptor database 2020
3.1	**Pathological conditions, signs and symptoms MeSH index number**	'Pathological conditions, signs and symptoms' schema index-reference under in MeSH descriptor database 2020
3.2	**Associated syndrome**	Clinical syndrome most equivalent to the listed descriptor term and most appropriate for syndromic interpretation
3.3	**Associated organ and body systems**	Organs and body systems of highest relevance to the listed descriptor term reflecting the diseases with which it might most likely be observed
3.4	**MeSH hierarchy body system disease schema**	Indexing and hierarchy of listed descriptor term to 'Disease descriptor headings' in MeSH descriptor database 2020 relating to Disease processes indexed by body system
3.5	**Diseases by body system MeSH index number**	Index-reference of most 'Diseases by body system' in MeSH descriptor database 2020
3.6	**Additional MeSH hierarchy**	Additional appropriate indexing in MeSH descriptor database 2020. (Selection of most relevant included only.)
3.7	**Associated MeSH disease categories ordered by frequency observed**	Disease categories of highest relevance to the listed descriptor term as listed in MeSH data base 2020 ordered by frequency observed in clinical equine practice Comprising: bacterial infections and mycoses/ virus diseases/ parasitic diseases/ neoplasia/ musculoskeletal diseases/ digestive system diseases/ stomatognathic diseases/ respiratory tract diseases/ otorhinolaryngologic diseases/ nervous system diseases/ eye diseases/ urological and male genital diseases/ female genital diseases and pregnancy complications/ cardiovascular diseases/ hemic and lymphatic diseases/ congenital, hereditary, and neonatal diseases and abnormalities/ skin and connective tissue diseases/ nutritional and metabolic diseases/ endocrine system diseases/ immune system diseases/ disorders of environmental origin"
3.8	**Lay term equivalents of associated MeSH disease categories ordered by frequency observed**	Lay term equivalents of Disease categories of highest relevance to the listed descriptor term as listed in MeSH data base 2020 ordered by frequency observed in clinical equine practice Comprising: Bacterial and fungal infections/ Viral infections/ Parasite burden/ Cancers/ Musculoskeletal diseases/ Digestive system diseases/ Oral and dental diseases/ Respiratory diseases/ Ear, nose and throat diseases/ Nervous system diseases/ Eye diseases/ Urinary system diseases/ Stallion genital diseases/ Mare genital diseases and pregnancy complications/ Heart and blood vessel diseases/ Blood and body fluid diseases/ Hereditary and congenital diseases/ Skin, coat, hoof wall and sole diseases / Nutritional and metabolic diseases / Hormonal system diseases/ Immune system diseases/ Trauma, injury, inflammation, toxicities and other diseases of environmental cause.
Description of columns in **Section 4 – Consideration of causes**		
4.0	**Description of causes other than HeV**	
4.1	**Most common causes**	
4.2	**Less common causes**	
4.3	**Rare or least common causes**	
4.4	**Considerations for clinical investigation of aetiology**	Considerations of causes in clinical veterinary practice
Description of columns in **Section 5 – Seasonal and geographical influences**		
5.0	**Seasonal influence**	
5.1	**Geographical influence**	
Description of columns in **Section 6 – Frequency in domestic horses**		
6.0	**Frequency observed in domestic equids**	
6.1	**How common is the condition described by the descriptor term in clinical practice?**	Very Common/ Common /Consistent but Uncommon / Rare/ Very Rare

This systematic approach to standardisation of descriptor terms informed the consolidation of terms (Table 6.2) as well as categorisation of each term as one of the following* (Table 6.3):

> Physiological phenomena/ Physiological parameter/ Altered physiological parameter partially specified in magnitude, direction or location/ Altered physiological parameter insufficiently specified in direction, magnitude or location/
> Patho-physiological phenomena/ Patho-physiological parameter specified in magnitude, direction or location/ Patho-physiological parameter insufficiently specified in magnitude, direction or location/ Pathological Syndrome/ Broad pathological syndrome/ General pathological descriptive term/ Clinical sign/ Clinical syndrome/ Non-scientific general term/ Non-specific scientific term, relatively specific non-scientific term

* Order listed: Most-common to least-common when used in equine practice in describing clinical condition

This allowed further systematic syndromic categorisation. Mapping to both disease as well as 'signs and symptoms' descriptor term schema in MeSH allowed for the systematic syndromic categorisation to be informed by systematic consideration of relevance to both clinical, anatomical (body system) and pathological contexts.

An extensive spread sheet representing this approach is offered as a supplementary pdf file (Appendix 7 - *Spread sheet example of systematic consideration of clinical descriptor terms listed in association with HeV infection and amongst a comparative cohort of severe equine disease events of lower likelihood of infection cause (adverse events following immunization AEFI*).

Table 6.2. Consolidated disease descriptor terms reported in association with HeV infection

Most representative and detailed clinical descriptor term observed with HeV disease in horses	Consolidated clinical term as listed for HeV
dependent_oedema	
oedema_of_the_head_and_neck	oedema head/face/ mandible/tongue/lips/neck
enlarged_mandibular_ln	
cyanosis_muddy_toxic_mm_endotoxaemia	reddened/congested MM sluggish capillary refill enterocolitis_colitis_without_diarrhoea**
petechiation_and_ecchymosis	
poor_circulatory_function	
dry_mm	
behaviour_changes	
depression_lethargy_or_dullness	somnolent head held low
seriously_ill	moribund or unresponsive weakness
pain and/or psychomotor agitation* and/or disorientation	aimless pacing or disorientation
pain and/or psychomotor agitation*	irritability/biting weaving
oral_discomfort_or_maniacal_and_erratic_behaviour	shaking head in bucket of water
Hyperaesthesia	hypersensitivity when approached
teeth_grinding_jaw_chomping**	oral discomfort**
neurological_signs	
teeth_grinding_jaw_chomping**	oral discomfort**
maniacal_and_erratic_behaviour	thrashing violently
seizure	vocalising
nystagmus	
head_pressing	
head_tilt	
muscle_trembling_spasms_fasciculations	twitching of eyes
blindness	
facial_nerve_paralysis_droopy_lips	
urinary_incontinence	
reduced_tongue_tone	
priampsm	penile erection/protrusion
opisthotonus	opisthotonus neck extension
ataxia	ataxia/unsteady on feet difficulty walking stumbling/staggering/loss of balance
hypermetric_gate_and_or_proprioceptions_defectis	altered gait/high stepping/short stepping
muscle_trembling_spasms_fasciculations	neurological muscle twitches rigid stance/stiffness
musculoskeletal pain and/or inflammation	lameness
recumbency_collapse	
ataxia_unsteady_on_feet	difficulty rising wide-based stance circling
musculoskeletal pain and/or inflammation	weight shifting
reduced_or_absent_gut_sounds	
hypermotile_gut_sounds	
abdominal_pain_or_colic_pawing_at_ground	abdominal pain or colic, pawing at ground rolling
abdominal_pain_or_colic_pawing_at_ground or neurological_signs or severe_illness	teeth grinding / jaw chomping**
reduced_or_absent_gut_sounds	
enterocolitis_colitis_without_diarrhoea**	blood in faeces
reduced_tongue_tone	salivation
icterus_yellow_mm	
lymphadomegaly	enlarged mandibular LN
respiratory_signs	
pleural_effusion	
pleural_and/or_pericardial_effusion	muffled heart sounds
tachypnoea	Dyspnoea/laboured respiration hyperpnoea#
abnormal_respiratory_auscultation	wet lung sounds auscultatory crackles and wheezes reduced resp sounds
cough	
nasal_discharge	foamy_nasal discharge with blood
lacrimation_ocular_discharge	lacrimation/ocular discharge
urinary_incontinence	bladder distension stranguria
pyrexia	
profuse_sweating	
tachycardia	distended jugular veins
acute_illness	
recumbency_collapse	
seriously_ill	weight loss
acute fatal illness	found dead

*** syndromic or pathologic descriptor term duplicated in table; ** most specific descriptor term duplicated in table**

Table 6.3. *Clinical descriptor term categories assigned* to facilitate systematic hierarchical consolidation enabling syndromic interpretation including UpSet plot by higher level term
Colour shading serves as key of that applied in Table 6.2 and Appendix 7 Spread sheet

'Broad clinical syndrome' attributed to HeV disease case/s **'listed descriptor term'** representative of a **'Broad clinical syndrome'** that encompasses detailed **'Clinical syndrome'**/s	**'Detailed clinical syndrome' attributed to HeV disease case/s** **'listed descriptor term'** representative of a **'Clinical syndrome'** encompassed by **'Broad clinical syndrome'**/s	**'Listed descriptor term' for HeV disease case/s with listed alternative syndromic equivalent** **'Listed descriptor term'** encompassed by '**Clinical syndrome**' of other **'listed descriptor term'**
Detailed 'Clinical syndrome' not attributed to HeV disease case/s **'clinical descriptor term'** not listed in association with HeV infection but representative of a **'Clinical syndrome'** encompassed by **'Broad clinical syndrome'**/s	**'Descriptor term' not listed for HeV disease case/s with alternative listed syndromic equivalent** **'Descriptor term'** not listed in association with HeV infection encompassed by a '**Clinical syndrome'** included as **'listed descriptor term'**	***Additional entry for analysis*** **Combined syndrome summary** Summary of multiple **'Clinical syndromes'**, considered useful only toward broadest syndromic interpretation of **HeV disease case/s**
Additional entry for analysis **Broadest clinical syndrome summary** **'Broadest clinical syndrome'** encompassing one or more **'Broad clinical syndrome'** which, in turn encompasses one or more detailed **'Clinical syndromes'**	***Additional entry for analysis*** **Broad clinical syndrome summary** **'Broad clinical syndrome'** encompassing one or more detailed **'Clinical syndromes'**	***Additional entry for analysis*** **Detailed clinical syndrome summary** **'Clinical syndrome'** summarising multiple **'listed descriptor terms'**
Unsuitable for clinical syndromic interpretation of disease Descriptor term considered unsuitable toward syndromic interpretation of **HeV infection** where referring to normal physiological phenomena or insufficiently defining of clinical condition		**Suitable for interpretation as Disease outcome** **'Listed descriptor term'** considered more suitable for interpretation as an outcome or effect of disease than towards a clinical syndrome referring to physiological phenomena or insufficiently defining of clinical condition

6.2.2 Categorisation of each clinical term into its most appropriate MeSH heading term

The clinical signs were standardised and categorise based on Medical Subject Headings (MeSH). MeSH is a controlled vocabulary or thesaurus developed by the United States National Library of Medicine. The list is based on a cascading tree structure and is used for indexing journal articles and books in the life sciences.[3] NIH NLM Medical Subject Headings (MeSH) medical descriptor term database (2020) was considered the most appropriate database because it includes human, equine, animal model and basic biomedical science peer-reviewed articles relating to henipaviruses and provides the hierarchical structure for mapping the clinical signs.

As some of the exact clinical descriptor terms listed in association with disease in horses did not appear in the MeSH database, the clinical term considered most relevant was used. For example, the listed clinical sign of 'lacrimation/ocular discharge' did not appear in searches

of the MeSH tree. The correct veterinary definition for a clinically evident ocular discharge is 'epiphora'. This is indexed under 'lacrimal apparatus diseases', which maps to 'eye diseases'. As this MeSH mapping relates to specific eye diseases, rather than a pathological response to inflammatory or infectious disease, thus it was considered less appropriate to apply for the context of HeV clinical signs. A further interpretation of 'lacrimation/ocular discharge' term is 'tears' or 'mucus'. Both terms mapped to 'bodily secretions' and then to 'fluids and secretions', which was selected as the best MeSH category to use as most representative of the clinical sign as it would be assessed holistically in potential consistency with HeV infection.

The MeSH categories identified were: neurobehavioral/gait disturbances, weakness and frailty, respiratory system, cardiovascular, gastrointestinal signs, body temperature, mucous membrane changes, secretions, critical/catastrophic disease, pathological clinical signs, blood clotting, and urological.

In total, the 94 clinical descriptor terms were used to report descriptions of HeV disease in 104 naturally occurring cases and three laboratory infections using the HeV REDLANDS 2008 strain (Table 6.2, Spread sheet S6.1 and Figure 6.1).

6.2.2.1 MeSH categories mapped of highest relevance to analysis of all clinical signs reported in equine HeV cases

Based on our understanding of the differential diagnoses (chapter 2), pathogenesis of disease (chapter 2), clinical case summaries (chapter 4) and the MeSH clinical descriptors defined here for HeV disease, we developed a structured framework for understanding the presenting clinical signs in the context of their underlying disease pathogenesis. The following schema demonstrates the varying different database mappings of highest relevance to horses presenting with severe acute HeV-like disease syndromes for the 'Clinical_disease_by _body/organ_system and related to pathophysiology' category MeSH mapping along with 'Microbiology/Virology/Parasitology' and 'Infectious disease core science and diagnoses' (Text box 6.1).

Text box 6.1. Overview of MeSH descriptor term schema to which listed terms were mapped

Microbiology

Viruses [B04]
- RNA Viruses [B04.820]
  - Mononegavirales [B04.820.455]
    - Paramyxoviridae [B04.820.455.600]
      - Paramyxovirinae [B04.820.455.600.650]
        - Henipavirus [B04.820.455.600.650.400]
        - Hendra Virus [B04.820.455.600.650.400.400]
        - Nipah Virus [B04.820.455.600.650.400.550]

Infectious disease core-science and diagnosis orientated

Infections [C01]
- Respiratory Tract Infections [C01.748]
  - Pneumonia [C01.748.610]
    - Pneumonia, Viral [C01.748.610.763]
- Virus Diseases [C01.925]
  - Pneumonia, Viral [C01.925.705]
  - RNA Virus Infections [C01.925.782]
    - Mononegavirales Infections [C01.925.782.580]
      - Paramyxoviridae Infections [C01.925.782.580.600]
      - Henipavirus Infections [C01.925.782.580.600.400]
      - Rubulavirus Infections [C01.925.782.580.600.680]
    - Nidovirales Infections [C01.925.782.600]
      - Coronaviridae Infections [C01.925.782.600.550]
      - Coronavirus Infections [C01.925.782.600.550.200]
        - Severe Acute Respiratory Syndrome [C01.925.782.600.550.200.750]

Clinical disease by body/organ system and related to pathophysiology

Respiratory Tract Diseases [C08]
- Lung Diseases [C08.381]
  - Pulmonary Edema [C08.381.742]
  - Respiratory Distress Syndrome, Adult [C08.381.840]
  - Pneumonia [C08.381.677]
    - Pneumonia, Viral [C08.381.677.807]

Cardiovascular Diseases [C14]
- Arteritis [C14.907.940.090]
  - INFLAMMATION of any ARTERIES

Cardiovascular Infections [C01.190]
- Communicable Diseases [C01.221]
  - Pathological conditions involving the CARDIOVASCULAR SYSTEM including the HEART, the BLOOD VESSELS or the PERICARDIUM
- Communicable Diseases, Emerging [C01.221.500]

Respiration Disorders [C08.618]
- Respiratory Distress Syndrome, Adult [C08.618.840]

Respiratory Tract Infections [C08.730]
- Pneumonia [C08.730.610]
  - Pneumonia, Viral [C08.730.610.763]
- Pleurisy [C08.730.582]
  - Pleuropneumonia [C08.730.582.473]
- Severe Acute Respiratory Syndrome [C08.730.730]

Fluids and Secretions [A12]
- Exudates and Transudates [A12.383]

6.2.3 Pathologic syndromic interpretations

Following this, most representative terms were collapsed into their most representative collective clinical manifestation term or disease syndrome. For example, 'behavioural changes' was used to represent many more specific terms (Table 6.2.1 subset of Table 6.2) and collective syndrome terms were used when greater detail was not available (Table 6.4). All terms could be grouped under seven higher level collective terms (three listed original and four additional merged or modified terms) as most appropriate.

Table 6.2.1. All and consolidated disease terms listed in association with HeV included in collective syndrome of 'Behaviour changes' (Subset of Table 6.2)

behaviour_changes	
Consolidated specific descriptor term	Additional listed descriptor terms
depression_lethargy_or_dullness	somnolent head held low
seriously_ill	moribund or unresponsive weakness
pain and/or psychomotor agitation* and/or disorientation	aimless pacing or disorientation
pain and/or psychomotor agitation*	irritability/biting weaving
oral_discomfort_or_maniacal_and_erratic_behaviour	shaking head in bucket of water
Hyperaesthesia	hypersensitivity when approached
teeth_grinding_jaw_chomping**	oral discomfort**

Table 6.4. Categorised terms for analysis of illnesses with an infectious cause

Listed and added collective terms	No. of collapsed individual descriptor terms
Behaviour changes	6
Lymphatic_immune_and_inflammatory_system_response	2
Neurological_signs	39
Non_specific_common_sign	5
Respiratory_signs	13
Severe_acute_illness	21
Systemic_vasculitis_and_circulatory_compromise	8
Total	94

While much attention is given to the respiratory and neurological signs, the summary highlights the relative importance of cardiovascular signs, which would be interpreted as resulting from the pathological process of vasculitis (Figure 6.1).

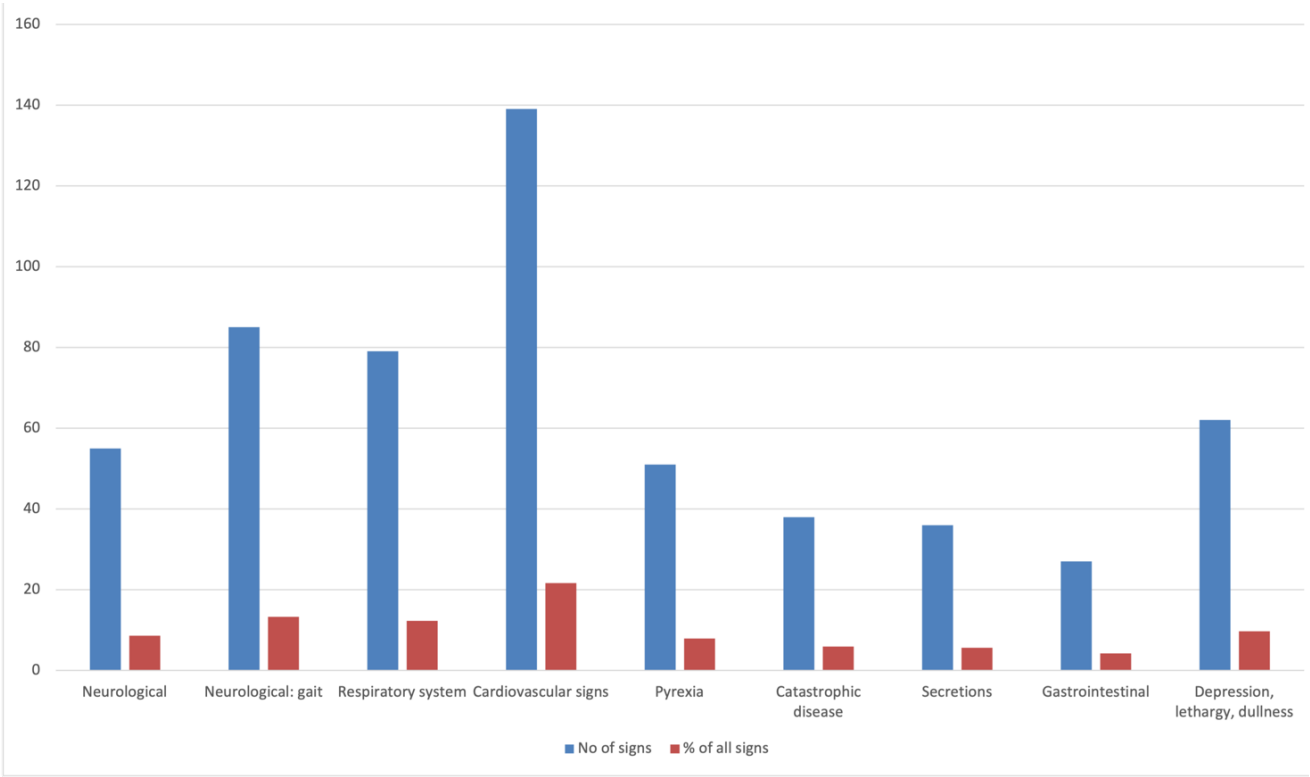

Figure 6.1: Summary of 'MeSH descriptor term database 2020' – syndromic categorisation of the 93 clinical descriptor terms that have been reported in description of HeV disease in 104 naturally occurring cases and three laboratory infections (HeV REDLANDS 2008 strain)

To better understand the syndromic clinical presentation of HeV, MeSH clinical descriptor terms were plotted as relative frequencies of combined occurrence (observed syndromes) among individual case presentations using Upset Plot in R. Figure 6.2. Figure 6.3 show the 10 most frequent combinations of disease descriptor terms reported in horses with HeV. The bars on the left show the frequency of occurrence of most frequently reported signs in any combination with other signs. The circles and lines indicate combinations of signs as observed in individual infected horses. The blue bars indicate the frequency of occurrence of the corresponding combination of signs. Two descriptor terms combine as indicative of Mucous membrane manifestations signifying haemo-circualtory compromise (outlined in red). These plots offer quantitative consolidation, of the understanding from chapter 2 and 4, that clinical manifestations of HeV infection in horses, while varying considerably in range of observed individual signs in any single time point assessment, are highly consistent with the expected chronological disease as understood in terms of known pathologic bases of HeV disease. Notably clinical signs describing mucous membrane changes were some of the most consistently reported (Figure 6.3) again consolidating understanding drawn from qualitative review (Chapter 2) and firsthand experiences (Chapter 4), while hitherto underemphasised in previously published reports, communications and scientific literature of lesser iterative clinical discipline engagement.

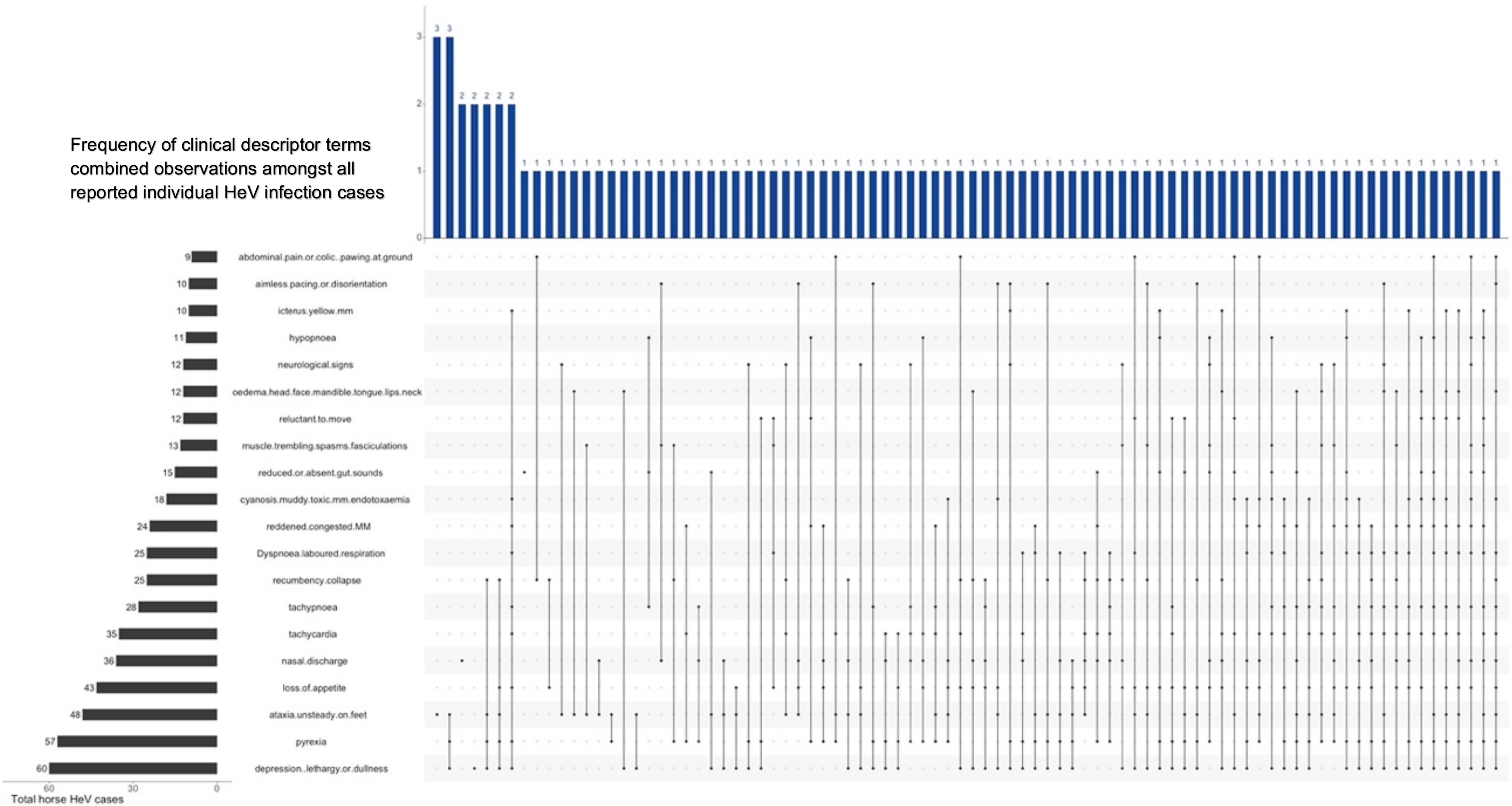

Figure 6.2. Upset plot generated in R highlighting syndromes associated with HeV disease in horses. The bars on the left show the frequency of occurrence of the twenty most frequently observed signs in combination with other signs. The circles and lines indicate combinations of signs as observed in individual infected horses. The blue bars indicate the frequency of occurrence of the corresponding combination of signs.

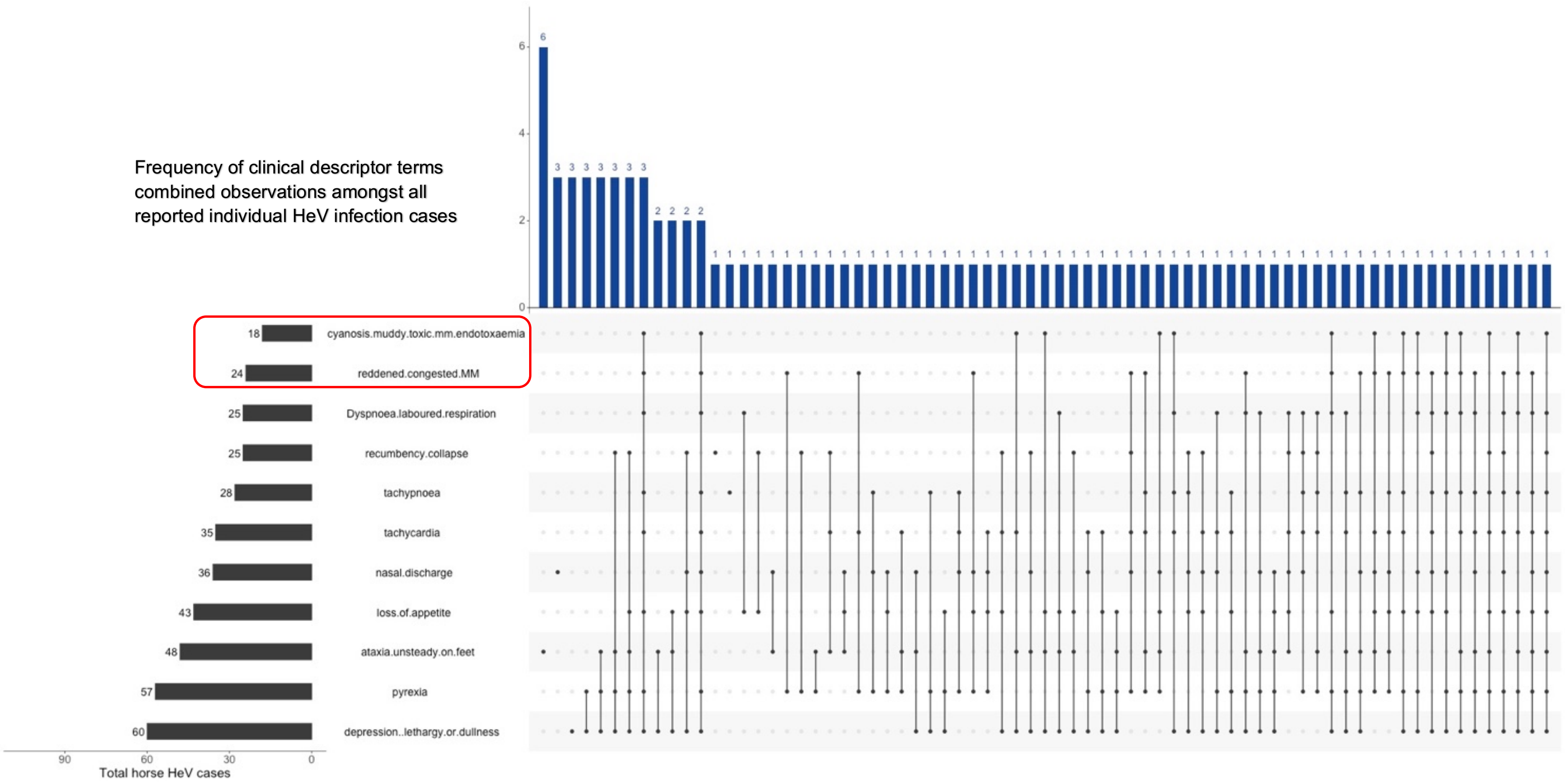

Figure 6.3. Upset plot generated in R highlighting syndromes associated with 10 most frequent HeV disease descriptor terms in horses. The bars on the left show the frequency of occurrence of the ten most frequently reported signs in any combination with other signs. The circles and lines indicate combinations of signs as observed in individual infected horses. The blue bars indicate the frequency of occurrence of the corresponding combination of signs. Two descriptor terms combine as indicative of Mucous membrane manifestations signifying haemo-circualtory compromise (outlined in red).

6.3 Development of a bespoke SQL database for sample management and prioritisation

Using this MeSH framework, we have developed the means to annotate and prioritise the clinical signs most likely to define cases of HeV-like illness in horses, thereby enabling targeted surveillance in cohorts of diseased horses. We built a de-identified SQL database to capture and compare subjects, geography, clinical features categorised by MeSH terms, samples, and results. Appropriately, sensitive horse and owner details were left with state biosecurity departments and local stakeholders (Figure 6.4). The SQL database was hosted in PostgreSQL (manipulated via pgAdmin 4), with systematic data capture via custom built forms as part of a linked-table receiver database (Microsoft Access). The design of the database aimed to allow for desired epidemiological analysis as well as animal focused diagnostic evaluation of cases and individuals, while permitting staged anonymity and protection of sensitive information best managed by local and state biosecurity sector partners.

The system configuration allowed for adaptable querying of 'sample event' information such as animal parameters, clinical signs, and geographical location, combined with details of sample and sample testing derivative location. In addition, characteristics could be added for efficient and comparative cohort analyses of multidirectional, repeated, and parallel laboratory test results. This research database allowed for the systematic prioritisation of retrospective samples to undergo testing. All cases were entered into the system and the cohorts on which parallel serology and molecular testing pipelines were applied (as reported in Chapter 7 and 8) were selected based on their assigned likelihood (Table 6.6) of emerging infectious HeV-like cause.

Such retrospective risk-based strategically-selected cohort testing aims to support, guide, and improve routine surveillance and biosecurity for significant diseases. The database served a key component of this proof of concept for what might be possible for further research and active surveillance activities, supporting and extending from routine government-based significant disease surveillance (via passive reporting). It could be updated and revised as information and priorities might change for ongoing passive, targeted and sentinel surveillance contexts.

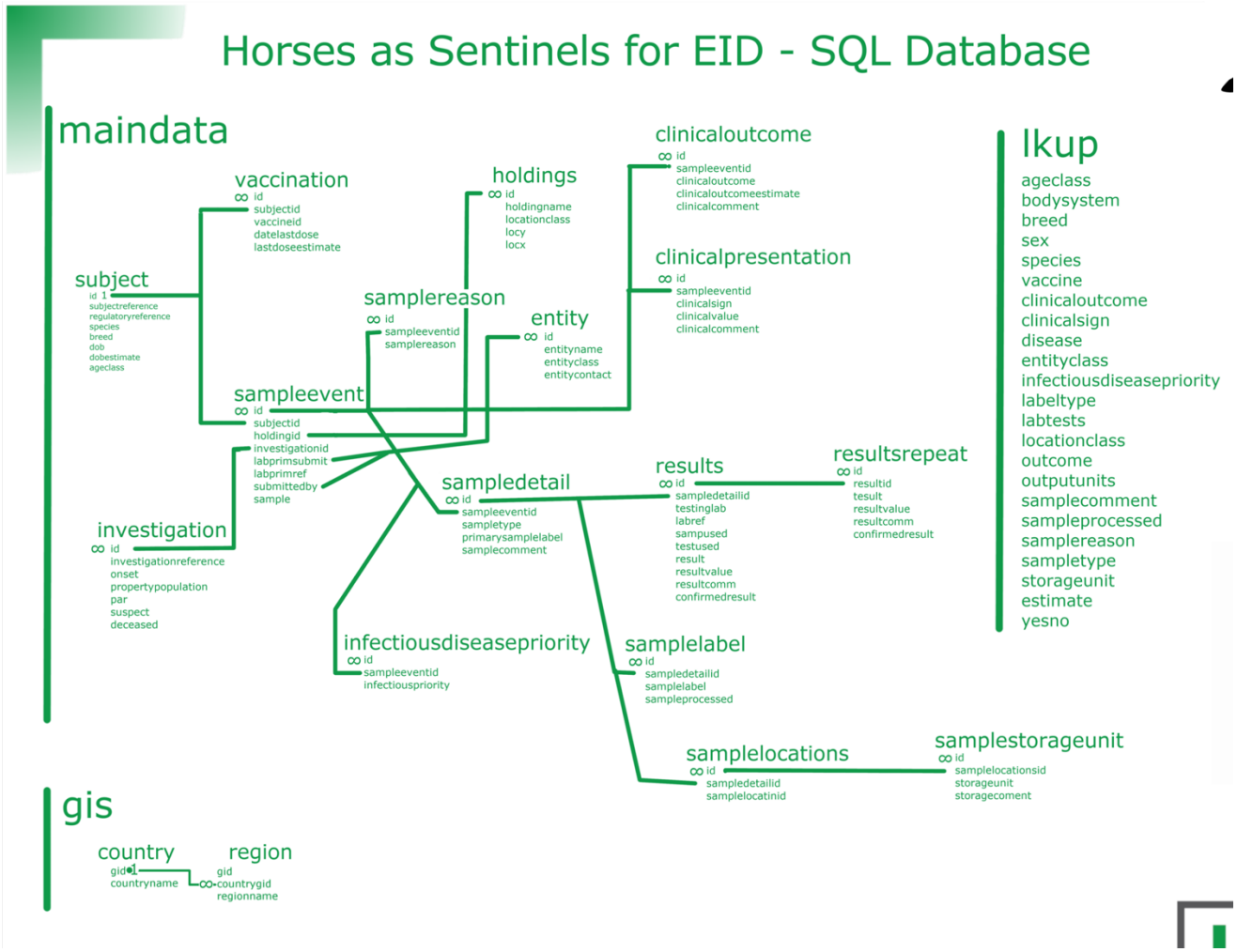

Figure 6.4. Horses as Sentinels research relational SQL database schema map Bespoke relational database design for efficient sentinel EID surveillance analyses combining of multiple information domains key to determination of diagnostic responsibility and highest suspect case selection with multiple case sample details and extensive parallel, series and duplicate results, with linkage with Government state laboratory records by unique sample detail and case investigation IDs, and protection of sensitive information associated with primary stakeholder by deidentification.

This research approach coupled this described transdisciplinary systematic assessment (qualitative and quantitative) of the consistency of pathologic basis and characteristics of HeV disease with clinical disease manifestations, with considerations of epidemiological and immunological consistency and applied a decision algorithm for categorizing each individual diseased horse case by its likelihood of having an infectious viral cause (Table 6.6). Based on the clinical details as reported on the submission form, cases were ranked into categories from highest likelihood of having an infectious cause, (Priority category 1) to least likely given alternative non-infectious cause realized (Priority category 6) (Table 6.6).

Table 6.5. Clinical terms (121) listed in the project database in association with cohort samples

Body system	Listed clinical terms in association with project cohort HeV testing submissions
non-specific	Pyrexia/effusion_pleural/leukocytosis/hypothermia/lethargy/hypothermia/colic/anorexia_innappetant/sweating/head_shaking/deceased/halitosis/weakness/bleeding_unspecified/abdominal_swelling/weightloss/pruritus/posture_abnormal/obtunded_demaenor/recumbency/moribund/dysphagia/abscess/hyperglycemia/torticollis/demeanor_agitated/neutropaenia/leukopaenia/fasciculation_muscle
nervous	head tilt/ptosis/ataxia/seizure/hypermetria/hypometria/nystagmus/hypereasthesia/aimless_walking/head_pressing/bruxism_teeth_grinding/neurological_other/proprioceptive_deficit/paralysis
musculoskeletal	Laminitis/effusion_joint_hydrarthrosis/lameness/muscular atrophy
digestive	quidding/tenesmus/prolapse_rectal/hepatomegaly/borborygmi_increased/hypersalivation/borborygmi_reduced_illeus/jaundice/diarrhoea/constipation
respiratory	discharge_nasal_unspecified/discharge_nasal_foamy_frothy/discharge_nasal_mucoid/cardiac_sounds_muffled/stridor/sneezing/ stertor/rr_hyperpnoea/rr_tachypnoea/rr_dyspnoea/ rr_hypopnoea# /bronchiovesicular_auscultation_increased/respiratory_unspecified/discharge_nasal_serosanguinous/sinus_purcussion_abnormal/discharge_nasal_mucopurulent/epistaxis/discharge_nasal_purulent/discharge_nasal_serous/cough
reproductive	Priapism/abortion/prolapse_vaginal/discharge_vaginal_unspecified/dystocia_other/uterine inertia/placental_abruption/placenta_abnormality_other/perinatal_death/discharge_vaginal_purulent
endocrine	hirsutism
integument	urticaria
ophthalmic/auditory/sinus	discharge_ocular_serous/uveitis/blindness_apparent/discharge_ocular_unspecified/corneal_ulcer/hyphema/conjunctivitis
Cardiovascular/ circulatory/ lymphatic	Endotoxaemia/thrombocytopaenia/lymphadenopathy/mucous_membranes_pale/mucous_membranes_injected/digital_pulse_amplitude_increased/dehydration/hr_tachycardia/hr_bradycardia/anaemia/heart_murmur/cyanosis/cardiovascular_other/mucous_membranes_tacky/petechial haemorrhage/cardiac_arrythmia/oedema
urinary	Polyuria/dysuria/haematuria/incontinence urinary

This enabled the prioritization of samples for application of extensive bespoke parallel serological or genomic viral discovery assays. Samples ranked as the highest likelihood of having an infectious cause (Priority category 1 & 2) (Table 6.6) were plated for serological screening and high-throughput RNA extraction (EDTA blood, serum, nasal swab, rectal swab) using both the MagMAX™ mirVana kit (Thermo Fisher, Australia). Remaining case samples from the entire cohort (categories 3-7) were extracted using the MagMAX™ CORE pathogen kit (only) to obtain total nucleic acid for confirmatory and comparative testing.

This database guided plating and aliquoting of bio-banked samples from >700 equine veterinary HeV-like disease sample events (>1500 samples). The information also allowed for systematic high-throughput plating and processing for infectious disease serology and molecular testing pipelines. For serology, a 33-plex fluorescent microbead immunoassay was used, targeting emerging zoonotic pathogens (Chapter 7). Molecular testing was performed by pan-PCR, NGS and custom pathogen discovery bioinformatics (Chapters 7, 8).

6.4 Conclusion

The systematic transdisciplinary approaches described here were developed by iterative consideration of the need to link observable disease manifestations in horses in their likelihood of arising from HeV infection and separately but similarly in defining Adverse Events Following Immunisation most accurately to both best serve horse health and their dependent stakeholders but also to promote public confidence in vaccine safety to support proactive consideration of immunisation as the single most reliable measure to reduce the risk of HeV infection in Australian horses in any region where horse are maintained within the range of any species of flying fox (regardless of species).

Bespoke design of the SQL relational database efficient linking of relevant case data and sample information while leaving sensitive data with primary stakeholders (vets and owners/ carers) and state level biosecurity departments and enabled syndromic classification of cases in order of prioritisation for an infectious cause. This enabled the biobank of over 1500 samples to be narrowed down to approximately 300 cases of highest likelihood of emerging infectious cause, allowing for systematic and efficient prioritisation for extensive parallel molecular and serological analyses to pursue hitherto unrecognised infectious cause most extensively.

Table 6.6. Infectious disease prioritisation categories (with examples) used in this study to identify Hendra-negative equine disease cases with highest likelihood of similar undiagnosed viral cause from larger cohort for further investigation

Infectious disease priority	Description	Example/s
Category 1 Highest infectious disease suspect	Case features 'pyrexia' or 'abnormal mucous membranes' AND one or more other clinical signs related to infectious disease OR the presence of either change AND 'epidemiological observation indicative of infectious cause' based on temporal and/or spatial relationship to similar disease cases	Pyrexia with tachycardia and acute onset respiratory consolidation and/or secretions. Pyrexia and neurological symptoms. Pyrexia and 'injected/congested' mucous membranes. 'Congested/injected mucous membranes' with acute severe respiratory dysfunction. Clustering of similar cases on same or neighbouring properties
Category 2 High infectious disease suspect	Pyrexia OR other clinical signs associated with infectious disease of interest	Acute onset abnormal respiratory secretions. Fever of unknown origin. Colic with the presence of neurological symptoms
Category 3 Moderate infectious disease suspect	Clinical signs may be associated equally with infectious and non-infectious causes	Colic with the presence of dehydration and mucous membrane changes. Ataxia with the absence of pyrexia or known trauma
Category 4 Low infectious disease suspect	Non-infectious aetiologies more common or most likely on differential diagnosis list, but infectious cause still possible	Ataxia following known traumatic event. Traumatic wounds following unusual behavioural event. Acute lethargy following chronic non-infectious disease condition
Category 5 No infectious disease suspect	No clinical signs of illness or no infectious cause considered likely	Traumatic wounds in the absence of underlying disease. Screening in unvaccinated horses to manage biosecurity risk prior to invasive procedures addressing non-infectious disease such as is a common requirement for dentistry or admission to equine hospitals in Australia
Category 6 Confirmed infectious disease	Other infectious disease confirmed via diagnostic testing	A case submitted for HeV testing, found negative and then testing positive for alternative known infectious disease such as ABLV, WNV, EHV or RRV*
Category 7 Asymptomatic in-contact with suspect or confirmed infectious disease case	Asymptomatic subjects in direct or close (on-farm) contact to a suspect or confirmed case of a significant infectious disease of interest	Clinically well horses in contact with clinically disease cases of emerging bat-borne Pararublavirus (related to MenPV) infection and Clinically well horses in contact with a clinically affected confirmed case of HeV

*ABLV, Australian bat lyssavirus; EHV, equine herpes virus; HeV, Hendra virus; RRV, Ross River virus; WNV, West Nile virus

Holistic consideration of both the consistency of HeV disease manifestations and the expansive divergence of differential cause for any individual clinical signs has generously consolidated understanding of the benefit for suspect HeV and EID horse case recognition afforded by proactive holistic assessment of the consistency disease manifestation with the expected chronological pathologic bases of HeV disease.

Combining such diverse disciplines in consideration of a problem focused objective is timely example of general systems thinking[4] enriched by lived and shared experiences amongst key disciplines, processes and stakeholders integrable or dividable through complex frameworks of biosecurity governance and great ethical scientific opportunity through purpose orientated collaborative sustained commitment. Furthermore the author has realised much of the most informative and guiding inference drawn and collated by this research via lived experience approaches consistent with ethnographic research[5] observed over the course of the research and considered specifically in benefit for One Health epidemiology by the extensive interdisciplinary research team and via workshop expert panel in another priority zoonotic viral disease context (HPAI) with which the author had fist hand experience early in the thesis research timeline (2017).[7,8] (see also Appendix 5) Specifically one key understanding drawn from these parallel zoonotic disease contexts (HPAI in LaoPDR and HeV in Australia) was that while any one agent or observer might never completely understand all the perspectives, operational limitations and priorities of all the relevant agents and stakeholders, any effort to understand such widely divergent perspectives, hear the unheard, identify covert barriers, acknowledge differences and support engagement toward a common purpose are likely to be of considerable benefit.

To relate this emphasising the benefit of ethnographically-inspired approaches to the focus of this chapter, I recall it was prolonged repeated efforts to understand the pain points limiting suspect case recognition (chapter 5) and proactive interpretation of diagnostic responsibility in relation to HeV and emerging zoonotic diseases, that repeatedly highlighted the need for clearer interpretation of both minimal or extensive clinical descriptions in their consistency with HeV or emerging RNA virus infection, by clinical practitioners, duty pathologists and government laboratory scientists alike, that supported the development, refinement and sentinel research application of the described approaches.

For such approaches to realise their full potential, integrated and sustained application is required, an application that in turn draws on all relevant disciplines and is supported by sustainable adequate funding and infrastructure, promoting seamless sharing and collation of most useful data for bolstered causative agent investigations, while protecting sensitive information relating the primary stakeholders (those to whom the horse matters most). The approaches decribed in this chapter are also highly suitable for many analogous syndromic surveillance activities across the broad range of species and agents of relevance to One health and biosecurity.

Note supporting file: **Appendix 7: -** ***Spread sheet example of systematic consideration of clinical descriptor terms listed in association with HeV infection and among a comparative cohort of severe equine disease events of lower likelihood of infection cause (AEFI)'***

6.5 References

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Horses as Sentinels of Emerging Infectious Disease

Chapter 7

EMPIRICAL SERO-EPIDEMIOLOGICAL INVESTIGATION FOR SPILLOVER OF FLYING FOX BORNE *PARARUBULAVIRUSES, HENIPAVIRUSES AND FILOVIRUSES* TO AUSTRALIAN HORSES –

Multiplex Fluorescent Microbead-based Immunoassay with Bayesian Estimation of Test Performance and Determination of Positive Test Classification in the Absence of Gold-Standard Comparison

Statement of personal contribution: I learnt and applied all serology assay techniques under the supervision of Dr Ina Smith between 2017 to 2020. The application of the Luminex assay on our large cohort in 2020/2021 was undertaken in 2020/21 with the support of Dr Smith and me, by a CSIRO laboratory technician (Ms Michelle Michie). This provided consistent results with a single operator using a single machine. This work was funded by Biosecurity Innovation Project 2020−21 Project ID 202043 'Metagenomic Investigation of Horses as Sentinels'. I learnt and applied the Bayesian approaches to analyses used in the manuscript under the supervision of my PhD supervisors and Dr Nagendrakumar Singanallur Balasubramanian. I learnt and applied the molecular approaches (Nested conventional PCR, RNA/ DNA extraction (various methods), preparation of RNA extracts for NGS and bioinformatical metatranscriptomic analyses (TRIM; BLAST; and De-Novo Assembly functions via HPC linex Batch script) under the supervision of PhD supervisors. I drafted all components of the original manuscript (2018−22), receiving and incorporating regular feedback from PhD supervisors.

Format: This chapter was initially prepared in a format for subsequent peer-reviewed journal submission. The presented findings and perspectives included in this thesis chapter are expected to be further presented as part of peer-reviewed scholarly research output following submission.

This thesis chapter extends the serology research published as a scientific abstract: **Spillover of bat borne Rubulavirus in Australian horses - Horses as sentinels for emerging infectious diseases.** Annand, E., Barr, J., Balasubramanian, N.S., Reid, P.A., Boyd, V., Burneikienė-Petraitytė, R., Žvirblienė, A., Grewar, J., E. Laing, Eden, J.S., Lynch, S.E., Diallo, I., and Yan, L., Secombe, C., Britton, P., Jones, C., Broder, C., Dhand, N.K., Smith I. International Journal of Infectious Diseases. 2020 Dec 1;101:401–2. The work was also presented at the national equine veterinary conference in 2018 and published in this organisation's scientific journal: **Serological evidence of bat borne *Rubulavirus* in Australian horses.** EJ Annand, PA Reid, J Barr, V Boyd, R Burneikienė-Petraitytė, A Žvirblienė, C Broder, CA Jones, N Dhand and I Smith. EVA Bain Fallon/ANZCVS absracts, The Australain Equine Veterinarian, 38, Vol 38, No 2, 2018.

7.1 Introduction

7.1.1 Background, justification and context

HeV and other EIDs of potential One Health significance, particularly the wide range of RNA viruses circulating among wild mammalian and avian species (reviewed in Chapter 2), may infect Australian horses via direct, indirect and insect transmission. Manifestations of infection may include asymptomatic infection, mild to moderate disease with recovery, to severe progressive disease and death.

The number of equine submissions for HeV testing nationally between July 2017 and July 2018 was 867, resulting in three positive cases being identified.(1) The state with the largest number of submissions in this period was Queensland with 586 submissions, followed by New South Wales with 247, Victoria with 13, Western Australia with nine, the Northern Territory with six and Tasmania with two.(1) Recognition of suspect HeV infection and case submission for government laboratory investigation has been subject to marked geographical sampling bias. There has been a strong sampling preference for locations in which cases have been diagnosed previously, despite scientific understanding of potential for spillover risk more broadly (see Chapter 1). Consideration of suspect HeV infection in cases displaying consistent disease manifestations in regions where spillover has not been recognised previously (southern NSW, Victoria, South Australia, and Western Australia), is subject to conscious and subconscious barriers. Examination of such currently unrecognised causes of the remaining horse disease cases consistent with HeV infection is of great value to veterinarians, horse owners, animal and human health care professionals, and government animal biosecurity agencies.

It is highly likely that HeV infections in horses have gone undiagnosed in Australia. Our discovery during this study (Chapter 8)(2) of the HeV variant (HeV-g2), a virus that had not been detected during routine molecular diagnostic analysis, highlighted this reality. Serology offers potential to diagnose additional cases that survive infection with related *Henipaviruses* (HNVs), yet fail detection due to:

- lack of appropriate sampling and laboratory testing
- limitations of highly specific routine PCR currently available

- where sampling has been outside the viraemic stage of infection, causing failure of detection by PCR
- where sampling is outside the detectable period for antibodies (IgM or rising IgG).

Additional paramyxoviruses (Henipavirus and Pararubulavirus*)* shed from flying foxes(3) could be transmitted to horses via similar pathways as HeV, causing disease in horses of similar morbidity and mortality, posing similar human health risk, and yet going undiagnosed. Such spillover from flying foxes is expected be similarly sporadic, occurring with low incidence across a broad geography, and manifesting disease mimicking many other diseases. It would also be expected that like most viral infections, a small percentage of infections would be asymptomatic or mild (Chapter 2).

The development of multiplex serological assays targeting both IgG and IgM to the extensive conceivable range of agents circulating in Australia, offers potential to detect antibodies in cases where the viraemic stage has been missed resulting in false negative molecular diagnostic determination. Antibody assays would be especially useful when paired sera is available and/or samples from in-contact subjects. However, interpretation of such exploratory testing, applied to single subject/case investigations of HeV like disease in horses, is challenging without knowledge of expected assay performance drawn from research testing of suitable background populations.

7.1.2. Traditional barriers to proactive use of serology turned to opportunities

Government animal health and biosecurity laboratory investigations prioritise diagnostic investigation of significant diseases by exclusion testing of reported suspect disease cases (passive syndromic surveillance) for a select list of well-characterised infectious agents of well-established significance, rather than open ended and exploratory testing. This applies in Australia and internationally, and has many logical justifications relating to the need for clear interpretation of diagnostic significance for:

- animal health
- human health (zoonotic transmission and disease risk)
- animal disease status/proof of freedom of exotic diseases relevant to trade negotiations and import policies that protect national biosecurity and food security.

Diagnostic responsibilities in relation to these three broad contexts act commonly to limit routine diagnostic assays and techniques to those offering most conclusive, prompt interpretation of biosecurity and disease significance at farm level and for management of onward animal and zoonotic transmission risks.

Assays and diagnostic activities capable of testing for causal agents more broadly and extensively, beyond those most well characterised both biologically and in their disease and biosecurity significance, are increasingly available. However, their incorporation into routine testing is delayed and challenging. Both the integration of clinical and research perspectives, and governance and infrastructure (supportive system frameworks) required to allow their clearest timely interpretation, are currently lacking. Government laboratories are restricted to the use of thoroughly validated assays accredited by the World Organisation for Animal Health (WOAH, formally OIE) and the National Association of Testing Authorities (NATA).

Traditionally, validation of serological assays, such as ELISAs − enzyme linked immunosorbent assays − used in routine biosecurity investigations, has required traditional analysis by comparison with a 'gold standard' assay. A gold standard is applied to the same sample in parallel, to provide conclusive inference of test performance and true animal infection/exposure status. For obvious reasons, such approaches are not available to target potentially pathogenic agents of emerging significance, novel pathogens, and infection/exposure in new species contexts.

Nevertheless, both the limitations of traditional approaches to assessing test performance relying on comparison to a 'gold standard', and indeed the unsuitability of some assays being considered 'gold standard' indications of true prevalence, are increasingly recognised, given the complexity of co-infection and cross reactivity. Experts in diagnostic assay performance and validation now argue that 'there is no gold standard'; every assay has the potential for an incorrect determination of subject status, even if not relating to the assay mechanistically. Such incorrect determinations can occur due to other contexts such as timing of the sampling event in relation to infection progression, sample type, deterioration of sample condition and handling limitations, or infection with divergent agents. These factors may elicit variable assay results or alter assay performance in sensitivity and specificity. Additionally, many disease cases involve unrecognised co-infections.

Advancement of information theory approaches to diagnostic performance assessment (4) and evaluation including probabilistic and Bayesian modelling approaches,(5–8) along with advancements in diagnostic testing platform technologies,(9–11) and computational interpretations,(12,13) are enabling ever-increasing possibilities for diagnostic application. These approaches may enable testing beyond the known for novel and emerging agents. (14)

Similarly, serology assays that measure circulating IgG or IgM are prone to cross-reactivity and non-specific binding, allowing for false positives interpreted for specific infectious agent/s for which exposure is assessed. Multiplexing Luminex microbead immunoassays that produce a continuous biomarker result (Mean Florescent Intensity, MFI) interpreted via Bayesian test performance and latent class analyses, allow interpretation of such cross reactivity to be converted into a strength, rather than a barrier. They offer inference of probable divergent agent exposure. Bayesian analyses allow best representation of data, (15) and interpretation of the likelihood of results to indicate exposure to a closely related virus, that could be of similar disease and biosecurity significance to a well-established agent.

Other barriers to extensive case-specific diagnostic testing for infectious aetiologies include lack of clarity of responsibility for the cost of testing; animal owners are required to cover the cost of testing of non-notifiable diseases. Also, laboratory testing resources are limited for the zoonotic disease context in non-production domestic species. This is due to the large testing loads for diseases amongst production animals and outbreak responses of industry, trade, and food security significance. Domestic companion (16) and performance animal testing for zoonotic and emerging disease purposes, and wildlife disease testing, rank lower in relative priority. They are given less bespoke resourcing generally to support both infrastructure and operational costs, and lower assignment of case/investigation specific government diagnostic cost remittance for specific cases.

Most suspect significant disease investigations, and almost all concerning domestic species, are instigated by the attending private veterinarians having recognised consistent clinical disease manifestations (syndromes), and contextual case features (epidemiology). Such investigations cover a wide range of diagnostic responsibility and are often challenging to interpret and/or communicate, both by submitting veterinarians and government duty pathology/laboratory scientists.

Case-specific serology targeting infectious cause poses additional diagnostic challenge, for which submissions are usually single sample events; paired samples (collected 10−14 days apart) allow for best interpretation and confirmation of an infection and are thus standard in human serology in investigation for infectious aetiology. Antibodies (both IgM and IgG) targeting related microorganisms, elicit cross-reactivity in many serology assays, further challenging conclusive diagnostic interpretation.

Nevertheless, employing serology in the diagnostic repertoire has great advantages, allowing for diagnostic inference of infection or exposure to disease agents when molecular diagnosis fails if the sample is taken outside time-critical viraemia; antibodies, however, are still detectable. (17) Importantly serology has the advantage of estimating population seroprevalence, characterising antibodies circulating in response to past infections and revealing asymptomatic infections.

This thesis primarily addresses the hypothesis that bat-borne paramyxoviruses related to HeV, circulating amongst Australian flying foxes and shed alongside HeV in urine, (the transmission pathway relevant for horses), (18) are spilling over to horses, causing HeV-like disease and posing similar but unrecognised One Health and biosecurity risks.

Other bat-borne RNA viruses such as those in the *Filoviridae* and *Coronaviridae* families may similarly be spilling over to Australian horses from flying foxes. There is increasing recognition of such spillover from *Pteropus* spp. flying foxes internationally, (19,20) and in recent research in Australia. (3,21–23) It is also expected that consistent proportional incidence of relatively asymptomatic infection is likely in horses and other domestic animals, even for many target viruses are of very high potential pathogenicity. There are also many viruses in the *Flaviviridae*, *Togaviridae* and *Bunyaviridae* families that are transmitted via biting arthropods (ticks, midges, and mosquitoes) with variably recognised potential for disease in humans and horses (see Chapter 2).

