Investigation Into the Diagnosis, Treatment and Risk Factors of *Macrorhabdus ornithogaster* in Australian Cage Birds

Hamish Robert Baron

BVSc (Hons), FANZCVS (Avian Medicine and Surgery)

Registered Avian Specialist Veterinarian

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Statement of originality

This thesis is submitted to The University of Sydney in fulfilment of the requirements of the degree of Doctor of Philosophy, Faculty of Science. I hereby declare that the contents of this thesis are the product of my own original work, and to the best of my knowledge and belief, it contains no material published previously or written by another source except where due acknowledgement has been made. The intellectual content of this thesis has not been submitted for the award of any other degree at The University of Sydney or any other academic institution.

Hamish Baron

May 25, 2023

Authorship attribution statement

Chapter 2 of this thesis is published as Baron HR, Stevenson BC, Phalen DN. Comparison of in-clinic diagnostic testing methods for *Macrorhabdus ornithogaster*. J Avian Med Surg. 2021;35(1):37-44. https://doi.org/10.1647/1082-6742-35.1.37.

I designed the study, collected, extracted, and assisted in analysis of the data and wrote the manuscript.

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I designed the study, collected, extracted, and assisted in the analysis of the data and wrote the manuscript.

Chapter 5 of this thesis is published as Baron HR, Stevenson BC, Phalen DN. Inconsistent efficacy of water-soluble amphotericin B for the treatment of *Macrorhabdus ornithogaster* in a budgerigar (*Melopsittacus undulatus*) aviary. Aust Vet J. 2020;98(7):333-337. https://doi.org/10.1111/avj.12936

I designed the study, collected, extracted, and assisted in the analysis of the data and wrote the manuscript.

Hamish Baron Candidate	David Phalen Supervisor	Ruth Zadoks Supervisor

Abstract

Macrorhabdus ornithogaster is a yeast that grows at the isthmus between the proventriculus and ventriculus in birds. It commonly infects budgerigars (*Melopsittacus undulatus*) in Australia. Infected budgerigars may show no signs while others die with melaena, malabsorption and emaciation despite what appears to be a ravenous appetite. The risk factors are not fully understood. Concurrent stressors or immunosuppressive co-infections have been postulated as potential triggers for the onset of disease, but these have not been investigated in the literature. In Australian cage birds, *Macrorhabdus ornithogaster* was first identified in the early 1990s. It is now likely endemic in the exhibition budgerigar community, and in populations of wild birds across most Australian states. Despite its significance as a pathogen, there is a significant lack of research surrounding the optimisation of the diagnostic tools available, both in the veterinary clinic, and in a laboratory setting. The standardisation of diagnostic tools, utilising only the most sensitive testing modalities, has the capacity to improve diagnosis and early detection as well as potentially improving treatment outcomes for individual avian patients and large collections. The treatment of choice is amphotericin B and it has been recommended in the literature at a range of dose rates and frequencies. Field experience indicates that its efficacy is inconsistent with some birds lacking a response to therapy, but since the establishment of a treatment protocol, efficacy has not been scientifically evaluated.

In this body of work, we evaluated the most common in-clinic diagnostic tools and established that the faecal suspension technique is the most likely to detect *Macrorhabdus ornithogaster* in the faeces of budgerigars ante-mortem. We then evaluated the common laboratory techniques, comparing the faecal suspension technique, faecal PCR, PCR of the isthmus scrapings, direct isthmus cytology and histopathology of the isthmus. Our work established that the PCR of the isthmus and direct isthmus cytology were the most sensitive laboratory diagnostic tools, but when both scientific and convenience criteria were evaluated, isthmus cytology was favoured. It is user-friendly, requires little training, has a rapid turnaround time, is cheap to perform but is as sensitive as PCR, making it the recommended diagnostic modality.

We evaluated amphotericin B for the treatment of *Macrorhabdus ornithogaster* using a variety of dose rates, administration techniques and durations. We established that there is a high treatment failure rate, when using the current recommended protocols and propose that, based on the pattern of organism shedding, the treatment failure is likely the result of a failure of protocol, rather than failure of active ingredient. Further evaluation of the treatment protocols should include *in vitro* evaluation of the organism susceptibility to the treatment compound and evaluation of the method of delivery. We explore a variety of novel treatment modalities for future use against *Macrorhabdus ornithogaster*.

Finally, we explored the potential role of co-infections with immunosuppressive viruses and evaluated the occurrence of *Macrorhabdus ornithogaster* in conjunction with psittacine beak and feather disease virus, avian polyomavirus and psittacine herpesvirus in budgerigars. We established that there was a very low viral burden in budgerigars testing positive for *Macrorhabdus ornithogaster* and that there was no link between infection with *Macrorhabdus ornithogaster* and the viruses that were targeted. Whilst performing the *Macrorhabdus ornithogaster* and viral DNA extraction, we evaluated the use of the BioSprint® 96 well One-For-All semi-automated DNA extraction technique for use on avian faecal samples. The BioSprint® performed favourably compared with a traditional commercial faecal DNA extraction kit, when measuring both the quality and the quantity of DNA extracted from faeces. This highlights the usefulness of semi-automated, high-throughput systems for the difficult application of DNA extraction from bird faeces.

Our work was able to establish the two most sensitive diagnostic tools, the faecal suspension technique and direct isthmus cytology, for the detection of *Macrorhabdus ornithogaster* in budgerigars ante- and post-mortem, respectively. We established that amphotericin B, when using the current treatment recommendations, fails to clear *Macrorhabdus ornithogaster* from most birds treated, and that no link could be established between several common immunosuppressive viruses of budgerigars, and infection with *Macrorhabdus ornithogaster.* We outline future directions for research and prioritise areas requiring deeper understanding. In doing so, we suggest novel drug delivery technologies and future directions for the investigation and treatment of *Macrorhabdus ornithogaster*.

Dedication

To my grandparents,

who taught me the value of hard work and the importance of hobbies.

To my mum and dad,

for your unconditional love and eternal support.

To my brothers,

for always showing up when it matters.

To my partner,

for inspiring and supporting my crazy dreams.

To my daughter,

may your world be full of exploration, love, and happiness.

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There are many people to acknowledge for their contribution to my life and to this thesis. I suppose it makes sense to start at the beginning, with my parents, Sheryl, and Rob. For instilling in us a desire to question the status quo, to investigate what we don't understand and to keep striving to be better. For always being there as a safe harbour, an inspiration, and a motivation, from the sidelines of sports fields across the globe, to the hours spent editing school projects and university assignments, your success as parents has made dreams like this possible. To my brothers, Malcolm, and Samuel, for helping create a bond where we knew the sky was the limit and providing the nurturing support that we all thrived on growing up. One could not wish for better people to have my back.

To Dr Deborah Monks, thanks for taking me under your wing and giving me foot in the door as a young vet student. Thank you for your friendship and wisdom and your tireless dedication to our profession and those within it. To Dr Bob Doneley for stepping up and taking on an orphaned resident, your mentorship and years of experience have helped to shape my perspectives and helped immeasurably in my career. Thanks also to Dr Mikel Sabater, my original residency supervisor, for providing the opportunity to experience a wider world view with your international experience. Thank you all for the contribution you have made both professionally and personally.

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To Dr David Phalen, my original PhD supervisor, mentor, friend, and wise-counsel – your input has been invaluable. Thanks for always putting in a bit more effort than was necessary, whether it was pushing me to be a better diagnostician, a more competent microscopist, or a better person. You are always a sounding board for constant improvement. Having you and Susan in Sydney during my residency and afterwards helped to make it a home, and I will always be grateful for your

contribution to my career and my life. To Dr Ruth Zadoks, I knew from our very first meeting that you were a force of nature and that your contribution to my supervisory team would make for a steep learning curve. You added a perspective and a body of experience that I had not been previously exposed to, you helped to improve my thinking, my writing and provided inspiration right when it was needed. It is humbling to work with someone with at the top of their game, thanks for reminding me that you can always be better. To Dr Ben Stevenson, my longtime friend, hockey goalkeeper, chicken dad and statistical hero, thanks for all your input. For answering my countless questions patiently and with kindness. Your statistical guidance has been invaluable.

To the teams at The Unusual Pet Vets, specifically Dr James Haberfield, my business partner and good friend, thanks for holding the fort whilst this research happened. Thanks for bringing me into the fold and sharing the vision of UPV and for sharing the journey through its ups and downs. It's been wild, but we've created something special. To our other partners, vets, nurses and support staff, you guys are the driving force for constant improvement. Thanks for helping to provide the motivation and inspiration to get this work finished. It is hard to keep up with all the great work you guys do, but I really feel like we are moving our little corner of the profession in the right direction. Thanks to each of you for your part in the journey.

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Preface

This thesis includes a compilation of seven chapters and is submitted with modifications of previously published manuscripts. I, Hamish Baron, am the primary author on all publications featured in this thesis.

Chapter 1 is the general introduction of the thesis. Chapters 2, 4, and 5 are bodies of work published in peer-reviewed scientific journals. Chapter 3 and 6 were in preparation for peer review at the time of thesis submission. Chapter 7 includes the general discussion and conclusion of the thesis.

An author attribution statement declaring each author's contributions are included at the beginning of every research chapter. The inclusion of published manuscripts follows the University of Sydney's Thesis and Examination of Higher Degrees by Research Policy 2015 guidelines for a thesis with publications.

Chapter 1 elucidates the general background to the scientific problem investigated, and introduces each of the five data chapters, and the aims and outcomes of each component of the work.

Chapter 2 is a research paper, published in the peer-reviewed scientific Journal of Avian Medicine and Surgery that describes the comparison of in-clinic diagnostic tools for the identification of birds infected with *Macrorhabdus ornithogaster*.

Chapter 3 is a research paper for submission to the peer-reviewed scientific Journal of Avian Medicine and Surgery that builds on the work of chapter 2 and compares the diagnostic tools available in a laboratory setting. This allows the comparison of the laboratory tools, with the 'gold standard' established in chapter 2 and provides the framework for robust diagnostic testing recommendations.

Chapter 4 is a research paper published in the peer-reviewed scientific Journal of Medical Mycology and combines data from three experiments: a retrospective review of treatment outcomes of birds with *Macrorhabdus ornithogaster* infections over a 9-year period, a pilot treatment trial evaluating treatment efficacy and shedding, and a randomised controlled trial evaluating treatment outcomes with two different treatment regimens.

Chapter 5 is a research paper published in the peer-reviewed scientific journal, The Australian Veterinary Journal and describes the variable treatment success when treating birds with *Macrorhabdus ornithogaster* with amphotericin B.

Chapter 6 is a research paper that evaluates three common budgerigar viruses and their relationship to infection with *Macrorhabdus ornithogaster* in budgerigars from Australian aviculturists' collections. This chapter also evaluates the extraction of viral and fungal DNA from avian droppings, to provide guidance on a new, automated, extraction technique, using the BioSprint®.

Chapter 7 provides a general discussion of the work presented within this thesis, including the significance and limitations of the research, and the directions for future research.

The COVID-19 global pandemic interrupted the candidature period significantly. Australia-wide lockdowns and interstate travel restrictions meant that laboratory time was limited in the last two years of the candidature. This impacted the ability to carry out further trials evaluating the efficacy of concurrent oral treatment with pro-oxidants, such as L-ascorbic acid, and AmB, outlined in Chapter 4. It also prevented the completion of *in vitro* susceptibility testing, for which a protocol was established, but further work could not be completed. Instead of omitting this chapter completely, I have included a reduced version as Appendix 1, to provide a baseline for future researchers.

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List of Publications

Publications Arising from the Candidature

Baron HR, Stevenson BC, Phalen DN. Comparison of in-clinic diagnostic testing methods for *Macrorhabdus* ornithogaster J Avian Med Surg. 2021;35(1):37-44. https://doi.org/10.1647/1082-6742-35.1.37

Baron HR, Stevenson B, Phalen D. Inconsistent efficacy of water-soluble amphotericin B for the treatment of *Macrorhabdus ornithogaster* in a budgerigar (*Melopsittacus undulatus*) aviary. Aust Vet J. 2020;98(7):333-337. https://doi.org/10.1111/avj.12936

Baron HR, Leung KL, Stevenson B, Sabater MS, Phalen DN. Evidence of amphotericin B resistance in *Macrorhabdus ornithogaster* in Australian cage birds. Med Mycol. 2019;57(4):421-428. https://doi.org/10.1093/mmy/myy062

Publications Arising from Collaborative Work During Candidature

Sollom HJ, **Baron HR**. Clinical presentation and disease prevalence of captive central bearded dragons (*Pogona vitticeps*) at veterinary clinics in Australia. Aust Vet J. 2023;101:200– 207. https://doi.org/10.1111/avj.13234

Taggers A, **Baron H**. A retrospective study of 333 emergency presentations and survival to discharge of backyard chickens in Australia from 2019 to 2022. Aust Vet J. 2023;101:212– 217. doi:10.1111/avj.13233

Schader JA, **Baron HR.** Investigation into the prevalence of *Serratospiculum* spp. in wild falcons of the greater Sydney region. Aust Vet J. 2021;99:363-367. doi:10.1111/avj.13091

Baron HR, Foo T, Phalen DN. Humeral air sac cystadenocarcinoma in a rainbow lorikeet (*Trichoglossus moluccanus*). Aust Vet J. 2020;98(4):168-171. doi: 10.1111/avj.12915. Epub 2020 Feb 4. PMID: 32017026.

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Baron HR, Šlapeta J, Donahoe SL, Doneley RTJ, Phalen DN. Compensatory gastric stretching following subtotal gastric resection due to gastric adenocarcinoma in a diamond python *(Morelia spilota spilota).* Aust Vet J. 2018;96(12): 481-486. doi: 10.1111/avj.12764.

Baron HR, Phalen D, Sabater M. A novel technique for extracapsular repair of the intertarsal joint in a duck. J Avian Med Surg. 2018;32(1):57-64. doi: 10.1647/2017-246.

List of Presentations

Domestic Conference Presentations Arising from Candidature Topic

Baron HR. Emergency medicine of Galliformes. The Australian Veterinary Association Annual Conference Proceedings, Darwin 2022

Baron HR. Emergency medicine of Psittacines. The Australian Veterinary Association Annual Conference Proceedings, Darwin 2022

Baron HR. *Macrorhabdus ornithogaster:* treatment trials and tribulations. Association of Avian Veterinarians Australasian Committee Annual Conference Proceedings, Adelaide 2018.

Baron HR. Common avian consultations: what I need and what I need to know. Australian Veterinary Association Annual Conference Proceedings, Brisbane 2018.

Baron HR. Circovirus in wild Australian Psittacines. Australian Wildlife Rehabilitation Conference Proceedings, Sydney 2018.

Baron HR. *Macrorhabdus ornithogaster:* what we know and what we need to know. Federation of Asian Small Animal Veterinary Associations Congress (FASAVA), Gold Coast 2017

Baron HR. Ligament luxation and subluxations in birds: a review of management options. Australian Veterinary Association Annual Conference Proceedings 2017.

Baron HR. Collateral ligament repair in a duck. International Conference on Avian Herpetological and Exotic Mammal Medicine Conference Proceedings, Venice, 2017.

Baron HR. Small mammal orthopaedic repairs 2015. Association of Avian Vets Australasian Chapter, Unusual and Exotics Pets Special Interest Group, combined proceedings

Baron HR. Avian beak trims: the science behind the art. 2015 Australian Veterinary Association Pan Pacific Veterinary Conference proceedings.

Baron HR. GnRH agonists: their role in avian reproductive disease. 2013 Association of Avian Vets Australasian Chapter, Unusual and Exotic Pets Special Interest Group, combined proceedings.

Contributions to the Scientific Community

2019–2023 Chapter President, Australia New Zealand College of Veterinary Scientists (ANZCVS) (Avian Medicine and Surgery)

2020 – 2023 Fellowship Supervisor, ANZCVS (Avian Medicine and Surgery)

2021 Fellowship Examiner, ANZCVS (Avian Medicine and Surgery)

2020 Membership Examiner, ANZCVS (Avian Medicine and Surgery)

2018-2022 Association of Avian Veterinarians Australasian Committee Student Liaison Officer

2017 Unusual Pets and Avian Veterinarians Committee Member

Association of Avian Veterinarians Australasian Committee Student Liaison Officer

2016–2023 Supervisor of Veterinary Science Students, Research and Enquiry 3

2016 Organising Committee – Association of Avian Veterinarians / Unusual Pet and Avian Veterinarians special interest group of the Australian Veterinary Association (AAVAC / UPAV) Combined Conference, Brisbane, QLD

2015 Organiser of the AAVAC / UPAV Combined Conference, Sydney, NSW

Unusual Pets and Avian Veterinarians Committee Member

Chapter 1. Introduction – *Macrorhabdus ornithogaster*, What We Know, What We Need To Know

Introduction

The budgerigar (Order Psittaformes: Family Psittaculidae: Subfamily Loriinae: Tribe Melopsittacini: Genus *Melopsittacus*: Species *Melopsittacus undulatus*)[1] is a small, light green and black native Australian parrot. In their natural environment, they are found in open scrublands, woodlands, and grasslands. They are normally found in small flocks of 3 – 100 birds that are nomadic, moving with the change in environmental conditions and availability of food and water.[1] During favourable conditions, populations explode, and budgerigars can be found in flocks of many tens of thousands. First described in 1805 and given its current binomial name by John Gould in 1840, the budgerigar has been bred in captivity since early in 1850.[2] Budgerigar breeders have established a variety of colour and feather mutations in the captive budgerigar population and there are now two very distinct phenotypes recognised in captivity, the pet or wild type budgerigar and the exhibition budgerigar. Exhibition budgerigars have been selected for size and desirable exhibition features including length of feather, so-called 'directional feather,' height, width at the shoulder and depth in the mask. As a result of these selection pressures, the exhibition budgerigar is now approximately twice the size of the wild type birds. Not only are these exhibition budgerigars substantially larger, but they are also significantly more expensive, both to purchase and to keep in an avicultural setting. To quantify the difference in purchase costs, top quality exhibition budgerigar pairs sold at auction for between AU$5000 and AU$17000 at the 2023 Australian National Budgerigar Society Show, whilst budgerigars in the pet store retail for between AU$30 and AU$50. Because of the potential financial returns, there are breeders in Australia and across the world that keep and breed budgerigars as their primary source of income. The health and disease states of these birds, therefore, is not only a welfare consideration, but a matter of the emotional wellbeing, and even livelihoods, of some breeders.

The exhibition budgerigar has a variety of disease conditions that are commonly seen by avian veterinarians. Many of these conditions are non-infectious; nutrition-

related obesity, hepatic lipidosis and cutaneous or subcutaneous lipomas are common.[2,4] Neoplastic changes of the uropygial gland and kidneys are seen frequently[4] and as the birds have got bigger and the feathers heavier, cystic changes to the primary flight feathers have become increasingly common (Baron, personal observation). A combination of avian polyomavirus (APV) and/or beak and feather disease virus (BFDV) cause budgerigar fledgling disease, which occurs intermittently in different collections around the time of breeding and results in feather dystrophy in young birds and nestling mortality.[5] Bacterial infections, such as *Chlamydia psittaci*, are becoming increasingly rare (Baron, personal observation), with the improved knowledge and routine treatment of large collections with in-water antibiotic therapy (often doxycycline) as a prophylactic measure. Similarly, ectoparasites, such as *Cnemidocopes pilae* and various feather lice and mites, are seen less frequently in veterinary practice, likely because of the availability of cheap and effective over-the-counter medications for the treatment of these conditions. Gastrointestinal pathogens, including *Trichomonas* spp. and nematodes, are managed well through prophylactic medication administration by aviculturists in conjunction with their avian veterinarian. Arguably the most significant pathogen seen in budgerigar collections in Australia, the gastric yeast *Macrorhabdus ornithogaster* (MO), is seen commonly in a clinical setting.[6,7]

Many of the breeders will present budgerigars having already unsuccessfully trialled treatments at home for the other common conditions above. Usually, the birds will be presented during a mortality event, where the breeder has been losing birds consistently and is seeking a definitive diagnosis to stem the flow of stock losses. In Australia, there are no over-the-counter medications available for the treatment of MO, and so it is possible that the budgerigars presenting to the veterinary clinic are an over-representation of the scale or severity of the infection in breeding facilities in Australia. To date, there has been no work carried out to evaluate the disease states of viruses, bacteria and fungi circulating in the budgerigar aviaries in Australia, but work carried out in the early 1990s demonstrated that MO was already widespread in exhibition budgerigar aviaries across South East Queensland.[6,7] Since the original work evaluating prevalence in budgerigar aviaries, no screening has been carried out to establish how the MO organism is currently circulating within the exhibition budgerigar communities. *Macrorhabdus ornithogaster* is now understood to be widespread in wild Australian

psittacine birds[8-10] as well as a small number of feral species,[11] which gives rise to concerns regarding spill-over events from wild populations into other captive or domestic bird species. It is well described that MO infects chickens, both naturally and experimentally.[12-14] The popularity of backyard and free-range poultry systems, where feeders and water systems are shared by wild bird species, mean that these spill-over events are increasingly likely to occur. Clinically, in avian veterinary practice in Australia it is becoming more common to see backyard laying hens infected with MO; these birds often show signs of diarrhoea, gastrointestinal (GIT) upset and weight loss, similar to those signs seen in their psittacine counterparts (Baron, personal observation). The emergence of MO in backyard poultry requires further investigation, but the endemic state of MO in budgerigar aviaries in Australia makes it a pathogen of significant concern, from both an animal welfare and economic standpoint.

Macrorhabdus ornithogaster

Introduction to Yeast

Yeasts are eukaryotic, single-celled microorganisms classified as members of the fungus kingdom. The yeast lineage originated hundreds of million years ago, and 1,500 species are currently identified.[15,16] Yeasts are characterised by their ability for unlimited clonal propagation by budding or fission in the absence of fruiting bodies.[17] They vary greatly in size, depending on species and environment, typically measuring 3–4μm in diameter, although some yeast can grow to 40μm in size.[18] Contrary to commonly held views, yeasts do not represent primitive unicellular eukaryotes, but instead have repeatedly emerged from distinct phylogenetic lineages of 'modern' fungi.[17,19] The phylogenetic diversity of yeasts is shown by their placement in two separate phyla: the Ascomycota and the Basidiomycota. The Ascomycota are a large and important group of fungi, distinguished from other fungi by a saclike ascus containing haploid ascospores.[20] The Ascomycota encompass over 1000 species, accounting for 40% of all described fungi.[21-23]

The Organism

Macrorhabdus ornithogaster is an ascomycete yeast (phylum Ascomycota: subphylum Saccharomycotina: class Saccharomycetes: order Saccharomycetales) that is the only member of its genus (*Macrorhabdus*)[24] and a common pathogen of

cage birds in Australia and worldwide.[25,26] *Macrorhabdus ornithogaster* is a long, straight, rod-shaped organism, measuring 20–80μm long and 1.5–4μm wide. When MO are viewed by direct microscopy in a wet mount preparation, they appear translucent, with small oblong refractile nuclei at regular intervals within the organism.[27,28] It only occurs naturally on the mucosal surface of the stomach of birds, at the junction between the proventriculus and ventriculus, a narrow transition zone termed the isthmus.[27]

Historical significance, geographical distribution, and introduction of *Macrorhabdus ornithogaster* in Australia

Macrorhabdus ornithogaster has been reported in more than 40 avian species, from passerines to poultry.[26,29] First referenced as a bacterium in canaries (*Serinus canarius domesticus*) in Utrecht in 1980,[28] it was Jones and Carol who described a wasting syndrome in budgerigars,[30] characterised by an apparent ravenous appetite, with seed ground to a fine powder and a proventricular yeast in a form resembling crystals. The organism was difficult to grow in traditional culture media.[30] These birds began gaining weight following treatment with nystatin. Based on the description provided in this report, it seems plausible, and likely with the benefit of hindsight, that this was the first report of a disease syndrome caused by MO in the UK.[30] Hargreaves was the first author to identify the organism as a fungus contributing to disease in 14 species of psittacine and passerine pet birds in the USA in 1981.[31] Shortly after this, it was classified more completely, but erroneously, as a bacterium, leading to much confusion, and the incorrect, but still most widely accepted colloquial name, 'Megabacteria'.[32] 'Megabacteria' was identified as a significant, emerging pathogen in exhibition budgerigars in the UK[33] following the rapid dissemination through the exhibition budgerigar studs, and was the cause of death in nearly 75% of budgerigar mortalities examined by 1992.[3] It is thought that MO was imported into Australia with exhibition budgerigars from the UK in 1989–90, and Filippich et al. were the first to report 'Megabacteria' in Australia when they found it in isthmus scrapings on necropsy in a variety of psittacine and passerine species.[7] These authors also recorded the presence of 'Megabacteria' in 23 budgerigar and two canary breeding colonies in South East Queensland by 1992.[6] Importantly from a historical point of view, Filippich et al. also evaluated the droppings of exhibition budgerigars imported from the UK immediately after being released from quarantine in Australia, and found

that 52% of these birds were 'Megabacteria'-positive.[6] The prevalence and incidence of infection in these studies was artificially inflated because aviaries with a high incidence of infection were chosen for screening; nonetheless, prevalence of infection in budgerigar aviaries ranged from 27% to 64%, with 47% of canaries examined also being faecal-positive for 'Megabacteria'.[7] Throughout the 1990s, so-called 'Megabacteria' caused significant disease in exhibition budgerigar aviaries and prompted investigations into treatment regimes[34] and a better understanding of the epidemiology in aviaries in Australia.[6,35]

While the investigations were being undertaken in cage birds, specifically, budgerigar aviaries, investigations also took place in wild birds in Australia. 'Megabacteria' was reported in 60% of wild caught European goldfinches (*Carduelis carduelis*) and 80% of wild caught young sulphur-crested cockatoos (*Cacatua galerita*) in Australia as early as 1994,[8] and is now well established in wild galah (*Eolophus roseicapilla*), *Cacatua* spp. and king parrot (*Alisterus scapularis*) populations, where seasonal wasting disease is reported in young birds on the east coast of Australia.[9,36] In Western Australia, *Cacatua* spp. and Australian ringneck parrots (*Barnardius zonarius*) and the red-capped parrot (*Purpureicephalus spurius*), demonstrated infection rates between 8% and 36%.[10] Based on the widespread distribution of MO in wild bird populations during the early 1990s, it seems unlikely that the importation of exhibition budgerigars was the sole source of introduction into birds in Australia, and it is plausible that the organism originated in Australia and was spread worldwide through the export of Australian psittacine species.

Disease Manifestations

Standard epidemiological classifications are challenging because some birds show no signs, instead, we can assess different stages of infection, starting with colonisation of the isthmus. It is likely colonisation of the isthmus occurs immediately upon exposure to MO from ingestion. This deduction is supported by experimentally infected chickens, that demonstrated the organism in their droppings shortly after inoculation and shed high numbers of MO by two weeks post infection.[14] Once colonisation of the isthmus has occurred, the bird is then infected. Infected but asymptomatic birds can be differentiated from diseased birds using two methods: signs of disease and histological changes. Histologically, birds that have recently become infected, or those that are infected but not showing signs of disease

typically have relatively few MO organisms. The organisms that are present are primarily confined to the surface of the isthmus mucosa and are not associated with an inflammatory response, or if one is present, it is usually heterophilic and mild.[37,38] In contrast, infected birds that have become diseased have a marked overgrowth of the mucosa of the isthmus. *Macrorhabdus ornithogaster* organisms will extend deep between the mucosal villi often into, and beneath, the koilin layer of the ventriculus.[39] These changes are accompanied by a severe chronic lymphoplasmacytic gastritis and ulceration of the proventricular and/or the ventricular mucosa.[37]

The *Macrorhabdus* Conundrum – The Development of Clinical Disease

The triggers that precipitate the transition of a bird from 'infected' to 'diseased' are very poorly understood, and to date only tenuous evidence supports the various theories. The most commonly proposed triggers include concurrent stressors such as co-infections with immunosuppressive organisms,[10,40] exposure to continuous light,[41] rapid change of diet and malnutrition.[42] Even the stress of capture and restraint during treatment[43] has been proposed as a trigger for disease development. Juvenile birds are reported as being over-represented,[43] possibly due to a variety of stressors (e.g., growth, weaning, new ownership, poorly developed immune system). Nutritional and reproductive status can affect gastrointestinal pH or bacterial microbiome,[42] a change that has also been reported to occur with, or result from, MO infections.[42,44] It is also plausible that variability between strains of MO influences the infection course.[26,45-48]

The suggestion that co-infection with one or more pathogens may result in immune suppression making some budgerigars more susceptible to MO associated disease has some support from the literature.[26,40,41] As discussed, APV and PBFDV are common pathogens in budgerigar aviaries and have the potential to cause lymphopaenia and in severe cases pancytopaenia. Psittacine adenovirus may also be immunosuppressive, but its prevalence in Australian budgerigars is unknown.[49-51] Avian polyomavirus causes a multisystemic infection in nestling budgerigars; often, antigen presenting cells are targeted, leaving survivors with a compromised immune system.[50,52] The PBFDV is well known for its impact on the immune system, specifically the bursa of Fabricius, and experimentally infected birds have been demonstrated to be immunosuppressed and therefore more susceptible to

infectious diseases.[53,54] Adenovirus infections in birds can target the spleen and therefore could also cause immunosuppression.[51,55,56] No work has been carried out to establish the prevalence of these viruses in budgerigar populations and whether there is an association between these viruses and co-infection with MO.

