MLA OJD HARVEST YEAR CONFERENCE
December 8-9, 2005

Co-sponsored by

Pfizer Animal Health
Contents

MLA Harvest Year Conference Programme.........................4
National and International Perspectives.........................6
History of OJD and research priorities in the NOJDP.......................7
A producer perspective – what has the research meant to me? .............11
An advisor’s view of OJD research ...............................................................15
Diagnosis – Existing Projects...............................................20
Hybridisation capture PCR and direct PCR on pooled faecal samples.....21
Validation of the gamma-interferon test for diagnosis of ovine Johne’s disease.................................................................28
Manipulation of the Interferon - gamma assay to maximise responses to M. ptb antigen in sheep .................................................................32
Evaluation of a Pourquier ELISA kit in relation to agar gel immunodiffusion (AGID) test for assessment of the humoral immune response in sheep and goats with and without Mycobacterium paratuberculosis infection ...34
Diagnosis – New Approaches.................................................39
Early cellular responses in ovine Johne’s disease ....................................40
Apoptotic responses during the pathogenesis of ovine Johne’s disease.44
ELISPOT – new methods for detecting IFN-γ...........................................48
Detection of Mycobacteria from blood..................................................51
Proteomic analysis of sheep serum by SELDI TOF-MS: Identification of putative biomarkers of ovine Johne’s disease.................................................55
Gene expression signals from the host ..................................................60
Molecular approaches to studying the host-parasite interaction ..........62
Review of Immunology, Pathology and Pathogenesis from 8th
International Colloquium on Paratuberculosis..................................65
Review of Molecular Biology, Microbiology, and
Culture from 8th International Colloquium on Paratuberculosis ..........70
Vaccination ...............................................................................77
Overview of OJD vaccination – international perspective .................79
Efficacy of a killed Mycobacterium paratuberculosis vaccine for the control of OJD in Australian sheep flocks ....................................................86
On-farm impacts of vaccination with GudairTM ....................................................98

Public Health and National Programs .........................105
Review of National Level Prevention and Control from 8th International Colloquium on Paratuberculosis .................................................................106
Review of Prevention and Control at a Herd Level from 8th International Colloquium on Paratuberculosis .................................................................112
Review of Implications for Public Health 8th International Colloquium on Paratuberculosis ...........................................................................................117
Crohn’s Treatment Trial ................................................................................120

Epidemiology, Economics .................................................121
Epidemiology of ovine Johne’s disease cross species transmission and survival of the organism in the environment ........................................122
Development of grazing management strategies for the control of ovine Johne’s disease ..................................................................................................128
Within flock spread of OJD - a case study from a sheep flock located in southeastern New South Wales ..............................................................................135
Risk factors for OJD prevalence in infected flocks ........................................144
Economic modelling of the impact of OJD ......................................................152
Genomic and proteomic comparative study of the sheep and cattle strains of Mycobacterium avium subsp. paratuberculosis ......................................159
A survey of potential wildlife reservoirs for Mycobacterium paratuberculosis ......................................................................................................................174
Review of Epidemiology from 8th International Colloquium on Paratuberculosis .................................................................................................183
Epidemiology of Johne’s disease: Recent developments and future trends ..........................................................................................................................189
# MLA Harvest Year Conference Programme

<table>
<thead>
<tr>
<th>Theme</th>
<th>Topic</th>
<th>Time</th>
<th>Speaker</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Day 1 - Thursday 8 December</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Welcome</td>
<td>Introduction &amp; Chair</td>
<td>9:00</td>
<td>Lorna Citer</td>
</tr>
<tr>
<td>Welcome</td>
<td>Welcome</td>
<td></td>
<td>Reuben Rose, Neil Buchanan</td>
</tr>
<tr>
<td>National &amp; International perspectives</td>
<td>History of OJD and research priorities in the NOJDP</td>
<td></td>
<td>David Kennedy</td>
</tr>
<tr>
<td></td>
<td>A Producer perspective - What has the research meant to me?</td>
<td></td>
<td>Steve Burgun</td>
</tr>
<tr>
<td></td>
<td>An advisor’s view of OJD research</td>
<td></td>
<td>David Sackett</td>
</tr>
<tr>
<td></td>
<td>Discussion/Questions</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Morning tea</strong></td>
<td></td>
<td>10:30 - 11:00</td>
<td></td>
</tr>
<tr>
<td>Theme 1</td>
<td>Introduction &amp; Chair</td>
<td></td>
<td>Evan Sergeant</td>
</tr>
<tr>
<td>Diagnosis – existing projects</td>
<td>Specificity of molecular diagnosis</td>
<td></td>
<td>Martin McLoon</td>
</tr>
<tr>
<td></td>
<td>HC and Direct PCR on pooled samples</td>
<td></td>
<td>Ian Marsh</td>
</tr>
<tr>
<td></td>
<td>Evaluation of gamma-interferon assay</td>
<td></td>
<td>David Stewart</td>
</tr>
<tr>
<td></td>
<td>Improvements to the IFNG-g assay</td>
<td></td>
<td>Richard Whittington</td>
</tr>
<tr>
<td></td>
<td>Evaluation of Pouquier ELISA</td>
<td></td>
<td>Sanjeev Gumber</td>
</tr>
<tr>
<td></td>
<td>Discussion/Questions</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Lunch</strong></td>
<td></td>
<td>12:30 - 1:30</td>
<td></td>
</tr>
<tr>
<td>Theme 2</td>
<td>Introduction &amp; Chair</td>
<td></td>
<td>Richard Whittington</td>
</tr>
<tr>
<td>Diagnosis – new approaches</td>
<td>Early cellular responses in OJD</td>
<td></td>
<td>Kumi De Silva</td>
</tr>
<tr>
<td></td>
<td>Apoptotic responses during the pathogenesis of OJD</td>
<td></td>
<td>Sally Browne</td>
</tr>
<tr>
<td></td>
<td>ELISPOT – new methods for detecting G-IFN</td>
<td></td>
<td>Doug Begg</td>
</tr>
<tr>
<td></td>
<td>Detection of Mptb in blood</td>
<td></td>
<td>Kate Goldsmith</td>
</tr>
<tr>
<td></td>
<td>Proteomic analysis of sheep serum</td>
<td></td>
<td>Ling Zhong</td>
</tr>
<tr>
<td></td>
<td>Gene expression signals from the host</td>
<td></td>
<td>Lyrissa Di Fiore</td>
</tr>
<tr>
<td></td>
<td>Molecular approaches to studying the host-pathogen interaction</td>
<td></td>
<td>Deb Taylor</td>
</tr>
<tr>
<td><strong>Afternoon Tea</strong></td>
<td></td>
<td>3:00 - 3:30</td>
<td></td>
</tr>
<tr>
<td>Theme 2 continued</td>
<td>Review of Immunology, pathology and pathogenesis stream from 8ICP</td>
<td></td>
<td>Mark Lanigan</td>
</tr>
<tr>
<td></td>
<td>Review of Molecular biology, Microbiology and Culture stream from 8ICP</td>
<td></td>
<td>Ian Marsh</td>
</tr>
<tr>
<td></td>
<td>Discussion/Questions</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Theme 4</strong></td>
<td>Introduction &amp; Chair</td>
<td></td>
<td>Joan Lloyd</td>
</tr>
<tr>
<td>Vaccination</td>
<td>Overview – international picture</td>
<td></td>
<td>Peter Windsor</td>
</tr>
<tr>
<td></td>
<td>Gudair field evaluation</td>
<td></td>
<td>Leslie Reddacliff</td>
</tr>
<tr>
<td></td>
<td>On-farm impact of vaccination</td>
<td></td>
<td>Jeff Eppleston</td>
</tr>
<tr>
<td></td>
<td>Vaccine safety</td>
<td></td>
<td>Dominic Dell'Osa, Pfizer</td>
</tr>
<tr>
<td></td>
<td>Discussion and questions</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Finish</strong></td>
<td></td>
<td>5:30</td>
<td></td>
</tr>
<tr>
<td><strong>Dinner</strong></td>
<td></td>
<td>7:00 for 7:30 pm</td>
<td></td>
</tr>
</tbody>
</table>
### Day 2 – Friday 9 December