Chapter 2 of this thesis highlights the high potential that viruses posing direct zoonotic risk, and/or causing similarly significant disease in humans, including divergent and emerging paramyxoviruses are causing HeV-like disease in Australian horses. Drawing from this consideration we constructed an extensive serological screening approach targeting an

extensive range of potentially zoonotic disease agents relevant to the Australian HeV-like horse disease context.

This study had the following aims:

- to determine whether Australian horses with perceived proximity to flying foxes demonstrate antibodies representing exposure to bat borne viruses; and
- to determine whether Australian horses are exposed to pathogens causing HeV-like disease of potential zoonotic relevance.

This chapter reports the approach and findings of that effort with emphasis on bat-borne Paramyxoviruses and Filoviruses. Select preliminary findings of parallel molecular findings are presented as relating to the priority Qld HeV-like disease sub cohort to support fullest interpretation of serology findings.

7.2 Materials and methods

7.2.1 Subject and sample cohort

7.2.1.1 Sample types, handing, storage, and processing

We constructed a biobank of diagnostic specimens collected from approximately 1,700 horses that underwent HeV testing in Queensland between 2015 and 2018. These samples had been found negative for HeV by RT-qPCR, and no causative diagnosis had been determined.

Given the susceptibility of target agent RNA, priority was placed on maintaining an ultra-cold (−80° C) chain throughout sample storage at the state laboratory following routine submission, throughout bio-secure transportation and through all research sample processing (performed on dry ice) and storage.

Clinical, epidemiological, and sample-related data were recorded, and samples archived at −80° C. Information collected on each case was drawn from primary laboratory submission documentation, variably including age, sex, breed, geographic location, clinical findings, samples submitted, vaccination status, number at risk, number affected, and treatments administered. Other notes made by submitting veterinarians were included, such as

perceived exposure to flying foxes. Such case-related information of primary concern to diagnostic aetiologic determination was entered into the research SQL database (Figure 6.4), with sensitive information such as attending veterinarian, animal owner and property details omitted and/or de-identified. This protected privacy and allowed for management of primary stakeholder sensitivities by state biosecurity departments and attending/submitting veterinarians appropriately.

Sample handling and processing was performed in a biosafety cabinet. Samples from priority suspect cases were determined for aliquoting by systematic assessment and classification algorithm (Table 6.6) drawn from the qualitative and quantitative approaches considering the expected clinical and case context most-consistent with HeV or related RNA viral infection described in Chapters 3, 4 and 6. Plate co-ordinates were assigned automatically based on prioritisation categories by bespoke R code linking SQL database sample detail IDs with those determined during physical sample cohort bio-banking.

7.2.1.2 Study cohorts, subjects, and sample events

Pilot serology investigation were undertaken in the initial years of this research as proof of concept, justification for further funding, and to develop and optimise the ultimate application of the multiplex assay on the larger priority cohort using the fullest antigen panel. Initial screening was performed on 374 sera and whole blood (EDTA) from Australian horses sampled from regions across Australia. (24) Animal ethics was granted from the *CSIRO wildlife and large animal AEC Project App. No. 2016−19*. Some of these sera had been collected from Australian horse subjects for previous research purposes (25) and some were submitted by practising veterinarians for the purpose of this research. Others were submitted to this research following routine government laboratory investigations, which included PCR and ELISA testing targeting HeV infection. The former two categories comprised serum (collected in serum clot tubes and separated from the cellular component by centrifugation), while those submitted following HeV investigation by government laboratories were whole blood (collected in EDTA tubes). Pilot cases/ samples did not undergo the same systematic categorisation of infectious likelihood but were selected nevertheless to be most representative of the target context – ie. Relative to geographic exposure potential and disease/ health/ vaccination status.

For the main study application of multiplex microbead immunoassay (MMIA), 412 serum or blood samples representing 409 horse subjects (three with paired samples) were included, (Table 7.5). Samples included:

- cases that had undergone Queensland government laboratory investigation for HeV infection (306 cases of highly suspect HeV-infection and 33 tested for biosecurity and occupational biosafety purposes in the absence of suspect infectious-disease)
- eight horses sampled in association with confirmed HeV infection spillover (NSW 2019)
- nine horses (10 sample events) that had experienced infectious-like disease of unknown aetiology in NSW
- eleven horses (13 sample events) that had suffered neurological disease as part of a 2020 cluster in SA
- 42 apparently healthy horses sampled by practising veterinarians for the purpose of this research serosurvey.

Control serum samples appropriate for each antigen were included. These comprised horses of previously established infection or infectious agent exposure status, and experimentally derived target antibody positive sera from rabbits.

7.2.2 Recombinant protein production and selection

Horse subject sera included in the main study application of MMIA, (412 samples representing 409 horses) were tested for both IgG and IgM against recombinant antigenic Paramyxovirus proteins that included: HeV RBP (G), NiV RBP (G), MojV RBP (G), GhV RBP (G), CedPV RBP (G), TioPV NP, MenPV NP, YepPV RBP (HN) and GroPV RBP (HN) (Table 7.1). Additional viral and bacterial antigens were included, representing other emerging infectious agents of potential bat-borne spillover and/or HeV-like horse disease context (Table 7.2). The same 412 blood/serum samples were tested against the same antigens for IgM.

The range of bacterial and viral antigens sourced for this multiplex format consisted of representation of potentially zoonotic pathogens sourced from commercial suppliers and collaborators within Australia and Lithuania, and an extensive panel of emerging viruses belonging to the *Paramyxoviridae*, *Coronaviridae* and *Filoviridae* families. The latter group was provided by collaborating researchers at the Uniformed Services University, USA. (Table 7.1, 7.2)

Table 7.1. Bat-borne Paramyxovirus recombinant protein antigens utilised in the fluorescent bead based multiplex serology immunoassay

Microorganism/ *Species*	Order	Family/subfamily	Genus/subgenus	Abbreviation	Antigen	Expression platform
Hendra virus *Hendra henipavirus*	Mononegavirales	*Paramyxoviridae*	*Henipavirus*	HeV	Receptor Binding Protein (RBP) or G glycoprotein (G)	Mammalian
Hendra virus *Hendra henipavirus*	Mononegavirales	*Paramyxoviridae*	*Henipavirus*	HeV	RBP/ G	Mammalian
Nipah virus *Nipah henipavirus*	Mononegavirales	*Paramyxoviridae*	*Henipavirus*	NiV	RBP/ G	Mammalian
Cedar virus *Cedar henipavirus*	Mononegavirales	*Paramyxoviridae*	*Henipavirus*	CedV	RBP/ G	Mammalian
Mojiang virus *Mojiang henipavirus*	Mononegavirales	*Paramyxoviridae*	*Henipavirus*	MojV	RBP/ G	Mammalian
Ghana virus or Kumasi virus *Ghanaian bat henipavirus*	Mononegavirales	*Paramyxoviridae*	*Henipavirus*	GhV or KV	RBP/ G	Mammalian
Menangle virus *Menangle pararubulavirus*	Mononegavirales	*Paramyxoviridae*	*Pararubulavirus*	MenPV	RBP or haemagglutinin-neuraminidase protein (sHN)	Mammalian
Grove virus *Grove pararubulavirus*	Mononegavirales	*Paramyxoviridae*	*Pararubulavirus*	GroPV	RBP/sHN	Mammalian
Yeppoon virus *Yeppoon pararubulavirus*	Mononegavirales	*Paramyxoviridae*	*Pararubulavirus*	YepPV	RBP/sHN	Mammalian
Tioman virus *Tioman pararubulavirus*	Mononegavirales	*Paramyxoviridae*	*Pararubulavirus*	TioPV	Nucleocapsid protein (NP)	Yeast
Menangle virus *Menangle pararubulavirus*	Mononegavirales	*Paramyxoviridae*	*Pararubulavirus*	MenPV	NP	Yeast

Table 7.2. Further antigens utilised in the fluorescent bead based multiplex serology immunoassay

Microorganism/Species	Order	Family/Subfamily	Genus/Subgenus	Abbreviation	Antigen	Supplier
Brucella suis	Hyphomicrobiales	*Brucellaceae*	*Brucella*	Brucella suis	repA	CSIRO
Brucella abortus	Hyphomicrobiales	*Brucellaceae*	*Brucella*	Brucella OMP	Outer membrane protein (OMP)	Commercial
Leptospira australis	Leptospirales	*Leptospiraceae*	*Leptospira*	Lepto aus.	Bacteria	Queensland Health
Leptospira pomona	Leptospirales	*Leptospiraceae*	*Leptospira*	Lepto pom.	Bacteria	Queensland Health
Chikungunya virus	Martellivirales	*Togaviridae*	*Alphavirus*	CHIKV	E1 protein	Commercial
Sindbis virus	Martellivirales	*Togaviridae*	*Alphavirus*	SINV	E1 protein	Commercial
Borna disease virus (1)	Mononegavirales	*Bornaviridae*	*Orthobornavirus*	BoDV	Envelope glycoprotein (G)	Commercial
La Crosse virus California encephalitis virus	Bunyavirales	*Peribunyaviridae*	*Orthobunyavirus*	CEV	NP	Commercial
Severe acute respiratory syndrome coronavirus 1	Nidovirales	*Coronaviridae*	*Betacoronavirus/ Sarbecovirus*	SARS-CoV-1	NP	Commercial
Severe acute respiratory syndrome coronavirus 2	Nidovirales	*Coronaviridae*	*Betacoronavirus/ Sarbecovirus*	SARS-CoV-2	NP	Commercial
Severe acute respiratory syndrome coronavirus 2	Nidovirales	*Coronaviridae*	*Betacoronavirus/ Sarbecovirus*	SARS-CoV-2	NP	Commercial
West Nile virus	Amarillovirales	*Flaviviridae*	*Flavivirus*	WNV	E	Commercial
Hepatitis E virus Orthohepevirus A	Hepelivirales	*Hepeviridae*	Orthohepevirus	HEV	Capsid protein	Commercial
Retrovirus SC110	Ortervirales	*Retroviridae*	*Gammaretrovirus*	Retro	E	Burnet Institute
Hervey pteropid gammaretrovirus	Ortervirales	*Retroviridae/ Orthoretrovirinae*	*Gammaretrovirus (unclassified)*	HPG	E	Burnet Institute
Ebolavirus *Zaire ebolavirus*	Mononegavirales	*Filoviridae*	*Ebolavirus*	EBOV	NP	CSIRO
Ebolavirus *Zaire ebolavirus*	Mononegavirales	*Filoviridae*	*Ebolavirus*	EBOV	E	USU
Bundibugyo virus *Bundibugyo ebolavirus*	Mononegavirales	*Filoviridae*	*Ebolavirus*	BDBV	E	USU
Bombali virus *Bombali ebolavirus*	Mononegavirales	*Filoviridae*	*Ebolavirus*	BOMV	E	USU
Reston virus *Reston ebolavirus*	Mononegavirales	*Filoviridae*	*Ebolavirus*	RESTV	E	USU
Marburg virus *Marburg Marburgvirus*	Mononegavirales	*Filoviridae*	*Marburgvirus*	MARV	E	USU
Ravn virus *Marburg Marburgvirus*	Mononegavirales	*Filoviridae*	*Marburgvirus*	RAVV	E	USU
Měnglà virus *Mengla dianlovirus*	Mononegavirales	*Filoviridae*	*Dianlovirus*	MLAV	E	USU
Lloviu virus *Lloviu cuevavirus*	Mononegavirales	*Filoviridae*	*Cuevavirus*	LLOV	E	USU
Middle East respiratory syndrome-like coronavirus	Nidovirales	*Coronaviridae*	*Betacoronavirus/ Sarbecovirus*	MERS-CoV	Spike	USU
Severe acute respiratory syndrome coronavirus 1	Nidovirales	*Coronaviridae*	*Betacoronavirus/ Sarbecovirus*	SARS-CoV-1	Spike	USU
Severe acute respiratory syndrome coronavirus 2	Nidovirales	*Coronaviridae*	*Betacoronavirus/ Sarbecovirus*	SARS-CoV-2	Spike	USU
Bat SARS-like Coronavirus Rs4874	Nidovirales	*Coronaviridae*	*Betacoronavirus/ Sarbecovirus*	Bat SARS-like CoV Rs4874	Spike	USU
Mock control	NA	NA	NA	NA	NA	USU

7.2.3 Multiplex microbead Immunoassays

Recombinant antigenic viral proteins were coupled to carboxylated magnetic microsphere bead sets, BioPlex® (BioRad) of an assigned number according to manufacturer's instructions as previously described. (20,26–28,36,53)

Briefly, Proteins were coupled onto Bio-Plex Pro magnetic COOH beads (Bio-Rad) according to manufacturer's instructions using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC: Thermofisher) and *N*-hydroxysulfosuccinimide **(**S-NHS: Thermofisher).

The presence of immunoglobulins against the proteins were measured using a Luminex xMAP-based multiplex microsphere immunoassay (MIA). Briefly, the beads were added to each well and then mixed with sera at a dilution of 1:100 in PBS and incubated with shaking for 30min at room temperature. The beads were washed using a magnetic plate washer (Tecan Flex) using PBS-T. The bound antibody was detected using biotinylated Protein A 2ug/mL (Thermofisher) together with biotinylated Protein G 2mg/mL (Thermofisher) in PBS-T, incubated and washed as above, followed by the addition of streptavidin–phycoerythrin 1ug/mL (Thermofisher) in PBS-T. The assay was read using a MagPix System (Luminex). The fluorescence results of 100 beads were recorded as the median florescent intensity (MFI).

Multiplex application of coupled viral protein assays were run as two separate panels due to limited range of bead numbers available and the numerous proteins applied. These included proteins of other emerging viral families and some emerging bacterial zoonoses of potential relevance to the study species and disease/exposure context (Tables 7.1,7.2).

7.2.3.1 Multiplex microbead immunoassays targeting IgG

Extensive multiplex screening of the main study cohort by MMIA was undertaken as previously described. (20,27,28,36,53) Briefly, each of the protein-coupled microsphere bead sets were pooled, then added to wells of a 96-well plate. The beads were washed three times in phosphate buffered saline with Tween™ (PBS-T) using a magnetic plate washer (Tecan). Subject sera diluted 1:100 in PBS (pH 7.4) were added (100 µL) to systematically assigned plate wells (Bespoke R code assigning plate co-ordinates based on SQL database-assigned *Figure 6.4* infectious disease priority *Table 6.6*) containing microsphere bead sets and incubated for 30 min at room temperature (RT) with shaking (800 rpm). Plates were then

washed as before adding 100 µL of biotinylated protein A (final concentration 2 µg/mL) (Pierce, Thermo Fisher Scientific, Waltham, MA, USA) and biotinylated protein G (final conc 2 µg/mL) (Pierce, Thermo Fisher Scientific, Waltham, MA, USA), and further incubated for 30 minutes at room temperature with shaking (800 rpm). Plated beads now exposed to subject sera were then washed again before adding 100 µL of streptavidin-phycoerythrin (1:1000) (Invitrogen) and incubating for a further 30 mins at room temperature with shaking (800 rpm). All incubation steps were performed with the plate wrapped in aluminium foil to avoid light degradation.

Median florescence intensities (MFI) for each coupled bead type and each well were determined using a MagPix (Luminex Corporation, Austin, Texas) configured to count 50−100 beads per region. Bio-Plex 200 (Biorad) machines were also employed in some cases to read plates in the pilot study application of the MMIA.

Sera case MFI results obtained using both machines tested in the pilot study were compared with Bayesian assessment of assay performance and binary predictor classification via mutual information ROC curves undertaken separately in parallel on results from each machine type. MFI levels and test performance varied between the two machine platforms, highlighting the importance of consistency, and of assessing test performance and interpreting binary test outcome specifically for result cohorts of equivalent assay conditions.

Extending from these pilot study experiences and given our desire to showcase diagnostic platforms readily available for government state laboratories to support subsequent integration of such testing, we used the MagPix platform exclusively to undertake the MMIA applied in the main study. Other measures to support maximum consistency and reliability in the main study cohort included: where possible using the same bead coupling batch for each antigen, ensuring consistent personnel for assay operation, and using an automatic plate washer. This latter precaution was especially important, given that notably variable results were obtained from some pilot attempts due to inadvertent partial loss of beads during the manual process using a handled magnetic bead washer. Importantly, this error was found to be most significant when using particular plate brands that featured a small gap between the magnets and the wells.

7.2.3.2 Multiplex Microbead Immunoassays targeting IgM

The assays were adapted for detection of equine IgM antibodies by substituting biotinylated protein A/G for biotinylated anti-equine IgM diluted 1:500 (US Biologicals). Multiple horse subjects that had received HeV vaccination at known time points in relation to sera sampling, included in the non-clinical cohort, provided suitable sera for establishing the IgM assay.

7.2.4 Bayesian and Information theory approaches to assessment of assay performance and binary predictor classification via mutual information ROC curves

There are inherent challenges in targeting serological responses that indicate infection and/or exposure to viruses for which species-specific control sera are unavailable from subjects of conclusively known infection or exposure status. To help address this challenge, a latent class analysis using Bayesian theorem utilising Markov Chain Monte Carlo simulation methods was used, informed by best estimates of characteristics of expected population epidemiology such as disease prevalence. A Bayesian latent class model (BLCM) was employed to determine threshold MFI values for categorising horse subject sera as seronegative or seropositive, and for determining thresholds for receiver operating characteristics (ROC) curve and area under curve (AUC) for each antigen included in the MMIA. The approach was consistent with that considered a robust analytical method applied in similarly challenged contexts across multiple studies. (5,8,27–31)

Computation was undertaken in R (version 4.2.0), (32) integrated with Markov Chain Monte Carlo simulation software OpenBUGS (version 3.2.3). (33) Briefly, raw MFI reads for each antigen were imported into R. Prior positive versus negative test result classification was set according to cut-off levels based on thrice the average of readings representing known negative subjects. These were determined in the context of geography − regions of low likelihood of flying fox exposure (southern WA) − and/or deduced by negativity on prior serology testing (applying a range of assays of known performance in flying fox and other species sera against the target agents). Summaries of cohort MFI distributions were determined using *base* R statistical packages. (32) Prior test classification cut-offs were applied most appropriately for each antigen, characterising bi-normal distribution assuming that the MFI outcomes for positive and negative subjects are independent and normally distributed, conditional on infection/exposure status. (Figures 7.1 and 7.2)

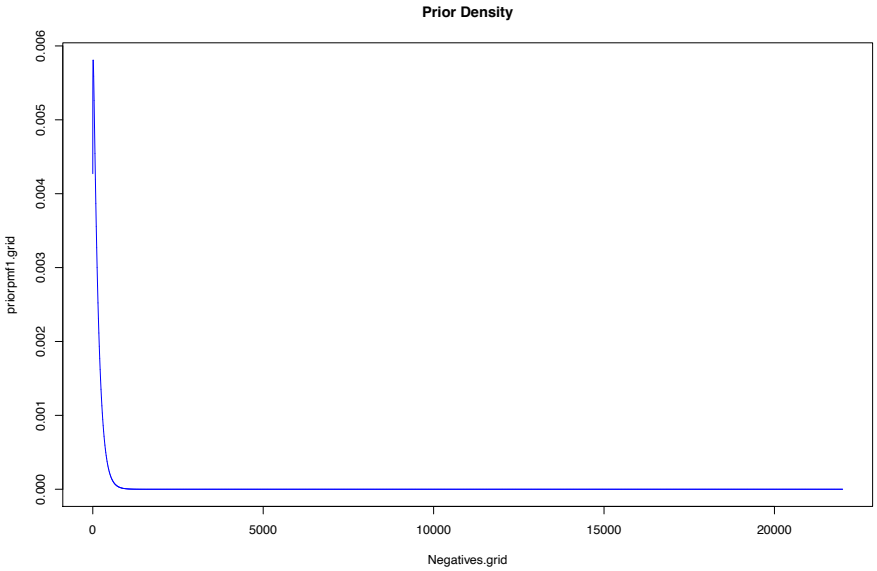


Figure 7.1. Example of MFI distribution plot of prior negative subjects

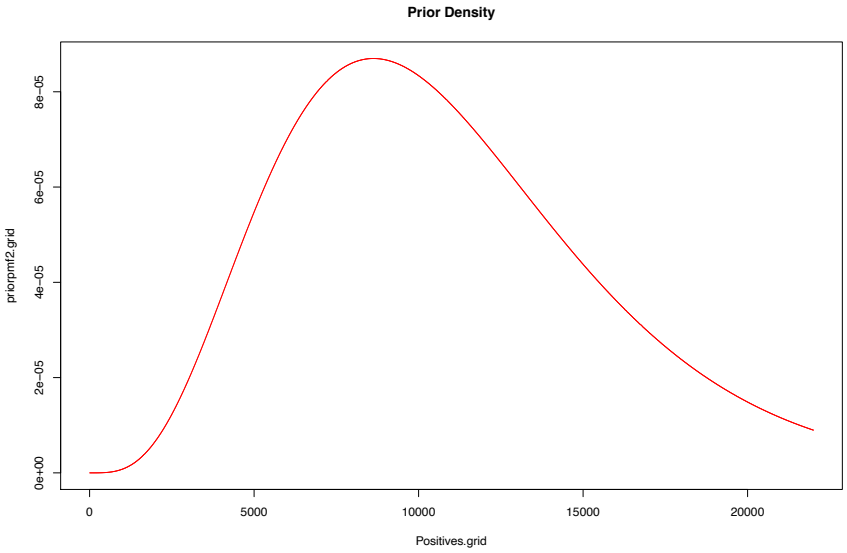


Figure 7.2. Example of MFI distribution plot of prior positive subjects

Models guiding Markov Chain Monte Carlo simulation applied to MFI results for each antigen were constructed, informed by anticipated prevalence given the subject cohort and epidemiology (Text box 7.1).

Model { for (i in 1:n[1])

{y[i] ~ dnorm(mu[1], tau[1])}

for (i in n[1] +1:n[1] + n[2])

{y[i] ~ dnorm(mu[2], tau[2])}

mu[1] ~ dnorm(100, 0.001)

mu[2] ~ dnorm(250, 0.0001)

for (i in 1:2) {sigma[i] < − 1/sqrt(tau[i])

tau[i] ~ dgamma(0.1, 0.1)}

AUC < −

Φ((mu[2] - mu[1])/sqrt(pow(sigma[1],2) + pow(sigma[2],2)))}

Where mu = mean MFI reads of positives [1] and negatives [1]; tau = variance for mean MFI reads of positives and negatives; AUC = Area Under Curve and Φ = standard normal cumulative distribution function.

Text box 7.1. Model informing Markov Chain Monte Carlo simulation for assessment of test performance and binary cut-off determination

Output in OpenBUGS was assessed for multiple parameter convergence over 100,000 simulations, and the first 1,000 were discarded as 'burnins' (Figure 7.3). Posterior cut-offs for results classification were determined in this way for all paramyxoviral antigens in the main study (informed also by prior cut-off estimation in the pilot study for comparable viral antigens). For the remaining antigens in the wider MMIA panel, positive result classification presented here is limited to conservative estimations without Bayesian optimisation based on Markov Chain Monte Carlo simulation. This allows for consideration of most-significant sero-reactivity of highest relevance to the interpretation of Paramyxovirus and Filovirus priorities of this manuscript.

	mean	sd	val2.5pc	median	val97.5pc	sample
AUC	0.9801	0.01109	0.9353	0.9813	0.979	99000
deviance	5246.0	6.752	5239.0	5244.0	5264.0	99000
k	237.3	3.961	229.8	237.1	245.3	99000
mu[1]	146.0	0.1	145.8	146.0	146.2	99000
mu[2]	10960.0	0.03159	10960.0	10960.0	10960.0	99000
sigma[1]	55.49	2.405	50.96	55.42	60.38	99000
sigma[2]	6152.0	456.5	5334.0	6125.0	7122.0	99000
tau[1]	3.266E-4	2.828E-5	-0.002893	3.262E-4	0.003541	99000
tau[2]	2.685E-8	3.939E-9	-0.003183	-2.156E-8	0.003183	99000

DIC monitor not set

History

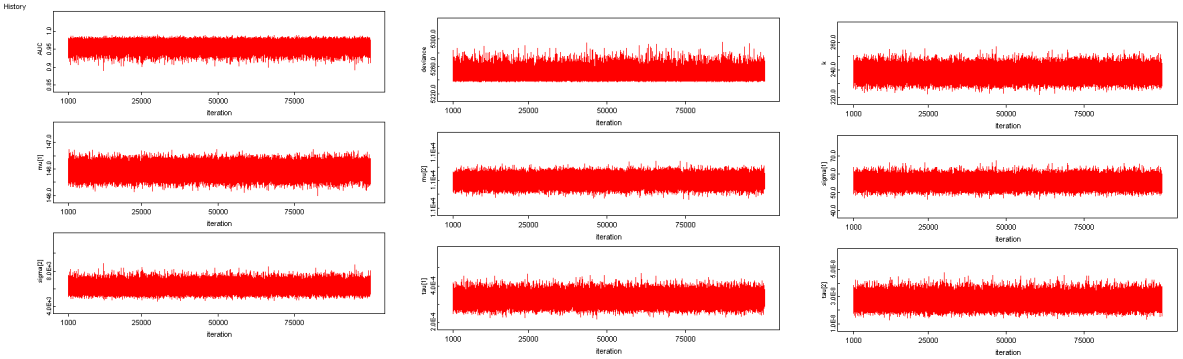


Figure 7.3. Example (HeV RBP IgG) of OpenBUGS output assessed for multiple parameter convergence over 99,000 simulations

Posterior outputs for the more than 100,000 simulations, were assessed by comparative distributions of predicted positive and negative MFIs (Figure 7.4), ROC curve (Figure 7.5), posterior estimations of AUC (Figure 7.6), and densities for posterior predicted positive and negative classification (Figure 7.7). Test classification cut-offs were determined as appropriate for each antigen assay performance at 95% specificity, given separation of at least two times the standard deviation of mean −ve MFI.

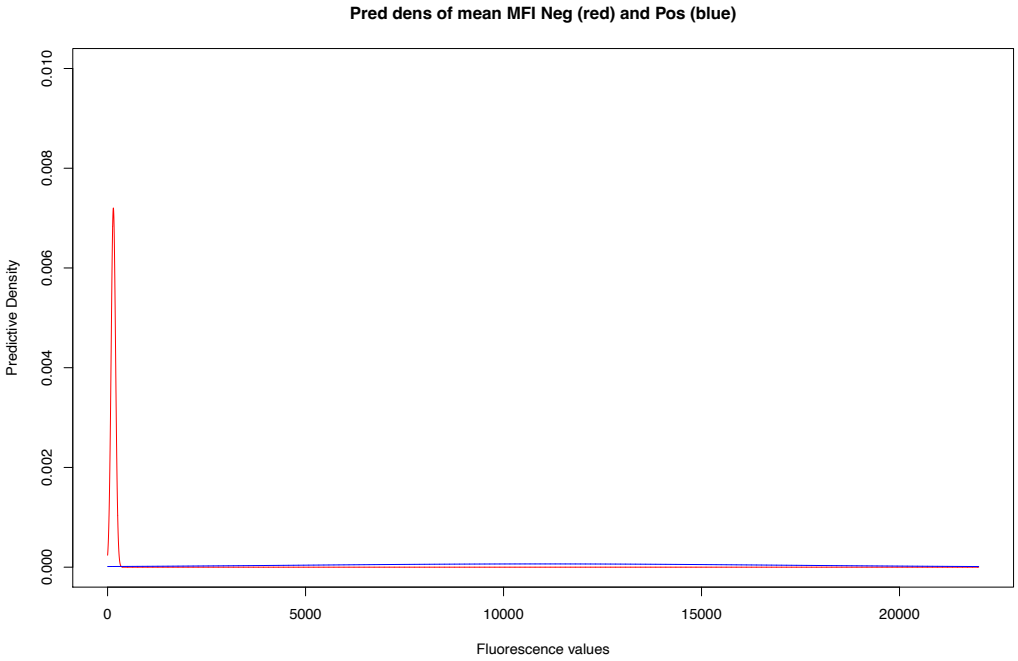


Figure 7.4. Example (HeV RBP IgG) of predicted positive and negative MFIs

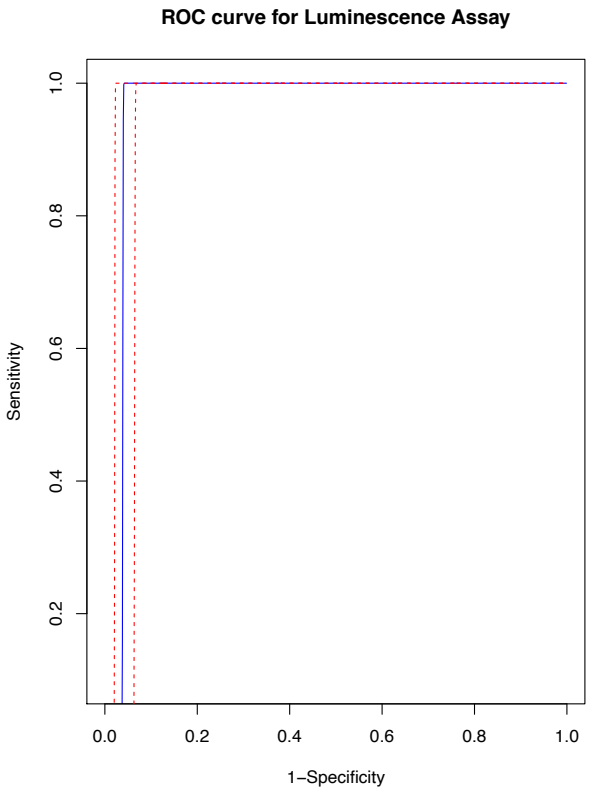


Figure 7.5. Example (HeV RBP IgG) ROC curve

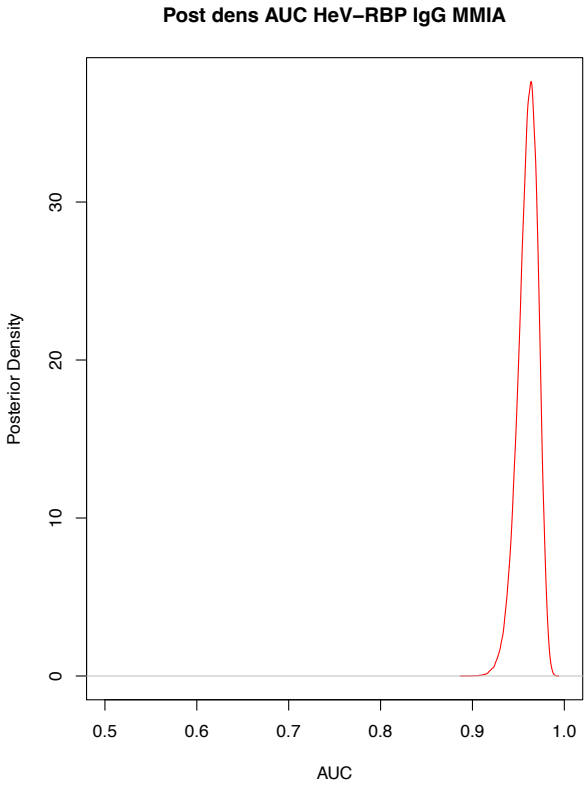


Figure 7.6. Example (HeV RBP IgG) posterior AUC plot

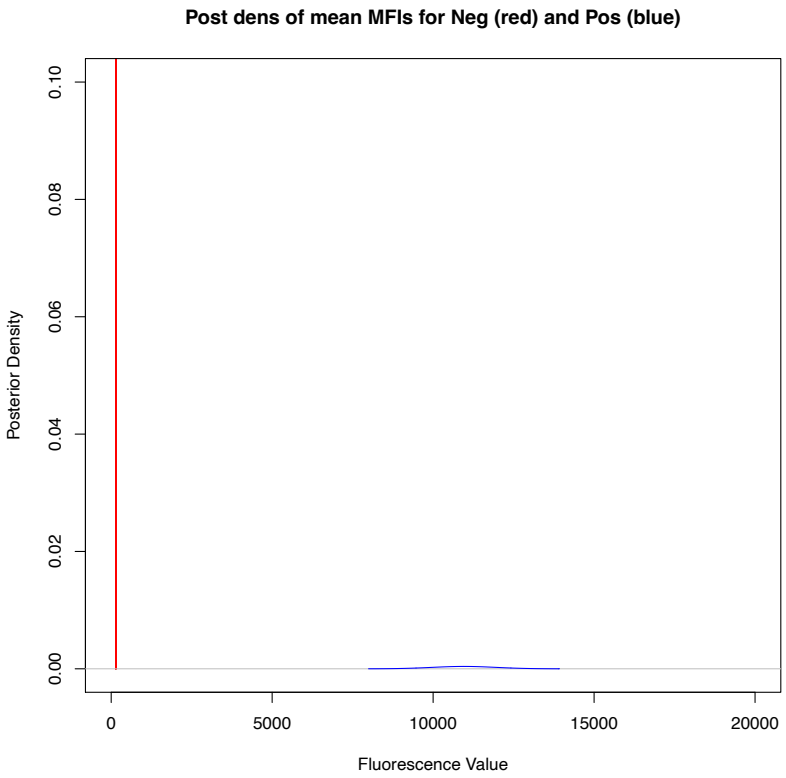


Figure 7.7. Example (HeV RBP IgG) densities for posterior predicted positive/ negative classification

7.2.5 Confirmatory assays (IFA) MenPV (other pararubulaviruses) and Filovirus

Horse subject sera amongst 374 subjects tested by multiplex microsphere-based immunoassay (MIA) in pilot investigations eliciting IgG reactivity against MenPV nucleocapsid (N) protein, Tioman virus (TioPV), N protein and MenPV haemagglutinin-neuraminidase (HN) protein were confirmed by immunofluorescence assay (IFA) utilising Vero (African green monkey) or *Pteropus alecto* kidney cells (PaKi)[5] cells. The cells were infected with MenPV, YepPV, Grove PV and TevPV under biosafety level 3 laboratory conditions at the Australian Animal Health Laboratory (AAHL), now the Australian Centre for Disease Preparedness. Convalescent samples from clinically affected animals were also tested when available. For the filovirus IFAs, Vero cells were infected with filoviruses (Bundibugyo, Marburg, Reston, Sudan, Tai Forest, and Zaire ebolaviruses) at biosafety level 4 (performed by J Barr).

Briefly, following fixation, the wells of the 96 well plates were treated with 0.1% Nonidet P-40 in PBS for 15 minutes at 37° C, then removed prior to the addition of test subject and control rabbit antisera for the dilution in 1% BSA and incubated for 1 hour at 37° C. The plates were washed in PBS-T four times and a combination of Protein A/G-488 (Invitrogen) 1:1000

with DAPI (1:50) in 1% BSA in PBS was added. The plates were incubated for 1 hour at 37° C. They were washed again in PBS-T, and PBS was added, before being viewed under an inverted fluorescence microscope.

Western blots were also performed at AAHL using a recombinant Zaire ebolavirus GP protein.

7.2.6 Metatranscriptomic Next generation Sequencing (RNA-seq)

While this chapter focuses on serology analyses, it is appropriate to briefly describe molecular testing findings from the priority Qld. HeV-like disease cohort to guide most proactive interpretation of the serology findings.

All high priority samples in the Qld. HeV-like disease cohort underwent total deep RNA sequencing (similar to that described in Chapter 8). Given this comprised more than 800 individual samples, to maximise the coverage of the cohort with available funds, RNA extracted products were pooled to represent 5 to 10 individual samples. Most samples were pooled as groups of 10. However, a reanalysis of all high priority samples was performed to identify those of high interest. High interest samples were then pooled into groups of 5 instead, to increase depth of sequencing on these samples. We identified these high interest and high priority samples from the original database by identifying those found to have 'mucous membrane injected or congested' and 'petechial haemorrhage' clinical signs.

All samples were pooled based on sample type: that is, we pooled samples from the blood only with other blood samples, those from swabs only with other swabs, and all rectal samples only with other rectal samples. As the general makeup of these samples will vary based on the commensal bacteria that differs in each area (etc), this limits the confounding affect this may have during sequencing and analysis.

Prior to pooling, we also performed Qubit analysis of each sample in order to determine the concentration of RNA in each sample. We then used this to roughly correct for the abundance of each sample in the pool. Samples with low concentration were present in a higher volume in the pool, while those with a higher RNA concentration were present at a lower volume. As such, coverage of each individual sample in each pool should be more equivalent during sequencing.

A total of 95 pools were generated, covering all high priority RNA samples.

Bioinformatical analyses and maximum likelihood phylogenetic tree construction, as well as individual case PCR detections reported here (Table 7.19) were undertaken as described in Chapter 8.

7.3 Results

During the initial phase of the project, when the Luminex multiplex immunoassays were being developed, testing was performed on selected horse serum samples to assess the performance of the assays against previous results in flying fox sera and other species. Initially 185 horses from Qld and 32 horses from NSW were tested. They were sampled in locations perceived as having significant flying fox exposure. Results were grouped according to the level of reactions as high >10,000 MFI, moderate 5,000-10,000, low 1,000-5,000 and negative <1,000. Cut-offs were subsequently determined by Bayesian analysis (see below). (24)

A summary of early findings relating to emerging MenPV-like bat-borne pararubulaviruses was communicated to government biosecurity officials and Australian equine veterinarians via scientific presentation and abstract publication in 2018. (34) Findings were published as a scientific abstract in the International Society for Infectious Diseases, International Journal of Infectious Diseases in 2020(24) with the following summary:

> *'Median fluorescence intensities (MFI) against MenPV N and a prior prevalence estimate of 20% were used in a Bayesian latent class model to determine appropriate cut-offs for positive test classification. Assay sensitivity was estimated assuming a specificity of both 95% and 99%. MFI reflecting potentially significant IgG to MenPV N protein was demonstrated in 34% (94/274) of horses with high perceived flying fox exposure (29% in QLD and 32% in NSW) whereas horses without plausible exposure recorded insignificant MFI. IFA confirmed antibodies to three of five related flying fox rubulaviruses tested (MenPV, Yeppoon virus and Grove virus).'*

During this initial screening of horse sera sampled from locations of perceived flying fox exposure, reactions were detected against the beads coupled with the MenPV N protein and

the ebolavirus Zaire N protein in the IgG assay. These reactions were investigated further (see section 7.3.1.2).

7.3.1 Notable pilot study results – paramyxoviruses and filoviruses

7.3.1.1 Outbreak of HeV-like disease in NSW 2016 with serology indicating MenPV-like Pararubulavirus infection

Case presentations: In 2016, two young adult geldings kept in adjacent small paddocks in western greater Sydney region presented with acute onset severe respiratory illness 11 days apart. Case 1 was a four-year-old Clydesdale gelding who presented with an obtunded demeanour, tachypnoea, tachycardia, congested/hyperaemic mucous membranes and pyrexia. This horse received supportive medical therapy (NSAIDs and antibiotics) and made a full clinical recovery in five days. The second (Case 3) was a three-year-old shire gelding who presented with the same symptoms and mild bilateral serous nasal discharge 11 days later. Case 3 recovered with supportive care in one week. Haematology and biochemistry analysis was performed on blood sampled during acute illness from both horses and demonstrated mild leukopaenia, neutropaenia and lymphocytosis, and mildly elevated creatine kinase. Sufficient convalescent sera were available only in Case 3.

A 25-year-old mare (Case 2) presented with acute fatal HeV-like disease the day following Case 1 and 10 days before Case 3 showed signs of disease. This mare had been residing in a large paddock adjacent to the yards in which the two geldings were kept, which was bordered by a tree-lined watercourse. Disease manifestations included pyrexia, severely obtunded and disorientated demeanour, severe tachypnoea/ respiratory distress, tachycardia, congested/injected hyperaemic mucous membranes and marked neurological signs (vision impairment, head tilt, wide based stance, walking into fences - Figure 7.8) The attending veterinarian (EJ Annand) elected to sample and submit for HeV testing and euthanasia due to the horse's moribund condition, however, the mare died naturally during the time preparing the lethal injection. HeV PCR testing by government state laboratory was negative for this case and Case 1; samples from Case 2 were not submitted for HEV testing. Flying foxes were not known to roost but were seen on the property visiting fruit trees near the residence and in the native eucalypt forest that ran along the back of the extensive paddock associated with the watercourse. A small dam on an adjacent property was also located near the stable shelters and yards in which Cases 1 and 3 were kept.

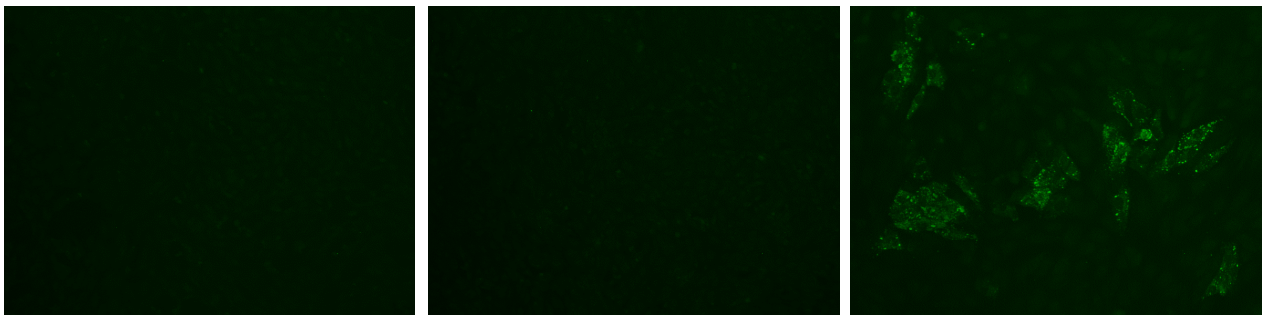
Figure 7.8. Photograph of Case 2 presenting with acute severe HeV-like disease in moribund condition with wide base stance and impaired mental awareness and vision

Approximately six months later a 22-year-old mare that had been maintained on the adjacent property, in the paddock containing the dam near the stable/yards containing the two geldings (Case 1 and 3), developed HeV-like disease featuring severe respiratory and neurological signs and was euthanased. Samples from this horse (Case 4) were also submitted for government biosecurity laboratory HeV testing and found negative.

Figure 7.9. Photograph showing: the extensive paddock where Case 2 was maintained (beyond the yard in which she was examined and died in the foreground), with minimal tree coverage but joined by a tree-lined watercourse in the distance. The paddock adjoined the stable shelters with adjoining yards housing Case 1 and Case 3. A small dam was located near the larger eucalypt tree to the right of the image. Case 4 (presenting with similarly fatal disease some months following) had been maintained in another extensive paddock on the neighbouring property with an adjoining fence-line running away from the photographer beginning near the stable shelters and large eucalypt tree.

Paired sera from Case 3 were tested with the Luminex multiplex immunoassay targeting IgG against a small panel of paramyxoviral proteins. A greater than 10-fold increase in MFI from insignificant to high (474 to 14,571) was detected between sera sampled during acute illness and that was collected 17 days later (Table 7.3). Low level reactivity was observed with the TioPV N protein but not at all with the MenV sHN protein (Table 7.3). Lack of reaction to the HN may suggest that seroprevalent antibodies were reacting to a related pararubulavirus rather than specifically MenPV (where the surface protein antigenicity is of greater species specificity), and/or that IgG targeting the N protein might be elicited earlier following acute agent exposure than those targeting the receptor binding protein (HN).

IFAs conducted on MenPV infected Vero cells confirmed a seroconversion with a rubulavirus similar to MenPV with fluorescence observed for the sera collected on the 16/5/2016 but not for the earlier sera collected on 29/4/2016 (Figure 7.10).



A **B** **C**

Figure 7.10. IFA assay conducted on paired samples from the horse for which paired sera were available (Case 3). (A) Control Vero cells with sera from 16/5/2016, (B) Vero cells infected with MenPV with sera from 29/4/2016 and (C)16/5/2016

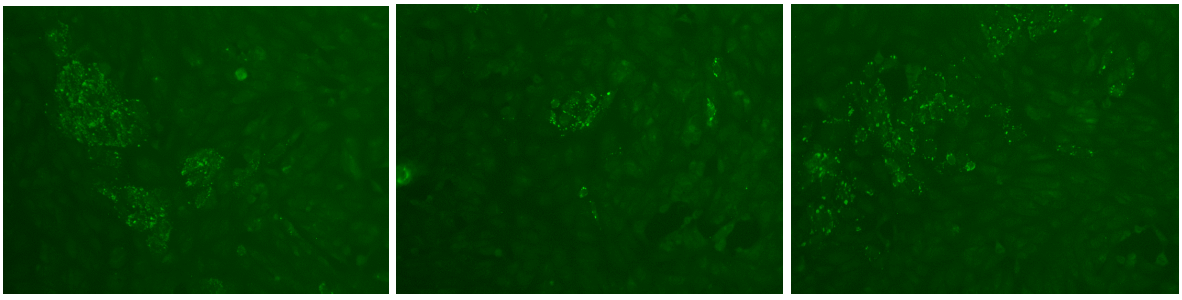
Another horse serum (serosurvey sample 15-008XX-4) that elicited notably high reactivity by IgG against Pararubulavirus N proteins in these initial investigations was a horse sampled near Brisbane, Qld in 2015 in a region of high perceived flying fox contact. This horse was vaccinated for HeV and sampled routinely as part of the serosurvey studies, without showing any overt contemporaneous signs of disease. Results demonstrated moderate MFI of 5807 to MenPV N protein coupled beads and low-level reactivity (MFI 1762) was also observed for the TioPV N protein coupled beads. No reaction was observed with the MenPV sHN coupled beads suggesting that the antibodies were reacting with a pararubulavirus related to MenPV but not MenPV.

Table 7.3. Reactions to Menangle (MenPV) and Tioman (TioPV) Pararubulavirus nucleoprotein (N) coupled Luminex beads

Sample	State & date of sera collection	Disease	State government HeV PCR	MenPV N (MFI) IgG	TioPV N (MFI) IgG	MenV sHN (MFI) IgG	MenPV N (MFI) IgM	TioPV N (MFI) IgM	MenV sHN (MFI) IgM
Case 1 4yo Clydesdale gelding	NSW 20/4/16	HeV-like recovered	–ve	1,100	472	NA	952	1,084	3,919
	NSW 29/4/16		NA	1,042	413	NA	1,994	1,899	6,841
Case 2 25yo Mare	NSW 21/4/16	HeV-like fatal	–ve	1,510*	188*	134	2,791	2,037	7,380
Case 3 3yo Shire gelding	NSW 29/4/16	HeV-like recovered	Not performed	474	474	232	801	600	4,983
	NSW 16/5/16			14,571	14,571	233	603	583	2,545
Case 4 22yo mare	NSW 8/12/16	HeV-like fatal	–ve	1,248	99	156	2,487	2,515	7,721
15-00836-4	Qld April 2015	None recognised – serosurvey	NA	6,156*	919*	277	NA	NA	NA

*Average MFI from 2 runs

IFAs confirmed the presence of antibodies to three of four related flying fox rubulaviruses tested (MenPV, Yeppoon virus and Grove virus) to the serosurvey horse sample 15-00836-4 (Figure 7.11).



A **B** **C**

Figure 7.11. Comparative immunofluorescence assays using various Australian flying fox derived pararubulaviruses on IgG positive equine sera 15-00836-4. Reactivity demonstrated against (A), MenPV, (B) Yeppoon virus (YepPV) and (C) Grove virus (GroPV) but not to Teviot virus (TevPV) (not shown here).

7.3.1.2 Luminex bead based Multiplex immunoassay results targeting IgG against *Filoviruses* N protein (EBOV)

Another horse sampled as part of the initial serosurvey targeting horses of high perceived flying fox exposure, a 15-year-old HeV vaccinated thoroughbred mare (submission 16-03301), displayed a strong reaction to the Zaire ebolavirus (EBOV) N protein (MFI 27201), with reactivity also observed for Reston ebolavirus (RESTV) N protein (MFI 10925). This horse was housed on a property in south-western greater Sydney in a paddock featuring a range of Eucalyptus trees as well as a dam where flying foxes (mostly GHFF with some LRFF and BFF) were observed regularly visiting by night. No significant reactions were recorded against EBOV GP, RESTV GP, Marburg (MARV) GP or Sudan ebolavirus (SUDV) GP (Table 7.4). The mare was experiencing disease manifestations at the time including mildly depressed or painful demeanour, unusual posturing of head, increased frequency of swallowing, mild, unilateral left sided mucoid nasal discharge, relatively decreased percussion over the left sinus (compared to the right) and mild diffuse increase in lung sounds evident only on 'rebreathing test' (consistent with previous diagnosis of IAD), with normal rectal temperature and hydration status. The mare was successfully treated for sinusitis of dental (tooth root infection) or idiopathic origin including antibiotics and sinus lavage via trephination. Subsequently a molar tooth of highest suspicion based on serial radiographs was extracted; however, it showed minimal gross signs of disease of the tooth root. While it is possible that

recent viral infection may have contributed to the onset of this mucoid sinusitis, it is considered unlikely to have been related to the Filovirus infection/exposure, which would most likely have occurred sometime previously given the high circulating IgG. A single severe infectious-like illness featured in the horse's medical history approximately two years earlier, featuring some respiratory signs and fever.

Sera was also collected from the mare's three paddock mates, who showed no signs of disease, as part routine serosurvey sampling. Of these, no further significantly high sero-reactivity was observed by MMIA using the EBOV N protein (in the limited pilot run in which they were included) however, sera from one additional horse (16-03301-2 sampled on the 23/9/2015), a 20-year-old gelding, demonstrated similarly significant sero-reactivity to EBOV and RESTV by IFA (Figure 7.13).

Table 7.4. Reactions to ebolavirus nucleoprotein (N) and glycoproteins (GP) coupled Luminex beads

Sample	EBOV GP	EBOV N	RESTV GP	SUDV GP	RESTV NP	MARV GP
16-03301-1 15-year-old mare 19/6/2015	355	27,201	125	131	10,926	771

Confirmatory testing using IFAs at AAHL were conducted with Vero cells infected with Zaire, Reston, Sudan, Tai Forest, Marburg and Bundibungyo ebolaviruses (Figure 7.12 & Figure 7.13). Reactions were observed for cells infected with Zaire and Reston ebolaviruses, but not for the other ebolaviruses.

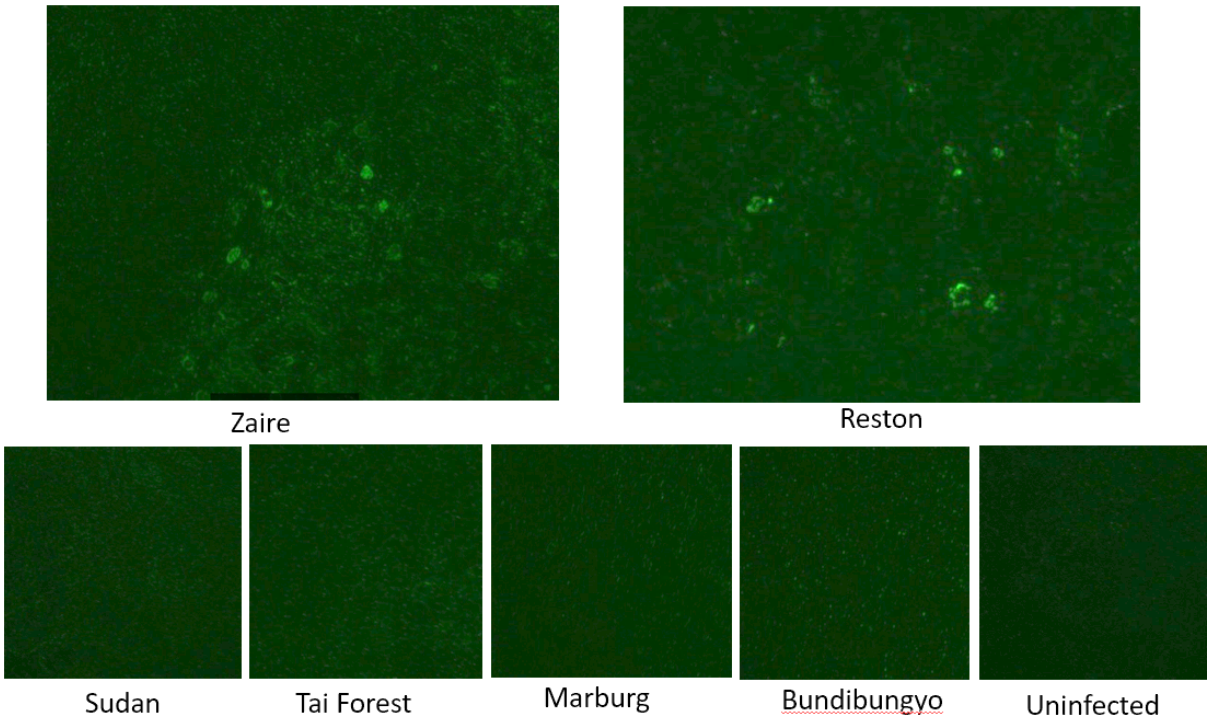


Figure 7.12. IFA of ebolavirus infected Vero cells with the horse sera 16-03301-1 15-year-old mare found to react in the Luminex immunoassay to the NP of EBOV and RESTV

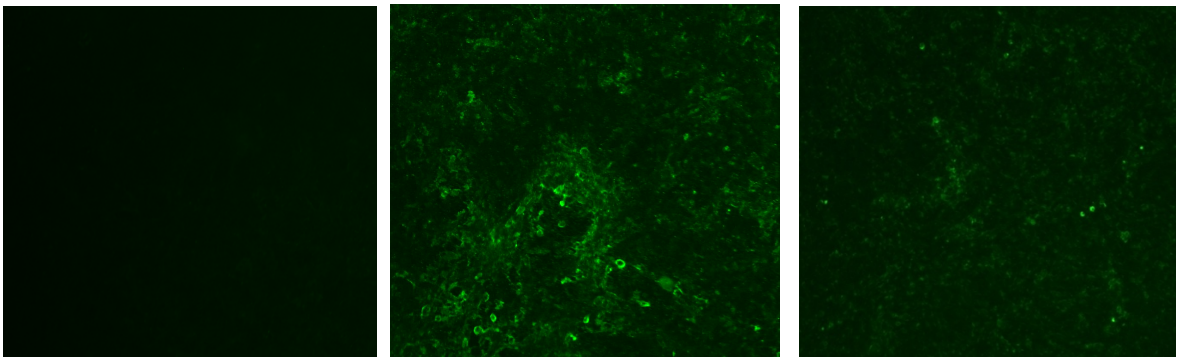


Figure 7.13. 16-03301-2 20-year-old gelding 23/9/2015 – Filovirus confirmatory IFA – left panel negative control, central panel EBOV, and right panel RESTV

Following these results, a western blot using the nucleoprotein of Zaire ebolavirus was performed on sera from the 15-year-old mare (16-03301-1) collected on 23/9/15 and 26/5/17 and shown to be reactive (Figure 7.14) for both. Notably higher reactivity was evident for the later sera sample potentially consistent with recent infection/exposure for the first sample and/or repeated exposure to a virus of high similarity to Zaire ebolavirus.

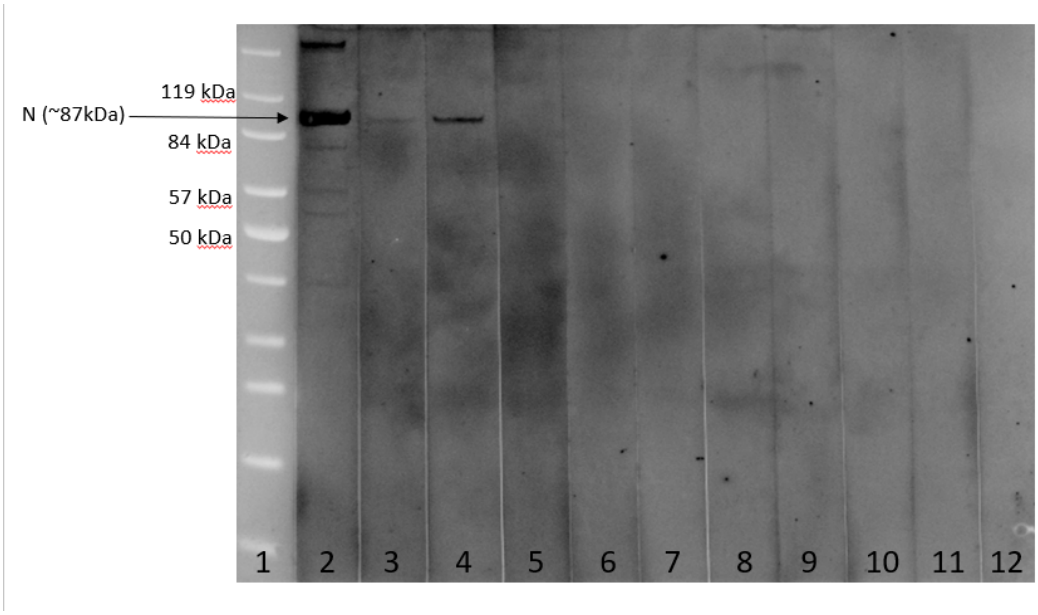


Figure 7.14. Western blot using Zaire ebolavirus Nucleoprotein. (1) Mwt marker, (2) ZEboV N rabbit sera, (3) 16-03301-1 15-year-old mare 23/9/15, (4) 16-03301-1 15-year-old mare 26/5/17, and (5-12) other horse sera

7.3.2. Presentation and preliminary interpretations of results drawn from application of the Horses as Sentinels extensive multiplex Australian Emerging Infectious Disease fluorescent microbead immunoassay

Large scale application of Luminex multiplex immunoassays for the main study cohort involved testing 412 subject sera (selected to represent key research case/ sample cohorts to support thesis hypothesis investigation and parallel molecular testing) with the Australian emerging infectious disease multiplex panel for both IgG and IgM (Table 7.5). The sera represented 409 horse subjects.