Epidemiology

The epidemiology of MO is also poorly understood, and a non-avian source for MO has not been identified.[27] The reservoir appears to be infected birds[57] and because asymptomatic carriers persistently spread the organism without showing signs of disease, they are often difficult to identify. It has been suggested that because of the success of the budgerigar as a cage bird, they may have acted as a reservoir for the global spread of the pathogen.[29] The disease spread appears to have occurred rapidly, both intranationally as reported by Baker[3] and internationally, and is likely to have a global distribution.[6,7,13,28,33,58]

It appears that most infections result from faecal-oral transmission, when organisms shed from infected birds contaminate the environment, food or water with their droppings.[14] Controlled clinical trials in chickens demonstrated the rapid transmission between birds inoculated orally with MO cultures and their non-infected cage mates.[14] The transmission of MO in altricial species via regurgitant feeding has been proposed as a second possible route of infection.[11] Transmission via regurgitant feeding – a route that is supported by MO occasionally being present in upper gastrointestinal samples collected from the crop or oesophagus[26] – whilst plausible, is yet to be proven as a transmission route.[59] It is unclear how long MO stays infectious outside the host or whether it remains viable in water, or in dry or desiccated faecal material. *In vitro* growth studies demonstrate that the organism remains viable, in culture media, for 24hrs at room temperature[60] and is minimally impacted by, or potentially benefits from, exposure to oxygen for a short period of time prior to culture.[12,60]

Macrorhabdus ornithogaster in Budgerigars

Although it has a wide host range across the globe,[13,61-66] MO is most commonly reported in budgerigars.[3,6,39,43,63,64] Infection occurs frequently in large breeding collections and is seen affecting pet birds in veterinary clinics in Australia and throughout the world.[4,6,33,39,43,65] In Australia, it appears that the organism is endemic

in the exhibition budgerigar population, and with most avicultural collections having been affected clinically, routine prophylactic treatment has become a mainstay of annual treatment regimens by exhibition budgerigar fanciers. As previously mentioned, some infected budgerigars will be asymptomatic,[6,39] whilst other infected birds will spend much of the day fluffed, lethargic, at the food bowl appearing to eat, but be emaciated.[39] Characteristically, these birds sit and grind seed or pellets in their beak, making a fine powder that is visible in the bottom of the food dish in infected aviaries.[39] Some of these birds go on to develop signs of regurgitation, melaena and diarrhoea and many that progress to this stage of the disease will die.[3] These deaths can occur either as disease outbreaks, with significant mortality events,[41,66] or as an insidious, chronic wasting disease with intermittent mortality but consistent debilitation and poor vigour in the birds in the collection.[4,9]

Despite its significance as a pathogen in budgerigars, little has been done to optimise the diagnosis and treatment or to establish a more complete understanding of the host-pathogen interaction. This gives rise to significant challenges in developing efficacious treatment and prevention recommendations.

Diagnosis

The accurate and repeatable diagnosis of MO is important from a veterinary perspective in that it informs treatment decisions, management planning for diseased collections and provides a first line of defense in biosecurity screening. To date, there are no robust and repeatable studies comparing the accuracy and practicality of the variety of different diagnostic modalities. We fill this knowledge gap by comparing in-clinic (ante-mortem) and laboratory (ante- and post-mortem) diagnostic tools, evaluating both scientific and convenience criteria to provide evidence-based diagnostic recommendations in a clinical setting.

Ante-mortem diagnosis of infection with MO has the potential to benefit the infected bird, the owner or fancier and the flock. A diagnosis has traditionally been carried out by evaluating faecal material via direct microscopy using a wet mount,[27] with or without Gram or quick stains (e.g., Diff Quik). When stained appropriately, MO are gram-positive and stain dark blue with Diff-Quik stains.[26,67] Faecal material may also be evaluated using one of two floatation-techniques, followed by direct microscopy, or by molecular techniques. The floatation techniques include use of the Mini-FLOTAC

(FLOTAC Group, University of Naples, 80138 Napoli NA, Italy) tool,[26,68] or a faecal suspension technique performed by combining faeces with 0.9% sodium chloride in a mini-Eppendorf tube to form a slurry, before a drop of suspension from the meniscus is evaluated.[26] All direct microscopic examination techniques require the microscope stage diaphragm to be completely down in order to maximise contrast and visualise the organisms most clearly.[26] Because of the large cigar-shape of MO,[69] it can be mistaken for long rod-shaped bacteria during direct microscopy, leading to false-positive results. This means that the detection of MO microscopically is dependent on the skills and experience of the person performing the cytology.

Post-mortem, a diagnosis serves to benefit the flock and the fancier or pet owner, but, despite the significant welfare and financial implications of getting a timely and correct diagnosis, the tools are yet to be comprehensively evaluated. At post-mortem examination, a diagnosis can be achieved by collecting faeces from the colon and utilising the ante-mortem diagnostic modalities. Scrapings can also be collected from the isthmus for direct wet mount microscopy or molecular diagnostics, and, finally, isthmus samples fixed in formalin can be evaluated using histopathology.[27,70] When evaluated histologically, MO are palely eosinophilic and found at the tips of the glands of the isthmus. Depending on the severity of infection, MO may be scattered and scarce or there may be multiple 'sheets' of MO lined atop one-another.[71]

Molecular techniques such as polymerase chain reaction (PCR), which uses DNA extracted from both faecal material and cloacal swabs, can be used as a diagnostic tool.[24,72] However, use of PCR is not without limitations, specifically cost, turnaround time, availability to the end user and confidence in the results (possibility of false negative results). The optimisation of DNA extraction from avian faeces requires further investigation, with recent investigations demonstrating many commercially available faecal kits extracting little or no DNA from avian faecal samples.[73]

Treatment

Once a diagnosis is obtained, the next major challenge for the avian veterinarian is the affordable and effective treatment of MO, both in the individual cage bird, and in large breeding facilities, where manual capture and medication of all birds is not practical. Various treatment trials have been performed,[38,45,46,57,60,67,74] using a variety of

antifungal and gastric pH modifying drugs, both in-water and by direct oral administration. In early trials, treatment was deemed a success if the organism could no longer be visualised in the faeces immediately following treatment.[74] It is unclear whether these treatment trials resulted in treatment success or simply a temporary cessation in shedding, but many of these birds were reported as being positive for MO on subsequent faecal smears.[6] This suggests that either the birds were being reinfected following treatment, or that treatment was unsuccessful in clearing the MO organisms.

Currently, the poor determination of infection status limits the interpretation and extrapolation of the results of treatment trials.[46-48,75] Importantly, for the evaluation of clinical outcomes, it is not necessary for birds to be euthanised at the conclusion of trials. Clinical observation is sufficient to inform clinical success (i.e., birds stop dying, begin to gain weight and are no longer sitting fluffed in the food bowl). For the fancier, clinical manifestation of disease is likely more important than infection status. For some, the tolerance of infection, so long as it does not cause signs in their collections, would be a welcome trade-off. For those that are interested in infection status, regardless of concerns about disease manifestation, the implementation of strict quarantine and testing practices can and should be undertaken. For these owners, clinical outcomes would not be a sufficient measure of success, and trials aimed at elimination of MO (to ensure biosecurity) would require euthanasia to confirm infection status[45,46,57,60] and provide more robust evidence of treatment outcomes.

The most successful and commonly implemented treatment regimens include the use of the ionophores, amphotericin B and nystatin, either in-water or given direct by mouth.[34,46] Recently, evidence for treatment success, both *in vitro* and *in vivo*, of sodium and potassium benzoate has provided hope for a novel treatment solution for MO infected birds.[45,47,60]

Nystatin

Nystatin, which binds to ergosterol in fungal cell membranes, has been used for the treatment of MO. Early trials resulted in the cessation of shedding when budgerigars were treated orally with nystatin.[74] Concentrations of 0.1IU/mL were effective in killing MO in vitro,[26,60] and when trialed in a flock of approximately 500 budgerigars, a dose of 3,500,000IU/L, for two days, then 2,000,000 IU/L of drinking water for 28

days was successful in resolving the clinical signs. Birds euthanised at the conclusion of treatment were found to be free of the organism.[46] Nystatin at this concentration in water is problematic because currently available powders contain only 100,000 IU/g and more concentrated powder will lead to issues with solubility.

Amphotericin B

Amphotericin B (AmB) is used as therapy for systemic fungal infections.[76] It binds to ergosterol, disrupting cell membrane integrity[77] via the formation of pores leading to fungal cell death.[78] Amphotericin B has a highly hydrophobic region which leads to the formation of micelles in an aqueous environment, resulting in very poor oral bioavailability.[79,80] The use of AmB for the successful treatment of infection with MO was first described in 1993.[34] Since then, it has been the mainstay of treatment in both individual cage birds and collections of birds. Various formulations are available, including water-soluble powders, lozenges and, more recently, an oral formulation for human consumption.[26,81-85]

Recommended oral doses range from 5mg/kg to 100mg/kg by mouth twice daily, and duration of treatment ranges from 10 to 30 days.[26,34,86] Administration is oral, by gavage tube, or in-water at a dose of 0.9mg/mL for 10 days. A formulation was commercially available (Amphotericin 2.5% powder, Vetafarm Australia Pty Ltd, Wagga Wagga 2650, NSW) and routinely used to treat individual birds and large avicultural collections until it was discontinued in 2021.[26,75,86] The in-water treatment has been reported anecdotally as being less efficacious than direct gavage or oral administration, perhaps due to the inconsistent water intake in sick birds. There is a single report of treatment failure, reported as resistance, to AmB treatment in budgerigars that required an increased dosing regimen, i.e., more than the initial dosage rate of 5mg/kg by gavage for 10 days.[34] In this report, resistance was not proven beyond the failure to respond to treatment with AmB. More recently, 47% treatment failure was reported after administering a dose of 100mg/kg PO BID for 30 days, calling into question the efficacy of AmB at the recommended dose rate.[43] The cause of AmB treatment failure is unclear, but it is likely the result of a combination of the route, frequency and rapid gastrointestinal transit time in budgerigars, as well as individual host factors, including variations of the stomach microbiome,[44] combined with the potential for MO resistance to AmB. On top of the concerns regarding treatment failure and appropriate dose administration, there

are significant limitations in the use of AmB as an oral formulation for treatment of fungal infections,[83] specifically availability, cost, potential toxicity, and administration difficulty. Assessment of the therapeutic value of AmB against MO requires robust modern clinical trials and is needed to continue to recommend AmB treatment as the standard of care for MO treatment.

Sodium and Potassium Benzoate

Sodium benzoate, the sodium salt of benzoic acid and widely used as a food preservative and a pickling agent, and potassium benzoate, the salt of benzoic acid and used as a food preservative that inhibits the growth of mould and yeast, are both extremely effective at limiting the growth of MO *in vitro*.[60] Various protocols have been described for use *in vivo*,[45,47] resulting in variable cessation of shedding and improvements in clinical signs. Importantly, mortalities, attributed to toxicity have been reported when using these protocols.[26,45] This toxicity is likely multifactorial due to excessive intake of either sodium or potassium in combination with impacts of temperature, reproductive status, duration of therapy and dose rate. Until the mortalities can be better controlled the risks of this treatment outweigh the benefits.

What We Need to Know

Despite nearly 45 years of research exploring the aetiology, pathogenesis, host interactions and treatment options for MO, there are still significant knowledge gaps in the literature. Many individual reports of diagnostic modalities exist, most with mixed success when it comes to the diagnosis of MO, but the literature lacks a standard against which we can compare diagnostic tests. We will aim to fill this gap and look to establish a standard by comparing and optimising both in-house and laboratory diagnostic testing. These chapters aim to guide clinicians in their selection of diagnostic tests, as well as their assessment, interpretation and understanding of the validity of results. They will provide a framework outlining the most sensitive and specific testing modalities, with the samples available. The laboratory tests are evaluated using scientific and convenience criteria to deliver real-world-applicable outcomes and guide evidence based clinical diagnostic decision-making.

With regards to treatment modalities, the literature is dotted with various reports of treatment trials against MO, with very few studies involving experimentally

infected birds and a confirmed treatment outcome, to fill this knowledge gap, we explored more deeply the success of AmB as a treatment modality against MO. Amphotericin B efficacy against MO was explored both as an orally administered liquid in a controlled trial, evaluating a range of dose regimes in experimentally infected chickens and in-water. This work provided the first robust experimental data regarding treatment efficacy of AmB against MO since 1996, when Filippich et al.[34] carried out their work in budgerigars in Southeast Queensland. Gaining a better understanding of treatment success with AmB will help to inform clinical decisions regarding treatment dose, frequency and allow clinicians the ability to prognosticate to owners.

Having established the most sensitive and specific ante-mortem and post-mortem diagnostic tests, and gaining a better understanding of the efficacy of AmB as a treatment tool, we sought to gain a deeper understanding about the links between co-infections and MO. The links between stressors, co-infection, age, and infection with MO are tenuous, but are mentioned frequently in the literature. In Chapter 6, we seek to better understand the viral disease states in budgerigars in Victoria, Australia, and to establish whether any of the commonly encountered immunosuppressive viruses are associated with MO infection status. By testing for these viruses and evaluating the relationship between viral infection and infection with MO, we look to expand the evidence base for co-infection as a trigger for MO disease. Simultaneously, we were able to explore a DNA extraction protocol that allows automated, high throughput processing of DNA from avian blood and faecal samples and compare this technique to the commonly utilised standard for avian DNA extractions. By evaluating this novel extraction technique, the data supporting more cost effective and efficient DNA extraction, with fewer opportunities for contamination, may help to make avian molecular diagnostic testing more accessible.

The need for a safe, efficacious, and cost-effective treatment modality is a priority of future research. We draw together the work in this thesis and the work carried out by others during the period of candidature. We explore a range of novel treatment modalities and new drug administration technologies that may be applied to the treatment of MO. We explore the future directions for MO research and suggest priorities for investigators. A major hurdle in the extrapolation of the MO literature

is the standardisation of techniques, in Appendix 1 we provide guidelines that will provide a framework for *in vitro* sensitivity testing for future investigators.

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Chapter 2. Comparison of In-Clinic Diagnostic Testing Methods for *Macrorhabdus ornithogaster*

Hamish R. Baron*, Ben C. Stevenson and David N. Phalen

Author Attribution Statement For Thesis With Publications

I am writing this letter to specify the role of Hamish Baron in the preparation and submission of the following manuscript (Chapter 2):

The convention for author placement in the list of contributing authors is ordered from highest to lowest contribution. The corresponding author of the manuscript is indicated by an asterix (*). The contribution to the manuscript of all authors is as follows:

Task	**Role of Author**
Conceptualisation	HB, DP
Investigation	HB
Methodology	HB, DP
Data Curation	HB, BS
Formal Analysis	BS
Resources	HB, DP
Writing – Original Draft	HB
Writing – Reviewing and editing	HB, DP, BS

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As the primary supervisor for the candidature upon which the thesis is based, I can confirm that the above authorship attributions statements are correct. I have sighted email or other forms of correspondence from all co-authors confirming their certifying authorship and permission for manuscript inclusion in this thesis.

Prof David Phalen

21 June 2023

Abstract

Macrorhabdus ornithogaster is an ascomycete yeast that is often found at the isthmus of the ventriculus and proventriculus of infected birds. Antemortem diagnosis has traditionally involved direct visualisation of organisms on wet-mount or Gram stained faecal preparations and/or cloacal and crop swabs; however, different in-clinic diagnostic techniques have never been compared to establish an optimum test for the identification of *M. ornithogaster* in an avian patient. We compared five microscopy-based diagnostic testing methods; faecal Gram stain, direct faecal wet preparation, faecal suspension technique, faecal suspension with Gram stain, and faecal suspension stained with new methylene blue. Each technique was performed on 96 faecal samples collected during the treatment of *M. ornithogaster* infected budgerigars with water-soluble amphotericin B. The faecal suspension technique produced statistically higher organism counts than the other four techniques and was always estimated to have the largest detection probability. We recommend that the faecal suspension technique be implemented as the most efficacious diagnostic test for in-clinic assessment of avian patients possibly infected *M. ornithogaster*.

Key words: Macrorhabdus ornithogaster, diagnosis, cytology, faeces, budgerigars, avian

Introduction

Macrorhabdus ornithogaster (MO) is a yeast that is found primarily in a narrow zone between the proventriculus and ventriculus (isthmus) of birds.[1] *Macrorhabdus ornithogaster* causes significant morbidity and mortality in pet birds and birds in avicultural collections worldwide. The microorganism has been identified in over 40 bird species including Psittaciformes, Passeriformes, Galliformes, Struthioniformes, Anseriformes, Pelecaniformes and Rheiformes.[1-4] *Macrorhabdus ornithogaster* ranges in size from 20 to 80µm long and 2 to 3µm wide, and are slender, straight rods with rounded ends when found in the faeces.[5,6] When the organism is viewed directly through a microscope in a wet mount preparation, small oblong refractile nuclei are found at regular intervals within the organism.[5,6] *Macrorhabdus ornithogaster* are readily identified in suspensions of scrapings of the isthmus obtained during post-mortem examination of infected birds. Histologically,

the organisms are palely eosinophilic and are present on the surface of the isthmus and between the glands of the proventriculus and isthmus, stacked in parallel. In severe cases, MO can grow deep between the glands of the proventricular isthmus and penetrate the koilin lining of the ventriculus, causing a lymphoplasmacytic inflammatory response.[5,7,8]

Macrorhabdus ornithogaster causes disease characterised by vomiting, dark tarry faeces, chronic wasting and, in severe cases, death.[2,3] Birds may also be infected with MO without exhibiting any clinical disease signs.[7,9] It is becoming increasingly evident that concurrent co-morbidities influence the host's immune competence and gastrointestinal flora, resulting in the manifestation of clinical signs associated with MO infection.[7,8,10,11] When disease does occur, MO can cause significant morbidity and mortality in avicultural collections. Therefore, it is essential to accurately detect infected birds early, so one can isolate and treat affected birds to prevent transmission through the avicultural collection. Similarly, birds that are infected without showing signs of disease may act as a persistent source of environmental contamination or expose progeny, perpetuating MO in future generations.[12]

Ante mortem, the most common diagnostic test used to obtain a diagnosis of MO is microscopic examination of the patient's droppings. Traditionally, a direct wet-mount slide is made using fresh faeces mixed with 0.9% sodium chloride solution and scanned at 40× magnification.[5] The Gram and quick stains (e.g., Diff Quik, Astral Diagnostics, Freeway Park, New Jersey) are often used to examine fixed faecal smears for the presence of MO, but poor stain uptake with variable staining characteristics has been reported.[10] When stained appropriately, the organisms are gram-positive and stain dark blue with Diff-Quik stains.[10] A rapid method of concentrating MO and separating the organisms from other solid faecal matter is based on homogenising the droppings with approximately 20 times their volume of physiologic saline in a small tube. After this the homogenised faeces are rested for three seconds prior to placing a small drop of the suspension collected from the meniscus on a microscope slide to examine through a microscope. Since MO takes longer to settle than the particulate matter, the organism is reportedly easier to see using this concentration technique.[10]

Recently, other diagnostic modalities including polymerase chain reaction (PCR) – amplifying MO DNA using MO-specific primers, in faecal or isthmus samples, and the Mini-FLOTAC device (FLOTAC Group, University of Naples, 80138 Napoli NA, Italy), which allows visualisation of MO via direct microscopy, have been evaluated.[10,13] The Mini-FLOTAC device was also compared with faecal Gram staining and found to be equally successful in the diagnosis of MO in a variety of species.[13] Cloacal swabs were tested using PCR technology and compared with faecal Gram stain in the diagnosis of MO in a flock of budgerigars. The PCR testing of the cloacal swabs was positive for MO in approximately 50% more birds than identified with the Gram stain,[14] but unfortunately PCR is not available in clinic. Necropsies were not performed on the sampled birds in either study; therefore, the sensitivity and specificity of the PCR, Mini-Flotac and faecal Gram stain diagnostic tests to identify MO in birds remain unknown.

Despite the general availability of in-clinic MO diagnostic tests to the veterinary community, there is no substantial information regarding each tests' sensitivity and specificity. This research investigation evaluated the efficacy of five commonly used in-clinic diagnostic tests for MO and provides recommendations for the most effective clinical diagnostic technique to identify infected avian patients.

Materials and Methods

Source of Samples

Faecal samples were collected from budgerigars maintained at an aviary in New South Wales, Australia, following reports of a mortality event attributed to MO. In the preceding two weeks 14 birds had died. Three out of 14 dead birds were submitted for post-mortem examination with only one having tissues submitted for histopathology. The two birds that were only examined grossly had large numbers of MO identified on direct wet-mount preparations of scrapings obtained from the proventricular isthmus. Histopathology of the remaining bird identified glandular dysplasia with mixed inflammatory cellular infiltration and large numbers of MO deep within the glands of the proventricular isthmus. These findings confirmed the presence of MO at a flock level and confirmed it as a disease-causing agent in at least one of the flock.

Ninety-six faecal samples were collected from 16 infected birds, birds were selected after being found to be positive on direct faecal analysis, none of the infected birds were showing signs of infection. Birds were selected in the order they were caught from both breeding cabinets and flight aviaries, and isolated in a standard budgerigar exhibition cage at the breeder's facility until faeces were passed onto a clean plastic liner within the cage. Birds with MO were identified and grouped together in a 1.2m × 1.0m × 0.6m flight cage to undergo treatment with in-water amphotericin B (AmB) 0.9mg/mL water-soluble powder (Megabac S, Vetafarm Pty Ltd, Wagga Wagga, New South Wales, AUS). Faeces were collected from each bird on day 1, 3, 4, 7, 9 and 10, during the ten-day treatment and then again as a single collection three weeks after the treatment with AmB was discontinued. To collect samples, the birds were housed individually in an exhibition cage to ensure each sample collected was confirmed to belong to the individual bird being tested at that time. The first faecal pellet passed after they were housed alone in the exhibition cage was collected using a cotton tip swab (Swispers, McPherson's Consumer Products, Kingsgrove, New South Wales, AUS).

Detection Techniques

Five microscopically evaluated detection techniques for MO were compared across the same faecal samples in this research study. Direct faecal wet mount preparations were made using a 2mm × 2mm piece of fresh faecal material that was placed on a glass slide with 0.03mL of 0.9% sodium chloride. A glass cover slip was placed over the faeces in the 0.9% sodium chloride and used to gently agitate the sample to facilitate mixing until the faeces were spread across the cover slip. Gram stains were performed on the faecal wet mount preparation samples by removing the cover slip and allowing the slides to air dry, after which the slides were heat-fixed and Gram stained (Australian Biostain Pty Ltd, Victoria, AUS). For the faecal suspension preparations, 0.3mL of 0.9% sodium chloride was placed in a 1.5mL microcentrifuge tube with 0.05g of faeces.[15] Care was taken to avoid the uric acid portion of the droppings when the faeces were collected. The microcentrifuge tube lid was closed, and a slurry formed by shaking the tube manually for 20 seconds. The slurry was placed in an upright position and 0.06mL from the meniscus was removed after three seconds, using a 1mL syringe (Fig 2.1). One 0.03mL aliquot was placed onto a glass slide and covered with a cover slip and microscopically examined without staining. Following assessment of the unstained suspension sample, the cover slip

was removed, and the sample was air-dried, heat-fixed and Gram stained. A second 0.03mL aliquot from the faecal suspension slurry was placed onto a glass slide and 0.01mL of methylene blue was aliquoted onto the sample prior to placement of a glass cover slip and microscopic evaluation. The wet mount samples (faecal wet preparation, faecal suspension preparation, and faecal suspension preparation with methylene blue) were microscopically examined immediately upon collection and the Gram stained samples were reviewed at the conclusion of the sample collection, by the same investigator who was blinded to the results of the previous MO counts from that faecal sample and who was experienced at examining samples for MO.

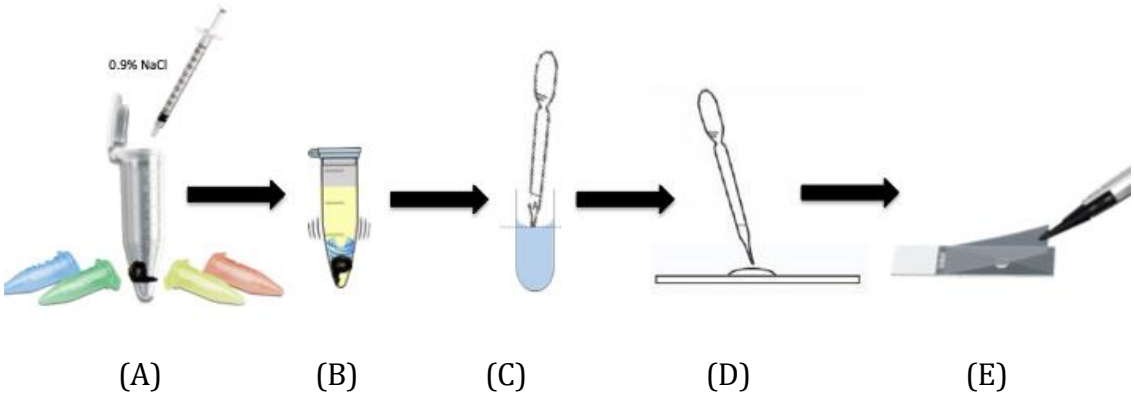

Figure 2.1. A pictorial representation of the faecal suspension technique: A) 0.3mL of 0.9% sodium chloride is placed in a microcentrifuge tube with 0.5g of faeces, care must be taken to avoid contaminating the suspension with the uric acid portion of the droppings. B) The lid is closed, and a slurry formed by shaking the microcentrifuge tube manually for 20 seconds. C) The slurry is placed in an upright position for 3 seconds to allow particulate matter to settle, prior to removing 0.06mL from the meniscus using a 1mL syringe. D) A drop is placed on a microscope slide, and E) covered with a cover slip for analysis.

Each slide was microscopically examined using the same technique, with the stage diaphragm reduced for unstained samples. Two passes of the 40× microscope objective over the coverslip were made, over the first quarter and the last quarter of the coverslip to ensure consistent evaluation of the same area of each slide, the edge of the cover slip was used to guide the examiners field of view. Each MO organism visualised was counted and recorded as a continuous numerical value.

Difficulties interpreting the slides were encountered for any of the diagnostic techniques evaluated when there was uric acid crystal contamination in the faecal sample. When contaminated, the uric acid crystals dominated the microscopic field,

thus reducing one's ability to evaluate the slides. Fresh slide samples that contained uric acid crystals were discarded and a second faecal pellet was immediately collected from the same bird, as previously described, to replace the non-diagnostic sample.

Data Collection and Statistical Analysis

It was not possible to apply standard regression models to the data because measurements from the diagnostic tests conducted on the same bird at the same time were not independent, nor were measurements obtained from the same bird at different points in time. If a bird was shedding many organisms, then all five tests were likely to detect many MO organisms; likewise, a bird shedding many MO will continue shedding high numbers of the organism at the next testing time point, consequently sequential measurements on different days would be correlated.

A generalised linear state-space model was fitted to account for these dependencies, using both fixed effects to explain the expected number of detected MO organisms with different tests, and random effects to explain within-bird variation in shedding across time, for example, due to treatment and underlying bird sickness. Our model assumed that the number of detected organisms by a test was a random variable with a negative binomial distribution, and we related the expected number of organisms detected to diagnostic test type via a log-link function.

For each bird, counts of MO using the five diagnostic tests were taken on selected days. Let y_{ijk} be the number of MO observed for the *i*th bird, on the *j*th day its samples were taken, using the *k*th test. The observed counts exhibit two types of correlation that must be modelled when it comes to statistical analysis. First, counts from the same bird and the same day but using different diagnostics tests are correlated, because both are related to the bird's unknown shedding rate; if the bird is shedding many MO organisms then large numbers are likely to be detected using all diagnostic tests. Second, counts from different samples taken soon after one another, but on different days, may be correlated due to the bird's unknown shedding rate varying slowly over time; a bird shedding many MO organisms on a particular day may still have a high shedding rate the following day.