<table>
<thead>
<tr>
<th>Time</th>
<th>Session</th>
<th>Presenter(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9:00</td>
<td>Introduction &amp; Chair</td>
<td>Joan Lloyd</td>
</tr>
<tr>
<td>9:30</td>
<td>Review of National Level Prevention and Control stream from 8ICP</td>
<td>Luzia Rast</td>
</tr>
<tr>
<td>10:00</td>
<td>Review of Herd level Prevention and Control stream from 8ICP</td>
<td>Peter Windsor &amp; Jeff Eppleston</td>
</tr>
<tr>
<td>10:30</td>
<td>Review of Public Health stream from 8ICP</td>
<td>Sally Spence</td>
</tr>
<tr>
<td>10:45</td>
<td>Crohn’s treatment trial</td>
<td>Warwick Selby</td>
</tr>
<tr>
<td>10:45</td>
<td>Discussion and questions</td>
<td></td>
</tr>
<tr>
<td>10:15 - 10:45</td>
<td>Morning Tea</td>
<td></td>
</tr>
<tr>
<td>10:15</td>
<td>Introduction &amp; Chair</td>
<td>Peter Windsor</td>
</tr>
<tr>
<td>10:45</td>
<td>Theme 3 Overview – epidemiology research including cross species transmission sheep-goat, sheep-cattle and survival in the environment</td>
<td>Richard Whittington</td>
</tr>
<tr>
<td>11:15</td>
<td>Effect of age and pasture contamination on OJD expression</td>
<td>Helen McGregor</td>
</tr>
<tr>
<td>11:30</td>
<td>Within-flock transmission of OJD</td>
<td>Luzia Rast</td>
</tr>
<tr>
<td>11:45</td>
<td>Risk factors for OJD prevalence in infected flocks</td>
<td>Navneet Dhand</td>
</tr>
<tr>
<td>12:00</td>
<td>Efficacy of OJD eradication by destocking</td>
<td>Pat Taylor</td>
</tr>
<tr>
<td>12:15</td>
<td>Economic modelling of the impact of OJD</td>
<td>Russell Bush</td>
</tr>
<tr>
<td>12:30 - 1:30</td>
<td>Lunch</td>
<td></td>
</tr>
<tr>
<td>12:30</td>
<td>Theme 3 continued</td>
<td></td>
</tr>
<tr>
<td>12:30</td>
<td>Molecular basis of host specificity</td>
<td>Ian Marsh</td>
</tr>
<tr>
<td>12:45</td>
<td>Wildlife reservoirs</td>
<td>Pat Kluver</td>
</tr>
<tr>
<td>12:50</td>
<td>Review of Epidemiology stream from 8ICP</td>
<td>Paul Freeman</td>
</tr>
<tr>
<td>12:55</td>
<td>Recent developments in JD epidemiology</td>
<td>Evan Sergeant</td>
</tr>
<tr>
<td>1:00</td>
<td>Discussion and questions</td>
<td></td>
</tr>
<tr>
<td>3:00 - 3:30</td>
<td>Afternoon Tea</td>
<td></td>
</tr>
<tr>
<td>3:00</td>
<td>Finish</td>
<td></td>
</tr>
</tbody>
</table>
National and International Perspectives
History of OJD and research priorities in the NOJDP

David Kennedy
Technical Adviser
Animal Health Australia’s National Johne’s Disease Programs
PO Box 2321 Orange NSW 2800 Australia
Phone: 02 6365 6016; Fax 02 6365 6088
E-mail: david@ausvet.com.au

The detection of ovine Johne’s disease (OJD) in the central tablelands of New South Wales in 1980 did not precipitate a coordinated response for over 15 years. In the mid-1990s not only was it realised that the infection was spreading more widely and causing heavy losses in some flocks in NSW, but OJD was also confirmed in other south-eastern States. During 1997, Animal Health Australia unsuccessfully sought support from the national sheep industry organisations to assist owners of infected flocks to control the disease. At the end of that year, the Commonwealth government commissioned an independent review of options available to deal with OJD. The *Hussey-Morris Report* recommended an approach that was translated into the six year $40.1 million dollar National OJD Control and Evaluation Program (NOJDP) in 1998.

It is pertinent that these developments took place shortly after the national cattle industries had initiated the first nationally coordinated approach to JD in 1995. The National Farmers Federation’s National JD Coordinating Committee aimed to restrict further spread of both forms of JD. Bovine Johne’s disease (BJD) had been largely confined to south-eastern Australia for over 70 years and regulatory control of movements and known infected herds had contributed to this situation. Much more was known about BJD than OJD and diagnostic tools and capabilities were more advanced. In comparison, it appeared that an opportunity existed to have an impact on OJD, as its distribution appeared to still be largely restricted. However there was still a lot to learn if it was going to be successfully controlled and possibly eradicated.

The NOJDP was managed under a Deed of Agreement\(^1\) with the purposes of:

- Providing, during a research and evaluation period, by 31 July 2004 sufficient information to allow an informed decision to be made on the national management of OJD, and especially on the feasibility and cost-effectiveness of eradication; and
- Controlling OJD during the research and evaluation period.

Research and Development was one of four sub-programs within the NOJDP, which also included Management, Operations and Communications sub-programs. Surveillance and Financial Assistance sub-programs were included subsequently.

Early research on OJD

Since 1995 the three main livestock industry Research and Development Corporations, Meat & Livestock Australia (MLA, formerly Meat Research Council), the Dairy Research and Development Corporation (DRDC) & Woolmark (latterly Australian Wool Innovation) had managed a collaborative approach to JD research in Australia. The McGarvie Smith Institute in NSW and the Rural Industries Research and Development Corporation (RIRDC) also funded complementary JD research. Research priorities were frequently reviewed and modified.

---

\(^1\) National Ovine Johne’s disease Control and Evaluation Program (NOJDP) Deed of Agreement signed in March 1999.
through extensive consultation with people involved in research and disease control and with industry representatives.