The infectious-like disease sample/subject cohort (334 horse subjects/337 sample events) included:

- 306 horses (306 sample events) that had been tested in Qld for HeV as part of highly suspect disease investigations (HeV-like Disease Qld)
- 17 horses from NSW in which HeV infection or other infectious cause was suspected, comprising:

- eight sampled in association with the confirmed HeV spillover of 2019 in the Hunter region (HeV Spillover 2019 − seven with direct contact to the confirmed case and one without)
- six sampled in association with the outbreak of HeV-like disease that had demonstrated seroconversion to a Menangle-like pararubulavirus in the pilot study (HeV-like disease outbreak NSW 2016)
- three additional horses (four sample events) with infectious-like disease, including two (three sample events) from an outbreak of viral-like disease in the Hume region in 2020, and one with 'sinusitis' in the greater Sydney region (other infectious-like disease, NSW)

- 11 horses (13 sample events) that had suffered neurological disease in a 2020 cluster in South Australia in 2020 (acute neuro disease cluster SA 2020).

The non-infectious disease cohort (75 horse subjects), acted as a control group and comprised:

- 33 horses (single sample events) tested for HeV for OHS or biosecurity reasons in the absence of infectious-like disease manifestations (infectious-like disease priority category 5) in Qld between 2015 and 2018 (HeV tested Qld)
- 42 horses (single sample events) without disease, sampled as part of the serosurvey undertaken by this research.

7.3.2.1 Determination of cut-offs for Binary outcome for the Luminex multiplex immunoassay

Table 7.6 presents key parameters and assay performance measures for Paramyxovirus antigens applied as part of the extensive multiplex florescent bead immune assay following pilot studies as part of this Horses as Sentinels research. Prior prevalence estimations (Table 7.6: 'Prior estimated prevalence') for each recombinant antigen were based on seroprevalence for IgG targeting divergent Paramyxoviruses amongst equivalent horse-subject sub cohorts' sera as part of this research's pilot investigations and when appropriate (HeV and NiV RBP antigens only), incidence of vaccination elicited seropositivity amongst the subject cohort in relation to the HeV vaccine utilising the same HeV RBP (G glycoprotein).

Table 7.5. Overview of the horse sera tested

Sample/subject sub-cohort	Infectious-like disease					No suspect infectious disease		Main study sample/subject cohort
	HeV-Like Disease Qld	HeV spillover 2019 7 direct HeV case contact/ 1 no direct HeV case contact	HeV-like disease outbreak NSW 2016 (MenPV-like outbreak 2016)	Other Infectious-like disease NSW	Acute neuro disease cluster SA 2020	HeV tested QLD * **for biosecurity clearance not suspect disease*	Non-diseased serosurvey	
HeV vaccine status								
subject count	306	8	6	3	11	*33*	42	409
sample count	306	8	6	4	13	33	42	412
unvaccinated	254	8	6	3	13	20	41	345
unknown	17					2		19
vaccinated_current	12			1		3		16
vaccinated_unknown_currency						3		3
vaccinated_not_current	15							15
partially_vaccinated	4					2		6
unknown_suspect_vaccinated	2					3		5
partially_vaccinated_not_current	2							2
unvaccinated foal* **Years since HeV vaccine current**	* passive transfer immunity from vaccinated (current) dam						1	1
NA	279	8	6	3	13	28	42	379
<1	15			1		4		20
1	3					1		4
2	5							5
3	3							3
4 **Infectious disease category**	1							1
1	144	1	6	3	11			165
2	161							161
3				1				1
5	1					33	42	76
6								0
7		7						7
1(7) **State**					2			2
Qld* ***viaBSLinc1+NT**	306					33		339
NSW		8	6	4			1	19
SA					13			13
WA **Sample type**							41	41
edta	282					32		314
serum	3	8	6	4	13		42	76
clot	20					1		21
clot_pm **Single or paired sample event**	1							1
paired_sampling_second				1	2			3
paired_sampling_primary				1	2			3
single sample event	306	8	6	2	9	33	42	406

Prior Boolean classifications were determined conservatively for each recombinant antigen in the IgG assay (Table 7.6: 'Prior cut-off (MFI)'), set at MFIs determined to be at least thrice the mean of horse-subject results of expected seronegative. Such examples included those recording low MFI readings in horses that had previously tested negative for equivalent agents or where epidemiology supported seronegative findings such as those from southern WA when tested for viruses of expected flying fox origin and horses of known unvaccinated HeV status when tested for HeV RBP (G glycoprotein). Similarly prior Boolean classifications were determined conservatively for each recombinant antigen in the IgM assay (Table 7.6: 'Prior cut-off (MFI)'), determined to be at least thrice the mean of horse-subject results of expected seronegative, such as those recording low MFI readings in horses that were sampled when clinically well. Conservative prior Boolean classifications for each antigen used in the IgM assay were further informed by both the distribution of results (described for each IgM antigen in the legend for Figure 7.6) in the context of expected IgM seroprevalence amongst horse-subject sub-cohorts and results from pilot applications of the fluorescent microbead immunoassay as part of this research.

Posterior cut-off MFIs for binary test result classification were determined, guided by median[4] over more than 100,000 model simulations, for each antigen (Table 7.6: 'Post k95[#] cut-off'), for intended assay performance at 95% sensitivity[5] (custom for general surveillance context) given separation of at least two times the standard deviation of mean −ve MFI. AUC and 1-pnorm value estimations for each antigen (Table 7.6: 'AUC', '1-pnorm') were similarly averaged on >100,000 simulations. Uncertainty relating to analytical versus diagnostic[6] sensitivity is inherent in this innovative explorative diagnostic assay application and further probability based latent class analyses using parallel relevant assay results to improve would improve confidence and precision of inference for individual antigens of key interest.

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[4] Mean has no value in BLCM, rather the Median and the Posterior Intervals that are 2.5 and 97.5 percentiles equivalent to 95% CI are informative.

[5] We use this function k95 to obtain the cut-off for a performance at 95% sensitivity and using the 1-pnorm function we estimate the specificity for the assay at that cut-off.

[6] *Analytical* refers to assay performance in set laboratory conditions versus *diagnostic* variably incorporating entire series of processes required for assay diagnosis for any individual case – incl. vet recognition, sampling, tissue/ sampling limitations, testing protocol and diagnostic criteria.

Table 7.6. Prior and posterior cut-offs for binary test outcome classification and Bayesian latent class estimation of test performance by ROCs and AUC for each Paramyxovirus antigen

Analyte	Prior estimated prevalence	Prior cut-off (MFI)	Post k95[#] cut-off	AUC[##]	Specificity[###]
HeV RBP (G) (Average of two equivalent proteins)	20%*	1323	237	0.96	0.962
HeV RBP (G) IgM	10%	1500*****	628	0.871	0.856
NiV RBP (G)	20%	1323	320	0.943	0.942
NiV RBP (G) IgM	10%	2975******	1590	0.924	0.899
MojV RBP	10%**	1323	335	0.983	0.979
MojV RBP IgM	10%	3888*******	3765	0.99996	1
GhV RBP	10%	1323	364	0.932	0.918
GhV RBP IgM	10%	3123********	1538	0.886	0.862
CedPV RBP	10%	1000	257	0.996	0.994
CedPV RBP IgM	10%	6239*********	5340	0.999	1
TioPV NP	15%***	1323	587	1	1
TioPV NP IgM	10%****	2360**********	1483	0.973	0.944
MenPV NP	15%	1323	857	0.99	0.979
MenPV NP IgM	10%	3588***********	3308	0.9996	1
MenPV RBP (HN)	15%	1323	427	1	1
MenPV RBP (HN) IgM	10%	7761************	7711	0.994	1
		11462*************	8061	1	1
YepPV RBP (HN)	15%	1323	366	1	1
YepPV RBP (HN) IgM	10%	1000**************	388	0.960	0.944
GrovPV RBP (HN)	15%	1323	166	1	0.962
GrovPV RBP (HN) IgM	10%	1497***************	770	0.842	0.817

[#] K95 equivalent to specificity 95% equivalent to Mean of negatives plus two standard deviations. K95 is used, as opposed to K99 (equivalent to specificity 99% is equivalent to the Mean-ve+3SD), for the desired higher sensitivity (K95) given the assay's research screening purpose

[##] Mean AUC of estimations from more than 100,000 model simulations

[###] Specificity estimated over >100,000 simulations given 95% sensitivity and assigned posterior cut-off (k95) for binary classification

*prior prevalence influenced by prevalence of HeV immunisation elicited seropositivity.

** as for pararubulaviruses given vaccine cross-reactivity considered less likely

*** prior prevalence for IgG targeting Pararubulaviruses drawn from seroprevalence (IgG TioPV and MenPV NPs) determined in the pilot study

****prior prevalence set for IgM targeting Pararubulaviruses a little lower than for IgG given considered less likely given indicative of recent exposure, while increased cross reactivity expected for IgM

*****Prior Boolean classification is set @ 1500 as mean was 490 and median was 302 max was 18402 min was 71 1st Q 226 3rd Q 445

******Prior Boolean classification is set @ # 2975 as mean was 1075 and median was 908 max was 16240 min was 222 1st Q 638 3rd Q 1217 and diff between thrice mean and thrice median added to thrice median was 2975 and well above 3rd Q

*******Prior Boolean classification is set @ 3888 as mean was 1472 and median was 1120 max was 18585 min was 126 1st Q 1164 3rd Q 2378 and diff between thrice mean and thrice median added to thrice median was 2975 and well above 3rd Q

********Prior Boolean classification is set @ 3123 as mean was 1041 and median was 725 max was 28279 min was 130 1st Q 494 3rd Q 1041 as both thrice mean and thrice median were 3123 and well above 3rd Q

*********Prior Boolean classification is set @ 6239 as the diff between thrice mean and thrice median added to thrice median (well above 3rd Q) where mean was 2378 and median was 1781 max was 18585 min was 126 1st Q 1164 3rd Q

**********Prior Boolean classification is set @ 2360 as the diff between thrice mean and thrice median added to thrice median (well above 3rd Q) where mean was 934 and median was 639 max was 9239 min was 63 1st Q 422 3rd Q 1075

***********Prior Boolean classification is set @ 3588 as the diff between thrice mean and thrice median added to thrice median (well above 3rd Q) where mean was 1378 and median was 1014 max was 11846 min was 52 1st Q 636 3rd Q 1647.

************Prior Boolean classification is set @ used 7761 as the diff between double mean and double median added to thrice median (well above 3rd Q) where mean was 4042 and median was 3719 max was 18388 min was 127 1st Q 2256 3rd Q 5127, or

*************Prior Boolean classification is set @ used 11642 as the diff between thrice mean and thrice median added to thrice median (well above 3rd Q) where mean was 4042 and median was 3719 max was 18388 min was 127 1st Q 2256 3rd Q 5127, or

**************Prior Boolean classification is set @ used 1000 where mean was 239 and median was 182 max was 3485 min was 13 1st Q 134 3rd Q 272

***************Prior Boolean classification is set @ 1497 as the diff between thrice mean and thrice median added to thrice median (well above 3rd Q) where mean was 616 and median was 382 max was 20769 min was 31 1st Q 238 3rd Q 568

Binary test result classifications are presented for the full main cohort along with positive test proportions using the posterior cut-offs (calculated as described above and shown in table 7.6) for each Paramyxovirus antigen (Table 7.7). Combined horse-subject test positivity is also presented as for most similar antigens as appropriate for expected comparative specific recombinant antigen assay performance and in relation to horse-subject vaccine status.

Table 7.7. Summary of the binary test outcome for the full cohort by Luminex multiplex immunoassays determined by Bayesian analysis

Analytical Summary	Positive	Pos test prop. (%)	Negative	Neg test prop. (%)	Total
hevrbppostk95class	81	26	225	74	306
hevrbpigmpostk95class	36	12	269	88	305
nivrbppostk95class	72	24	234	76	306
nivrbpigmpostk95class	37	12	268	88	305
mojvrbppostk95class	29	9	277	91	306
mojvrbpigmpostk95class	9	3	296	97	305
ghvrbppostk95class	42	14	264	86	306
ghvrbpigmpostk95class	35	11	270	89	305
cedpvrbppostk95class	19	6	287	94	306
cedpvrbpigmpostk95class	14	5	291	95	305
HeV.NiViggpostk95classcombined	85	28	221	72	306
HeV.NiViggpostk95classcombinedunvaccinated	33	11	273	89	306
otherhnviggpostk95classcombined	60	20	246	80	306
moj.ghv.iggpostk95classcombined	56	18	250	82	306
otherhnviggpostk95classcombinedunvaccinated	59	19	247	81	306
moj.ghv.iggpostk95classcombinedunvaccinated	49	16	257	84	306
HeV.NiV.CedPV.iggpostk95classcombined	88	29	218	71	306
HeV.NiV.CedPViggpostk95classcombinedunvaccinated	88	29	218	71	306
HeV.NiV.negCedPViggpostk95classcombinedunvaccinated	24	8	282	92	306
HeV.NiV.negmoj.ghv.iggpostk95classcombinedunvaccinated	22	7	284	93	306
HeV.NiV.negotherhnv.iggpostk95classcombinedunvaccinated	22	7	284	93	306
HeV.NiVigmpostk95classcombined	48	16	257	84	305
HeV.NiVigmpostk95classcombinedunvaccinated	47	15	258	85	305
otherhnvigmpostk95classcombined	42	14	263	86	305
otherhnvigmpostk95classcombinedunvaccinated	34	11	271	89	305
hev.nivigm.neg.igg.postk95classcombined	33	11	272	89	305
otherhnvigm.neg.igg.postk95classcombined	37	12	268	88	305
tiopvnpostk95class	11	4	295	96	306
tiopvnigmpostk95class	32	11	270	89	302
menpvnpostk95class	42	14	264	86	306
menpvnigmpostk95class	17	6	285	94	302
menpvhnpostk95class	6	2	300	98	306
menpvhnigmpostk95class_4prior7761	27	9	278	91	305
menpvhnigmpostk95class_4prior11462	26	9	279	91	305
yeppvrbppostk95class	2	1	304	99	306
yeppvhnigmpostk95class	26	9	279	91	305
grovpvrbppostk95class	31	10	275	90	306
grovpvhnigmpostk95class	43	14	262	86	305
prvnpiggpostk95classcombined	49	16	257	84	306
prvshniggpostk95classcombined	35	11	271	89	306
prvnpigmpostk95classcombined	39	13	263	87	302
prvshnigmpostk95classcombined	63	21	242	79	305
prvn.shn.igmpostk95classcombined	22	7	283	93	305
prvn.shn.iggpostk95classcombined	12	4	294	96	306
prvn.shn.igm.neg.igg.postk95classcombined	21	7	284	93	305

7.3.2.2 Summary of IgG and IgM Paramyxovirus antigen results for the full cohort included in the main study application of the extensive Horses as Sentinels multiplex Australian Emerging Infectious Disease fluorescent microbead immunoassay

The following is a summary of results for the HeV-like disease cohort from Queensland - 306 serum subjects tested against all proteins in the full MIA IgG panel, other than the Pararubulavirus NPs (TioPV and MenPV), and 305 were tested against RBP antigens and 302 NP antigens in IgM. These variations in sera sample numbers relate to inclusion of single sample only, representing most relevant sample event instance in relation timing of observed disease versus expected immunoglobulin response, for Subjects for which paired sera samples were included in the full tested sample cohort (convalescent sample included for IgG and acute sample included for IgM).

Median fluorescence intensities (MFI) against MenPV N and a prior prevalence estimate of 20% were used in a Bayesian latent class model to determine appropriate cut-offs for positive test classification. Assay sensitivity was estimated assuming a specificity of both 95% and 99%. MFI reflecting potentially significant IgG to MenPV N protein was demonstrated in 34% (94/274) of horses with high perceived flying fox exposure (29% in QLD and 32% in NSW), whereas horses without plausible exposure recorded insignificant MFI.

7.3.2.2.1 Henipaviruses

The HeV sG protein was coupled for the study locally as well as being provided by collaborators at USU. Therefore, differences in the MFI obtained for some proteins of the same type coupled in different laboratories reflected the amount of protein coupled to the beads and was accounted for by parallel separate analyses and standardising to consolidate to one (Table 7.8 Eg. HeV RBP Bead 28 & 43 ave).

Preliminary analysis identified numerous sera from horses understood to be unvaccinated showing significant reactivity to the HeV RBP (Table 7.8). Of the 73 horses from the main study cohorts (406 subjects) that had detectable antibodies against the HeV RBP by Luminex microsphere immunoassay, 14 (19%) were verifiable as unvaccinated (from available records). Note antibody cross-reactivity is expected between the HeV and NiV sG proteins due to high RBP phenotypical consistency between these viruses (≈ 80% shared amino-acid sequence).

Table 7.8. Vaccination status of the sera in the Luminex assay most highly reactive with Hendra virus and Nipah Virus sG proteins targeting IgG antibodies.

Values expressed as MFI and colour coded: red; orange; green and black as: highest (>10,000); high (5,000-10,000); moderate (1,000-5000); and low (<1000) MFI respectively

Case ID	HeV RBP IgG Bead 28	HeV RBP IgG Bead 43	HeV RBP Bead 28 & 43 ave	NiV RBP IgG Bead 46	Vaccination status	Years since current HeV Vaccine status
p16-0099X	16452	3724	10609	2383	unknown	N/A
p18-0456X	18613	5085	12461	7052.5	unknown	N/A
p18-0460X	17882.5	6839	12898	10319	unknown	N/A
p18-0461X	6815.5	1435	4163	2494	unknown	N/A
p18-0495X	15838	5594	10716	6259	unknown	N/A
p18-0468X	14524	4253	9388	5964	unknown	N/A
p17-1393X	7565	1820	4692	1995	unknown	N/A
p17-1432X	13763.5	3913	8838	5992	unknown	N/A
p17-1459X	13076	3596	8336	3540.5	unknown	N/A
p17-1459X	25588.5	13230.5	19410	13926	unknown	N/A
p17-1666X	4907	1056	2982	1031	unknown	N/A
p17-1550X	17903	4631	11267	7711	unknown	N/A
p17-1543X	24734.5	11963	18349	13918	unknown	N/A
p17-1440X	16719	6976.5	11848	8674.5	unknown	N/A
p17-1372X	4439	601	2520	464	unknown	N/A
p17-1491X	13898	4405	9152	1608	unknown	N/A
p16-0103X	14925.5	1345	9005	2090.5	unvaccinated	N/A
p16-0186X	24798.5	12572	19437	13339	unvaccinated	N/A
p18-0507X	22612	8914	16555	12408	unvaccinated	N/A
p18-0466X	14786	3985	9386	3659	unvaccinated	N/A
p17-1585X	5192.5	853	3023	832	unvaccinated	N/A
p17-1406X	8907	5511	7209	8429	unvaccinated	N/A
p17-1406X	6160	3557	4858	5651	unvaccinated	N/A
p17-1430X	26725	16842	21784	18496	unvaccinated	N/A
p17-1454X	13620	2794	8207	4453	unvaccinated	N/A
p17-14X40	10080	1628	5854	2452	unvaccinated	N/A
p17-1576X	4898.5	845	2872	1781	unvaccinated	N/A
p17-1570X	17555	5931.5	11743	5051	unvaccinated	N/A
p17-1480X	16710	4275.5	10493	3240	unvaccinated	N/A
p17-1663X	11758	2926	7342	5177.5	unvaccinated	N/A
p17-1495X	10761	7861.5	9311	9368	unknown-suspected-vaccinated	N/A
p17-1574X	12465	2896	7680	4469	unknown-suspected-vaccinated	N/A
p17-1574X	12310	3459	7884	2160	unknown-suspected-vaccinated	N/A
BMXXXXX	9204	4912	7058	6468	unvaccinated-foal_passive-transfer-immunity-via-vaccinated-dam	N/A
p17-1601X	25581	11431	18506	14222	unknown-suspected-vaccinated (vaccinated-not-specified-HeV)	N/A
p17-1418X	14625	3731	9178	6437	vaccinated-unknown-currency	0
p17-141X5	18404	6847	12626	8227	vaccinated-unknown-currency	0
p17-14X85	23622	14539	19080	14035	vaccinated-unknown-currency	0
p18-0027X	1048.5	407	728	405	partially-vaccinated-not-current	3
p17-1406X	6422	4890	5656	2238.5	partially-vaccinated	0
p16-0134X	15485	3086.5	9714	3770	vaccinated-not-current	2
p18-0461X	20995.5	7111.5	14603	7801.5	vaccinated-not-current	1
p18-0484X	25036	13868	20185	14281	vaccinated-not-current	2
p18-0495X	14982	3940	9461	4136	vaccinated-not-current	2
p18-0488X	2544.5	539	1542	378	vaccinated-not-current	1
p17-1336X	1244	1021	1132	1136	vaccinated-not-current	3
p17-1453X	20743	8792	14768	10379	vaccinated-not-current	NA
p17-14X39	18674	7518	13096	9127.5	vaccinated-not-current	NA
p17-1570X	11421	2796	7108	3908	vaccinated-not-current	0.5
p17-1483X	15059	2703	8881	4272.5	vaccinated-not-current	NA
p17-1550X	21430	9117	15274	9948	vaccinated-not-current	1
p18-0026X	24045	11358	17702	13452.5	vaccinated-not-current	0.2
p17-1343X	14734	5561.5	10148	7221	vaccinated-not-current	2
p18-0496X	15977	4634	10306	5705.5	vaccinated-not-current	NA
p17-1769X	17867	6562	12214	9378.5	vaccinated-current	0
BM2020-5-XX	13967.5	12579.5	13274	11999.5	vaccinated-current	0
BM2020-5-XXX	17160	11435	14298	11296	vaccinated-current	0
p17-1464X	11515.5	7366.5	9441	9869	vaccinated-current	0
p16-0154X	25378.5	10612.5	19261	14072.5	vaccinated-current	0
p16-0200X	17070	5113.5	11569	8190	vaccinated-current	0
p18-0449X	18232	5051	12101	6744	vaccinated-current	0
p18-0510X	7176	866	4021	622	vaccinated-current	0
p17-1400X	19496	6839	13168	10084	vaccinated-current	0
16-0330X	12875.5	9073	10974	12158.5	vaccinated-current	0
p17-149X6	12061	9352	10706	7071.5	vaccinated-current	0
p17-1729X	15285	13194.5	14240	14192	vaccinated-current	0
p17-172X1	13391	10769	12080	11892.5	vaccinated-current	0
p17-1571X	23818	16548.5	20183	17247	vaccinated-current	0
p17-1485X	18261	7861	13061	9264.5	vaccinated-current	0
p17-148X0	13120	2469.5	7795	6088	vaccinated-current	0
p17-14X50	23880	13891	18886	13717.5	vaccinated-current	0
p17-1X502	22211	9238.5	15725	9366	vaccinated-current	0

HeV nucleocapsid (N) protein can be utilised to confirm natural infections as reactions with only the N protein would indicate a natural infection. Preliminary results using a yeast expressed HeV N are shown in Table 7.9. The HeV N was provided by collaborators at Vilnius University in Lithuania to investigate the reactors with the sG protein. Please note that due to time constraints this work is preliminary, and the assay did not undergo any optimisation. Ten unvaccinated and 10 vaccinated horse sera were used as controls. Average MFI value for the negative and vaccinated for the HeV N protein was 196. 'Tamworth', a naturally infected horse, was used as a positive control (tested in duplicate) and a serum sample from a naturally infected person was also used to gauge reactivity. There was one serum sample that showed similar reactivity as the positive controls with reaction to the HeV N at an MFI of 2,589 (Table 7.9), however, another sample (C12) showed a reaction with an MFI of 894. This was over four times the MFI value of the known unvaccinated and vaccinated cohort, which may constitute a low positive/indeterminate sample.

Further work with optimisation with the N protein is required. The N protein in conjunction with the HeV F protein when available could be utilised in a 'differentiating infected from vaccinated animals' (DIVA) assay to determine natural infections with HeV.

Table 7.9. Luminex assay with Hendra virus nucleocapsid and glycoprotein proteins to determine natural infection

Subject/ Sample Description	HeV NP	HeV RBP (G)
Tamworth HeV Pos horse sera	2564	14858
Tamworth HeV Pos horse sera (repeat)	2480.5	14829
Human HeV infected sera	1527	8118
Horse subject Sera p17_1430X	2589	24205.5
Horse subject Sera p17_1491X	894	6107
Blank	135	66
Vaccinated horse	146.5	17574
Unvaccinated horse sera control (known seronegative)	138	75

7.3.2.3 Sub cohort summaries

Full MMIA panel results summaries were generated for each sample event context cohort to provide an estimation of suitable cut-offs and test performance, for cases that had duplicate sera sample test results (three horses representing six serum samples). Only the earlier sample event sample was included when analysing IgG, and the later sample event

exclusively for IgM. Conservative prior positive test classifications were determined based on assay optimisation results including with reference to experimental use of other species (humans and rabbits). In the pilot studies, a cohort putatively lacking flying fox exposure were used to inform the MFI distributions. Non-diseased horses from southern regions in Western Australia that would be expected to have a lower exposure to bat-borne agents were used (exposed only to the LRFF, and likely less frequently and/or in association with lower flying fox population density). Proportions positive by binary test outcome classification are only reported for cohorts with larger subject numbers (more than 15 horse subjects).

7.3.2.3.1. Infectious-like disease cohorts

7.3.2.3.1.1. Overview of serology screening of Qld HeV-Like Disease 2015 – 2018 sub cohort

Sample type, infectious disease likelihood category, vaccination status for 306 unique horse-subject sample events from Qld HeV-like disease cohort are described in Table 7.10

Tables 7.11 – 7.16 present summary of binary test outcome and positive test outcome proportions for individual and logically combined antigens both in IgG and IgM for this key Qld HeV-like disease sub cohort.

7.3.2.3.1.1.1. Overview of Pararubulavirus antigen results for HeV-Like Disease Qld 2015 – 2018 sub cohort

Positive sub cohort proportion (PSCP) for IgG targeting MenPV NP (14%) was higher for the Qld. HeV-like disease sub-cohort (Table 7.11) than for that targeting TioPV NP (4%) which is expected given MenPV is endemic in Australian flying foxes while TioPV is a Pararubulavirus detected internationally (while closely related to MenPV and numerous Pararubulaviruses identified in Australia). Combined PSCP was higher for IgG targeting Pararubulaviruses receptor binding proteins RBPs (16%) than nucleoproteins (NPs) (11%) as expected given higher target agent immunological specificity. These seroprevalences of IgG targeting either Pararubulavirus RBPs was also greater than amongst sera from the sub-cohort drawn WA (Table 7.49) (49/257 or 16% compared with 3/38 or 7%) considered of geographically lower likelihood of flying fox exposure. As part of the pilot study sero-reactors from this cohort of relatively lower likelihood of flying fox exposure were investigated further with many having spent time in regions of higher perceived flying fox exposure such as coastal NSW before residing in WA, where the samples included in the study were obtained. Such extensive was

unfortunately unavailable for many individual horse subjects. The relatively high IgG sero-reactivity to the Pararubulavirus NPs (Table 7.49) amongst the WA cohort (12/29 or 29%) is difficult to interpret but may relate to cross-reactivity with antibodies targeting Paramyxoviruses circulating amongst horses without requiring Flying fox exposure (NPs are more widely conserved between greatly divergent but related viruses), or to exposure to bat-borne Paramyxoviruses in WA relating to the distribution of the little red flying fox in which infection with HeV-g2 has been determined similarly as for Grey-headed and Black flying foxes.(35) The high sensitivity of the assay when using the k95 cut-off for 95% sensitivity as a screening research tool is also expected to have influenced this finding and interpretation should note that the maximum MFI readings for MenPV NP IgG were considerably lower in the WA cohort (Table 7.49) than in the Qld HeV-like disease cohort (Table 7.11) – specifically 2307 compared with 6279 respectively. This could be consistent with lower or less recent Flying fox borne Pararubulavirus exposure amongst this cohort.

7.3.2.3.1.1.2. Overview of Henipavirus antigen results for HeV-Like Disease Qld 2015 – 2018 sub cohort

Positive sub cohort proportion (PSCP) for IgG targeting HeV or NiV RBPs (Gs) (28%) was higher for the Qld. HeV-like disease sub-cohort (Table 7.12) than for RBP of divergent Henipaviruses (MojV and GhV) (18%) which is expected given the equivalent IgG against this protein elicited from target viral infection and HeV immunisation with the HeV vaccine based on the same recombinant G glycoprotein. Seropositivity for either prototypic Henipavirus RBP (HeV or NiV) was 11% (33) amongst unvaccinated horses (303) in the HeV-like disease cohort (Table 7.12), considerably higher than amongst horses in the comparative WA cohort (7/45 or 3%) (Table 7.50), consistent with higher likelihood of immunity elicited to natural virus exposure (mostly prior to the presenting disease condition for which the case was sampled given the delay in IgG response in acute infection without prior exposure) (Figure 7.23).

Table 7.10. Infectious-like disease horse sub-cohort summary: HeV-Like Disease Qld 2015 - 2018

HeV_vaccine_status	Unvaccinated: 254 |unknown: 17 |vaccinated not current: 15|vaccinated current: 12 |partially vaccinated: 4 |partially vaccinated not current: 2 |unknown suspect vaccinated: 2					
Sample type	Serum					
Years since vaccinated	NA: 279	<1: 15	1: 3	2: 5	3: 3	4: 1
Sample reason	Hendra-like disease: 306					
State	Qld : 306					
infect_prior	1: 144 | 2: 161 | 5: 1					
plating_unique_v_duplicate	paired_sampling_second: 0		paired_sampling_primary: 0		single sample event: 306	

Table 7.11

HeV-like disease Qld 2015−2018, Pararubulavirus antigens

	Binary test classification based on K95* Bayesian analyses		Proportion positive (%)	Antigen coupled bead No./s	Sub-cohort MFI distribution					
	Negative	Positive			Min	1st quartile	Median	Mean	3rd quartile	Maximum
TioPV NP IgG	295	11	4	30	69	106	135	197	203	2742
TioPV NP IgM	270	32	11	30	63	362	578	783	886	9239
MenPV NP IgG	264	42	14	62 or 48	26	142	235	471	525	6479
MenPV NP IgM	285	17	6	63 or 48	52	582	1007	1281	1570	6610
Pararubulavirus NPs IgG combined	257	49	16							
Pararubulavirus NPs IgM combined	263	39	13							
MenPV sHN (RBP) IgG	300	6	2	28	109	134	151	179	169	2544
MenPV sHN (RBP) IgM	278	27	9	28	127	2147	3464	4019	5214	18388
	279	26	9	*prior7761 | **prior11462						
YepPV sHN (RBP) IgG	304	2	1	13	13	17	19	44	24	3530
YepPV sHN (RBP) IgM	279	26	9	13	13	126	177	217	263	1804
GrovPV sHN (RBP) IgG	275	31	10	18	27	38	48	104	70	2506
GrovPV sHN (RBP) IgM	262	43	14	18	31	221	333	584	520	20769
Pararubulavirus sHNs (RBPs) IgG combined	271	35	11							
Pararubulavirus sHNs (RBPs) IgM combined	242	63	21							
Pararubulavirus NPs & sHNs (RBPs) IgG combined	294	12	4							
Pararubulavirus NPs & sHNs (RBPs) IgM combined	283	22	7							
Pararubulavirus NPs & sHNs (RBPs) IgM combined & −ve IgG	284	21	7							

Table 7.12

HeV-like disease Qld 2015–2018, *Henipavirus* antigens

	Binary test classification based on K95* Bayesian analyses		Proportion positive (%)	Antigen coupled bead No./s	Sub-cohort MFI distribution					
	Negative	Positive			Min	1st quartile	Median	Mean	3rd quartile	Maximum
HeV RBP IgG	225	81	26	28 & 43 ave.	66	99	112.0	2098	303	21784
NiV RBP IgG	234	72	24	46	122	179	216	1471	293	18496
NiV & HeV IgG combined	221	85	28							
unvaccinated NiV & HeV IgG combined	273	33	11							
HeV RBP IgM	269	36	12	43	71	210	293	460	438	18402
NiV RBP IgM	268	37	12	46	222	620	898	1059	1245	16240
NiV & HeV IgM combined	257	48	16							
NiV & HeV IgM combined & −ve IgG	272	33	11							
unvaccinated NiV & HeV IgM combined	258	47	15							
CedPV RBP IgG	287	19	6	53	106	159	186	202	211	1534
CedPV RBP IgM	291	14	5	53	126	1027	1634	2179	2818	18585
NiV & HeV & CedPV IgG combined	218	88	29							
unvaccinated NiV & HeV & CedPV IgG combined	218	88	29							
NiV & HeV pos but CedPV neg IgG	282	24	8							
MojV RBP IgG	277	29	9	29	90	145	175	236	224	2644
MojV RBP IgM	296	9	3	29	92	694	1074	1429	1720	20378
GhV RBP IgG	264	42	14	35	73	105	129	248	191	5499
GhV RBP IgM	270	35	11	35	130	478	693	1007	1007	28279
MojV & GhV RBP IgG combined	250	56	18							
MojV & GhV RBP IgG combined unvaccinated	257	49	16							
Non-prototypic HNVs IgG combined	246	60	20							
Non-prototypic HNVs IgG combined unvaccinated	247	59	19							
Non-prototypic HNVs IgM combined	263	42	14							
Non-prototypic HNVs IgM combined unvaccinated	271	34	11							
Non-prototypic HNVs IgM combined & −ve IgG	268	37	12							
NiV & HeV IgG combined but negative for Non-prototypic HNVs & unvaccinated	284	22	7							
NiV & HeV IgG combined but negative for MojV & GhV & unvaccinated	284	22	7							

Table 7.13. HeV-like disease Qld 2015 - 2018, SARS-like CoV antigens

	Binary classification Prior to Bayesian estimation		Proportion positive (%)	Antigen coupled bead No./s	Sub-cohort MFI distribution						Conservative MFI cut-off applied for Binary classification
	Negative	Positive			Min	1st quartile	Median	Mean	3rd quartile	Maximum	
MERS CoV IgG	298	8	3	15	12	16	20	129	47	4620	1000
MERS CoV IgM	285	20	7	15	12	178	275	456	499	8556	1196
SARS CoV-1 NP IgG	301	5	2	39	12	15	17	98	24	2626	1000
SARS CoV-1 NP IgM	305	0	0	39	13	85	129	146	181	770	1000
SARS CoV-1 NP IgM	302	3	1	39							500
SARS CoV-1 NP IgM	294	9	3	65	68	1308	1749	2354	2716	17708	6876
SARS CoV-2 NP IgG	283	23	8	42	40	54	60	250	82	5472	1000
SARS CoV-2 NP IgM	303	2	1	42	43	293	374	409	493	1321	1217
SARS CoV-2 NP IgM	293	12	4	42							811
SARS CoV-2 NP IgG	288 / 71	18 / 5	6 / 7	77	103	180	232	370	339	2242	2000
	217	13	6	77	92	183	284	645	549	12500	9867
SARS CoV-2 NP IgM	292	11	4	77	71	1714	2440	3331	3988	16544	9867
rs4874 IgG	301	5	2	54	68	87	94	129	102	1958	1000
rs4874 IgM	299	6	2	54	151	496	674	795	952	2834	2372

Table 7.14. HeV-like disease Qld 2015 - 2018, *Filovirus* antigens

	Binary classification Prior to Bayesian estimation		Proportion positive (%)	Antigen coupled bead No./s	Sub-cohort MFI distribution						Conservative MFI cut-off applied for Binary classification
	Negative	Positive			Min	1st quartile	Median	Mean	3rd quartile	Maximum	
Zaire ebolavirus EBOV NP IgG	290	16	5	72	80	122	182	370	377	4594	1323
Zaire ebolavirus EBOV NP IgM	294	9	3	72	76	639	932	1296	1411	16969	3861
Zaire ebolavirus EBOV EGP IgG	289	17	6	34	41	192	219	573	277	20171	1323
Zaire ebolavirus EBOV EGP IgM	294	11	4	34	87	611	1003	1326	1596	10352	3974
Bundibugyo ebolavirus BDBV EGP IgG	292	14	5	64	56	93	138	372	260	7531	1323
Bundibugyo ebolavirus BDBV EGP IgM	290	15	5	64	76	551	833	1186	1374	13101	3285
Bombali ebolavirus BOMV EGP IgG	298	8	3	55	116	156	178	267	200	7206	1000
Bombali ebolavirus BOMV EGP IgM	304	1	0	55	247	452	586	617	730	2109	1844
Reston ebolavirus RESTV EGP IgG	298	8	3	72	21.0	26	30	122	44	6652	1000
Reston ebolavirus RESTV EGP IgM	297	8	3	72	33	1240	2239	2785	3819	13670	8741
Marburg Marburgvirus MARV EGP IgG	301	5	2	37	34	44.0	50.0	100	73	3031	1000
Marburg Marburgvirus MARV EGP IgM	300	5	2	37	39	332	506	566	691	2583	1802
Marburg Marburgvirus RAVV EGP IgG	304	2	1	62	30	48	63	123	102	4262	1000
Marburg Marburgvirus RAVV EGP IgM	292	13	4	62	32	214	335	426	461	3150	1304
Mengla dianlovirus MLAV EGP IgG	304	2	1	22	43	56	61	82	66	1236	1000
Mengla dianlovirus MLAV EGP IgM	304	1	0	22	98	265	382	420	515	1781	1268
Lloviu cuevavirus LLOV EGP IgG	299	7	2	66	121	211.2	241	293	261	4534	1000
Lloviu cuevavirus LLOV EGP IgM	301	4	1	66	278	651	902	1018	1160	6040	3086

Table 7.15

HeV-like disease Qld 2015 - 2018, Borna Disease virus & Hepatitis E virus antigens

	Binary classification Prior to Bayesian estimation		Proportion positive (%)	Antigen coupled bead No./s	Sub-cohort MFI distribution						Conservative MFI cut-off applied for Binary classification
	Negative	Positive			Min	1st quartile	Median	Mean	3rd quartile	Maximum	
Borna disease virus BoDV EGP IgG	275	31	10	48	71	198	458	1008	1068	11966	2573
Borna disease virus BoDV EGP IgM	298	3	1	73	57	3660	4918	5662	7061	19718	17655
Borna disease virus BoDV EGP IgM	294	7	2								14713
Borna disease virus BoDV EGP IgM	287	14	5								11770
Orthohepevirus A CP HEV IgG	283	23	8	30	73	181	345	1066	742	20793	2480
Orthohepevirus A CP HEV IgM	283	18	6	64	63	1210	2042	3523	4255	26606	10456
Orthohepevirus A CP HEV IgM	202	99	33								3306
Orthohepevirus A CP HEV IgM	178	123	41								2480

Table 7.16

HeV-like disease Qld 2015 - 2018, Arbovirus and other antigens

	Binary classification Prior to Bayesian estimation		Proportion positive (%)	Antigen coupled bead No./s	Sub-cohort MFI distribution						Conservative MFI cut-off applied for Binary classification
	Negative	Positive			Min	1st quartile	Median	Mean	3rd quartile	Maximum	
West Nile virus WNV E IgG	303	3	1	38	80	99	117	192	156	7237	k95 by Bayesian estimation
West Nile virus WNV E IgM	292	6	2	38	79	743	1039	1239	1412	8860	3680
Chikungunya virus CHIKV E1 IgG	239	67	22	48	77	237	815	1934	2402	17294	3000
Chikungunya virus CHIKV E1 IgM	288	13	4	48	60	822	1392	1733	2112	8556	4977
Sindbis virus SINV E1 IgG	266	40	13	55	75	171	344	901	768	10359	2000
California encephalitis virus (La Crosse) CEV NP IgG	251	9	3	72	76	94	107	234	134	4870	1000
California encephalitis virus (La Crosse) CEV NP IgM	291	10	3	76	72	1343	1871	2350	2779	12408	6563
Retrovirus SC110 Retro E IgG	304	2	1	38	67	83	87	115	92	3704	1000
Retrovirus SC110 Retro E IgM	164	1	1	38	32	246	333	372	445	1485	1187
Hervey pteropid gammaretrovirus HPG E IgG	304	2	1	45	64	84	88	103	94	2257	1000
Hervey pteropid gammaretrovirus HPG E IgM	300	0	0	45	36	194	262	288	367	834	1000
Hervey pteropid gammaretrovirus HPG E IgM	300	0	0								845
Brucella suis RepA IgG	272	34	11	26	84	141	233	982	624	20655	2000
Brucella suis RepA IgM	293	8	3	26	57	1638	2600	3415	4272	18324	9900
Brucella abortus OMP IgG	284	22	7	28 or 55	73	121	165	500	367	12742	1323
Brucella abortus OMP IgM	287	14	5	55	64	1454	2409	3306	4319	24034	8339
Leptospira australis (whole organism) IgG	302	4	1	26	54	73	78	154	84	10074	1000
Leptospira pomona (whole organism) IgG	302	4	1	77	50	70	75	150	82	9885	1000

7.3.2.3.1.2 Brief description of most-significant preliminary molecular findings from Qld HeV-like disease sample cohort (2015 – 2018)

7.3.2.3.1.2.1 Novel paramyxoviruses

Several novel paramyxoviruses were identified from Qld HeV-like disease cohort samples by RNAseq. This includes the novel variant of Hendra virus (Chapter 8) and canine morbilliviruses (Section 7.3.3.1.2.2). Initial analyses of the RNAseq data uncovered an additional two potentially important paramyxoviruses (Figure 7.15). One is a novel orthorubulavirus from a horse with fever and respiratory infection (Case p17_14213), as well as another highly divergent virus present in multiple cases with respiratory disease (p17_14401 & p17_15710). The genetic divergent of both makes assessments of their origins and pathogenicity difficult; however, both are potentially zoonotic. That said parallel research investigating flying fox urine samples has identified equivalent viruses, highlighting likelihood of flying fox origin. Further work is ongoing to characterise these viruses.

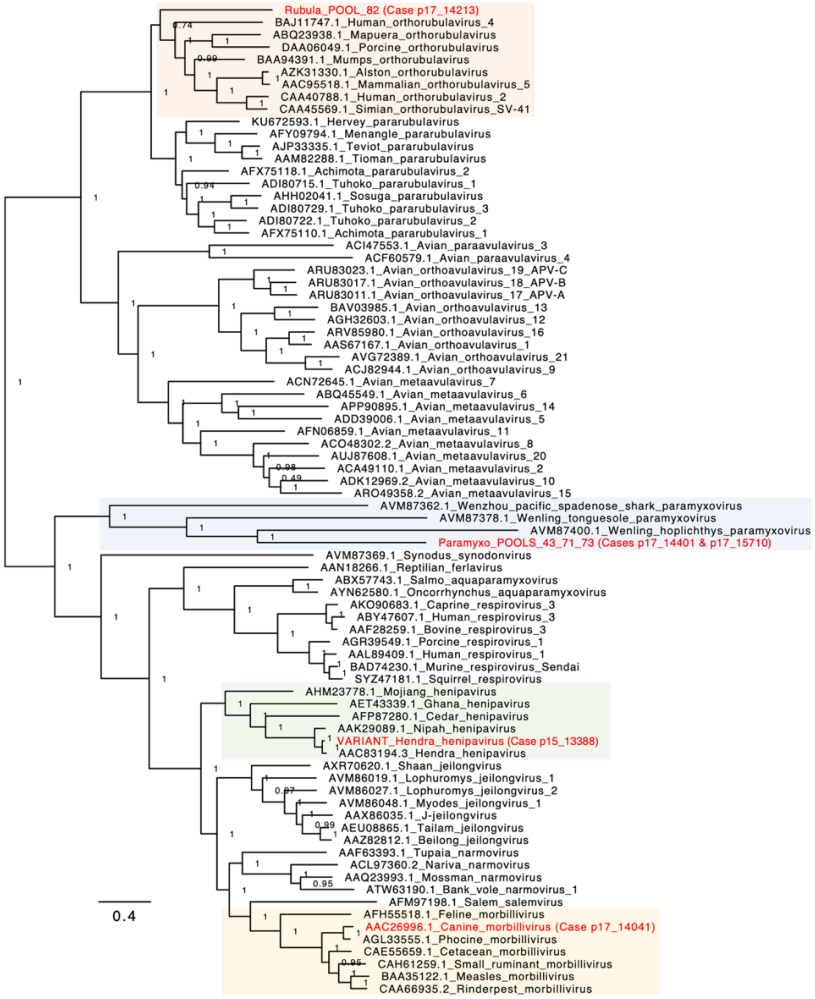

Figure 7.15. Maximum-likelihood phylogeny of paramyxoviruses using L protein sequences.
Phylogeny generated with PhyML using the GTR model. Branch labels represent SH-like supports. Tip labels in red indicate strains identified in this study. The colors are: Orange = orthorubulaviruses; Blue = highly divergent, uncharacterised PMX; Green = Henipaviruses & Yellow = Morbilliviruses. Scale is proportional to substitutions per site.

7.3.2.3.1.2.2 Canine morbilliviruses

Two distinct canine morbilliviruses were identified (libraries BIP006 and POOL76), from rectal and nasal sample pools respectively. Figure 7.16 shows a phylogeny of the relationship of these viruses to reference strains. BIP006 is phylogenetically clustered with known vaccine strains and is likely derived from transmission of a vaccine escape mutant from a vaccinated dog, or perhaps vaccination of the horse itself. Interestingly, the POOL76 virus is appears to be a novel genotype and is unlikely to be vaccine derived since those strains are well described. This suggests that a natural and diverse lineage of canine morbillivirus is present in the Australian population. The source of the infection and possibility of it being zoonotic is yet to be determined.

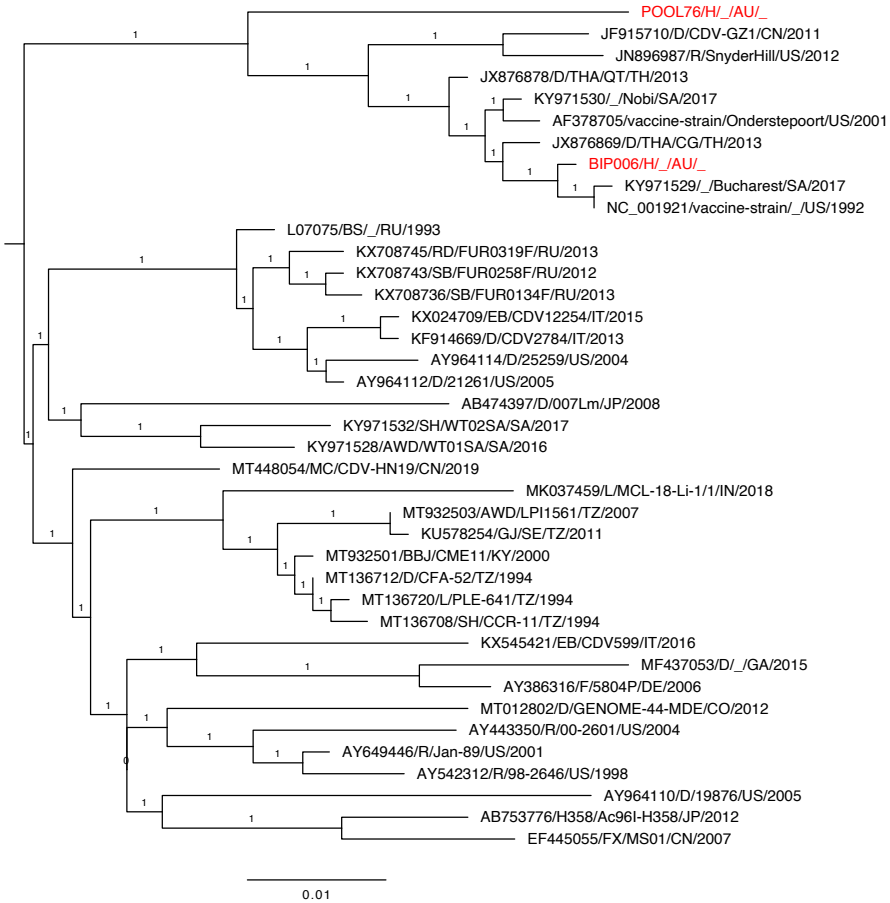

Figure 7.16. Maximum-likelihood phylogeny of canine morbillivirus partial fusion protein CDS based on a 775 bp nucleotide alignment. Phylogeny generated with PhyML using the GTR model. Branch labels represent SH-like supports. Tip labels in red indicate sequences identified in equine nasal-oral samples. The vaccine strains are included and indicated by tip labels. Tip labels show GenBank accession ID/host abbreviation/isolate/country/year.

Table 7.17 matches the most-significant molecular Paramyxovirus findings that had been confirmed by separate PCR detection to identify positive individual case/s (following pooled detections) from the Qld HeV-like disease cohort with their paramyxoviral serology results.

Table 7.17. Most-significant Paramyxovirus molecular findings from Qld HeV-like disease cohort identified to individual disease-case events

Case ID	Significant molecular agents detected	Interpretation relative to known significant pathogens	Disease description	Initial detection method	Paramyxovirus Serology	Signalment	Affected property region/ Month/ Year	Field epidemiology submission form comments details
p15-133XX	Novel Hendra virus variant (HeVg2) as per published(2)	Determined to be genomically divergent and distinct second lineage yet sufficiently conserved phenotypically so as to be of equivalent pathogenicity and immunogenicity (prototypic) as previously known HeV	depressed (obtunded) demeanor, injected/ congested gingival mucous membranes (darkened red/ purple change with darker periapical line and prolonged capillary refill time), tachycardia (75 beats/min), tachypnoea (60 breaths/min), normal rectal temperature (38.0°C), muscle fasciculations, head pressing and collapse. The moribund condition justified euthanased on humane grounds.	Adapted nested conventional pan-paramyxovirus RT-PCR coupled with RNA seq metatranscriptomics. Deep RNA seq Metatranscriptomics afforded further genome.	Seronegative (-ve) for all in both IgG and IgM as per published description(2) see Chapter 8	12-year-old gelding	South-east QLD - sunshine coast hinterland/ 2015	Homebred horse - always resided on property. Attending veterinarian perceived high flying fox colony numbers and potential exposure. A near-by roost was known to host BFFs, GHFFs and little red flying-fox species with numbers varying seasonally and annually.
p17-142XX	Novel Rubula-like Paramyxovirus	Menangle virus spillover to pigs causing severe reproductive loss and influenza-like illness in in-contact humans. Horses as sentinels detection of seroconversion (IgG) and IgM positivity to bat-borne Rubulavirus in outbreak of severe respiratory equine disease cases near Sydney (1 death two with HeV like illness but recover) – See Section 7.3.1.1	Presented in morning following collapse (lay down in stocks while was being tacked up for ridden exercise). Trainer recorded pyrexia (39.5C), pale mucous membranes and tachycardia (58bpm) at time. Examination by attending veterinarian revealed rectal temperature 38.4 (high normal range), mildly elevated heart rate (44 bpm) and slightly injected mucous membranes. Persistent recumbency while observed over 45 minutes (lateral recumbency with intermittent sternal recumbency and some flank watching). Stood and walked to stable after non-steroidal anti-inflammatory medication (flunixin). Forelimb proprioceptive deficits observed. Combination of signs prompted consideration of HeV as DDx, appropriate sampling and biosecurity management.	Adapted nested conventional pan-paramyxovirus RT-PCR coupled with RNA seq metatranscriptomics	Seropositive (+ve) for MenPV N by IgM (3910) & RBP (9074) only. -ve for all other Paramyxo antigens for both IgG & IgM	2 yo filly	Darling downs, Near Toowoomba/ May/ 2017	
p17-1404XX	Canine Morbillivirus - vaccine strain		Outbreak of acute severe illness featuring neurological signs and lethargy: 3 affected (2 fatal) of 25 at-risk. Case 1 (not sampled) died 3 weeks before sample event – euthanased with profound ataxia and proprioceptive defectis) | Case 2 – died overnight preceding sample event (not seen by owner day prior) (sampled post-mortem); Case 3 (sampled) Paddock mate of Case 2, mild to moderate illness featuring lethargic demeanour.	Adapted nested conventional pan-paramyxovirus RT-PCR coupled with RNA seq metatranscriptomics	Case 2: HeV and NiV IgG low +ve (276 & 775 MFI) & also for MojV (1064), GhV (1192) and CedPV (583) but -ve IgM for all HNVs; -ve for Pararubulavirus IgGs other than GrovPV HN (797) & +ve for IgM to YepPV RBP, MenPV and TioPV NPs Case 3: Low +ve NiV(490), MojV (705), GhV (804) & CedPV (376) and ProvPV RBPs IgG only -ve for all by IgM	unspecified	South-east Qld – Scenic Rim region/ May/ 2018	Case 2 and 3 paddock mates. Case 1 in contact horse euthanased in moribund condition 3 weeks earlier.
p17-144XX	Novel highly divergent Paramyxovirus		Nasal discharge and lethargy. Pleuropneumonia indicated by fluid on thoracic ultrasonography	Deep RNA seq metatranscriptomic NGS as pools confirmed to individual case samples by PCR	HeV and NiV IgG low +ve (405 & 452 MFI) & also for GhV (378) +ve for TioPV N (1813) IgM & -ve for all other Paramyxovirus antigens by IgM	Young adult TB mare	Darling downs, Near Toowoomba/ June/ 2017	athletic injury (tendon) approximately 1 week ago. Returned to property on which was bred to rehabilitate.
p17-157XX			Acute disease (2 days) featuring inappetence, obtunded demeanour, slightly icteric mucous membranes and ophthalmic sclera, Heart rate within normal range (40bpm); mildly increased respiratory rate with increased respiratory effort noted (abdominal respiratory pattern) and marked pyrexia (39.4C)	Deep RNA seq metatranscriptomic NGS as pools confirmed to individual case samples by PCR	HeV and NiV IgG +ve relating to immunisation (20183 & 17247 MFI) low +ve for GhV (678), CedPV (413) and GorvPV (300) RBPs IgG but -ve for all Paramyxovirus antigens by IgM	20-year-old standardbred mare. HeV Vaccinated (current)	South-east QLD - sunshine coast hinterland/ August/ 2017	

7.3.2.3.1.2.3 Novel vertebrate associated viruses

Co-infection was identified across multiple sample types, particularly for novel astroviruses (Figure 7.19), hepatoviruses (Figure 7.17) and caliciviruses (norovirus-like) (Figure 7.18). The multiple detections makes linking specific causes to signs of disease difficult. Of note, this is the same case highlighted previously (BIP 17_1744X), and shows the additional detection of an heptatovirus new from the RNA sequencing and the remarkable diversity of viruses and individual animal may carry.

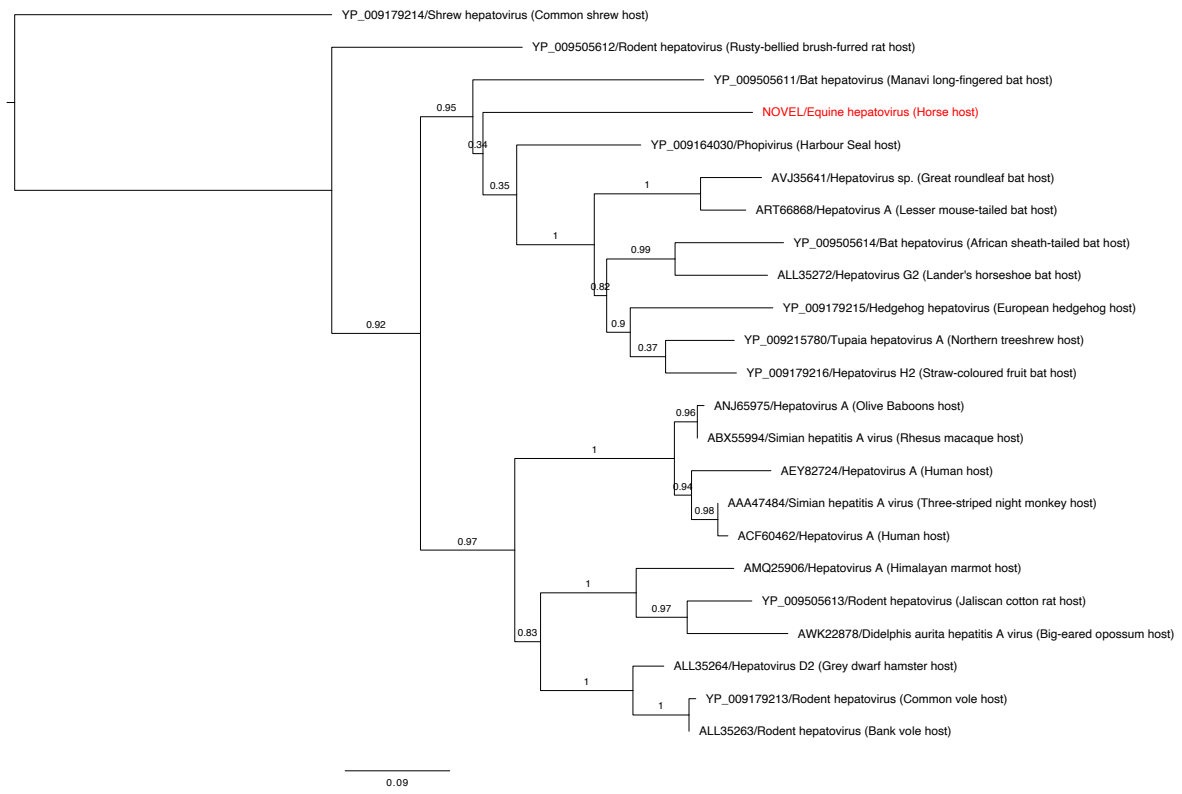

Figure 7.17. Maximum-likelihood phylogeny of novel Hepatovirus.
Phylogeny was generated with PhyML using the GTR model and is based on a 367 amino acid alignment of the capsid protein CDS. Tip labels denote GenPept Accession/Organism (Host organism common name).
Novel virus is indicated in red. Branch labels depict SH-like support values.