Our model accounts for both forms of correlation by fitting a state-space model, where the state process represents a latent variable related to the unknown shedding rates of the birds, which we hereafter refer to as a shedding measure. We fit a negative binomial distribution to each observed count, which is a distribution commonly used for count data with high variance like ours. The observed count y_{ijk} has the expectation

$$\lambda_{ijk}=\exp\{\beta_{0k}+s_{ij}\},$$

where s_{ij} is a latent variable related to the shedding measure of the *i*th bird on the *j*th day its samples were taken, and β_{0k} controls the relationship between this shedding measure and the expected number of organisms detected using the *k*th method. A diagnostic test with a large β_{0k} is capable of detecing many organisms, and may therefore be more effective at diagnosing an *M. ornithogaster* infection.

The latent shedding measure of a bird may vary over time due to both treatment and random day-to-day fluctuations caused by other processes, such as bird health and immune response. Our model allows the shedding measure to change over time differently during treatment and non-treatment periods, for example to accommodate a decrease in shedding during treatment but a potential increase upon its cessation. We use

$$s_{ij}=\alpha_1 t_j+u_j$$

for measurements taken during the treatment period, where t_j is the number of days since the beginning of treatment when the *j*th day the bird's samples were taken, and

$$s_{ij}=\alpha_1 t_e+\alpha_2(t_j-t_e)+u_j$$

for measurements following the cessation of treatment, where t_e is the total number of treatment days. The parameters α_1 and α_2 control the per-day change in the bird's shedding measure during treatment and following cessation of treatment, respectively. Finally, u_j accounts for changes in shedding due to other factors, for

example bird health, which we model using a Gaussian process to account for correlation between two counts from samples from the same bird taken close together in time.

Comparing β_{0k} parameters between different diagnostic tests allows us to determine if some tests tend to detect larger numbers of organisms than others. Moreover, our model allows calculation of the probability of successfully diagnosing a *M. ornithogaster* infection given a particular shedding measure and diagnostic test, achieved by calculating the probability of a nonzero count under the fitted negative binomial distribution. To further assess the diagnostic performance of the tests, we calculated the probability of successful diagnosis using the different tests, based on the estimated shedding measures at end-of-treatment and follow up of the birds that remained infected throughout the experiment.

The model was fitted in R using the package Template Model Builder (GitHub Inc., San Francisco, CA, USA).[16] Code used to fit the model is available at https://github.com/b-steve/macrorhabdus, where further technical details about the model can also be found.

Results

Detection Techniques

The faecal suspension technique provided the clearest identification field for visualising MO compared with all other techniques because the preparations contained little background debris. Indeed, this technique consistently visualised more MO organisms when compared with all other techniques (Fig 2.2). When the methylene blue stain was added, many of the MO organisms were found to be associated with small accumulations of debris, making direct visualisation more difficult. When using the direct faecal wet mount and Gram stain techniques, most of the MO organisms were visualised on the periphery of the smear where there was less faecal material. A total of three faecal samples were contaminated with uric acid crystals and required selection of a second fresh faecal sample to replace the non-diagnostic samples.

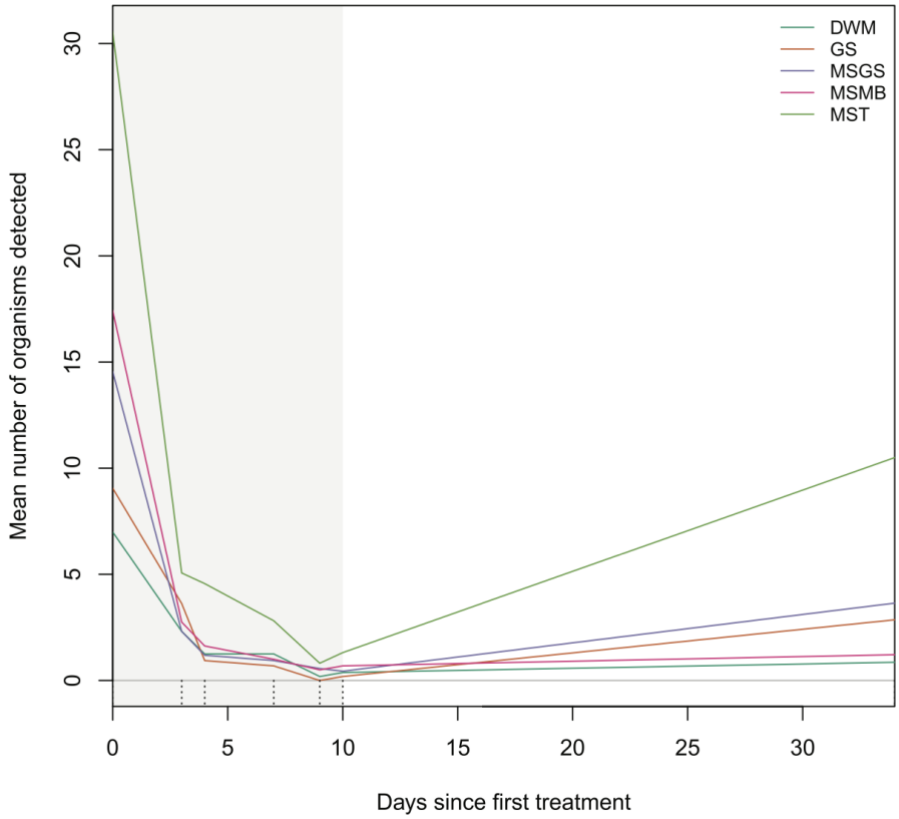

Figure 2.2. The average number of *Macrorhabdus ornithogaster* organisms detected across six persistently positive birds for each day samples were taken with each of the different diagnostic testing methods (direct faecal wet mount [DWM], faecal Gram stain [GS], faecal suspension technique Gram stain [MSGS], faecal suspension new methylene blue stain [MSMB], and faecal suspension technique [MST]) demonstrating that the faecal suspension technique, on average, identified more organisms than any of the other diagnostic techniques. The grey region represents the treatment period, and the vertical dotted lines show the days on which samples were taken between first treatment (day 0) and follow-up (day 34).

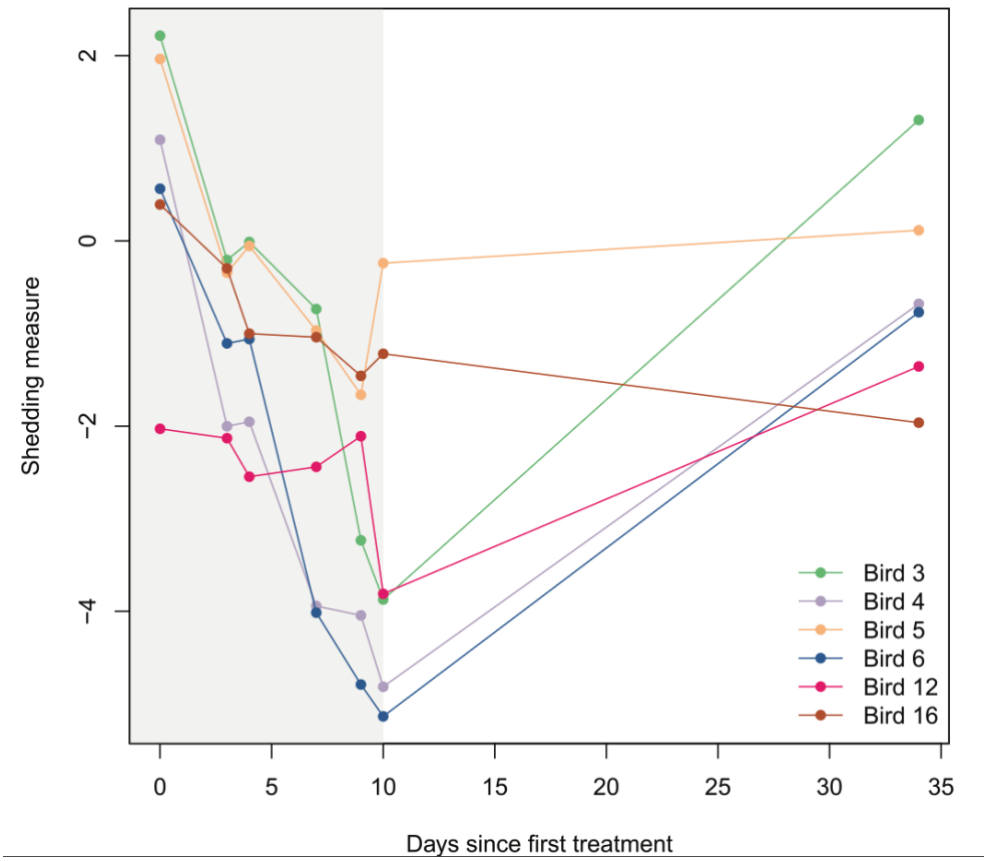

Figure 2.3. Shedding measures based on the faecal suspension technique, estimated by the model for the 6 birds that returned positive tests at the conclusion of the study on days samples were taken. A shedding measure of 0 corresponds to the average shedding rate (number of MO / microscopic slide) across birds at first treatment. The shedding measure can be interpreted multiplicatively following exponentiation; for example, a shedding measure of −0.5 indicates that expected detection counts are exp (−0.5) = 0.61 times as high as that of the average bird at first treatment. Estimated shedding rates for all birds decreased during the treatment period, shown in grey, although birds 5 and 16 were still shedding at moderate rates at end-of-treatment. The remaining 4 birds were shedding at low rates at end-of-treatment but had returned to moderate levels by follow-up.

The estimate of α_1 was negative and statistically significantly different from zero ($p < .001$), confirming that shedding decreased during the treatment period. The change in counts between the end-of-treatment and follow-up varied between birds: Some did not shed any further organisms, whereas others saw a substantial increase in shedding rates (number of MO / microscope slide) (e.g., Fig 2.3).

The expected number of organisms detected was statistically significantly different ($p < .001$) between different diagnostic modalities. In particular, the expected number of organisms detected using the faecal suspension technique was more than that of all other diagnostic tests evaluated: direct faecal wet mount ($p < .001$), faecal

suspension Gram stain ($p < .001$), faecal suspension with methylene blue ($p < .001$), and faecal Gram stain ($p < .001$). In addition, the expected number of organisms detected by faecal suspension with methylene blue was more than that using direct faecal wet mount ($p < .001$) and Gram stain ($p = .006$). All other pairwise comparisons between diagnostic testing methods were not statistically significant ($p > .05$).

Six birds returned positive results based on parallel testing at follow-up, with four estimated to have low shedding rates at the end of treatment, despite remaining infected (Fig 2.3). Consequently, the estimated probabilities of detecting MO organisms were low (<.5) at these shedding rates for all diagnostic tests (Fig 2.4), indicating that false negative diagnoses are likely for some infected birds immediately after treatment. All four birds with low shedding rates at end-of-treatment had substantially higher estimated shedding rates at the end of follow-up (Fig 2.3), with correspondingly higher probabilities of detecting MO organisms, close to 1 for the macro suspension technique (Fig 2.4), indicating that for some birds, diagnosis of infection based on shedding status, is more sensitive at follow-up than at the end of treatment.

The remaining two birds had relatively high estimated shedding measures at the end of treatment, but the shedding rate did not change substantially during the follow-up period (Fig 2.3). Consequently, estimated MO detection probabilities were similar at the two points in time (Fig 2.4).

The faecal suspension technique method had estimated MO detection probabilities that were higher than the other diagnostic tests, and the 95% confidence intervals around the estimates indicate significant differences with faecal Gram stain, faecal suspension Gram stain, and direct faecal wet mount methods for many of the scenarios (bird 3 at end-of treatment, birds 4 and 6 at follow-up, and birds 12 and 16 at both end-of-treatment and follow-up) (Fig 2.4). However, evidence was not found to suggest a difference in detection probabilities between the faecal suspension test and faecal suspension stained with methylene blue or between any other pairwise comparisons.

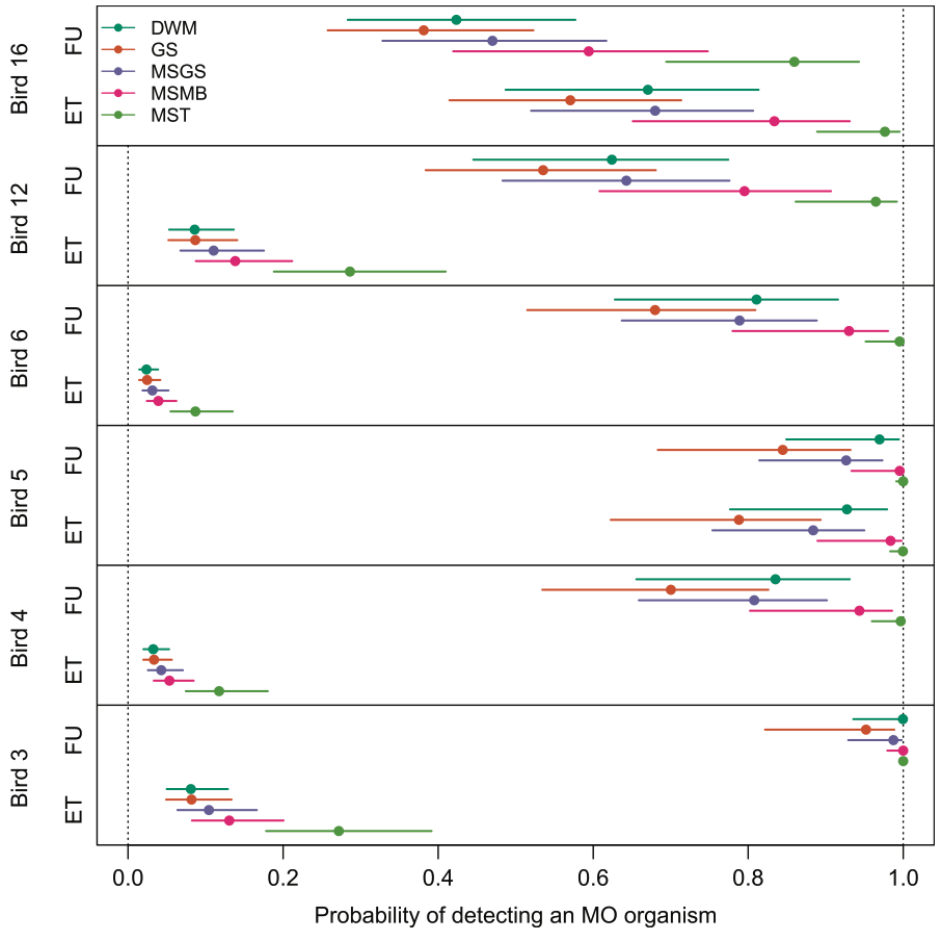

Figure 2.4. Point estimates and 95% confidence limits for the probabilities of detecting at least 1 organism, when shedding measures (shedding measure of 0 corresponds to the average shedding rate) were set at values estimated by the model for the 6 birds that returned positive tests at the conclusion of the study. Birds' shedding measures for both end-of-treatment (ET) and follow-up (FU) were considered. Probabilities are shown separately for direct faecal wet mount (DWM), faecal Gram stain (GS), faecal suspension Gram stain (MSGS), faecal suspension new methylene blue stain (MSMB), and faecal suspension technique (MST). Estimated detection probabilities were highest by MST. Birds 5 and 16 were shedding at high rates at end-of-treatment and thus had high detection probabilities at that stage, whereas the remaining 4 birds' shedding rates were low, making a successful diagnosis unlikely by any method. These 4 birds had higher detection probabilities at follow-up, after their shedding rates had increased.

In some cases, the difference between the faecal suspension technique and other methods had substantial practical significance: at shedding rates like bird 16 at follow-up, for example, the probability of detecting an MO organism was estimated (with 95% confidence limits) to be 0.86 (0.69, 0.94), whereas that for the faecal Gram stain was 0.38 (0.25, 0.52).

Discussion

Macrorhabdus ornithogaster has a worldwide distribution and is a significant pathogen of pet, aviculture, and wild birds. At present, there is no accepted 'best practice' for veterinary clinicians attempting to diagnose MO in avian patients. We have evaluated the most common in-clinic diagnostic tests to establish the most effective technique for identifying MO organisms in infected budgerigars. Of the 5 diagnostic tests evaluated in this study, the faecal suspension technique was found to identify a greater number of MO organisms than the others, and, with stained methylene blue faecal suspension, was the most likely to identify at least 1 organism in the faeces. We therefore recommend using the faecal suspension technique over faecal Gram stain, faecal suspension Gram stain, direct faecal wet mount, and the faecal suspension technique stained with methylene blue.

Not only did the faecal suspension technique consistently identify the highest number of organisms, but they were easily visualised using this technique because there was minimal background interference of digested plant material and other particulate matter. The methylene blue stained the background liquid, but also clearly highlighted the background of bacteria and digested plant material complicating the diagnosis of MO.

The direct faecal wet mount preparation technique is the most commonly used in-clinic diagnostic modality recommended for the visualisation of MO in faeces,[10] but proved less effective in identifying MO organisms. This method consistently recorded lower counts, and more importantly, often missed birds that were found to be positive using the faecal suspension technique. Despite the faecal smear preparation being very thin, the dense consolidation of faecal material and digested plant cell wall, feathers and other ingesta prevented clear visualisation of MO organisms and was likely the reason for fewer organisms being detected using the direct faecal wet mount preparation technique.

The faecal Gram stained samples consistently produced lower MO counts than most of the wet mount techniques. This was despite the fact that faecal Gram stained slides were made from the same faeces and preparations as the wet preparation techniques. The reason for this is unclear but it is postulated that removing the cover slip from the previously examined slide may have also removed some MO organisms held in

suspension and decreased the number of MO on the stained slides. It is also possible that because the birds were undergoing treatment when samples were being collected, the MO organisms may have stained differently or not at all with Gram stain. This is supported by the findings of Gestier,[17] who identified variable staining MO organisms during a treatment trial with amphotericin B, and Phalen,[10] who confirmed the difficulty in variable stain uptake and proposed that the organism sticks poorly to glass slides, something that is partially resolved with heat fixing. It remains poorly understood how the results from these treated birds, will relate to birds that are not undergoing treatment, whether treatment has an effect on the detection of the MO organism in any of the other tests has not been explored.

Our statistical model was used to investigate the probability of each diagnostic test detecting at least one shed MO organism in birds that remained infected throughout the study. The better the diagnostic testing method, the higher the probability of detecting at least one MO organism will be, although detection probabilities for the same test vary depending on the number of organisms a bird is shedding. For example, any diagnostic test is likely to detect MO in birds shedding many organisms (e.g., birds 3 and 5), but unlikely to detect MO in birds that are shedding few organisms (e.g., 4 and 6). However, the differences are more pronounced for birds between these two extremes (i.e., birds 12 and 16 at follow-up). It is important to note that a larger than expected number of organisms detected from one sample does not imply that a diagnostic test is more likely to routinely detect at least one shed organism from an infected bird; the model fitted different mean-to-variance relationships across the different tests, and it is possible for a diagnostic test with a larger expectation to nevertheless have a lower probability of detecting at least one organism if its variance is also larger.

All samples in this study were evaluated by the same observer (HB), who was experienced in the evaluation of each of the diagnostic modalities. Observer experience is important when evaluating samples for the presence of *Macrorhabdus ornithogaster* because the organism can be mistaken for large bacterial rods (false positive) or missed during direct microscopy (false negative). Low number of organisms on a sample may also lead to false negative results if the organism is not observed in the fields that were examined. A limitation of this work was that the fresh samples were required to be evaluated sequentially, meaning that the

observer was not blinded for the testing of the non-Gram stained samples from a single bird, leading to potential interpretation biases that may have influenced the results.

Previous studies evaluating faecal Gram stain and its efficacy compared with a diagnostic test using PCR technology and the Mini-FLOTAC method found that PCR test identified significantly more positive results, while the Mini-FLOTAC method only had a slight increase in the likelihood of positive birds being identified.[13,14] Cloacal swabs tested using PCR technology returned 57% positive results for MO birds in a mixed budgerigar aviary, while the same birds evaluated by faecal Gram stain were found to be 24% positive for the presence of MO, an absolute difference of 33%.[14] In comparison, our study found that 27/81 (33.3%) of the samples tested, which were positive based on the macro suspension technique, were negative using faecal Gram stain, similar to the difference between faecal samples tested for MO using PCR technology and Gram stain.[14]

In conclusion, this study evaluated five different in-clinic diagnostic testing methods to determine which is the most effective in making a diagnosis of MO in budgerigars. We established that the faecal suspension technique is superior in identifying the greatest number of organisms as well as the most effective at isolating organisms if they are present. The results of this study should aid veterinarians in their effort to detect and diagnose MO in budgerigars and may also apply to avian patients of other species. In Chapter 3 we look to better understand the sensitivity and specificity of assays using faeces for the detection of MO in birds known to be infected and compare the success of the faecal suspension technique with diagnostic tests that utilise PCR technology.

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Chapter 3. Comparison of Five Laboratory Diagnostic Modalities for the Detection of *Macrorhabdus ornithogaster* in Budgerigars

Hamish R. Baron*, Ruth N. Zadoks, Ben C. Stevenson and David N. Phalen

Author Attribution Statement For Thesis With Publications

I am writing this letter to specify the role of Hamish Baron in the preparation and submission of the following manuscript (Chapter 3):

The convention for author placement in the list of contributing authors is ordered from highest to lowest contribution. The corresponding author of the manuscript is indicated by an asterix (*). The contribution to the manuscript of all authors is as follows:

Task	**Role of Author**
Conceptualisation	HB, RZ, DP
Investigation	HB, RZ
Methodology	HB, RZ, DP
Data Curation	HB, RZ, BS
Formal Analysis	HB, BS
Resources	HB, RZ, DP
Writing – Original Draft	HB
Writing – Reviewing and editing	HB, RZ, DP, BS

As the primary supervisor for the candidature upon which the thesis is based, I can confirm that the above authorship attributions statements are correct. I have sighted email or other forms of correspondence from all co-authors confirming their certifying authorship and permission for manuscript inclusion in this thesis.

Prof David Phalen

21 June 2023

Abstract

Macrorhabdus ornithogaster (MO) is a pathogenic yeast that causes morbidity and mortality in several bird species. To date, no studies have evaluated the sensitivity and specificity of commonly employed laboratory diagnostic tools for the detection of MO. This study compared five diagnostic modalities for the detection of MO based on scientific and convenience criteria using post-mortem samples from 20 budgerigars that were presented as part of a disease outbreak investigation. Faeces were collected from the colon during necropsy and subsequently tested for the presence of MO by a suspension technique and polymerase chain reaction (PCR). The isthmus of the proventriculus was tested for the presence of MO by direct cytology on scrapings, by PCR, and by histopathological examination of sections of formalin-fixed tissues. *Macrorhabdus ornithogaster* was detected in 55% of the budgerigars by one or more techniques. Relative to detection of MO by any of the modalities used, testing of the isthmus by cytology or PCR was the most sensitive diagnostic test evaluated. Direct scrapings of the isthmus are recommended for the diagnosis of MO at necropsy in avian practice because this test performed as well as PCR in terms of scientific criteria but was superior in terms of the convenience criteria of being affordable, user friendly, with a rapid turnaround time and relatively equipment-free.

Introduction

Macrorhabdus ornithogaster (MO) is a yeast that colonises the mucosa of the isthmus (transition zone) between the proventriculus and ventriculus in infected birds. Infection can be clinical or inapparent and is difficult to diagnose. *Macrorhabdus ornithogaster* affects individual birds but can also occur in flocks or large collections either as a chronic wasting syndrome,[1,2] or as an outbreak and large mortality event.[3,4] Rapid, correct diagnosis allows the initial management and treatment of infected birds to take place and improves welfare and survival outcomes. Aviculturists that seek veterinary attention for individual bird or flock health issues are often constrained by the cost of diagnostic testing, so tests should ideally also be affordable and, from the veterinarian's perspective, user-friendly. Convenience criteria (speed, cost, ease of use), however, need to be balanced against scientific criteria, notably sensitivity and specificity. To date, there has been no peer-

reviewed study comparing the post-mortem diagnostic modalities available to avian clinicians for the diagnosis of MO based on both sets of criteria.[5]

Post-mortem testing allows for use of methods that can be used in live birds, including testing of faeces using microscopic or polymerase chain reaction (PCR)-based methods, as well as for microscopic examination and/or PCR of scrapings of the ventricular isthmus where MO is concentrated. In the faecal suspension technique, the faecal portion of the droppings is suspended in saline and a sample of the supernatant is examined microscopically, making it convenient, rapid, and affordable. It compares favourably with other ante-mortem on-site tests in terms of sensitivity, making it the current recommendation for the point-of-care, ante-mortem diagnosis of MO.[6]

Polymerase chain reaction of the faeces has also been investigated as a diagnostic tool for MO[7,8] and is more sensitive and specific than faecal Gram stain,[8] but has not been compared with the faecal suspension technique.[9] Although potentially superior to faecal suspension based on scientific criteria, PCR has limitations in terms of convenience, as it is more expensive and has a longer turnaround time than microscopic techniques. Because the test is not available commercially in some countries (e.g., Australia), testing currently relies on shipment of samples to an external molecular laboratory, sometimes internationally. Sample degradation during shipment, combined with the challenges of extracting quality DNA from avian faeces,[10] may cause false-negative PCR results, what effect this has on results remains unknown and requires further exploration.

Post-mortem, the diagnosis of MO is traditionally made using histopathology because tissues are received by the pathologist fixed in formalin.[11] The avian clinician can also make a post-mortem diagnosis based on direct microscopic evaluation of mucosal scrapings of the isthmus between the proventriculus and ventriculus during the necropsy. However, because of its large cigar-shape,[12] MO can be confused for long rod-shaped bacteria that are present at the isthmus, leading to false-positive results, which means that the detection of MO microscopically is dependent on the skills and experience of the person performing the cytology. The potential lack of specificity when testing isthmus samples can be overcome by PCR, whereby PCR on isthmus samples may have greater sensitivity than PCR on faecal samples due to the ease of DNA extraction.

Here, we compare five commonly used tests for the diagnosis of MO in post-mortem specimens using both scientific and convenience criteria and make recommendations for their use in avian practice.

Materials and Methods

The 20 budgerigars included in this study were from an aviary in New South Wales, Australia, and were presented for diagnostic necropsy. The aviculturist reported that birds in their collection were experiencing feather loss, feather dystrophy, and weight loss despite a ravenous appetite. In some cases, birds showing signs of disease had died. The submitted birds were euthanised using gaseous carbon dioxide and routinely necropsied using instruments that had been cleaned and soaked in 3% sodium hypochlorite for five minutes and rinsed with sterile water in-between birds to prevent DNA carry-over.

Faeces were collected from the distal 2cm of the colon by milking the contents manually into a 1.5mL micro-Eppendorf tube (Sigma-Aldrich, NSW 2154, Australia). They were then suspended in 0.3mL of 0.9% sodium chloride. The faecal suspension was examined by direct microscopy by a single investigator, experienced in the detection of MO (HB) using the suspension technique.[6] A 0.2mL sample from the meniscus of the same suspension was collected immediately and evaluated via PCR. DNA from the faecal suspension was extracted as per the manufacturer's instructions for tissue using the Qiagen DNeasy Blood and Tissue kit (Qaigen, Germantown MD, 20874, USA), using 100µL for the final resuspension step. Polymerase chain reaction was then performed using 1mL of [0.1µM] MO-specific forward primer AGY1 (GGACTTATATTACTAGTCAGATGG) and 1mL of [0.1µM] of the generic *Saccharomyces* spp. reverse primer, SM4 (CTTCGATCCCCTAACTTTCGTTC), combined with 11.87mL of nuclease-free water, 4mL of 5× buffer and 0.13mL of MyTaq; to this master mix, 2mL of extracted DNA was added.[9] The PCR reactions were carried out using a Palm-Cycler Thermal Cycler (Corbett Research, NSW 2137, Australia). The program included an initial denaturation cycle of 3 minutes at 95°C, followed by 40 cycles whose steps included a 30-second denaturation phase at 95°C, an annealing phase of 53°C for 30 seconds and an extension phase at 70°C for 30 seconds, and finally a 10-minute extension cycle at 70°C. Amplified DNA was separated according to its size, on a 1.5%, agarose gel stained with RedSafe (iNtRON Biotechnology, Gyeonggi-do, 13202, Korea), by 30

minutes of electrophoresis (110 volts). Amplicons were visualised under UV light (BioRad Geldoc; Bio-Rad Laboratories Pty Ltd, NSW 2142, Australia). DNA extracted from a pure culture of MO was used as a positive control in the PCR reactions and nuclease-free water (UltraPure, Thermo Fisher Scientific Australia Pty Ltd, VIC 2179, Australia) was used as the negative control.