Between 1995 and 1998, about $1.4 million had been invested on OJD research in the areas of,

- **Diagnostic tests** – including culture methods, an ELISA test, a gamma interferon assay and direct polymerase chain reaction (PCR) tests on faeces.
- **Molecular and applied epidemiology** – including an initial assessment of survival of the organism, DNA typing of isolates from varying locations and host species and role of wildlife in transmission of the disease.

The NOJDP initially allocated $10.5 million to the R&D sub-program. Half of the research funding was provided by the national sheep industry and half from the Commonwealth. MLA managed the NOJDP research sub-programs on behalf of Animal Health Australia (then the Australian Animal Health Council, AAHC).

It is important to appreciate the context in which the NOJDP and its research sub-program were developed in 1998. As Australia was faced with controlling a spreading epidemic, possibly with a view to eradicating it, the initial research program focused on addressing questions relevant to the immediate challenge of controlling OJD. Hence, research projects were largely of an applied nature, as is evident from the objectives of the projects below that were initiated or proposed during the first year of the NOJDP. Some of these projects were not formally part of the NOJDP but were complementary projects that were funded by MLA and/or AWI.

- **Evaluation of Eradication Strategies**
  To determine if destocking and decontamination over a 15 month period could eradicate OJD from infected properties and if it was an economic option.

- **Control Strategy Evaluation**
  To evaluate strategies to control and limit the impact of OJD in infected flocks
  a) by management
  b) by vaccination.

- **Genetic Preservation**
  To identify means of ‘safely’ retaining valued genetics from sheep flocks or goat herds that were infected with JD.

- **Cross Species Infection**
  To determine whether OJD can be controlled or eradicated from sheep in the presence of other
  a) farmed species such as goats and cattle; and
  b) wildlife such as kangaroos, rabbits, wallabies and possums.

- **Survival of M. paratuberculosis in the Environment**
  To determine how long *M. ptb* survives in the environment and to assess the influence on *M. ptb* survival times of factors such as soil pH, UV light, temperature, moisture and organic matter.

---

2 Summary of Current (& Proposed) R&D For OJD, WE Sykes, August 1999.
a) laboratory tray trials
b) field plots

- **Tracer Weaner Model**
  To demonstrate that Australian ovine *M. ptb* isolates are capable of colonising the intestines of newly weaned merino lambs after oral dosing at levels likely to be representative of those in the field, and that *M. ptb* can be detected in these lambs four to six weeks after dosing.

- **Hybridisation Capture PCR (HC-PCR)**
  To develop a rapid, cost effective test for the diagnosis of OJD utilising pooled faecal samples.

- **Faecal Culture**
  To complete validation of the Pooled Faecal Culture Test.

- **Sensitivity of Abattoir Surveillance**
  To evaluate the sensitivity of abattoir surveillance in detecting infected flocks.

During the course of the NOJDP, extensive consultation was maintained with people involved in research and disease control and with industry representatives and as a result research priorities were reviewed and modified. About $5.7 million was committed to projects in the first three years. After the Mid-Term Review of the NOJDP, another $2.7 million was earmarked for research, leaving approximately $2 million of the original budget unallocated⁴.

### Later research focus

The research focus for the second three years was on diagnosis, vaccination, control and epidemiology with an increased emphasis also on more fundamental research on pathogenesis and immunity. The sub-program aimed to:

- Add value to the current knowledge where there was a clear and direct benefit to the development of control strategies (knowledge gaps).
- Assist providers of advice and those that develop policy to apply the outcomes of research through a better understanding of the disease.
- Seek understanding of the cellular pathogenesis in early stages of the disease, leading to the development of improved diagnostic tests and potentially detecting other points of intervention to limit the expression of the disease.
- Application of new and relevant technology applications to diagnose the disease at an early stage in the infection process and possibly define vaccine targets⁵.

### Impact of OJD research

This Harvest Year Conference highlights the consolidated outcomes of the research sub-program but, very importantly, the outcomes were also communicated during the NOJDP through a range of advisory media. Primarily MLA’s excellent series of *Tips and Tools* and *Ovine Johne’s Disease R&D Updates* kept industry, farmers and policy makers abreast of developments so that improvements in knowledge and tools could be incorporated into control programs.

---

Moreover the application and implementation of many of these research outcomes in the current National Approach to OJD demonstrates the significance and long-term contribution of the research to managing OJD in Australia. The Assurance Based Credit (ABC) Scheme is based on a quantitative risk assessment that was commissioned by AWI to bring together much of the knowledge gained through research. For instance, it incorporates area prevalence based on abattoir surveillance, various levels of flock vaccination and flock testing utilising pooled faecal culture and abattoir surveillance.

**Conclusion**

The applied R&D sub-program of the NOJDP and the complementary OJD research that was undertaken from 1995 to 2004 dramatically increased Australia’s (and the world’s) understanding of OJD and its capacity to manage the disease. The Australian sheep industry and those involved in managing OJD in Australia owe a great deal to the research scientists and their institutions. Many of them are participating in this Harvest Year Conference. Some of the key people who are not participating but whose enthusiasm and commitment was also critical to the success of the research program, especially in the early years, were Drs David Skerman, Bill Sykes and Bruce Allworth.
A producer perspective – what has the research meant to me?

Stephen Burgun
Arthursleigh Farm
Marulan, NSW, 2579
Phone: 02 4884 1514; Fax: 02 4884 1514
Email: sjburgun@bigpond.com

This can definitely not be considered as a paper but rather a short story about my take on MLA’s research into OJD, what I needed to learn and what I believe was delivered.

Firstly to introduce myself. I am a third generation ‘Farm Manager’ and while I am uniquely employed in that field by the University of Sydney I am definitely not an academic, scientist, researcher or technical assistant just a ‘Working Farm Manager’ employed to run the University’s commercial grazing property ‘Arthursleigh Farm’ at Marulan in the Southern Tablelands of NSW.

I was literally born into large commercial merino sheep operations. I have Jackerooed, Overseen, been a Livestock Selling Agent and am an accredited sheep and lamb assessor. I have seen and handled enough sheep to consider myself a genuine sheep man but when we discovered OJD on the property I was managing I knew ‘absolutely bloody nothing’ about it, had hardly known of its existence and could not (and actually still have trouble) pronouncing Mycobacterium paratuberculosis .

Now despite everyone’s perception of what some one with reasonably extensive knowledge in sheep husbandry should have known about such a disease at least I wasn’t on my own, my neighbours knew nothing, my ram suppliers new nothing and almost everyone I came across new nothing of the disease. Speculation on the topic had become a hobby.

Anyone who has ever run sheep (in particular Merino sheep) will know that there are always those ‘poor doing bastards’, you know ‘the bastards like nothing better than to die’. You could drench them, draft them off and feed them but no matter what you did they were always there. Did we finally have the answer to our ever present dilemma? We assumed so and until better informed myself and other sheep producers were proclaiming ‘it was bloody OJD all the time’.

But we had to learn something about this disease and very quickly as it had (or was about to) drastically effect our viability, not to mention relationships with neighbours, clients and ram suppliers. And what did we need to learn about it? Well frankly everything.

I remember going to the Ag-Facts (or such) looking for available information and at the time gathered these two (& sorry unsourced) paragraphs which I had included in a report to our business’s superiors (who are essentially accountants) to explain our dilemma.