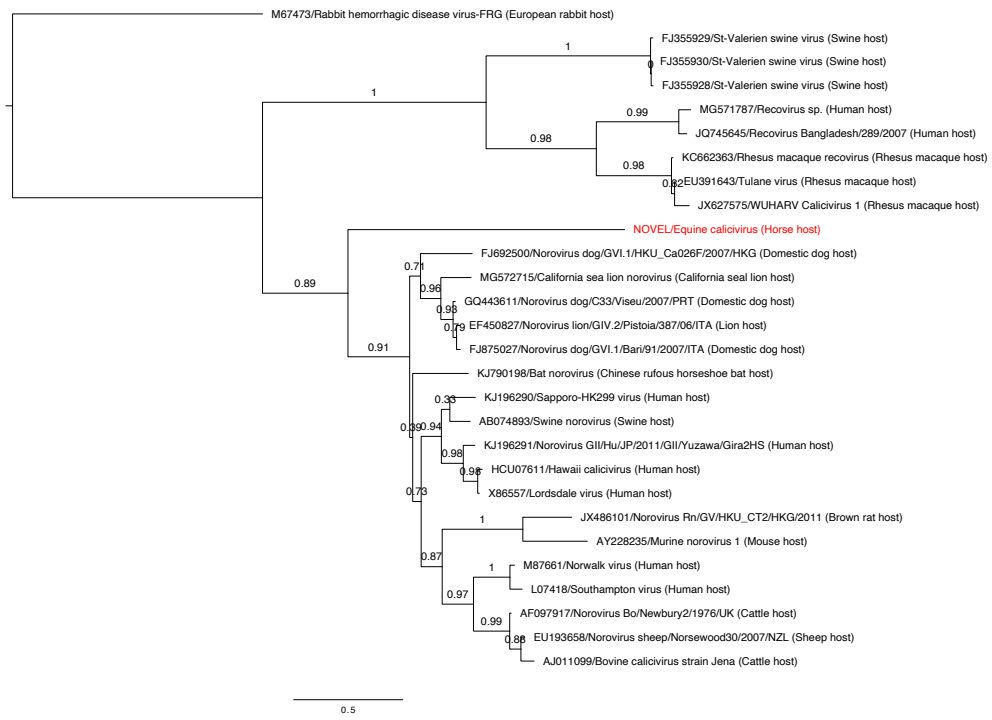

Figure 7.18. Maximum-likelihood phylogenies of novel Noro-like Calicivirus.
Phylogeny is based on a 256 amino acid alignment of the ORF1 CDS and generated using PhyML with a GTR model. Tip labels denote GenPept Accession/Organism (Host organism common name). Novel virus is indicated in red and was identified from a rectal swab sample. Branch labels depict SH-like support values.

Novel astro-like viruses (Figure 7.19) were also detected related to viruses detected in diseased humans, pigs, cattle, rodents and those detected in bats.

Table 7.18 matches the most-significant molecular novel vertebrate-associated virus findings to date from the Qld HeV-like disease cohort that have been confirmed in individual horse-case samples by separate PCR detection following pooled RNA detections.

Initial detection method descriptions are included - varying in order and combination of Pan-viral PCR, Benchtop RNA seq and/or Deep RNA seq (with metatranscriptomics) such as: '*RNA seq metatranscriptomic NGS applied to cDNA as part of Pan-viral family conventional PCR screening - parallel detections in multiple assays - with follow up 'Deep RNA seq'*.

The Novel beta coronavirus molecular finding (table7.18) along with the serology reactivity to CoVs (such as in Table 7.13) suggests that divergent and emerging viruses in this family are also infecting horses and contributing to disease – justifying further similar research to guide proactive One health and biosecurity management.

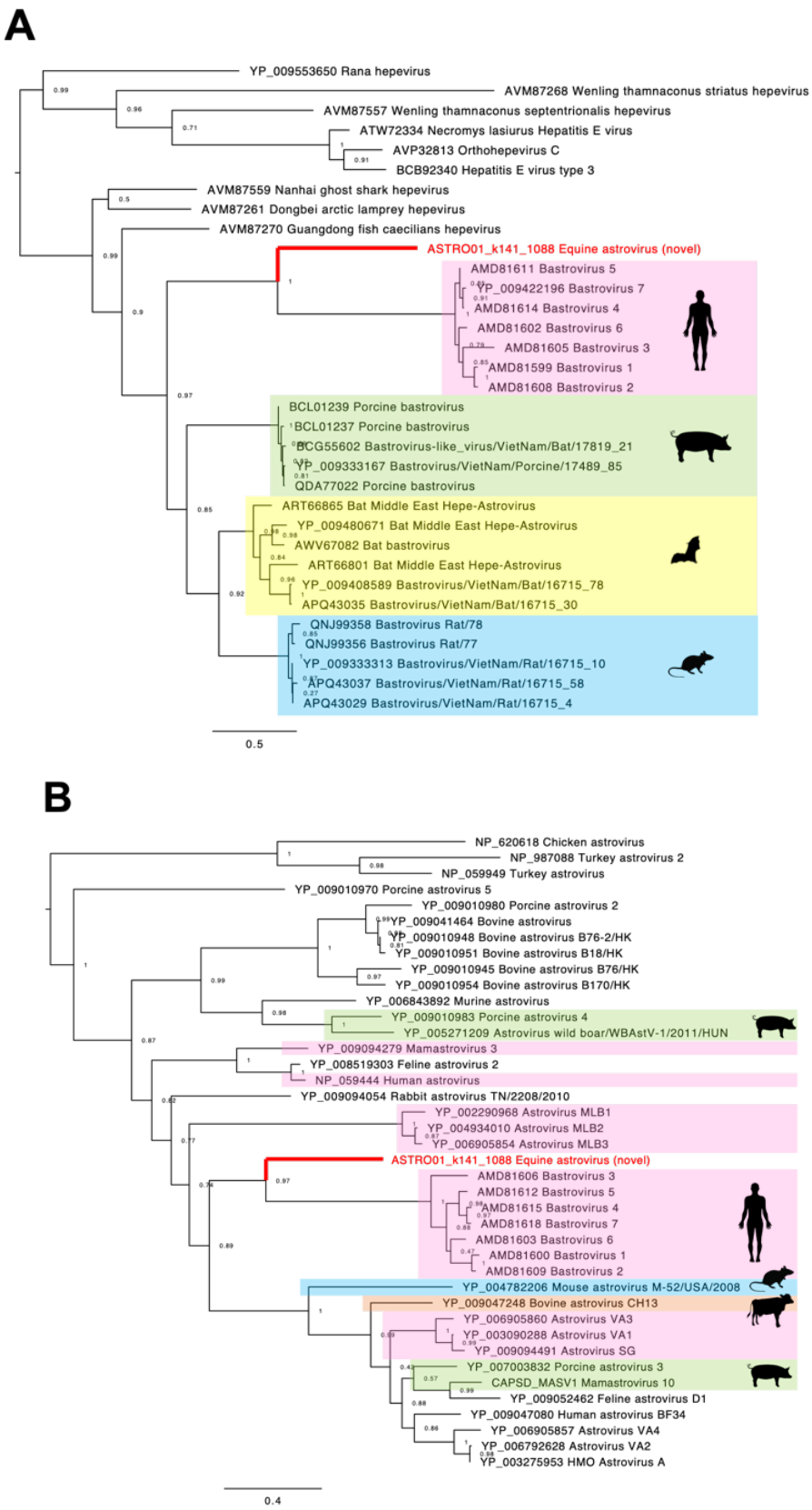

Figure 7.19. Maximum-likelihood phylogenies of novel astro-like virus.
Panel A shows phylogeny based on 269 amino acid alignment of RNA-dependent RNA polymerase CDS. **Panel B** shows phylogeny based on 343 amino acid alignment of capsid CDS. Novel virus is indicated in red. Tip labels denote GenPept Accession ID and organism name. Relevant virus hosts are indicated by shading. Branch labels represent SH-like supports.

Table 7.18. Most-significant molecular novel vertebrate-associated virus findings from Qld HeV-like disease cohort identified to individual disease events

Case ID	Significant molecular agents detected	Interpretation relative to known significant pathogens	Disease description	Initial detection method	affected property region	Signalment	Month/ year	Field epidemiology submission form comments details
p17-174XX	Novel Astrovirus and Novel Norovirus	Noroviruses associated with severe and highly transmissible acute diarrheal disease in humans and other species.	inappetent/ anorexic; pyrexic and diarrhoea (indicative of colitis)	RNA seq metatranscriptomic NGS applied to cDNA as part of Pan-viral family conventional PCR screening - parallel detections in multiple assays. Deep RNA seq Metatranscriptomics afforded further genome.	Darling downs	16-month-old TB colt	February/ 2017	
p18-043XX	Novel beta coronavirus	98% similar to Embecoviruses (betacoronaviruses - previously group 2a coronavirus) detected in bats, camels and bovine.	Dull and lethargic (obtunded demeanour); pyrexic (39.1C); hyperpnoea and gums blueish (congested and cyanotic mucous membranes)	Pan-coronavirus RT-PCR screening paired with RNA sequencing	Central QLD - Near Roma	6-month-old QH filly	August/ 2018	Affected horse recently introduced to property kept on a river paddock with flying foxes present

7.3.2.3.1.2.4 Novel nidovirus

Numerous respiratory pools were found to be infected by a novel nidovirus that was genetically similar (although still divergent >50% protein difference) to a bovine nidovirus causes severe repository disease in US cattle (Figure 7.20). The identification of this virus in multiple libraries and samples suggests relative high abundance, and we are currently working to identify specific cases to see the clinical disease in the relevant cases. We believe this has high pathogenetic potential.

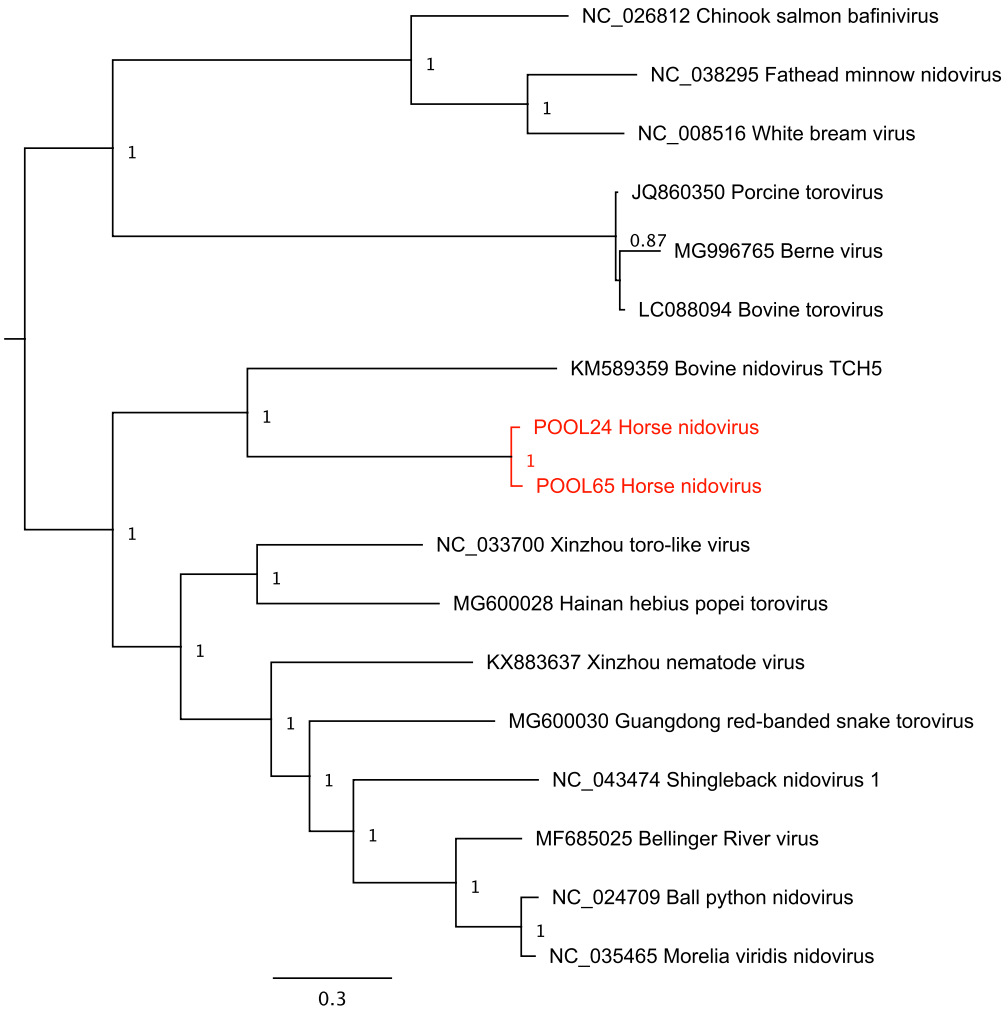

Figure 7.20. Maximum-likelihood phylogeny of nidoviruses using L protein sequences.
Phylogeny generated with PhyML using the GTR model. Branch labels represent SH-like supports. Tip labels in red indicate strains identified in this study.

Table 7.19 matches the most-significant molecular novel nidovirus findings to date from the Qld HeV-like disease cohort that have been confirmed by separate PCR detection from samples from individual horse sample events.

Table 7.19. Most-significant molecular novel Nidovirus findings from Qld HeV-like disease cohort identified to individual disease events

Case ID	Significant molecular agents detected	Interpretation relative to known significant pathogens	Disease description	Initial detection method	affected property region	Signalment	Month/ year	Field epidemiology submission form comments details
p17-160XX	Novel Nidovirus 1	A novel torovirus-like virus of the Nidovirales order. Nidoviruses infect broad host species and ranges, are often highly pathogenic causing very severe respiratory, neurological and disease and mortality outbreaks. This novel virus is very widespread amongst our prioritised disease cases with detections in 19/34 respiratory libraries (nasal/oral swabs). Closest relative is another Nidovirus from bovine with severe respiratory illness. A similar novel Nidovirus was determined as the likely cause large scale mortalities in the endangered Bellinger River snapping turtle (Myuchelys georgesi): doi: 10.1371/journal.pone.0205209. 1983 detection in Switzerland in horses: Purification and Partial Characterization of a New Enveloped RNA Virus (Berne Virus) https://doi.org/10.1099/0022-1317-64-9-1849	Acute onset (12hours) severe disease featuring: inappetence/anorexia; lethargy; marked pyrexia (39.6C), tachycardia (80BPM), tachypnoea (36bpm); reduced GIT borborygmi; hind-limb ataxia. Absence of 'typical colic signs' and any abnormal nasal discharge. administered supportive medical therapy including intravenous fluids, non-steroidal anti-inflammatory (flunixin) and gastrointestinal anti-spasmodic (buscopan). Some clinical improvement noted.	Deep RNA seq metatranscriptomic NGS as pools confirmed to individual case samples by PCR	South-east QLD - sunshine coast hinterland	7-year-old gelding	August/ 2017	
p18-044XX		presented (6/9/18) with signs suggestive of severe colic (tachycardia, rolling and absent GIT borborygmi) symmetrical swelling of temporal muscles (likely oedema), mild hind-limb ataxia. Initially responded to supportive and anti-inflammatory medical treatment with heart rate returning to normal range (40-bpm) and normal rectal temperature. deteriorated with prolonged recumbency overnight and gradual deterioration over 36 hours until moribund condition (persistent disorientation and circling; severe tachycardia 180bpm; circulatory shock indicated suspected (indicated by marked tachycardia and mucous membranes pallor) justified euthanasia.	As above	South-east QLD - sunshine coast hinterland	15-year-old TB mare	Septem ber	A case of sudden horse death of unknown cause on same property 4 to 8 weeks prior to this cases sample event
p18-046XX		No clinical disease description on submission form. Included in cohort as possible non-diseased case *(Likely in error – where lack of description interpreted as no sig. disease during categorisation highlighting need for improved case descriptions)*	As above	Near Gladstone, North Qld.	8-year-old pony gelding	Septem ber	Affected pony introduced to property 12 days prior
p17-160XX	Novel Nidovirus 2	On average there are 3-5% difference between the samples across the region sequences obtained so far. We had another PCR product that only worked for p17_160XX and p18_045XX. The genetic difference was 15% for these, showing that there is a fair level of diversity amongst the few cases found so far, and we'll need to make our primers carefully for a larger screen as above	Ataxia and neurological disease progressively more severe over a 3-day period resulting in euthanasia	As above	South-east Qld - Moreton bay region	14 year-old gelding	Septem ber	
p18-045XX			Depressed and anorexic justifying referral to vet clinic on the 5/9/18. Vital signs had been within normal limits so the horse was treated as a 'colic'. 5pm 6/9/18 recorded severe pyrexia – 40.3C	As above	Katherine NT	4-year-old male		Homebred and kept on extensive pasture

7.3.2.3.1.2.5 Hepaci and Pegiviruses

RNA sequenced libraires from blood samples of the Qld HeV-like disease cohort, contained a high abundance of equine hepaci- and pegiviruses (Figure 7.21), suggesting they are commonly infecting this species. Their association with disease is not well understood.

Figure 7.21. Maximum-likelihood phylogeny of hepaci and pegiviruses using NS5B (RdRp) protein sequences.
Phylogeny generated with PhyML using the GTR model. Branch labels represent SH-like supports. Tip labels in red indicate strains identified in this study.

7.3.2.3.1.2.6 Viral abundance across libraries and relative to sample type

To understand the prevalence of these viruses amongst the cohort, we re-mapped all the sequences onto the genomes of the key viral pathogens identified, focusing on those likely causing disease. The abundance of each virus was quantified and shown as a heat map, split according to the libraries (pools) as sample type (Figure 7.22 below). This illustrates not only the great diversity of potentially significant emerging RNA pathogens associated with HeV-like disease in Australian horses and the suitability of 'Horses as Sentinels' approach to bolstering routine surveillance but also the surprising diagnostic potential of even such minimally invasive sample types as the swabs and blood samples analysed.

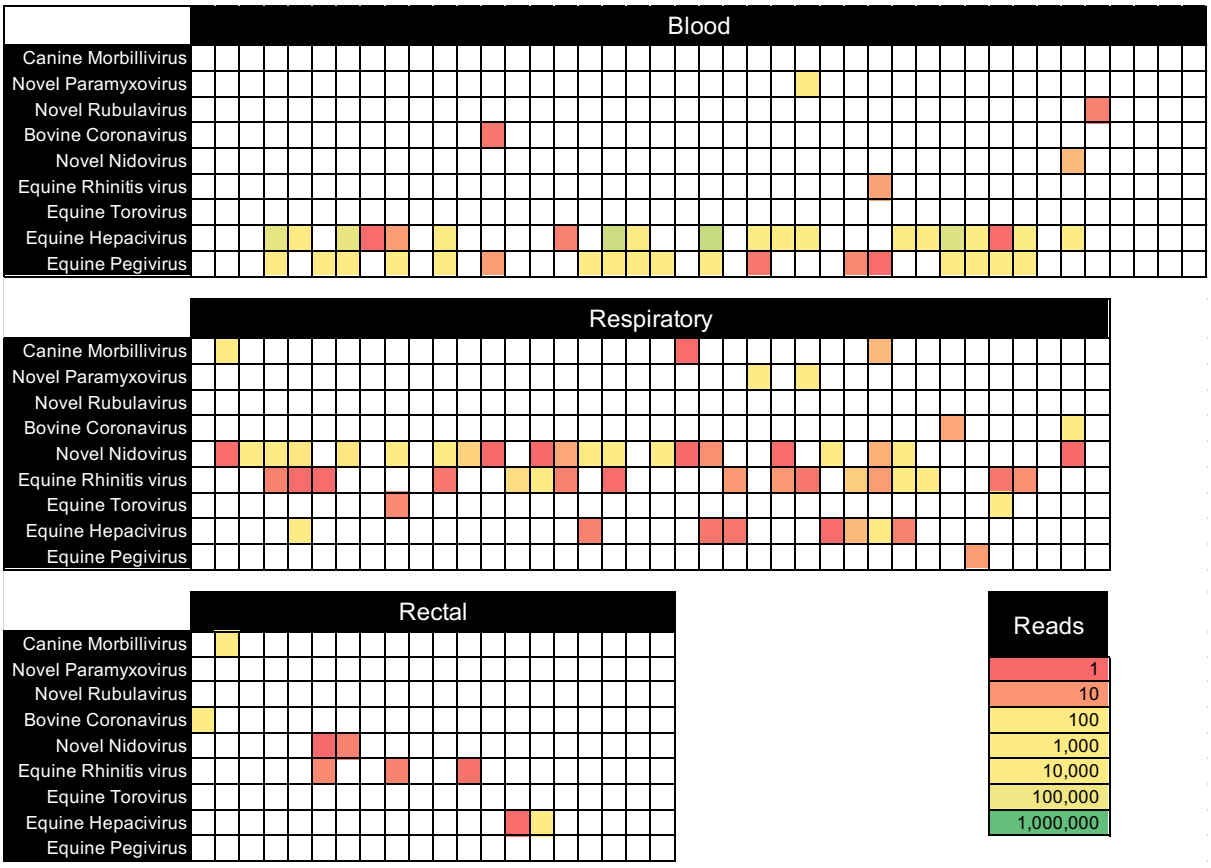

Figure 7.22. Heatmap of viral abundance across RNAseq libraries by sample type.
RNA reads were mapped to each virus and plotted for each key viral pathogen. The amount of mapped reads was recorded and coloured according to the key provided.

7.3.2.3.1.2.7 Additional metatranscriptomic findings for the Qld HeV-like disease cohort

The high throughput metatranscriptomic molecular pipeline applied in this research, focusing on approximately 300 of the highest suspect cases amongst the Qld HeV-like

disease cohort, has identified hundreds additional viruses. The RNAseq data is undergoing further detailed analysis and future research will look to extend these findings. Some initial findings beyond those reported above include known and novel lineages of previously undescribed viruses such as equine rotaviruses, rhinitis viruses, herpesviruses & toroviruses.

Rectal swab sample libraries posed challenge for analyses due to extreme abundance of invertebrate viruses likely from bacteria, protists and plant matter present. For this reason, it was critical to avoid pooling them with blood or nasal/oral swab samples.

7.3.2.3.1.3 HeV spillover 2019 seven direct HeV case contact/one no direct HeV case contact

Sample type, infectious disease likelihood category, vaccination status for 8 unique horse-subjects sampled (single sample events) in association with the 2019 HeV spillover event that was then the furthest southern detected (Hunter NSW) are described in Table 7.20.

Tables 7.21 – 7.26 present summary of binary test outcome and positive test outcome proportions for individual and logically combined antigens both in IgG and IgM for this HeV spillover event sub cohort. 7 of the 8 horse subjects were perceived as having direct contact with the confirmed HeV case. All were unvaccinated and while none were seropositive for HeV or NiV IgG 2 of the 7 horse subjects with direct exposure demonstrated seropositivity for HeV RBP IgM (Table 7.22), highlighting the potential benefit of sampling and testing horses in association with confirmed outbreaks for both IgG and IgM as part of routine responses to both best manage onward animal and human transmission risks and biosecurity.

Furthermore, this sub cohort demonstrated some seropositivity for IgG against Pararubulavirus NPs (3/8) and RBPs (2/8) and IgM against Pararubulavirus NPs (5/8) but not RBPs (0/8) (Table 7.21), highlighting both potential for previous unrecognised divergent paramyxovirus spillover in horses with exposure to flying foxes as for HeV. Additionally, the relatively high Pararubulavirus NP seropositivity in this cohort exposed to a confirmed HeV infected horse, illustrate the potential for cross reactivity of IgM and IgG (while to a lesser extent) elicited in response to exposure to HNVs to Pararubulavirus NPs.

Table 7.20. Infectious-like disease horse sub-cohort summary: HeV spillover event NSW 2019

HeV_vaccine_status	unvaccinated: 8
Sample	Serum: 8
Sample Reason	7 direct HeV case contact/ 1 no direct HeV case contact
State	NS: 8
infect_prior	1: 1 7: 7
plating_unique_v_duplicate	single sample event: 8

Table 7.21

HeV spillover event NSW 2019, *Pararubulavirus* antigens

	Binary test classification based on K95* Bayesian analyses		Proportion positive (%)	Antigen coupled bead No./s	Sub-cohort MFI distribution					
	Negative	Positive			Min	1st quartile	Median	Mean	3rd quartile	Maximum
TioPV NP IgG	8	0	0	30	82	99	104	152.5	139	444
TioPV NP IgM	3	5	63	30	748	1157	1692	1777	1840	4072
MenPV NP IgG	5	3	38	62 or 48	231	319	381	782	1040	2443
MenPV NP IgM	8	0	0	63 or 48	829	1360	1694	1762	2328	2674
Pararubulavirus NPs IgG combined	5	3	38							
Pararubulavirus NPs IgM combined	3	5	63							
MenPV sHN (RBP) IgG	8	0	0	28	124	130	133	148	144	237
MenPV sHN (RBP) IgM	8	0	0	28	2383	4496	4894	4746	5608	5814
YepPV sHN (RBP) IgG	8	0	0	13						
YepPV sHN (RBP) IgM	8	0	0	13	14	17	18	19	19	26
GrovPV sHN (RBP) IgG	8	0	0	18	217	240	255	270	308	340
GrovPV sHN (RBP) IgM	8	0	0	18	41	46	49	52	58	66
Pararubulavirus sHNs (RBPs) IgG combined	6	2	25							
Pararubulavirus sHNs (RBPs) IgM combined	8	0	0							
Pararubulavirus NPs & sHNs (RBPs) IgG combined	6	2	25							
Pararubulavirus NPs & sHNs (RBPs) IgM combined	8	0	0							
Pararubulavirus NPs & sHNs (RBPs) IgM combined & −ve IgG	7	1	13							

Table 7.22

HeV spillover event NSW 2019, *Henipavirus* antigens

	Binary test classification based on K95* Bayesian analyses		Proportion positive (%)	Antigen coupled bead No./s	Sub-cohort MFI distribution					
	Negative	Positive			Min	1st quartile	Median	Mean	3rd quartile	Maximum
HeV RBP IgG	8	0	0	28 & 43 ave.	82	85	88	90	90	111
NiV RBP IgG	8	0	0	46	218	229	243	240	248	263
NiV & HeV IgG combined	8	0	0							
unvaccinated NiV & HeV IgG combined	8	0	0							
HeV RBP IgM	6	2	25	43	270	317	401	492	617	918
NiV RBP IgM	8	0	0	46	808	1084	1134	1133	1213	1364
NiV & HeV IgM combined	6	2	25							
NiV & HeV IgM combined & −ve IgG	6	2	25							
unvaccinated NiV & HeV IgM combined	6	2	25							
CedPV RBP IgG	8	0	0	53	132	136	138	139	141	149
CedPV RBP IgM	4	4	50	53	2671	3161	5934	5895	7480	11117
NiV & HeV & CedPV IgG combined	8	0	0							
unvaccinated NiV & HeV & CedPV IgG combined	8	0	0							
NiV & HeV pos but CedPV neg IgG	8	0	0							
MojV RBP IgG	8	0	0	29	132	157	161	164	169	204
MojV RBP IgM	8	0	0	29	803	1128	1380	1471	1760	2484
GhV RBP IgG	8	0	0	35	132	145	165	166	189	199
GhV RBP IgM	8	0	0	35	620	766	957	938	1066	1345
MojV & GhV RBP IgG combined	8	0	0							
MojV & GhV RBP IgG combined unvaccinated	8	0	0							
Non-prototypic HNVs IgG combined	8	0	0							
Non-prototypic HNVs IgG combined unvaccinated	8	0	0							
Non-prototypic HNVs IgM combined	4	4	50							
Non-prototypic HNVs IgM combined unvaccinated	4	4	50							
Non-prototypic HNVs IgM combined & −ve IgG	4	4	50							
NiV & HeV IgG combined but negative for Non-prototypic HNVs & unvaccinated	8	0	0							
NiV & HeV IgG combined but negative for MojV & GhV & unvaccinated	8	0	0							

Table 7.23. HeV spillover event NSW 2019, SARS-like CoV antigens	Binary classification Prior to Bayesian estimation		Proportion positive (%)	Antigen coupled bead No./s	Sub-cohort MFI distribution						Conservative MFI cut-off applied for Binary classification
	Negative	Positive			Min	1st quartile	Median	Mean	3rd quartile	Maximum	
MERS CoV IgG	8	0	0	15	14	19	20	23	23	40	1000
MERS CoV IgM	7	1	13	15	315	443	569	668	722	1343	1196
SARS CoV-1 NP IgG	8	0	0	39	14	14	14	15	16	20	1000
SARS CoV-1 NP IgM	8	0	0	39	117	133	173	164	183	215	1000
SARS CoV-1 NP IgM	8	0	0	39							500
SARS CoV-1 NP IgM	8	0	0	65	1244	1854	2264	2274	2592	3256	6876
SARS CoV-2 NP IgG	8	0	0	42	54	55	56	59.5	66	68	1000
SARS CoV-2 NP IgM	8	0	0	42	425	457	501	522	550	717	1217
SARS CoV-2 NP IgM	8	0	0	42							811
SARS CoV-2 NP IgG	6	2	25	77	254	319	493	827	1047	2036	2000
SARS CoV-2 NP IgM	8	0	0	77	1470	3186	4321	3977	5038	5312	9867
rs4874 CoV IgG	8	0	0	54	90	95	97	101	101	132	1000
rs4874 CoV IgM	8	0	0	54	652	904	1163	1226	1456	2195	2372

Table 7.24. HeV spillover event NSW 2019, *Filovirus* antigens	Binary classification Prior to Bayesian estimation		Proportion positive (%)	Antigen coupled bead No./s	Sub-cohort MFI distribution						Conservative MFI cut-off applied for Binary classification
	Negative	Positive			Min	1st quartile	Median	Mean	3rd quartile	Maximum	
Zaire ebolavirus EBOV NP IgG	7	1	13	72	124	190	231.5	533	431.5	2191	1323
Zaire ebolavirus EBOV NP IgM	8	0	0	72	618	1290	1790	1778	2097	3320	3861
Zaire ebolavirus EBOV EGP IgG	7	1	13	34	56	62	78	1609	101	12332	1323
Zaire ebolavirus EBOV EGP IgM	6	2	25	34	1515	2269	2872	3685	3754	10156	3974
Bundibugyo ebolavirus BDBV EGP IgG	8	0	0	64	148	181	248	250	290	402	1323
Bundibugyo ebolavirus BDBV EGP IgM	7	1	13	64	1002	1281	1738	1915	2080	4020	3285
Bombali ebolavirus BOMV EGP IgG	8	0	0	55	132	133	135	193	140	591	1000
Bombali ebolavirus BOMV EGP IgM	7	1	13	55	584	749	850	1063	920	2928	1844
Reston ebolavirus RESTV EGP IgG	8	0	0	72	28	31	34	143	42	899	1000
Reston ebolavirus RESTV EGP IgM	7	1	13	72	5236	6128	6892	7400	7716	12314	8741
Marburg Marburgvirus MARV EGP IgG	8	0	0	37	39	42	47	64	62	142	1000
Marburg Marburgvirus MARV EGP IgM	7	1	13	37	677	1121	1255	1335	1324	2686	1802
Marburg Marburgvirus RAVV EGP IgG	8	0	0	62	49	58	69	74	83	124	1000
Marburg Marburgvirus RAVV EGP IgM	7	1	13	62	368	628	807	895	968	1971	1304
Mengla dianlovirus MLAV EGP IgG	8	0	0	22	57	58	59	77	67	188	1000
Mengla dianlovirus MLAV EGP IgM	6	2	25	22	514	666	809	922	1000	1735	1268
Lloviu cuevavirus LLOV EGP IgG	8	0	0	66	142	145	150	168	153	306	1000
Lloviu cuevavirus LLOV EGP IgM	6	2	25	66	1510	1696	2033	2427	2700	4318	3086

Table 7.25

HeV spillover event NSW 2019, Borna Disease virus & Hepatitis E virus antigens

	Binary classification Prior to Bayesian estimation		Proportion positive (%)	Antigen coupled bead No./s	Sub-cohort MFI distribution						Conservative MFI cut-off applied for Binary classification
	Negative	Positive			Min	1st quartile	Median	Mean	3rd quartile	Maximum	
Borna disease virus BoDV EGP IgG	7	1	13	48	457	508	771	947	918	2707	2573
Borna disease virus BoDV EGP IgM	8	0	0	73	2441	5194	6745	6338	7788	8818	17655
Borna disease virus BoDV EGP IgM	8	0	0								14713
Borna disease virus BoDV EGP IgM	8	0	0								11770
Orthohepevirus A CP HEV IgG	6	2	25	30	162	234	693	2990	2775	13106	2480
Orthohepevirus A CP HEV IgM	8	0	0	64	1474	3886	7782	6594	9278	10274	10456
	2	6	75	64							3306
	1	7	88	64							2480

Table 7.26

HeV spillover event NSW 2019, Arbovirus and other antigens

	Binary classification Prior to Bayesian estimation		Proportion positive (%)	Antigen coupled bead No./s	Sub-cohort MFI distribution						Conservative MFI cut-off applied for Binary classification
	Negative	Positive			Min	1st quartile	Median	Mean	3rd quartile	Maximum	
West Nile virus WNV E IgG	8	0	0	38	88	93	97	100	107	115	k95 by Bayesian estimation
West Nile virus WNV E IgM	8	0	0	38	591	1170	1334	1306	1578	1703	3680
Chikungunya virus CHIKV E1 IgG	8	0	0	48	74	77	82	122	99	366	3000
Chikungunya virus CHIKV E1 IgM	7	1	13	48	769	1936	2983	3484	3691	9947	4977
Sindbis virus SINV E1 IgG	7	1	13	55	599	634	801	1214	990	4256	2000
California encephalitis virus (La Crosse) CEV NP IgM	8	0	0	76	1159	2298	2511	2356	2726	2872	6563
Retrovirus SC110 Retro E IgG	8	0	0	38	80	82	85	87.5	91	102	1000
Retrovirus SC110 Retro E IgM	8	0	0	38	600	638	738	786	915	1063	1187
Hervey pteropid gammaretrovirus HPG E IgG	8	0	0	45	80	83	83	86	89	96	1000
Hervey pteropid gammaretrovirus HPG E IgM	8	0	0	45	334	355	419	422	455	580	1000
Hervey pteropid gammaretrovirus HPG E IgM	8	0	0								845
Brucella suis RepA IgG	7	1	13	26	136	148	168	436	209	2257	2000
Brucella suis RepA IgM	8	0	0	26	2511	6378	8923	7730	9560	9796	9900
Brucella abortus OMP IgG	8	0	0	28 or 55	96	103	109	145	154	279	1323
Brucella abortus OMP IgM	7	1	13	55	1626	4449	5161	5011	5380	8630	8339
Leptospira australis (whole organism) IgG	8	0	0	26	70	73	73	77	80	92	1000
Leptospira pomona (whole organism) IgG	8	0	0	77	70	70	72	74	76	86	1000

7.3.2.3.1.4 HeV-like disease outbreak NSW 2016 (MenPV-like outbreak 2016) and other infectious-like disease NSW

Sample type, infectious disease likelihood category, vaccination status for 9 sera samples drawn from 8 horse-subjects sampled (paired sera drawn from one) in association with two HeV-like disease clusters in NSW are described in Table 7.27. These events were HeV-like the MenPV-like disease outbreak of 2016 described in Section 7.3.1.1 (5 subjects, 6 samples) and anther Infectious-like disease event in Hume region of NSW in July 2020 (3 sera representing 3 subjects).

Tables 7.28 – 7.33 present summary of binary test outcome and positive test outcome proportions for individual and logically combined antigens both in IgG and IgM for this HeV spillover event sub cohort.

Table 7.27. Infectious-like disease horse sub-cohort summary: HeV or other viral-like suspect infectious disease NSW 2016 and 2020

HeV_vaccine_status	unvaccinated: 7 | vaccinated current: 2
Sample	Serum: 9
Sample Reason	HeV-like disease outbreak NSW 2016 (MenPV-like outbreak 2016): 6 Other Infectious-like disease event NSW (2020): 3
State	NSW: 9
infect_prior	5: 9
plating_unique_v_duplicate	paired_sampling_second: 1 paired_sampling_primary: 1 single sample event: 7

Table 7.28

HeV or other viral-like suspect infectious disease NSW, *Pararubulavirus* antigens

	Binary test classification based on K95* Bayesian analyses		Proportion positive (%)	Antigen coupled bead No./s	Sub-cohort MFI distribution					
	Negative	Positive			Min	1st quartile	Median	Mean	3rd quartile	Maximum
TioPV NP IgG	9	0	0	30	104	139	203	200	213	339
TioPV NP IgM	6	3	33	30	582	748	1084	1325	1899	2514
MenPV NP IgG	5	4	44	62 or 48	136	238	335	1646	1248	9551
MenPV NP IgM	9	0	0	63 or 48	603	805	1249	1489	1994	2790
Pararubulavirus NPs IgG combined	5	4	44							
Pararubulavirus NPs IgM combined	6	3	33							
MenPV sHN (RBP) IgG	9	0	0	28	126	134	143	148	156	182
MenPV sHN (RBP) IgM	8	1	11	28	2545	3918	5116	5323	6842	7721
YepPV sHN (RBP) IgG	9	0	0	13						
YepPV sHN (RBP) IgM	9	0	0	13	17	18	20	24	27	44
GrovPV sHN (RBP) IgG	7	2	22	18	138	208	296	291	340	506
GrovPV sHN (RBP) IgM	8	1	11	18	53	58	68	95	131	185
Pararubulavirus sHNs (RBPs) IgG combined	6	3	33							
Pararubulavirus sHNs (RBPs) IgM combined	8	1	11							
Pararubulavirus NPs & sHNs (RBPs) IgG combined	5	4	44							
Pararubulavirus NPs & sHNs (RBPs) IgM combined	7	2	22							
Pararubulavirus NPs & sHNs (RBPs) IgM combined & −ve IgG	9	0	0							

Table 7.29

HeV or other viral-like suspect infectious disease NSW, *Henipavirus* antigens

	Binary test classification based on K95* Bayesian analyses		Proportion positive (%)	Antigen coupled bead No./s	Sub-cohort MFI distribution					
	Negative	Positive			Min	1st quartile	Median	Mean	3rd quartile	Maximum
HeV RBP IgG	7	2	22	28 & 43 ave.	96	116	3146	127	14298	96
NiV RBP IgG	7	2	22	46	248	262	2785	271	12000	248
NiV & HeV IgG combined	7	2	22							
unvaccinated NiV & HeV IgG combined	9	0	0							
HeV RBP IgM	6	3	33	43	435	457	639	826	1264	435
NiV RBP IgM	9	0	0	46	966	1159	1095	1250	1530	966
NiV & HeV IgM combined	6	3	33							
NiV & HeV IgM combined & −ve IgG	7	2	22							
unvaccinated NiV & HeV IgM combined	6	3	33							
CedPV RBP IgG	9	0	0	53	138	150	164	166	227	138
CedPV RBP IgM	6	3	33	53	2608	3456	4439	6690	7814	2608
NiV & HeV & CedPV IgG combined	7	2	22							
unvaccinated NiV & HeV & CedPV IgG combined	7	2	22							
NiV & HeV pos but CedPV neg IgG	9	0	0							
MojV RBP IgG	9	0	0	29	157	165	161	168	179	157
MojV RBP IgM	9	0	0	29	1155	1557	1528	1668	2544	1155
GhV RBP IgG	8	1	11	35	189	194	243	205	565	189
GhV RBP IgM	6	3	33	35	974	1082	1371	1575	2432	974
MojV & GhV RBP IgG combined	8	1	11							
MojV & GhV RBP IgG combined unvaccinated	8	1	11							
Non-prototypic HNVs IgG combined	8	1	11							
Non-prototypic HNVs IgG combined unvaccinated	8	1	11							
Non-prototypic HNVs IgM combined	3	6	67							
Non-prototypic HNVs IgM combined unvaccinated	4	5	56							
Non-prototypic HNVs IgM combined & −ve IgG	4	5	56							
NiV & HeV IgG combined but negative for Non-prototypic HNVs & unvaccinated	9	0	0							
NiV & HeV IgG combined but negative for MojV & GhV & unvaccinated	9	0	0							

Table 7.30. HeV or other viral-like suspect infectious disease NSW, SARS-like CoV antigens	Binary classification Prior to Bayesian estimation		Proportion positive (%)	Antigen coupled bead No./s	Sub-cohort MFI distribution						Conservative MFI cut-off applied for Binary classification
	Negative	Positive			Min	1st quartile	Median	Mean	3rd quartile	Maximum	
MERS CoV IgG	9	0	0	15	18	22	48	123	58	524	1000
MERS CoV IgM	7	2	22	15	212	331	447	685	880	1644	1196
SARS CoV-1 NP IgG	9	0	0	39	14	15	15	124	16	604	1000
SARS CoV-1 NP IgM	9	0	0	39	104	126	180	176	213	269	1000
SARS CoV-1 NP IgM	9	0	0	39							500
SARS CoV-1 NP IgM	9	0	0	65	1172	1938	2217	2515	3499	3654	6876
SARS CoV-2 NP IgG	7	2	22	42	55	62	62	495	66	2345	1000
SARS CoV-2 NP IgM	9	0	0	42	239	434	476	495	600	680	1217
SARS CoV-2 NP IgM	9	0	0	42							811
SARS CoV-2 NP IgG	8	1	11	77	250	337	912	982	1278	2006	2000
SARS CoV-2 NP IgM	9	0	0	77	1224	2946	3480	3541	4463	5010	9867
rs4874 CoV IgG	9	0	0	54	96	99	105	104	107	109	1000
rs4874 CoV IgM	9	0	0	54	552	875	1071	1135	1432	1998	2372

Table 7.31. HeV or other viral-like suspect infectious disease NSW, *Filovirus* antigens	Binary classification Prior to Bayesian estimation		Proportion positive (%)	Antigen coupled bead No./s	Sub-cohort MFI distribution						Conservative MFI cut-off applied for Binary classification
	Negative	Positive			Min	1st quartile	Median	Mean	3rd quartile	Maximum	
Zaire ebolavirus EBOV NP IgG	9	0	0	72	122	146	182	306	188	984	1323
Zaire ebolavirus EBOV NP IgM	8	1	11	72	939	1241	1478	1859	2017	3992	3861
Zaire ebolavirus EBOV EGP IgG	8	1	11	34	49	50	84	400	105	2928	1323
Zaire ebolavirus EBOV EGP IgM	7	2	22	34	894	1473	2122	2480	3120	4720	3974
Bundibugyo ebolavirus BDBV EGP IgG	7	2	22	64	111	151	170	443	193	1446	1323
Bundibugyo ebolavirus BDBV EGP IgM	9	0	0	64	994	1412	1521	1595	1874	2069	3285
Bombali ebolavirus BOMV EGP IgG	9	0	0	55	133	136	138	147	157	183	1000
Bombali ebolavirus BOMV EGP IgM	9	0	0	55	470	610	702	712	830	1011	1844
Reston ebolavirus RESTV EGP IgG	9	0	0	72	27	36	37	39	39	63	1000
Reston ebolavirus RESTV EGP IgM	8	1	11	72	2788	4194	5620	5596	6172	9721	8741
Marburg Marburgvirus MARV EGP IgG	9	0	0	37	48	50	51	124	120	538	1000
Marburg Marburgvirus MARV EGP IgM	8	1	11	37	471	736	1004	1046	1394	1819	1802
Marburg Marburgvirus RAVV EGP IgG	9	0	0	62	46	60	71	99	87	214	1000
Marburg Marburgvirus RAVV EGP IgM	8	1	11	62	362	573	796	787	883	1645	1304
Mengla dianlovirus MLAV EGP IgG	9	0	0	22	59	63	73	75	82	105	1000
Mengla dianlovirus MLAV EGP IgM	8	1	11	22	303	522	664	768	1026	1505	1268
Lloviu cuevavirus LLOV EGP IgG	9	0	0	66	143	144	146	148	150	158	1000
Lloviu cuevavirus LLOV EGP IgM	8	1	11	66	826	1268	1713	1882	2465	3487	3086

Table 7.32

HeV or other viral-like suspect infectious disease NSW, Borna Disease virus & Hepatitis E virus antigens	Binary classification Prior to Bayesian estimation		Proportion positive (%)	Antigen coupled bead No./s	Sub-cohort MFI distribution						Conservative MFI cut-off applied for Binary classification
	Negative	Positive			Min	1st quartile	Median	Mean	3rd quartile	Maximum	
Borna disease virus BoDV EGP IgG	7	2	22	48	236	747	1524	1609	1619	3743	2573
Borna disease virus BoDV EGP IgM	9	0	0	73	4782	6804	7882	7908	8468	13596	17655
Borna disease virus BoDV EGP IgM	9	0	0								14713
Borna disease virus BoDV EGP IgM	8	1	11								11770
Orthohepevirus A CP HEV IgG	9	0	0	30	158	325	388	380	504	577	2480
Orthohepevirus A CP HEV IgM	9	0	0	64	1560	1736	2299	3261	3450	7590	10456
Orthohepevirus A CP HEV IgM	6	3	33								3306

Table 7.33

HeV or other viral-like suspect infectious disease NSW, Arbovirus and other antigens	Binary classification Prior to Bayesian estimation		Proportion positive (%)	Antigen coupled bead No./s	Sub-cohort MFI distribution						Conservative MFI cut-off applied for Binary classification
	Negative	Positive			Min	1st quartile	Median	Mean	3rd quartile	Maximum	
West Nile virus WNV E IgG	9	0	0	38	83	96	134	129	156	166	k95 by Bayesian estimation
West Nile virus WNV E IgM	9	0	0	38	790	943	981	1284	1597	2352	3680
Chikungunya virus CHIKV E1 IgG	9	0	0	48	128	146	314	286	402	440	3000
Chikungunya virus CHIKV E1 IgM	8	1	11	48	860	1103	1512	2230	2451	5179	4977
Sindbis virus SINV E1 IgG	9	0	0	55	235	358	647	922	1769	1898	2000
California encephalitis virus (La Crosse) CEV NP IgM	9	0	0	76	1153	1744	2770	2599	3234	4168	6563
Retrovirus SC110 Retro E IgG	9	0	0	38	83	87	92	90	94	96	1000
Retrovirus SC110 Retro E IgM	9	0	0	38	338	399	642	606	759	916	1187
Hervey pteropid gammaretrovirus HPG E IgG	9	0	0	45	83	86	87	95	102	133	1000
Hervey pteropid gammaretrovirus HPG E IgM	9	0	0	45	232	258	352	379	431	582	1000
Hervey pteropid gammaretrovirus HPG E IgM	9	0	0								845
Brucella suis RepA IgG	8	1	11	26	106	151	228	1782	246	13451	2000
Brucella suis RepA IgM	9	0	0	26	938	2372	2964	3153	4180	4747	9900
Brucella abortus OMP IgG	7	2	22	28 or 55	136	141	144	710	230	3337	1323
Brucella abortus OMP IgM	9	0	0	55	1026	2419	3071	3017	3762	4502	8339
Leptospira australis (whole organism) IgG	9	0	0	26	68	71	73	87	77	147	1000
Leptospira pomona (whole organism) IgG	9	0	0	77	66	68	71	87	78	147	1000

7.3.2.3.1.5 Acute neuro disease cluster SA 2020 sub-cohort

Sample type, infectious disease likelihood category, vaccination status for 11 horse-subjects sampled (paired sera drawn from one) in association with a cluster of neurological HeV-like disease in South Australia in July 2020 are described in Table 7.34.

Tables 7.35 – 7.40 present summaries of binary test outcome and positive test outcome proportions for individual and logically combined antigens both in IgG and IgM for this infectious-like disease sub cohort.

Table 7.34. Infectious-like disease horse sub-cohort summary: Acute neuro disease cluster SA 2020 Sub-Cohort

HeV_vaccine_status	Unvaccinated: 11		
Sample	Serum: 11		
Sample Reason	viral-like_cluster_acute neuro_SA: 11		
State	SA: 11		
infect_prior	1: 9	1 (7): 2	
plating_unique_v_duplicate	paired_sampling_second: 2	paired_sampling_primary: 0	single sample event: 9

Table 7.35

Acute neuro disease cluster SA 2020, *Pararubulavirus* antigens

	Binary test classification based on K95* Bayesian analyses		Antigen coupled bead No./s	Sub-cohort MFI distribution					
	Negative	Positive		Min	1st quartile	Median	Mean	3rd quartile	Maximum
TioPV NP IgG	9	2	30	100	166	253	512	365	2450
TioPV NP IgM	5	6	30	550	1018	1548	2294	2067	7988
MenPV NP IgG	8	3	62 or 48	149	251	671	1150	1104	6228
MenPV NP IgM	9	2	63 or 48	465	1396	1776	2825	2692	9009
Pararubulavirus NPs IgG combined	7	4							
Pararubulavirus NPs IgM combined	5	6							
MenPV sHN (RBP) IgG	11	0	28	123	126	128	136	139	169
MenPV sHN (RBP) IgM	10	1	28	4026	4828	5403	5812	6406	9490
YepPV sHN (RBP) IgG	11	0	13	14	17	18	21	20	50
YepPV sHN (RBP) IgM	8	3	13	168	238	275	650	406	3485
GrovPV sHN (RBP) IgG	11	0	18	38	42	48	68	71	157
GrovPV sHN (RBP) IgM	8	3	18	323	425	646	1340	737	8541
Pararubulavirus sHNs (RBPs) IgG combined	11	0							
Pararubulavirus sHNs (RBPs) IgM combined	7	4							
Pararubulavirus NPs & sHNs (RBPs) IgG combined	8	3							
Pararubulavirus NPs & sHNs (RBPs) IgM combined	11	0							
Pararubulavirus NPs & sHNs (RBPs) IgM combined & -ve IgG	8	3							

Table 7.36

Acute neuro disease cluster SA 2020, *Henipavirus* antigens

	Binary test classification based on K95* Bayesian analysis		Antigen coupled bead No./s	Sub-cohort MFI distribution					
	Negative	Positive		Min	1st quartile	Median	Mean	3rd quartile	Maximum
HeV RBP IgG	11	0	28 & 43 ave.	82	85	92	104	108	188
NiV RBP IgG	10	1	46	216	238	247	286	271	664
NiV & HeV IgG combined	10	1							
unvaccinated NiV & HeV IgG combined	10	1							
HeV RBP IgM	8	3	43	282	373	497	1122	627	7232
NiV RBP IgM	10	1	46	944	1020	1098	1804	1232	8495
NiV & HeV IgM combined	8	3							
NiV & HeV IgM combined & -ve IgG	8	3							
unvaccinated NiV & HeV IgM combined	8	3							
CedPV RBP IgG	11	0	53	121	133	140	143	149	187
CedPV RBP IgM	8	3	53	1747	2463	2838	3676	4960	6684
NiV & HeV & CedPV IgG combined	10	1							
Unvaccinated NiV & HeV & CedPV IgG combined	10	1							
NiV & HeV pos but CedPV neg IgG	10	1							
MojV RBP IgG	10	1	29	105	111	122	150	148	398
MojV RBP IgM	10	1	29	820	1366	1957	2657	2794	9239
GhV RBP IgG	11	0	35	129	152	179	193	200	322
GhV RBP IgM	8	3	35	652	881	1038	2126	1906	10624
MojV & GhV RBP IgG combined	10	1							
MojV & GhV RBP IgG combined unvaccinated	10	1							
Non-prototypic HNVs IgG combined	10	1							
Non-prototypic HNVs IgG combined unvaccinated	10	1							
Non-prototypic HNVs IgM combined	6	5							
Non-prototypic HNVs IgM combined unvaccinated	6	5							
Non-prototypic HNVs IgM combined & -ve IgG	6	5							
NiV & HeV IgG combined but negative for Non-prototypic HNVs & unvaccinated	10	1							
NiV & HeV IgG combined but negative for MojV & GhV & unvaccinated	10	1							

Table 7.37. Acute neuro disease cluster SA 2020, SARS-like CoV antigens	Binary classification prior to Bayesian estimation		Antigen coupled bead no./s	Sub-cohort MFI distribution						Conservative MFI cut-off applied for binary classification
	Negative	Positive		Min	1st quartile	Median	Mean	3rd quartile	Maximum	
MERS CoV IgG	11	0	15	16	17	22	85	32	600	1000
MERS CoV IgM	9	2	15	264	399	758	877	862	2527	1196
SARS CoV-1 NP IgG	11	0	39	14	14	15	18	17	42	1000
SARS CoV-1 NP IgM	10	1	39	133	173	187	312	222	1161	1000
SARS CoV-1 NP IgM	9	2	39							500
SARS CoV-1 NP IgM	9	2	65	1984	2624	3445	5099	5197	14065	6876
SARS CoV-2 NP IgG	11	0	42	56	58	61	60	62	66	1000
SARS CoV-2 NP IgM	10	1	42	430	448	618	675	639	1816	1217
SARS CoV-2 NP IgM	9	2	42							811
SARS CoV-2 NP IgG	10	1	77	162	258	500	1299	631	9783	2000
SARS CoV-2 NP IgM	9	2	77	2929	4579	4709	7088	7500	20262	9867

Table 7.38. Acute neuro disease cluster SA 2020, *Filovirus* antigens	Binary classification prior to Bayesian estimation		Antigen coupled bead No./s	Sub-cohort MFI distribution						Conservative MFI cut-off applied for binary classification
	Negative	Positive		Min	1st quartile	Median	Mean	3rd quartile	Maximum	
Zaire ebolavirus EBOV NP IgG	10	1	72	132	221	295	602	587	2754	1323
Zaire ebolavirus EBOV NP IgM	9	2	72	1044	1518	2079	3583	2936	14601	3861
Zaire ebolavirus EBOV EGP IgG	9	2	34	50	54	67	1751	220	14014	1323
Zaire ebolavirus EBOV EGP IgM	10	1	34	904	1381	2133	2520	3506	5458	3974
Bundibugyo ebolavirus BDBV EGP IgG	10	1	64	72	146	221	485	443	2273	1323
Bundibugyo ebolavirus BDBV EGP IgM	9	2	64	667	1403	1634	2839	2353	9422	3285
Bombali ebolavirus BOMV EGP IgG	10	1	55	133	138	144	381	257	2290	1000
Bombali ebolavirus BOMV EGP IgM	11	0	55	610	715	751	867	891	1466	1844
Reston ebolavirus RESTV EGP IgG	10	1	72	27	28	37	207	140	1411	1000
Reston ebolavirus RESTV EGP IgM	10	1	72	4060	4824	5378	6215	7655	10134	8741
Marburg Marburgvirus MARV EGP IgG	11	0	37	41	44	47	69	66	236	1000
Marburg Marburgvirus MARV EGP IgM	10	1	37	708	845	974	1234	1331	3117	1802
Marburg Marburgvirus RAVV EGP IgG	11	0	62	36	77	94	142	115	682	1000
Marburg Marburgvirus RAVV EGP IgM	9	2	62	445	586	691	1270	950	5076	1304
Mengla dianlovirus MLAV EGP IgG	11	0	22	59	61	63	66	70	82	1000
Mengla dianlovirus MLAV EGP IgM	11	0	22	469	533	579	653	715	1054	1268
Lloviu cuevavirus LLOV EGP IgG	11	0	66	141	145	147	216	181	749	1000
Lloviu cuevavirus LLOV EGP IgM	10	1	66	199	1404	1447	1734	1579	3882	3086

Table 7.39

Acute neuro disease cluster SA 2020, Borna Disease virus & Hepatitis E virus antigens	Binary classification prior to Bayesian estimation		Antigen coupled bead no./s	Sub-cohort MFI distribution						Conservative MFI cut-off applied for binary classification
	Negative	Positive		Min	1st quartile	Median	Mean	3rd quartile	Maximum	
Borna disease virus BoDV EGP IgG	10	1	48	312	387	471	760	871	2618	2573
Borna disease virus BoDV EGP IgM	11	0	73	7555	7756	9971	10166	11082	16160	17655
Borna disease virus BoDV EGP IgM	9	2								14713
Borna disease virus BoDV EGP IgM	9	2								11770
Orthohepevirus A CP HEV IgG	10	1	30	175	488	644	1290	1420	5490	2480
Orthohepevirus A CP HEV IgM	8	3	64	1739	3973	7631	7819	10912	17636	10456
Orthohepevirus A CP HEV IgM	2	9								3306

Table 7.40

Acute neuro disease cluster SA 2020, Arbovirus and other antigens	Binary classification prior to Bayesian estimation		Antigen coupled bead no./s	Sub-cohort MFI distribution						Conservative MFI cut-off applied for binary classification
	Negative	Positive		Min	1st quartile	Median	Mean	3rd quartile	Maximum	
West Nile virus WNV E IgG	11	0	38	92	99	116	124	130	237	k95 by Bayesian estimation
West Nile virus WNV E IgM	10	1	38	158	1462	1721	2286	2281	7567	3680
Chikungunya virus CHIKV E1 IgG	11	0	48	92	94	120	180	156	699	3000
Chikungunya virus CHIKV E1 IgM	9	2	48	1133	1675	2761	2919	3450	5994	4977
Sindbis virus SINV E1 IgG	11	0	55	120	191	261	397	578	972	2000
California encephalitis virus (La Crosse) CEV NP IgM	8	3	76	1542	2239	2452	4958	5187	21313	6563
Retrovirus SC110 Retro E IgM	10	1	38	477	606	645	768	843	1614	1187
Hervey pteropid *Gammaretrovirus* HPG E IgG	11	0	45	84	86	91	90	94	99	1000
Hervey pteropid *Gammaretrovirus* HPG E IgM	10	1	45	320	390	468	516	564	1058	1000
Hervey pteropid *Gammaretrovirus* HPG E IgM	10	1								845
Brucella suis RepA IgG	10	1	26	102	167	234	576	807	2007	2000
Brucella suis RepA IgM	11	0	26	2682	4438	7512	6396	8408	9314	9900
Brucella abortus OMP IgG	10	1	28 or 55	97	123	167	530	290	3660	1323
Brucella abortus OMP IgM	9	2	55	1926	3352	5404	6365	7320	17263	8339
Leptospira australis (whole organism) IgG	11	0	26	68	75	77	82	85	110	1000
Leptospira pomona (whole organism) IgG	11	0	77	66	73	75	79	79	106	1000

7.3.2.3.2 No suspect infectious disease cause cohorts

7.3.2.3.2.1 HeV tested in Queensland without infectious-like disease sample cohort (2015 – 2018)

Sample type, infectious disease likelihood category, vaccination status for 33 sera samples drawn from the same number of horse-subjects that underwent Government laboratory HeV testing in Queensland for biosecurity clearance context without suspicion of infectious-like disease are described in Table 7.41. Such context typically involved unvaccinated horses in areas of established HeV spillover risk at the time of veterinary hospital admission and/or invasive surgical or diagnostic procedures involving relatively high human exposure.