The proventriculus and ventriculus were removed from the bird and sectioned longitudinally in a sterile Petri dish (Thermo Fisher Scientific Australia Pty Ltd, VIC 2179, Australia) using a new, sterile surgical blade for each case to prevent carry-over, and samples were prepared for cytology, PCR, and histopathology. For cytology, the mucosa of one half of the isthmus was scraped using a number 15 scalpel blade. The scraping was placed onto a microscope slide and suspended in 0.05mL of 0.9% sodium chloride over which a coverslip was placed. The sample was then examined immediately under direct light microscopy with the stage diaphragm 90% closed to increase contrast. Slides were examined using the 40× objective. The coverslip was divided into four sections (left lateral, left middle, right middle, and right lateral, the lateral portions were examined using two passes or until at least two MO organisms were detected, and the slide was then graded as positive or negative. Identification of at least two organisms confirmed infection status, and helped to decrease the possibility of false positive results given the challenges differentiating MO from the surrounding long bacterial rods present in some samples.

A 3mm × 3mm full-thickness section of isthmus was then removed from the second half of the previously sectioned isthmus, using a new sterile surgical blade for each sample, and placed in a 1.5 Micro-Eppendorf tube. DNA extraction and PCR were carried out using the protocol described above for faeces. The remainder of the proventriculus and ventriculus, including the isthmus, was fixed in 10% neutral-buffered formalin, a longitudinal section was paraffin-embedded, and sectioned at 4mm. One section was stained with haematoxylin and eosin (H&E) and another with periodic acid-Schiff (PAS) stain. One slide with sections was examined by an experienced avian pathologist (DP) for the presence of MO, using the same selection criteria as were applied to the cytological samples above. If the histological section examined did not contain isthmus a second, deeper section of the sample was

stained to ensure a diagnostic sample (i.e., enabling visualisation of the isthmus) was obtained (n = 4 birds). The pathologist was blinded to the other test results.

Statistical Analysis

Birds were defined as positive if they tested positive to any of the diagnostic modalities because the risk of false positive results was deemed minimal due to the specificity of PCR primers, and the expertise and experience of the microscopists. Sensitivity of individual tests was calculated against this reference standard using the formula, sensitivity = true positive / (true positive + false negative) × 100. Confidence intervals for the sensitivities were computed using Wilson's method,[13] and sensitives were calculated as proportions. The sensitivity of the five diagnostic techniques was compared using McNemar's test with the mid-p adjustment.[14] All analyses were carried out using v4.2.2 of the R software environment for statistical computing.[15] All data and code are available at https://github.com/b-steve/macrorhabdus/tree/master/lab-modality-comparison.

Results

Of the 20 birds, 12 (60%) had gross feather abnormalities, including a mixture of missing primary flight and tail feathers, with a range of severities. Two birds had a large spleen, and one bird had a serpentine keel bone consistent with a history of metabolic bone disease. The prevalence of MO infection in this study was 55% (11 of 20) (Table 3.1) based on parallel testing.

Table 3.1. Detection of *Macrorhabdus ornithogaster* infection based on different testing modalities in budgerigars.

Bird ID	Faeces		Isthmus		Histopathology		MO Status
	PCR	Microscopy	PCR	Microscopy	H&E	PAS	
1	–	–	–	–	–	–	–
2	+	+	+	+	+	+	+
3	–	–	+	+	–	–	+
4	+	+	+	+	+	+	+
5	–	–	–	–	–	–	–
6	+	+	+	+	–	–	+
7	–	–	–	–	–	–	–
8	–	–	–	–	–	–	–
9	–	–	+	+	+	+	+
10	+	+	+	+	–	–	+
11	–	+	+	+	+	+	+
12	–	–	–	–	–	–	–
13	–	–	–	–	–	–	–
14	–	–	–	–	–	–	–
15	–	–	+	+	+	+	+
16	–	–	–	–	–	–	–
17	–	–	–	–	–	–	–
18	+	+	+	+	–	–	+
19	+	+	+	+	+	+	+
20	+	+	+	+	–	–	+

Isthmus cytology and PCR were in full agreement and more sensitive (SE = 1.00; 95% CI = 0.74, 1.00) than isthmus histopathology (SE = 0.55; 95% CI = 0.28, 0.79; p = 0.031) or faecal PCR (SE = 0.27; 95% CI = 0.10, 0.57; p = 0.004) whereas sensitivity of the faecal suspension technique was numerically but not significantly lower (SE = 0.73; 95% CI = 0.43, 0.90; p = 0.125). A table of p-values for pairwise comparison is below (Table 3.2).

Table 3.2. p-values for pairwise comparison of diagnostic methods for detection of *Macrorhabdus ornithogaster* from budgerigars postmortem. MST (faecal suspension technique), PVS (proventricular isthmus scraping), PCRT (PCR isthmus), PCRF (PCR faeces), Hist (Histopathology). Significant values (p > .05) are in bold.

	PVF	PCRT	PCRF	HIST
MST	0.125	0.125	**0.031**	0.453
PVS		1.000	**0.004**	**0.031**
PCRT			**0.004**	**0.031**
PCRF				0.289

Discussion

This study evaluated the five post-mortem tools available to avian clinicians to diagnose MO infections and established that isthmus testing by means of cytology or PCR is more sensitive than use of alternative methods or sample types. Based on convenience criteria, cytology is superior to other tests, regardless of sample type, because it is readily available, requires very little equipment, is cheap to perform and has a rapid turnaround time. By combining the scientific criteria and the convenience criteria, we recommend direct isthmus cytology as the most accurate, rapid, and least expensive diagnostic modality for the diagnosis of MO, in necropsy specimens (Table 3.3).

Table 3.3. The diagnostic tools compared in this study, evaluated based on convenience criteria, showing relative strengths and weaknesses of the different diagnostic modalities as judged by the author.

Test parameter	Isthmus			Faeces	
	Cytology	PCR	Histopathology	Cytology	PCR
Affordable	+++	+	+	+++	+
User-friendly	++	+	+	+++	+
Rapid (turnaround time)	+++	+	+	+++	+
Equipment free	++	+	+	++	+
Deliverable	Related to the clinic–laboratory–client interface rather than the test				

Histopathology, traditionally considered the post-mortem diagnostic modality of choice, was less sensitive than other tests using isthmus samples, producing 25% false negative results. This is thought to be because the tissue section selected for histopathology represents only a small portion of the isthmus and may contain only a small number of MO organisms. In a clinical setting, it is also challenging to position the stomach so sections through the ventriculus, proventriculus and isthmus are obtained in the same plane, but this was ruled out as a cause of false-negative results in the current study by evaluating new sections if the originals were incomplete. To increase sensitivity of histopathology, it may be possible to routinely cut serial slices as is also done for avian ganglioneuritis. If information on which portions of the stomach are of interest is provided to the pathologist selecting the tissues and histopathology technician processing the samples, the need to request new sections could be reduced.

For the first time, we have assessed birds with known infection status post-mortem and confirmed that different diagnostic modalities have different sensitivities. This allows a better understanding of the sensitivity of the faecal suspension technique commonly employed in practice in live birds. The results demonstrate that in our laboratory, with experienced investigators, finding MO in faeces from a live budgerigar means that it is infected; however, not finding MO does not mean it is negative. This has implications for the negative predictive value of biosecurity screening, especially if such screening is conducted on individual animals. At flock or aviary level, sensitivity of detection could be improved by testing multiple birds, as is customary in other avian species.[16] The variability in prevalence of MO in budgerigars[2,6,8,11,17,18] makes it difficult to develop standard recommendations for the number of animals to be tested.

Faecal PCR had much lower sensitivity than isthmus PCR. There are various possible explanations for false-negative results using faecal PCR. It has been postulated previously that intermittent shedding of the organism may result in infected birds going undetected. [8,19] Although our results provide robust evidence that infected birds may go undetected with faecal testing, which would be consistent with intermittent shedding, they do not explain negative faecal PCR-results for birds that are positive on faecal microscopy (Table 3.1). Such discrepancies could be due to presence of inhibitors in the avian droppings preventing the amplification of MO

DNA,[20] because of primer selection, storage of the samples between collection and DNA extraction or because of poor MO DNA extraction from the faecal sample, with many commercially available faecal kits extracting little or no DNA from avian faecal samples[10]. In this study, we used the Qiagen DNeasy Blood and Tissue kit, which, whilst not specifically designed for faecal DNA extraction, performed favourably when compared with six other DNA extraction kits using avian faeces.[10]

This study highlights that a scraping of the isthmus is a superior sample compared to faeces when attempting to diagnose MO infection in the dead bird. We established that histopathology and PCR are unnecessary in the post-mortem diagnosis of MO and that direct microscopy, based on the right sample preparation, performs just as well, but gives an answer in real time and at low cost. Our work on budgerigars at post-mortem also has implications for diagnosis of MO in live birds because we have demonstrated conclusively that infection may go undetected when droppings are tested.

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Chapter 4. Treatment Trials and Tribulations Using Amphotericin B for the Treatment of *Macrorhabdus ornithogaster* in Australian Cage-Birds

Hamish R. Baron*, Katerina CL. Leung, Ben C. Stevenson, Mikel S. Gonzalez, and David N. Phalen

Author Attribution Statement For Thesis With Publications

I am writing this letter to specify the role of Hamish Baron in the preparation and submission of the following manuscript (Chapter 4):

The convention for author placement in the list of contributing authors is ordered from highest to lowest contribution. The corresponding author of the manuscript is indicated by an asterix (*). The contribution to the manuscript of all authors is as follows:

Task	**Role of Author**
Conceptualisation	HB, MS, DP
Investigation	HB, KL
Methodology	HB, MS, DP
Data Curation	HB, BS
Formal Analysis	HB, BS
Resources	HB, KL, MS, DP
Writing – Original Draft	HB
Writing – Reviewing and editing	HB, DP, MS

A modified version of Chapter 4 was previously published in the Journal of Medical Mycology and is reproduced here with permission of the publisher.

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As the primary supervisor for the candidature upon which the thesis is based, I can confirm that the above authorship attributions statements are correct. I have sighted email or other forms of correspondence from all co-authors confirming their certifying authorship and permission for manuscript inclusion in this thesis.

Prof David Phalen

21 June 2023

Abstract

Amphotericin B (AmB) is widely used for the treatment of *Macrorhabdus ornithogaster* (MO) infections. To date, however, there have been no randomised controlled trials confirming its efficacy in birds where cure was confirmed by post-mortem examination. To determine the efficacy of AmB against MO, a three-part study was undertaken. Initially, treatment outcomes of MO infected birds that presented to the Sydney School of Veterinary Science over a nine-year period and were treated with AmB were reviewed. This was followed by a pilot treatment trial with two naturally infected birds (*Melopsittacus undulatus* and *Agapornis roseicollis*) administering AmB at 100mg/kg twice daily for 30 days. Finally, a randomised controlled trial was performed using experimentally infected chickens that were treated with AmB at 25mg/kg and 100mg/kg twice daily for 10 days. Nine years of clinical records indicated treatment failure in 80.4% of 36 cases that met the inclusion criteria. The pilot study in naturally infected birds showed that AmB given twice daily for 30 days at 100mg/kg did not clear, but significantly decreased, MO burden, followed by a profound rebound effect of the number of organisms shed in the faeces following treatment cessation. Finally, the randomised controlled trial found that AmB given at 100mg/kg did not clear, but significantly decreased, the burden of MO compared with both the 25mg/kg group ($p = 0.037$) and the no treatment control group ($p = 0.001$), whilst AmB at 25mg/kg twice daily showed no statistical difference to the no-treatment control group. A strong curvilinear correlation between body weight and MO infection burden was present in the infected chickens. These findings represent treatment failure in three scenarios and indicate that the current treatment recommendations using AmB for the treatment of MO require revision.

Keywords: Amphotericin B, *Macrorhabdus ornithogaster*, treatment

Introduction

Macrorhabdus ornithogaster (MO) is an anamorphic (asexual reproductive stage) ascomycete yeast that grows at the isthmus of the ventriculus and proventriculus in birds.[1] Infection may remain subclinical, but is commonly implicated in disease, causing proventriculitis and ventriculitis manifesting as vomiting, diarrhea, chronic wasting disease and death.[2,3] Disease occurs in individual birds and can also cause significant mortality in avicultural collections. Susceptible species include wild birds, pet and aviary birds, chickens, and other birds raised commercially. MO has a global distribution.[3-12]

Filippich first described the use of amphotericin B (AmB) for the treatment of MO infection in 1993,[13] and it has become the mainstay of treatment in individual cage birds.[14] Recommended dosage rates range from 5mg/kg by mouth twice daily to 100mg/kg by mouth twice daily, and recommended durations of treatment range from 10 to 30 days.[13-15] A water-based formula with a dosage rate of 0.9–1.0mg/mL for 10 days is also commercially available and is routinely used to treat individual birds and avicultural collections.[14,15,13,16] Filippich was also the first to report resistance to AmB treatment when administered at a dosage rate of 5mg/kg by gavage for 10 days. Of 30 birds treated for MO, two were treated twice with 5mg/kg PO for 10 days but remained persistently infected. Of the birds initially reported as being treated successfully, at least 5/24 birds were faecal positive for MO by 10 weeks following treatment.[13] It is unclear whether this was a true resistance to treatment, a failure of treatment protocol or drug delivery, or whether the birds became re-infected post treatment. Recently, a report assessing clinical outcomes of budgerigars treated with AmB for macrorhabdosis (the clinical disease caused by MO), found that only 53% of birds treated with 100mg/kg PO BID for 30 days were treated successfully, and of these, 17% re-presented with MO infection within 2.5 years, suggesting that relapse or reinfection and apparent treatment failure also occurs in Germany.[17]

In this study, we investigate the efficacy of AmB for the treatment of macrorhabdosis. Efficacy is assessed through a retrospective case study, treatment trials in a naturally infected budgerigar (*Melopsittacus undulatus*) and peach-faced lovebird (*Agapornis roseicollis*) and in a controlled trial using day-old infected chickens (*Gallus gallus domesticus)*.

Materials and methods

Retrospective Case Series

Case selection and classification

One hundred and forty cases with a diagnosis of macrorhabdosis were retrieved from the medical records from an avian and exotics veterinary hospital in Camden, NSW, Australia, from January 2008 to June 2017 by searching for them using the keywords *Macrorhabdus*, *ornithogaster*, megabacteria, amphotericin and AGY (an abbreviation of avian gastric yeast, used by veterinarians in the clinic). Birds that had a confirmed ante-mortem diagnosis of MO infection based on a positive wet mount or Gram stain received treatment with AmB and had at least one follow-up wet mount, faecal examination or, for deceased birds, ventricular scraping were included in the study. Thirty-six birds met the inclusion criteria and had complete clinical records available for assessment.

Treatment success was defined as consistent improvement or resolution of the patient's pre-treatment signs for more than 14 days after treatment and the absence of MO in the faeces 14 days or more after treatment. Treatment failures were defined as birds that died during the treatment period, birds whose signs did not resolve and birds that were still shedding MO two weeks after treatment ceased.

Data collection and statistical analysis

Data was extracted from the medical records into a secure data portal, for analysis with statistical management software R.[18]

Initial Treatment Trial

A budgerigar and a peach-faced lovebird were surrendered from private breeding facilities after treatment was initially declined on cost-basis. Both birds came from large collections experiencing signs consistent with macrorhabdosis. The lovebird aviary had been treated with AmB at 100mg/kg PO every 12 hours by gavage for 30 days. The lovebird was three years old according to the closed ID band on its leg and was of unknown sex. It was last treated with AmB 90 days earlier. It had a pectoral muscle score of 3/5 with no overt signs of disease. The budgerigar was three years old and was a female based on her closed leg band and the colour of her cere respectively. She had a pectoral muscle score of 2/5 and was lethargic, with a

ravenous appetite and dark faeces, consistent with the signs of macrorhabdosis. Both birds were shedding MO in their faeces based on the faecal suspension technique.

The budgerigar and lovebird were housed separately in standard 60cm x 40cm x 60cm wire flight cages (AviOne, Kongs Australia Pty, Ingleburn, NSW, Australia) under natural day light conditions. The birds were fed a high-quality seed mix appropriate for the species and allowed access to clean water *ad libitum*. All animal care practices and procedures were in accordance with the University of Sydney Ethics Committee approval 2016/1069.

Amphotericin B Preparation

Amphotericin B 1000mg/g powder (PCCA, Houston, Texas, USA) was mixed with lactulose (Dulose, Aspen Australia Ltd, St Leonards NSW, Australia) to make a suspension that had a concentration of 50mg/mL. The suspension was made fresh every third day and stored in the refrigerator at 4°C in a lightproof container between treatments. The birds were treated with AmB solution in lactulose at the dose of 100mg/kg PO BID for 30 days.[14]

Detection and Quantification of M. ornithogaster in Faeces

Faecal samples and body weights were collected twice weekly for two months prior to treatment, during the one-month treatment trial and for one month after treatment. To collect faeces, the birds were weighed and then isolated in an all wire holding cage. The first faecal pellet produced was collected using a cotton tip applicator off a clean plastic tray, with care taken to avoid the urate portion of the faeces, and placed into a conical 1.5 microcentrifuge tube.

The faecal samples were processed within 20 minutes of collection. Samples were thoroughly mixed by agitation for 30 seconds in 0.5mL of sterile 0.9% NaCl (Provet Pty Ltd, Eastern Creek, NSW, Australia). The top 0.03mL of the meniscus was placed onto a glass slide and covered with a cover slip. The slide was examined with direct light microscopy with the stage diaphragm down at 400× magnification, the slide was divided in four as previously described and the two lateral divisions were

examined using two passes across the width of the cover slip, and the number of organisms was recorded from each field.

Blinded Randomised Controlled Treatment Trial

Forty, day-old white leghorn chickens (*Gallus gallus domesticus*) were obtained from a local producer (Red Lea Chickens Pty Ltd, Blacktown, NSW, Australia). Chicks were randomly assigned to four groups of ten individuals using a random numbers table. The chicks were housed in raised, galvanised wire cages (AviOne, Kongs Australia Pty, Ingleburn, NSW, Australia) measuring 77cm × 46cm × 46cm. Chicks were fed a granular chicken starter crumble (Barastoc, Ridley Corporation, Melbourne, VIC, Australia) that did not contain antibiotics. Food and water were provided *ad libitum.* The chicks were maintained at an ambient temperature of 26–28°C and supplementary heat was provided using a 250W ceramic heat globe that remained on 24hr a day. Chicks were humanely euthanised with carbon dioxide at the conclusion of the trial. All animal care practices and procedures were in accordance with the University of Sydney Ethics Committee approval 2016/1069.

Infection Trial

Macrorhabdus ornithogaster was isolated from a peach-faced lovebird (*Agapornis roseicollis*) showing signs suggestive of disease caused by an MO infection and grown *in vitro* as previously described.[19] The MO organisms were centrifuged to create a concentrated pellet. The growth media was decanted, and the remaining pellet was diluted in sterile phosphate buffered saline (PBS) to make a concentration of 10^5 MO/mL.[10] The chicks from the three infection groups (1, 2 and 3) were inoculated with 1mL of the suspension by gavage tube into their crop. The negative control group (group 4) was inoculated with 1mL of phosphate buffered saline (PBS) using the same technique.

The chicks had faeces collected following inoculation, seven days after inoculation, five infected chicks were randomly selected and euthanised, and scrapings were collected from the proventricular isthmus and examined by direct wet mount microscopy to confirm infection. Once infection was confirmed in all the euthanised individuals, treatment of the remaining infected chicks commenced.

Group 1 was treated with AmB at 25mg/kg PO BID, group 2 was treated with AmB at 100mg/kg PO BID, group 3 was administered sterile water at an equivalent

volume to AmB given to groups one and two PO BID and group 4 (uninoculated) was not given either AmB or sterile water.

The chicks were weighed twice daily prior to treatment and dose calculations were performed to adjust dose volume to match growth rate. All treatments were administered directly into the crop by the same investigator twice daily for ten days.

Faecal Preparation

Faecal samples were collected from three birds in each group, twice weekly prior to and during the treatment trial and processed as described in "Detection and Quantification of M. ornithogaster in Faeces" above.

Post-mortem Assessment of Treatment Efficacy

Ten days after treatment commenced, all chicks were euthanised. The proventriculus and ventriculus were removed and opened. The isthmus was divided in half longitudinally; the two sections were placed in 1mL of PBS and formalin (Fronine, Thermo Fisher Scientific Australia Pty Ltd, Riverstone, NSW, Australia) respectively. Within five hours of collection, the PBS suspension was agitated vigorously for 30 seconds and a wet mount of 0.3mL of the PBS solution was drawn from the meniscus and the concentration of the organisms was determined as described for the faecal samples and expressed as the number of MO organisms per high-powered (40x) microscope field (MO/HPF). A total of ten fields were examined from four quadrants of the slide as described previously.

Data Collection and Statistical Analysis

A generalised linear mixed model was used to estimate the effects of treatment group and bird weight on the number of MO organisms per slide, which was modelled as a Poisson random variable. The treatment and weight variables were included as fixed effects. The negative control group was excluded from the analysis as these chickens remained uninfected on cytology and on post-mortem examination. Random effects due to individual birds and slides (because multiple fields were recorded from the same slide, using random fields) were included to account for both between-bird variations in MO infection levels (e.g., due to differences in immune response) and inhomogeneous density of MO organisms across slides respectively. The model was fitted in R using the package TMB.[18,20] A t-test was used to compare the average weight of the birds in the negative control

group to those that were infected with MO. The null hypothesis of no difference in average weight was challenged. All statistical modelling is available at https://github.com/b-steve/macrorhabdus

Results

Retrospective Case Series

A total of 36 birds met the inclusion criteria. Of these, 23 (63.8%) died or were euthanised within three months of diagnosis. From the time of diagnosis, the mean survival time was 22 days with a median survival time of 14 days. Of the birds that died, eight had specific morbidities occurring in conjunction with macrorhabdosis mentioned in their history, including primary gastrointestinal disease (e.g., melena, enteritis, trichomoniasis) and other infectious diseases (e.g., *Cnemidocoptes* sp. and ataxia); one of the birds had moderate anaemia (14%) on presentation and was treated but subsequently died and was found to have a myxosarcoma of the ventricular wall with no evidence of MO following treatment. Of the birds that survived, seven (19.4%) remained positive following treatment with a median follow-up of 21 days and a mean of 53.9 days (range 14 – 130 days). There were six (19.6%) treatment successes, with negative faecal cytology results following treatment with a median follow-up of 20 days and a mean of 20.5 days (range 14 – 60 days). Treatment success was defined as negative faecal cytology based on our previous work. The birds that met the inclusion criteria are represented in Table 4.1.

Table 4.1. Species representation of birds that underwent treatment for *Macrorhabdus ornithogaster* with amphotericin B, highlighting the sex ratio and the clinical outcomes following treatment.

Species	Number	Male	Female	Unknown	MO Cytology Positive	MO Cytology Negative	Died
Melopsittacus undulatus	22	10	7	4	5	3	14
Nymphicus hollandicus	3	1	0	2	0	1	2
Eolophus roseicapilla	2	0	0	2	0	0	2
Agapornis spp.	7	0	0	7	2	2	3

Alisterus scapularis	1	0	0	1	0	0	1
Polytelis alexandrae	1	0	0	1	1	0	0

All birds were treated with AmB at doses ranging from 5mg/kg to 100mg/kg PO BID. The following doses were used; 5mg/kg (1 bird), 25mg/kg (26 birds), 50mg/kg (2 birds) and 100mg/kg (5 birds). Three birds were treated in the water at 1mg/mL due to the owners' inability to medicate the bird. The treatment period varied from 1 day (those that died shortly after treatment) to 30 days. Results are summarised in Table 4.2. The variety of doses presented in table two highlight the change in dose rate over time, in 2008 a dose of 5mg/kg PO BID was used (like the initial drug trials by Filippich in 1992),[13] during the ensuing 5 years 25mg/kg PO BID was the mainstay of treatment and in the last two years, the dosage regime was consistently 100mg/kg PO BID.

Table 4.2. The variety of dose rates used in the retrospective case series and their corresponding treatment successes when using amphotericin B to treat *Macrorhabdus ornithogaster*.

Dose Rate	Number of Birds	Treatment Success	Percentage of Treatment Success (%)
5mg/kg	1	0	0/1 (0.00)
25mg/kg	25	4	4/25 (16.00)
50mg/kg	2	0	0/2 (0.00)
100mg/kg	5	1	1/5 (20.00)
1mg/mL in water	3	1	1/3 (33.33)
Total	36	6	6/36 (16.67)

Two models were fitted to compare the treatment groups: (1) a logistic regression model was fitted to test the null hypothesis that there was no difference in treatment success probabilities, and (2) a parametric survival analysis regression model was fitted to test the null hypothesis that there was no difference in mean survival times. Neither null hypothesis was rejected, with p-values of 0.82 and 0.33, respectively.

Pilot Case Study

Treatment decreased the number of MO shed in the faeces in both birds, and both birds had screening days during treatment when no MO were observed in their

faeces (Figure 4.1). By day three follwing the cessation of treatment both birds experienced a rebound in faecal shedding and began shedding higher numbers of MO than before treatment. In the month following treatment the infected lovebird lost weight, displayed a ravenous appetite, and became progressively lethargic and was humanely euthanised one month after the completion of treatment. Both birds had post-mortem examinations performed and MO was identified at the isthmus of the proventriculus and ventriculus, in both birds gastric ulceration was present on the luminal surface of the proventriculus. No other abnormalities were detected in either bird.

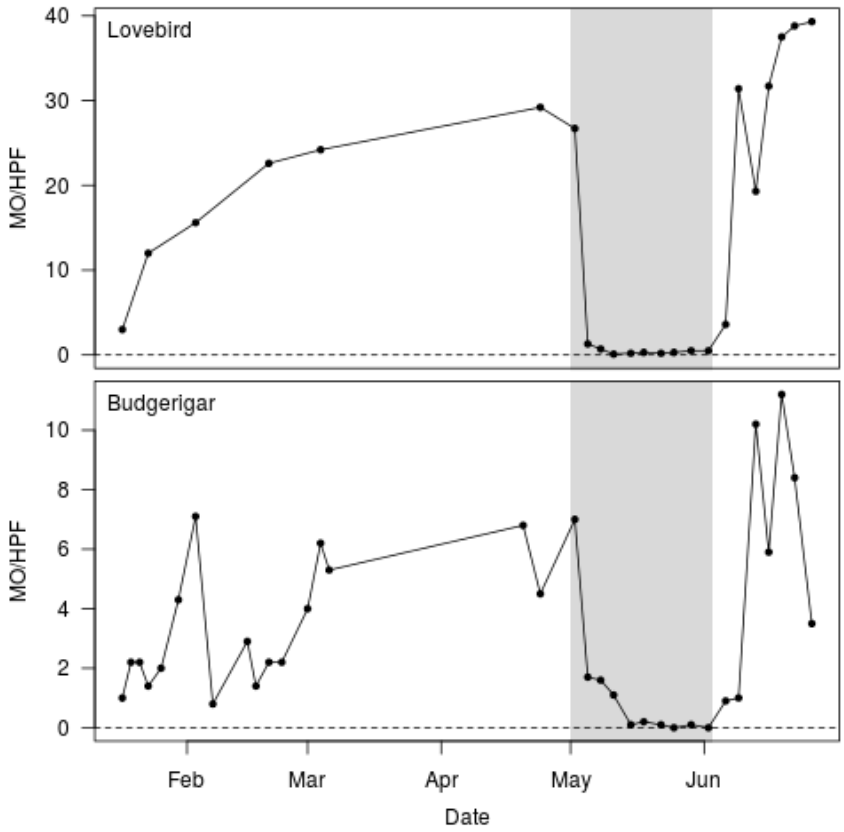

Figure 4.1. Budgerigar and lovebird *Macrorhabdus ornithogaster* per high-powered field in faeces measured using the faecal suspension technique over a five-month period. The shaded area represents the treatment period when amphotericin B was administered twice daily at 100mg/kg by gavage tube.

Infection Trial

Seven days after inoculation, the five birds (two from group 2 and three from group 3) that were euthanised had positive isthmus scrapes for MO. The remaining birds completed the treatment trial; of these, three birds from the 100mg/kg AmB group

were negative for MO on isthmus cytology. The remaining birds in the three infected groups (seven birds in group 1, eight birds in group 2 and seven birds in group 3) were positive at the completion of the treatment period, while the negative control group remained negative. The average MO/HPF and average weight in each group are shown in Table 4.3.

Table 4.3. Four treatment groups demonstrating the average *Macrorhabdus ornithogaster* / high powered field (MO/HPF) and average bodyweight, with the body weight range in each group in grams.

Treatment Group	MO/HPF	Average weight (g)	Range (g)
1 – Amphotericin B 25mg/kg PO BID	1.15	457.12	393–572
2 – Amphotericin B 100mg/kg PO BID	0.78	492.10	378–543
3 – Sterile water (equivalent volume) PO BID	4.4	506.14	463–550
4 – Negative Control	0	538.2	345–624

Likelihood-ratio tests provided strong evidence of differences between treatment groups ($p = 0.004$) and for a quadratic weight effect in the linear predictor ($p = 0.016$). These effects are shown in Figure 4.2.