“Johne’s disease is a disease of older sheep, characterised by progressive loss of body condition, leading to emaciation. Once symptoms have developed, animals will not respond to any treatment and eventually die. Mortality is rarely seen as an acute outbreak. However, up to 5% may die in older mobs of sheep 5 years and older and in more severely affected flocks, younger sheep die. Deaths of 15% in mobs of 2 year old sheep have been reported.”
“Apart from trade restrictions, most economic loss from OJD results from the death of infected sheep. Although difficult to measure, the production of infected animals is likely to be reduced before symptoms become obvious.”

O.K, we know what it is and we know the bastards die, but?

- How did we get it?
- How do we diagnose it?
- Who else had it and why were we so badly disadvantaged by regulations?
- Could we get rid of it?
- What was it costing us?
- If we de-stocked could we?
  a. In fact clean country after the magic one, two or who knows how many summers? Did the ‘roos’ carry it? Wombats? or Ducks? Car tyres and boots?.
  b. Could we use cattle as an alternative species to graze on pastures during that period of de-stocking to remain commercially viable? Would the cattle get OJD?
  c. Could we find clean sheep after country was deemed clean?
  d. So on and so on………..

As I had mentioned my bosses are essentially accountants and when they were presented with the initial ramifications of our status i.e.

- It was no longer possible to sell sheep other than for slaughter.
- While it was possible to continue to operate a commercial wool producing enterprise despite having OJD, the disease introduced additional costs from mortalities and restricted selling options would reduce overall income.
- The real estate market (at the time) had demonstrated a reduction in capital value of land infected with OJD and by association there was a negative social stigma attached to the status of the flock and its owners - OJD flocks were decidedly ‘second class’.

At that stage, weighing up the negative impacts an interim plan to eradicate OJD by de-stocking parts of the property serially over six years was commenced in 1998. At the time it was believed that two summers (a period of 15 months at least) was necessary for contaminated land to become safe following the removal of sheep.

Land de-stocked of sheep during the eradication program was to be grazed by cattle because they are considered unlikely to contract and spread OJD. Alternative grazing of cattle was aimed at maintaining an income from infected country (straight de-stocking was not considered a financially viable option). Following the two year de-stocking period, if the eradication program was deemed effective, new sheep from a clean source were to be purchased to re-stock.

So where were we heading? – down an unknown track, with unknown outcomes and no science to back it up.

What did the research (science) do for us and how has it impacted on what we were doing?

Well firstly the stigma of being OJD infected started to wain as the surveillance research exposed just how widespread (certainly in our area) the disease was. The Southern Tablelands was rife with the disease. Pooled faecal cultures are now the main test for OJD detection in
flocks and more sensitive than blood tests (MLA, 2002) but when you are infected you are infected and testing becomes of little importance.

Neighbours started talking to each other again, as we tend to forget just how emotive that early period was, with sheep farmers looking for someone to blame, either for being the source of their infection, being a threat to them becoming infected or as the minority group that had resulted in harsh trade restrictions being put on them, an innocent third party.

Research told us that it was unlikely that we contracted OJD from kangaroos, wombats or wood ducks, although ‘Marsupials may be infected in unique and unusual situations, they were not considered to pose a threat to OJD on most farms” (MLA, 2002). O.K throw out the way out theories, we most probably contracted OJD from our ram suppliers, from strays or contaminated run-off from neighbouring infected farms.

“Evaluations of Eradication Strategies for OJD (Project code: OJD.001)” results raised severe doubts about de-stocking as those that attempted had failed. In short why would we then continue a de-stocking policy in a high prevalence area and attempt to purchase in (very expensive) clean sheep when it was most likely deemed to fail?

An easy decision - we gave up on our de-stocking program, threw in the rams, vaccinated the new crop of lambs as recommended (“vaccination at weaning reduces subsequent deaths from OJD and delays the onset of bacterial shedding in dung” (MLA, 2002)) and it was back to business as usual.

The cumulative research (and it is unclear to me which projects or combinations of projects) delivered us a best practice management for the disease, that with vaccination was extremely compatible to best practice internal parasite management and weaning practices that we really should be employing regardless of OJD.

You see, MLA (2002) research suggests that infection status of ewes and lambing paddocks has been found to be critical in the transmission of OJD and recommends that we prepare ‘low risk’ lambing paddocks to reduce transmission within flocks. Again, what would be a low risk pasture? MLA (2002) research suggests that “most OJD bacteria die within six weeks, with a small number surviving over a year in shaded areas”.

This practice would fit perfectly (at least in good seasons) for sensible pasture and ewe nutritional management. Paddocks spelled this way would build up a residual feed source for ewes to be put onto just prior to the commencement of lambing so as to better match their nutritional requirements.

As lambs are susceptible to infection before and after weaning (MLA OJD Update, 2004) the practice of cleaning pastures of internal parasites by alternate grazing by cattle for 6 months prior to weaning is also compatible to providing ‘low risk’ OJD pastures as well.

Research (MLA OJD Update, 2004) suggests that cattle can very rarely be infected with the sheep strain of Johne’s disease, however we should avoid grazing young calves on pastures grazed by infected sheep. Therefore when we are preparing ‘low risk’ paddocks for lambing and weaning with cattle we should use dry cattle if they are available, or at worst cows with bigger calves at foot.

So, in short for me its ‘happy days’. While I haven’t had enough time to gauge the effectiveness of the vaccine on our hoggets, I can be a sheep farmer again. It appears the disease is manageable using sensible management strategies that are not radical but actually complement or mirror existing sheep management strategies employed for internal parasite control and pasture allocation.
All I want now is for the price of wool to go up and we are back in business.

On completion I would just like to present to you all of my cards. As Manager of The Sydney University Farm ‘Arthursleigh’ we were host to two major MLA funded epidemiological studies and while I was not involved in the project other than outsourcing staff from time to time for site construction, feed supplementation, shearing and crutching etc. I was able to observe first hand some of the findings as they unfolded.

As a University Farm manager please don’t get the impression that I have a vested interest in endorsing the research conducted, as I am a born sceptic who is not that fond of ‘some’ academics impractical and unworkable ideas (believe me I have had some beauties). However as an observer, the projects run on our farm were exemplary and should be complemented. They were well set up and thought out, and well (sometimes painful in detail and intensity) managed by extremely dedicated people.

If research generated from these projects is part of what we are now using as ‘best practice’ and of a standard of the other OJD research then I am a believer until better science tells us something different.

And by the way those poor doing sheep at the back of the mob that we grew up seeing and were always there, well they’re ‘poor doing bastards’ or ‘wormy’. By the time you notice a sheep sick with clinical signs of OJD that sheep doesn’t come back into the yards he’s as good as dead, I know MLA research taught me that.
An advisor’s view of OJD research

David Sackett
Holmes Sackett & Associates Pty Ltd
112 Fitzmaurice Street
Wagga Wagga NSW 2650
Phone: 02 6931 7110; Fax: 02 6931 7113
Email: david@hs-a.com.au

Considerable effort has gone into research on OJD since it emerged as an important issue in the mid 1990’s. The value of the findings of that research are highlighted by the differences in approach to managing OJD both at an industry and at the farm level.