Tables 7.42 – 7.47 present summary of binary test outcome and positive test outcome proportions for individual and logically combined antigens both in IgG and IgM for this sub cohort of comparative background geographical disease agent exposure, sample conditions, storage, and processing to the priority Qld. HeV-like disease cohort.

Results from this cohort are especially well suited to strengthening interpretations of IgM seropositivity amongst the Qld. HeV-like disease cohort as described briefly in relation to interpretations of Paramyxovirus antigen IgM findings under sections

7.3.2.2.1.1.1 and 7.3.2.2.1.1.1 2.

Table 7.41. Non-Infectious-like disease horse sub-cohort summary: HeV tested without disease Qld 2015 - 2018

HeV_vaccine_status	Unvaccinated: 20 | vaccinated current: 3 | vaccinated unknown currency: 3 | unknown suspect vaccinated: 3 | partially vaccinated: 2 | unknown: 2		
Sample	edta: 32 | Clot:: 1		
Sample Reason	HeV-Tested without suspect infection: 33		
State	Qld: 33		
infect_prior	5: 33		
plating_unique_v_duplicate	paired_sampling_second: 0	paired_sampling_primary: 0	single sample event: 33

Table 7.42

HeV tested without disease Qld 2015 - 2018, *Pararubulavirus* antigens

	Binary test classification based on K95* Bayesian analysis		Proportion positive (%)	Antigen coupled bead no./s	Sub-cohort MFI distribution					
	Negative	Positive			Min	1st quartile	Median	Mean	3rd quartile	Maximum
TioPV NP IgG	31	2	6	30	72	107	153	233	215	1005
TioPV NP IgM	29	4	12	30	188	323	579	715	890	2464
MenPV NP IgG	26	7	21	62 or 48	37	136	332	431	540	1190
MenPV NP IgM	31	2	6	63 or 48	188	418	798	1175	1184	8247
Pararubulavirus NPs IgG combined	24	9	27							
Pararubulavirus NPs IgM combined	27	6	18							
MenPV sHN (RBP) IgG	33	0	0	28	108	146	162	166	172	378
MenPV sHN (RBP) IgM	32	1	3*	28	563	1568	2320	2700	3658	7889
	33	0	0**	*prior7761 | **prior11462						
YepPV sHN (RBP) IgG	33	0	0	13	13	19	22	32	29	168
YepPV sHN (RBP) IgM	31	2	6	13	54	110	155	190	234	790
GrovPV sHN (RBP) IgG	25	8	24	18	30	45	61.0	249	143	2944
GrovPV sHN (RBP) IgM	29	4	12	18	126	221	375	674	530	8463
Pararubulavirus sHNs (RBPs) IgG combined	25	8	24							
Pararubulavirus sHNs (RBPs) IgM combined	28	5	15							
Pararubulavirus NPs & sHNs (RBPs) IgG combined	30	3	9							
Pararubulavirus NPs & sHNs (RBPs) IgM combined	31	2	6							
Pararubulavirus NPs & sHNs (RBPs) IgM combined & -ve IgG	31	2	6							

Table 7.43

HeV tested without disease Qld 2015 - 2018, *Henipavirus* antigens

	Binary test classification based on K95* Bayesian analysis		Proportion positive (%)	Antigen coupled bead no./s	Sub-cohort MFI distribution					
	Negative	Positive			Min	1st quartile	Median	Mean	3rd quartile	Maximum
HeV RBP IgG	18	15	45	28 & 43 ave.	80	98	117	4053	7795	19080
NiV RBP IgG	20	13	39	46	153	201	219	2892	4469	14035
NiV & HeV IgG combined	18	15	45							
unvaccinated NiV & HeV IgG combined	31	2	6							
HeV RBP IgM	32	1	3	43	116	173	272	537	424	8362
NiV RBP IgM	31	2	6	46	354	560	711	977	982	7417
NiV & HeV IgM combined	31	2	6							
NiV & HeV IgM combined & -ve IgG	31	2	6							
unvaccinated NiV & HeV IgM combined	31	2	6							
CedPV RBP IgG	27	6	18	53	135	173	191	213	218	479
CedPV RBP IgM	31	2	6	53	444	1195	1584	2059	2172	7009
NiV & HeV & CedPV IgG combined	18	15	45							
unvaccinated NiV & HeV & CedPV IgG combined	18	15	45							
NiV & HeV pos but CedPV neg IgG	31	2	6							
MojV RBP IgG	29	4	12	29	102	163	201	225	278	542
MojV RBP IgM	32	1	3	29	326	620	920	1205	1238	6838
GhV RBP IgG	26	7	21	35	81	122	155	259	279	815
GhV RBP IgM	32	1	3	35	234	391	690	960	861	9989
MojV & GhV RBP IgG combined	25	8	24							
MojV & GhV RBP IgG combined unvaccinated	28	5	15							
Non-prototypic HNVs IgG combined	22	11	33							
Non-prototypic HNVs IgG combined unvaccinated	25	8	24							
Non-prototypic HNVs IgM combined	31	2	6							
Non-prototypic HNVs IgM combined unvaccinated	31	2	6							
Non-prototypic HNVs IgM combined & -ve IgG	31	2	6							
NiV & HeV IgG combined but negative for Non-prototypic HNVs & unvaccinated	31	2	6							
NiV & HeV IgG combined but negative for MojV & GhV & unvaccinated	31	2	6							

Table 7.44 HeV tested without disease Qld 2015–2018, SARS-like CoV antigens

	Binary classification prior to Bayesian estimation		Proportion positive (%)	Antigen coupled bead no./s	Sub-cohort MFI distribution						Conservative MFI cut-off applied for Binary classification
	Negative	Positive			Min	1st quartile	Median	Mean	3rd quartile	Maximum	
MERS CoV IgG	33	0	0	15	14	18	26	120	100	709	1000
MERS CoV IgM	32	1	3	15	77.0	126	220	328	416	1590	1196
SARS CoV-1 NP IgG	33	0	0	39	13	17	20	95	69	877	1000
SARS CoV-1 NP IgM	33	0	0	39	44	70	108	128	151	595	1000
SARS CoV-1 NP IgM	32	1	3	39							500
SARS CoV-1 NP IgM	32	1	3	65	545	1335	2052	2751	4052	8032	6876
SARS CoV-2 NP IgG	29	4	12	42	52	58	72	299	237	2108	1000
SARS CoV-2 NP IgM	33	0	0	42	165	252	334	337	421	613	1217
SARS CoV-2 NP IgM	33	0	0	42							811
SARS CoV-2 NP IgG	32	0	0	77	98	162	280	389	376	1658	2000
SARS CoV-2 NP IgM	31	2	6	77	564	1638	2491	3462	4284	10978	9867
rs4874 IgG	33	0	0	54	80	91	96	100	100	172	1000
rs4874 IgM	31	2	6	54	264	415	714	817	969	2966	2372

Table 7.45 HeV tested without disease Qld2015–2018, *Filovirus* antigens

	Binary classification prior to Bayesian estimation		Proportion positive (%)	Antigen coupled bead no./s	Sub-cohort MFI distribution						Conservative MFI cut-off applied for Binary classification
	Negative	Positive			Min	1st quartile	Median	Mean	3rd quartile	Maximum	
Zaire ebolavirus EBOV NP IgG	32	1	3	72	80	135	216	296	260	1518	1323
Zaire ebolavirus EBOV NP IgM	32	1	3	72	292	549	928	1341	1497	10591	3861
Zaire ebolavirus EBOV EGP IgG	29	4	12	34	45	201	220	813	289	9240	1323
Zaire ebolavirus EBOV EGP IgM	30	3	9	34	300	490	1047	1820	1797	15182	3974
Bundibugyo ebolavirus BDBV EGP IgG	31	2	6	64	56	106	203	620	406	7564	1323
Bundibugyo ebolavirus BDBV EGP IgM	31	2	6	64	247	563	875	1340	1397	8805	3285
Bombali ebolavirus BOMV EGP IgG	32	1	3	55	123	166	184	238	200	1715	1000
Bombali ebolavirus BOMV EGP IgM	32	1	3	55	304	415	488	608	636	2038	1844
Reston ebolavirus RESTV EGP IgG	32	1	3	72	23	27	30	91	39	1294	1000
Reston ebolavirus RESTV EGP IgM	33	0	0	72	412	1477	1950	2632	3488	6411	8741
Marburg Marburgvirus MARV EGP IgG	33	0	0	37	38	45	54	93	77	937	1000
Marburg Marburgvirus MARV EGP IgM	32	1	3	37	175	294	441	575	704	1950	1802
Marburg Marburgvirus RAVV EGP IgG	32	1	3	62	38	52	88	167	182	1438	1000
Marburg Marburgvirus RAVV EGP IgM	31	2	6	62	111	196	308	539	508	5034	1304
Mengla dianlovirus MLAV EGP IgG	33	0	0	22	51	57	60	70	67	206	1000
Mengla dianlovirus MLAV EGP IgM	33	0	0	22	154	233	319	394	478	1168	1268
Lloviu cuevavirus LLOV EGP IgG	33	0	0	66	130	236	251	263	267	659	1000
Lloviu cuevavirus LLOV EGP IgM	33	0	0	66	415	547	897	1020	1210	2832	3086

Table 7.46

HeV tested without disease Qld 2015 - 2018, Borna Disease virus & Hepatitis E virus antigens

	Binary classification prior to Bayesian estimation		Proportion positive (%)	Antigen coupled bead no./s	Sub-cohort MFI distribution						Conservative MFI cut-off applied for binary classification
	Negative	Positive			Min	1st quartile	Median	Mean	3rd quartile	Maximum	
Borna disease virus BoDV EGP IgG	23	10	30	48	94	406	856	2010	3152	7433	2573
Borna disease virus BoDV EGP IgM	33	0	0	73	1402	2961	5334	5410	7248	14185	17655
Borna disease virus BoDV EGP IgM	33	0	0								14713
Borna disease virus BoDV EGP IgM	32	1	3								11770
Orthohepevirus A CP HEV IgG	27	6	18	30	102	228	644	1815	1785	16724	2480
Orthohepevirus A CP HEV IgM	29	4	12	64	551	1110	2754	4408	5886	18696	10456
Orthohepevirus A CP HEV IgM	21	12	36								3306

Table 7.47. HeV tested without disease Qld 2015 - 2018, Arbovirus and other antigens

	Binary classification prior to Bayesian estimation		Proportion positive (%)	Antigen coupled bead no./s	Sub-cohort MFI distribution						Conservative MFI cut-off applied for binary classification
	Negative	Positive			Min	1st quartile	Median	Mean	3rd quartile	Maximum	
West Nile virus WNV E IgG	32	1	3	38	83	99	132	214	227	1135	k95 by Bayesian estimation
West Nile virus WNV E IgM	32	1	3	38	391	764	1074	1387	1878	4326	3680
Chikungunya virus CHIKV E1 IgG	28	5	15	48	77	213	509	1521	1561	10718	3000
Chikungunya virus CHIKV E1 IgM	32	1	3	48	334	796	1312	1530	1719	6274	4977
Sindbis virus SINV E1 IgG	25	8	24	55	93	241	684	1746	1712	8482	2000
California encephalitis virus (La Crosse) CEV NP IgG	29	0	0	72	83	100	122	136	151	488	1000
California encephalitis virus (La Crosse) CEV NP IgM	31	2	6	76	430	1291	1951	2326	2666	7812	6563
Retrovirus SC110 Retro E IgG	32	1	3	38	80	86	90	157	94	2285	1000
Retrovirus SC110 Retro E IgM	25	0	0	38	106	216	293	310	406	594	1187
Hervey pteropid *Gammaretrovirus* HPG E IgG	33	0	0	45	76	87	92	94	97	120	1000
Hervey pteropid *Gammaretrovirus* HPG E IgM	33	0	0	45	116	160	210	241	295	614	1000
Hervey pteropid *Gammaretrovirus* HPG E IgM	33	0	0	45							845
Brucella suis RepA IgG	29	4	12	26	94	144	315	1140	819	14906	2000
Brucella suis RepA IgM	33	0	0	26	553	1658	2018	3119	3667	9204	9900
Brucella abortus OMP IgG	30	3	9	28 or 55	80	120	153	536	372	6872	1323
Brucella abortus OMP IgM	30	3	9	55	697	1484	2247	2986	3140	12513	8339
Leptospira australis (whole organism) IgG	33	0	0	26	67	74	80	84	85	167	1000
Leptospira pomona (whole organism) IgG	33	0	0	77	65	73	76	83	83	182	1000

7.3.2.3.2.2 Non-diseased Horses as Sentinel serosurvey

Sample type, infectious disease likelihood category, vaccination status for 41 sera samples drawn from the same number of horse-subjects that were accrued as part of this research serosurvey to provide a comparative serology sample bank with least suspicion of infection with, or exposure to, target paramyxoviruses and other bat-borne RNA viruses are described in Table 7.48. Many of these sera were obtained at the time of routine veterinary health procedures such as dentistry and routine health checks, importantly without perceived association with overt infectious-like disease.

One foal subject sera included in this category in the main study application of the main study multiplex fluorescent microbead immunoassay was omitted from these summaries given its remarkably higher target bat borne paramyxovirus agent epidemiology and immunology context. This foal was 'out of' a HeV vaccinated mare and resided in paddocks with overt exposure to Grey-headed flying foxes occupying the same waterway associated roosts from which MenPV is understood to have spilled over to pigs previously. Perhaps unsurprisingly sera drawn from this foal, at approximately one month age, demonstrated seropositivity to IgG targeting HeV (7058) and NiV (6468) RBPs, was seronegative for IgM targeting RBPs of these and the other HNVs, was seronegative for IgG targeting all paramyxovirus NPs and RBPs yet demonstrated moderate seropositive for IgM targeting TioPV NP (1548).

Tables 7.49 – 7.54 present summary of binary test outcome and positive test outcome proportions for individual and logically combined antigens both in IgG and IgM for this sub cohort of comparatively lesser background geographical disease agent exposure and infectious-like disease sample event status to the priority Qld. HeV-like disease cohort and other infectious like disease sub-cohorts.

Results from this cohort are especially well suited to strengthening interpretations of IgM seropositivity amongst the Qld. HeV-like disease cohort and other infectious like disease sub-cohorts.

Table 7.48. Non-Infectious-like disease horse sub-cohort summary: non-diseased serosurvey, limited to WA samples – excluding unique NSW (foal) sample

HeV_vaccine_status	unvaccinated: 41
Sample	Serum: 41
Sample Reason	Serosurvey
State	WA: 41
infect_prior	5: 41
plating_unique_v_duplicate	single sample event: 41

Table 7.49

Non-diseased serosurvey sampled in WA, *Pararubulavirus* antigens – *excl. unique NSW (foal) sample*

	Binary test classification based on K95* Bayesian analysis		Proportion positive (%)	Antigen coupled bead No./s	Sub-cohort MFI distribution					
	Negative	Positive			Min	1st Quartile	Median	Mean	3rd Quartile	Maximum
TioPV NP IgG	40	1	2	30	112	145	248	297	444	1016
TioPV NP IgM	30	11	27	30	431	684	1115	1270	1485	4328
MenPV NP IgG	30	11	27	62 or 48	116	288	616	682	902	2307
MenPV NP IgM	38	3	7	63 or 48	523	778	1092	1559	1520	11846
Pararubulavirus NPs IgG combined	29	12	29							
Pararubulavirus NPs IgM combined	28	13	32							
MenPV sHN (RBP) IgG	40	1	2	28	123	140	152	164	170	503
MenPV sHN (RBP) IgM	39	2	5	28	2172	3264	4366	4411	4986	9748
	39	2	5	*prior7761 | **prior11462						
YepPV sHN (RBP) IgG	40	1	2	13	14	20	23	38	27	597
YepPV sHN (RBP) IgM	37	4	10	13	108	162	214	247	291	1158
GrovPV sHN (RBP) IgG	38	3	7	18	36	44	60	114	73	1064
GrovPV sHN (RBP) IgM	35	6	15	18	189	285	391	492.2	548	1460
Pararubulavirus sHNs (RBPs) IgG combined	38	3	7							
Pararubulavirus sHNs (RBPs) IgM combined	33	8	20							
Pararubulavirus NPs & sHNs (RBPs) IgG combined	39	2	5							
Pararubulavirus NPs & sHNs (RBPs) IgM combined	35	6	15							
Pararubulavirus NPs & sHNs (RBPs) IgM combined & -ve IgG	36	5	12							

Table 7.50

Non-diseased serosurvey sampled in WA, *Henipavirus* antigens – *excl. unique NSW (foal) sample*

	Binary test classification based on K95* Bayesian analysis		Proportion positive (%)	Antigen coupled bead no./s	Sub-cohort MFI distribution					
	Negative	Positive			Min	1st Quartile	Median	Mean	3rd Quartile	Maximum
HeV RBP IgG	38	3	7	28 & 43 ave.	78	95	102	125	132	298
NiV RBP IgG	40	1	2	46	168	191	226	221	234	400
NiV & HeV IgG combined	38	3	7							
unvaccinated NiV & HeV IgG combined	38	3	7							
HeV RBP IgM	36	5	12	43	192	246	317	392	416	1209
NiV RBP IgM	39	2	5	46	576	762	891	966	1052	2326
NiV & HeV IgM combined	36	5	12							
NiV & HeV IgM combined & -ve IgG	36	5	12							
unvaccinated NiV & HeV IgM combined	36	5	12							
CedPV RBP IgG	38	3	7	53	128	172	204	227	226	1055
CedPV RBP IgM	39	2	5	53	805	1638	2228	2597	3054	7002
NiV & HeV & CedPV IgG combined	37	4	10							
unvaccinated NiV & HeV & CedPV IgG combined	37	4	10							
NiV & HeV pos but CedPV neg IgG	40	1	2							
MojV RBP IgG	37	4	10	29	101	145	164	221	220	866
MojV RBP IgM	38	3	7	29	490	926	1304	1531	1849	4436
GhV RBP IgG	36	5	12	35	93	112	130	211	195	1200
GhV RBP IgM	38	3	7	35	436	582	728	889	969	2410
MojV & GhV RBP IgG combined	33	8	20							
MojV & GhV RBP IgG combined unvaccinated	33	8	20							
Non-prototypic HNVs IgG combined	32	9	22							
Non-prototypic HNVs IgG combined unvaccinated	32	9	22							
Non-prototypic HNVs IgM combined	35	6	15							
Non-prototypic HNVs IgM combined unvaccinated	35	6	15							
Non-prototypic HNVs IgM combined & -ve IgG	35	6	15							
NiV & HeV IgG combined but negative for Non-prototypic HNVs & unvaccinated	40	1	2							
NiV & HeV IgG combined but negative for MojV & GhV & unvaccinated	40	1	2							

Table 7.51. Non-diseased serosurvey WA, SARS-like CoV antigens	Binary classification prior to Bayesian estimation		Proportion positive (%)	Antigen coupled bead no./s	Sub-cohort MFI distribution						Conservative MFI cut-off applied for binary classification
	Negative	Positive			Min	1st quartile	Median	Mean	3rd quartile	Maximum	
MERS CoV IgG	41	0	0	15	14	15	19	53	30	14	1000
MERS CoV IgM	33	8	20	15	107	254	359	669	678	107	1196
SARS CoV-1 NP IgG	41	0	0	39	13	17	19	26	21	13	1000
SARS CoV-1 NP IgM	41	0	0	39	76	102	128	167	175	76	1000
SARS CoV-1 NP IgM	39	2	5	39							500
SARS CoV-1 NP IgM	40	1	2	65	1640	2196	3096	3348	3884	1640	6876
SARS CoV-2 NP IgG	41	0	0	42	52	55	63	99	67	52	1000
SARS CoV-2 NP IgM	41	0	0	42	247	351	419	459	514	247	1217
SARS CoV-2 NP IgM	38	3	7	42							811
SARS CoV-2 NP IgG	38	3	7	77	194	311	456	776	608	194	2000
SARS CoV-2 NP IgM	39	2	5	77	2234	3362	4381	5021	6069	2234	9867
rs4874 IgG	41	0	0	54	87	95	101	122	114	87	1000
rs4874 IgM	40	1	2	54	450	615	778	1035	1220	450	2372

Table 7.52 Non-diseased serosurvey WA, *Filovirus* antigens	Binary classification prior to Bayesian estimation		Proportion positive (%)	Antigen coupled bead no./s	Sub-cohort MFI distribution						Conservative MFI cut-off applied for Binary classification
	Negative	Positive			Min	1st quartile	Median	Mean	3rd quartile	Maximum	
Zaire ebolavirus EBOV NP IgG	37	4	10	72	107	188	342	520	540	3710	1323
Zaire ebolavirus EBOV NP IgM	39	2	5	72	803	1456	1756	2156	2580	6911	3861
Zaire ebolavirus EBOV EGP IgG	38	3	7	34	46	206	234	660	296	13838	1323
Zaire ebolavirus EBOV EGP IgM	38	3	7	34	605	980	1294	1746	1874	7040	3974
Bundibugyo ebolavirus BDBV EGP IgG	39	2	5	64	81	126	198	295.6	281	1990	1323
Bundibugyo ebolavirus BDBV EGP IgM	39	2	5	64	510	818	1078	1260	1285	4590	3285
Bombali ebolavirus BOMV EGP IgG	40	1	2	55	135	176	191	297	205	4392	1000
Bombali ebolavirus BOMV EGP IgM	41	0	0	55	394	511	563	624	734	1083	1844
Reston ebolavirus RESTV EGP IgG	40	1	2	72	26	31	34	125	43	2963	1000
Reston ebolavirus RESTV EGP IgM	38	3	7	72	1856	2885	3258	4086	4724	10508	8741
Marburg Marburgvirus MARV EGP IgG	41	0	0	37	39	50	54	81	71	458	1000
Marburg Marburgvirus MARV EGP IgM	38	3	7	37	301	584	717	851	1020	2644	1802
Marburg Marburgvirus RAVV EGP IgG	41	0	0	62	43	56	74	138	152	661	1000
Marburg Marburgvirus RAVV EGP IgM	38	3	7	62	172	344	493	612	738	1790	1304
Mengla dianlovirus MLAV EGP IgG	41	0	0	22	56	61	65	72	68	153	1000
Mengla dianlovirus MLAV EGP IgM	41	0	0	22	193	319	393	466	515	1151	1268
Lloviu cuevavirus LLOV EGP IgG	40	1	2	66	140	236	258	284	270	1281	1000
Lloviu cuevavirus LLOV EGP IgM	40	1	2	66	529	894	1080	1283	1518	4142	3086

Table 7.53

Non-diseased serosurvey WA, Borna Disease virus & Hepatitis E virus antigens	Binary classification prior to Bayesian estimation		Proportion positive (%)	Antigen coupled bead no./s	Sub-cohort MFI distribution						Conservative MFI cut-off applied for binary classification
	Negative	Positive			Min	1st quartile	Median	Mean	3rd quartile	Maximum	
Borna disease virus BoDV EGP IgG	43	7	14	48	201	566	944	1558	1458	8704	2573
Borna disease virus BoDV EGP IgM	40	1	2	73	3583	7009	8669	8704	9943	17946	17655
Borna disease virus BoDV EGP IgM	40	1	2								14713
Borna disease virus BoDV EGP IgM	38	3	7								11770
Orthohepevirus A CP HEV IgG	28	13	32	30	133	364	1051	2201	3172	13404	2480
Orthohepevirus A CP HEV IgM	36	5	12	64	1419	3104	5200	5952	7386	22792	10456
	11	30	73								3306
	3	38	93								2480

Table 7.54

Non-diseased serosurvey WA, Arbovirus and other antigens	Binary classification prior to Bayesian estimation		Proportion positive (%)	Antigen coupled bead no./s	Sub-cohort MFI distribution						Conservative MFI cut-off applied for binary classification
	Negative	Positive			Min	1st quartile	Median	Mean	3rd quartile	Maximum	
West Nile virus WNV E IgG	40	1	2	38	82.0	100	136	233.4	181	2868	Post k95 by BLCA
West Nile virus WNV E IgM	41	0	0	38	840	1189	1460	1636	1918	3578	3680
Chikungunya virus CHIKV E1 IgG	36	5	12	48	84	221	545	1476	1874	8632	3000
Chikungunya virus CHIKV E1 IgM	39	2	5	48	735	1382	2032	2234	2648	6069	4977
Sindbis virus SINV E1 IgG	33	8	20	55	112	260	552	1331	1474	10448	2000
California encephalitis virus (La Crosse) CEV NP IgG	32	3	9	72	84.0	95.5	104	329.7	160	4172	1000
California encephalitis virus (La Crosse) CEV NP IgM	41	0	0	76	1167	1582	1914	2202	3019	4398	6563
Retrovirus SC110 Retro E IgG	40	1	2	38	81	86	88	124	92	1260	1000
Retrovirus SC110 Retro E IgM	39	2	5	38	195	322	410	498.2	547	2079	1187
Hervey pteropid *Gammaretrovirus* HPG E IgG	41	0	0	45	82	86	90	97.88	92	442	1000
Hervey pteropid *Gammaretrovirus* HPG E IgM	41	0	0	45	156	234	271	292	298	722	1000
Hervey pteropid *Gammaretrovirus* HPG E IgM	41	0	0								845
Brucella suis RepA IgG	38	3	7	26	96	137	254	615	660	3476	2000
Brucella suis RepA IgM	39	2	5	26	1003	2711	4256	4733	6277	11959	9900
Brucella abortus OMP IgG	36	5	12	28 or 55	92.0	151	247	667.6	538	7622	1323
Brucella abortus OMP IgM	35	6	15	55	1867	3350	4458	5383	6989	17283	8339
Leptospira australis (whole organism) IgG	40	1	2	26	65.0	72.0	78.0	191.1	84.0	4680	1000
Leptospira pomona (whole organism) IgG	40	1	2	77	66.0	70.0	74.0	187.7	81.0	4610	1000

7.3.2.3.3 Filoviruses

A selection of significant serological results (raw MFI) of testing from the filovirus panel provided by the USU (Table 7.2) (36) are shown below (Table 7.55). The background MFI values for the mock antigen were below 200. The MLAV antigen did not show any reactivity with antibodies present in the blood of any of the horses shown below. Antibodies to the EBOV G protein were observed for 16 of the horses, with the highest one having an MFI of above 20,000. This serum (p17-1362xx) also showed reactions to the BoMV (7206), LLOV (4534) and RESTV (6652) proteins.

Specific reactions to only the EBOV protein were observed for nine serum samples. Specific reactions were only observed to BDBV protein in five blood samples. Multiple sera displayed cross-reactions to at least four of the filoviruses such as P17-145aa positive for EBOV, MARV, BoMV, LLOV and RestVP while highest for EBOV. Interpreted together with similar seropositivity for Ebolavirus group filoviruses in flying foxes in Australia(23) and internationally(20) supports that filoviruses are circulating amongst flying foxes in Australia and resulting in sporadic spillover infection in horses.

Table 7.55. Filovirus reactivities with the panel of antigens from Uniformed Services University with raw MFI values*

Sample	Mock	MLAV (1000)	EBOV (1323)	MARV (1000)	BoMV (1000)	RAVV (1000)	BDBV (1323)	LLOV (1000)	RestVP (1000)
p15_135xx	66	117	**6475**	44	**2024**	60	116	741	**1648**
p18_45xx	86	65	**6502**	58	250	112	692	254	88
p17_141xx	102	62	**5848**	94	200	481	**4383**	244	38
p17_141xx	169	533	**6120**	54	**2629**	172	935	**1887**	**2547**
p17_141xx	155	655	**6939**	53	**3089**	174	1061	**2208**	**2904**
p17_1362xx	151	963	**20171**	144	**7206**	66	553	**4534**	**6652**
BM20-6.2xx	145	70	**14013**	72	**2290**	134	**2273**	749	**1411**
BM 7-2	139	63	**4402**	41	331	90	997	243	215.5
p17_172xx	166	62	**6124.5**	73	530	173	**1900**	300.5	554
BM2020-1 xx	154	76	**12332**	142	591	63	310	306	899
p18_3xx	133	77	**1539**	86	436	136	**4898**	320	332
p18_49xx	105	66	285	53	209	965	**5019**	257	33
17-01670-x	95	152	**13838**	44	**4391**	45	122	**1281**	**2963**
p17_145xx	120	66	**3328**	213	187	100	**1547**	243	101
p17_145aa	83	**1235**	**14352**	**3031**	**4485**	47	523	**3476**	**2899**
p17_137xx	85	143	**8541**	376	**1608**	**4262**	**6311**	577	**1079**
p18_1xx	89	61	277	62	197	71	**7531**	248	28
p17_155xx	113	71	256	103.5	198	104	**3315**	261	38
p17_144xx	126	71	225	252	182	**2075**	**5498**	243	36
p17_144xx	92	320	**6336**	51	**2623**	164	794	**1241**	**2140**
p17_135xx	102	115	**9239**	115	**1715**	**1438**	**7563**	659	**1294**

*Prior cut-offs for positive test classification (see table 7.52) are included in brackets under each antigen in column headings and subject sera results exceeding these are shown in bold.

7.3.2.3.4 SARS coronaviruses

The SARS-2 CoV nucleoprotein (N) antigen was selected due to the known cross-reactivity that the N protein usually displays; thus it can be utilised to detect antibodies targeting coronavirus in sera from mammals. Nine blood samples had detectable coronavirus antibodies to the N protein, with the highest one having an MFI of 12,500 (Table 7.56). Interestingly, paired sera collected three months apart from one horse-subject, that suffered infectious-like neurological disease amongst the 2020 South Australian cluster, showed an increase in MFI when tested in parallel from 4404 to 9783 (Table 7.56).

Table 7.56. Horse samples showing seropositivity to SARS2 CoV-2 nucleocapsid protein*

Sample	SARS2 N MFI (prior cut-off set at 2000)
p18_45xx	**5774**
p17_140xx	**3091**
p17_140xx	**4411**
BM20 6-a 21/4/20	**4404**
BM20 6-b 16/7/20	**9783**
p17_172xx	**8394**
p18_3xx	**5455**
p17_168xx	**12500**
17-1670-xx	**5308**

*Prior cut-offs for positive test classification (see table 7.52) are included in brackets under each antigen in column headings and subject sera results exceeding these are shown in bold (all included).

7.3.2.3.5 Hepatitis E virus

Hepatitis E virus (HepE) is a potentially zoonotic virus that is transmitted via water or food contaminated with faecal matter, causing liver disease in susceptible mammals. This virus has previously been detected in horses in Egypt,(37) Spain(38) and Korea.(39) In Australia, HepE has been associated with liver disease in a human following consumption of uncooked pork.(40) Serological evidence of disease in pigs(41) and recent molecular detection recently in rabbits has provided further evidence of HepE circulation in Australia.(42) The HepE recombinant protein used to determine the seroprevalence of the virus in rabbits(43) was used to determine the seroprevalence of HepE in horses. Reactive IgG antibodies to HepE

were detected in 52 of the 418 horse blood samples tested, representing a seropositivity of 12% (Table 7.59). A selection of significant serological results (raw MFI) of testing with the HepE virus capsid protein (Table 7.2) are shown below (Table 7.57). This represents the first evidence of HepE virus infection in horses in Australia.

Interestingly, as well as being associated with hepatic disease, this virus has been associated with neurological disease in humans (reviewed in 2012 by Cheung et al.)(44) and infection has been associated with the handling of horse manure.(45)

Table 7.57. Horse samples with detectable IgG to the capsid protein of Hepatitis E virus

Sample	Hepatitis E virus capsid MFI (prior cut-off set at 2573 MFI)
p15_133xx	5490
p18_46xx	6985
p18_42xx	8870
p18_44 xx	6763
p18_51 xx	4330
p18_51 xx	18094
p18_51 xx	20793
p18_46 xx	10620
p17_139 xx	18013
17-01670- xx	4580
p18_3 xx	4011
6-11	5490
BM2020-1 xx	7658
p17_140xx	9980
p17_140xx	12838
p17_172xx	4402
BM2020-1 xx	13106
17-01670-x	5436
17-01670-x	13404
17-01670-xx	4068
17-01670-xx	6569
p17_157xx	6669
p17_157xx	5540
p17_162xx	4152
p17_137xx	6441
17-01670-xx	5188
p17_167xx	10752
17-1670-xx	9302
17-1670-xx	6498
p17_155xx	5427
p17_154xx	16724
17-1670-xx	4004
p17_135xx	13382

7.3.2.3.6 Bornavirus

Borna viruses, belonging to the *Bornaviridae* family of the order Mononegavirales, are known to cause encephalitic disease in horses. The bornaviral envelope protein used in this assay was obtained commercially, and the reactions observed require further testing for confirmation. Twenty-one horses had detectable antibodies against the Bornavirus recombinant protein with the highest MFI at 11,966.5 (Table 7.58).

Table 7.59 presents proportions HepE seropositivity determined by single prior cut-off estimation for IgG and multiple for IgM for the full main study cohort as well as sub cohort positive proportions for IgG and IgM (including for each varied IgM prior MFI cut-off).

The only prior evidence of borna disease virus infection in Australian horses was a recent seroepidemiology survey of NSW horses (See Chapter 2).(46) These findings interpreted together with this sole published study support that bornaviruses likely spill over from small mammal wild reservoirs to horses in Australia, similarly as for where ever they have been detected globally.

Table 7.58. Horse samples showing seropositivity to Bornavirus

Sample	Borna MFI
p18_49xx	11966
17-01670-xx	5205
p17_135xx	5160
p17_141	5758
p18_3xx	5333
p17_157xx	4784
17-01670-xx	4789
p17_157xx	7433
p17_157xx	5834
p17_157xx	7285
p17_173xx	8799
p17_163xx	5265
p18_1xx	4997
p17_148xx	8360
p17_148xx	5073
p17_154xx	5253
17-1670-xx	8704
p17_144xx	4184
p17_133xx	5151
17-1670-xx	5549
p17_149xx	8745

Table 7.59. Overall main study and sub cohort results summaries and seropositive proportions for IgG and IgM targeting Borna disease virus

Borna disease virus BoDV EGP IgG (cut-off 2573 MFI)	Negative	Positive	Prop. positive (%)	Sub cohort total tested	Overall proportion positive (%)
HeV-like disease Qld 2015 - 2018	275	31	10	306	
HeV spillover event NSW 2019	7	1	13	8	
HeV or other viral-like suspect infectious disease NSW	7	2	22	9	
Acute neuro disease cluster SA 2020	10	1	9	11	
HeV tested without disease Qld 2015 - 2018	23	10	30	33	
Non-diseased serosurvey sampled in WA	43	7	14	50	
Non-diseased 1 month old foal NSW	1	0	0	1	
totals	366	52		418	12
Borna disease virus BoDV EGP IgM (for most conservative prior cut-off: 17655 MFI)					
HeV-like disease Qld 2015 - 2019	298	3	1	301	
HeV spillover event NSW 2020	8	0	0	8	
HeV or other viral-like suspect infectious disease NSW	9	0	0	9	
HeV tested without disease Qld 2015 - 2019	33	0	0	33	
Acute neuro disease cluster SA 2021	11	0		11	
Non-diseased serosurvey sampled in WA	40	1	2	41	
Non-diseased 1 month old foal NSW	1	0	0	1	
totals	400	4		404	1
Borna disease virus BoDV EGP IgM (for mid-level prior cut-off: 14713 MFI)					
HeV-like disease Qld 2015 - 2020	294	7	2	301	
HeV spillover event NSW 2021	8	0	0	8	
HeV or other viral-like suspect infectious disease NSW	9	0	0	9	
Acute neuro disease cluster SA 2022	9	2		11	
HeV tested without disease Qld 2015 - 2020	33	0	0	33	
Non-diseased serosurvey sampled in WA	40	1	2	41	
Non-diseased 1 month old foal NSW	1	0	0	1	
totals	394	10		404	2
Borna disease virus BoDV EGP IgM (for lower-conservative prior cut-off: 11770 MFI)					
HeV-like disease Qld 2015 - 2021	287	14	5	301	
HeV spillover event NSW 2022	8	0	0	8	
HeV or other viral-like suspect infectious disease NSW	8	1	11	9	
Acute neuro disease cluster SA 2023	9	2		11	
HeV tested without disease Qld 2015 - 2021	32	1	3	33	
Non-diseased serosurvey sampled in WA	38	3	7	41	
Non-diseased 1 month old foal NSW	1	0	0	1	
totals	383	21		404	5

7.4 Discussion

In response to an infection, the first antibodies produced are immunoglobulin M (IgM), which generally occurs at day 5 post infection. As the immune system matures, IgG antibodies are produced that are more specific to control the infection. The presence of G (IgG) in the blood or serum of a mammal is usually considered to be an indication of past infection, where the presence of IgM is considered to be indicative of a recent infection (Figure 7.23).

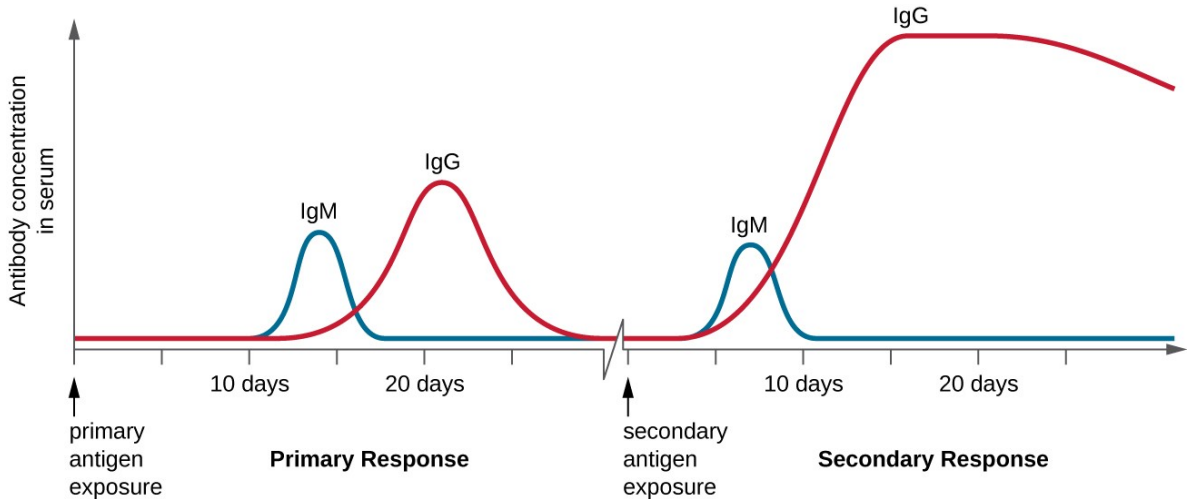

Figure 7.23. Schematic of IgG and IgM antibody responses associated with primary and secondary exposure to viral antigens. Source: Lumenlearning.com – B Lymphocytes and Humoral Immunity | Microbiology (https://www.coursehero.com/study-guides/microbiology/b-lymphocytes-and-humoral-immunity/)

Luminex bead-based immunoassays have many advantages, including the potential for multiplexing – simultaneously using multiple related viral and bacterial proteins to detect antibodies present in sera and blood increasing the potential for interpretation of seropositivity relative to related agent antigens. Multiple proteins of same viral groups can be multiplexed to improve the positive predictive value and help to determine which virus is most likely to be the virus to which the subject was exposed and mounted immunity. The ability to use very small sample volumes allows for use in wildlife species and smaller animals, but also allows larger volumes of sera to be preserved for confirmatory assays and other purposes. This includes use as positive control sera of the target species in further assays. The use of the combination of biotinylated Protein A and G allows for the detection of IgG antibodies to a range of infectious agents. These assays can therefore be easily adapted for the detection of IgG antibodies in a wide range of mammals such as humans, equines, bats,

rodents, bovines, canines, caprines, felines, ovines, porcines and lagomorphs. including humans, horses, bats and livestock. The machine counts between 50 to 100 beads to give an MFI, and this gives a statically accurate result.

7.4.1 Discussion on approaches to cut-off estimation and performance assessment of assay for best interpretation in absence of gold standard.

Information theory approaches to assessment of test performance and optinal binary outcome cut-off for application to continuous biomarker results (including estimation of receiver operating characteristic (ROC) curve averaged on more than 100,000 simulations and Predictive receiver operating characteristic (PROC) curves) differ from more traditional approaches by allowing for evaluation of key parameters defined conditionally on test outcome rather than actual true pathogen exposure status. These approaches are highly appropriate when confirmation of exposure status is not practical or possible, such as in the absence of a gold standard.

The study population, approach and purpose of this study met these criteria on a number of counts. Firstly, testing targeted emerging and divergent viruses beyond those for which fully validated assays are available. Secondly the epidemiology of emerging bat-borne viruses features sporadic and complex spillover dynamics and pathways that are not verifiable or directly observable. Thirdly, henipaviruses and pararubulaviruses of pteropid origin are considered highly biohazardous (level 3 and 4 biocontainment requirements), with high cost and risk involved in culturing live virus. Incidentally, probabilistic Bayesian approaches applying information theory to infectious disease diagnostics and epidemiology are understood to offer the most accurate interpretation of empirical data, allowing for superior inference of desired parameters applying to a target population. (7,47)

7.4.2 Current limitations but great potential for more use

The assays that were developed in this research had variable validation. There has been a long history of work with the HeV sG protein(28,48–50) and optimisation of the paramyxoviruses and filovirus proteins.(36) However, the other proteins used in this study were acquired for this work and had varying amounts of validation, depending on the availably of samples for assessment. In cases where the results have produced interesting preliminary findings, further testing is required to confirm the observed reactions.

7.4.3 Final interpretation of results in the context of emerging zoonotic disease significance

The route of transmission of bat-borne pararubulaviruses is assumed to be like that of HeV and other emerging paramyxoviruses in flying foxes − via aerosol droplet inhalation during inquisitive grazing behaviour, coupled with a high surface area in the upper respiratory tract. This behaviour and anatomy may provide horses greater opportunity for spillover virus exposure/infection. Epidemiologically, if this is occurring, we would be most likely to see significant exposure in a minority of horses with perceived proximity to flying fox colonies and feeding habitats.

Based on the geographic overlap of flying fox populations, the epidemiology is consistent with sporadic and low frequency spillover. The findings included an abundance of convincing negatives and low reactivity consistent with minimal or no exposure in the majority of the horse population but consistent proportion of clear high antibody positives consistent with sporadic exposure and infection as for HeV. This is in contrast to the results of these same assays in flying foxes, (27) in which a far higher proportion of the population will be expected to have some past exposure and antibody levels (and consequently MFIs). Thus, the variation in MFI would be expected to relate to time since exposure and we would expect a wider distribution of lower MFI positives.

From a biobank of approximately 1,700 cases from Queensland, collected between 2015 and 2018, 306 cases were selected based on their clinical manifestations (reported on laboratory submission forms in variable detail) consistent with the disease presentation of HeV (See Chapters 3, 5 and 6). This research made parallel molecular detections of divergent paramyxoviruses, including the novel HeV variant (HeV-g2) (Chapter 8), (2) canine morbilliviruses, pararubulavirus-like viruses, and a further divergent virus, most closely related to both henipaviruses and pararubulaviruses. These findings were made among minimally invasive samples (blood and swabs) rescued from government-based disease investigations. Considering the molecular detections of paramyxoviruses, the interpretation of the reactions in the Luminex immunoassay and in the IFA observed may represent exposure to known and unknown related agents (i.e. cross-reactivity). Cross-reactivity is especially likely for both IgG and IgM directed against the viral NP antigens (somewhat less

so for envelope proteins and RBPs), and in IgM assays in general (given the less specific antigenic binding of these larger immune molecules).

Most of the novel paramyxoviruses occurred sporadically across the cohort and were mostly present in blood and respiratory samples, and occasionally stools. This trend was also observed for the Bovine coronavirus.

Indeed, detection of IgM seropositivity for pararubulavirus NPs and MenPV sHN among horses sampled in association with PCR confirmed HeV infection (sub-cohort HeV event NSW 2019) confirms the potential that some seropositivity targeting these pararubulavirus proteins may result from alternative related viral exposure. This is especially so for the nucleocapsid proteins (NPs) that are more highly conserved between related viruses. Further infectious disease surveillance-based research in both wild and domestic species will provide crucial improved understanding of immuno-biology, ecology, transmission, animal disease, One Health and biosecurity risks relating to these viruses that circulate among Australian flying foxes and might spill over to horses similarly as for HeV.

Similarly, the IgM assay (as for IgM assays in general) exhibited higher levels of cross reactivity than the IgG equivalent using the same antigens. The Luminex microbead immunoassay approach supported extensive multiplexing to efficiently run parallel assays for a range of differential diagnoses and related viruses. This approach allowed for both proactive and conservative interpretation and minimised the misinterpretation of cross-reactive positivity.

Nevertheless, the assays performed remarkably as AUC and ROC descriptions of test performance via the information theory approach of Bayesian estimation utilising informed distribution models over more than 100,000 Markov Chain Monte Carlo simulations. Conclusive seronegativity was seen in majority frequencies expected given the epidemiological understanding of targeted viral ecology and conceivable transmission pathways. Similar optimisation of equivalent microbead immunoassays using the same antigenic proteins on sera from other mammalian species, including flying foxes, rabbits, pigs and humans in related research in the same CSIRO laboratory has demonstrated similarly diagnostic performance and coherency of findings. (11, 42-43) Many known individual case epidemiological contexts aligned with seropositivity. This included IgM targeting the HeV and NiV RBP in sera from horses sampled in temporal association with both primary and booster

HeV vaccination amongst the nonclinical disease cohort, demonstrating IgM positivity to HeV and NiV, as expected. Many positive IgM antibodies detections aligned with high known exposure to flying foxes and/or disease manifestations and outbreaks consistent with infectious causes. Ideally, the positive detections would be further characterised with suitable confirmatory assays; however, there was insufficient time for this to be done within the project.

Additional RNA sequencing findings performed in parallel to this serology highlighted that novel Nidovirus and equine rhinitis viruses are highly common respiratory pathogens. This is not unexpected for the rhinitis viruses (analogous to human rhinoviruses in humans), however, the abundance and unknown disease potential for the novel Nidovirus suggests urgent follow up work is required to understand the role of this virus in HeV-like disease in horses. Furthermore, the equine hepaci- and pegiviruses were found to be common blood pathogens in Australian horses, and more should be done to understand their contribution to disease. The novel Astrovirus and Norovirus identified in a case featuring acute severe diarrhoea along with molecular detection of divergent paramyxoviruses in cases featuring pleuropneumonia- like signs highlight the need to consider such significant RNA viral causes in these two relatively common disease syndromes that have hitherto been attributed to stress (such as from travel) and bacterial cause.

The Filovirus serology findings highlight the likelihood that one or more of these viruses circulating among Australian flying foxes indeed sporadically transmit to horses. The disease significance of such infections has not yet been possible to characterised in this study and neither has molecular detection and characterisation occurred. It is likely that the sample type and tissue will greatly influence the detectability of these viruses (and many others). Recent clarification that filoviruses maintain persistent replication in testicular tissue in humans (empirical),(55-56) non-human primates(57) and bats(58-59) (both experimental/ laboratory), pose potentially profound significance both for epidemiology and diagnostic testing. This perspective prompts consideration that targeted active surveillance efforts extending from routine disease investigations and other opportunistic sample bio-banks might be best informed by potential for variable and select tissue tropism. It is plausibility that a gonadotropic tissue tropism/ latency effect may be potentiating covert reservoir maintenance among the putative Australian flying fox hosts - and/or among other, hitherto

unrecognised, mammalian reservoirs - failing molecular detection to date. As such, testing testicular tissue/ and/or semen such as might be collected via epididymal harvest postmortem (as performed in deceased stallions for cryopreservation) in Flying Foxes, suspect horse disease cases and other species, might offer greater chance of molecular detection and characterisation, which would in turn open possibility for further interpretation of One Health and biosecurity significance of these findings and the recently reported serological evidence for circulation among Australian Flying-foxes (23) and the emerging filovirus epidemiology internationally.(20,36,54) Indeed studies have found MARV and EBOV antigen in experimentally infected bat testes, in the absence of histological changes.(55,56) Interestingly, among the growing number of metagenomic studies of bats, filoviruses are uncommonly detected – comprising ~1% of all bat viruses detected (~30% are coronaviruses).(60)

Taken together, this raises an important question, of whether or not the right tissues for detecting nucleic acid are sampled and tested, across a broad range of emerging multi-species viral contexts of one Health significance… Given many target viral agents manifest with only-transient viremia (Flaviviruses like JEV for eg.),(61) and many virus-host infection patho-physiology feature organ-specific specialised immunity (privileged), such as testes, liver and eyes, does risk-based sampling and research testing supporting routine biosecurity, need to focus on sights of viral persistence? Certainly in the complex, as yet understood epidemiology of Filoviruses and their potential spillover from Flying foxes to horses and other mammals, highlight these potential diagnostic limitations of sample type,(62) confounded by expected rare/ sporadic occurrence only for spillover infections (as for HeV and ABLV), with likelihood of relatively lower and widely varying disease morbidity - as for Reston for example – further reducing likelihood of spontaneous suspect case investigation all confound as barriers to molecular detection and progressing critical understanding to guide biosecurity and One health risk management. Another great example of the importance of iterative problem-focused and hypothesis driven, transdisciplinary scientific undertakings in close integration with numerous procedural and system actors across broad ranging disciplines and sectors

The findings also highlight the need for paired serology sampling (initial and convalescent samples) in horses, greatly support the interpretation of significance of disease and biosecurity in case findings. The paired sample cases available to this study (both in the pilot

testing and the few in the large cohort) proved highly informative in diagnostic interpretations. Veterinarians in equine practice are less familiar with the extent of diagnostic benefit of paired sera for infectious causes, compared with production animal species contexts, in which herd testing is common. Similarly, the acutely fatal context of HeV (both naturally resulting from disease and veterinary euthanasia justified by moribund condition) limit the availability of convalescent sera that would allow greater application and interpretation of serology supporting the extent of molecular biosecurity testing as part of government laboratory investigations of horses with HeV-like disease.

This innovative serology example (as for the molecular activities of this research) highlights the suitability of utilising horse disease and serosurveys to inform One Health epidemiology and biosecurity. The study also highlights the highly suitable sentinel function of horses for early detection of viruses of human health significance circulating in often seasonal wildlife reservoirs.

7.5 Conclusion

Over 1,000 Australian horses are investigated for Hendra virus (HeV) annually, with very few (<1%) found positive.(1) Other bat-borne viruses with potential zoonotic consequences could spill over to horses and pose a risk to humans. Menangle virus (MenPV) (*Paramyxovirus, Pararubulavirus*) caused severe reproductive failure in pigs and influenza-like illness in two humans (1997),(51) and was subsequently isolated from flying fox urine along with novel related paramyxoviruses.(52) Here we described serological evidence for natural exposure to emerging and divergent bat-borne henipaviruses and pararubulaviruses in Australian horses, along with seroconversion and IgM detection in an outbreak of severe respiratory illness. We also described the detection of antibodies to filoviruses in horses. This further justifies routine and research sentinel surveillance for emerging infectious diseases beyond the limited range of established pathogens. Future research should determine the significance of pararubulavirus spillover to horses and associated zoonotic risk.

We highlighted the potential of optimised syndromic surveillance for emerging zoonoses and One Health benefit of horses as sentinels of emerging infectious disease. Future research

should determine the significance of bat-borne pararubulavirus spillover to horses and any associated human health risks.

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Chapter 8

NOVEL HENDRA VIRUS VARIANT DETECTED BY SENTINEL SURVEILLANCE OF AUSTRALIAN HORSES (PUBLISHED MANUSCRIPT)

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Transcript also amended to this thesis as **Appendix 4: US CDC EID Journal Podcast interview transcript**

Novel Hendra Virus Variant Detected by Sentinel Surveillance of Horses in Australia

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We identified and isolated a novel Hendra virus (HeV) variant not detected by routine testing from a horse in Queensland, Australia, that died from acute illness with signs consistent with HeV infection. Using whole-genome sequencing and phylogenetic analysis, we determined the variant had ≈83% nt identity with prototypic HeV. In silico and in vitro comparisons of the receptor-binding protein with prototypic HeV support that the human monoclonal antibody m102.4 used for postexposure prophylaxis and current equine vaccine will be effective against this variant. An updated quantitative PCR developed for routine surveillance resulted in subsequent case detection. Genetic sequence consistency with virus detected in grey-headed flying foxes suggests the variant circulates at least among this species. Studies are needed to determine infection kinetics, pathogenicity, reservoir-species associations, viral-host coevolution, and spillover dynamics for this virus. Surveillance and biosecurity practices should be updated to acknowledge HeV spillover risk across all regions frequented by flying foxes.

Highly pathogenic zoonotic Hendra virus (HeV) and Nipah virus (NiV) are prototypic members of the genus *Henipavirus*, family *Paramyxoviridae*, that have natural reservoirs in pteropodid flying foxes (*1*). These viruses exhibit wide mammalian host tropism, cause severe acute respiratory and encephalitic disease mediated by endothelial vasculitis, have high case-fatality rates, and cause chronic encephalitis among survivors (*2*–*4*). By March 2021, a total of 63 natural HeV spillovers had been recognized in horses in Australia,

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resulting in 105 horse deaths (*5*,*6*) and 4 deaths among 7 confirmed human cases (*7*). In southern Asia, NiV has caused zoonotic outbreaks with 70%–91% case-fatality rates, resulting in >700 human deaths (*8*–*10*). In response to the fatal disease threat posed by henipaviruses to humans and domestic animals, vaccines and postexposure prophylaxis (PEP) have been developed (*11*). A subunit vaccine, Equivac HeV (Zoetis, https://www.zoetis.com.au), based on the soluble recombinant G-attachment glycoprotein (receptor-binding protein [RBP]) of HeV (HeV-sG), that has been used for horses in Australia since 2012 (*12*). The human monoclonal antibody (mAb) m102.4 has been administered as emergency PEP in 16 human cases and has demonstrated safety, tolerability, intended pharmacokinetics, and no immunogenicity in a phase 1 trial (*13*). Combinations of cross-reactive humanized fusion (F) protein and RBP mABS have also been described for clinical development as PEP (*14*–*16*), and a human vaccine using HeV-sG is now in phase 1 clinical trials (*17*).

Horses are the predominant species known to be infected with HeV by natural spillover from flying foxes; 2 canine (*18*) and all known human infections having resulted from close contact with infected horses. HeV transmission from *Pteropus* spp. (flying foxes) to horses is thought to occur primarily through contaminated urine (*19*). The spatial distribution of previously detected spillovers to horses and molecular HeV testing of flying fox urine suggested that transmission was predominantly from black flying foxes (BFF; *P. alecto*) and spectacled flying foxes (SFF; *P. conspicillatus*) (*19*,*20*). However, serologic testing has detected antibodies to HeV or related henipaviruses among all 4 flying fox species in Australia (*20*–*23*). Of note, seroprevalence of IgG targeting the HeV RBP has been reported in 43% of grey-headed flying foxes (GHFF; *P. poliocephalus*) in South Australia and Victoria (*22*) and 60% (169/284) in southeastern Queensland (*21*).

Australia hosts >1 million horses. Their grazing behavior, large respiratory tidal volume, and extensive highly vascularized upper respiratory epithelium may contribute to their vulnerability for HeV spillover (*23*). Detecting spillover to horses relies on attending veterinarians recognizing clinical manifestations consistent with HeV disease, sampling appropriately, and submitting samples for priority state laboratory testing (*24*). Passive surveillance using suspected disease testing is affected by a strong regional bias for areas where HeV has previously been detected and where domestic horse populations overlap with BFF distribution ranges, from eastern coastal Queensland to northern New South Wales (*25*). Testing for HeV is less commonly performed on horses with similar disease manifestations farther south in Australia because of a perception that spillover infection is less likely to occur in regions without BFF (*26*). Among >1,000 horses with manifestations consistent with HeV disease tested annually across regions of established risk, <1% are found to be positive (*25*,*27*).