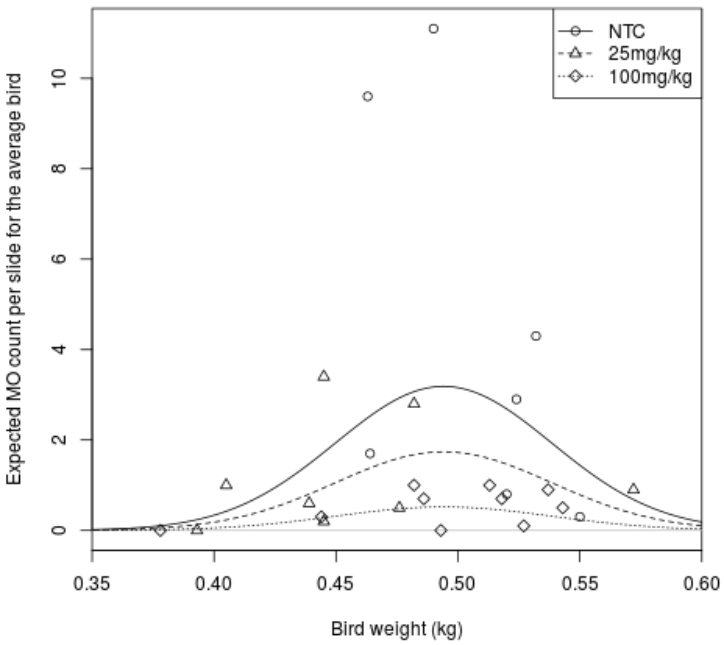

Figure 4.2. Quadratic effect highlighting the finding that regardless of group, chickens, experimentally infected with *Macrorhabdus ornithogaster*, have the largest number of *Macrorhabdus*

ornithogaster organisms per slide when they are of average weight (495g) compared with the cohort. (p = 0.016). Ten birds from Group 1, eight birds from Group 2 and seven birds from Group 3 were included in the analysis.

The number of MO organisms / HPF in the 100mg/kg group was significantly different from both the 25mg/kg group (p = 0.037) and the no-treatment control group (p = 0.001). In comparison to the 100mg/kg group, the average bird in the control and 25mg/kg groups were estimated to have 6.1 and 3.3 times the number of MO organisms per slide respectively. These estimates had corresponding 95% confidence intervals of (2.2, 17.6) and (1.1, 11.6). The difference between the control and the 25mg/kg group was not statistically significant.

The final model included a quadratic effect, whereby birds of approximately 495g were estimated to have the largest number of MO organisms per slide, on average (Figure 4.2). The coefficient of the quadratic term was strongly significant (p = 0.016), providing support for this model over one with a linear relationship. The quadratic model was also preferred by the Akaike information criterion (AIC).

The faecal samples that were collected from all birds on day 0 (prior to inoculation) were negative for MO. From day 2, all samples collected from inoculated groups contained >10 organisms/HPF, confirming active infection and shedding of the organism. During the treatment trial, shedding results varied between groups, with three birds in the 100mg/kg group having samples that returned <1 organism/HPF and two birds that returned negative faecal cytology results on day eight of treatment. Because samples were collected randomly from different chicks during the treatment trials, further statistical analysis at an individual animal level was not carried out, but is explored further in Chapter 5.

A t-test was used to compare the average weight of the birds in the negative control group to those that were infected with MO. The null hypothesis of no difference in average weight was rejected (p = 0.022); uninfected birds were estimated to be 53g heavier than infected birds, with a 95% confidence interval of (8, 99) g.

Additional Findings

This is the second study to report the impact of MO infection on chickens infected at one day of age. In the first study, MO infection had a significant impact on food conversion ratios and infected birds did not gain weight as fast as control birds, results supported in part by this study, which found a significant difference in weight between the infected birds and the non-infected control group.[10] In our study, a non-linear relationship was observed between weight gain and the number of MO observed cytologically at the isthmus, post-mortem. This indicates that chicks with the heaviest burden were in the middle of the average weight range. This curvilinear weight relationship has not been previously described. Chicks with low and high body weights were likely to be infected with low numbers of MO/HPF, while the chicks with average body weights were likely to have higher numbers of MO/HPF. The chicks with the heaviest burden were those nearest the weight 495g. The reason for this relationship is unclear but may be related to the median weight range chicks having the most optimal growth environment for MO or the smaller chicks, with lower burdens, being bullied away from food by those chicks with a higher MO burden and a more ravenous appetite. The heavier chicks may have begun to mount an immune response and were therefore harbouring fewer organisms. Because birds clinically affected by macrorhabdosis often present emaciated and with a ravenous appetite, the curvilinear nature of the weight to MO burden requires further investigation.

Discussion

Macrorhabdus ornithogaster is a significant pathogen of avicultural and wild birds worldwide. Amphotericin B is the treatment that is most recommended for MO infection. However, since Filippich first demonstrated AmB's efficacy against MO there has been only one additional study on its clinical efficacy.[13,17] In the present study, we assessed the efficacy of AmB for the treatment of MO by reviewing the outcomes of clinical cases of MO, performing controlled treatment trials in two naturally infected birds, and experimentally infected chickens.

The retrospective study of client-owned birds diagnosed with MO infection treated with AmB demonstrated that AmB treatment was largely unsuccessful, curing only 6/36 (17%) of the treated birds. The cure rate may have been even lower, as

repeated faecal examinations at the end of treatment were not always performed and some infections may have relapsed, as seen in the budgerigar and lovebird in this study and in a recent study where a re-presentation rate of 17% of the treated budgerigars occurred.[17] The cure rate may also have been higher, as not all birds were confirmed to be persistently infected with MO: clinical signs could be attributed to another cause (i.e. not definitively ruled out) or death could have occurred due to an unrelated pathology.

The success of AmB treatment in the current study (17%) is considerably lower than the efficacy demonstrated by the study performed in Germany, where 36% of budgerigars infected with MO recovered from the infection with AmB treatment at 100mg/kg PO BID for 30 days.[17] Many of the birds treated in our retrospective cohort received a lower dose of AmB and it is likely that the differences in the response to therapy are a result of the different dose regime. The route of administration as well as duration of therapy may have negatively impacted the treatment success.

Possible causes for treatment failure in these client-owned birds could include one or more of the following: reinfection, infection with AmB resistant MO strains, problems with owner compliance or failure to obtain therapeutic concentrations of active AmB. Failure to obtain therapeutic concentrations at the site of infection may be due to AmB's solubility in water. Amphotericin B is reported as being highly hydrophobic, making it insoluble in water at pH 6–7 and with a bioavailability of 0.1mg/mL at pH 2 and 11.[21] It is probable that poor water solubility when in suspension contributed to these treatment failures. Various modifiers and suspensions have been trialed using lipid emulsification and solubilising agents to increase bioavailability of AmB in oral formulations, which may lead to better treatment efficacy, and these warrant future investigation in avian patients.[21,22,23]

To rule out the variables of owner compliance, route of administration and dosage rate, a preliminary treatment trial of a naturally infected budgerigar and lovebird was undertaken. While shedding stopped during treatment, it rebounded immediately after treatment was stopped, strongly suggesting that the infection was not completely cleared, even when given at the highest recommended dosage rate (100mg/kg, every 12 hours) for the longest recommended duration of treatment (30 days). This is very likely because of the nature of the growth environment of MO.

The deep glandular crypts of the proventriculus and isthmus often harbour organisms that are stacked atop one another in multiple rows.[14] These crypts likely prevent some of the MO from contacting the AmB, and the rapid transit time of the GIT, in combination with the 12hrs between oral dosing, means that penetration of the AmB and opportunity to cause cell death is minimal. Based on the profound rebound in the MO numbers, it seems likely, that AmB is effective at killing the MO in the lumen of the GIT, which explains the lack of organisms being shed in the droppings. However, the convoluted nature of the isthmus surface could prevent killing of all MO during treatment, resulting in a treatment that is effective to suppress shedding, but inadequate to clear the infection completely. This highlights the importance of repeat faecal testing at least three days after the cessation of treatment to determine if the treatment was effective rather than assuming that the cessation of shedding during treatment represents a cure. Why such a marked increase in shedding, and presumed regrowth in the isthmus, occurred in the budgerigar and lovebird treated in this study following the cessation of treatment is not known. In addition, this rebound was seen in these two birds, but further investigation in a larger cohort is required to evaluate the time that shedding reoccurs and any relationship that is present between recurrence and the severity of infection.

To better understand the treatment success of AmB against MO, a randomised controlled trial using experimentally infected chickens was undertaken using an isolate from a lovebird flock that had failed to respond to treatment. The results of this trial showed that treatment with AmB at 25mg/kg every 12 hours for 10 days, was ineffective based on its failure to cure any of the birds or to significantly reduce concentrations of the organism in the isthmus as compared to the infected untreated control birds. Treatment with AmB every 12 hours at 100mg/kg for 10 days had a negative impact on the MO, reducing its concentration at the isthmus, but only cleared infection in three of 10 birds. The rapid growth rate and heavy body weight made treatment for longer than 10 days cost-prohibitive (daily cost of the solution/day was approximately AU$100 for all chickens). Importantly, these results in chickens may not necessarily reflect the results in other bird species, considering the anatomical and physiological differences between these species.

Combined, the three phases of this investigation show that treating birds with MO infection directly with oral AmB, even at the maximum recommended dose rates and strict adherence to dosage regimes, does not result in the definitive cure of infected birds in most cases. Based on the controlled treatment trials in chickens, it appears as though a dose-response relationship exists that supports susceptibility to treatment: more drug results in a better effect. There are multiple other possible explanations for poor treatment success, including a short duration of therapy, the rapid transit time of the avian GIT resulting in short contact time at the isthmus (regardless of species), the hydrophobic nature and available formulation of AmB or the protection afforded by the crypts of the isthmus where MO thrives. Lastly, there is the possibility that MO has developed some resistance to AmB, given it has been the mainstay of treatment in cage birds for the last 30 years.

There is limited information about the pharmacokinetics, dynamics, or efficacy of AmB in different bird species. Amphotericin B is a fungicidal heptaene macrolide antimycotic that exerts a powerful and broad activity against a vast array of fungi and has a remarkably low rate of microbial resistance.[24] Resistance is reported most in *Aspergillus terreus* and *Candida* spp. Despite extensive research, the mechanism for resistance remains poorly understood.[25,26] Amphotericin B-ergosterol binding was found to play a minor role in intrinsic AmB resistance by forming aqueous pores in the lipid bi-layer of yeast or fungi, causing leakage of proteins and amino acids and disrupting membrane proton gradients.[25] Researchers examining *A. terreus* strains resistant to AmB found that the oxidative stress response plays a major role in modulating resistance. Resistant strains possessed an almost doubled basal superoxide dismutase activity when compared with susceptible strains, as well as exhibiting enhanced oxidative stress response when treated with AmB. This work concluded that superoxide dismutase activity and oxidative stress response are crucial in the resistance of *A. terreus* to AmB.[27] Appendix 1 outlines a proposed *in vitro* susceptibility testing protocol that will help to standardise sensitivity testing in MO and provide a framework for future investigators.

If the current protocols for treating MO with AmB prove to be ineffective globally, then avian veterinarians may be left with no proven effective and safe treatments for MO infection. All other potential therapeutic options have been shown to be ineffective, potentially toxic or have unknown efficacy. Fluconazole is effective at

treating chickens experimentally infected with MO when administered at 100mg/kg, but this dosage rate was toxic in budgerigars and lower dosage rates in budgerigars were not effective.[14] An *in vitro* investigation, where growing MO was exposed to constant concentrations of sodium benzoate, potassium benzoate and potassium sorbate, showed that all of these chemicals could inhibit their growth.[28] Flock treatment using sodium benzoate was reported to be effective in treating a budgerigar aviary with MO however, the authors have seen both sodium toxicity in budgerigars and lovebirds treated with sodium benzoate and potassium toxicity in lovebirds treated with potassium benzoate (Baron and Phalen, unpublished 2015), indicating that if these chemicals are to be used they need to be used cautiously. The potential efficacy of nystatin against MO is not known. *In vitro*, under conditions of constant contact, nystatin at a concentration of 10 units per mL, inhibited growth of MO[28] and there is a report in the literature of treatment using nystatin resulting in the cessation of MO shedding in budgerigars at a dose of 3,500,000IU/mL for two days, followed by 2,000,000IU/mL for 28 days.[29] Filippich, however, was unable to stop MO shedding with nystatin in budgerigars.[13] The combination of treatment with nystatin and AmB is a novel concept that may have a synergistic effect and warrants further investigation.

A potentially novel approach to treating MO infections involves concurrent oral treatment with pro-oxidants, such as L-ascorbic acid, and AmB. *In vitro* studies using L-ascorbic acid resulted in an increased radical oxygen species generation and enhanced AmB susceptibility in resistant strains of *A. terreus*, *Candida albicans* and *Aspergillus flavus* at classically therapeutic doses. All investigated strains displayed an *in vitro* increase of AmB susceptibility with increasing L-ascorbic acid concentrations.[24] These same results were mirrored when assessed using an insect model *in vivo*, where L-ascorbic acid in combination with AmB significantly improved the survival of larvae infected with resistant *A. terreus* compared with AmB treatment alone.[24]

Amphotericin B is the recommended treatment for cage birds with macrorhabdosis and, despite reports of treatment failure anecdotally, this report is the first to combine broad treatment failure in a retrospective case series analysis, case report and blinded, randomised controlled trial. We present AmB treatment failure in three scenarios, despite being administered orally at the accepted dose and duration. This

report highlights the need for further investigation into effective treatment modalities, duration of treatment, other dose regimes, other formulations, strain specific resistance to AmB and the importance of complete, consistent cessation of faecal shedding before withdrawal of treatment for MO. A water-soluble formulation of AmB is commercially available in Australia and is commonly used by aviculturists for the treatment of MO in large budgerigar collections. Our work demonstrates a need to investigate whether this AmB formulation is effective in eliminating MO infection, or whether suppression during treatment, like was seen here, is the result. Further *in vitro* susceptibility testing assessing treatment success outside the host and larger-scale naturally infected controlled trials with treatment success confirmed by necropsy are warranted and a push for novel, successful and cost-effective treatment modalities is required to better treat and eliminate MO from infected birds.

In conclusion, our work suggests that in at least one geographic area of Australia, treatment with AmB for 10 days, even at the highest recommended dosage rate reported in the literature, has limited efficacy. This indicates an urgent need to determine if this is a pattern that is being seen in other countries and to develop alternative treatment protocols.

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Conflict of interest

The authors declare no conflicts of interest.

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Chapter 5. Inconsistent Efficacy of Water Soluble Amphotericin B for the Treatment of *Macrorhabdus ornithogaster* in a Budgerigar (*Melopsittacus undulatus*) Aviary

Hamish R. Baron, Ben C. Stevenson, and David N. Phalen

Author Attribution Statement For Thesis With Publications

I am writing this letter to specify the role of Hamish Baron in the preparation and submission of the following manuscript (Chapter 5):

The convention for author placement in the list of contributing authors is ordered from highest to lowest contribution. The corresponding author of the manuscript is indicated by an asterix (*). The contribution to the manuscript of all authors is as follows:

Task	**Role of Author**
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Investigation	HB
Methodology	HB, DP
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As the primary supervisor for the candidature upon which the thesis is based, I can confirm that the above authorship attributions statements are correct. I have sighted email or other forms of correspondence from all co-authors confirming their certifying authorship and permission for manuscript inclusion in this thesis.

Prof David Phalen

21 June 2023

Abstract

Amphotericin B is widely used for the treatment of *Macrorhabdus ornithogaster* infections. Recent work has identified treatment failure using amphotericin B in Australian and German cage birds. A water-soluble formulation of amphotericin B is commercially available and commonly used for the treatment of *M. ornithogaster*. The formulation's efficacy has not been evaluated since it was originally developed in 1998. To determine the efficacy of the commercially available, water-soluble amphotericin B against *M. ornithogaster*, faecal shedding of 16 naturally infected budgerigars (*Melopsittacus undulatus*) was monitored while being treated using in-water amphotericin B as per the manufacturer's instructions for 10 days. At the conclusion of treatment, 11 birds had stopped shedding *M. ornithogaster* and five birds were still shedding. All birds were then housed individually. Treatment was continued for an additional 10 days for the five shedding birds. All birds were rechecked 16 days after the end of the second treatment period. At this time, four birds that were negative after 10 days of treatment were shedding again and two of the birds that were treated for 20 days were shedding. Additionally, one bird from each treatment group died after treatment and before follow-up testing. These findings represent a 36% treatment failure suggesting that treatment with the commercially available, water-soluble amphotericin B for a ten day treatment course as recommended by the manufacturer, has inconsistent efficacy against *M. ornithogaster* in some budgerigars in Australia and would not be effective for eliminating it from budgerigar aviaries.

Keywords: Amphotericin B, efficacy, *Macrorhabdus ornithogaster*, treatment, Macrorhabdus

Introduction

Macrorhabdus ornithogaster (MO) is an anamorphic ascomycete yeast that grows mostly at the isthmus of the ventriculus and proventriculus in birds.[1] Infection can occur with or without clinical signs, but MO is commonly implicated in causing significant disease characterised by vomiting, diarrhoea, chronic wasting disease and death.[2,3] Because of its impact on avicultural collections and individual birds, there is a need for effective treatment protocols.

Filippich first described the use of amphotericin B (AmB) for the treatment of MO infection in 1993.[4] Since then, it has become the mainstay of treatment in individual cage birds and collections of birds. There is a myriad of formulations available, including an oral formulation in development for human consumption,[5-10] water-soluble powders and human oral lozenges. Recommended oral doses range from 5mg/kg by mouth twice daily to 100mg/kg by mouth twice daily, and recommended durations of treatment range from 10 to 30 days.[4,6,11] The water-based formula has a dosage rate of 0.1mg/mL as the only source of drinking water for 10 days and is commercially available (Amphotericin 2.5% powder, Vetafarm Australia Pty Ltd, Wagga Wagga 2650, NSW) and routinely used to treat individual birds and aviculture collections.[6,11,12] This product has formally been marketed under the trade name of Megabac-S, referring to the name 'Megabacteria' that has been used erroneously for this yeast as described in Chapter 1.

Amphotericin B is used as therapy for systemic fungal infections in other host species;[12] it binds to the ergosterol in the cell membranes of fungi, disrupting cell membrane integrity[13] via the formation of pores, which upsets membrane permeability leading to fungal cell death.[14] In many currently available formulations, the poor systemic absorption of AmB limits its application as an oral formulation for treatment of systemic fungal infections. However, the poor systemic absorption makes AmB a good option for infections in the GI tract with limited risk of side effects due to this feature. Amphotericin B has a highly hydrophobic region causing the formation of micelles in an aqueous environment, resulting in very poor oral bioavailability.[15,16]

In 1998, Gestier reported on the efficacy of a novel, water-soluble AmB formulation (Megabac-S) to treat MO in 10 naturally-infected budgerigars when administered in the drinking water.[17] The amphotericin was administered at a dosage rate of 0.9mg/mL of drinking water changed fresh daily for 10 days and all 10 birds studied remained negative on faecal Gram stain for at least 10 days following treatment.[17] Another in-water suspension was used recently and was found to be effective in 56% of cases, with budgerigars and cockatiels (*Nymphicus hollandicus*) testing negative to MO in faeces via polymerase chain reaction (PCR) after 28 days of treatment.[18] Megabac-S is a water-soluble AmB powder manufactured for the treatment of MO in cage birds.[17] The manufacturer instructions result in a solution

that is 0.1mg/mL, nine times less than the 0.9mg/mL solution administered by Gestier in the original studies. Since this water-soluble product became available, it has been used by veterinary practitioners to treat individual birds and collections of aviary birds, but has also been used, at times indiscriminately, by aviculturists without veterinary guidance. Widespread and inappropriate use raises the possibility that MO may be developing resistance to AmB.

Filippich was the first to report treatment failure using AmB administered at a dosage rate of 5mg/kg by gavage for 10 days.[4] Two further studies have evaluated treatment outcomes and revealed high rates of treatment failure in cage birds; the first, in Germany, found that only 53% of birds treated with 100mg/kg PO BID for 30 days were treated successfully, and of these 17% re-presented with MO infection within 2.5 years.[19] Recently, in Australia, a retrospective analysis found that treatment with AmB using variable treatment protocols, was only successful in 17% of cases. In the same study, treatment with 100mg/kg PO BID for 10 days in experimentally infected chickens resulted in a significant decrease in number, but not elimination of MO as determined by post-mortem examination.[20]

In this study, we investigate the efficacy of water soluble AmB for the treatment of MO in a budgerigar aviary experiencing deaths attributed to MO. Because of the study setting, efficacy could not be determined based on post-mortem examination, but was assessed by monitoring faecal shedding.

Materials and methods

Faecal samples were collected from an exhibition budgerigar aviary in New South Wales, Australia following reports of a mortality event attributed MO (See also Chapter 2). The aviary contained 450 budgerigars ranging in age from newborn to six years old. In the two weeks prior to testing, 14 deaths had occurred and post-mortem examinations in three birds revealed large numbers of MO on direct wet-preparations of the proventricular isthmus. Histopathology of two of the dead birds identified glandular dysplasia with mixed inflammatory cellular infiltration with a massive number of MO deep within the glands of the proventriculus.

Prior to seeking veterinary assistance, the budgerigar fancier had administered various treatments including in-water trimethoprim-sulphonamide (Sulpha AVS, Carlingford Veterinary Hospital, NSW 2118, Australia), a five-in-one in water

medication containing amprolium, ronidazole, tylosine, levamisole and probiotics (Vita King 5 in 1, Vita King Inc, Granger IN 46530, USA) and an iodophore-based germicide (Sani-Chick, Ruakura Pty Ltd, Narellan 2567, NSW) in water at unknown dose rates. These treatments did not appear to change mortality rates.

Bird Selection, Treatment and Assessment of Faecal Shedding

Faeces were collected from 54 birds as part of an initial diagnostic investigation at the aviary; 16 were found to be shedding MO and were included in the treatment evaluation described here, as well as in the diagnostic evaluation described in Chapter 2. The first faecal pellet was collected from the floor of a standard exhibition cage after the bird was isolated in there alone. The samples processed using the faecal suspension technique and evaluated by the same investigator (HB) who was experienced in identification of MO using direct microscopy.

Initial AmB treatment was for 10 days. Birds had faeces collected and were evaluated on day 10 and 12 following treatment. Birds that were still shedding MO based on the faecal suspension technique after 10 days, were treated for an additional 10 days using the same dose regime. During the first 10 days of treatment the birds were housed together in a single flight cage; no other birds were housed with the treatment group. Amphotericin B powder was added to the drinking water at a rate of 1200mg of 20g/kg AmB powder to 240mL of drinking water to make a solution of 0.1mg/mL, as per manufacturer's instructions. The unmixed powder was kept at 4°C (39°F) between treatments. Water was administered in standup canister drinkers with a narrow trough for the birds to allow drinking, but prevent bathing. Each day, the volume of medicated water consumed was determined by the change of weight in the water source. The same style of drinker was filled with medicated water and attached to an adjacent empty cage and the volume of water lost was measured during the same period to act as a control for evaporation and other losses.

At the conclusion of treatment, all birds were housed individually in 0.6m × 0.4m × 0.6m flight cages and samples were collected 26 days after the cessation of the first treatment period (16 days after a second treatment period ended). Treatment success was defined as a cessation of faecal shedding as inferred from failure to detect MO organisms visible in two consecutive faecal samples.

Data Collection and Statistical Analysis

Generalised linear mixed-effects models were fitted to the MO shedding data. The full model (available at https://github.com/b-steve/macrorhabdus) estimated the following effects on the expected number of MO organisms detected; an effect of time on treatment, to investigate whether MO shedding decreased during the treatment period; an effect of bird weight at the beginning of treatment, to investigate the relationship between bird body mass and MO shedding; an interaction effect between time on treatment and bird weight, to investigate the relationship between the rate of decrease in MO shedding during the treatment period and bird body mass; an interaction effect between time on treatment and initial shedding rate, to investigate the relationship between the rate of decrease in MO shedding during the treatment period and the quantity of MO shed at the commencement of treatment; an effect of time off treatment, to determine whether MO shedding changed during the follow-up period, and whether this change was different to that during the treatment period; and an interaction effect between time off treatment and weight change during the study, to investigate the relationship between the number of MO shed at follow-up and weight change during the study.

Additionally, the model incorporated random effects via a latent Gaussian process for each bird, accounting for temporal correlation in the data. The numbers of MO shed by the same bird on different dates cannot be considered statistically independent, because a bird that shed a large number of MO organisms on one sampling occasion was likely to shed many organisms at the next sampling occasion, for example.

The model was fitted in R using the package TMB.[21] Code to fit the model can be found at https://github.com/b-steve/macrorhabdus/tree/master/treatment-effects.

Results

Sixteen (29%) of the 54 birds examined were initially faecal positive for MO. Faecal shedding ceased in 11/16 (69%) birds after 10 days of treatment. Of 11 faecal negative birds, seven (64%) remained negative when sampled three weeks after the conclusion of treatment, demonstrating a 10-day treatment success of 7/16 (44%). The five birds that remained positive following 10 days of treatment were treated for an additional 10 days. Of these birds, two became negative and two remained

positive 16 days after the conclusion of a second 10-day treatment course, the last bird passed away before the end of treatment. In the time between the end of treatment and follow-up, one bird that had remained positive and one bird that was negative after 10 days of treatment died after showing signs of weight loss and a ravenous appetite. The bodies of these birds were not made available for post-mortem examination, so it is unknown whether they died from MO. The two dead birds were removed from statistical analysis. Overall, treatment was considered a success in in 9/14 birds. Details for individual birds are presented in Table 5.1.

Table 5.1. Complete data for faecal testing results each day samples were collected from budgerigars (n=16) undergoing treatment for *Macrorhabdus ornithogaster* infection using Amphotericin B in drinking water.

#	10.12.18 (Prior to treatment)					12.12.18					15.12.18					17.12.18					19.12.18					20.12.18 (Following treatment)					13.01.19 (Three weeks after treatment)				
	Direct Wet Mount	Gram Stain	Faecal Suspension Technique	Faecal Suspension with Methylene Blue	Faecal Suspension Gram Stained	Direct Wet Mount	Gram Stain	Faecal Suspension Technique	Faecal Suspension with Methylene Blue	Faecal Suspension Gram Stained	Direct Wet Mount	Gram Stain	Faecal Suspension Technique	Faecal Suspension with Methylene Blue	Faecal Suspension Gram Stained	Direct Wet Mount	Gram Stain	Faecal Suspension Technique	Faecal Suspension with Methylene Blue	Faecal Suspension Gram Stained	Direct Wet Mount	Gram Stain	Faecal Suspension Technique	Faecal Suspension with Methylene Blue	Faecal Suspension Gram Stained	Direct Wet Mount	Gram Stain	Faecal Suspension Technique	Faecal Suspension with Methylene Blue	Faecal Suspension Gram Stained	Direct Wet Mount	Gram Stain	Faecal Suspension Technique	Faecal Suspension with Methylene Blue	Faecal Suspension Gram Stained
1	4	1	3	2	1	5	2	5	5	1	0	0	0	0	0	3	0	3	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	2	3	11	8	7	6	3	7	4	2	0	0	0	0	0	0	1	6	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	32	27	96	87	143	4	8	6	7	6	6	1	18	6	4	2	6	7	2	5	0	0	0	0	1	0	0	0	0	0	7	27	86	13	32
4	16	0	54	23	1	0	0	2	1	1	1	0	2	2	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	2	13	1	3
5	25	93	108	45	62	1	16	8	2	6	4	10	14	4	8	1	2	7	2	2	1	0	3	1	0	3	2	14	8	2	3	7	26	2	10
6	5	2	24	23	0	3	4	2	2	1	2	0	6	3	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	2	14	0	1
7	2	4	6	5	5	1	3	6	5	2	2	0	8	3	0	6	0	3	2	0	0	0	0	0	0	0	0	0	0	0	-	-	-	-	-
8	6	4	63	34	2	2	2	7	3	3	1	0	6	2	0	4	0	1	1	1	0	0	0	3	1	0	0	2	0	0	-	-	-	-	-
9	0	0	5	4	0	0	0	3	2	1	0	0	2	2	0	0	0	4	1	1	1	0	1	0	0	0	0	0	0	2	0	0	0	0	0
10	0	0	2	0	2	7	7	5	3	2	0	3	0	0	1	0	0	3	1	1	0	0	2	1	2	0	0	2	1	1	0	0	0	0	0
11	0	1	2	2	0	1	1	0	0	0	0	0	0	0	1	1	0	1	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
12	0	2	1	0	0	0	1	2	0	1	0	0	2	0	0	0	0	2	1	0	0	0	4	1	1	0	0	0	0	0	0	1	6	1	2
13	4	1	16	15	0	2	2	8	1	3	1	1	3	1	2	1	2	2	1	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
14	2	0	6	5	2	1	2	5	4	2	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15	6	4	72	14	1	1	3	3	0	2	1	0	3	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
16	8	3	20	12	7	3	4	12	5	4	1	0	8	2	1	2	0	6	3	1	1	0	3	2	2	3	1	3	2	2	0	1	2	0	3

Likelihood-ratio tests were used to test for presence of the effects described above in statistical analysis. There was strong evidence that shedding decreased during the treatment period ($\chi_1^2 = 15.40, p < 0.001$) and that the initial shedding rate was associated with this decrease ($\chi_1^2 = 6.10, p = 0.014$). The more organisms a bird was shedding at the beginning of treatment, the smaller the percentage decrease in expected number of organisms shed over the course of the treatment. For example, Bird 5 shed 333 organisms in the faecal sample collected on the first day of treatment and the model estimated (with a 95% confidence interval provided in parentheses) that each day of treatment was associated with a 17% (7, 26%) decrease in the expected number of MO shed, while the corresponding estimated effect for Bird 14, which shed only 15 organisms in the faeces collected on the first day of treatment, was a reduction of 46% (33, 56%) per day. Every bird had an estimated treatment effect that was significantly different from zero, regardless of their initial shedding, providing evidence that time on the treatment was associated with decreases in MO shedding for all birds and indicating that increasing length of treatment is likely to improve treatment outcomes.