Industry Issues

It is now 10 years since NSW embarked upon the development of the Strategic Plan for the control and possible eradication of OJD. This was closely followed by Victoria with its own equally flawed version and other states adopted various strategies depending on their degree of naivety and regulatory zeal.

It is always a challenge to try to manage a disease when one has less than ideal information on which to develop a strategy. It is quite apparent that one cannot always stand back and do nothing while waiting for all the key issues to be addressed by research. In many instances, to do so, would be wrong and not in the interests of the industries affected or at risk.

There were two key pieces of research that were missing in the early days of policy development for OJD.

The first was to seriously review the probability of success of any control or eradication program against accepted criteria for successful disease and control programs. Had such a review been undertaken in the early days of policy development, it is quite likely that programs aimed at control or eradication would have taken quite different directions.

The second was an assessment of the economic impact of OJD on both individual businesses and for the broader sheep industry. There is no doubt that such an assessment would have had to be done with less than perfect knowledge about the impact of the disease at the flock level but this could have been taken into account with sensitivity analyses. It was not until the study of Hassall & Associates (2003) that it began to be widely accepted that the majority of the cost of the disease related to the regulatory strategies (quarantine, zoning) rather than the costs associated with the direct effects of OJD on flock productivity.

Instead of good research, we had well meaning but naïve people attempting to produce good policy in what would be best described as a vacuum of good quality research on which to base decisions. The objective was the eradication of disease rather than the improvement of industry productivity and profitability. As mentioned above, it is not realistic that all questions should be answered before a program can be commenced, because it would inevitably lead to inaction which in many cases would be an undesirable outcome. However there are some key issues that should have been addressed with greater rigour from the start.
Flock Issues

During most of the 1990’s advice to producers on the management of OJD was, at best, based on experience in the other industries or other countries and could, at best, be described as a ‘best guess’. Field observations of differences in estimated mortality rates could not be adequately explained and hence there were no sound recommendations on strategies that could be adopted to minimise the impact of the disease at the flock level.

A comparison of knowledge now, compared to 10 years ago, shows the extent of improvement in our knowledge of issues related to the epidemiology of OJD (Table 1). This improved knowledge has enabled much more appropriate advice to be provided to clients and more cost effective management strategies to be implemented. Much of what was recommended ten years ago, such as eradication by de-stocking despite its high cost (Sackett & Holmes 1997) is now, with results of research rarely recommended.

Table 1: Examples of how knowledge on OJD has improved since 1995

<table>
<thead>
<tr>
<th></th>
<th>1995</th>
<th>2005</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteria survival</td>
<td>1-2 years</td>
<td>90% die within 6 weeks</td>
</tr>
<tr>
<td>Death rates</td>
<td>Low</td>
<td>2-17% pa</td>
</tr>
<tr>
<td>Testing (mob)</td>
<td>Pooled Faecal Culture</td>
<td>Movement of sheep ‘Clusters’</td>
</tr>
<tr>
<td>Spread</td>
<td>Movement of sheep</td>
<td>'Clusters'</td>
</tr>
<tr>
<td>Other species</td>
<td>Goats ☑</td>
<td>Goats ☑</td>
</tr>
<tr>
<td></td>
<td>Cattle ×</td>
<td>Cattle – young cattle, high challenge</td>
</tr>
<tr>
<td></td>
<td>Wildlife ?</td>
<td>Wildlife ×</td>
</tr>
<tr>
<td>Vaccination</td>
<td>Not available</td>
<td>Highly effective but not 100%</td>
</tr>
<tr>
<td>Eradication by destocking</td>
<td>Yes</td>
<td>&lt;50% success rate</td>
</tr>
</tbody>
</table>

Much of the current advice is based on the use of vaccination, to either minimise the risk of introduction or to manage the incidence of OJD in the flock. Risk, and hence the likely benefit, is determined with assistance of a matrix such as the one shown in Figure 1.
However at a cost of $1.70 per dose it is important that the vaccine is used appropriately to ensure flock profitability is improved with vaccine use. The proportion of wethers in the national flock is now down to 13% (Curtis & Croker 2005) as a consequence of the high prices for sheep meat and drought in some regions of Australia. In flocks that are selling wethers by 1-2 years of age, vaccination of the wether portion is not required unless the flock has an extremely high incidence of OJD or wethers are sold as store sheep for wool production. This will halve the cost of flock vaccination.

**Cost Effectiveness of Vaccination**

The use of vaccine is now widely recommended as an OJD control strategy. However, its cost benefit will vary between flocks depending on the mortality rate in the absence of vaccine, vaccine efficacy, flock structure and value of the wool and sheep produced.

An analysis of the economics of vaccine use was undertaken for a self-replacing merino flock with varying mortality rates due to OJD. The mortality rates are due to OJD only, and are averages across the whole flock. Mortalities were assumed to commence in two year old sheep and peaked in three year olds, then declined until five years at which stage sheep were sold. The flock was assumed to sell wethers at 2.5 years of age, and had a clip average fibre diameter of 19.3 microns. Historical ten year average (July 1995 – June 2005) prices for surplus sheep and wool were used. Sheep sale prices were assumed to remain constant regardless of vaccination strategy, so all benefits would be derived from reduced mortality rates. The results of the analysis over six years, the time taken for all sheep to be vaccinated and the flock to approach a steady state, are shown in Table 2.
Table 2: Financial impact of vaccination with varying OJD mortality rates

<table>
<thead>
<tr>
<th>Flock Mortality Rate Due to OJD</th>
<th>Cumulative Cashflow/DSE (Six Years)</th>
<th>Years to Break Even</th>
<th>Internal Rate of Return*</th>
</tr>
</thead>
<tbody>
<tr>
<td>7%</td>
<td>+ $5.54</td>
<td>2</td>
<td>110%</td>
</tr>
<tr>
<td>5%</td>
<td>+ $1.94</td>
<td>2</td>
<td>60%</td>
</tr>
<tr>
<td>2% (Ewes &amp; Wether Lambs vaccinated)</td>
<td>+ $0.15</td>
<td>4</td>
<td>-6%</td>
</tr>
<tr>
<td>2% (Ewes only vaccinated)</td>
<td>+ $0.43</td>
<td>3</td>
<td>44%</td>
</tr>
</tbody>
</table>

* Assumes a terminal value which is based on the benefit in year six capitalised at 20%

As expected the benefit from vaccination is greater in flocks with higher OJD mortality rates due to the combination of reduced mortalities and a shorter time to breakeven because of the reduction in mortalities in younger sheep compared to flocks with lower OJD mortalities. Investment in vaccination for flocks with 5% and 7% mortality provides a high return. At 2% mortality rate vaccination is uneconomic based on reduction in deaths alone but premiums for OJD vaccinated sheep may change that. If the mortality rate was to increase above 2%, vaccination is likely to be become economically viable but the risk factors that are likely to increase the mortality are not well understood, making the optimum strategy uncertain. A strategy of vaccinating ewe lambs only provides acceptable returns assuming no effect on wether sale prices which would be the case if sold for slaughter but might not apply if sold as store sheep.