Routine testing for equine HeV infection as part of priority disease investigation is specific for the matrix (M) gene (*28*). Additional nucleoprotein (N) gene–specific testing (*29*) is limited to HeV-positive samples that undergo confirmatory testing (*30*) or in the minority (<7% nationally) of suspected equine HeV cases submitted directly to the national reference laboratory from states where spillover is considered less likely (*25*) and state testing is unavailable. This distinction is notable because it means that most horse-disease cases found negative for HeV are not investigated further, despite evidence that other viruses with potential spillover risk to horses, including novel related batborne paramyxoviruses, circulate in Australia (*27*,*31*–*35*). Likewise, animal health surveillance worldwide prioritizes targeted testing to exclude pathogens of established importance over open-ended diagnostic approaches, which are inherently more challenging to put in place and interpret.

Employing a transdisciplinary, interagency approach combining clinical-syndromic analysis and molecular and serologic testing, we explored the hypothesis that some severe viral disease–like manifestations in horses that are consistent with HeV, despite the horse testing negative, could be caused by undetected spillover of novel paramyxoviruses from flying foxes that potentially pose similar zoonotic risk. Here we report the identification of a previously unrecognized variant of HeV (HeV-var), circulating as a second genotype lineage (HeV-g2), clinically indistinguishable from prototypic HeV infection, that resulted in severe neurologic and respiratory disease in a horse.

Materials and Methods

Study Cohort

A biobank of diagnostic specimens collected in Queensland during 2015–2018 was developed from horses that underwent quantitative reverse transcription PCR (RT-PCR) testing but were negative for HeV (*28*). We recorded clinical, epidemiologic, and sample-related data, including vaccination status and perceived exposure to flying foxes (inconsistently reported by submitting veterinarians). All samples were archived at –80°C. We applied a decision algorithm based on systematic interpretation of pathologic basis and syndromic analysis of clinical disease

descriptions to categorize each case by likelihood of infectious viral cause (Appendix Table, https://wwwnc.cdc.gov/EID/article/28/3/21-1245-App1.pdf). We plated samples (EDTA blood, serum, nasal swab, rectal swab) from cases assigned priority category 1 or 2 status, considered as having the highest likelihood of infectious cause, for serologic screening and high-throughput nucleic acid extraction using the MagMAX mirVANA and CORE pathogen kits (ThermoFisher, https://www.thermofisher.com).

Pan-paramyxovirus RT-PCR Screening

We prepared cDNA from extracted RNA using Invitrogen SuperScript IV VILO Master Mix with ezDNase (ThermoFisher). A nested RT-PCR assay targeting the paramyxovirus L protein gene was adapted using primers developed elsewhere (*36*) and an AllTaq PCR Core kit (QIAGEN, https://www.qiagen.com). We identified amplicons corresponding to the expected size (584 bp) by gel electrophoresis before purification with AMPure XP (Beckman Coulter, https://www.beckmancoulter.com). To capture any weak detections, we also prepared pools by equal-volume mixing all PCR products across plated rows. We performed next-generation sequencing using an Illumina iSeq with the Nextera XT DNA library preparation kit (Illumina, https://www.illumina.com). For analysis, we assembled reads with MEGAHIT (*37*) before identifying them by comparison to GenBank entries using BLAST (*38*).

HeV-var Whole-Genome Sequencing

We subjected samples positive for HeV-var by paramyxovirus RT-PCR to meta-transcriptomic sequencing to determine the complete genome sequence and identify any co-infecting agents. RNA was reverse transcribed with Invitrogen SSIV VILO Master Mix (ThermoFisher) and FastSelect reagent (QIAGEN). We performed second-strand synthesis with Sequenase 2.0 (ThermoFisher) before DNA library preparation with Nextera XT (Illumina) and unique dual indexes. We performed sequencing on an Illumina NextSeq system to generate 100 million paired reads (75 bp) per library.

Assembly and Comparative Genomic and Phylogenetic Analyses

For genome assembly, we trimmed RNA sequencing reads and mapped them to a horse reference genome (GenBank GCA_002863925.1) using STAR aligner to remove host sequences. We assembled nonhost reads de novo with MEGAHIT (*37*) and compared them with the GenBank nucleotide and protein databases using blastn and blastx (*38*). We extracted the putative virus contig and remapped reads to this draft genome using bbmap version 37.98 (https://sourceforge.net/projects/bbmap) to examine sequence coverage and identify misaligned reads. We extracted, aligned, and annotated the majority consensus sequence by reference to the prototype HeV strain using Geneious Prime version 2021.1.1 (https://www.geneious.com) and submitted it to GenBank (accession no. MZ318101).

For classification, we aligned the paramyxovirus polymerase (L) protein sequence according to International Committee on Taxonomy of Viruses (ICTV) guidelines (*39*). We prepared alignments of partial nucleocapsid (N) and phosphoprotein (P) nucleotide sequences with known HeV strains from the GenBank database. Phylogenies were prepared using a maximum likelihood approach in MEGA X (https://www.megasoftware.net) according to the best-fit substitution model and 500 bootstrap replicates.

Quantitative RT-PCR Development

We adapted quantitative RT-PCR targeting the HeV M gene (*28*) to target HeV-var. The duplex assay used the Applied Biosystems AgPath-ID One-Step RT-PCR kit (ThermoFisher), and distinguishes prototype and variant HeV strains. In brief, we combined 4 µL RNA with 10 µL 2× RT-PCR buffer, 0.8 µL 25× RT-PCR enzyme mix, 2 µL nuclease-free water, and 3.2 µL primer/probe mix (0.6 µL each primer, 0.3 µL each probe from 10 µmol stock; Table 1). We generated the reaction using 10 min at 50°C for cDNA synthesis, 10 min at 95°C for RT inactivation, and 50 cycles of 95°C for 15 s and 60°C for 30 s with FAM and HEX channels captured at the end of each cycle. As positive control, we synthesized

Table 1. Oligonucleotides used for duplex quantitative reverse transcription PCR targeting the matrix gene of novel Hendra virus variant from horse in Australia

Virus	Name	Sequence, 5′ → 3′*	Reference
Prototype	Mr_fwd_1	CTTCGACAAAGACGGAACCAA	(*34*)
	Mr_rev_1	CCAGCTCGTCGGACAAAATT	
	Mr_prb_1	FAM-TGGCATCTT-ZEN-TCATGCTCCATCTCGG-IABk	
Variant	Mv_fwd_1	TCTCGACAAGGACGGAGCTAA	Referent
	Mv_rev_1	CCGGCTCGTCGAACAAAATT	
	Mv_prb_1	HEX-TGGCATCCT-ZEN-TCATGCTTCACCTTGG-IABk	

*FAM and HEX 5′ reporter dyes were combined with ZEN Internal Quencher and the 3′ quencher Iowa Black, and supplied by Integrated DNA Technologies (https://www.idtdna.com).

gene fragments encoding a T7 promoter upstream of the partial M gene for both prototypic and variant HeV (Appendix Figure 1). We expressed RNA transcripts using the NEB HiScribe T7 High Yield RNA Synthesis kit (New England Biolabs, https://www.neb.com).

Virus Isolation, Confirmation, and Neutralization Studies

We attempted isolations in Vero cells (ATCC CCL-81) and primary kidney cells derived from black flying foxes (*40*). We confirmed them by cytopathogenic effect formation, quantitative RT-PCR, RNA sequencing, electron microscopy, and viral neutralization studies using HeV and isolated HeV-var mAb m102.4 (Appendix).

Serologic Analysis

We performed serologic analysis using multiplex microsphere immunoassays with a Luminex MAGPIX system (https://www.luminexcorp.com). We performed initial screening for IgG using an extensive panel of bacterial (*Leptospira*, *Brucella*) and viral antigens (paramyxovirus, filovirus, coronavirus, flavivirus, alphavirus) coupled to MagPlex beads (Bio-Rad, https://www.bio-rad.com) for multiplex screening. We added blood or serum diluted 1:100 to the beads, with binding detected following the addition of a combination of biotinylated-protein-G and -A and streptavidin-R-phycoerythrin. We read median fluorescence intensity on the MAGPIX system (Luminex) targeting 100 beads per antigen and used a Bayesian latent class model to assess test performance and determine appropriate cutoffs for positive test classification (*32*). We also applied an IgM assay in which biotinylated equine IgM was used in place of biotinylated proteins A and G.

In Silico Analysis of the RBP Homology and mAb Binding

We compared the translated protein sequence of the HeV-var RBP sequence with established x-ray crystallography-derived structures of the HeV RBP protein bound to mAb m102.4 (*41*) and to ephrin-B2 using SWISS-MODEL (https://swissmodel.expasy.org). We used the results to assess the ability of m102.4 to neutralize this variant and further establish the likelihood of antibodies produced by immunization with the HeV vaccine being protective against this variant.

Results

Case Report

In September 2015, veterinary care was sought for a 12-year-old Arabian gelding in southeastern Queensland for severe disease consistent with HeV infection. The horse had always resided on the same property. Disease onset was acute; rapid deterioration occurred over 24 hours. Clinical assessment determined depressed (obtunded) demeanor, darkened red-to-purple change of the gingival mucous membranes with darker periapical line and prolonged capillary refill time, tachycardia (heart rate 75 beats/min), tachypnoea (60 breaths/min), normal rectal temperature (38.0°C), muscle fasciculations, head pressing, and collapse.

HeV infection was suspected by the attending veterinarian, who had previously managed a confirmed case, on the basis of consistency with clinical disease manifestations and perception of plausible flying fox exposure. A nearby roost was known to host BFFs, GHFFs, and little red flying foxes (LRFF) of population sizes that varied seasonally and annually (*42*). Because of its moribund condition, the horse was humanely killed. We obtained postmortem nasal, oral, and rectal swab samples and combined them in 50 mL of sterile saline; we collected blood in an EDTA tube. Pooled swabs and blood samples were submitted to the Queensland Biosecurity Sciences Laboratory (Coopers Plains, Queensland, Australia) for priority HeV testing. Quantitative RT-PCR testing targeting the M gene did not detect viral RNA and ELISA testing did not detect HeV RBP IgG (*28*,*43*).

Identification of Novel HeV-var

Given the high assigned likelihood of a zoonotic infectious cause (Appendix Table), we screened both the EDTA blood and pooled swab samples using pan-paramyxovirus RT-PCR (*36*). This identified the partial polymerase sequence of a novel paramyxovirus, most closely related to HeV (≈89% nt identity). Deep sequencing (mean coverage depth: 46.9×) of blood RNA generated the near–full-length genome of a novel HeV (Figure 1, panel A). The virus was less abundant in the pooled swab sample; mean coverage depth was 0.6× reads, spanning only 9.9% of the genome (Figure 1, panel B). No other viruses were present in either sample, and other microbial reads assembled were from common microflora, including *Staphylococcus aureus* and *Aeromonas*, *Veillonella*, *Pseudarthrobacter*, *Streptococcus*, *Acinetobacter*, and *Psychrobacter* spp.

Confirmation of HeV Infection

A comparison of the primer and probe sequences used for the routine diagnostic PCR (*28*,*29*) revealed multiple mismatches in the binding sites, explaining the failure of routine surveillance to

detect this variant (Figure 2). A quantitative RT-PCR assay was designed to detect both prototype and variant HeV strains in duplex (Table 1; Appendix Figures 1, 2), which amplified the templates of each virus with similarly high efficiency (>94%) and sensitivity, capable of detecting <100 copies of target RNA (Appendix Figure 2). The assay quantified results from the EDTA blood and pooled swabs samples, confirming RNA sequencing; the virus was more abundant in the EDTA blood (quantification cycle 26.87) than in the pooled swab samples (quantification cycle 30.67). We rescreened the priority cohort (864 samples from 350 cases in Queensland) using this novel assay but identified no additional cases. We successfully isolated virus from the EDTA blood sample of the case-animal in Vero cells. Electron microscopy of infected cells revealed cytoplasmic inclusion bodies (nucleocapsid aggregations; Figure 3, panel A) and enveloped viral-particle budding (Figure 3, panel B), consistent with HeV (Figure 3, panels A–D) (*44*).

Blood was tested for IgM and IgG against a panel of 33 antigens representative of bacterial and viral zoonoses (*32*,*45*), including RBPs of paramyxoviruses: HeV, NiV, Cedar henipavirus (CedV), Mojiang henipavirus (MojV), Ghana bat henipavirus (GhV), Menangle, Grove, and Yeppoon and pararubula viruses. We observed no notable reactions for this animal-case blood in either the IgG or IgM assays, indicating a lack of detectable antibodies consistent with acute viremia.

Genomic Analysis of Novel HeV-var

We performed phylogenetic analyses of the novel HeV-var with other known paramyxoviruses (Figure 4, panels A–C). Comparison of the nucleotide similarity of the novel HeV-var to the HeV prototype strain (GenBank accession no. NC_001906) revealed an 83.5% pairwise identity across the genome (Figure 4, panel D). The L protein phylogeny revealed that the branch lengths of prototype and variant HeV to their common node did not exceed 0.03 substitutions/site (Figure 4, panels A, B). Therefore, the viruses were considered to be of the same species according to ICTV criteria (*39*). However, this HeV-var is clearly well outside known HeV diversity (Figure 4, panel C).

After this finding, comparison with a partial novel henipavirus M gene sequence derived from a GHFF from South Australia in 2013 (*46*) revealed 99% similarity to this HeV-var. This, along with additional subsequent flying fox detections (*47*), suggests that this HeV-var represents a previously undescribed lineage (HeV-g2), with reservoir-host infection across at least the range of this flying fox species.

Analysis of the RBP

Genomic sequencing showed greatest variability in the noncoding regions with mean pairwise genome identity higher (86.9%) across coding regions (Figure 4, panel D). At the protein level, this HeV-var shared 82.3%–95.7% (mean 92.5%) aa identity to the HeV prototype (Table 2). Of note, the HeV-var RBP shared 92.7% aa identity with prototypic HeV. Modeling of the novel HeV-var RBP structure based on the translated protein sequence using the x-ray crystal structure of the prototypic HeV RBP published elsewhere (*40*) supports that the epitopes for binding ephrin-B2 receptor and mAb m102.4 remain functionally unchanged because of consistency between key residues (Figure 5). Indeed, mAb m102.4 neutralization assays revealed equivalent neutralization potency of m102.4 (2.3 μg/mL of m102.4 neutralized 30 median tissue culture infectious dose of HeV-var and 4.6 μg/mL of m102.4 neutralized 300 median tissue culture infectious dose of HeV).

Discussion

We describe use of an innovative, syndromic risk-based targeted active sentinel surveillance activity for diagnostic investigation, extending from routine priority disease investigations, to identify a consequential virus. Based on ICTV criteria (*39*), this HeV-var is a novel genotypic variant of HeV, not a new *Henipavirus* species, but it evaded detection by routine diagnostic testing for HeV because of genomic divergence. Our findings highlight the potential of sentinel surveillance through One Health interagency and transdisciplinary syndromic infectious disease research to improve detection of emerging pathogens. We also describe a new assay for laboratory diagnosis and surveillance of this virus among humans and animals.

Comparing the translated amino acid sequences of this HeV-var and prototypic HeV RBP in silico revealed no change in the mAb m102.4 or ephrin-B2 entry receptor binding sites. Similarly, we confirmed equivalent m102.4 neutralization in vitro for this HeV-var and HeV. As such, it is expected that current PEP using mAb m102.4 will also be effective against this HeV-var. We emphasize that although the HeV RBP shares only 79% aa identity with NiV RBP, the HeV-sG subunit vaccine provided 100% protection against lethal challenge with both HeV and NiV in animal models (*11*). The high similarity between this HeV-var and HeV RBP (92.5% aa identity), structural consistency of

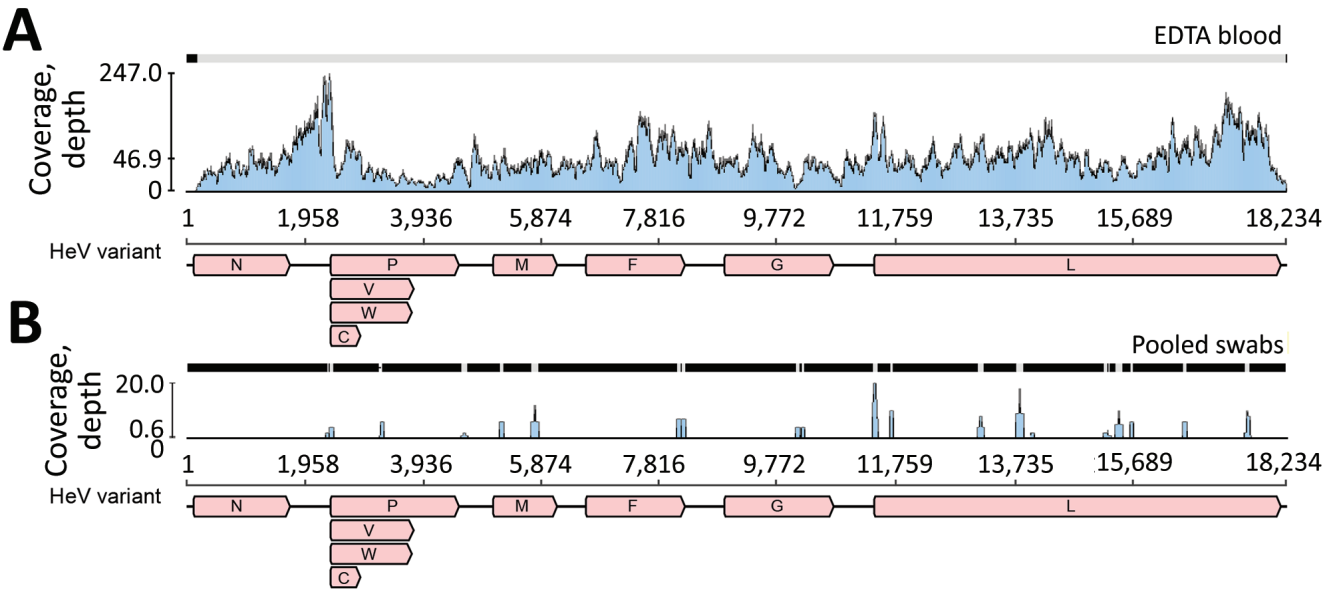

Figure 1. Sequence coverage of novel HeV variant from horse in Australia. The RNA sequencing reads were mapped to the novel HeV variant genome to examine coverage across the genome and depth for EDTA blood (A) and pooled swab samples (B). The x-axis shows the genome position with genes annotated and the y-axis shows the sequence read coverage (depth). Mean coverage depths were 46.9 for EDTA blood and 0.6 for pooled swab samples. V, W, and C indicate variably transcribed nonstructural proteins. F, fusion; G, glycoprotein; HeV, Hendra virus; M, matrix protein; N, nucleoprotein; P, phosphoprotein.

critical epitopes, and equivalent in vivo viral neutralization assays also support that current vaccination using the HEV RBP will elicit similarly protective antibodies against HeV-var.

The 99% similarity between HeV-var and a partial M-gene sequence detected in a GHFF from Adelaide in 2013 highlights that a greater diversity of HeV strains than previously recognized circulates among flying fox species in Australia and that this novel variant likely circulates as a relatively consistent sublineage (HeV-g2), at least across the range of GHFF. Subsequent identification of HeV-g2 in GHFF and LRFF from regions without previous molecular HeV detection further support this understanding (*47*).

Our findings indicate the urgent need for prompt reassessment of HeV spillover risk for horses and handlers living in southern New South Wales, Victoria, and South Australia, where risk for HeV infection has been perceived as substantially lower than that in regions within the range of BFF distribution. Our findings indicate a need to update current molecular assays, which are not expected to distinguish between HeV and HeV-var (HeV-g2), and increase surveillance testing in horses and screening of flying foxes for HeV-g2 in these areas. These might further resolve the previously reported anomaly of high seropositivity despite low HeV detection within these species reported elsewhere (*20*–*22*).

Despite relatively high genetic divergence, the phenotypic similarity of this variant to prototypic HeV, combined with the observed consistency of disease manifestations in horses, suggests that the 2 strains have equivalent pathogenicity and spillover potential. Further characterization of HeV genomic diversity and any host-species associations will increase our understanding of transmission dynamics as well as virus-host coevolution features, such as possible codivergence or founder effects. Indeed, as climate change and anthropogenic habitat loss alter the extent and nature of interspecies interactions, BFFs have rapidly expanded their range

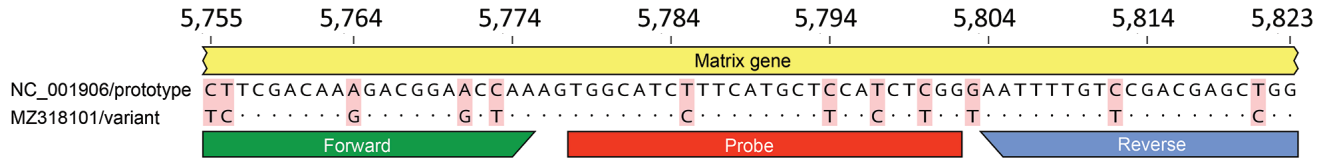

Figure 2. Genomic variation in the Hendra virus (HeV) matrix gene assay primer/probe binding sites for novel HeV variant from horse in Australia. The genomic region targeted by the commonly used HeV matrix gene quantitative RT-PCR assay (*28*) was aligned and compared for the prototype and variant HeV strains. The genomic positions relative to the prototype strain (GenBank accession no. NC_001906) are shown at the top. Primers (forward and reverse) and probe binding sites are indicated by the colored bars. Mismatches between the sequences are highlighted with red shading; dots indicate identical bases.

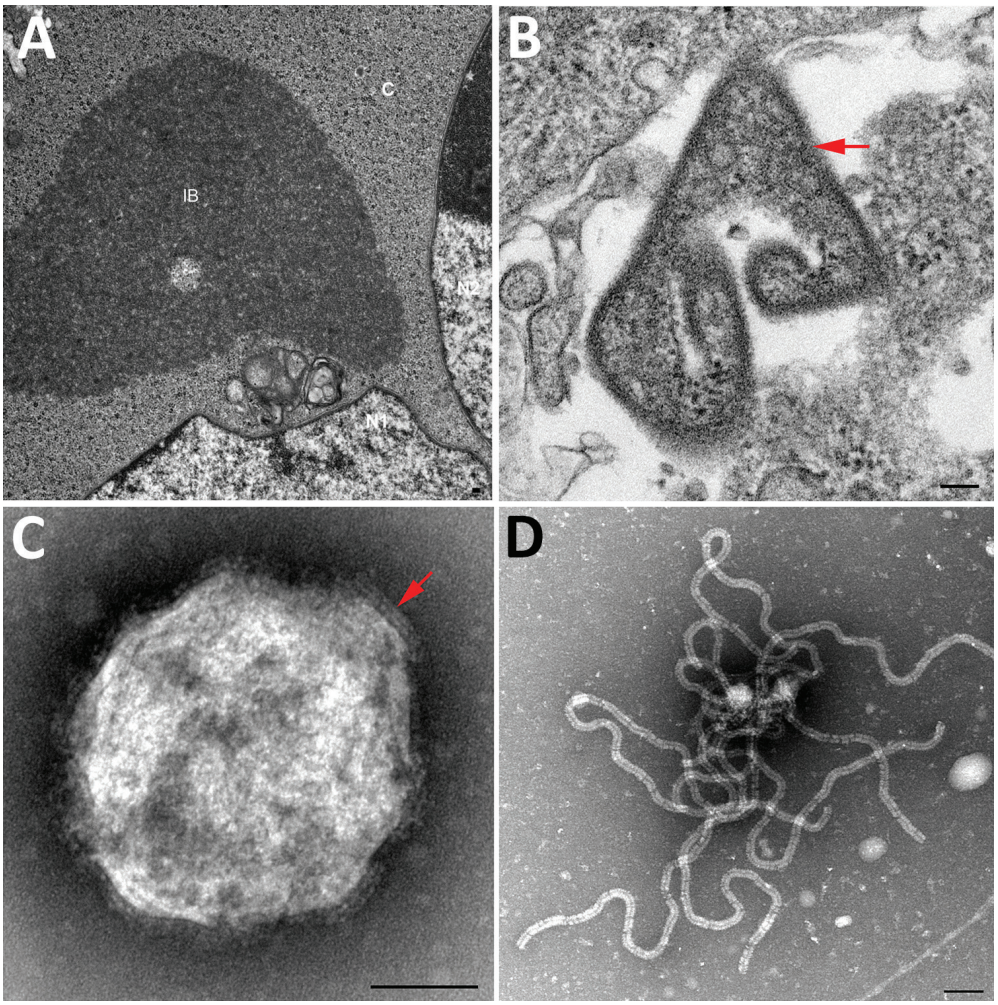

Figure 3. Transmission electron micrographs of Vero cells inoculated with the EDTA blood sample in study of novel Hendra virus variant from horse in Australia. A) Thin section showing inclusion body (IB) within the cytoplasm (C) of multinucleated (N1 and N2) syncytial cell. The nonmembrane bound IB consists of hollow nucleocapsids. B) Thin section showing virion (red arrow) with egress occurring at the plasma membrane. C) Negative contrast analysis shows a double-fringed envelope of the virion (red arrow). D) Negative contrast analysis shows strands of ribonucleic protein characteristic of the family *Paramyxoviridae*. Scale bars represent 100 nm.

southward, increasing their overlap with GHFFs (*48*). Sampling multiple species across time and space should inform how this variant strain circulates within and among flying fox species. Clearly, biosecurity practices should be updated to acknowledge spillover risk in all regions frequented by any species of flying fox.

Passive disease surveillance and biosecurity risk management for emerging diseases relies on recognition of suspected disease cases by clinical veterinarians, who play crucial roles relevant to animal and human health (*24*). Sporadic incidence of HeV and rare occurrence of Australian bat lyssavirus, yet high zoonotic consequence of both and lack of pathognomonic disease signs, inherently challenge surveillance of horses in Australia for these viruses. Critical and timely human postexposure management relies on confirmed animal-case diagnosis yet missed cases are inevitable, resulting in unmanaged risk of fatal zoonotic disease. Veterinarians are challenged in performing disease recognition by simultaneous obligations to serve both animals and animal owners, manage biosecurity risks, and meet Workplace Health and Safety Act and Biosecurity Act requirements (*24*,*49*,*50*). Veterinary description of disease manifestations most consistent with HeV led us to prioritize this case in our research testing pathway. This research detection of HeV-var highlights potential for improving emerging infectious disease surveillance through extending veterinarian-initiated risk-based suspect significant disease investigation, by selecting cases of highest likelihood of related viral cause and employing parallel serology and molecular testing pathways constructed to suit the available sample types and target diseases of highest clinical, species, and geographic relevance. These strategies build on the existing strength of systematic interpretation of clinical and field observations made by clinical veterinarians as part of existing submission and biosecurity protocols. This example serves as proof-of-concept for other disease contexts, highlighting the benefit of integrated transdisciplinary inquiry-based research approaches with routine biosecurity operations. Indeed, in

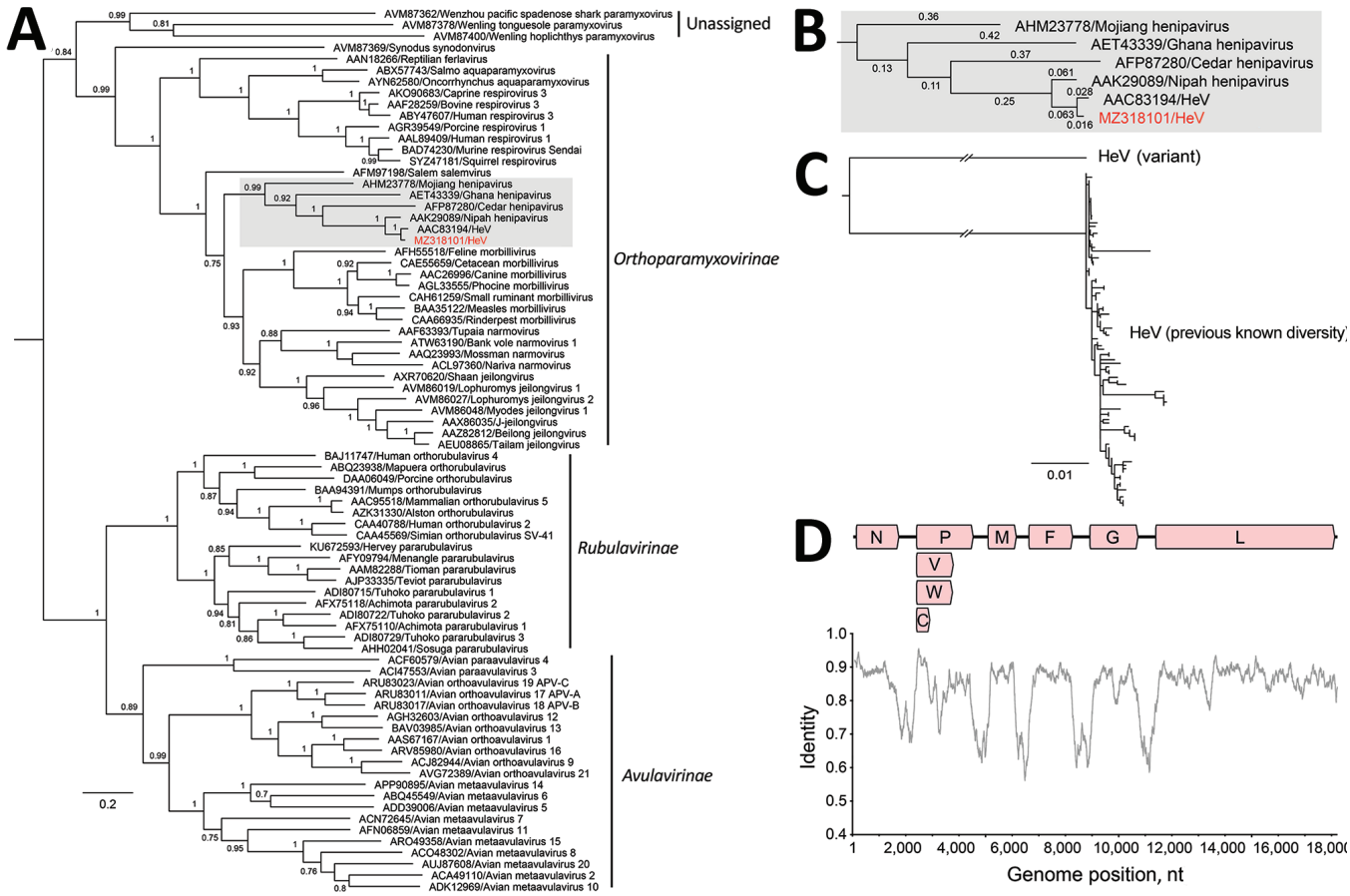

Figure 4. Phylogenomics of novel HeV variant from horse in Australia. A) Maximum-likelihood phylogeny of paramyxoviruses using complete L protein sequences. Gray shading indicates henipaviruses, and red text indicates the novel HeV variant, which groups with the prototypic HeV. Bootstrap support values as proportions of 500 replicates are shown at nodes; values <0.7 are hidden. Scale bar indicates substitutions per site. B) Enlarged gray area from panel A shows branch lengths for the henipavirus clade. The branch leading back to the common ancestor of all known HeVs and the novel HeV variant does not exceed 0.03; thus, they are considered variants of the same species. C) Maximum-likelihood phylogeny of partial N and P where deep branch lengths have been collapsed for visualization only to demonstrate that the variant is well outside the known diversity of HeV. Scale bar indicates substitutions per site. D) Nucleotide genomic similarity of the variant compared with the prototypic HeV strain. V, W, and C indicate variably transcribed nonstructural proteins. F, fusion; G, glycoprotein; HeV, Hendra virus; L, paramyxovirus polymerase; M, matrix protein; N, nucleoprotein; P, phosphoprotein.

October 2021, a fatal horse-case of HeV-g2 infection near Newcastle, New South Wales, was detected through an updated quantitative RT-PCR incorporated into routine priority disease testing.

Acknowledging the limitations of this single case, which lacked tissue for histopathology and immunohistochemistry, it is nonetheless appropriate that this HeV-var (Hev-g2) be considered equally pathogenic to prototype HeV based on coherent and consistent clinical signs of disease and pathology, evidence of viraemia, the phylogenetic analysis indicating that the variant belongs to the HeV species, and the modeling of the interactions of the functional RBP domain to the virus entry ephrin-B2 receptor. Moreover, this case fits the case definition for HeV infection in Australia's AUSVETPLAN, which is that an animal tests positive to HeV using ≥1 of PCR, virus isolation, or immunohistochemistry (*50*).

Updated PCR diagnostics suitable for routine priority detection of this HeV-var (Hev-g2) have been developed and are now used in many animal and human health laboratories in Australia. These findings demonstrate the limitation of exclusion-based testing

Table 2. Protein lengths of novel Hendra virus variant from horse in Australia and similarity to prototype strain*

Protein	Length, aa	Similarity, %
Nucleoprotein	532	96.6
Phosphoprotein	707	82.3
Matrix	352	95.7
Fusion	546	95.4
Glycoprotein	603	92.5
Large	2,244	95.7

*Prototype strain: GenBank accession no. NC_001906.

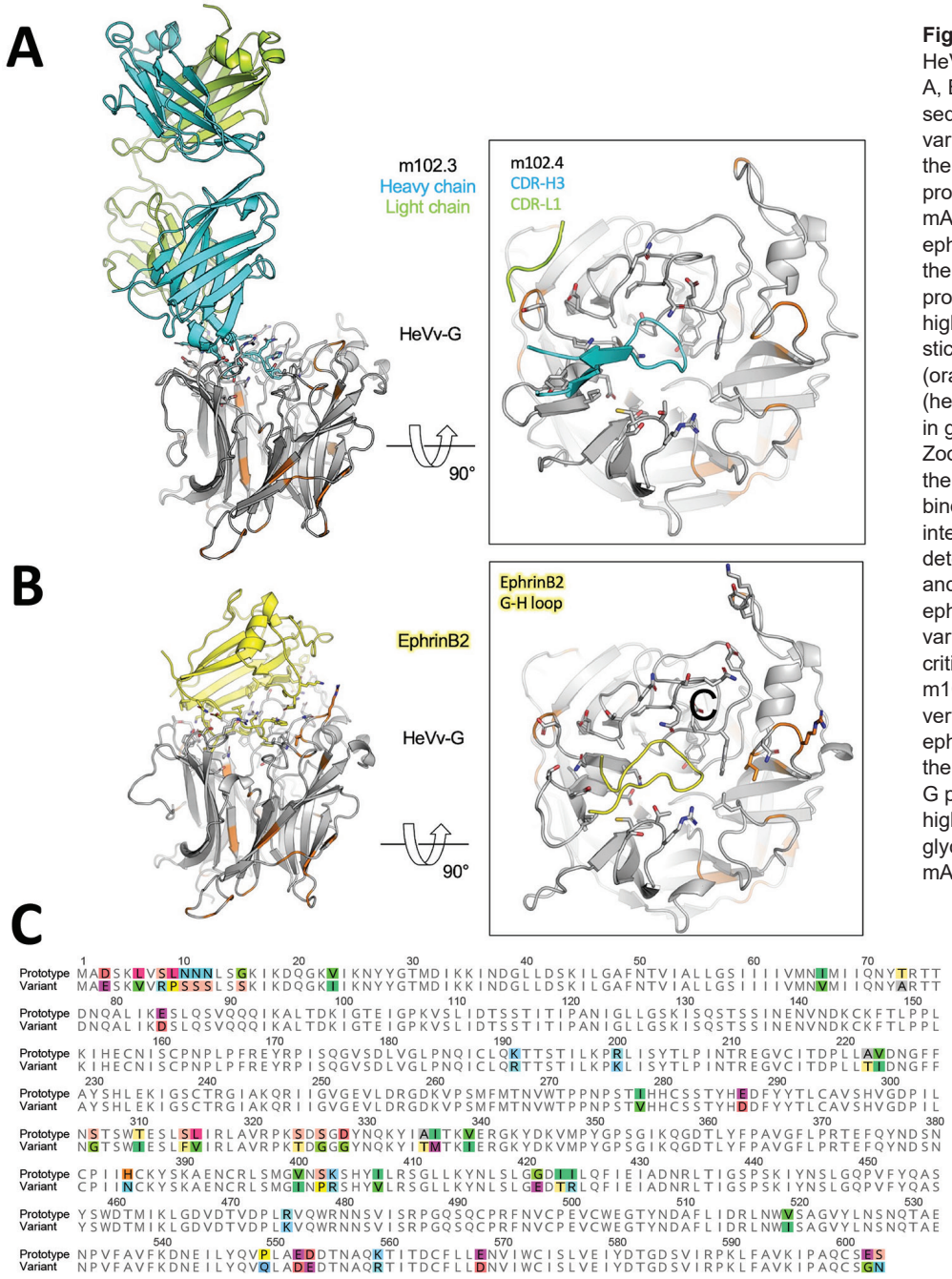

Figure 5. In silico modeling of HeV-var from a horse in Australia. A, B) The translated protein sequence encoded by the HeV variant G gene was modeled using the known protein structure of the prototype virus bound to the human mAb m102.4 (A) and the receptor ephrinB2 (B). Side views (at left) of the interactions between the HeV G protein and the 2 binding partners highlight key binding residues (as sticks) and the variant positions (orange) relative to the m102.4 (heavy chain in teal and light chain in green) and ephrinB2 (in yellow). Zoomed top views (at right) of the HeV G and m102.4/ephrinB2 binding interface highlight specific interactions by the complementarity-determining regions of the mAb and G-H loop of the receptor ephrinB2. These data show that variable positions do not occur at critical epitopes at the HeV G and m102.4 binding interface and have very minor effect on the receptor ephrinB2 binding. C) Alignment of the prototypic and variant HeV strain G proteins. Variable positions are highlighted in color. F, fusion; G, glycoprotein; HeV, Hendra virus; mAb, monoclonal antibody.

for emerging zoonoses and a gap in our understanding of how frequently detection of known zoonoses across a broad range of systems are missed because of the diagnostic tools used. Further investigations to determine the prevalence of HeV-g2 circulation among and excretion from all flying fox species in Australia should be prioritized. The risk of zoonotic HeV disease in horses and human contacts should be interpreted across all regions frequented by all species of flying foxes, particularly those areas previously considered to be at low risk for HeV spillover.

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C.C.B. is a United States federal employee and inventor on US and foreign patents pertaining to soluble forms of Hendra virus and Nipah virus G glycoproteins and monoclonal antibodies against Hendra and Nipah viruses, whose assignees are the US Department of Health and Human Services (Washington, DC, USA) and Henry M. Jackson Foundation for the Advancement of Military Medicine Inc. (Bethesda, Maryland, USA). Remaining authors declare no competing interests. Opinions or assertions contained herein are the private ones of the author(s) and are not to be construed as official or reflecting the views of any of the Australia or international affiliated government or research agencies or official policy or position of the Uniformed Services University, US Department of Defense, or Henry M. Jackson Foundation for the Advancement of Military Medicine, Inc.

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Novel Hendra Virus Variant Detected by Sentinel Surveillance of Horses in Australia

Appendix

Supplementary Methods

Viral Isolation

Positive case samples for the novel HeV were sent to the Australian Centre for Disease Preparedness (ACDP), a World Organization for Animal Health Reference Laboratory for Hendra and Nipah virus diseases, in line with established national arrangements for confirmatory testing of notifiable diseases of animals. Virus isolation was attempted in Vero cells (ATCC CCL-81) and primary kidney cells derived from *Pteropus alecto* (PaKi; *39*) on whole blood and pooled nasal, oral and rectal swab samples. Vero cells were grown at 37°C in EMEM (ThermoFisher; https://www.thermofisher.com) containing 10% fetal calf serum (FCS; ThermoFisher), supplemented with 1% v/v L-glutamine, 10 mM HEPES, 0.25% v/v penicillin–streptomycin and 0.5% v/v amphotericin B (Sigma-Aldrich; https://www.sigmaaldrich.com). PaKi cells were cultured in DMEM/F-12 media (ThermoFisher) with 5% FCS and supplemented with 1% v/v L-glutamine, 10 mM HEPES, 0.25% v/v penicillin–streptomycin and 0.5% v/v amphotericin B (Sigma-Aldrich).

For virus isolation, washed monolayers of cells were inoculated with 500 µL of whole blood diluted 1:5 in culture media or 500 µL of pooled swab sample prefiltered (0.45-µm cellulose acetate) to remove bacteria and any residual solid particles. Inoculum was removed after 45 min and cell monolayers were washed with phosphate-buffered saline, then overlaid with culture media containing 1% (v/v) FCS. Flasks were incubated at 37°C for 6–7 days and regularly monitored for cytopathic effect by light microscopy. Cells were then frozen, thawed and the cell suspension clarified by centrifugation (1000*g* at 4°C). Supernatant (500 µL) was then passaged onto fresh cell monolayers. A maximum of three passages per sample were

performed on each cell line. Final pass samples were tested by qRT-PCR to detect the presence of replicating HeV genome.

Electron Microscopy

For negative contrast EM, the clarified supernatant from Vero cell cultures, infected with HeV-var, were inactivated with 4% formaldehyde overnight. After adsorption of the inactivated supernatant onto formvar/carbon coated Cu400 grids, the preparation was then stained with Nano-W (Nano-probes) for 1 min. For thin section EM, the pelleted cells were fixed with modified Karnovsky fixative (4% formaldehyde and 2.5% glutaraldehyde in 0.1 M Sorensen's phosphate buffer) at 4ºC overnight. The pellet was rinsed in analogous buffer, fixed with 1% osmium tetroxide for 1 hr and dehydrated with a graded ethanol series prior to being embedded in Spurr's resin (ProSciTech; https://proscitech.com.au) according to manufacturer instructions. A Leica UC7 microtome was used to produce ultrathin sections, which were then stained in saturated uranyl acetate in 50% ethanol followed by lead citrate. Grids were examined and images acquired using a JEOL JEM-1400 transmission electron microscope at 120V.

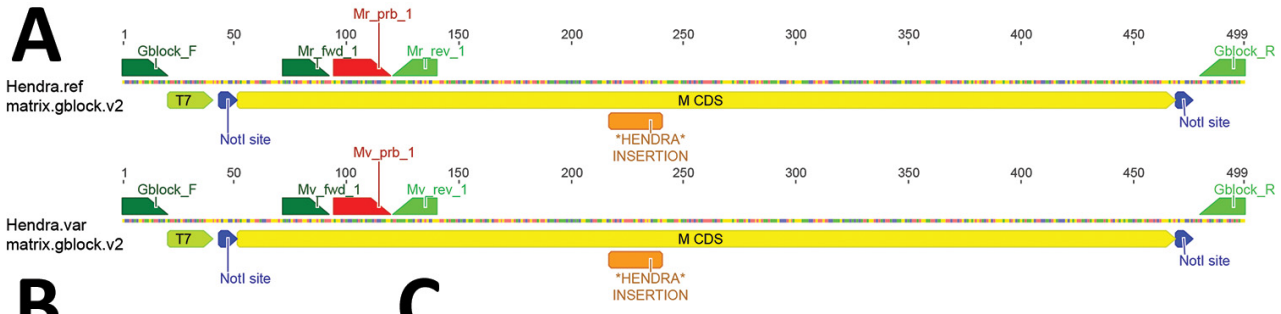
Neutralization Assay

Serial dilutions of the mAb m102.4 and Hendra virus (isolate Hendra virus/Australia/Horse/2008/Redlands) or the HeV-var (Hendra-var/Australia/Horse/2015/Gympie) diluted to contain 100 $TCID_{50}$/well were incubated for 45–60 min at 37°C in a 96 well plate. A suspension of Vero cells was added to every well at a concentration of 4×10^5 cells per mL. Positive and negative serum controls and virus-only controls were included. Plates were incubated in a humid atmosphere containing 5% CO_2. Cells were examined after 3 days under an inverted microscope for cytopathic effect.

Appendix Table. Infectious disease prioritization categories (with examples) used in this study to identify Hendra-negative equine disease cases with highest likelihood of similar undiagnosed viral cause from larger cohort for further investigation

Infectious disease priority	Description	Example
Category 1 Highest infectious disease suspect	Case features 'pyrexia' or 'abnormal mucous membranes AND one or more other clinical signs related to infectious disease OR the presence of either change AND 'epidemiological observation indicative of infectious cause' based on temporal and/or spatial relationship to similar disease cases	Pyrexia with tachycardia and acute onset respiratory consolidation and/or secretions. Pyrexia and neurological symptoms. Pyrexia and 'injected/congested' mucous membranes. 'Congested/injected mucous membranes' with acute severe respiratory dysfunction. Clustering of similar cases on same or neighboring properties
Category 2 High infectious disease suspect	Pyrexia OR other clinical signs associated with infectious disease of interest	Acute onset abnormal respiratory secretions. Fever of unknown origin. Colic with the presence of neurological symptoms
Category 3 Moderate infectious disease suspect	Clinical signs may be associated equally with infectious and noninfectious causes	Colic with the presence of dehydration and mucous membrane changes. Ataxia with the absence of pyrexia or known trauma
Category 4 Low infectious disease suspect	Non-infectious etiologies more common or most likely on differential diagnosis list, but infectious cause still possible	Ataxia following known traumatic event. Traumatic wounds following unusual behavioral event. Acute lethargy following chronic noninfectious disease condition
Category 5 No infectious disease suspect	No clinical signs of illness or no infectious cause considered likely	Traumatic wounds in the absence of underlying disease. Screening in unvaccinated horses to manage biosecurity risk prior to invasive procedures addressing non-infectious disease such as is a common requirement for dentistry or admission to equine hospitals in Australia
Category 6 Confirmed infectious disease	Other infectious disease confirmed via diagnostic testing	A case submitted for HeV testing, found negative and then testing positive for alternative known infectious disease such as ABLV, WNV, EHV or RRV*

*ABLV, Australian bat lyssavirus; EHV, Equine herpes virus; HeV, Hendra virus; RRV, Ross River Virus; WNV, West Nile virus.



B

Name	Feature	Start	End
Gblock_F	Primer bind	1	20
T7	Promoter	21	40
NotI site	Restriction site	44	51
M CDS	Matrix gene	52	468
Mr/v_fwd_1	Primer bind	72	92
Mr/v_prb_1	Probe bind	95	119
Mr/v_rev_1	Primer bind	121	140
HENDRA	Sequence insertion	217	240
NotI site	Restriction site	469	476
Gblock_R	Primer bind	480	499

C

>Hendra.ref.matrix.gblock.v2
GCCACTCGGATTGTAACTCGTAATACGACTCACTATAGGGAGAGCGGCCGCATGGCTG-
GAATCCAAGGAAGCTTCGACAAAGACGGAACCAAAGTGGCATCTTTCATGCTCCATCTCGGGAATTTTGTCCGACGAGCTGGGAAGT
ACTATTCTGTCGAATACTGCAAGAGAAAGATTGACAGAATGAAGCTCCAGTTCTCTCTTGGTTCAATTGGGTGACACGAAAATGACAGA
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GCCACTCGGATTGTAACTCGTAATACGACTCACTATAGGGAGAGCGGCCGCATGGCCG-
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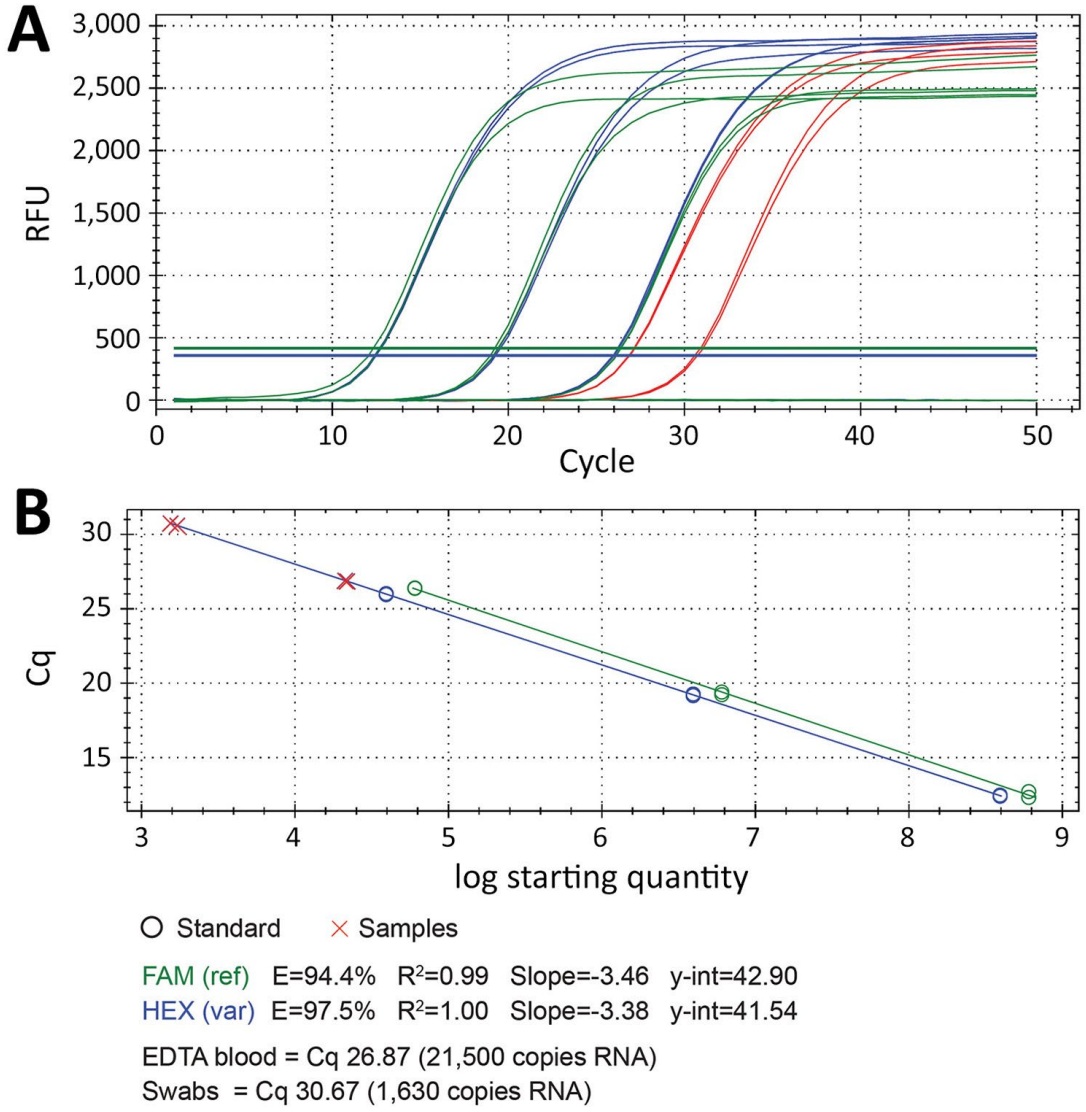

Appendix Figure 2. Quantitative reverse transcription PCR (qRT-PCR) performance and results for clinical samples. A) Amplification plot for duplex qRT-PCR assay reporting both reference (FAM [green]) and variant (HEX [blue]) HeV–strain channels with the synthetic RNA controls. Red traces show detections in the HEX channel for animal-case clinical EDTA blood and swab samples. B) Standard curve plot for duplex qRT-PCR assay reporting both FAM and HEX channels with the synthetic RNA controls.

Horses as Sentinels of Emerging Infectious Disease

Chapter 9

DISCUSSION AND CONCLUSION: 'HORSES AS SENTINELS' RESEARCH INSIGHTS, CONCLUSIONS AND FUTURE DIRECTIONS

Statement of personal contribution: I drafted the first complete manuscript (2019−21) and received feedback from PhD supervisors. I received support in the system process analyses from epistemology scholar Tim Jackson and technical support from project research assistants Nicole Popovic and Anna Gonzalez.

Format: This chapter has been prepared primarily for this purpose (rather than for peer review journal submission).

9.1 'Horses as Sentinels for Emerging Infectious Diseases': Research insights, conclusions, and future directions

Many horses throughout Australia display signs of severe disease indistinguishable from that caused by infection with Hendra virus, based on clinical manifestations alone, such as severe respiratory and neurological illness. Many cases, however, remain undiagnosed.

A variety of emerging RNA viruses are being shed from flying foxes in their urine similarly to HeV,(1) with potential to follow the same established infection transmission pathway to horses.

Combining these understandings, a hypothesis was developed. This hypothesis was:

> ***Bat-borne viruses are shed along with Hendra virus from flying foxes, are transmitted to horses via the same mechanism as Hendra virus, causing similar disease in horses, potentially posing similar risks of zoonotic infection*.**

This research aimed to test this hypothesis by:

1. collating and characterising a cohort of suitable cases of highest suspect HeV-like disease cases and biobanking their suitable biological samples for causative infectious diagnostic investigations beyond those routinely available
2. coupling innovative diagnostic pipelines with epidemiology and traditional virological approaches, to identify pathogens of significance to Australia's human and animal health, beyond those currently screened for as part of priority disease investigations of horses with severe Hendra virus-like illness (Chapters 7 and 8)
3. developing an understanding of the disease significance to horses and zoonotic diagnostic responsibility of the novel infectious agents identified.

First-hand perspectives from clinical practice, animal ownership and production were integrated with core scientific and One Health epidemiological perspectives to extend the capacity of routine disease surveillance in this emerging infectious disease context.

Early in this research project, serological findings (Chapter 7) supporting that a Pararubulavirus (related to MenPV previously attributed to disease in pigs and in-contact human, maintaining amongst and shedding from flying foxes similarly to HeV) caused an

outbreak of HeV-like disease in horses near Sydney in New South Wales in 2016.(2) One of three horses affected, the sole for which sufficiently convalescent sera was available, demonstrated greater than 10-fold increase in reactivity of IgG targeting the Pararubulavirus N protein (seroconversion), confirmed by IFA, and all three cases demonstrated IgM reactivity consistent with current or recent infection. These novel findings, in the absence of molecular detection, further virus characterisation (partially due to sample type limitations), and/or extensive population level contextual findings, raised perceived challenges (as well as opportunity) for veterinarians and government biosecurity alike. These related to inadequately conclusive interpretation inherent for detection as an isolated disease event of an emerging or novel diagnosis in the absence of molecular information and extensive epidemiological knowledge. Some clinically consulting veterinarians were justifiably concerned that this detection could impact WPHS, while others were intrigued that this virus could be the cause of disease outbreaks, including pregnancy loss, for which a diagnosis had not been achieved. Nevertheless, many clinical veterinarians perceived great potential benefit in capability to test for such a novel disease of potential significance to horse and human health and overall, the equine veterinarians of Australia responded appropriately recognising the real possibility of zoonotic infections beyond those known for which testing was available including by increased messaging on appropriate staged PPE in infectious like disease cases regardless of diagnostic conclusivity. In the absence of molecular detection, viral isolation and characterisation, government laboratory scientists were unsure of the validity of the finding. In addition, government sector policy leaders felt unable to act without an understanding of the significance of the disease in terms of Australia's disease-free status, international animal movements and freedom of trade. Furthermore, wildlife health scientists were concerned that this finding would create negative impact by heightening anti-bat sentiments.

The added molecular evidence followed some years later as part of this same research initiative (Table 7.17). The finding is not surprising given the prior knowledge of higher spillover frequency of these viruses in Australian flying foxes compared to HeV gained from research analysing flying fox urine. And given the established wide species tropism for these genera of paramyxovirus, biological plausibility of an equivalent spillover pathway

established for HeV, and consistency of observed disease in affected horses with expected pathogenesis. (2,3)

This veterinary epidemiology and virology research aspires to more proactive surveillance for improved One Health and biosecurity management in response to findings of this nature. However, it is necessarily complex to gain a wider appreciation, in such novel zoonotic disease contexts, of the numerous considerations and wide range of discipline perspectives required in guiding meaningful operative surveillance operations and responses.

This project aimed to demonstrate how three innovative pillars may be applied to existing resources to significantly enhance Australia's biosecurity and emerging infectious disease preparedness:

1. A purpose-built, de-identified relational database (SQL) was developed (Chapter 5) to capture and compare subjects, geography, clinical features (chapters 3, 4 & 6) sample information and results while appropriately leaving sensitive horse and owner details with state biosecurity departments and local stakeholders.
2. Advanced serological testing was applied to samples from horses collected during infectious-like clinical syndromes consistent with, yet found negative for, HeV (Chapter 7). These samples had been tested as part of routine biosecurity. Samples of equivalent type storage and processing, from horses that were free of infectious-like disease at the time of sampling, were subjected to the same testing. These samples acted as comparative controls, to aid assay development and interpretation. Such comparative analysis allowed for insights into the significance of novel and emerging infectious agents and their likelihood of causal association with disease.
3. Both targeted (Pan-viral-family nested conventional PCR) and unbiased (Metatranscriptomic Next Generation Sequencing) molecular testing was applied in parallel as for serological testing. (Chapters 7 and 8)

Research application of this three pillared 'Horses as Sentinels' approach demonstrated a proof of concept for targeted sentinel surveillance extending routine passive disease surveillance. This proved highly successful in identifying infectious agents that are novel and/or currently going undetected, but which appear to be contributing to severe disease in

horses. The approach is also suitable for improved emerging infectious disease surveillance in other species and disease syndrome contexts.

Each component of the three-pillar system offers significant value. However, their combined application and appropriate integration with multiple routine processes (clinical veterinary, state biosecurity, national biosecurity and epidemiology and national reference laboratory) provided further benefit, demonstrating the ultimate proof of concept for integrated sentinel surveillance.