The rate of decrease in shedding during treatment did not persist following its conclusion, with many birds showing an increase in shedding between the end of treatment and follow-up. There was no evidence that the bird's weight at the start of the treatment was associated with the number of MO organisms shed in the droppings ($p = 0.585$), and a trend towards an association between weight and the effectiveness of the treatment ($p = 0.069$); however, there was evidence that shedding at follow-up was related to a bird's weight change during the study ($\chi_1^2 = 8.8, p = 0.003$). Gaining more weight (or losing less) was associated with decreased shedding at the end of the study. Each 1g increase in weight during the study was associated with a 24% (8, 38%) decrease in the expected number of organisms shed at follow-up.

Medicated Water Consumption

Sampling was carried out in December and outside temperatures were between 24°C and 40°C (75°F and 99°F) during the day. The average daily volume of water consumed by the group over the 10-day treatment period was 67mL (range 61 – 80ml). This accounted for an average volume consumed per bird of 4mL (approximately 7mL/100g of body weight based on the average body weight of 61g)

or 0.4mg (0.7mg/100g of body weight) of AmB each day during the treatment trial. The water drinker in the empty cage that acted as a control for evaporation consistently lost 1g in weight (1mL) in a 24hr period, which allowed accurate calculation of the daily water intake.

Discussion

This study evaluated the treatment efficacy of in-water AmB for the treatment of MO. Our study found that treatment was unsuccessful in 36% (5/14) of the birds treated. These findings are like the findings of three recent publications that have called into question the efficacy of AmB for the treatment of MO. All three studies demonstrated poor treatment outcomes in retrospective case series, with AmB treatment successes in naturally infected birds ranging from 17% to 56%.[18-20] As well as a retrospective analysis, the Australian study evaluated the efficacy of pure AmB powder prospectively. It was gavage fed, in suspension with lactulose to experimentally infected chickens. It found that a dose of 100mg/kg PO BID was effective at decreasing, but not eliminating, the infection in all chickens, while treatment with 25mg/kg AmB caused no statistical difference in faecal shedding compared with the control group.[20]

The treatment failure in some birds in our study may be the result of infection with AmB-resistant MO strains,[20] a failure to maintain therapeutic concentrations of AmB at the site of infection, drug formulation, treatment duration or failure to achieve therapeutic levels in severely unwell birds that were not drinking normally. In the original trials published by Gestier, a concentration of 0.9mg/mL was used and resulted in 100% cessation in shedding after 10 days of treatment.[17] The current manufacturer recommendations result in a concentration of 0.1mg/mL of the AmB solution. It is unclear whether this is a deliberate recommendation based on unpublished literature, or a calculation error that results in sub-therapeutic dosing of the in-water medication. Sub-therapeutic doses at the isthmus are considered possible because of rapid drug passage through the stomach or because of deep-seated infection between the gastric glands, with MO organisms being afforded some 'protection' by their position deep within the proventricular wall. Histologically, the depth with which the MO organism penetrates the gastric glands in severe cases is evident,[22] and may go some way to explaining the reports of cessation of shedding during treatment that is followed by a return to positive MO

faecal samples when treatment ceases.[4,20] An alternative explanation for failure to respond to treatment could include immunosuppression as a result of viral infection. In a previous study, zebra finches had a higher prevalence of MO if they were infected with the zebra finch circovirus.[23] Changes to gastrointestinal pH or bacterial flora have both been shown to occur with, or result from, MO infection. They are commonly related to diet, but may also be the result of gastrointestinal ulceration or administration of previous medications, which had taken place repeatedly in our study animals, suggesting that individual variation or response to previous therapies may explain the variable response to AmB seen within this treatment group.[24,25]

Treatment failure with in-water medications is often attributed to birds that fail to drink, or are drinking sub-normal amounts of liquid, when they are sick.[4] In this study we evaluated water consumption to ensure that the birds were drinking the medicated water. The manufacturer's guidelines for water-soluble AmB stipulate that the therapeutic dose depends on water consumption of 6mL per 100g of body weight. On average, the birds in this trial consumed 7mL/100g of body weight, because of individual variation, it is likely that some birds drank a lot, others only a small amount, with the average intake being close to the recommend minimum intake. It is quite likely that a proportion of birds did not ingest a therapeutic dose and acts as another potential contributor to individual response to treatment. The water intake in our birds was higher than that reported by Filippich et al. (1993), who found that the daily water intake was 5.1 +/− 2.1mL/100g of body weight in the budgerigars in their trial.[4] The sampling and treatment was carried out in December 2018, summer in New South Wales, Australia. Outside temperatures were between 24°C and 40°C (75°F and 99°F) during the day. The aviary was well insulated and temperature inside the building fluctuated between 24°C and 30°C; if the inside temperature rose above 30°C an evaporative cooler provided climate control. Because of these warm, dry temperatures it is possible that the birds drank more readily than they may do during the winter, ensuring average AmB intake reached the recommended level.

Weight has not previously been established as a prognostic indicator for the elimination of MO in the faeces following treatment. This study established that shedding at follow-up was related to a bird's weight change during the study. Based

on these results, practitioners should expect that birds that are responding to treatment will gain weight and that failure to gain weight suggests treatment failure.

This report highlights the partial efficacy when treating MO infections with a commercially available water-soluble AmB treatment. It illustrates clearly, the need for further investigation into effective treatment modalities, includes other types of medication but also different treatment regimens (higher dosages, longer treatment, and other formulations), investigation into strain-specific resistance to AmB and the importance of complete, consistent cessation of faecal shedding before withdrawal of treatment for MO. Further *in vitro* sensitivity testing assessing treatment success outside the host and larger-scale naturally infected controlled trials with treatment success confirmed by necropsy are warranted. Ultimately, a push for novel, successful and cost-effective treatment modalities is required to better treat and eliminate MO from infected birds.

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Chapter 6. *Macrorhabdus ornithogaster* and Viral Co-Infections in Exhibition Budgerigars (*Melopsittacus undulatus*) in Victoria, Australia and the Evaluation of the BioSprint 96 One-For-All Automated Analyser for Extraction of DNA from Budgerigar Faeces

Hamish R. Baron*, David N. Phalen and Ruth N. Zadoks

Author Attribution Statement for Thesis With Publications

I am writing this letter to specify the role of Hamish Baron in the preparation and submission of the following manuscript (Chapter 6):

The convention for author placement in the list of contributing authors is ordered from highest to lowest contribution. The corresponding author of the manuscript is indicated by an asterix (*). The contribution to the manuscript of all authors is as follows:

Task	**Role of Author**
Conceptualisation	HB, RZ, DP
Investigation	HB, RZ
Methodology	HB, RZ, DP
Data Curation	HB
Formal Analysis	HB
Resources	HB, RZ, DP
Writing – Original Draft	HB
Writing – Reviewing and editing	HB, DP, RZ

As the primary supervisor for the candidature upon which the thesis is based, I can confirm that the above authorship attributions statements are correct. I have sighted email or other forms of correspondence from all co-authors confirming their certifying authorship and permission for manuscript inclusion in this thesis.

Prof Ruth Zadoks

21 June 2023

Introduction

Macrorhabdus ornithogaster (MO) is a common pathogen of budgerigars (*Melopsittacus undulatus*) in Australia and worldwide.[1,2] It is an ascomycetous yeast found at the isthmus between the proventriculus and ventriculus. Signs of infection range from subclinical to causing a ravenous appetite with severe emaciation, black tarry stools, regurgitation, and death. The pathogenesis of macrorhabdosis is poorly understood, and the difference between a bird that is infected but asymptomatic and one that goes on to develop clinical disease has not been elucidated. It has been postulated that stress, immunosuppression, co-infections, and poor genetic diversity may play a role in the development of disease,[2,3] but to date these theories have been only superficially explored. A recent retrospective review of mortality events in 221 captive budgerigars identified MO in 56 birds. In 38 of 56 cases (68%), MO was identified alongside a range of co-morbidities, but several birds experienced MO infections with no other gross or histological disease.[4] *Macrorhabdus ornithogaster* was thought to have been the primary cause of death in 22 of 56 cases (39%), highlighting its significance as both a primary and secondary pathogen in captive budgerigar populations.[4] The role of viruses as cause of co-infections or co-morbidities in budgerigars in Australia has not been previously explored.

The budgerigar fledgling disease virus (BFDV, a polyomavirus) the beak and feather disease virus (PBFDV, a circovirus), and the budgerigar adenovirus (PsAdV) are three viruses that have the potential to cause disease of the immune system. Budgerigar fledgling disease virus is a significant pathogen of budgerigars globally and is recognised as one of the major challenges faced by the budgerigar fancier.[4] The severity of disease caused by BFDV depends on the age and condition of the bird when exposure occurs. The clinical presentation also varies, with some birds dying acutely at 10–15 days old with no clinical signs, while other fledglings may develop abdominal distension, subcutaneous haemorrhage, ataxia, hydropericardium, ascites and feather abnormalities, including missing primary flights and tail feathers, and die.[6] Avian polyomaviruses cause a multisystemic infection in nestling budgerigars. In many infected budgerigars, antigen-presenting cells are targeted and therefore survivors of infection may have a compromised immune system.[7] In Victoria, Australia, APV was detected in faecal material via polymerase chain reaction (PCR), demonstrating a prevalence of 10% in clinically normal budgerigars

sampled. The APV PCR products that were sequenced showed 99% sequence homology with budgerigar fledgling disease (BFD) polyomavirus strain WF-GM01 (GenBank accession no. GU452537).[8] In budgerigars in New Zealand, 22% were PCR positive on whole blood for a novel polyomavirus, with 32% sequence divergence to the BFD strain. In this study, no association between feather abnormalities and the novel polyomavirus was noted, despite many birds in the cohort presenting with feather abnormalities.[9]

Adenovirus infections in birds can target the spleen and therefore could also cause immunosuppression. Both clinical and subclinical infections with a siadenovirus (the Budgerigar adenovirus) have been documented in budgerigar aviaries.[10,11] Psittacine adenovirus was described in budgerigars in the USA when a novel siadenovirus strain BuAdV-1 USA-IA43444-2016 was identified in a zoological collection.[10] The strain had 99% genomic homology to budgerigar adenovirus 1 (BuAdV-1), previously reported in Japan. Clinical disease varied significantly between these two reports, with birds in the American collection being identified as subclinical carriers, while the five birds in the Japanese study died.[11] An adenovirus has been reported once in the droppings of a budgerigar in Victoria, but no sequencing was carried out on DNA from the positive sample to further characterise the virus. Because of the nature of the sample collection, the disease state of the budgerigar in question was unknown.[8]

Finally, a common virus thought to cause immunosuppressive disease in budgerigars is PBFDV. The virus is highly infectious, and, like many species, budgerigars appear highly susceptible to PBFDV infections. The virus causes progressive feather dystrophy, feather loss, immunosuppression and sometimes death in budgerigars. The virus is enzootic in wild Australian psittacine species, and it is widespread amongst avicultural collections in Australia.[8,12] The PBFDV infection results in necrosis and atrophy of the bursa of Fabricius and thymus as juveniles,[13] which may lead to increased disease susceptibility and infections with pathogens such as MO. In Victoria, budgerigar faecal samples were evaluated for the presence of PBFDV, and the virus was identified in 18 of 38 (47%) samples based on PCR. The prevalence in budgerigar aviaries was similar to that in lorikeets (*Trichoglossus* spp.) (54%), but lower than that in lovebirds (*Agapornis* spp.) (67%). Each of these bird species had a PBFDV prevalence higher than in the collections with other

psittacine species (ranging from 0% to 25%) – many of which, however, had sample sizes below statistical power. [8]

Although their presence in wild avian populations in Australia is well characterised, MO, APV, BFDV and PsAdV presence and prevalence are poorly understood in captive avicultural collections.[14] Detailed information on the co-occurrence between the four pathogens is not available, particularly within the captive budgerigar populations. Little is known about pathogen prevalence, or, indeed, about optimal methods for pathogen detection. The first objective of this study was to determine the prevalence of infection with BFDV, PBFDV, psittacine adenoviruses and the recently discovered polyomavirus reported from New Zealand, in budgerigar collections in Victoria. The second objective was to determine if budgerigars sub clinically infected with these viruses were more likely to be infected with MO.

In this study we also were able to compare the effectiveness of two different DNA extraction techniques for extracting MO DNA from faeces. The challenges of extracting DNA from avian droppings are becoming better understood and variability between test kits has been reported, with many commercially available faecal kits extracting little or no DNA from avian faecal samples.[9] The results from Chapter 2 in this thesis, which showed poor sensitivity of PCR as a diagnostic test when conducted on faecal DNA extracts, highlighted the importance of optimising DNA extraction and so we evaluate the use of the BioSprint 96 One-For-All Vet (Qiagen, VIC 3168, Australia), automated extraction method in more detail in this chapter. Automatic high-throughput procedures significantly decrease time and costs[15] and allow for standardisation of extracting DNA from diagnostic samples. However, these approaches have not yet been evaluated for avian faecal samples.

Materials and Methods

Sample Collection

Forty budgerigars were sampled from each of three breeding aviaries in Victoria, Australia. Aviaries belonged to exhibition fanciers who had all bred and exhibited birds in Victoria in the last 12 months. Birds were sampled from a large flight aviary, where captures were carried out using a 2mm woven nylon hand bird net and placed into a cleaned exhibition cage to await examination. After the birds had

passed two droppings, band numbers were recorded, droppings were collected using a sterile cotton-tip applicator and stored in a micro-Eppendorf tube with 0.3mL of 0.9% NaCl. The birds were weighed, examined for feather condition, evidence of skin lesions and external injuries. Feathers were regarded as abnormal if, on physical examination, any feathers were missing or dystrophic. Once appropriately restrained in hand, a blood sample of 0.1mL was collected from the jugular vein of each bird using 0.33mm (29G) needle on a BD Ultra-FineTM 0.3mL insulin syringe (Provet, Victoria 3202, Australia). Blood was spotted onto two pieces of grade-one filter paper (Advantec, NSW 2164, Australia) and dried in individually labelled micro-Eppendorf tubes. To avoid cross contamination between samples, hands were washed with F10 Hand Gel (Chemical Essentials, Victoria 3132, Australia), exhibition cage liners were soaked sprayed with F10 solution, and soaked in a 4% sodium hypochlorite solution between birds. Samples were stored at −80°C until processed.

Microscopic Detection of *Macrorhabdus ornithogaster*

Samples were collected with permission of the collection owners and the University of Sydney Animal Ethics Committee (Production and Companion Animal) project number 2021/1984, and faecal samples were examined using the faecal suspension technique evaluated previously.[16] One 0.03mL aliquot was placed onto a glass slide and covered with a coverslip and microscopically examined without staining. Each slide was microscopically examined with the stage diaphragm reduced, two passes of the 40× microscope objective over the coverslip were made over the first and the last portions of the coverslip and the samples reported as either positive (two or more organisms counted) or negative.

Methods Evaluation

Spiking

The spiking experiments were performed to evaluate the sensitivity for each DNA extraction method. Two faecal samples were selected from healthy budgerigars that were negative on faecal suspension for MO and mixed. The droppings were spiked with pure MO culture samples. One hundred microlitres of each serial dilution ($\sim 10^1$ to $\sim 10^6$CFU/mL of MO) was added to 20mg of faeces, and the DNA was extracted from the co-mingled, spiked, faecal samples using two extraction methods

– BioSprint and QIAamp DNA Stool Mini Kit (Qiagen, VIC 3168, Australia), as described below.

DNA Extraction

DNA from the spiked samples and 20 unspiked pilot samples was extracted from the faeces and blood spot using the BioSprint (Qiagen Pty Ltd, VIC 3168, AUS) 96 Vet One-For-All Kit using a modified protocol (Buffer RTL and not AL used) for animal whole blood. A second extraction was carried out on the same samples using the QIAamp DNA Stool Mini Kit (Qiagen, VIC 3168, Australia) for faeces, and the Qiagen DNeasy Blood and Tissue kit (Qaigen, Germantown MD 20874 USA) for blood. Following assessment of the pilot samples, the remaining samples had DNA extracted from the blood spot on filter paper and the faecal samples using the BioSprint 96 well extraction protocol for animal whole blood.

Assessment of DNA Yield

The quantity and quality of the extracted DNA was assessed using the Nano Drop spectrophotometer (ThermoFisher Scientific, VIC 3179, Australia). The yield of the extracted DNA was calculated by the amount of light absorbed by 1µl of the DNA at 260 nm and the purity of DNA was based on the A260/A280 ratio. DNA was defined as pure if the 260/280 absorbance ratio ranged between 2.0 and 2.3. To evaluate for inhibiting substances, PCR was run using three dilutions of the DNA extracted from unspiked samples using the BioSprint, alongside faecal samples spiked with known-positive MO cultures. To do this, DNA extracts were diluted 1:10, 1:100 and 1:1000 and PCR was run using the MO protocol described below. To further quantify the inhibitory substances in the avian droppings, DNA collected from a PBFDV-positive lorikeet liver was added to budgerigar faecal samples, the DNA was diluted as described for MO and PCR was run using the PBFDV protocol described below.

Pathogen Detection

Molecular Detection of MO

All samples that were evaluated microscopically, had DNA extracted for evaluation via PCR. Polymerase chain reaction was then performed using MO-specific forward primer AGY1 (GGACTTATATTACTAGTCAGATGG) and the generic *Saccharomyces* spp yeast reverse primer, SM2 (CAATACGCCTGCTTTGAACACTC) as previously reported.[17] A pure culture of MO was used as the positive control by spiking

budgerigar faeces with 0.1mL of pure MO slurry, separate from the spiking experiment above, prior to DNA extraction. Nuclease-free water (UltraPure, Thermo Fisher Scientific Australia Pty Ltd, VIC 2179, Australia) was used as the negative control.

Molecular Screening of Polyomavirus Infection

DNA from the blood spot samples was evaluated for the presence of the polyomavirus major capsid protein VP1 gene using a broad-spectrum nested PCR as described by Johne et al.[18] A DNA sample from a known APV-positive control, collected from the liver of an infected budgerigar, confirmed by sequencing was used as a positive control, and nuclease-free water was included as a negative control.

Molecular Screening for Adenovirus Infection

DNA from the faeces was evaluated for the presence of PsAdV using a broad-spectrum nested PCR with degenerate primers (5'-TNMGNGGNGGNMGNTGYTAYCC-3' (forward), 5'-GTDGCRAANSHNCCRTABARNGMRTT-3' (reverse).[19] The first step was run for 6 touchdown cycles with 30-second cycles an annealing temperature ranging from 62°C to 50°C (−2°C temperature drop each cycle), and an extension step at 72°C for 30 seconds in each cycle. This was followed by 44 cycles with denaturation at 95°C for 30 seconds, 30-second cycles at 50°C and a final extension phase at 72°C for 10 minutes. The second PCR was run using the same PCR cycle with the nested primers 5'-GTNTWYGAYATHTGYGGHATGTAYGC-3' (forward) and 5'-CCANCCBCDRTTRTGNARNGTRA-3' (reverse), at the same temperatures and cycle duration and number. A known PsAdV positive DNA sample, extracted from the liver of an infected tawny frogmouth (*Podargus strigoides*), confirmed by amplicon sequencing, was used as a positive control, and nuclease-free water was included as a negative control.

Molecular Detection of PBFDV

DNA extracted from the blood spot and the faeces were evaluated using a single step PCR for the presence of PBFDV major capsid protein gene, using primers described by Das et al.[20] A known PBFDV positive DNA sample, extracted from the combined liver and spleen of a rainbow lorikeet, this sample was used as the positive control, and nuclease-free water was included as a negative control.

For all PCR reactions, amplified DNA was separated according to its size, on a 1.5%, agarose gel stained with RedSafe (iNtRON Biotechnology, Gyeonggi-do, 13202, Korea), by 30 minutes of electrophoresis (110V). Amplicons were visualised under UV light (BioRad Geldoc; Bio-Rad Laboratories Pty Ltd, NSW 2142, Australia).

Results

DNA Quantity and Quality

The DNA quantity and quality was compared, and the results are outlined in Table 6.1

Table 6.1. Summary of the comparison between BioSprint and QIAamp DNA Stool Mini Kit for the extraction of DNA from avian faeces and blood using the NanoDrop.

DNA Extraction Method	BioSprint		QIAamp DNA Blood and Tissue / Stool Mini Kit	
Type of sample	Blood	Faeces	Blood	Faeces
ng/ul Median	36.20	42.99	19.56	0.64
ng/mL Interquartile range (IQR)	32.13 – 40.23	37.31 – 47.10	8.73 – 36.24	0.45 – 1.13
260/280 Median	2.32	2.30	1.74	0.65
260/280 IQR	2.22 – 2.48	2.15 – 2.61	1.66 – 1.83	0 – 1.01

With regards to the DNA purity, the BioSprint achieved a higher A260/280 ratio compared to the QIamp Mini Kit, with a median of 2.32 and 1.74 respectively for blood and 2.30 and 0.65 for faeces based on the first 20 samples tested. The faecal extraction using the QIAamp DNA Stool Mini Kit gave the lowest 260/280 ratio and returned many samples that had little, or no DNA extracted. Based on these results, the remainder of the DNA samples were extracted from both blood spot and faeces using the BioSprint extraction. A summary of the DNA yields is found in Table 6.1. All dilutions of the PBFDV-spiked faecal samples extracted using the BioSprint returned positive results, demonstrating no evidence for the presence of inhibiting substances in the DNA. Similarly, PCR on the DNA extracted from faeces with the BioSprint was able to amplify MO DNA at all six concentrations, while the DNA

extracted using the QIAamp DNA Stool Mini Kit amplified the MO DNA only from the sample with the highest spike concentration (~10^6 CFU/mL).

Pathogen Testing

The budgerigars that were sampled in this cohort were not showing signs of disease. All birds were examined and were in robust body condition, despite shedding MO in their faeces. *Macrorhabdus ornithogaster* was detected in 27 faecal samples using PCR and in 29 samples using direct microscopy. Pathogen testing results are summarised in Table 6.2. The PCR and direct microscopy results agreed in 25/27 of the PCR positive birds; there were four samples that were positive on microscopy and not on PCR and two samples that were positive on PCR and not positive on microscopy. A total of 31 samples (25.8%) were positive for MO when using parallel testing, i.e., a positive on either diagnostic modality is considered positive (Table 6.3). Three faecal samples were positive for PBFDV whilst no blood samples were positive. None of the birds that tested positive for PFDV were also positive for MO. There were no birds that were positive for APV or PsAdV on any sample.

Table 6.2. Summary of the results of the PCR testing of samples from 120 budgerigars for *Macrorhabdus ornithogaster*, psittacine beak and feather disease virus, avian polyomaviruses, psittacine adenoviruses and microscopic analysis of faeces for MO.

	Macrorhabdus ornithogaster		Beak and feather disease virus		Avian polyomaviruses		Psittacine adenoviruses
	Microscopy	PCR - Faeces	PCR - Blood	PCR - Faeces	PCR – Blood	PCR - Faeces	PCR - Faeces
Number of samples	130	130	130	130	130	130	130
Positive	29	27	0	3	0	0	0
% Positive	22.3	20.8	0	2.3	0	0	0

Table 6.3. Summary of the positive results of *Macrorhabdus ornithogaster* infections in budgerigars, broken down by collection and the diagnostic modality.

	Collection 1	*Collection 2*	*Collection 3*
PCR	7	8	12
Microscopy	8	9	12
Parallel Testing Total	8 (20.0%)	10 (25.0%)	13 (32.5%)

Discussion

We set out to evaluate the presence and prevalence of MO and common viral infections in a population of exhibition budgerigars in Australia. This study demonstrated a very low or absent viral load in the budgerigars that we evaluated. The budgerigars in this study do not appear to have APV or PsAdV circulating at detectable levels and a very low prevalence of PBFDV. By contrast, the birds had an MO prevalence of 25.8% with a range of 20.0%–32.5% between the colonies, using ante-mortem diagnostic tools (faecal suspension microscopy and faecal PCR). This is in line with previously reported prevalence of 27% and 40% using direct wet mount microscopy as reported in two colonies of exhibition budgerigars in Australia.[21] It is likely that the true number of birds infected with MO in both studies is higher, but the sensitivity of the diagnostic testing modalities available ante-mortem is less than 100% (Chapter 2). Because none of the birds in this study were showing signs of macrorhabdosis, it is not possible to rule out an association between viral load and infected birds developing clinical disease and future work should focus on evaluating the viral load in infected birds with clinical macrorhabdosis.

This study adds further weight to previous work (Chapter 2), that demonstrated that no ante-mortem technique detects all infected birds although there was very strong agreement (Cohen's Kappa = 0.8632) between positive results by direct microscopy using the faecal suspension technique and faecal PCR in the current study. PCR-positive samples that are negative on cytology may occur when organisms are damaged and not visible cytologically, yet the DNA persists, or, more likely, because the organisms were not visualised on direct microscopic evaluation of the faecal slides. These results highlight the possibility of false negative results using direct microscopy. Similarly, samples were positive using direct microscopy that were negative on PCR. This may be the result of false positive results, where long rod-shaped bacteria were mistaken for MO organisms by the evaluator, or because of false negative results on PCR. We know that false negative results occur in faecal PCR samples that were confirmed positive using the gold-standard isthmus scraping in birds that received a necropsy, (Chapter 2), but further evaluation of the true status of the birds in this study that showed discrepancies between microscopy and PCR was not possible given that all birds remained alive throughout the study.

False negative results may be the result of poor DNA extraction from bird droppings, or because of inhibitors in the avian faecal samples.[22] The presence of PCR inhibitors in faecal samples is well documented and variation in faecal samples in different species can affect the quality of extracted DNA.[23] It has been suggested that avian faeces may contain more inhibitors compared to their mammalian counterparts because of the common cloaca and the excretion of urofaeces.[24] The application of rigorous quality controls in avian faecal DNA extraction is necessary, especially because of the lower moisture content in avian faeces, resulting in difficult dissolution.[25] We controlled for the quality of the DNA extraction by evaluating the DNA quantity and purity using the Nano Drop as well as using the spiking experiments. Dilution series were prepared in faecal samples prior to DNA extraction (using MO) and in DNA extracts from faeces (using PBFDV), to inform extraction efficiency and PCR inhibition, respectively. Using the DNA extracted with the BioSprint we detected MO DNA, even at the lowest dilution, but were only able to detect MO DNA in the most concentrated MO sample extracted using the Qiagen kit, demonstrating far greater sensitivity in the DNA extraction protocol using the BioSprint technique. To evaluate for inhibiting substances affecting the sensitivity of the assay, the PCR protocols for PBFDV were run using serial dilutions of the DNA samples alongside DNA extracted from faeces spiked with known-positive PBFDV DNA. All PCR reactions were positive with DNA extracted using the BioSprint method. The variability between the two protocols and the complicated nature of avian DNA extraction from faeces highlights the importance of robust internal quality controls. The inclusion of pre- and post-extraction dilution series not only helps to optimise protocols, but allows confidence that negative results are not artefacts of the sample type or sample preparation method.