Future Research Questions

1. What are the geographic limits of OJD in Australia? Where do we not have to worry that OJD will be established or if it does establish, it will be of no economic significance?
2. What are the factors that result in some flocks having high mortality rates where others remain low?
3. What is the optimum vaccination strategy in low prevalence flocks or flocks that have, through vaccination, reduced the prevalence to a low level? Does it remain annual vaccination, vaccination every 2nd year or vaccination of every 2nd sheep each year?
4. Will the use of vaccine ultimately lead to the eradication of OJD from infected flocks? If so, how long will it take and what are the factors that are associated with success?
5. Is vaccination sufficiently effective that other control strategies (culling early cases, use of low risk pastures) are not required?
6. Can we develop a vaccine with a similar or better high level of efficacy as Gudair, but without the risk to those administering the vaccine?
References
Diagnosis – Existing Projects
Hybridisation capture PCR and direct PCR on pooled faecal samples

Ian Marsh\textsuperscript{a}, Richard Whittington, Leslie Reddacliff and Graeme Eamens

\textsuperscript{a}Elizabeth Macarthur Agricultural Institute
PMB 8 Camden, NSW 2570, Australia,
Phone: 02 4640 6502; Fax: 02 4640 6384
Email: ian.marsh@agric.nsw.gov.au

Summary
Hybridisation capture-PCR (HC-PCR) was first reported as a method for the detection of \textit{Mycobacterium avium} subsp. paratuberculosis (\textit{M. ptb}) in 1995 and was successfully trialled on a small number of faecal samples from cattle with Johne’s disease. In our laboratory HC-PCR was found to be capable of detecting \textit{M. ptb} in pellets from infected sheep diluted at rates of up to 1 in 100 in normal faeces, suggesting that the technique should be evaluated further as a potential low-cost diagnostic technique for flocks/herds using pooled samples. A locally optimised HC-PCR method was evaluated on faeces from infected and non-infected sheep using faecal samples pooled from 50 sheep and individual faecal samples. The status of each of the faecal samples was determined by radiometric culture. A simpler direct-PCR (D-PCR) technique was evaluated on the same samples and was found to be more sensitive than HC-PCR. Initial results indicated that D-PCR from faeces can be used as a rapid means of screening pooled faecal samples for flock diagnosis of Johne’s disease in sheep, improving detection of \textit{M. ptb} infection in the early stages of disease, or where prevalence is low, which is critical for the management of ovine Johne’s disease (OJD). An improved D-PCR test, with similar or better sensitivity than current culture techniques and which would be acceptable for regulatory purposes, would be of immense benefit to the industry. This is a potentially achievable short term outcome, building on years of previous and on-going research in the EMAI Microbiology Laboratory.

Introduction and background
The difficulty of diagnosis of \textit{M. ptb} infection in the early stages of disease, or where prevalence is low, is critical for the management of OJD [15]. The two most useful diagnostic tools currently available to the industry are abattoir surveillance and pooled faecal culture (PFC). Abattoir inspection is an extremely efficient and economical method of regional surveillance [1] but it is not applicable to live animals, and can only ever detect animals that have progressed to have gross lesions. Sheep without gross lesions can shed \textit{M. ptb} in their faeces and are a risk to other livestock. PFC is the most sensitive practical current test to detect OJD infection in a living mob of sheep [13] [14]. However, PFC is expensive, and may take many months for definitive results.

The use of molecular techniques to directly detect the DNA of \textit{M. ptb} in faeces, without having to first grow the organism in culture would theoretically allow the rapid (several days) and sensitive diagnosis of OJD in living sheep. Hybridisation capture-PCR (HC-PCR) was first reported as a method for the detection of \textit{M. ptb} in 1995 and was successfully trialled on a small number of faecal samples from cattle with Johne’s disease [12]. However, sample to sample cross contamination during the DNA purification step highlighted that the original format of the test was unsuitable for routine diagnostic use. A modified HC-PCR was reported with analytical sensitivity of 5000 organisms per 200 mg faecal sample and capable of detecting \textit{M. ptb} in pellets from infected sheep diluted at rates of up to 1 in 100 in normal faeces, suggesting that...
the technique should be evaluated further as a low-cost diagnostic technique for flocks/herds using pooled samples [9]. HC-PCR was evaluated on faecal samples pooled from 50 sheep and individual faecal samples as a rapid diagnostic test for *M. ptb*. The status of each of the faecal samples was determined by radiometric culture. A simpler direct-PCR (D-PCR) technique was evaluated on the same samples and was found to be more sensitive than HC-PCR [10]. Direct-PCR using primers from the 5’ region of IS900 was evaluated in a blind trial on 502 pooled faecal samples which were concurrently examined by culture. Twenty one (64%) of the 33 culture positive pools were detected by direct PCR, representing 11 (79%) of the 14 farms with infected sheep. Direct-PCR was also more sensitive than the immunomagnetic bead capture-PCR designed for the same purpose [11]. Using individual faecal samples, 74% of culture positive samples were detected with direct-PCR compared to 44% with immunomagnetic bead capture-PCR. Direct-PCR from faeces can be used as a rapid means of screening pooled faecal samples for flock diagnosis of Johne’s disease in sheep.

The D-PCR test developed at EMAI under an MLA-funded project was completed in 2000. The test was further evaluated at EMAI in 2004 under routine diagnostic conditions in our laboratory and at VIAS in Victoria, and was also included in several on-going OJD research projects.

The sensitivity of the current test is less than faecal culture. At the level of the pooled sample, the sensitivity for D-PCR compared to PFC varied from 64% in the early trials to about 40% in the most recent investigations. At the farm level (more than one pool is normally examined), sensitivity was 79% in the early trials and 66% in the recent work. It is, however, always misleading to quote sensitivity estimates in isolation – they depend on the population being tested, and this has varied over the years. For example, the early trials probably included more farms with a higher prevalence of infection, and D-PCR detected all the high prevalence farms in the recent trials. When looked at from the view of number of *M. ptb* organisms in the sample examined, D-PCR appears to require about ten times as many to yield a positive result – about $10^4$ for D-PCR compared to about $10^3$ for culture. In practical terms, this all means that D-PCR will readily detect pools with large numbers of *M. ptb* organisms. This translates to sheep with severe multibacillary disease, and these are the animals of greatest immediate risk of transmitting infection.

The cost of D-PCR is similar to culture, but its great advantage is the short time for results. The current D-PCR test is now approved for use in NSW and is proving of use to some producers in the management of infected flocks, by providing a rapid assessment of whether sheep are shedding large numbers of *M. a. paratuberculosis*. However, because of the lower sensitivity compared to culture, negative results cannot be used for ABC points or to remove suspicion of infection.

There is opportunity to improve the current D-PCR test, and some of the avenues for this were already suggested in the final report from the above project and in the peer reviewed publications generated from the project [9] [10]. Several papers on *M. ptb* -specific D-PCR from faeces or tissues (often from humans) have been published, and their techniques can also be investigated for adaptation to D-PCR from sheep faeces [2] [3] [4] [5] [6] [7] [8]. Many of the techniques as published, while apparently sensitive, are too time consuming, costly or at risk of cross-contamination, for use as routine diagnostic tests. MLA has provided further funding to investigate improvements to the D-PCR procedure.