Most conclusively, this was showcased by the identification of a novel variant of HeV (HeVg2) in multiple samples from a horse that was euthanased in moribund condition following a severe acute disease clinically indistinguishable from HeV but having tested negative by routine surveillance testing at the time. The research developed and optimised a novel duplex qPCR assay to detect both the discovered variant of HeV (HeVg2) and the formerly known lineage (HeV) with priority. Sequence instruction and reagents for the novel assay was shared, just thirty-four days (Table 9.1) after the initial molecular detection (well before scientific publication) for efficient integration into existing state biosecurity diagnostic approaches. This directly resulted in a prospective contemporary detection of HeVg2 infection, again causing prototypic deadly disease in a horse (Table 9.1),(4) as part of routine significant disease investigation by state government laboratory testing. This remarkable research translation occurred within the project timeline.

Table 9.1. Timeline of significant advances in knowledge and management of Hendra virus infection and the Hendra virus g2 variant detection

Year	Event
1994 September	First recognised spillover of HeV involving human fatality
1994 August	Retrospective diagnosis of Mackay case involving a human fatality
1999−2007	Sporadic cases identified
2008 July	Redlands outbreak involving a human fatality and recognition of wider clinical manifestations, with death of five horses
2009 July	Cawarral outbreak involving a human fatality with the death of four horses
2011 June to October	Large number of spillover (more than all previous identified)
2012	Equine HeV vaccine becomes available
2013 January	**Partial genome detection of HeV variant in grey headed flying fox samples from Adelaide – unpublished**
2014	Horses as Sentinels research founded extending from recognition of potential for EIDs beyond HeV in horses extending from detection of ABLV in two horses (2013) (5,6) with in-kind support (only) from QDAF and Equine Veterinarians Australia Group
2015	Horse from Gympie, Qld euthanased following HeV-like signs and rapid deterioration. HeV is not detected in samples submitted to the state laboratory for testing
2016 December	Conference abstract describing partial genome molecular detections of variant HeV in flying foxes – unpublished and not publicly available (7)
2016	Philanthropic funding obtained – DALARA foundation for human and horse health
2018	Early serological findings confirmed the hypothesis of divergent paramyxovirus spillover to Australian horses – communicated to government agencies and practising veterinarians and published as abstract in international journal (2)
2020	Biosecurity Innovation Program funding obtained from Department of Agriculture, Water, and the Environment, allowing for large scale application of methods (8)
2021 January 21	**First molecular detection HeV variant in horse** as part of Horses as Sentinels research
2021 February 24	Communication of **updated assay, extended geographic spillover risk and expected vaccine efficacy** to all human and animal health government agencies (9) (Appendix 1)
2021 March	AVA media release (10) with parallel state government (11) veterinary communication
2021 July 17	Full manuscript hosted as preprint on BioRxiv (12)
2021 September	Manuscript published in the Australian Equine Veterinarian journal detailing HeV variant infection as consistent with prototypic HeV and guiding practicing veterinarians on recognition of suspect cases based on pathologic understanding (13) (Appendix 2)
2021 October 8	**Contemporary detection of HeV variant causing prototypic fatal disease in a horse** in Newcastle NSW, made possible by sharing of data, interpretations, and updated assay (14) (Appendix 3)
2021	Peer-reviewed manuscript published detailing multiple detections of HeV variant in grey-headed flying foxes and a little red flying fox, circulating as consistent second genotypic lineage (HeVg2) across broad geography previously considered low risk for HeV (Victoria, South Australia, and Western Australia) (15)
2022 March	Final manuscript publication in Emerging Infectious Diseases journal (3) with podcast (16)
2022 May	Peer-Reviewed publication describing detections of HeVg2 in urine of grey headed flying foxes and little red flying foxes by Horses as Sentinels and Bat one health research collaboration in Emerging Infectious Diseases journal (17)
2022 May	Peer-reviewed publication detailing equivalent cellular entry (in both Humans and Flying foxes) and immunology between HeV and HeV g2 along with bolstered monoclonal antibody therapeutic approach afforded by proactive government approved international research collaboration (18)

This retrospective research detection of HeVg2 expediated transdisciplinary interpretation of disease significance and expected immunology as well as calculation of geographical spillover risk. All were communicated promptly along with sharing of developed testing capacity for routine testing (duplex HeV/HeVg2 qPCR). (9) These advances clearly contributed in a meaningful way to improvement of One Health and biosecurity risk management for this deadly disease.

The further facilitated prospective spillover detection of HeVg2 in a horse, just nine months after the initial discovery, was testament to proactive communication for practicing veterinarians, (10,13) allowing for the appropriate One Health management of the case. This detection demonstrated the proactive responses that are possible when the disease context can be clearly established by priority collaborative transdisciplinary and multisectoral consideration, a vaccine is available, and a test developed and made available. In this example, meaningful improvements for One Health and biosecurity risk management of this deadly disease were achieved, while avoiding significant costs to industry or disruption to freedom of trade and international animal movement. (8)

The discovery highlighted the reality that cases will have been missed across a broad geography, resulting in unmanaged severe zoonotic disease risk in each sporadic spillover event. The updated scientific knowledge and capacity offered by this research is proven in efficacy to detect and manage such cases in the future.

For the transdisciplinary analysis of the zoonotic and EID surveillance system processes we engaged with stakeholders in eight individual workshops. We considered and discussed existing and desired processes from the recognition of disease in an animal to its diagnosis, appropriate timely One Health responses and governance, funding and policy frameworks required to support such emerging disease detection, preparedness, and management. The perspectives, findings and flow diagrams offered by this research report could be used to guide a strategic review of guidelines, procedures, funding, and governance of EIDs, biosecurity and surveillance. The system processes framework and critical stakeholder engagement consolidated and extended the value offered by our project's findings and are well-placed to guide interpretation and response to further novel disease agents across a broad range of species and disease syndrome contexts.

9.2 Key messages and future directions

There is a need for national recognition of the seriousness of the risk of potential zoonotic disease transmission and spillover from wildlife via horses (and other animals) to people. We also need to acknowledge the key roles played by veterinarians in the field in recognition of disease and in sampling for this human health priority that are often at odds with animal health, primary stakeholder, industry, and trade priorities.
Appropriately funded and nationally co-ordinated governance integrated with human health is required to support improved sentinel surveillance mechanisms and more comprehensive laboratory analysis of samples beyond exclusion testing for such emerging zoonotic disease contexts.

The key suggestions for improving surveillance for EIDs in Australia are summarised below:

1. Bolster syndromic sentinel surveillance of diseases in animals. This improvement is expected to identify high consequence pathogens, allowing for assays to be developed and mitigation strategies to be implemented to lessen their impact.
2. Strengthen state laboratories to be able to respond to emerging infectious disease threats by increasing their capabilities to perform NGS by partnering with research laboratories for capability transfer beyond the routine significant disease exclusions.
3. Develop a restricted access database for sharing of molecular sequences before publication. It is suggested that the sharing of sequences would benefit response and interpretation and could be a requirement of any government funded research.
4. Develop a policy regarding reporting of potentially significant pathogens of as-yet uncertain pathogenicity, allowing proactive timely interpretation to guide risk management and improve Australia's significant disease surveillance and biosecurity.
5. Strengthen national co-ordination in supporting uniformity between states in surveillance, especially for border towns but also to ensure uniformity in communication of risks, veterinary training, stakeholder education etc.

6. Establish strong interagency working groups and pathways to engage with relevant governance and policy to improve the strengths in the current system but also address various challenges. This may be achieved through building on established interagency relationships to improve surveillance and communication of relevant findings to all key stakeholders.
7. Develop a nationally co-ordinated **Emerging and/or Zoonotic Significant Disease Investigation Program** with information system capacity, interagency governance, core scientific research integration and funding (including engagement and funding support from human health). Such a program would allow effective surveillance to be coordinated nationally, even where priorities are in conflict, and to be shared without burdening primary stakeholders.

Greater definition, communication, and support across the veterinary, laboratory and research disciplines in the future will be required in order to recognise the next emerging infectious disease that could impact the health of Australia's people and animals.

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24 February 2021
Final version following preliminary sharing with Qld CVO late 23 February 2021

Priority National Update from: **'Horses as Sentinels' Research**

Innovative metagenomic, multiplex serology and custom database combined in testing cases of priority infectious clinical syndromes to improve diagnosis of infectious diseases and Australia's biosecurity.

Co-Hosted by: The University of Sydney (USYD) (Sydney School of Veterinary Science and Faculty of Medicine and Health-Marie Bashir Institute for Biosecurity and Infectious Diseases) and CSIRO (Health and Biosecurity Business unit).
Funded by: Biosecurity Innovation Project 2020-21 Project ID 202043 - Metagenomic Investigation of Horses as Sentinels Research; Dalara Foundation, philanthropic donation - Horses and Human Health; Marie Bashir Institute of Biosecurity and Infectious Diseases - Internal USYD seed funding; USYD on behalf of Australian Government – Department of Education Skills and Employment - Research Training Program scholarship.
Supported by: Westmead Institute for Medical Research**:** Queensland Government Biosecurity Sciences Laboratory and Queensland Department of Agriculture and Fisheries**;** Australian Centre of Disease Preparedness Diagnostic Surveillance and Response Laboratory**;** University of Sydney, Sydney Medical School; Griffith University (nationally), and; Broder lab - Uniformed Services University of Health Sciences, USA with specific support from Dr Kai Xu of National Institute of Allergy and Infectious Diseases, NIH, USA.

Dear Chief Veterinary Officers of Australia, Chief Health-Officers, and other key stakeholders most-relevant to our nation's Hendra virus disease surveillance and biosecurity. Please receive the following **'Horses as Sentinels'** research project notification extended to you by Dr Allison Crook (Qld CVO).

We consider this information and document the most effective way for sharing **IN-CONFIDENCE** to the relevant animal and human health authorities to enable the formulation of policies to ensure human and animal wellbeing, while considering primary stakeholder and industry sensitivities. Please understand that much of the content of this update is privileged, confidential and intended for scientific publication.

Please respect this and share only as required for optimal animal and public health response. When communicating and sharing this information for this purpose please make myself and the project team aware by email correspondence. We are preparing a scientific manuscript under the following drafted title with priority. We will share the final draft with you for your consideration, interpretation and support prior to journal submission.

Draft manuscript title: **'A previously unacknowledged, highly pathogenic Henipavirus, most closely related to Hendra virus and putatively an additional prototypic variant associated with grey-headed flying foxes.'**
Intended journal for submission: **Emerging Infectious Diseases. Authorship:** EJ Annand, B Horsburgh, PA Reid, JS Eden, I Smith et al.

Summary
We have identified a variant of Hendra (HeV) virus (*Hendra-virus-variant Horse GXXXXX 2015*) from a 2015 case of fatal equine disease in south-east Queensland that was found negative by routine PCR testing for HeV at BSL in Queensland in 2015.
The equine HeV disease caused by this variant was clinically indistinguishable from the most severe reported form of acute HeV disease. This variant was not detected by the molecular methods routinely relied upon due to sufficient mismatches in genomic sequence (approx. 15% at a nucleotide level).
This finding is important for national HeV surveillance in humans and animals. We have determined that the 2015 horse HeV-variant shares near-identical genomic sequence to that detected in a grey-headed flying fox

(*Pteropus poliocephalus*) from Adelaide in 2013 *(Grey-headed flying fox Adelaide 2013)*. This sequence was shared with us in good will by relevant CSIRO scientists and is yet to be published.
We have redesigned the PCR assays suitable for routine biosecurity screening, have shared these assays with ACDP DSR and QDAF BSL. We wish to extend these updated assays with all relevant state and national animal and human health laboratories as soon as possible.
Our analysis of this HeV-variant support the understanding that immune-protection should be afforded as for the prototypic HeV by both the Equivac HeV® vaccine in horses and the mono-clonal antibody m102.4 post-exposure therapy in humans.

Case History:
The equine disease incident occurred in September 2015 in GXXXXX, Qld. EDTA blood and Swab (Nasal, rectal and oral pooled by attending veterinarian) samples were received by 'Horses as Sentinels' research in 2017. *(Qld CVO to update town once attending veterinarian has been contacted.)*

Affected horse: A 12-year-old male Arabian of local origin ('homebred').

Clinical disease: Acute severe disease featuring severely 'injected' mucous membranes, tachycardia (75), tachypnoea (60), normal rectal temperature, muscle fasciculations, head pressing and recumbency with rapid deterioration over 24 hours resulting in euthanasia on humane grounds.

Hypotheses

Some members of our core and extended research team have formed the following hypotheses which we feel pose a great many implications to former understanding and risk management for Henipaviruses in Australia.

1. We hypothesise that Australian flying fox populations are reservoir species for multiple strains (genotypic variation) of Henipavirus that intermittently cause consistent Hendra virus (HeV) disease in incidental and intermediate spillover hosts such as horses and humans (minimal phenotypic variation). We speculate that each virus (genotypic strain) may predominantly circulate and be excreted from specific flying fox species. While grey-headed flying foxes (GHFF) appear susceptible to infection with the 'classic' Hendra virus species currently described (perhaps, in future, referred to as '*Hendra virus - Black flying fox strain*' (HeV-BFF)), they may be a primary reservoir host for this HeV-variant strain that may predominantly circulating in GHFF populations (currently referred to as '*HeV-variant horse GXXXXX 2015/ HeV-variant GHFF Adelaide 2013*'). **Ed Annand, Ina Smith, JS Eden, Alison Peel, Raina Plowright, Andrew Breed, Kai Xu, Christopher Broder and Peter Reid. Feb 2021. *'Horses as Sentinels' National Research Update* by email-letter care of CVOs of Australia. 23/02/21**

2. Such species-predominance may result from early viral genotype diversification in populations of bat species that were previously more isolated geographically. In this way, if there is determined to be predominance of this HeV-variant in the more-southernly distributed GHFF and classic-HeV in more-northerly distributed BFF may be due to a 'founder effect'. **JS Eden, Ed Annand, Ina Smith, Peter Reid. *'Horses as Sentinels' National Research Update* by email-letter care of CVOs of Australia. 23/02/21**

3. Alternatively, or additionally, flying fox species variation in susceptibility, viral load and shedding may relate to small differences in variant phenotype (in regions other than the Ephrin-B2/-B3 receptor binding domain) and may contribute to a species association for each strain. **Ed Annand, Ina Smith, Kai Xu, Christopher Broder and Peter Reid. *'Horses as Sentinels' National Research Update* by email-letter care of CVOs of Australia. 23/02/21**

Investigation of these hypotheses might be achieved by prioritised research and suitable multi-species One-health surveillance. Such future work may provide evidence for host-virus co-evolution of species-specific viral species/strains.

Scientific discovery

The initial detection resulted from our batched molecular research testing pathway that combines next-generation-sequencing with the use of nested-conventional-PAN-Paramyxovirus PCR screening to samples from disease cases prioritised utilising our SQL database which aims to facilitate this research support of routine biosecurity while leaving sensitive information with the state laboratory. Cases were prioritised based on their likelihood of infectious cause following our pathogenic-basis-of-disease syndromic analysis.

From this we identified the polymerase (L protein) RNA sequence of a novel Paramyxovirus most closely related to HeV (approx. 11% nucleotide sequence difference in this region).

A phylogenetic tree generated using the partial L gene shows the relationship of the new variant (in red) to other paramyxoviruses (figure 1). Following RNA-based next generation sequencing of the EDTA blood sample, ~99% of the genome of the new variant was generated. The comparison of the nucleotides of this variant to the genome to the HeV reference strain revealed that a 82.9% nucleotide similarity. The variant shares 82.3% to 95.7% amino acid identity (mean 92.5%) when compared with the proteins from the HeV reference strain.

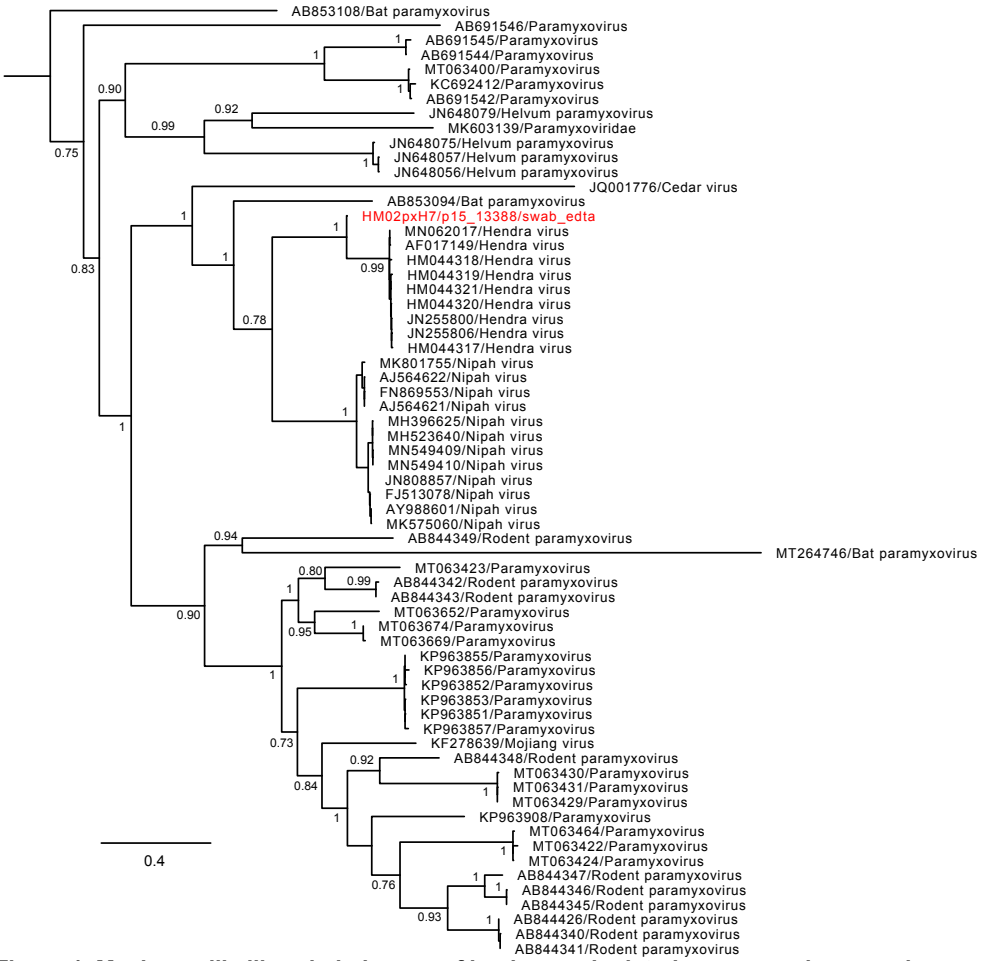

Figure 1. Maximum-likelihood phylogeny of henipa- and related paramyxoviruses using partial polymerase gene. The genetic relationship of the virus from 2015 fatal equine case was compared to reference sequences sourced from NCBI GenBank (case coloured in red). This analysis revealed the newly described virus to be a previously unsampled lineage of Hendra virus. The node labels show the branching support, and the scale bar represents the number of substitutions per site.

Considerations of priority to national biosecurity

Our intent in sharing our significant findings in this way, prior to their publication, is to enable the most-appropriate national and inter-sectorial response to these findings by human and animal health relating to HeV disease.

The most-important scientific considerations currently relating to this variant's identification from the perspective of horse veterinarians and public health are:

a) Will currently available immune-prophylaxis (Equivac® HeV) and post-exposure therapy (m102.4) be effective for this variant?

The *HeV-variant 2015 horse* G glycoprotein, known also as the receptor binding protein (RBP) has a 92.5% identity to the reference prototypic HeV RBP. An *in-silico* analysis of the variant RBP, using previously determined x-ray structures of the reference HeV RBP, afforded through collaboration with Prof Christopher Broder and Dr Kai Xu has shown that the neutralizing human monoclonal antibody (m102.4), should bind effectively to the RBP of the variant. The variant RBP also shares the Ephrin-B2 & -B3 virus entry receptors binding domain elements with the reference HeV RPB, and thus supports the clinical manifestation of HeV-disease observed in the case described above. Importantly, these findings, support understanding that equivalent immune-protection should be afforded by the Equivac HeV® vaccine against this HeV-variant.

b) Can this variant be reliably detected via routine biosecurity surveillance?

There are sufficient mismatches in genomic sequence (approx. 15%) that would result in this variant not being detected by the molecular detection methods relied upon by surveillance in humans and animals.

i. In response to this we have redesigned the M and P qPCR approaches (N gene assay currently in development) to detect the variant and in such a way to be incorporated most easily and rapidly into routine use.

ii. We are in the process of optimising their use in collaboration with CSIRO ACDP and QDAF BSL scientists and wish to share this capacity ASAP with all state and national laboratories responsible for the screening of HeV disease.

iii. Based on the above HeV-variant RBP characterisation, serological assays that use the reference HeV RBP (the soluble G glycoprotein antigen (sG)) are expected to readily detect target immunoglobulins (IgG and IgM) in horses with exposure to this HeV variant. This has implications for human, flying fox and horse testing and importantly offers an understanding for the previously observed mismatch between seropositivity in GHFF and relatively lower viral detections and spillover events from this species.

Our research program, "*Horses as Sentinels",* testing in horses, humans and flying foxes*"* (via collaborating projects) as well as planned collaborative contribution to testing of horses as part of the validation of a HeV DIVA assay by CSIRO ACDP should assist in clarifying some of these unknowns relating to serology testing for HeV.

c) How consistently is this HeV-variant associated with GHFF and does this change the geographical range of relatively higher spillover risk for HeV disease?

We have shared the full genomic sequence of this HeV-variant (*HeV-variant Horse GXXXXX 2015*) with scientists at ACDP who are currently attempting its isolation and who shared with us partial genome (6500bp) of a HeV-variant characterised by CSIRO scientists from a diseased GHFF submitted to ACDP (then AAHL) from Adelaide in 2013. Our comparison of these two viruses demonstrated their sharing 99% of their genome. The Flying fox roost most proximal to the spillover event in South-east Queensland is known to host both GHFFs and BFFs.

Please see the nucleotide sequence alignment (Figure 2) showing our newly designed matrix primers and probe set (Mv_fwd_1, Mv_prb_1 and Mv_rev_1) with mismatches against the prototype HeV highlighted. The redesigned primers match both *HeV-variant horse GXXXXX 2015* and *HeV-variant GHFF Adelaide 2013'*sequences, and we have confirmed they perform well. We recommend they be incorporated into routine testing in parallel with current assays.

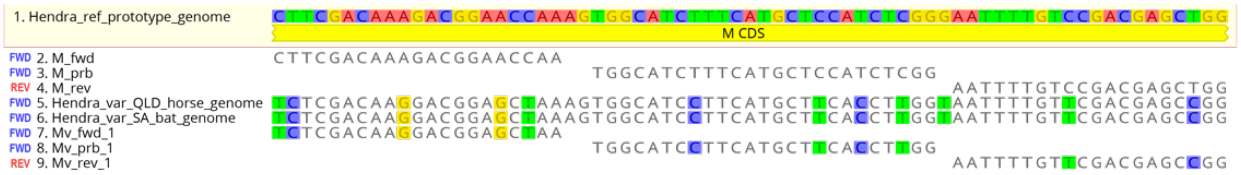

Figure 2: Hendra virus variant primers courtesy of *'Horses as Sentinels Research' group in recommendation for urgent prepublication use on compassionate grounds. Primer design by JS Eden WIMR and I Smith CSIRO* - Feb 2021.

Until such time that we publish these scientific findings including these PCR approaches, please use the caption associated with Figure 2 (above) in acknowledgement and please update us on their use to preserve IP and ensure expediate scientific appraisal of evidence.

We look forward to supporting the national response extending from this finding.

We will communicate any further findings as they become available, with those relevant.

I and other 'Horses as Sentinels' research contributors are available by phone and email.

Yours sincerely,

Dr Ed Annand *on behalf of 'Horses as Sentinels' research*
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Appendix 2: Australian Equine Veterinarian journal publication detailing HeVg2 infection as consistent with prototypic HeV, guiding veterinarians on recognition of suspect cases

Hendra virus - Two Viruses, Same Fatal Disease

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Hendra Virus and Flying Foxes

Pteropodid flying foxes are the natural reservoir for the highly pathogenic Hendra virus (HeV). [1] HeV causes severe acute respiratory and encephalitic disease mediated by endothelial vasculitis, with high fatality and chronic encephalitis. [2–4] There have been 64 known natural spillovers of HeV in Australian horses, resulting in 106 horse deaths, with 4 of the seven confirmed human cases being fatal. [5–9]

Anti-HeV antibodies are detected in all Australian mainland flying foxes, with seroprevalence between ~20% and ~65%. [10,11] In the grey-headed flying-fox (GHFF), seroprevalence was ~40% from roosts sampled in SA and Vic, [12] and 57% from one in southeast Qld. [10] HeV transmission from flying foxes to horses occurs primarily via urine; [13] mainly black (BFF) and spectacled flying foxes are thought to play active roles in disease transmission. [13,14]

Horses are likely to be vulnerable to respiratory droplet HeV transmission because of their grazing behaviour, large respiratory tidal volume and large surface area ratio of their highly-vascularised upper respiratory epithelium. [15,16]

Veterinary disease detection and testing

Passive disease surveillance and biosecurity risk management for HeV and EIDs in Australian animals largely relies on clinical syndromic recognition and management of suspect disease cases by private veterinarians who play crucial roles relevant to animal and human health. [17]

Surveillance via suspect disease testing is subject to strong regional bias for regions where HeV has previously been detected and where high horse populations overlap with the distribution of black and spectacled flying foxes. [18] Testing is less commonly performed on horses with similar clinical signs in southern Australia because of the perception that the likelihood of disease occurrence is lower outside the distribution of these species. [19] Black flying foxes have expanded their range south in response to anthropogenic changes, increasing their overlap with GHFFs. [20]

Clinical decision making should be based on consideration of clinical disease in the context of disease pathogenesis. Given that HeV manifests as a systemic vasculitis, a broad range of clinical signs may be seen in horses. These depend on the 'window' in time that clinical observations are made, the infectious dose of virus and route of infection, individual animal variation in immune and inflammation response, the completeness of the examination, and reporting accuracy.

Clinical presentation of Hendra virus disease in Horses

HeV infection in horses usually results in an acute fulminating disease course with rapid progression over 2-3 days and a case mortality rate of approximately 80%. In acute cases, the most common signs observed in all infected horses from 1994 to 2018 have been a rapid onset of illness, anorexia, tachycardia, pyrexia, depression, mucous membrane injection/ congestion, and a rapid deterioration with either overt respiratory and/or neurologic signs. [7, 21] Respiratory signs include tachypnoea, hyperpnoea (with deep inspiratory or expiratory effort), dyspnoea and nasal discharge (shortly ante-mortem and/ or post-mortem). Reduced respiratory sounds due to pulmonary consolidation can feature on auscultation in the later stages of the disease. Clinical signs of neurological dysfunction are also apparent, including encephalitic signs. Mucous membrane changes feature hyperaemia initially around incisor gingival margins, progressing to hyperaemic petechial/ecchymotic areas across

gingivae, which coalesce, then become more deeply reddened and eventually cyanosed. A minority of infections have been asymptomatic or mildly symptomatic, whilst some horses which have survived acute infection have developed persistent neurological signs consistent with non-suppurative meningoencephalitis.[7,21,22]

Testing

Over 1000 horses are tested for HeV annually; many of these horses suffer diseases clinically consistent with HeV, such as acute severe respiratory and/or neurological disease, pyrexia and other signs of disease mediated via endothelial vasculitis. However, less than 1% of horses tested annually are found to be positive.[23]

Routinely, alternative PCR diagnostic approaches are only applied to HeV-positive samples that undergo further molecular characterisation as part of confirmatory testing.[24] Importantly, this means that most horse-disease cases found negative for HeV are not investigated further.

Research

Our 'Horses as Sentinels' research developed an innovative approach to targeted sentinel surveillance extending from routine state biosecurity priority significant-disease-investigation (passive HeV disease surveillance) to improve HeV disease risk management and Australia's biosecurity. A transdisciplinary (virology, pathology, clinical equine veterinary science, epidemiology) systematic approach to the syndromic interpretation of HeV-like disease was undertaken.

We constructed a biobank of diagnostic specimens collected from horses that underwent HeV testing in Queensland between 2015 and 2018, were negative by RT-qPCR and for which no causative diagnosis had been determined. Clinical, epidemiological and sample-related data were recorded, and samples archived at -80 °C. Information collected on each individual case was drawn from primary laboratory submission documentation.

We reviewed and analysed the pathogenic, clinical, and syndromic characteristics of HeV disease and differential diagnoses to guide clinical recognition of severe HeV-like disease most likely to be of similar viral cause in the Australian context. We then combined (via SQL database) systematic clinical-syndromic-pathogenic analyses with molecular, bioinformatical and serological pathogen-discovery pipelines.

Using this approach, we identified a HeV variant that failed detection by routine diagnostic testing.[9] Prioritisation of this case in our research testing pathway was based on the clear description of disease consistent with HeV by the attending veterinarian.[8,9]

The virus identified is considered a novel variant of HeV (HeV-var), based on the ICTV criteria, [25] of apparent equivalent pathogenicity and zoonotic spillover potential.[9] Due to the high similarity at the protein level between this variant and prototype HeV, it is expected that the current HeV horse vaccine will elicit protective antibodies, with current serological assays unable to distinguish between exposure to the variants.[9]

The similarity of this virus (99%) to partial sequence detected from a SA GHFF, along with case exposure to this species in QLD, suggest circulation at least across the range of this flying-fox species.[9] Further investigation should be prioritised, and biosecurity practices updated to appreciate HeV spillover risk across all regions frequented by flying foxes regardless of species.

The research has led to the identification of multiple novel disease agents of variable significance to horse, human and ecological health. We are currently interpreting results from extensive testing of our priority cohort of 300 cases categorised as the highest likelihood of infectious cause drawn from over 1700 HeV excluded bio-banked cases.

Conclusion

Our findings demonstrate the limitation of exclusion-based testing for emerging zoonoses, and a gap in understanding how frequently the detection of known zoonoses across a broad range of systems is missed because of the diagnostic tools used.[9] We have described a new assay suitable for routine human and animal health laboratory diagnosis and surveillance of HeV.[9]

This research has demonstrated the potential for improved significant zoonotic disease detection when ground-level veterinary observations are optimally interpreted and coupled with advanced 'One Health' multidisciplinary epidemiology interagency collaborative approaches to targeted sentinel surveillance.

These findings prompt an urgent reassessment of HeV spillover risk for zoonotic HeV disease in horses and in-contact humans living in southern New South Wales, Victoria and South Australia, where previously the risk has been considered substantially lower or negligible compared to regions within the distribution of BFFs. Biosecurity practices should be reviewed and updated to encompass an appreciation of spillover risk to include all regions frequented by flying foxes regardless of species. Further investigations should be prioritised to determine the prevalence of HeV-var circulation among and excretion from all Australian flying fox species.

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Novel variant Hendra virus infection in a horse in Newcastle, New South Wales

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1. Introduction

- Hendra virus (HeV) is one of Australia's highest profile zoonotic diseases, with high consequences for both horses and humans
- 65 spill-over events have been recognised since its discovery in 1994, with 107 horse deaths[1]
- HeV has also caused 4 human deaths amongst 7 confirmed cases[2]
- HeV is endotheliotropic and disease results from vascular compromise that affects many tissues[3]
- A wide variety of clinical manifestations may therefore be observed depending on the stage of the disease process
  - Typically acute onset with severe rapid deterioration to fatal disease[3,4]
  - The disease syndrome is well understood to feature pyrexia and mucous membrane changes (injection, congestion and/or petechial haemorrhage) with overt respiratory and/or neurological signs
  - Cases may also show colic, vague signs of anorexia and lethargy, or may be found dead
- The natural reservoir is the *Pteropus* spp. fruit bats (flying foxes), of which there are four species in mainland Australia[5]
- HeV spill-over infections in horses have been previously recognised from Far North Queensland to Scone in NSW[1] and the southern limit is moving progressively south
- Human infection is via direct contact with bodily fluids and respiratory droplets from infected horses, with an estimated attack rate of 10% for people exposed[3]
- In early 2021, a novel variant Hendra virus genotype 2 (HeV-g2) was identified during retrospective surveillance testing of samples from a horse that died in Queensland in 2015[4]
- In October 2021, the first antemortem equine case of HeV-g2 was detected in Newcastle, NSW via updated PCR testing
  - This is the southernmost HeV (of either genotype) detection to date

Map showing locations of HeV-g1 and HeV-g2 cases in horses and HeV-g2 detections in flying foxes in Australia

2. The novel HeV-g2 variant

- After its discovery, diagnostic tests were promptly updated by June 2021[4]
- Clinically indistinguishable and equivalent pathogenicity to HeV-g1[4,6]
- Sufficiently different genome sequence to fail detection by qPCR but similar enough to be susceptible to the current vaccine and pose equivalent One Health disease risks[4]
- Detections in samples from flying foxes in Victoria, South Australia and Western Australia[7] support that HeV-g2 has a broader distribution and highlights the risk of spill-over events in all regions with flying-foxes, broadening the previously known at-risk geographic areas

3. The Newcastle HeV-g2 case

In October 2021, the first contemporary detection of HeV-g2 in a horse was made in Newcastle, NSW. The horse was a seven year old unvaccinated Clydesdale gelding and was euthanised. 11 people and 9 horses were close contacts, no further infections occurred. This is the southernmost Hendra virus (HeV) detection to date, extending the southern limit by 95km.

Timeline of events of the Newcastle HeV-g2 case from first recognition of clinical signs to positive PCR result

Owner noted anorexia	Owner noted ataxia and circling	Clinical examination consistent with HeV	Rapid progression to moribund condition	Euthanasia and sample collection	**HeV-g2 positive**
• Morning of 5th October 2021 • No intervention • Horse left unattended	• Afternoon of 5th October 2021 • Veterinary attention sought immediately	• Obtunded • Mydriasis + negative menace response • Profuse sweating • Purple mucous membranes • Tachycardia (100bpm) • Tachypnoea (28brpm)*	• During examination • Collapsed into lateral recumbency • Non-responsive • Unable to rise • Nystagmus	• Euthanised with lethal injection via jugular catheter • With owner consent • EDTA blood sample taken for HeV PCR exclusion testing	• Midday on 6th October 2021 • Reported by EMAI, confirmed by ACDP • HeV-g2 RNA detected by qPCR, negative for HeV-g1

*Relatively minimal respiratory signs with no overt secretions

4. Public Health Response

- The gelding lived alone in a one-hectare paddock but shared a fence line with nose-to-nose contact
- The owner was directed to leave the body untouched, and the Emergency Animal Disease biosecurity procedures were explained
  - Despite this, farewells and burial took place overnight with a total of 11 people
- An urgent multi-agency outbreak response took place involving risk assessment of exposed people and at-risk animals
- 9 at-risk horses were identified
  - Owners given PPE and infection control training
- Biosecurity Direction placed to prevent animal movement on and off the property for 21 days
- 12 human contacts were identified
  - 3 low-moderate, 8 low and 1 nil likelihood of transmissible exposure
  - Interviewed daily to screen for any symptoms
- No testing or post-exposure therapy was required
- No human or additional animal cases occurred

5. Discussion

- This was the first HeV-g2 detection in equine specimens submitted for routine government biosecurity priority testing
- Its detection was a testament to rapid implementation of a new diagnostic test following the discovery of the new variant and subsequent proactive interpretation of expanded disease risk by both clinical and biosecurity sector veterinarians
- A fast and effective public health response was carried out, which resulted in no further human or animal infections
- It is also the southernmost detection of either HeV variant in Australia
  - This supports the expanded geographical risk associated with HeV-g2[4,7]
- The case was also outside the typical winter seasonal window in which most cases of HeV-g1 are recognised [1]
  - 69% of HeV-g1 infections are in winter[1], coinciding with the seasonality of detection of HeV in flying fox excreta
  - Proactive wildlife surveillance may help to determine if the seasonality of HeV-g2 is different
- The clinical presentation was similar to the 2015 HeV-g2 retrospective case in Queensland, with acute onset and deterioration, congested mucous membranes, tachycardia, neurological signs and normal rectal temperature. Respiratory signs were mild and increased oronasal secretions were not recognised[4]
- Clinical manifestations for HeV infection are broad and it should therefore be considered as a differential diagnosis with any of the expected range of clinical signs, especially neurological and/or respiratory, as well as sudden death cases
  - It should be considered in all regions that host flying foxes, not just the traditional at-risk areas
- The Newcastle case occurred near an urban centre, with a large diverse equine population and low HeV vaccination rates
  - The previously southernmost case in Scone prompted vaccine uptake amongst the local Thoroughbred breeding community including implementation of prescriptive vaccination policies[8]
  - The pleasure horse demographic involved in this case highlights the need to tailor risk communication strategies and vaccine promotion to a variety of horse owners
- Unidentified historical HeV-g2 infections are expected to have resulted in horse deaths and unmanaged risks for humans
  - On the same property in Newcastle, HeV testing had been performed in 2016 for two cases of sudden death
    - qPCR was negative for HeV-g1, the samples were not archived so retrospective HeV-g2 testing is not possible

6. Conclusions

- The discovery of the HeV-g2 variant and its detection in Newcastle confirms the greatly extended geographical risk of HeV spill-over to horses
  - Any region with flying-foxes should consider the possibility of HeV infection
    - Vaccinate horses, implement precautions and perform testing in suspect cases
  - Vigilance is also needed outside the winter season
- The case demonstrates the real-world benefits of sentinel emerging infectious disease research
- It also emphasises the importance of rapid interagency response and effective integration of relevant sectors
- In the absence of a human vaccine, vaccination of horses remains a mainstay to prevent zoonosis
- Subsequent research may inform further on:
  - Owner risk perception, communication strategies and barriers to vaccine uptake
  - Transmission and spill-over risk for each genotype combined with proactive surveillance
  - Related and emerging viruses of similar significance

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Sentinel Surveillance Shows Novel Hendra Virus in Horses in Australia

[Announcer] This program is presented by the Centers for Disease Control and Prevention.

[Sarah Gregory] Hello, I'm Sarah Gregory, and today I'm talking with Dr. Edward Annand, an equine veterinarian epidemiologist and a research associate at the University of Sydney School of Veterinary Science in Australia. We'll be discussing the detection of a novel Hendra virus variant from a horse in Australia. Welcome, Dr. Annand.

[Edward Annand] Thanks very much for having me, Sarah. It's a pleasure to be here.

[Sarah Gregory] Well, it's a pleasure to have you. Actually...you're my first Australian guest, so I'm quite excited to have you here. Lot of things going on here in this study of yours, so let's start with a real basic. What is Hendra virus? This is new, right?

[Edward Annand] Well, it's a paramyxovirus, so that's the same family as measles, mumps and Newcastle disease, say, in chickens. And the genus is the *Henipavirus* genus. So...the Hendra virus... may seem a little new, and we do think of it as an emerging disease, but I think it might be more appropriate to think of that word "emerging" as relating to our understanding, not so much the virus. It's very likely an ancient virus that has co-evolved with flying foxes. They seem to have an incredible capacity to live with Hendra virus and many other paramyxoviruses, whereas when Hendra virus spills over into domestic animal species and then, unfortunately, sometimes also into humans as well, it is highly fatal.

[Sarah Gregory] Alright. So emerging but been around for a long time, recognized as emerging. When was it first recognized?

[Edward Annand] So Hendra virus was first recognized when it spilled over into some Thoroughbred racehorses in 1994. And a very good friend of mine, Dr. Peter Reid, who's also one of our co-authors on the paper, was the attending veterinarian in '94 in Hendra, which is a suburb of Brisbane - a really lovely suburb near the river - a very unique suburb where there's a sort of culture of training the racehorses almost from sort of backyard stables and taking them down to the little beach nearby and swimming them - passionate horse owners. Wonderful horses have been raised and trained in Hendra.

It was a mare called Drama Series, incidentally enough, who had been spelling (out in a paddock not within the city, nearby) and there had been some flying foxes frequenting the trees there next to that paddock. She developed the illness and was brought into to a stable, and actually, there was 13 horses that died within a 12-hour period… Peter described those horses as sort of having drowned in their own lung fluid. So it was very spectacular for Peter.

Also for our biosecurity and infectious disease department because we had what appeared to be a highly contagious disease, especially when it's also affected a horse in the neighbouring stable, and it affected a strapper (who became very ill) and unfortunately the trainer, who died. So it was recognized that this was very likely an infectious disease and something we hadn't seen or recognized before. There was a big effort to work out what it was, and they ended up isolating a novel virus and originally called it 'equine morbillivirus', and it was later named the Hendra virus. And also, the flying foxes were identified as that reservoir host and the source of the infection.

[Sarah Gregory] So Hendra was actually the location, sort of like Ebola virus is named after the river.

[Edward Annand] Yeah. Most viruses have been named, I guess, after their location. A close relative of Hendra virus in the pararubulavirus family is called Menangle virus - also from Australian flying foxes. Again, that's a suburb as well. So Hendra...the people of Hendra are a little bit sensitive, of course, to the name, but...and they prefer it to be called Hendra virus disease, not Hendra disease. But, yeah, that's where it started.

[Sarah Gregory] And apparently, as you were saying, it affects horses predominantly. Why are horses more susceptible?

[Edward Annand] So this is really more theorized than known for sure, because we don't know, perhaps, the full range of species that this virus might have affected without us realizing. But horses are the only domestic animal species in which Hendra virus has been detected. Internationally, there is Nipah virus, which has affected pigs, and we can talk a bit more about that.

Horses are very inquisitive animals, and quite inquisitive in their grazing behaviour. They have very well...like, overdeveloped airway (upper airway) spaces—including sinuses, guttural pouches—and so there's a lot of surface area of highly vascularized epithelium. And they have a very high tidal volume. So when they're going around grazing, they're sniffing in and out what they're about to eat - to see if it's good or not. Then they also have a quite well-developed olfactory nervous system - running down into the front of their lip area there. This is something that's involved when they do that 'flehmen response'. Those people who've had horses, particularly stallions - after they smell a mare when she's in heat, they will flip the lip up - that's the flehmen response.

So there is a thought there that, given Hendra virus is both neurotropic, and can be pneumotropic (which means...the first one is it can infect the nervous tissue and the second one is, you know, can infect the respiratory tissue), there's two possible entry pathways for the virus: It can enter via the respiratory system, having been inhaled as a droplet from a contaminated urine deposit that's on the ground there; or it may also track its way, beginning in the epithelium of the lip and olfactory areas in the upper airway system, up through the nervous system as well. These have been shown via histology to be possible viral entry pathways.

They are also very closely monitored by their owners. So compared to a cow say, who's one of the herd, or other production animals, where, you know, the veterinarian may not be called for every sick animal, horses—while they sometimes are run very extensively and not checked so regularly—there are many horses that the veterinarians attend regularly. And many owners will, get a horse out more readily than they'd go to the doctor themselves, you know? Veterinarians are attending, even for minor illnesses. And so...they're a very appropriate sentinel species in that way.

[Sarah Gregory] I see, okay.
So you've mentioned Nipah virus now a couple of times. What is Nipah virus?

[Edward Annand] So Nipah virus is very closely related to Hendra virus. It's similar enough that the available vaccine, which was made for Hendra virus for horses, and now has been developed for humans, is cross-protective. That vaccine targets the G glycoprotein (now called the receptor-binding protein). Even though there's about only about 70% similarity between the HeV G glycoprotein (this protein is the way they attach into the cell) and the Nipah virus G glycoprotein, the immunity that an immunized mammal gains is effective against both viruses. So they're that similar. They're also quite similar in their

pathogenicity and tend to cause the same range of disease manifestations. They are both circulating in flying foxes - so pteropodid bats (fruit bats) are the reservoir hosts for both viruses.

So the biggest difference is ***where*** they are occurring. Nipah virus has been identified spilling over into pigs and to humans, without domestic animal intermediate hosts, via the date palm. This has happened in Malaysia and Bangladesh. We haven't detected Nipah virus in Australia, but we've got a sort of equivalent, you know, a virus that's so similar that that vaccine works in the same way, that shares spillover reservoir species and shows the same manifestation of disease. So it's really that their geographic distribution that's the difference, there.

[Sarah Gregory] I see, okay. Yeah, I actually did a podcast on Nipah virus and the date palm trees and how they were infecting the sap being collected below it several years ago. These viruses sound like they're pretty deadly, is that right?

[Edward Annand] Yeah. They are considered, in a way, the deadliest viruses that we know of. But… while in an individual patient, that becomes sick, in those that become infected with these viruses (both horses and humans), they are highly lethal. There's been recorded fatality rates ranging from sort of 70% to even 100%. But there is another consideration—I think coronavirus (SARS- coronavirus-2) perhaps even in comparison to SARS-coronavirus-1—reminded us that how deadly a pathogen is, also has to take into account how many…. how contagious it is between humans…. it's a bit of a balance. If you've got something that is really deadly, then you tend to detect it… and it doesn't spread as far and as quickly. And that was sort of the case for the SARS-coronavirus-1, whereas SARS-coronavirus-2, being much more mild disease in the majority of people, managed to spread across the world - as we know too well. And it's a little bit similar for Hendra virus (henipaviruses). They are deadly and they're a big concern for veterinarians, and for those...at highest risk. But perhaps what would be even more deadly, would be a version of these viruses, that we might discover one day, that was even more transmissible and even perhaps slightly less deadly in the individual.

[Sarah Gregory] I seem to remember with SARS-1 that that's basically why it died out so quickly, because it was so deadly it killed off its hosts and didn't...and wasn't so transmissible because it didn't have anywhere to move to. Is that sort of a fair assessment?

[Edward Annand] Yeah, that's kind of what I was saying. That's the idea there, and it's good to be reminded of that. But these henipaviruses have been listed consistently in those top priority diseases (along with highly pathogenic avian influenza, SARS coronaviruses and Ebola viruses) as global priorities and potentially pandemic threats. But as we discussed, for Hendra virus or Nipah virus, the true pandemic threat would be a virus that was more transmissible. In fact, the canine distemper virus is in the same family and quite closely related, and it's an example of a virus that is more...far more transmissible. So something between Hendra and canine distemper virus (a divergent or emerging morbillivirus) would be the ultimate threat, perhaps.

When we talk about those fatality rates—say, 70%, 80%, 100%—a lot of the time we won't detect the milder cases, particularly in an extensive outbreak. Serology can help determine that, but it's always a challenge to determine the true fatality rate because it's always a challenge to know the true prevalence of many outbreaks. In the case of HeV, it's not so much a challenge because we tend to be right on top of it when we've detected it, and monitor all of the horses that are in contact. But nevertheless, it's clear that there will... In that first outbreak, there were 20% of horses that didn't show severe disease and that...but did seroconvert, and so obviously had become infected.

[Sarah Gregory] I see. Okay, so what are the symptoms? You talked about two different ways it can transmit earlier, but how does it manifest in a horse, and is it the same or different than in people?

[Edward Annand] So yes, thanks, Sarah. We thought a lot about this as part of our research, because it is recognizing the consistency...the disease manifestations in the horse as being consistent with HeV infection, that enables detection, testing, and laboratory confirmation. And without all of that, then the humans that are exposed will not receive appropriate, timely assessment for exposure and/or infection and healthcare… including - you know - potentially lifesaving treatment…

So we've tried to think a lot about it… We've looked at all of the manifestations...the previous cases… and the symptoms that they showed…, and what we found is that: there is a lot of variation in any one time point… So a lot of the time, vets will look at horses and only have a small window to examine the horse. But what is consistent is the manifestation through time…: and what tends to happen is that the horse becomes very depressed; it may have a high fever in that early stage - So in a way, it's kind of influenza-like symptoms without so much of the runny nose…

And then it will usually progress quite rapidly (it could be within a 12-hour period) to overt respiratory manifestations (respiratory distress) and/or neurological, sort of central nervous system encephalitic signs, including changes in the gait, head pressing, even seizure and nystagmus. There's also a lot of sweating, and that's a typical progression of disease. And including the changes in the gingival mucous membranes, they become quite congested, early on they might be a little bit injected, we say, which is when they have prominent vasculature and it's something that we look at in the horse.

And in humans, it's quite similar in that they have influenza-like illness early on, usually progressing to encephalitic signs - without so much of that overt respiratory distress being sort of reported. There are, of course, very few cases. There's been seven cases of confirmed Hendra infection, of which four have passed away, and a number have been veterinarians – sadly. So we realize there is a large risk for veterinarians…

[Sarah Gregory] You mentioned flying foxes. So horses are getting Hendra virus from spillover from flying foxes. Tell us how that happens.

[Edward Annand] So the flying foxes, as we mentioned earlier, are understood to have co-evolved with these viruses. They live in dense communities (when roosting); they have very high metabolism - of course bats being the only flying mammals. So they're sort of perfect host for RNA viruses to co-evolve with, and they've evolved with the RNA viruses. So they...they're own immunity/ immune systems have evolved in ways that we're only just beginning to understand to be very resilient against severe disease or disease (at all) from these viruses.

With the formally known HeV, which we now call Hendra virus genotype 1 (before this discovery), there tended to be a seasonal trend (winter months), and certain regions that were thought to be the predominant areas where we were going to expect spillover. But ultimately, it is a sporadic event. All we need to happen is for an infected deposit of urine to land into a paddock where there's a horse that is either not vaccinated or doesn't have appropriate immunity, and that horse to engage with that—as we discussed earlier, usually smelling it or sniffing it as part of their grazing behaviour. And that's sort of what we need to happen....in order to see infection in a horse, and it is very unpredictable as to exactly where that's going to happen. It's a sporadic event..., occurring at relatively low frequency compared with other viruses (such as those endemic among horses), and yet it happens consistently… sporadically with low frequency, where we've seen more than 60 spillover events resulting in more than 100 horse fatalities since we've known about this virus. So it is a very big challenge to know we are catching all cases. We actually have been testing around about a thousand suspect cases each year but finding less than 1% have Hendra. And a lot of horse diseases can look a little bit similar to Hendra, particularly at a particular point in time. Even some non-infectious causes of disease, such as colic, which is acute abdominal disease, say, from a twisted bowel, that can result in the horses staggering, changing their mucus membrane and sweating a lot. And so, it can be a really big challenge to decide when we think that Hendra might be involved, to take the right samples, and to go through that process. There's a fantastic response system set up where veterinarians and owners can get an answer, usually within a day - sometimes it can take a bit longer, but the rapid screening turnaround allows them (attending veterinarians) to critically manage the human health risks and the biosecurity risks.

[Sarah Gregory] And rapidity is very important, because apparently this virus is...it goes from zero to 60 very quickly, is what I'm hearing, right?

[Edward Annand] Right. So just the progression of disease, in an unvaccinated animal, in an area where there are flying foxes, even if the veterinarians don't often perceive/ realise the potential contact with flying foxes…: sometimes it might not be obvious that they are on the same property, they may just be flying overhead or visiting a dam on the property, and it might not be known to the veterinarian, and sometimes not even to the owner. So we tend to try to err on the side of caution, and veterinarians test any case that is not vaccinated, and could be a HeV case, even if Hendra virus might not be our top differential at the time that we're looking at the horse.

[Sarah Gregory] So flying foxes are not foxes, they are actually bats, yes?

[Edward Annand] Right. So they are Old World fruit bats (pteropodid fruit bats). Their face is a little bit fox-like, and if anyone finds them something that's not so nice to look at, it's useful just to turn the photo around so that they are kind of upside down, but they look kind of the right way up for us. And it's amazing how cute they look straight away, I think, even for people that may have not found them so cute - may have found them a little bit scary.

[Sarah Gregory] I see, okay. So horses are getting it from snuffling around in the grass where there's urine, most likely. How are the people getting it from the horses?

[Edward Annand] Good question, Sarah. So all of the known cases of Hendra have occurred where there's been significant exposure to bodily fluids or through direct exposure to the respiratory excretions, or even just close proximity of the human face to the airway of the horse. When horses are ill, veterinarians often are obliged to perform pretty invasive procedures to help them, it might be passing a nasogastric tube to administer fluids, passing an endoscope to check the respiratory function (upper respiratory), or doing a rectal examination to check for colic. And especially if horses are staggering around… there again - there can be a lot of fluid involved, be it blood or excretions, so it can be quite hard to control that. So veterinarians in areas that perceive a risk for HeV have been trained in both themselves applying personal protective equipment and biosecurity protocols, but also in training the owners, on the spot, in quarantine practice, safe biosecurity, and personal protective equipment use, as well.

[Sarah Gregory] So when you're examining a horse, you're using PPEs (the vets)?

[Edward Annand] Right. In a perfect world, from a HeV point of view, we'd all have our PPE on every time we see a horse that even could conceivably have HeV. But as you can imagine, in the broad geography of Australia, in some areas that are hot and dusty (over 40°C temperature at times) —also busy veterinarians, stud veterinarians—we tend not to be in PPE as a rule. We have the concept of staged PPE. So we might always try to put on some gloves, particularly if go look at an ill animal or if we go look at a vulnerable animal, like a foal. And then the next stage up, we're going to be putting on a mask as well, overalls. Another high-risk scenario is examining foetal loss (abortion) for zoonotic disease, where we use a higher tier of PPE. But for Hendra virus, it's our highest tier of PPE.

When we feel that it could be the case...and as I said, we don't often realize that before we get there, we might not even realize it at the start of the examination, and sometimes we might not even realize it on the first examination. It may be a subsequent examination where we realize it. But once we sort of pull that trigger of suspicion, of that virus being involved, then we're going to put on the full...what do they call it...hazmat suits. You know, the goggles, the mask, and the gloves, and take full precaution. And also, we're sort of a little bit...we have to minimize the treatments, and this is very sad actually - we're very minimized then as to what we can do for the horse. We can't do all those usual treatments that might be necessary or might really help. And that's been a big issue, including for welfare, for personal indemnity, liability, and there have even been horses that have passed away for otherwise saveable conditions, because of the need to exclude the possibility of Hendra virus, and that delaying the timeliness of treatment or restricting the range of treatment that can be afforded to the horse until we get the negative result.

[Sarah Gregory] Oh, that's a shame, yeah. So since Hendra (Hendra virus) is so deadly, it seems that treatments and vaccines are rapidly in the works, or there are some already. How's that going? Tell us about that.

[Edward Annand] Yeah. So we're very lucky to have a highly effective vaccine for use in horses. This was developed as a priority because of how deadly this virus is for humans. Particularly upon realizing around 2011, and even from 2009, we started to see more cases identified. So after '94, there was just sporadic case identification. Unfortunately, some more fatalities occurred. And then in 2009, there was another larger disease event that involved an equine hospital and the loss of another veterinarian's life. This prompted *(both increased awareness among veterinarians resulting in increased suspect case submissions and)* the urgent development of a vaccine for horses, as a sort of One Health approach to protecting humans and of course protecting horses. It's been available since 2012, and has been used across Australia, but predominantly where there is perceived to be the greatest risk, so that's the areas of Eastern Queensland and North-eastern New South Wales.

In experimental trials using laboratory animals, that vaccine has shown equal efficacy for Nipah virus (both Bangladesh and Malaysian strain). And because of the high priority of the henipaviruses as global disease threats, there's been good support and funding, particularly in the US, to see the development of that vaccine for humans. That is now at a point where the vaccine using the same mechanism, same antigenic protein, is undergoing Phase 1 human trial. There is also a monoclonal antibody that has been developed. It's Professor Chris Broder (who is one of our wonderful co-authors and has been a huge supporter of this research) from the Uniformed Services University of the Health Sciences in Bethesda, who has been the driver and leader of the science behind the development of the vaccine and of the monoclonal antibody. We've seen that monoclonal antibody used, I think, around 13 times now. It tends to be used on compassionate grounds (it's not fully registered yet), but it is a lifesaving treatment and has been used with great success. But it needs to be used promptly after the recognition of disease, and that's why it's so important for us to recognize this disease in horses.

[Sarah Gregory] So we have this vaccine, and now we have a vaccine that's in trial for people. But you mentioned unvaccinated horses. Why would there be unvaccinated horses?

[Edward Annand] Ah. Well, that's a very good question, Sarah. So it is to do with the perception of risk, I guess, and that differing quite heavily between, say, a veterinarian who understands and has thought a lot about this virus and has had to really think about the...this very imminent threat that can happen in an unpredictable way and an unpredictable time (could be the middle of the night). And the veterinarians are really sort of caught between a rock and a hard place because, you know, they get the call in the middle of the night—okay, fine if they know the property and the owner and the horses and everything quite well. Say they don't… say they don't know the property, or the situation really very well at all. All they know is this horse is staggering around, it could just have a colic (non-infectious) or it could have Hendra virus. If they don't go, which some veterinarians unfortunately have had to opt for that decision, then they would realize that there's a missed opportunity (if it would be HeV) for those owners, and that might even include children, who may be quite closely exposed to the horse. As we know, horses can be...there can be great sentimental value to people's horses in some circumstances -they're kept for a wide variety of purposes. So the veterinarian is caught between: them being the only way that there can be a timely diagnosis potentially saving people involved; and their own personal risk - which are very many—their own personal health risk, for their family as well, and for their liability, financial issues, workspace health and safety liability, and this sort of thing. There really is quite a challenging scenario, but the perception of risk is clear for the veterinarian. It's also clear for a very, very highly valuable horse where the insurance policy might say, you know, if your horse does not receive the right timely treatment, then we won't be able to pay out on loss of the horse. Or just someone who's got a particularly valuable horse that they really don't want to lose, and a lot of people will be taking that...opt-in to

take the vaccination. But the owner is realizing that the actual likelihood of their individual horse becoming infected with Hendra virus...the likelihood of it becoming infected is quite low. There's only been, you know, a hundred horses since we've known about this virus that have become infected and died there. So it's unlikely, on the basis of probability, that their individual horse will get Hendra virus. So they've sort of realized that.