It is the first time that the BioSprint has been used to extract DNA from avian faeces and blood as part of a robust quality assurance protocol. The extraction technique was compared to that of a standard QIAamp DNA Stool Mini Kit. The quality and quantity of the DNA extracted from the blood spot samples was similar between the two kits, but the BioSprint extracted significantly more high-quality DNA when compared with the QIAamp DNA Stool Mini Kit. There was a slightly higher 260/280 ratio when using the BioSprint than the generally accepted ratio of 1.8–2.0, with a median value of ~2.3; however, the spectral profiles were reviewed for each sample and these demonstrated normal trough and peak profiles, consistent with a pure

sample. The QIAamp DNA Stool Mini Kit had several samples with very low 260/280 ratios, indicating residual reagents or a very low concentration of DNA, making the reliability of any downstream application of PCR questionable when using the kit according to manufacturer instructions for budgerigar faeces.

It remains poorly understood which host factors lead budgerigars to become infected with MO, or to progress from infection to disease. Further investigation, including longitudinal studies, is required to evaluate stressors and how they impact the host susceptibility to MO infection and transition from infection to clinical macrorhabdosis. There is evidence to suggest that stressors play a role in mortality events attributed to MO;[3] these stressors may be the result of concurrent disease states,[26] stocking density, reproduction, and continuous administration of medications or nutrition.[27-29] Nutritional and reproductive status may result in changes to the gastrointestinal pH or bacterial microbiome, these changes have also been shown to occur with, or result from, MO infections.[28,30] One consideration that has not been evaluated in the literature and should be investigated is the genetic predisposition to infection and disease. In poultry, birds are selected for disease resistance and the resultant progeny are more resilient in following generations. This is something that is not routinely practiced in budgerigar breeding facilities, with exhibition traits given preference over all others. A shift in focus to encompass selection for resistance to infection and disease caused by MO may prove to be a viable solution in the long term. In future studies, close attention should be paid to sample processing protocols that may affect apparent pathogen prevalence, and use of the BioSprint protocol described in this study may be a good option for DNA extraction from budgerigar blood and faecal samples.

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Chapter 7. Future Directions and Research Priorities for *Macrorhabdus Ornithogaster*

Hamish R. Baron*, David N. Phalen and Ruth N. Zadoks

Author Attribution Statement for Thesis With Publications

I am writing this letter to specify the role of Hamish Baron in the preparation and submission of the following manuscript (Chapter 7):

The convention for author placement in the list of contributing authors is ordered from highest to lowest contribution. The corresponding author of the manuscript is indicated by an asterix (*). The contribution to the manuscript of all authors is as follows:

Task	**Role of Author**
Conceptualisation	HB
Investigation	HB
Methodology	HB
Data Curation	HB
Formal Analysis	HB
Resources	HB, DP, RZ
Writing – Original Draft	HB
Writing – Reviewing and editing	HB, DP, RZ

As the primary supervisor for the candidature upon which the thesis is based, I can confirm that the above authorship attributions statements are correct. I have sighted email or other forms of correspondence from all co-authors confirming their certifying authorship and permission for manuscript inclusion in this thesis.

Prof Ruth Zadoks

21 June 2023

Introduction

This body of work set out to better understand and characterise the diagnosis, treatment, and the role of viral co-infection for MO-infection and macrorhabdosis in Australian cage birds. We identified knowledge gaps in clinical practice and in the literature and set out to answer the key questions: what is the most sensitive and specific tool for the diagnosis of MO, in-clinic and in the laboratory? How effective is AmB at treating MO in the avian patient? And, finally, do common viral infections play a role in the rate of infection with MO? The research in this thesis encompasses work done over a period of six years, with interruptions caused by the COVID-19 global pandemic. However, the timeline means that there have been further investigations and advancements by other investigators during this time, and these should be considered alongside our research findings as we look to provide future directions for investigators and research priorities for the future.

Comparison of In-Clinic Diagnostic Testing Methods for Macrorhabdus ornithogaster

A major conundrum facing avian veterinarians is the accurate and cost-effective diagnosis of MO in-clinic. This informed the initial stages of our diagnostic investigation. We compared the five most common in-clinic diagnostic tools, and identified the faecal suspension technique as the test that identified the greatest number of MO organisms.[1] This was likely because of the lack of background debris (plant material, particulate matter etc.) which often hinders the visualisation of MO organisms using the other four methods. We evaluated these tests using scientific criteria, utilising 'detection probability', which is an estimate of the proportion of animals in the population that would test positive using the specific test. The detection probability is the product of sensitivity, specificity, and prevalence. Our inability to confirm a definitive infection status in live birds meant that we could not obtain sensitivity directly; instead, if detection probability is higher for one method compared with another, we inferred that the sensitivity is also higher, based on the assumption that false-positive diagnoses are rare with any of the methods when experienced microscopists are making the diagnosis.

However, the diagnosis of MO is difficult for inexperienced clinicians using direct microscopy (Personal communication, D. Hsu, 2023), with many confusing large

bacterial rods, feather debris or digested food material for MO. It is common to receive photographic enquiries asking to verify a microscopic organism as MO, many of which are large bacterial rods. It is likely, therefore, that false positive diagnoses occur, but in this study the use of parallel testing confirmed infection status of these birds giving more surety that the positive microscopic faecal samples were true positives. The faecal suspension technique that had the highest detection probability is a technique that is not well described in the literature[2] but has been utilised for the diagnosis of MO in our clinic for many years.[3] There are specific features of this diagnostic test that are essential to its success, most importantly the avoidance of the urate portion of the faecal material when selecting the sample. Some clinicians have reported difficulty with this technique after selecting the whole dropping and suspending it in 0.9% NaCl, this causes the uric acid to become suspended and renders the test un-readable. The technique also requires the user to allow the faecal suspension to settle before collecting a sample from the meniscus. We have not compared the results when samples are collected from the fluid meniscus at different time points, but optimising this test by carrying out this work will provide the end user with more certainty surrounding timing of collection of the meniscus fluid.

Although convenience criteria (being affordable, user friendly, with a rapid turnaround time and relatively equipment-free) were not formally evaluated in this study, they are an important consideration when discussing tools for use in-clinic.[4] The faecal suspension technique requires very little equipment (micro-Eppendorf tube, 1ml syringe, a slide and slide cover), and does not require equipment associated with performing quick stains, which may not be available in all veterinary clinics. These results position the faecal suspension technique strongly when considering convenience and combined with its higher sensitivity, makes it the diagnostic modality of choice in-clinic.

In this chapter, two of the diagnostic tools were evaluated using the same slide (i.e., a single aliquot of meniscus or faeces was used for examination without staining, and subsequently the same slide was used for Gram-stained examination) whereas some diagnostic tools were evaluated on different slides (wet mount sample with methylene blue). Because of this, the results for tests done on a single slide are non-independent, and will be more closely correlated, than results from tests done on

different slides from the same sample. If this research was to be repeated, correction of this potential bias can be performed simply by evaluating each diagnostic modality using a single test per aliquot, with all aliquots coming from the same faecal sample.

One of the major limitations of many veterinary studies is power, and this study is no exception. Whilst we evaluated 480 individual tests on 288 separate slides, the samples were collected from only 16 individuals, from one aviary of budgerigars.[1] In our statistical analysis, we corrected for non-independence within birds, both across tests and over time, but not for the re-use of slides. Also, this statistical correction did not address generalisability or external validity of our results, which would have been greater if samples were collected from different birds across different budgerigar flocks and, ideally, also from different bird species. A major limitation in this study, and indeed across much of the literature evaluating MO treatment outcomes and diagnostic tools, was the inability to perform necropsy examinations, to confirm the true infection status either following diagnostic testing, or following treatment, limiting the data we could collect relating to sensitivity and specificity.

Comparison of Five Laboratory Diagnostic Modalities for the Detection of Macrorhabdus ornithogaster in Budgerigars (Melopsittacus undulatus)

The lack of definitive information on infection status when evaluating sensitivity and specificity of commonly employed laboratory diagnostic tools for the detection of MO has prevented the establishment of a 'gold standard' diagnostic protocol. We addressed this by comparing five diagnostic tools using samples from 20 dead budgerigars. During necropsy, faeces were collected from the colon and tested for MO using the faecal suspension technique established as having the highest detection probability in Chapter 2, and via PCR. The isthmus of the proventriculus was tested for the presence of MO by direct cytology of scrapings, by PCR, and by histopathological examination of sections of formalin-fixed tissues. This was the first time that histopathology had been evaluated against any of the molecular diagnostic techniques and revealed that it performed poorly when compared with direct cytology and isthmus PCR, despite traditionally being considered the gold-standard post-mortem diagnostic tool.[5] The laboratory diagnostic tools in this study were not only evaluated using scientific criteria (sensitivity and specificity) but

were also considered in terms of the convenience criteria This holistic approach to the diagnosis of MO allowed us to compare the most successful in-clinic ante-mortem testing (Chapter 2) with the commonly available laboratory tools.

Relative to detection of MO by any of the modalities used, testing of the isthmus by cytology or PCR was the most sensitive diagnostic test evaluated, results which are similar to those of a study of 100 psittacine and passerine birds in the Czech Republic,[6] where qPCR accuracy was evaluated for the diagnosis of MO in avian droppings, using a very similar model to that described in Chapter 3. In contrast to our study, which had 100% agreement between PCR and cytology of the isthmus, the authors identified 4% more qPCR positive MO samples using DNA extracted from faeces when compared isthmus mucosal cytology. In agreement with our work, both techniques revealed significantly higher numbers of positive MO results than cytology of the faecal samples revealed (34%) and although the faecal cytology method was not described in detail, it is assumed that this was a direct wet mount, rather than the suspension technique described in Chapter 2.[6] All the samples evaluated using qPCR of the isthmus in the Czech study agreed with the qPCR of the faeces and the authors concluded that faecal qPCR is able to detect all infected individuals, including those with negative stool or gastric cytology.[6] This study found faecal qPCR more sensitive than the conventional PCR of the faecal material in our study.[1,6] The reason for this is unclear but it may be to do with the different faecal DNA extraction protocols. The Czech study utilised the NucleoSpin DNA Kit (Macherey-Nagel, Düren, Germany) which includes lysis beads as part of the cell lysis protocol. This lysis step may have been more effective at breaking up the thick cell wall of the MO organisms and releasing DNA for amplification. Comparison between these two extraction techniques warrants further investigation. The results of our study, show that finding MO in faeces from a live budgerigar using the protocol we described, whether by microscopic or PCR evaluation, means that it is infected (unless the result is a false positive diagnosis), however, not finding MO does not mean that budgerigar is negative. This has implications for the negative predictive power of biosecurity screening at a collection level, as well as for the evaluation of individual pet birds in the veterinary clinical setting. Unfortunately, the variability in prevalence and shedding of MO in infected budgerigars[1,3,7-10] makes it difficult standardise recommendations for quarantine or intake screening protocols.

Histopathology has traditionally captured many of the diagnoses of MO, simply because the birds were received by the pathologist in formaldehyde, and the diagnosis was made either incidentally, or as part of a disease investigation.[11] Our work has identified that histopathology was the least effective diagnostic tool, although in rare cases, histopathology may show MO infection when an isthmus scraping does not (see discussion of Treatment Trials and Tribulations of Amphotericin B). This is likely because of the difficulties associated with selecting and processing the isthmus in the same plane so they can be prepared on a glass slide for histopathological assessment. In our study, despite tissues being selected specifically for evaluation of the isthmus, it was missed on initial processing in 4/20 samples. This highlights, that when formalin-fixed tissues are the only sample available, clear instructions must be given to the diagnostic laboratory, specifically requesting the tissue planes required, to maximise diagnostic efficacy using histology.

When it comes to sample selection, Chapter 3 highlights that a scraping of the isthmus is a superior sample compared to faeces when attempting to diagnose MO infection in the dead bird. Based on our findings, PCR and histopathology are unnecessary for the post-mortem diagnosis of MO. When isthmus scrapings are performed by an experienced clinician, direct microscopy performs just as well as molecular techniques but gives an answer in real time, is inexpensive and requires very little equipment. We recommend that direct isthmus scrapings become part of a routine necropsy screening in any clinic that performs necropsies on avian specimens.

Treatment Trials and Tribulations of Amphotericin B for use Against Macrorhabdus ornithogaster in Australian Cage-Birds

Having established the most appropriate diagnostic samples, both ante- and post-mortem, the focus of the thesis shifted to evaluating the current therapy recommendations. The pilot study in Chapter 4 evaluated the MO load in the droppings of two birds undergoing treatment with AmB. We highlighted the dramatic reduction in shedding in birds undergoing treatment with AmB. This is likely because the AmB is effective at killing the MO organisms in the gut and that during the passage from isthmus to faeces, the organisms that are within the lumen, are mostly eliminated. The profound rebound effect that we illustrated is very likely

the result of AmB's inability to kill the MO organisms that are deep within the glandular crypts of the isthmus. This may be because of the short contact time caused by rapid ingesta / AmB transit through the gut, poor penetration deep into the glands or because the BID dosing allows proliferation of the MO when there is little or no AmB within the gastrointestinal lumen. The penetration of the MO deep into the gastric crypts, and in severe cases beneath the koilin layer of the ventriculus provides a protective barrier not only to treatment, but also to diagnosis. This highlights the importance of timing when evaluating treatment efficacy, which should be evaluated at least three days after the cessation of therapy, rather than immediately at the end of treatment. In Chapter 2 we evaluated a budgerigar submitted for post-mortem examination that did not have overt MO infection based on isthmus scrapings but had severe glandular dysplasia and large numbers of MO deep within the gastric glands of the proventricular isthmus,[1] a finding that is supported consistently in the literature.[2,7,11,12] This suggests that in rare cases, histopathology may be more sensitive than isthmus scraping. Specifically in cases where severe infection has resulted in remodeling of the mucosa, but recent treatment has cleared the MO organisms from the mucosal surface. The inflammation, mucosal hyperplasia and glandular dysplasia that results from a severe MO infection goes some way to explaining how, when drugs are administered intermittently, or in low doses in-water, that penetration and efficacy will be significantly reduced. At the same time, the severe mucosal damage explains why, as experienced so often following treatment, the budgerigars fail to gain weight, continue to regurgitate, and pass tarry droppings despite a reduction in MO organisms being shed. In severe cases, it has been reported that these isthmus lesions can spontaneously progress to gastric adenocarcinoma lesions,[7] highlighting the inflammatory response, and subsequent remodeling power of the MO. Interestingly, the profound rebound effect that we described in Chapter 4, where shedding post-treatment exceeded shedding pre-treatment, was not mirrored by the budgerigars undergoing treatment in Chapter 5. The budgerigars that remained infected varied in the severity of their shedding following cessation of treatment, whereby three of the birds returned to pre-treatment levels, whilst the remainder were only shedding low numbers of MO, and none showed increased shedding levels. There are a variety of possible reasons for the variation in MO shedding following treatment. It is possible that growth environment may be superior in some

patients, because of the existing microbiome[13] or the pH at the isthmus.[14] Additionally, the number of MO in the droppings following treatment may be associated with the degree of inflammation or hyperplasia caused by the infection with MO, more chronically affected cases are likely to have more inflammatory response and crypts that afford more protection to MO organisms, resulting in more persistent shedding in the face of therapy.[7,11,15] It is not clear what role chronicity of infection plays in response to treatment in a bird with MO, however, the chickens in our randomised controlled trial began therapy for MO shortly after being infected, and yet only 3/10 birds were negative for MO on isthmus scraping post-mortem after 10 days of treatment with 100mg/kg.[3] Based on the current therapeutic recommendations, AmB has limited efficacy, even in birds that have only recently been infected and who have not developed secondary histological characteristics associated with MO infection. In the absence of alternative treatment options, more research is required to establish what, if any, risk factors exist for treatment failure. These studies will require long-term monitoring both pre- and post- treatment, to maximise understanding of what results in poor response to therapy in some birds, but not others.

The negative MO faecal results demonstrated during treatment in both chickens and lovebirds in these trials suggest that MO is susceptible to AmB *in vivo*, but in both cases, AmB failed to eliminate the infection. This is a common complication experienced by budgerigar fanciers, veterinarians and owners following treatment with AmB and is well described in retrospective reviews of treatment success.[3,8,16] The failure to clear infection has previously been implied, but MO shedding and rebound following treatment with AmB had not been clearly demonstrated prior to this work. Having now evaluated AmB in multiple treatment trials, the recurrence in shedding, (sometimes at a higher level than prior to treatment) is considered more likely the result of a failure of protocol, rather than a genuine resistance to AmB. Amphotericin B has a remarkably low rate of microbial resistance,[17] and the mechanism of resistance remains poorly understood.[17,18] In fungal strains resistant to AmB the oxidative stress response plays a major role in modulating resistance,[19] and treatment of resistant strains with AmB plus L-ascorbic acid demonstrated increased AmB susceptibility with increasing L-ascorbic acid concentrations.[17] These results indicate that co-treatment using L-ascorbic acid or other pro-oxidants in conjunction with AmB, may provide a novel and efficacious treatment modality

for infection with AmB resistant MO, although the addition of L-ascorbic acid may not overcome the inherent limitations of AmB treatment, such as poor water solubility or inadequate contact time in the isthmus.

Strain-specific susceptibly to AmB was investigated briefly as part of a chapter in this thesis. The interruption of the COVID-19 pandemic meant that laboratory time was limited, and the fastidious growth requirements of MO and manpower required to perform susceptibility testing made it impossible to complete. However, the *in vitro* susceptibility testing protocol that was adapted from the EUCAST Antifungal susceptibility guidelines[20,21] as part of this investigation, has been included as Appendix 1. No susceptibility testing protocols exist for MO, and despite no results being available, Appendix 1 outlines the culture requirements and growth characteristics of the organism, something that has been collated poorly in the literature in the past, making the repetition of culture, and susceptibility testing difficult.[2,22,23]

Inconsistent Efficacy of Water-Soluble Amphotericin B for the Treatment of Macrorhabdus ornithogaster in a Budgerigar (Melopsittacus undulatus) Aviary

The use of AmB water-soluble medications is commonplace in budgerigar aviaries across Australia. The efficacy of this solution required evaluation, given the variable response to treatment reported anecdotally, and because the formulation's efficacy has not been evaluated since it was originally developed in 1998. Given the poor efficacy of AmB against MO in the controlled treatment of two naturally infected psittacine birds, and a group of experimentally infected chickens in Chapter 4, we needed to evaluate controlled treatment on naturally infected birds on a larger scale. To do this, we evaluated a commercially available water-soluble AmB formulation on a colony of infected budgerigars. Our results demonstrated treatment success in 64% of cases, a result that is inferior to the original 100% clearance rate reported,[24] but that was superior to a similar study that compared water-soluble AmB with oral dosing of 100mg/kg BID in conjunction with in-water medication.[16] Both studies provide robust evidence that water-soluble, in-water treatment with AmB at the current recommended dose rate and duration is an inconsistent, and only partially effective delivery method and confirm that it is not effective for eliminating MO from budgerigar aviaries. Interestingly, there was no difference identified between

infection rates in birds that were administered AmB by mouth twice daily alongside in-water AmB, when compared with those only administered in-water medication.[16] From a welfare perspective, the capture and restraint of birds, twice daily for medication administration causes stress and anxiety, and is not a benign process. The authors concluded that, based on treatment outcomes in their retrospective cohort, in-water medication was favoured when compared with twice daily oral administration of AmB.[16] Given the potential role of stressors and the development of infection and disease discussed in Chapter 6, it is important to take a holistic view, including balancing the welfare and stress considerations of capture and restraint, with the optimisation of treatment outcomes.

In our study, there were birds that tested negative following 10 days of treatment but returned positive results two weeks post-treatment. This apparent treatment failure may in fact be due to false-negative results from faecal cytology or reinfection after treatment success. Treatment failure with AmB has been reported previously[25-27] and each trial had birds that were co-housed, suggesting reinfection as a possible explanation for return to faecal shedding following treatment. Only those birds that remained infected after the initial 10-day treatment period in our study (Chapter 3) were housed individually during the extended treatment period, so birds that remained positive after 20 days represent genuine treatment failure as there was no chance of reinfection from other birds occurring in this cohort.[3] Where treatment failure continues to be a concern, although culling of treatment-unresponsive birds to reduce environmental shedding and risk to other birds might appear a logical approach, culling of positive birds was evaluated previously and did not result in an intergenerational decrease in the rate of MO infection in the ensuing years.[27] However, the long-term selection of birds that are able to tolerate infection, without signs of disease, or those birds that do not become infected, is an approach that warrants further investigation.

Treatment failure with in-water medications can be attributed to birds that are drinking less than normal when they are sick.[27] The average daily water intake of the birds in our study was 7 ml / 100 g of body weight, close to the lower limit required to fulfill the treatment recommendations (6 ml / 100 g of body weight per day). Individual variability between birds implies that it is likely that some birds would have consumed less than the recommended daily volume. The water intake

of the budgerigars in our study was greater than that reported previously in another treatment trial for MO, where the budgerigars drank 5.1 +/- 2.13 ml / 100 g of body weight.[27] Our study took place during the height of summer, where outside temperatures were between 24 °C – 40 °C (75 °F – 99 °F) and budgerigars would be drinking readily. The lack of water consumed, raises concerns about the therapeutic value of in-water AmB in areas where the climatic conditions are cooler, and the birds are naturally drinking less. The requirement for the birds to drink a significant volume of medicated water, may partially explain the variable treatment success reported anecdotally by avian veterinarians across Australia. The variable water intake, combined with the 10-day treatment recommendation by the manufacturer, likely contributed to a failure of protocol rather than a failure of the product. It is possible that the persistent use of AmB at potentially subtherapeutic doses in aviculture since 1998,[24] has resulted in an increase in the number of MO strains that are more resistant to the active ingredient. To explore strain resistance, *in vitro* susceptibility testing is required, and a protocol is yet to be standardised. A proposed protocol is provided for future investigations in Appendix 1.

Macrorhabdus ornithogaster and Viral Co-Infections in Exhibition Budgerigars (Melopsittacus undulatus) in Victoria, Australia

Studies focusing on avian gut bacteria and faecal analysis have traditionally been performed using microbial culture techniques.[28] However, the advent of nucleic acid-based analysis techniques has largely replaced culture techniques. Pathogen screening in faecal samples and evaluation of experimental infections in the gut is now commonly performed using molecular techniques such as PCR.[29] The evaluation of the microbiome using culture-independent methods has been applied in canaries with or without MO and demonstrated significant changes to the gut microbes of infected birds,[13] although such cross-sectional studies cannot differentiate cause and effect of such changes. As the technology has moved away from culture-based techniques, the focus has shifted to the extraction and purification of nucleic acid, from various samples.[30] The extraction of DNA from faeces has proved particularly challenging and today there are many commercially available DNA isolation kits, designed and marketed specifically for faeces. To date, most of the studies evaluating DNA extraction have been performed on mammalian species,[31-35] with some comparing extraction techniques across classes.[35] Faecal

samples vary greatly depending on their host, this is especially evident when comparing avian and mammalian faecal samples.[36] The anatomy and physiology of the avian gut is unique, specifically the crop, ventriculus, proventriculus and cloaca. Food is stored in the crop for future digestion, allowing the bird to move to safety, where the food is then crushed and mechanically degraded in the ventriculus. Prior to excretion, solid residues of ingesta arrive at the avian cloaca, where it is mixed with urinary secretions from the kidneys. The resorption of fluid and water conservation that takes place in the avian cloaca and colon means that droppings are passed as a semi-solid macerate that has a high concentration of uric acid, making DNA extraction from avian faeces challenging.[30,37]

Some samples may need to undergo a pre-treatment process, which may include degradation of the tissue or sample material. This can be achieved by a combination of mechanical, enzymatic, or chemical treatment. The pre-treatment process is a delicate balance between lysing cells to facilitate the subsequent yield of DNA, and the breakdown of the material of interest. This is particularly important in the case of MO, where the thick cell wall appears more robust than much of the surrounding faecal sample material. It is for this reason, that DNA extraction, particularly from avian faecal material, requires a process of optimisation and quality control tailored to the origin of the sample and the target organism.[30,37]

To date, the DNA extraction kits that have been compared for use with avian faeces are limited to the commercially available faecal analysis kits (PowerSoil DNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, CA, USA), Maxwell 16 Tissue DNA Purification Kit (Promega Biotech AB, Stockholm, Sweden), DNeasy Blood & Tissue Kit (Qiagen AB, Sollentuna, Sweden), QIAamp Fast DNA Stool Mini Kit (Qiagen), QIAamp DNA Stool Mini Kit (Qiagen) and QIAamp cador Pathogen Kit (Qiagen)).[37] Traditionally in our laboratory, we have used the DNeasy Blood and Tissue Kit or the QIAamp DNA Stool Mini Kit, but these kits have required various modifications, including the addition of glass beads for vortexing and freeze-thaw lysis cycles to yield appropriate levels of MO DNA from both faecal and pure MO culture samples. In response to these challenges, in Chapter 6 we evaluated the use of the BioSprint® 96 One-For-All Vet Analyser (Qiagen) and compared the DNA quality and yield with the DNeasy Blood and Tissue kit.

The BioSprint® uses MagAttract magnetic-particle technology for nucleic acid purification. The kit uses chaotropic salts and proteinase K to lyse samples in a single step, the nucleic acids then bind to the silica surface of the magnetic particles. DNA and RNA that are bound to these particles are then washed in a series of wash buffers, followed by an air-drying step, improving the purity of the nucleic acids.[38] The BioSprint® kit does not have a protocol for faecal material and has not been evaluated in the literature for use with avian droppings. In Chapter 6 we utilised the 'Animal Whole Blood' protocol (for blood samples) and 'Tissue Protocol' (for faecal samples). The BioSprint® extracted significantly more, high quality DNA from both the blood and faecal samples, especially for faeces. There are many examples of avian literature that use the traditional faecal extraction kits, many of which report good success in identifying their target pathogen;[16,34,39-40] however, many of these studies carry out very little quality control or evaluation of the DNA quality that was extracted as part of their protocol. It is possible that these studies have been returning false negative results because the DNA extraction protocols result in low yield or poor quality DNA from the avian faecal samples. This is yet to be explored in detail, but based on the mediocre results of most of the commonly used commercial extraction kits,[37] it seems plausible, that with optimisation of extraction protocols, improved results will occur. Recently two techniques utilising bead beating, as part of a pre-treatment step have demonstrated good success extracting quality DNA from avian faeces. The extraction of DNA from broiler chicken faeces for evaluation of *Escherichia coli* underwent robust optimsation[41] and the work by Vrbasova et al., specifically evaluated the MO DNA extracted from avian faeces by qPCR. The authors demonstrated repeatable results on qPCR using the NucleoSpin DNA Kit, which utilises mechanical lysis beads in the initial mechanical breakdown step.[6] The thick cell wall of the MO organism provides some resistance to quality DNA extraction, and in our laboratory, we have experienced the challenges of MO DNA extraction using traditional faecal kits. The BioSprint®, whilst not relying on mechanical breakdown, does have hundreds of tiny magnetic beads in each well, that will naturally assist in damaging the cell wall for DNA release. It appears that mechanical breakdown of the cell wall may provide an avenue for further optimisation of traditional faecal extraction kits, when it comes to the release of high-quality DNA from avian droppings, at least for tough targets like MO.

The initial outlay of expenditure to be able to run the BioSprint® 96-well system is significant. But once set up, the cost savings in a high-throughput lab will quickly be recouped. In our study, the time spent extracting the DNA from the 240 samples using the BioSprint®, was the same as the time it took to extract 40 samples using the DNeasy® kits. This represented a significant cost saving, for a superior quality DNA product. Furthermore, the risk of carry-over contamination is decreased when using the BioSprint®. Samples were only opened once, to be transferred into the 96-well plate, this contrasts with the DNeasy Kit protocol, where samples must be opened six times to add new reagents. These findings are supported by a study that assessed the extraction of DNA for evaluation of dietary samples. Samples collected from cormorant gastrointestinal tract had DNA extracted using the BioSprint®, which was more effective at isolating amplifiable DNA and had higher PCR amplification success than a traditional Cetyl Trimethyl Ammonium Bromide (CTAB) protocol.[42] This study too, found the BioSprint® more efficient, by eight times, when compared with the CTAB protocol and despite the higher material costs (1/3 higher for each BioSprint® extraction), the labour component results in a significantly more affordable extraction process when evaluating bulk samples.[42]

Our work has outlined how the BioSprint®, a semi-automated paramagnetic particle-based DNA purification systems achieves high rates of quality DNA amplification. The results align with others in the literature[42] but provide a novel, rapid, efficient, and reliable method for DNA extraction from avian blood and faeces. Further optimisation of this protocol should take place, but this preliminary investigation provides a robust framework for large-scale avian faecal DNA extractions, with drastically reduced costs, time, effort, and risk of contamination.