**Use of the existing D-PCR test in diagnostic investigations**

In current diagnostic procedures at EMAI, pooled faeces are subjected to DNA extraction based on a commercial resin and then tested in the PCR. The test can be completed within a week and relies on two phases. The initial phase involves a ‘conventional’ PCR assay on the DNA
extract based on the IS900 gene sequence of *M. ptb*, using a special combination of IS900 primers, and two sample dilution rates to reduce the risk of PCR inhibition. The resultant PCR product is assessed on an agarose gel for molecular size to ensure it conforms to the specific molecular size expected for *M. ptb*. The second (confirmatory) phase then involves a restriction endonuclease assay (REA), but requires a sufficiently strong DNA gel band from the first phase before it can be run. If there is a product of the correct size but too weak to run in the REA, the result of the D-PCR testing is classified as a trace PCR reaction and is considered inconclusive. A positive D-PCR result requires both an IS900 PCR product of the correct size and an REA result to confirm that *M. ptb* is present.

To date, routine diagnostic application has been limited, with just 61 pools from 10 accessions in the past 2 years. Concurrent PFC testing was carried out on some accessions. Six accessions containing 31 pools were D-PCR negative. Two of these submissions (representing 15 pools) were also tested by PFC and found to be negative. From the remaining 4 accessions, reactions were detected in the D-PCR as shown in Table 1. In two flocks (Table 1) rapid confirmation of infection was possible by detection of a positive D-PCR.

**Table 1:** Diagnostic ovine faecal pool accessions yielding a reaction in the *M. avium paratuberculosis* direct PCR.

<table>
<thead>
<tr>
<th>Flock</th>
<th>Reason</th>
<th>No pools submitted</th>
<th>D-PCR result (pools)</th>
<th>PFC result relevant to D-PCR results on specific pools</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Show testing</td>
<td>5</td>
<td>1 Trace* 4 Neg</td>
<td>1 Neg 4 Neg</td>
</tr>
<tr>
<td>2</td>
<td>Trace forward Introductions from high risk SA flock</td>
<td>9</td>
<td>1 Pos 3 Trace 5 Neg</td>
<td>1 Pos 1 Pos, 2 Neg 5 Neg</td>
</tr>
<tr>
<td>2</td>
<td>As above</td>
<td>9</td>
<td>1 Pos 1 Trace 7 Neg</td>
<td>1 Pos 1 Neg 7 Neg</td>
</tr>
<tr>
<td>3</td>
<td>Surveillance testing</td>
<td>7</td>
<td>3 Pos 4 Neg</td>
<td>3 Not done 4 Not done</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30</td>
<td>5 Pos 5 Trace 20 neg</td>
<td>2 Pos, 3 Not done 1 Pos, 4 Neg 20 Neg</td>
</tr>
</tbody>
</table>

* indicates trace reaction in PCR, insufficient for confirmatory REA test; classified inconclusive

**Proposed improvements to the D-PCR test**

Specific areas for improvement of the existing D-PCR test which will be examined include:

- Increasing the starting sample size

  Culture uses a 2g sample, D-PCR currently uses only 0.2g. Increasing this to 2g has the possibility of increasing sensitivity (theoretically up to 10 fold). A preliminary examination of this in the previous project did not show marked improvement, but it was not further investigated. Digestion techniques to concentrate *M. ptb* organisms from the larger faecal samples will be trialled. Similar techniques have already been attempted with meat samples and material suitable for preliminary testing in the proposed project has been stored from a current MLA-funded project on *M. ptb* in meat – Project number: PRMS.044.)
Removing competing DNA from the reaction

Because of the very resistant cell wall of mycobacteria, a pre-treatment targeted at more labile micro-organisms may be effective. This seems not to have been used in any of the molecular work published to date. Restriction enzyme digests prior to PCR could also be helpful to avoid non-specific bands. Digestion techniques as above may also be helpful.

Improving the lysis of the resistant mycobacteria

In the current protocol it is likely that many \textit{M. ptb} cells remain intact, and thus their DNA is unavailable to the subsequent PCR. Alternate techniques to the simple boiling used in the current protocol will be trialled to improve DNA extraction. We have experience with several potentially useful procedures through the proteomics work done by Ian Marsh during his MLA-funded PhD studies.

Removal of DNA inhibitors

One of the difficulties with performing PCR on materials such as faeces is the loss of sensitivity due to inhibition of the PCR reaction by many normal faecal components. At present, the D-PCR attempts to overcome this problem using a commercial product to separate the target organisms from such inhibitory components. However, 4 years in molecular biology is a long time, and there are now many newer commercial kits or materials for DNA extraction from faecal, soil and other contaminated samples which might be trialled. Such kits are not targeted at mycobacteria, and will not give optimum results with ‘off-the-shelf’ use.

Developing an internal control target

This would be used in each test to identify PCR inhibition. Currently, when we get a negative result in D-PCR, we have no way of knowing whether the negative result is because no \textit{M. ptb} DNA was present (i.e a true negative) or whether inhibitors in that particular sample prevented the PCR from amplifying any specific DNA that was present. A control target that can be added to each reaction, and which gives a distinct band of different size to specific bands, allows assessment of possible inhibition. Such a modification would allow more confidence in a negative D-PCR result for \textit{M. ptb}. This confidence is important if D-PCR is to be acceptable for ‘negative’ regulatory purposes.

Examination of DNA losses

Several stages of the extraction process may be susceptible to loss of target DNA thus resulting in reduced sensitivity. Minimising the amount of loss of this DNA will ultimately lead to a more sensitive diagnostic test.

Use of a second \textit{M. a. paratuberculosis}-specific PCR target

Currently, even using PCR/REA as confirmation of growth in Bactec medium, the identification of \textit{M. ptb} DNA is not considered to be fully confirmatory of infection unless sub-culture to solid media/mycobactin dependence is also positive. We are at present investigating the use of other targets in multiplex PCR with IS900, that would satisfy regulators as definitive diagnostic criteria for \textit{M. ptb}. These techniques would be applicable to D-PCR.

The use of real-time PCR

This can further reduce turn-around time by a day, and would facilitate the quantification of a positive result. We are at present conducting preliminary investigations into the use of this technology for confirmation of growth from Bactec cultures. If successful, we could also apply this to D-PCR.
Results to date

Work to date has involved the preparation of large quantity of diluted positive OJD faeces. This was undertaken such that all optimisation and validation experiments in this project could be performed using aliquots of the same faecal preparations thus providing more meaningful inter-experimental comparisons of the results. The faecal samples were prepared from OJD positive faeces and diluted 1 in 100,000. All samples were then evaluated by routine PFC, standard D-PCR and a modified D-PCR. The later incorporated a Qiagen QIAamp DNA stool mini kit for DNA extraction. The results to date indicate that the new DNA extraction kit may have already increased the sensitivity by at least 10 fold (Table 2). Improvements in one or more of the other areas being investigated will hopefully further improve the test.

Table 2: Summary of the results to date comparing routine PFC, standard D-PCR and a modified D-PCR using a Qiagen QIAamp DNA stool mini kit on diluted OJD positive faecal samples.