There are then cost issues. Some owners will have a sort of vaccine hesitancy in general. And in Australia, there is also an interesting thing where the owners can pick up vaccines from non-veterinary suppliers, and they haven't always needed to have a veterinarian administer the vaccine – this is different in the US. In the US and the UK, most horses have got a passport, and the veterinarian has to come and administer the vaccine and write in there. And in the US, of course, you've got vaccines for West Nile virus, for quite a bunch of diseases for horses. Whereas here, in the past before the HeV vaccine, we really only vaccinated horses for tetanus, some might vaccinate as well for Strangles, and some with breeding horses would vaccinate for equine herpes virus. Particularly the tetanus vaccine, they can be picking this up from their feed store or pet supply store. There's a big difference where the HeV vaccine must be administered by a veterinarian.

There's this challenge where many owners won't perceive the risk as very direct or probable. It's hard for them to realize the risk matrix where, even though it's a low likelihood, if it's a really high consequence (fatal), it's still high risk. So a lot of owners will not understand the justification in getting horses vaccinated - that's why it's been so important to educate them on the risks and where there is a risk.

[Sarah Gregory] I see, okay. Are there particular flying foxes causing these transmissions?

[Edward Annand] This is a really interesting question, Sarah. So after they identified the reservoir host of HeV to be pteropodid flying foxes, there was effort to identify the distribution and which flying foxes carried the virus. So serology has consistently shown that all of the four flying fox species that we have on mainland Australia—the black flying fox, the grey-headed flying fox, the little red flying fox, and the spectacled flying fox—all have immunity (seropositivity) to the HeV receptor binding protein (the G glycoprotein that our vaccine is made of). So serology would suggest that they all carry and could be shedding the virus, and spillover could come from all of them. So initially, it was thought that wherever there was flying foxes in Australia, there was a risk for Hendra virus. But over time, there was a failure to identify Hendra-diseased horses (HeV infected horses via PCR) outside of the range of the black flying fox and the spectacled flying fox. So even though, not only has the serology shown that it was in the other species, but there had been isolation and molecular detection of HeV in the grey-headed flying fox, for example, detections in horses had only occurred in those areas where the black flying fox is circulating and is living. And that is an area that has extended over time—we might talk more about that, if you're interested. But it is Queensland (in the eastern coastal areas of Queensland; so the northeast of Australia) and the north-eastern region of New South Wales (so the next state down). So coastal, tropical, and subtropical regions on our East Coast, and of course this is also where there are plenty of horses living as well.

The other thing that added to what we now see as a misnomer that the distribution of Hendra spillover was limited to there, was that there had been a failure to detect HeV in the urine of flying foxes. Some researchers, including some of the wonderful bat One Health researchers on this paper (Raina Plowright and Alison Peel) have been collecting urine from underneath the roost of the bat with a big plastic sheet, and there had been a failure to identify via PCR HeV in that urine outside of that region, as well. So this was adding to the evidence that maybe the spillover, for some unknown reason, might have only occurred from the black flying fox and the spectacled flying fox. But what our paper shows is that there was an equivalent virus - sufficiently divergent in its genotype sequence (around a 15% difference, there) to fail detection on the established PCR - yet sufficiently conserved in form and function (much less variation in translated amino-acid sequence), and importantly, no difference - no change at all - in the critical epitope on the receptor-binding protein, and no difference in observed disease as well. So we're talking about an equivalent virus that was missing molecular surveillance.

[Sarah Gregory] Okay, that's very interesting. The way horses are sometimes tested involves dead-end testing if the horse is negative for Hendra virus. But are there dangers in this?

[Edward Annand] Thanks, Sarah. This is another very good question. So Australia is not unique in its animal health surveillance and approach to disease management and biosecurity in focusing on a select few, in a way, well-established diseases. This is really the challenge globally for animal health. We know a lot about a range of diseases and we have tests that are really reliable in detecting those diseases. And that is important because it helps us to give rapid, confident diagnostic answers to guide the health of animals, biosecurity of our countries, trade agreements, and the health of humans (when a zoonotic disease.)

The challenge is that we are realizing over time that there is a much greater range of organisms out there, and we are only really looking at the tips of the icebergs. They're very important - the tips of the icebergs that we've been looking - because they are the ones that over all of time, we have understood of causing significant disease. But we are realizing - with the evolution of molecular techniques, next generation sequencing, whole-genome sequencing techniques, open- ended diagnostic approaches - that there is a far greater biodiversity out there. And that this is very important to understanding both health and disease. So, yeah. It has been necessary that the test is really specific and reliable and can give a very quick, conclusive answer of whether or not a horse has Hendra virus (for example). But this study has really highlighted for this context—and of course by analogy, many others—that it's also very important that we consider divergent and emerging disease agents and apply additional testing approaches when indicated. The challenge is, when do we do that? What's the justification for that diagnostic responsibility - to go beyond what we know?

[Sarah Gregory] Looking at your study now, what time-period were you looking at?

[Edward Annand] The time-period we were focusing on with our cases was really from 2015-2018. Our study was actually conceived and began in 2014, off the back of my experiences with the detection of Australian bat lyssavirus, which is a relative of rabies virus, in its first time being detected in domestic mammals (so it had only previously been detected in bats and had caused three known human fatalities). So off the back of those important cases (two horses dying of that) and interpreting/ communicating the science, writing that up with Dr. Peter Reid, as well as many wonderful scientists from the state laboratories and national lab, I became familiar with some incredible virologists, particularly Dr. Ina Smith, that were looking into the wider diversity of bat-borne viruses, and we were sort of realizing that they had a lot more viruses than the disease significance was known for. And as veterinarians, we had a lot more severe, concerning cases than we were getting diagnoses for. So it was really a matchup of that. So, by 2015, we had support of the Australian veterinarians, and particularly the Queensland state laboratory (Biosecurity Sciences Laboratory) and their biosecurity department. We were beginning the sample bank of sort of rescued samples after they'd gone through their window of investigations within the usual system with the usual tests.

[Sarah Gregory] And on that note, what is different about how you suggest surveillance be done?

[Edward Annand] I think that the key difference is that we need to incorporate active surveillance activities, in addition to passive disease reporting and investigation, and that we involve very much transdisciplinary teams, involve the researchers, so that we're getting that cutting edge, forefront approaches from each of the relevant disciplines and sort of combining that in constructive, proactive ways with routine government-based disease surveillance. It's very challenging to do this, but if this study highlights that it can be done and can be very successful... The active surveillance activity here might be described, in a technical way, as risk-based - because we were sort of focusing on the most concerning cases, both targeted and general—so we were targeting a range of related viruses with some of our approaches, but we also had an open-ended aspect to our approach, and so we could detect completely novel viruses—and

sentinel, because we are detecting...like the canary in the mine, you know, we're detecting something earlier than it would otherwise be detected and getting that early warning both for One Health and animal biosecurity benefits.

These sort of active surveillance initiatives can be coupled and integrated with the routine surveillance and disease management approaches that happen in a government-run laboratory (system) if you have the right framework, network, funding and collaboration to enable that to happen - importantly while protecting all of those things that are so important to keeping engagement from the stakeholders at ground level—those to whom the horse matters most, the owners and the vets. They have to be confident that by engaging with that surveillance system (/ activity), they're not going to have too much burden or negative repercussions. So, we focused on that as well - you know, leaving the sensitive information with that state lab and just taking what we needed to do the research, and then giving back to the state lab updated assays, updated information, updated capacity.

[Sarah Gregory] So you had an unusual surveillance employed to detect this case in your study. Is... sort of that everything you had just talked about, how you went about detecting this case?

[Edward Annand] Yeah. We gave a pretty good summary… There's been a lot of talk about...well, I should just say that another form of active surveillance has been wildlife surveillance - we've talked a bit about that through this interview as well. The under roost testing of urine, is an active surveillance activity, again, designed to guide routine surveillance. There's been a lot of talk about the benefit of interdisciplinarity trends, disciplinary approaches both in research and in surveillance generally, including in the government sector. I really like the term "convergence" and convergence research, which is something that the United States research bodies have highlighted should be a big aim, now, since 2016. And actually, the term "convergence", really sums up that transdisciplinary approach, that's drawing from deep within each discipline that's relevant to the problem (to usually to a vexing problem) persistently over time and reviewing their focus on that problem over time. So sort of inquiry-focused or hypothesis-driven research that is transdisciplinary, not just the benefit of the junction and meeting of the disciplines, but actually forming those interdisciplinary teams and drawing from deep within them to use our best tools towards a vaccine problem that is a real significance for society.

[Sarah Gregory] Now briefly, tell us about your study and why you did it.

[Edward Annand] So we called it Horses as Sentinels for Emerging Infectious Diseases, and as I mentioned, it really came out of my personal experiences on the farm (ground level experience) with the detection of Australian bat lyssavirus (the relative of rabies virus there in horses). And then, as a result of that, meeting with the bat virologists, including Dr. Ina Smith, and also being connected very generously with Dr. Peter Reid, who extended his support to me when he heard about the detection - and he knew firsthand, of course, what it could be like to consider such a diagnosis. We formed a group, initially the three of us. We found great support within the state laboratory and amongst our colleagues as veterinarians, who saw this problem and felt it firsthand as well.

So then we formed a team, and we basically were looking to resolve that hypothesis—could there be similar viruses - closely related, likely, to Hendra virus - causing Hendra virus-like disease in Australian horses, posing similar zoonotic human health risk but failing detection via routine surveillance? So that was our hypothesis.

And we looked to resolve that by extending the known assays, developing new assays, both serology approaches - where we used some innovative approaches, such as both the nucleocapsid proteins (which are far more conserved), so actually looking for cross-reactivity as well as more specific proteins like the G glycoprotein or the receptor-binding protein - and then using the latest, best methods of Bayesian latent class analysis, information theory approaches to epidemiology and to test performance, to interpret those results. Because of course we were sort of forging ahead a new path here, and there wasn't always going to be a Gold Standard reference test to check out our work with. So we were using these novel assays as tools, interpreting them as best we could.

And on the molecular side, we have a lot of challenges there for viral discovery when it comes to open-ended, next-generation sequencing because the actual amount of genetic material in the sample, particularly a clinical sample of variable quality, will be at most, in an infected animal, 1%. So you really are looking for a needle in a haystack. The RNA itself is very, very fragile, so we have to really take care of the samples, keep them at negative 80, think about the biosafety issues, and then develop a pipeline. We tried viral enrichment methods as part of our extraction, but in the end, we found a great high-throughput extraction approach and coupled up with the leaders in that sort of research, particularly Dr. John-Sebastian Eden, who had been working with Eddie Holmes around that...the full biodiversity, but also with paediatricians Professor Cheryl Jones and Dr. Philip Britton that had done very similar research in children looking for novel causes of encephalitis in Australia.

So we formed a team, and then there was a lot of early career members of our team, as well that joined. And we sort of focused on this issue, kept revising our approaches, and also gaining funding. So we had various sources of funding, philanthropic donation from the Dalara Foundation for Horse and Human Health, which was our big opportunity to develop all those assays, trial our approaches. And once we had some initial findings, particularly serology findings, that the hypothesis was likely correct, that there was other concerning viruses. Of course, we communicated that (with our government's approval) to our veterinarians, so they became aware. But everyone realized we had to keep going and try to get that molecular evidence, and so we were very grateful our national government agency recognized the value in this research and funded it as part of their Biosecurity Innovation Program.

We then used all our approaches that we developed on our 300 most priority, higher suspect cases from a biobank of 1,700 cases, and that effort happened in 2020-2021, and we had the viral discovery here January 21st, 2021 (my son's birthday, actually). And then I think on the 24th of February, we had communicated to the government agencies everything about the finding and the interpretation, as well, of expected vaccine efficacy being equal. Thanks to the amazing collaboration afforded through Chris Broder with Kai Xu, we were able to model the protein based on our sequence. And then we were able (sometime in March) to notify veterinarians and the public via media release.

We had also shared the capacity for the updated testing to the state and national laboratories for animal and human health. And quite incredibly, in October, prior to the ultimate publication in your wonderful journal...so we did host the manuscript in pre-print as well, on bioRxiv, to make sure everyone could read it while it was going through the rigorous process of peer review - which I commend your journal on, again. You have wonderful reviewers; they were really engaging and wonderful. We then found that there was another prospective case detection, so a contemporary detection of a case, and it was further south than ever before, near Newcastle. And it was of this novel, previously unrecognized HeV variant genotype, now called Hendra virus G2. So extraordinary to see that happen as well in the timeline, and a great example of the benefit of proactive, transdisciplinary, convergent research that is supporting the government sector and routine surveillance.

[Sarah Gregory] Why is this all so important?

[Edward Annand] For us, it was so important because of the very real risk for our colleagues (veterinarians on the frontlines). It is just really difficult for the veterinarians that, while the chances of a single individual horse, farm or owner experiencing Hendra infection, and being confronted by that deadly risk (might be relatively low) - for veterinarians it's really not at all such a low probability. They are spending their time going around and seeing all the sick horses in the area and having to treat them, sometimes in the middle of the night, sometimes without any prior knowledge of the likelihood of HeV spilling over on that particular property due to say flowering trees, waterways or bat roosting areas. So it's very, very difficult, and that was really our justification. It was about saving the lives of our colleagues and of those that love working and caring for horses in Australia - that's why it's been important to us.

But of course, then we realized, over time, that the research had broader importance - that can extend from routine surveillance and give back to it. Looking back, it seems to be a really good example of convergence research, of active surveillance activities coupled with routine surveillance. But, I mean, it's important to realize that Peter Reid and I didn't really know the meaning of any of these terms when we set out on our journey. We see that they apply now, having studied epidemiology in my case, and him having come all the way along this journey. But for us, it was just about making the job safer. We've actually seen a lot of veterinarians leaving equine practice just because of this region or going and practicing somewhere where there was negligible perceived risk for HeV.

[Sarah Gregory] Which is a shame for everybody—for the horses, for the vets, everybody.

[Edward Annand] Yeah. And very sadly, we've also seen a loss of the relationship between owners and vets. The relationship between horse owners and vets is different for different horses and in different countries - they are all unique. But we had something special here, as a relationship between veterinarians and horse owners, trainers, carers and breeders. And that relationship has suffered due to the challenges in managing HeV and the difference in perceived risk, and the perceived pressure to vaccinate, and all of this sort of thing...

[Sarah Gregory] Yeah, that is a shame. Are there environmental factors that are affecting the spread of this virus?

[Edward Annand] So the distribution of the flying foxes has been changing over time and also the pressure on the flying foxes. The main reasons for this are anthropogenic - you know, human related causes - particularly land clearing, both for development of housing and also for farming. This has meant that those crucial East Coast forests (coastal forests) that host the native blossoms that the flying foxes seek, have greatly diminished. And actually, (this is all stuff I've learned from these incredible flying fox researchers, such as Ali Peel and Raina Plowright - on the paper), it's not just like any tree would work for them… They really used to be quite nomadic, so they would go up and down the coast with the seasons and they would actually visit particular species of flowering trees. We've also had bush fires, and there's been a lot of changes...even changes in the climate and microclimates. Really, (these many factors) have changed the way that the flying foxes are living and where they are living. So the black flying foxes have been coming south, also the grey flying foxes (have changed their distributions and behaviours), where we now have flying foxes in our most southern areas. Even black flying foxes, which traditionally or typically were not coming so far south, are coming all the way down (in some numbers, while the numbers will remain higher further up) - we're seeing that sort of change. And also, the way that they're living, so they're living now in larger roosts and really close to cities - let's say, the outskirts of cities and even in the middle of little towns - and they're less nomadic. So populations would meet perhaps less frequently. And the seasons themselves will influence all this.

In 2011, we had an extraordinary (unprecedented) number of individual spillover events to horses of Hendra virus. One theory is that bats that hadn't seen each other for a while, had caught up with populations there, and then that virus had spread around them. Certainly, they're also sometimes less well- nourished than they were in the past. They may be, in fact, more likely to visit and use as food sources trees in the gardens of homes, in cities, in sort of commercial fruit-type orchards, and in the trees (including non-natives) where horses are also living. That type of thing is happening. And these, apparently, are not their preferred foods, and so they can also be quite stressed. We have also had heat waves. So there's a lot of influences, and we're just beginning to understand all that, but it's incredible to watch the research that people like Alison Peel and Raina Plowright have been doing on that.

[Sarah Gregory] Do you have any recommendations for further investigations?

[Edward Annand] We have some proposals in to do further research on the viruses we've found. So we have found some additional paramyxoviruses that we're working on interpreting, that are more divergent than this one but seem to have been found in diseased horses, and have also been found in flying foxes. We're looking to investigate those viruses with some further research. We are also definitely—regarding recommendations, as well—looking to involve veterinarians on the frontline in research, and involving wildlife and ecology researchers in EID research. And so, acting the One Health aim in a very meaningful way. It's not easy to do, so I think there's got to be a lot of thought on how to do that, including funding, and also making sure that we meet the various aims of the different disciplines, while also meeting a common aim. Actually, I drew quite a lot in my research inspiration from my wife, who is an ethnographic anthropologist. They're very different disciplines, but there is incredible benefit to considering what is valuable from a very broad range of perspectives to a problem like this.

[Sarah Gregory] Tell us about your job, what you do, and how you got there and what you like most about it.

[Edward Annand] Well, I definitely thought of myself as an equine veterinarian. I've been practicing as an equine veterinarian in England, a little bit in France, and all the three eastern states of Australia since 2007. I absolutely love horses and really enjoy working with horse owners, carers, producers, breeders and trainers - and just the breadth of the purpose of the horse. I really enjoy that job. Because of this research, I was obliged to study epidemiology, and I gained a qualification in that. Also, to have studied virology and pathology - I felt I probably should put some of that stuff to use. I really am pleased to have been employed by the same federal Department of Agriculture, Water, and the Environment that funded our research here, in a very exciting role that is sitting within their epidemiology and One Health section, but also engaging with a very promising section that they have that is all about biosecurity strategy and reform. This new role is really an exciting opportunity for me, perhaps, to try to integrate some of the perspective we gained through this research into other disease scenarios for the benefit of animal health and One Health and our nation's biosecurity.

[Sarah Gregory] And what part of Australia do you live in—I know you're teaching in Sydney— and what are some of your favourite activities there?

[Edward Annand] Right. So I was born in Sydney, as it happened, but I grew up in Queensland. I met my wife in Sydney, and we've raised our children there. But recently, we've moved to the Great Ocean Road. So my role for Sydney and for the federal department are remote, which is lovely to be able to be around the children as much as possible. Actually, you know, my eldest son is only eight years old, so he doesn't really know what it would be like to have me as his dad without this enormous research project. So I'm really looking forward to spending more time with my kids - they're just wonderful - two boys, there. We live in a little town called Aireys Inlet on the Great Ocean Road - it's called "the surf coast" and it is some of the best surf in Australia… So I love surfing, the sea and the water. But I also love working with horses, and while my wife made sure I've downsized my herd a little bit, (I've only got, I think, six left now - I had 24 at one point) I love seeing them every day. I also love brass instruments—the trombone, the cornet, and even the flugabone… and jazz.

[Sarah Gregory] You play those? [Edward Annand] Yeah, yeah. [Sarah Gregory] Nice, nice...jazz. Yeah, I play in a folk orchestra. Not even remotely similar...

[Edward Annand] I think that my playing developed alongside my research as a little bit of a... what do they say...for mental health.

[Sarah Gregory] Oh, absolutely. Well, thank you for taking the time to talk with me today, Dr. Annand.

[Edward Annand] Oh, it was an absolute pleasure, Sarah. We covered a lot of ground, but one thing we probably missed out on was just the gratitude to each and every one of those co-authors, in particularly my co-lead author, Bethany Horsburgh, who is an incredible early career scientist who made this discovery possible.

[Sarah Gregory] And thanks for joining me out there. You can read the March 2022 article, Novel Hendra Virus Variant Detected by Sentinel Surveillance of Horses in Australia, online at cdc.gov/eid. I'm Sarah Gregory for *Emerging Infectious Diseases*.

[Announcer] For the most accurate health information, visit cdc.gov or call 1-800-CDC-INFO.

Happold J, Fostering inter-disciplinary collaboration in One Health. Ausvet blog post Jun 2019. https://www.ausvet.com.au/fostering-inter-disciplinary-collaboration-in-one-health/

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Fostering inter-disciplinary collaboration in One Health

Anthropologist Margaret Mead once said: Never doubt that a small group of thoughtful, committed citizens can change the world; indeed, it's the only thing that ever has.

This is an inspiring quote. It resonates whenever we have the opportunity to work with others to address 'wicked' health problems.

This week in Sydney, a 'thoughtful and committed' group met to explore ways in which ethnographers and applied social scientists might work with epidemiologists to address the threats posed by infectious diseases.

Among perspectives shaped by our disciplines, desires and experiences, we found some common ground.

- We recognise that disease is not simply a biological phenomenon: social, cultural, economic and political factors are equally important in understanding how and why disease occurs…and figuring out how best to take action to improve health.
- We appreciate that people have different perspectives, different priorities and different understandings about the causes of diseases.
- We accept that people differ in the beliefs or evidence that they consider valid in informing decision and actions.
- Perhaps most importantly, we agree that a richer understanding of people, cultures, context and 'why we do what we do' will help us achieve better health outcomes, locally and globally.

However, we also identified some impediments to working across disciplines and challenges in moving from multi-disciplinary projects to true inter-disciplinary collaboration.

- Ethnographers and epidemiologist may hold different assumptions and beliefs about what is reality and the nature of knowing.
- There are practical constraints and competing priorities in our day-to-day work.
- Ethnographic research takes time, yet policy-makers and outbreak responders may demand insights with a sense of urgency.
- And there is the perennial challenge of communicating our ideas to audiences outside our discipline.

These challenges and constraints are not trivial, but we are excited by the opportunities that interdisciplinary collaboration provides. To paraphrase Margaret Mead; thoughtful, committed collaboration between social scientists and epidemiologists (and others!) may be the only way to understand infectious diseases in ways that lead to effective, sustainable and culturally acceptable actions to improve health.

This meeting was part of a larger multidisciplinary project on Ethnography meets One Health Epidemiology led by anthropologist Dr Holly High, infectious disease paediatrician Dr Phillip Britton and veterinarian/epidemiologist Dr Ed Annand. The project is hosted by the University of Sydney (USYD) Marie Bashir Institute for Infectious Diseases and Biosecurity (MBI) and Social Sciences and Humanities Advanced Research Centre (SSSHARC).

Jonathan Happold, an epidemiologist and Senior Consultant at Ausvet, is a Visiting Fellow in the Department of Anthropology at the University of Sydney.

Appendix 6: An update on Hendra virus (HeV), HeV-like illnesses and horses as sentinels for emerging infectious disease, *The Northern Territory Disease Control Bulletin Vol 24, No. 2, June 2017.*

An update on Hendra virus (HeV), HeV-like illnesses and horses as sentinels for emerging infectious disease

Edward Annand,[1] Peter Reid,[2] Ina Smith,[3] Ibrahim Diallo,[5] Stacey Lynch,[6] Richard Weir,[7] Lorna Melville,[7] Cristy Secombe,[8] Philip N. Britton,[9] James Gilkerson,[4] Cheryl Jones,[10] Navneet Dhand[1]

1. University of Sydney, School of Veterinary Science and Marie Bashir Institute for Infectious Diseases and Biosecurity; Private Equine Veterinarian and The Australian Veterinary Association; 3. CSIRO, Australian Animal Health Laboratory; 4.University of Melbourne, Faculty of Veterinary Science, Centre for Equine Infectious Disease; 5. Biosecurity Sciences Laboratory, Biosecurity Queensland, QDAF; 6. AgriBio, Victorian Arbovirus control program, Agriculture Victoria, DEDJTR; 7. Berrimah Veterinary Laboratory, NT DPIR; 8. College of Veterinary Medicine, Murdoch University; 9. University of Sydney, Faculty of Medicine and Marie Bashir Institute for Infectious Diseases and Biosecurity; 10. University of Melbourne, Department of Paediatrics and Murdoch Children's Research Institute

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Abstract

Hendra virus (HeV) causes a usually fatal acute disease in horses mediated by an endothelial vasculitis. It also has a high case fatality rate in humans who may become infected via exposure to an infected horse. We are conducting a project that aims to identify infectious causes of severe acute equine illness presentations other than HeV and evaluate the respective zoonotic potential of any pathogens identified. This will provide much needed additional knowledge for health professionals and the public and will clarify the potential human health risk associated with contact with unwell horses.

Key words: Hendra virus; Australian bat lyssavirus; horse; zoonoses; emerging infectious disease.

Background

Hendra virus (HeV) causes a usually fatal acute disease in horses mediated by an endothelial vasculitis. Following its initial diagnosis, and based on the 1994 cases attended by Dr Peter Reid, HeV was originally thought of as an acute equine respiratory syndrome featuring marked pyrexia[1] which resulted in death predominantly from pulmonary oedema and hypoxia. Most cases had featured terminal, frothy, clear or bloody nasal discharge with interstitial pneumonia and pulmonary haemorrhage. Neurological signs, while present, were *not* the major clinical features. This emergence of a fatal zoonotic disease in horses has changed the veterinary approach to the investigation of sick horses in Australia to more carefully consider zoonotic risk.

In 2008, the cases at Redlands in Queensland were interpreted and described with more emphasis on neurological signs and other nonspecific signs that included dull demeanor and mild colic with less emphasis on respiratory signs. A review of these cases published in *Emerging Infectious Diseases* prompted veterinarians and horse owners to consider HeV infection in any horse exhibiting acute-onset febrile illness, regardless of clinical manifestations, and to implement appropriate risk assessment and management strategies in sick horses and in horses in the pre-clinical stage of infection.[2] Subsequently, experimental live virus challenge studies at the Australian Animal Health Laboratory (AAHL) using the Redlands isolate demonstrated pulmonary pathology and respiratory signs in all horses consistent with that shown previously in 1994.[3]

Clinical presentations of naturally infected acute field cases since 1994 have shown either or both of these signs. Consequently, an appropriate 'typical presentation for HeV' might be considered an ' acute severe and often rapidly progressing fatal illness usually featuring pyrexia, with respiratory and/or neurological signs.'

A recent, retrospective clinical review of 11 New South Wales cases of HeV between 2006 and 2012 described 5 cases as being found dead or dying along a fence line. This suggested neurological dysfunction likely featured as part of the unobserved clinical deterioration. In most cases, disease presented as an acute illness leading to death within 48 hours. These cases further highlight that HeV should be considered

in cases of acute unexplained equine fatality. Full autopsies were not conducted so therefore pulmonary involvement was not able to be described. When signs of disease were observed, neurological signs predominated.[4]

A review of the emergence of both HeV and another emergent virus in the same family, Nipah virus (NiV), has been recently published in *Vaccine* by Broder, Weir and Reid.[5] The review discusses virus tissue tropism and cellular entry, replication strategies, pathogenesis, clinical features of human and animal infection, and the development of an effective, safe vaccine and post-exposure prophylaxis.

Veterinarians are reminded of the appropriate samples to collect from suspect animals for HeV testing as described by the Queensland (QLD) Department of Agriculture and Fisheries 'Guidelines for veterinarians handling potential HeV infection in horses.'[6] The samples include nasal, oral, rectal mucosal, vaginal swabs and where possible urine placed into viral transport medium (obtainable from your government laboratory) or 1mL of saline (in a serum blood tube), as well as blood collected into standard 10.0mL EDTA and 8.5mL serum clot tubes.

Since 2009 testing for HeV has increased throughout QLD,[7] likely in part, due to increased awareness of the disease. Many practicing and government veterinarians have been all too aware of a considerable number of cases that have concerning HeV-like clinical signs involving acute respiratory and or neurological presentation, which are often fatal. Strikingly, the majority of these cases return negative results for HeV testing and yet many are suggestive of an infectious cause. In 2013, 2 such cases that underwent additional testing were found to have died from Australian bat lyssavirus (ABLV), which causes a disease indistinguishable in clinical presentation and outcome to rabies in mammals including in humans.[8] Could more of these cases featuring HeV-like clinical signs, yet testing negative for HeV be posing further zoonotic disease threats? The authors are conducting a project aiming to answer this question and identify other known and unknown pathogens that may be causing such equine illnesses.

The differential diagnoses for acute severe illness in horses includes a long list of both infectious and non-infectious aetiologies (see Box).

Case features or symptoms that may increase the suspicion of infectious causes in the differential

Box. Examples of differential diagnoses that could be considered for acute severe illness in horses by category

Infectious: Bacterial meningitis / abscessation; Bacterial pneumonia; Bacterial systemic toxaemia; Anthrax; Viral infection (encephalitis / meningitis, vasculitis, severe respiratory)##; Mycotic infection - particularly Cryptococcus (pneumonia / encephalitis); Equine protozoal myeloencephalitis*/ Amoebic encephalitis*; Trypanosomiasis* #

** Not known to occur in Australia.*

 # Surra (Trypanosoma evansi) is not found in Australia but is endemic in neighboring countries. Native trypanosome species are of unknown presence or clinical significance in horses.

See tables for lists of viruses potentially involved in acute equine illness.

Colic due to acute abdominal conditions (examples include strangulating intestinal or infarctive lesions)

Toxicity: Snake envenomation – brown, tiger, taipan; Tick paralysis – *Ixodes holocyclus*; Tetanus; Botulism; Metaldehyde; Ergot alkaloidosis,

Plant toxicities: Avocado; Pyrrolizidine alkaloids (in the NT *Crotolaria* spp. especially *C. crispate*); Annual ryegrass toxicity; Cardiac Glycosides Eg. Indigofera.

Poisons: 1080; Paraquat; Monensin; Lead

Trauma: Traumatic encephalopathy

Neoplastic: Acute clinical signs due to progression (Rare in horse - examples include: Cholesterol granuloma; Adenocarcinoma; Lymphoma; Pituitary adenoma)

Iatrogenic: Air embolism; Intracarotid injection; Drug overdoses (Moxidectin, Metronidazole, Trimethoprim sulphonamide, Lignocaine)

Other: Cardiac – ruptured chordae tendinae; aortic root rupture; Metabolic derangement (Hypocalcemia; Hyponatremia; Hypoglycemia); Hypo-/hyperosmolality disorders; Hyperammonemia; severe haemorrhage into a body cavity; Hepatotoxic encephalopathy.

diagnosis include pyrexia, respiratory and/or neurological signs as well as multiple cases occurring in an apparent epidemiological relationship. In such cases, and where HeV testing is negative, the following viruses already detected in Australia could be involved (see Table 1), many of which pose direct or indirect (arthropod vector) human health threats. The viruses in Table 2 are known to cause similar disease in horses internationally. In addition to these viruses those in Table 3 have been recently identified in Australian bats[10] and potentially may follow a similar 'spill-over' pathway to horses as HeV with unknown clinical significance.[11]

Our project aims to investigate the possibility of infectious causes of severe acute equine illness presentations other than HeV and evaluate the respective zoonotic potential of any pathogens identified. This will provide much needed additional knowledge for health professionals and the public and will clarify the potential human health risk associated with contact with unwell horses. It is also expected to improve treatment and outcomes for horses which may be compromised by uncertainty surrounding these presentations.

Horses, monitored closely for individual illness that are often heavily exposed to biting insects and featuring a proven spill-over risk for bat borne viruses (HeV and ABLV) are a highly suitable sentinel species for early detection of emerging infectious diseases of potential human and livestock significance.

For veterinarians attending suitable cases please mark submissions for forwarding to AAHL following timely HeV +/- ABLV testing at the Berrimah Veterinary Laboratory, NT Department of Primary Industries and Resources for inclusion in this additional research testing. Please do not hesitate to contact Dr Ed Annand for further information or to discuss case suitability:
Email: ed.annand@sydney.edu.au or Mobile: 0439572329.

Table 1. Viruses that could be involved

Virus	Genus / Family	Reservoir	Insect vector	Confirmed infection in Horses	Humans
Australian bat lyssavirus (ABLV)	*Lyssavirus* / *Rhabdoviridae*	All bats	None	Yes	Yes
Menangle	*Rubulavirus* / *Paramyxoviridae*	Flying foxes	None	No#	Yes
Elsey ELSV	*Orbivirus* / *Reoviridae*	Unknown	*Culicoides*, mosquitoe	Yes	No
Murray valley encephalitis virus	*Flavivirus*/ *Flaviviridae*	Birds / mosquitoes	Mosquitoes	Yes	Yes
West Nile virus WNV (Kunjin)				Yes	Yes
Japanese Encephalitis				Yes	Yes
Ross River Virus	*Alphavirus* / *Togaviridae*	Macropods	Mosquitoes	Yes	Yes
Equine Herpes viruses1	*Varicellovirus* / *Alphaherpesvirus*	Horse	None	Yes	No

Equine seropositivity for this or a very closely related virus has recently been identified as part of this research. . Further testing is underway.

Table 2. Viruses known to cause similar disease in horses internationally

Virus	Genus / family	Reservoir/vector	Confirmed infection in Horses	Humans
Nipah (NiPV)	*Henipavirus* / *Paramyxoviridae*	Flying foxes	Yes*	Yes
Rabies virus	*Lyssavirus* / *Rhabdoviridae*	Terrestrial carnivores and bats	Yes	Yes
African horse sickness virus	*Orbivirus* / *Reoviridae*	*Culicoides*, mosquitoes and ticks	Yes	No
Equine Encephalosis virus		*Culicoides*	Yes	No
Peruvian horse sickness (PHSV)**		Mosquitoes	Yes	Yes
Eastern equine encephalitis virus	*Alphavirus* / *Togaviridae*		Yes	Yes
Getah virus			Yes	Yes
Shuni virus	*Orthobunyavirus* / *Bunyaviridae*	*Culicoides*, mosquitoes	Yes	No
Borna disease virus	*Bornavirus* / *Bornaviriadae*	Rodents suspected	Yes	Yes
St. Louise encephalitis virus (SLEV)	*Flavivirus* / *Flaviviridae*	Birds / mosquitoes	Yes	Yes

*Confirmed in 1998/1999 Malaysia / Singapore outbreak. Also a henipavirus outbreak occurred in 2014 in the Philippines that caused fatalities in horses, humans, dogs and a cat and featured human to human transmission-it is thought to have been very closely related to HeV and NiV[9]
**This virus is considered practically identical to ELSV.

Table 3. Viruses recently identified in Australian bats

Virus	Genus / family	Reservoir/vector	Confirmed infection in Horses	Humans
Cedar virus	*Henipavirus* / *Paramyxoviridae*	Flying foxes	no#	no
Hervey virus	*Rubulavirus* / *Paramyxoviridae*		no	no
Grove virus			no	no
Teviot virus			no#	no
Yepoon virus			no	no

Equine seropositivity for this or a very closely related virus has recently been identified as part of this research. Further testing is currently underway.

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Kevin De Witte, Chief Veterinary Officer, Department of Primary Industry and Resources, NTG, current Chair of the NT Zoonosis Committee.

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Appendix 7: Spread sheet example of systematic consideration of clinical descriptor terms listed in association with HeV infection and among a comparative cohort of equine disease events of lower likelihood of infection cause (AEFI).

Appendix 7 Spread sheet example of systematic consideration of clinical descriptor terms listed in association with HeV infection and amongst a comparative cohort of severe equine disease events of lower likelihood of infection cause (adverse events following immunization AEFI).

1.0 Clinical term standardised	1.1 Clinical descriptor term observed with HeV disease in horses (most representative and detailed)	1.2 Clinical descriptor term as listed in association with HeV infection	1.3 Clinical descriptor term as listed in association with AEFI	1.4 Listed equivalent descriptor term as listed in association with AEFI	1.5 Listed collective terms	1.6 Pathologic syndromic term	1.7 Collective disease or body system terms	2.0 Term defined	2.1 Descriptor term category/ies	2.2 Specificity in clinical description of disease and causal consideration	3.0 MeSH hierarchical indexing in 'Pathological conditions, signs and symptoms' schema	3.1 Pathological conditions, signs and symptoms MeSH index number	3.2 Associated syndrome	3.3 Associated organ and body systems	3.4 MeSH hierarchy body system disease schema	3.5 Diseases by body system MeSH index number	3.6 Additional MeSH hierarchy	3.7 Associated MeSH disease categories ordered by frequency observed	3.8 Lay term equivalents of associated MeSH disease categories ordered by frequency observed	4.1 Description of causes other than HeV	4.1 Most common causes	4.2 Less common causes	4.3 Rare or least common causes	4.4 Considerations for clinical investigation of aetiology	5.0 seasonal-influence	5.1 geographical-influence	6.0 frequency-observed-in-domestic-equids	6.1 How common is the condition described by the clinical descriptor term in clinical practice?
Inflammation	NA		Inflammation					Inflammation' is a broad term describing the pathological process characterized by the responses of tissue to harmful stimuli or injury. It may be either acute or chronic and it may be generalised or specific to a region, organ or body system. The term also encompasses many variations and phases in physiopathology of immune response and disease severity and all may have diverse primary and secondary causes.	Patho-physiological phenomena/ Altered physiological parameter without specified direction, magnitude or location/ General pathological descriptive term/ Broad pathological syndrome.	High in describing a wide encompassing category of biophysiological and disease processes. Very low specificity for type, structures, severity and cause where may occur in all body tissues, organs and fluids as part of normal or intended physiological responses and/or as part of disease and response to injury, nutritional or circulatory insult, cell damage, parasitism or infection.	Pathological Conditions, Signs and Symptoms/ Pathological Processes/ Inflammation	C23.550.470	NA	immune/ Non-specific generalised systemic	NA	NA	Pathological conditions, signs and symptoms Pathologic Processes/Systemic Inflammatory Response Syndrome/Cytokine Release Syndrome [C23.550.470.790.250] characterized by marked elevations of various inflammatory CYTOKINES and by the following associated conditions: DYSPNEA; FEVER; HEADACHE; HYPOTENSION; NAUSEA; RASH; TACHYCARDIA and/or HYPOXIA. and Acute-Phase Reaction [C23.550.470.099] An early local inflammatory reaction to insult or injury that consists of fever, an increase in inflammatory humoral factors, and an increased synthesis by hepatocytes of a number of proteins or glycoproteins usually found in the plasma.	Immune System Diseases/ bacterial infections and mycoses/ virus diseases/ musculoskeletal diseases/ digestive system diseases/ stomatognathic diseases/ respiratory tract diseases/ otorhinolaryngologic diseases/ nervous system diseases/ eye diseases/ urological and male genital diseases/ female genital diseases and pregnancy complications/ hemic and lymphatic diseases/ skin and connective tissue diseases/ disorders of environmental origin	Immune system diseases/ Bacterial and fungal infections/ Viral infections/ Parasite burden/ Musculoskeletal diseases/ Digestive system diseases/ Oral and dental diseases/ Respiratory diseases/ Sinus (nose), laryngeal (throat), and ear diseases/ Eye diseases/ Urinary system diseases/ Stallion genital diseases/ Mare genital diseases and pregnancy complications/ Blood and body fluid diseases/ Skin, coat, hoof wall and sole diseases (including laminitis)/ Trauma, injury, inflammation, toxicities and other diseases of environmental cause.	Very broad term with almost infinite locations, types and severities. Requires additional characterisation/ description before causes may be considered. Degenerative processes such as osteoarthritis due to 'wear and tear' of musculoskeletal tissues over a performance career. Infectious processes such as due to parasites (gastrointestinal worms), bacteria, or viruses which are all very common. Might be most commonly referred to in association with vaccine administration to describe localised/ focal inflammation associated with vaccine site reaction when considered 'Acute-Phase Reaction' or signs of targeted (desired) or generalised when considered 'Cytokine Release Syndrome' inflammatory response to the vaccine product (Adjuvant, antigen, carrier and preservative) - ideally minimised.	Degenerative processes such as osteoarthritis due to 'wear and tear' of musculoskeletal tissues over a performance career. Infectious processes such as due to parasites (gastrointestinal worms), bacteria, or viruses which are all very common. In association with vaccine administration most commonly inflammation would be observed as part of an injection site reaction or general immune system response to vaccination (systemic/ innate, cell mediated and targeted/memory immune system inflammatory response to vaccination). It is also appropriate to consider both whether vaccine associated inflammation is: intended and/or expected or excessive, unusual and/ or prolonged; and to what extent it is influenced by underlying, pre-existing, concurrent or alternative processes, causes or diseases. Consideration requires further characterisation of affected body systems structures and/or signs and severity.	Consideration requires further characterisation of affected body systems structures and/or signs and severity.	Consideration requires further characterisation of affected body systems structures and/or signs and severity.	Further characterisation as to location, nature and severity. In assessing localised or generalised inflammatory responses that exceed the most commonly expected and/or intended response to vaccination thorough consideration of pre-existing, concurrent, confounded and additional causes should be undertaken. When the condition is considered a problem requiring clinical diagnostic assessment and management, veterinarians undertake 'optimal contextualised clinical appreciation of the pathogenic basis of disease' (See glossary) which aims to interpret the disease clinically as contextualised by its occurrence: through time, amongst immediate and general horse populations; in its immediate and wider environment associations and significance; and in its significance to immediate and wider stakeholders (cultural significance). Body systems most affected and the pathophysiology of the presenting inflammation should be established. A through individual, farm and herd health history should be established and considered together with assessment of nutrition, husbandry and environment and findings derived via a thorough clinical examination. Commonly informative and utilised laboratory assessments include Haematology and Biochemistry (immune blood cells individually and comparatively and circulating levels of inflammatory proteins such as 'Serum reactive protein/ Serum amyloid A, immunoglobulins and fibrinogen').	Yes, many common causes of inflammation in the horse are influenced by season such as insect bites, virus infections, plant toxicities, sun burn, hoof conditions such as laminitis, seedy toe and abscessation, also causes of dermatitis - see more specific descriptor term entries.	Yes, many common causes of inflammation in the horse are influenced by geographical conditions such as insect bites, virus infections, plant toxicities, sun burn, hoof conditions such as laminitis, seedy toe and abscessation, also causes of dermatitis - see more specific descriptor term entries.	Most horses will experience activation of the immune system and response with varying manifestations of local and /or generalised inflammation above normal levels at least once per month such as that resulting from sole bruising or other musculoskeletal strain, gastric ulceration or respiratory or gastrointestinal irritant/ antigen exposure. Common viruses are the predominant cause of generalised systemic inflammation and are very common. Also an expected response to immunisations.	Very Common
Oedema generalised or localised	NA		NA					Encompasses: Oedema/ Facial oedema/ Swollen testes/ Periorbital swelling/ Scrotitis	NA	NA	Pathological Conditions, Signs and Symptoms/ Signs and Symptoms/ Oedema	C23.888.227	Oedema	immune/ lymphatic/ cardiovascular/ musculoskeletal	NA	NA	NA	hemic and lymphatic diseases/ immune system diseases/ disorders of environmental origin/ skin and connective tissue diseases/ nutritional and metabolic diseases/ parasitic diseases/ bacterial infections and mycoses/ virus diseases/ musculoskeletal diseases/ digestive system diseases/ stomatognathic diseases/ respiratory tract diseases/ otorhinolaryngologic diseases/ urological and male genital diseases/ female genital diseases and pregnancy complications/ cardiovascular diseases/ neoplasia	Blood and body fluid diseases/ Immune system diseases/ Trauma, injury, inflammation, toxicities and other diseases of environmental cause/ Skin, coat, hoof wall and sole diseases (including laminitis) / Nutritional and metabolic diseases/ Parasite burden/ Bacterial and fungal infections/ Viral infections/ Musculoskeletal diseases/ Digestive system diseases/ Oral and dental diseases/ Respiratory diseases/ Sinus (nose), laryngeal (throat), and ear diseases/ Urinary system diseases/ Stallion genital diseases/ Mare genital diseases and pregnancy complications/ Heart and blood vessel diseases/ Cancers	NA	NA	NA	NA	NA	NA	NA	NA	NA
Dependent oedema	dependent_oedema	dependent oedema	Oedema	dependent_oedema	dependent_oedema	dependent_oedema	systemic_vasculitis_and_circulatory_compromise	Abnormal fluid accumulation in tissues or body cavities. Most cases of oedema are present under the skin in subcutaneous tissue. May result from inflammation that accompanies local tissue insult or may have generalised systemic causes such as haem-osmotic and or circulatory disruption or systemic inflammatory condition resulting in altered fluid dynamics through altered vascular permeability, compromised lymphatic return or both. In cases featuring systemic or generalised cause oedema will be evident in gravitationally dependent anatomy	clinical sign/Patho-physiological phenomena/ General pathological descriptive term/ Broad pathological syndrome.	Moderately specific in broadly defining conditions of increased fluid infiltration of tissue. Wide ranging manifestations and causes.	Pathological Conditions, Signs and Symptoms/ Signs and Symptoms/ Oedema	C23.888.227	Oedema	immune/ lymphatic/ cardiovascular/ musculoskeletal	NA	NA	NA	hemic and lymphatic diseases/ immune system diseases/ disorders of environmental origin/ skin and connective tissue diseases/ nutritional and metabolic diseases/ parasitic diseases/ bacterial infections and mycoses/ virus diseases/ musculoskeletal diseases/ digestive system diseases/ stomatognathic diseases/ respiratory tract diseases/ otorhinolaryngologic diseases/ urological and male genital diseases/ female genital diseases and pregnancy complications/ cardiovascular diseases/ neoplasia	Blood and body fluid diseases/ Immune system diseases/ Trauma, injury, inflammation, toxicities and other diseases of environmental cause/ Skin, coat, hoof wall and sole diseases (including laminitis) / Nutritional and metabolic diseases/ Parasite burden/ Bacterial and fungal infections/ Viral infections/ Musculoskeletal diseases/ Digestive system diseases/ Oral and dental diseases/ Respiratory diseases/ Sinus (nose), laryngeal (throat), and ear diseases/ Urinary system diseases/ Stallion genital diseases/ Mare genital diseases and pregnancy complications/ Heart and blood vessel diseases/ Cancers	Very broad term with almost infinite locations, manifestations causes and severities. Requires additional characterisation/ description before causes may be considered. Might be most commonly referred to in association with vaccine administration to describe oedema in the neck region resulting from localised/ focal inflammation associated with vaccine site reaction or oedema observed in gravitationally dependent sites (distal limbs, head and jaw in horses kept grazing at pasture, ventral abdomen/chest/brisket or preputial/ mammary region that might result from generalised (ideally minimised) inflammatory response to the vaccine product (Adjuvant, antigen, carrier and preservative) and which will often have additional pre-existing, confounding or co-contributing causes such as intestinal parasitism and associated gastrointestinal disease.	In association with vaccine administration most commonly oedema would be observed resulting from localised inflammation as part of an injection site reaction or diffusely evident in gravitationally dependent sites resulting from general immune system response to vaccination (systemic/ innate, cell mediated and targeted/memory immune system inflammatory response to vaccination). It is also appropriate to consider both whether vaccine associated inflammation is: intended and/or expected or excessive, unusual and/ or prolonged; and to what extent it is influenced by underlying, pre-existing, concurrent or alternative processes, causes or diseases. Consideration requires further characterisation of affected body systems structures and/or signs and severity. Common causes of oedema in horses apart from association with injections and inflammatory reactions to 'veterinary-chemical-products' include insect bite reactions, reactions to any inflammation of the soft tissues, disruption of lymphatic function and focally targeted inflammatory response.	Low circulating serum protein resulting in disruption of functional osmotic homeostasis such as occurs in many protein losing enteropathies (any condition of the gastrointestinal tract that results in a net loss of protein from the body usually due to damage to the gut wall) such as colitis and diarrhoeal diseases, pleuropneumonia (travel sickness), some chronic diseases such as associated with intestinal parasite disease and Chronic liver diseases.	Granulomatous enteritis of infectious and/or immune mediated in cause and neoplastic conditions such as the rare but consistent neoplastic condition lymphoma which presents in horses of all ages and most commonly in young adult horses being either multicentric, alimentary or mediastinal in location. Lymphoma in turn is understood to be potentiated by infection with a recently appreciated Herpes virus. Liver fluke infestation (regionally relevant). Congestive heart failure.	Further characterisation as to location, nature and severity. Body systems and pathophysiology of the presenting inflammation should be established. A through individual, farm and herd health history should be established and considered together with assessment of nutrition, husbandry and environment and findings derived via a thorough clinical examination. Commonly informative and utilised laboratory assessments include Haematology and Biochemistry (immune blood cells individually and comparatively and circulating levels of inflammatory proteins such as 'Serum reactive protein/ Serum amyloid A, immunoglobulins and fibrinogen'). When considering the extent to which responses to vaccination through localised or generalised inflammation response and the extent to which it is considered to be within the expected range of normal or seemingly excessive all possible pre-existing, concurrent, indirect and confounding additional causal factors should be investigated.	No other than where underlying causes might be influenced by seasons.	No other than where underlying causes might be influenced by seasons.	Very common both in response to any injection and to vaccine administrations as part of injection site reactions as well as in response to the great variety of regional and generalised inflammatory causes.	Very Common
Oedema of the head and neck	oedema_of_the_head_and_neck	oedema head/face/ mandible/tongue/lips/neck	Facial oedema	oedema_head_face_mandible_tongue_lips_neck	dependent_oedema	dependent_oedema	systemic_vasculitis_and_circulatory_compromise	Abnormal fluid accumulation in the skin and subcutaneous tissues of the head and/or jaw. May result from inflammation that accompanies local tissue insult such as tooth root infection or may have generalised systemic causes such as haem-osmotic and or circulatory disruption or systemic inflammatory condition resulting in altered fluid dynamics through altered vascular permeability, compromised lymphatic return or both. In cases featuring systemic or generalised cause oedema will be evident in gravitationally dependent anatomy such as lower limbs and head (where head is positioned low for grazing or due to obtunded demeanour).	Patho-physiological parameter partially specified in magnitude, direction or location/ Clinical sign/broad pathological syndrome	Moderately specific in broadly defining conditions of increased fluid infiltration of tissue observed in the head region. low specificity for underlying cause.	Pathological Conditions, Signs and Symptoms/ Signs and Symptoms/ Oedema	C23.888.227	Oedema	immune/ lymphatic/ cardiovascular/ musculoskeletal	NA	NA	NA	hemic and lymphatic diseases/ immune system diseases/ disorders of environmental origin/ skin and connective tissue diseases/ nutritional and metabolic diseases/ parasitic diseases/ bacterial infections and mycoses/ virus diseases/ musculoskeletal diseases/ digestive system diseases/ stomatognathic diseases/ respiratory tract diseases/ otorhinolaryngologic diseases/ urological and male genital diseases/ female genital diseases and pregnancy complications/ cardiovascular diseases/ neoplasia	Blood and body fluid diseases/ Immune system diseases/ Trauma, injury, inflammation, toxicities and other diseases of environmental cause/ Skin, coat, hoof wall and sole diseases (including laminitis) / Nutritional and metabolic diseases/ Parasite burden/ Bacterial and fungal infections/ Viral infections/ Musculoskeletal diseases/ Digestive system diseases/ Oral and dental diseases/ Respiratory diseases/ Sinus (nose), laryngeal (throat), and ear diseases/ Urinary system diseases/ Stallion genital diseases/ Mare genital diseases and pregnancy complications/ Heart and blood vessel diseases/ Cancers	Wide ranging causes can include: - Inflammation and disease involving tissues in the head (including dental disease) or upper neck region; - focal inflammation or disease that has caused oedema that has migrated to the head region due to its relative gravitational dependency when persistently carried low either for grazing (the majority of the time for horses living at pasture) or due to obtunded demeanour /weakness. - Similarly oedema usually localises to this region due to its gravitational dependency when due to generalised inflammation or immune mediated disease such as hypersensitivity and allergy and - also in cases of generalised oedema due to disruption of osmotic function of the vascular/ circulatory system usually due to low serum protein and the resulting extravascular fluid leakage.	When oedema occurs in association with localised inflammation following vaccine administration in the neck, facial oedema commonly follows due to migration of extravascular fluid downward to the closest most gravitationally dependent site.	Extravascular fluid accumulation in response to generalised inflammation or immune mediated disease such as viral infections, hypersensitivity and allergy, and also in cases due to disruption of osmotic function of vascular system usually due to low serum protein and the resulting extravascular fluid leakage.	Rarer conditions resulting in accumulation of fluid in the tissues of the head region such as neoplasia and liver disease such as liver fluke infection with liver fluke (horses are infrequent but consistent incidental hosts for liver fluke that rely on sheep in their usual life cycle).	As for oedema with specific attention to regionally relevant causes.	As for oedema	As for oedema	As for oedema	Very Common
	enlarged_mandibular_ln					lymphatic_immune_and_inflammatory_system_response	lymphatic_immune_and_inflammatory_system_response																					
	cyanosis_muddy_toxic_mm_endotoxaemia		NOT LISTED			cyanosis_muddy_toxic_mm_endotoxaemia	systemic_vasculitis_circulatory_compromise																					
	petechiation_and_ecchymosis	petechiation and ecchymosis	NOT LISTED	petechiation_and_ecchymosis	petechiation_and_ecchymosis	petechiation_and_ecchymosis	systemic_vasculitis_and_circulatory_compromise																					
	petechiation_and_ecchymosis		NOT LISTED			petechiation_and_ecchymosis	systemic_vasculitis_and_circulatory_compromise																					
	petechiation_and_ecchymosis		NOT LISTED			petechiation_and_ecchymosis	systemic_vasculitis_and_circulatory_compromise																					
	poor_circulatory_function		NOT LISTED			poor_circulatory_function	systemic_vasculitis_and_circulatory_compromise																					
	poor_circulatory_function		NOT LISTED			poor_circulatory_function	systemic_vasculitis_and_circulatory_compromise																					
	dry_mm		NOT LISTED			dehydration/ systemic_vasculitis_and_circulatory_compromise	systemic_vasculitis_and_circulatory_compromise																					
Behavioural Symptoms	NA		NA					Encompasses: Behavioural change/ Hormonal issues/ Adipsia/ Pain/ Restlessness/ Distress/ Aggression/ Agitation/ Hypersensitive to stimuli/ Hyperactivity/ Disorientation/ Confusion/ Lethargy/ Weakness/ Low energy/ Depression/ Listless/ Malaise/ Recumbency	NA	NA	Behaviour and Behaviour Mechanisms/ Behaviour/ Behavioural Symptoms	F01.145.126	Behavioural symptoms	nervous, endocrine, immune	NA	NA	NA	disorders of environmental origin/ nutritional and metabolic diseases/ endocrine system diseases/ immune system diseases/ nervous system diseases/ skin and connective tissue diseases/ musculoskeletal diseases/ digestive system diseases/ stomatognathic diseases/ bacterial infections and mycoses/ virus diseases/ nervous system diseases/ female genital diseases and pregnancy complications/ respiratory tract diseases/ otorhinolaryngologic diseases/ eye diseases/ parasitic diseases/ neoplasia/urological and male genital diseases	Trauma, injury, inflammation, toxicities and other diseases of environmental cause/ Nutritional and metabolic diseases/ Immune system diseases/ Hormonal system diseases/ Skin, coat, hoof wall and sole diseases (including laminitis)/ Musculoskeletal diseases/ Digestive system diseases/ Oral and dental diseases/ Bacterial and fungal infections/ Viral infections/ Nervous system diseases/ Mare genital diseases and pregnancy complications Respiratory diseases/ Sinus (nose), laryngeal (throat), and ear diseases/ Cancers/ Eye diseases/ Urinary system diseases/ Stallion genital diseases	NA	NA	NA	NA	NA	NA	NA	NA	NA
Behavioural Symptoms	behaviour_changes	behaviour changes	Behavioural change	behaviour_changes	behaviour_changes	behaviour_changes	neurological_signs	Change from normal behaviour - diverse potential manifestations and causes.	Altered physiological parameter insufficiently specified in direction, magnitude or location/ clinical sign/non-specific scientific term	**Very General** and **Common** category of signs with many possible specifying descriptions and almost infinite causes.	Behaviour and Behaviour Mechanisms/ Behaviour/ Behavioural Symptoms	F01.145.126	Behavioural symptoms	nervous, endocrine, immune	NA	NA	NA	disorders of environmental origin/ nutritional and metabolic diseases/ endocrine system diseases/ immune system diseases/ nervous system diseases/ skin and connective tissue diseases/ musculoskeletal diseases/ digestive system diseases/ stomatognathic diseases/ bacterial infections and mycoses/ virus diseases/ nervous system diseases/ female genital diseases and pregnancy complications/ respiratory tract diseases/ otorhinolaryngologic diseases/ eye diseases/ parasitic diseases/ neoplasia/urological and male genital diseases	Trauma, injury, inflammation, toxicities and other diseases of environmental cause/ Nutritional and metabolic diseases/ Immune system diseases/ Hormonal system diseases/ Skin, coat, hoof wall and sole diseases (including laminitis)/ Musculoskeletal diseases/ Digestive system diseases/ Oral and dental diseases/ Bacterial and fungal infections/ Viral infections/ Nervous system diseases/ Mare genital diseases and pregnancy complications Respiratory diseases/ Sinus (nose), laryngeal (throat), and ear diseases/ Cancers/ Eye diseases/ Urinary system diseases	Almost infinite - this term should be accompanied by further description for meaningful consideration.	Pain or altered metabolism due to varying degrees of mild transient localised and/or generalised inflammation expected in response to immunisation. Mild transient 'head ache' as is common side effect of many immunisations (experienced by 15% of people who receive Yellow fever vaccine).	Hypersensitivity responses to immunisation.	Exacerbation of underlying inflammatory diseases such as laminitis that cause behavioural changes due to pain and that may be exacerbated by immune responses such as those expected following immunisations.	Further description of the nature of the behavioural change is required to consider appropriate further diagnostic procedures towards establishing cause. Many cases may result from pain - see requirements for further investigation of pain.	No	No	**Very General and Common** category of signs with many possible specifying descriptions and almost infinite causes.	Very Common