In Australia, the adoption of molecular diagnostics by the avian veterinary community is improving, but many of the tests remain costly, with a slow turnaround time. Despite this, the commercial application of avian molecular faecal analysis is becoming more available to the end user, and more attractive for molecular diagnostic laboratories to offer. Optimisation of DNA extraction and high-throughput analysis using systems such as the BioSprint® will facilitate an accurate and low-cost system that ultimately benefits all stakeholders. This is especially true of samples that can be collected non-invasively, such as faecal material, which provides an opportunity for aviculturists and breeders to carry out sample

collection, and potentially even submission, in the same way that livestock producers submit faecal samples to district laboratories for evaluation. A move towards improving accessibility of testing for common avian pathogens, especially those that result in morbidity and mortality such as MO and the common viral conditions, will have significant welfare implications for avifauna in Australia.

DNA extracts from faeces were also used to survey potential viral co-infections. The percentage of virus-positive birds in our study was in stark contrast to work carried out previously across Port Phillip Bay in Victoria, which found budgerigars were infected with APV, PBFDV in moderate numbers.[39] Neither study evaluated disease status, but it may be that the population infection status differs, or that the opportunistic sampling carried out in the previous study, where faeces were collected from the floor of flight aviaries, resulted in a higher prevalence due to contamination of the original samples.[39] A cohort of budgerigars from New Zealand demonstrated a 22% prevalence of APV but did not amplify PBFDV DNA from blood samples.[43] These studies highlight the variation that may be present between populations, which could be regional or aviary-specific, and illustrate the importance of careful data interpretation.

The budgerigars that were infected with MO in Chapter 6 were not showing signs of disease. All birds were examined and were in robust body condition, despite shedding MO in their faeces. This is a finding that was consistent throughout our research in Chapters 2, 3 and 5, where many of the birds that were infected and actively shedding did not show signs, despite, in some cases, having very high numbers of organisms in their droppings.[1,3,44] Chapter 6 set out to investigate whether there was a link between immunosuppressive viruses and co-infection with MO. Whether immunomodulation precipitates the disease state, macrorhabdosis, remains unclear, but there was no evidence that viral infections were associated with the MO status of the budgerigars in our study. The link between co-infections and disease associated with MO remains unproven; some investigators have published reports of mortality events, attributed to MO, following stressful environmental conditions.[45] A colony of zebra finches experienced mass mortality caused by MO following exposure to continuous light for 30 days,[46] and MO was reported in 48.3% of budgerigars transitioned onto a commercially formulated diet compared with 3.4% of budgerigars that were left on a seed

mixture.[14] These results may be caused by stress associated with the transition to the pelleted diet,[45] as the budgerigars in this cohort struggled with the transition away from seed and lost body condition, with one bird dying during the transition process.[14] The overgrowth of MO may equally be caused by a change in gastric microbiome as a result of the pelleted diet,[13] or the lower energy pelleted diet, leaving the birds immunocompromised. Finally, as pointed out before, there is considerable variation between regions and aviaries and, in the close confines of the study population, the budgerigars in this cohort may have become more heavily infected by chance when one heavy-shedding bird was included in their cohort.

Further investigation is required to better understand whether any of the postulated stressors may be risk factors for the development of disease.

General Discussion

This thesis has focused on MO in cage birds, specifically exhibition budgerigars in Australia. We have provided data to better characterise the most accurate diagnostic and treatment options available to avian veterinary clinicians. However, there much still to understand about MO as a pathogen. What is clearly understood, based on our work and that of others, is that MO is a pathogen that can cause significant disease, with no current effective treatment options. It is a pathogen that is well established in the avicultural and wild birds in Australia and, whilst not specifically explored in this body of work, it is a pathogen that is increasingly reported in backyard poultry worldwide.[47,48] It is unclear whether this is because poultry are being presented more frequently to avian veterinarians as pets, or because infected wild avian reservoir species are transmitting infection more frequently. The welfare-driven shift towards free-range egg production systems in Australia,[49] combined with the tendency of these systems to attract wild birds, means that MO is an emerging pathogen of concern in free-range layer hen and meat chicken flocks. The welfare, financial and disease implications of MO for these systems is not characterised beyond diarrhoea and altered growth rates reported in the few experimental infection studies in chickens.[3,15,50] It is essential that priority be placed on investigating the impacts of MO in chickens with evaluation of signs associated with disease, and follow-up metrics evaluating egg production, meat yields and histological changes in the stomach.

Further work is required to optimise the diagnostic process for MO so that molecular techniques, PCR and qPCR[6] may become useful as a reliable and effective screening tool, for avicultural and pet birds shedding the organism. qPCR has recently been identified as a feasible and accurate method of detecting MO in faecal material, which has provided a framework for further optimisation.[6] Much of this optimisation will centre around the successful and repeatable extraction of MO DNA from faecal material, potentially using high-throughput semi-automated extraction techniques such as the BioSprint® outlined in this thesis. Advances in technology, in conjunction with the affordable, customisable, and accessible nature of modern molecular diagnostic tools, makes it plausible that qPCR machines will become part of an in-clinic diagnostic suite in the coming years. Instruments that can run qPCR panels, like the TrueMark Infectious Disease Research Panels from ThermoFisher Scientific (Scoresby, 3179, VIC Australia), are customisable and allow selection of specific target organisms via a custom panel.[51] The creation of a customisable quarantine screening test or a flock health profile for large aviculturists using this technology will allow rapid and accurate detection of pathogens specifically concerning for that collection or species, and will not require extensive laboratory training given the robust internal quality control mechanisms.

As with many of the studies in the literature evaluating MO, some of the results in our work raise the question of external validity, or how the results may be applied to collections outside of our study population. Other methodological limitations that we have discussed include the use of the same samples with multiple diagnostic tests, and the evaluation of detection probability as a measure of sensitivity. Robust epidemiological aspects of study design can help to address the issue of generalisability. Had the opportunity arisen, it would have been beneficial to collect fewer samples per flock across a larger number of flocks, to increase the representativeness of our dataset and to make more general inferences about how these tests can be applied across a wider study population. There is a huge appetite for robust scientific data within the budgerigar fancier community and many fanciers were happy to open their aviaries for research. Difficulties arise for researchers when it comes to sourcing funding for research into avian species other than poultry. Above all, in this research, the difficulties surrounded travel and access to these aviaries during the COVID-19 travel restrictions. The access to funding in non-chicken species will remove one of the major roadblocks for future

investigators and provide the opportunity to establish more data specific to MO management and treatment, that will ultimately benefit the wider avian community, including veterinarians working with various avian species and poultry producers.

Alternative Treatment Modalities

Many of the challenges faced in treatment of avian patient are also faced by clinicians and researchers aiming to treat humans with AmB, including patient gastric pH, temperature, and the amphipathic nature of AmB. The oral administration of AmB in birds will ultimately benefit from the race to develop a thermally stable, orally bioavailable AmB formulation for use in humans. Novel nanoparticulate drug delivery systems have been under development for oral administration with formulations such as AmbiOnp,[52] nanosuspensions, lipid-based drug delivery systems including cochleates, self-emulsifying drug delivery systems,[53] solid lipid nanoparticles[52] and polymeric nanoparticles[54] demonstrating potential for oral drug delivery, in clinical trials in humans.[55]

The most promising of these formulations for application against MO in avian patients, is the oral liposomal formulations of AmB (LAmB).[56] These liposomal formulations decrease toxicity *in vitro* and *in vivo*[56] and show outstanding systemic bioavailability in rodent models[57-60] as well as in stage one human trials. The systemic bioavailability may facilitate longer GIT exposure and more efficacious treatment against MO, especially where inflammation and hyperplasia of the glandular tissue has afforded protection against transient luminal drugs. The novel AmB suspension utilises a mixture of vitamin E; an anti-oxidant for formulation stability, gelucire; a nonionic water-dispersible surfactant for lipid-based formulations to improve wettability and active solubility and peceol; an oil to act as an emulsifier, mixed with AmB at a variety of concentrations.[57] These formulations were created in an attempt to produce a stable drug for use in tropical locations for the treatment of leishmaniasis and fungal infections; it was therefore necessary to have excellent stability at high temperatures, something that is going to be extremely important in avian patients.[61] Following exposure to high temperatures of up to 43°C for 60 days, the liposomal formulation displayed no decrease in efficacy,[57] allowing extrapolation that this same formulation may be suitable for oral application in avian species that have similar body temperatures. The same trial assessed efficacy of the formulation in a simulated fasted-state intestinal fluid model

with a pH of 6.5. The formulation remained unchanged with few or no crystalline features, and the drug was found to be 95% available after 4hrs at 37°C.[57] The gastrointestinal fluid pH in the trial was significantly higher than that of the avian proventriculus,[62] and the growth requirements for MO of pH 3–4,[50] but provides some evidence that a significant proportion of the drug remains available in an acidic environment. The oral formulation iCO–010 was the most thermally stable and displayed treatment efficacy against systemic candidiasis and visceral leishmaniasis, with the highest affinity for deposition in the liver, kidney and spleen in both rat and mouse models, which gives rise to concerns regarding toxicity, following systemic absorption in avian patients.[59,61] Thankfully, no evidence of toxicity occurred during the rodent trials using iCO–010.[57,59,61]

Given the apparent efficacy and safety of liposomal formulations of AmB, it is possible that this, or a close modification of the formulation may be significantly more effective against MO infections when given orally. Work with these novel drug delivery technologies is ongoing and aims to standardise a successful, cost effective and safe formulation for administration in humans. It is hoped that the oral formulations may become commercially available and can be trialled for the treatment of MO in cage birds under dispensation from the APVMA.

Ventricular Beads

Macrorhabdus ornithogaster is unique in that it grows in such a narrow zone, with very strict growth conditions. This specific growth environment, combined with the unique anatomy and physiology of birds, makes local treatment using ventricular beads a treatment modality that warrants exploration. The gastric contents move between the ventriculus and proventriculus in a rhythmic motion under autonomic control.[63] It is this transfer of ingesta that makes it theoretically possible, to achieve high drug concentrations at the proventricular isthmus by having a constant source of drug product within the ventriculus. The ventriculus functions as a grinding stomach, which naturally stores rocks, grit, and sand to help with the mechanical breakdown of food. This unique adaptation means that the ventriculus will store appropriately sized drug elution particles, without having to worry about them passing via the small intestine, one of the major problems with sustained release drugs in humans.[64] In theory, sustained release particles can sit in the ventriculus, and attrition of antifungal or pH modifying agents under the activity of the

ventricular contraction will release continuous, local pharmacological agents, achieving high concentrations of the desired drug at the proventricular isthmus. Cornerstones of success in using such a delivery technique include the ability to administer the slow-release particles, the stable state of the active ingredient at high temperatures and low pH, and the ability to control the medication's rate of release from the ventricular particles to prevent over- or under-dosing. There are currently no published attempts outlining the administration of antibacterial or antifungal continuous release beads in the ventriculus or proventriculus of birds. However, the literature does provide some framework to guide future research. Appropriate ventricular bead diameter has been examined in budgerigars, with 1mm metallic spheres remaining present in the ventriculus for an average of between 33 and 75 days.[65] Barium-impregnated spheres (BIPS) were used to examine gastrointestinal transit time and established that 1.5mm BIPS remained in the ventriculus for 368.0 ± 176.8 hours post administration in the domestic pigeon (*Columba livia*).[66] When compared with liquid barium, it was concluded that BIPS were not useful in determining gastrointestinal transit time in birds with a muscular grinding stomach. This highlights that spheres administered orally do not just pass through the ventriculus and that retention of granular material administered therapeutically is possible. This retention is essential for the success of a slow-release bead product, where the large particles will remain, as grit does, in the ventriculus.

The use of antifungal agents in polymethylmethacylate (PMMA) beads[67-74] has been explored in the human literature. Of these, AmB, anidulafungin and voriconazole appear to be the most suitable active ingredients when considering the treatment of MO. Each of these drugs has been shown to have consistent elution from the PMMA for more than two weeks *in vitro*.[67] Various studies have examined AmB and its elution from PMMA beads with conflicting results.[68-71,75] The delivery of AmB using bone cements showed excellent long-term elution, with a constant concentration present *in vitro* in foetal bovine serum for 45 days.[71] However, the authors had concerns about the bioavailability of AmB, with only 3.7% of the AmB in the beads available in the serum.[71] The concentration of serum AmB in this study did not prevent growth of *Candida albicans in vitro*, further compounding concerns about whether the AmB remained active in the serum.[71] This is in contrast to *in vivo* case reports of concentrations of AmB up to 3.2µg/ml in fluid drained from a surgical wound[70] and successful surgical correction involving the use of AmB-impregnated

beads for fungal osteomyelitis, demonstrating high concentrations and prolonged AmB activity at human body temperature.[68] Calcium sulphate (Osteoset, Write Medical Technology Inc, Arlington, Tennessee) impregnated with voriconazole has been shown to have equivalent elution characteristics to PMMA beads.[67] Osteoset is a calcium sulphate matrix and will likely degrade in the ventriculus of the bird without causing adverse effects and potentially even providing some benefit in calcium availability.

When it comes to the make-up of the ventricular beads, it is established that adding a soluble poragen or insoluble filler increases the porosity and improves the release of hydrophilic antimicrobials such as aminoglycosides or vancomycin.[69] It remains unclear what effect this would have on a hydrophobic compound such as AmB. A high poragen load (greater than 10% volume) is considered necessary to give PMMA extensive interconnecting pores. It is unclear what dose rate or range would be required in avian patients to achieve therapeutic levels against MO; however, high doses of AmB can be applied to cement beads and have been reported to achieve therapeutic levels in other species. Marra et al. used a formulation of 750mg of AmB in four batches of PMMA beads and this was considered sufficient to be detectable in serum and to achieve local therapeutic levels when used to treat osteomyelitis in a human.[70]

Further research is warranted to investigate the feasibility and efficacy of orally administered ventricular beads for the treatment of MO. Bone cement packs are available commercially in a variety of diameters, and creation of the beads is done via a ready-to-mix sterile bone cement pack. Appropriate ventricular bead size would need to be established for the various avian species, and variations are likely to occur based on size, age, and the natural diet of the bird. This is an important area of future study and will facilitate successful storage of the beads within the ventriculus. Various molds are available and can be adjusted based on the diameter of the bead required; this gives the flexibility to treat multiple species. Hand mixing and rolling the beads is also a feasible, albeit labour-intensive way to achieve the appropriate size, and this may be the most viable option given the 1mm ventricular particle size reported previously in budgerigars.

Future Directions and Priorities for Investigation

The knowledge base around MO is continuing to grow at a steady pace; however, there are some key priorities that should be addressed. Firstly, the investigation of *in vitro* susceptibility of a variety of therapeutic agents, including antifungal drugs not previously utilised against MO, should be investigated. Appendix 1 provides a discussion and guide, outlining a standardised approach to susceptibility testing based on the EUCAST guidelines. With this knowledge and any novel agents identified as being effective against MO, investigation into the novel drug delivery technology mentioned above should be carried out. During this investigation, the potentiation of drugs through addition of antioxidants such as L-ascorbic acid should be investigated as a cheap, but potentially efficacious, method of improving antifungal effects against MO. It is likely that the application of an effective therapeutic agent at an appropriate dose, combined with the effective delivery of drug to the isthmus, will result in treatment success, despite all the challenges mentioned in this thesis. Investigation of MO should be expanded to include species outside the scope of the literature encompassing traditionally affected species. In chickens specifically, the effects of MO on brown layer strain chickens should be investigated and the implications weighed against the perceived welfare benefits to free-range poultry systems. This research will help inform prevention, diagnostic and treatment efforts when the eventual spillover from wildlife reservoirs to free-range poultry occurs. Finally, continued evaluation and optimisation of DNA extraction techniques from avian droppings is needed. This will benefit research extending far beyond MO; however, the thick cell wall and robust nature of the organism provides a unique challenge to researchers and so MO may act as a guide to the quality and quantity of difficult-to-extract DNA. The improved extraction techniques will then inform the development of in-clinic or accessible laboratory molecular techniques, the most promising of which is qPCR as reported recently by Vrbasova et al.[6]

Conclusion

Macrorhabdus ornithogaster is an important pathogen of pet and commercially kept birds. Its high prevalence in Australian native birds, combined with the result from our challenge trial in chickens, suggests that spill-over into free range poultry might be possible and add to its importance. Prior to our work, the diagnostic tools, both in-clinic and in the laboratory, lacked a gold standard. We have established that the

faecal suspension technique performs better than any other microscopic technique when utilising faecal samples, and that direct cytology of a sample collected from the isthmus is as good as PCR of DNA extracted from the isthmus at detecting MO. Direct cytology of the isthmus and the faecal suspension technique are quick, cheap to perform and user-friendly tests that have better sensitivity when compared with the other ante-mortem and post-mortem diagnostic tools respectively. Improvements to DNA extraction methods may improve the sensitivity and large-scale feasibility of faecal diagnostics in live birds.

We presented multiple sequences of treatment failure with AmB at various dose rates and delivery methods. Treatment failure appeared to be the result of a failure of protocol, rather than a failure of drug efficacy. Differentiation of those options requires consistent application and assessment of treatments, and *in vitro* susceptibility testing. Meanwhile, there is also an urgent need for the formulation of a more effective treatment regime or delivery modality. We have presented multiple novel treatment options and drug delivery technologies, many of which, if successful, will change the treatment regime for MO, but may also have far-reaching consequences for the way avian patients are medicated.

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Appendix 1. In-Vitro Antimicrobial Sensitivity Testing – A Framework For Future Investigations

Introduction

Culture and sensitivity testing is now commonplace for yeasts, moulds and fungi, with various standardised protocols, minimum inhibitory concentration (MIC) values and breakpoints established for the more common organisms.[1,2] However, *Macrorhabdus ornithogaster* (MO) understanding and culture techniques have not experienced the same trajectory in growth of the knowledge base and there is only a single publication examining the methodology and efficacy of culture techniques.[3] Formerly thought to be a large bacterial rod, it was not until 2003 that phylogenetic analysis revealed it as a novel, ascomycetous yeast that remains the only known member of its family.[4]

The lack of standardised laboratory techniques is due, in part, to the relatively immature nature of the literature and our understanding surrounding this unique organism, but also likely due to the current lack of economic or human health implications caused by MO. The first publication mentioning a filamentous bacterial rod causing a wasting disease syndrome in budgerigars was published in 1977[5] and this was followed by a report in canaries in 1980[6] that coined the term 'megabacteria.' In the last 30 years, various groups have attempted to culture the organism. Initially, a low number of passages were achieved using MRS-Medium (agar and broth; Marck, Darmstadt, Germany), a medium used to isolate *Lactobacillus* spp., but the organism became increasingly degenerate using this technique.[7] In 1990, Scanlan and Graham reported success culturing the organism on blood agar in a facultative anaerobic environment with 10% CO_2. The authors reported an extensive variety of standard bacterial culture and sensitivity profiles as well as reporting eight different strains of an organism, initially exhibiting the morphology typical of MO.[8] However, it appears likely based on the conclusions of this study and the variety of results obtained, that the organisms being cultured were not MO and were instead Gram-positive bacterial rods. More recently, Ravelhofer et al. described a successful culture technique using two different liquid media. The media contained minimal essential medium (MEM; Seromed Biochron KG, Berlin, Germany) or MRS broth (Merck), 20% foetal bovine serum, saccharose 5% and enrofloxacin 100µg/mL or penicillin-streptomycin. This was the first report successfully quantifying the growth of MO and the authors found that the number of microorganisms doubled over 11 days.[9] During the passage, the authors observed the organisms morphologically and identified Y-branching chains and club-like

condensation of one end of the organism, characteristics consistent with MO.[10] Despite successfully growing MO, the slow rate at which the organisms grew suggests that the culture technique required optimisation and that the growth of the organism may have been erroneously identified as successful, based on future publications.[10]

Hanafusa et al. optimised the very specific, fastidious growth requirements in 2007.[3] In order to consistently passage the organism, enriched culture medium, made up of RPMI, 5% glucose, 20% foetal bovine serum and with a pH of 3–4, was used. The authors performed the culture under microaerophilic conditions at 38–42°C and were successful in maintaining a cell line for 22 passages over 90 days.[3] The organism also demonstrated improved growth characteristics when exposed to oxygen at between 5°C and 42°C for 24hrs prior to microaerophilic culture. For the first time, the authors were able to fulfil Koch's postulates by infecting a group of white leghorn chickens (*Gallus gallus domesticus*) with a strain cultured *in vitro* but originally collected from a budgerigar.[3] The chickens developed soft faeces and showed decreased weight gain and heterophilic inflammation of the proventricular isthmus compared with the control group, indicating the MO was responsible for the clinical signs.[3]

The fastidious growth requirements have hampered attempts at further characterisation and *in vitro* culture and sensitivity testing, and, to date, all published sensitivity trials have been carried out *in vivo*.[11-13] An unpublished *in vitro* culture and sensitivity trial was carried out on two isolates of the organism from budgerigars.[14] A variety of antifungal and low-toxicity antifungal chemicals were trialled to establish an effective *in vitro* treatment for MO. The authors sampled AmB, potassium sorbate, gentian violet, sodium and potassium benzoate, sodium acetate, fluconazole, nystatin and fumagillin in methanol using serial dilutions. Growth was measured spectrophotometrically by assessing optical density before and after incubation. The authors found that gentian violet, potassium sorbate, sodium benzoate, and potassium benzoate inhibited growth of MO at all concentrations tested.[14] This work performed sensitivity testing at a variety of dose rates and provides a framework for appropriate *in vitro* sensitivities but did not manage to establish MIC for any of the antifungals tested.

The protocols for establishment of antifungal MICs for yeasts are well described. The European Committee on Antimicrobial Susceptibility Testing (EUCAST) outlines the

current accepted gold standard for performing antifungal broth dilution minimum inhibitory concentrations.[2] Whilst the technical aspects of the culture media require modifications, the same principles can be applied to culture and sensitivity testing for MO. Because many treatment trials and clinical reports of successful treatment have been carried out *in vivo*, it is difficult to extrapolate these results to an *in vitro* setting.[10-13,15-18] Nonetheless, these reports help to inform the antifungal medications selected for analysis.

Macrorhabdus ornithogaster has been reported in a huge variety of avian hosts, from passerines to poultry, and has been identified in over 40 species.[10,19] Because of the difficulty in performing culture, most studies have only identified the organism morphologically. It has been postulated previously that a variety of MO strains may exist, and these strains may vary between species and taxa,[19] but more recently very little genetic variation was reported in the ITS and IGS regions of MO in Japan. The authors found that MO collected from pet birds in Japan showed little genetic variation, with no ability to distinguish between host species, signs associated with infection or MO shape.[20] Genetic analysis has identified MO definitively as a yeast[4] but, despite this, in the last 15 years very few nucleotide sequences isolated from only a handful of bird species are recorded in GenBank.[4,20,21] A small number of single nucleotide polymorphisms are reported, with at least five genotypes present in passeriform birds[21] and two different genotypes, A, isolated from Passeriformes and group B, isolated from Psittaciformes, based on the genotyping of the ITS region,[22] while the host of each group can be divided into Passeriformes and Psittaciformes, a trend that was also observed recently in Germany.[3] Work carried out in Japan, evaluating the ITS, D1/D2 regions as well as the IGS1 region (a region commonly used in the phylogenetic analysis of other pathogenic fungi) revealed a small number of single nucleotide polymorphisms in both passerine and psittacine birds. This work also found that MO isolated from Passeriformes was included in group B, refuting the notion that the groups proposed by Abdi-Hachesoo et al.[22] are completely separated by taxonomic order of the host bird.[20] The authors also found no association between the MO genotype, morphology or disease states of the birds in the study and they conclude that the ITS and IGS1 regions contain little genetic variation and are unsuitable for molecular epidemiological studies of MO.[20] Because identical gene fingerprints have been identified in multiple different avian species, MO is not

considered to be species-specific, but it remains possible that a variety of MO strains may exist.

Reports of successful treatment using amphotericin B (AmB) vary from 5mg/kg PO BID[11] to 200mg/kg PO BID,[15] and treatment failure has been reported using 100mg/kg.[12,23] It is unclear whether the cause of this treatment failure is drug bioavailability, poor AmB activity at the site of infection or strain-specific variability in response to treatment. Variability between strains may explain the mixed results when MO is treated with AmB medication during treatment trials and clinical reports in the literature.[11-13,23]

This work aims to establish a standardised culture and sensitivity protocol for MO, that allows genotyping of pure isolates to assess inter-strain variability in relation to their susceptibility to commonly used antifungal agents. With good record keeping and assessment of the avian hosts prior to collection, this will allow the end user to establish whether there is inter-species strain variability, whether these strains vary in pathogenicity and how the various strains respond to therapy with common antifungal agents.

Culture Media

The composition of the MO culture media is outlined in Table A1.

Table A1. Culture media formula

Step	Reagent	Volume	Instructions
1	Basal Medium Eagle (BME)	800mL	
2	Sterile foetal bovine or chicken serum	200mL	Mix well
3	Penicillin G Sodium	100 U/mL	Mix well
4	Streptomycin	100mg/mL	Mix well
5	Glucose / sucrose crystal	50g	Agitate until dissolved
6	Hydrochloric acid	To effect	Real time measurements of pH until pH reaches 3–4. Mix well and re-test and aliquot.
7	Filter sterilise	0.22 µm pore size filter	

Important Notes

Store at 4°C or lower for up to a month – ensure sterilising when aliquoting for quality control purposes, use one aliquot of the sterilised medium for sterility

checks, for retesting the pH (2.9–4.1 is acceptable) and as a growth control with an untreated strain during each passage of *Macrorhabdus ornithogaster* growth.

Antifungal agents

Preparation of stock solutions

The amount of powder or diluent required to prepare a standard solution is calculated as follows:

$$\text{Volume (L)} \times \text{Concentration (mg/L)}$$

$$\text{Weight (g)} = \text{Potency (mg/g)}$$

The pure antifungal powder should be weighed on an analytic balance and antifungal drug stock solutions prepared at concentrations 200× the highest concentration to be tested in the microdilution plate. Follow the manufacturer's instructions regarding appropriate diluents; if water is not deemed appropriate, alternative solvents should be sought to dissolve antifungal drugs. See Table A2 for a list of drugs requiring solvents other than water. If the drugs are failing to dissolve, a rocking table can be utilised. Once the stock solution has been formulated, it can be stored in sterile polypropylene vials at −70°C for no more than six months.[1]

Table A2. Solvents and diluents for preparation of stock solutions for antifungal agents requiring solvents other than water.[1]

Antifungal Agent	Solvent	Diluent
Amphotericin B	Dimethyl sulphoxide	Growth Medium
Ketoconazole	Dimethyl sulphoxide	Growth Medium
Itraconazole	Dimethyl sulphoxide	Growth Medium
Flucytosine	Dimethyl sulphoxide or 50:50, acetone: water	Growth Medium

Preparation of working solutions

The range of concentrations tested should be determined based on the antifungal drug. The only breakpoints for MO susceptibility in the literature were obtained from a poster presentation[14] and from unpublished data from our laboratory, these provide the only framework for guiding future in vitro drug trials. The breakpoints that were established are in Table A3.

Table A3. Breakpoint guides for *Macrorhabdus ornithogaster* susceptibility established previously from our work and from the literature.[14]

Antifungal agent	Drug concentration breakpoint	Unit
Amphotericin B	0.5	µg/mL
Potassium sorbate	0.1	mg/mL
Gentian violet	0.5	mg/mL
Sodium benzoate	0.05	mg/mL
Potassium benzoate	0.5	mg/mL
Sodium acetate	2	mg/mL
Nystatin	10	IU/mL
Fluconazole	45	µg/mL

Preparation of inoculum

The ability to culture and then create viable, standardised inoculum colonies is imperative for accurate and reproducible MO susceptibility testing. The inoculum should be prepared by suspending five representative samples of 10µL, obtained from a 24hr culture in a fresh inoculum of BME media. The final inoculum must be between 0.5×10^5 and 2.5×10^5 CFU/mL.

Preparation of microdilution plates

Culture media should be added to either 24 or 96 well plates, with serial dilutions of the antifungal agents in each well. To ensure MO viability, the plates should be inoculated within 30 min of preparing the inoculum suspension. Prior to inoculation of each well with 100µL of the $1–5 \times 10^5$ CFU/mL MO suspension, the inoculum suspension should be vortexed to maintain consistent homogenisation throughout the microdilution plates and prevent settling. All inoculation should occur without touching the contents of the well. In each plate, there should be control wells, that contain 100µL of sterile drug-free medium, with 100µL of the same inoculum suspension, alongside 100µL of sterile culture media from the lot used to prepare the inoculum that acts as a control for sterility of the medium.

Incubation of Microdilution Plates

The microdilution plates should be incubated without agitation at 40 ± 2°C in a microaerophilic sleeve for 24 ± 2 hrs.

Reading Results

The microdilution plates should be read using a microdilution plate reader via spectrophotometry. The recommended wavelength for measuring the absorbance of the plate is 530nm.[1] Establishing a baseline value (blank wells) allows this value to be subtracted from the readings in the sample wells.

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