<table>
<thead>
<tr>
<th>Sample (dilution)</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PFC</td>
</tr>
<tr>
<td>Positive faeces (neat)</td>
<td>+</td>
</tr>
<tr>
<td>Positive faeces (1 in 5)</td>
<td>+</td>
</tr>
<tr>
<td>Positive faeces (1 in 10)</td>
<td>+</td>
</tr>
<tr>
<td>Positive faeces (1 in 50)</td>
<td>+</td>
</tr>
<tr>
<td>Positive faeces (1 in 100)</td>
<td>+</td>
</tr>
<tr>
<td>Positive faeces (1 in 500)</td>
<td>+</td>
</tr>
<tr>
<td>Positive faeces (1 in 1000)</td>
<td>+</td>
</tr>
<tr>
<td>Positive faeces (1 in 5000)</td>
<td>+</td>
</tr>
<tr>
<td>Positive faeces (1 in 10,000)</td>
<td>-</td>
</tr>
<tr>
<td>Positive faeces (1 in 50,000)</td>
<td>-</td>
</tr>
<tr>
<td>Positive faeces (1 in 100,000)</td>
<td>-</td>
</tr>
<tr>
<td>Negative faeces</td>
<td>-</td>
</tr>
<tr>
<td>Extraction control</td>
<td>-</td>
</tr>
<tr>
<td>Process control</td>
<td>-</td>
</tr>
</tbody>
</table>

Conclusions

In all the above investigations it is important to keep the ultimate goal in mind – a fast, practical, reliable and robust, and cost-effective test. Some procedures may give improvements in sensitivity, but may be too expensive or unreliable to include in a useful diagnostic protocol.
References


Validation of the gamma-interferon test for diagnosis of ovine Johne’s disease


aCSIRO Livestock Industries, Australian Animal Health Laboratory
Private Bag 24, Geelong, 3220, Victoria
Phone 03 5227 5749; Fax: 03 5227 5000
E-mail: david.j.stewart@csiro.au

Abstract

In theory, the interferon-γ (IFN) test has potential for improved control of ovine Johne’s disease (OJD) by early detection before contamination of pasture and transmission of infection. To validate the IFN test, a project has been completed for determining specificity and sensitivity. Because of non-specific IFN responses, raised cut-points were required to achieve high specificity (≥98%). This resulted in reduction of sensitivity to below 50%, limiting its application for early detection or certification from disease freedom. A major limitation for adoption is the widespread use of vaccination precluding the use of immunological tests for diagnosis. Other limitations, apart from relatively low sensitivity, include cost of the test as well as a narrow time window for laboratory initiation of the assay so that test result validity is not compromised. The assay may have application in a test and cull program as a surrogate test for faecal shedding and the removal of sheep with severe disease but this approach to control of OJD will require further confirmation.

Background

For an effective OJD control program, it is important that diagnostic tests can identify infected sheep prior to commencement of bacterial shedding, pasture contamination and exposure of other livestock to infection. In theory, the interferon-γ (IFN) test has the potential to fill this role since the assay detects a cell-mediated immune (CMI) reaction, which occurs earlier than the humoral antibody response in mycobacterial infections. Antibody assays have low sensitivity in OJD and seroconversion usually only occurs after the commencement of shedding.

Objectives

1. Complete IFN test development by standardising potency of PPD antigens and determine the criteria defining a cut-point for a positive reaction.

2. Determine the specificity and sensitivity of the IFN test in sheep flocks.

Specificity trial

The specificity of the IFN test was evaluated on 6 different unexposed flocks. Blood samples were collected from individual sheep on each of the properties in 3 NSW Rural Lands Protection Boards (Riverina, Dubbo and Armidale) and one property each in WA, SA and Vic. The interpretation criteria for the IFN test included: the mean optical densities (OD) values for the Avian PPD stimulation minus the nil antigen mean ODs with a difference of 0.05 or 0.10 being required for a positive result (Avian PPD-Nil antigen ≥ 0.05 or A-N ≥ 0.10); the mean OD values
for the Johnin stimulation minus the nil antigen mean ODs with a difference of 0.05 or 0.10 being required for a positive result (Johnin-N ≥ 0.05 or J-N ≥ 0.10) and the OD values for J-N minus A-N with a difference of 0.05 or 0.10 being required for a positive result (J-A ≥ 0.05 or J-A ≥ 0.10). Specificity varied according to the geographical location of the property and the age of the sheep and IFN reactors were most evident in the Armidale flock. It is probable that these non-specific IFN responses were due to false positive reactions induced by environmental bacteria that shared antigens with *Mycobacterium avium* subspecies *paratuberculosis* (M. *ptb*). The location of the flocks, their history and diagnostic testing (pooled faecal culture and serology) were not indicative of OJD. For lambs, aggregate specificity was high (>99% CI ≥ 97.7%) irrespective of the scale (A-N, J-N, J-A) or the cut-point (≥ 0.05, ≥ 0.010). In the current study, specificities for yearlings and adult ewes using the A-N ≥ 0.05 and J-N ≥ 0.05 diagnostic criteria were 94-95% and 92-94%, respectively. Specificities improved when an OD difference of 0.10 is exploited for A-N and J-N, with the aggregate specificity for yearlings increasing to 99% (CI 97.0-99.9, CI 96.4-99.7) and for adult ewes to 98% (CI 95.8-99.5) and 99% (CI 96.4-99.7), respectively. Alternatively, if the IFN cross-reactive responses to *M. avium* PPD were subtracted from the Johnin PPD, specificity was >99% (CI ≥ 97.7%). Increasing the specificity reduces the sensitivity of the test but the apparent false positive rate is also decreased. The Johnin PPDs, used for this trial and provided by Pfizer Animal Health, were from source A (J_A) and source B (J_B).

**Sensitivity trials**

**Flock A**

The flock, consisting of 145 adult pregnant Merino ewes, was part of another NSW Department of Primary Industries project (OJD.024) run by Dr Richard Whittington. In flock A, of 145 sheep 46 (31.7%) were tissue culture positive, 36 (24.8%) were histopathology test positive and 54 (37.2%) were positive to either reference test. The sensitivity of the IFN test was 67% (CI 52.5-78.9) and 52% (CI 37.8-65.7) for the cut-points, J-N ≥ 0.05 and J-A ≥ 0.05, respectively. Specificities improved when an OD difference of 0.10 is exploited for A-N and J-N, with the aggregate specificity for yearlings increasing to 99% (CI 97.0-99.9, CI 96.4-99.7) and for adult ewes to 98% (CI 95.8-99.5) and 99% (CI 96.4-99.7), respectively. For J-N ≥ 0.05, sensitivity was 65% for all culture positives and 81% for all histopathology positives. J_A Johnin PPD was used for this trial.

**Flock B**

In flock B (part of University of Sydney project OJD.002 run by Professor Richard Whittington) there were 48 (22.9%) culture positive sheep out of 210 and 8 (3.8%) histopathology positive sheep. For both A-N and J-N ≥ 0.05, sensitivity was 8.3% (4 reactors out of 48) for the combined reference standards. For culture alone, A-N and J-N sensitivity were both 8.3% (4 reactors) and for histopathology alone, A-N and J-N sensitivity were both 25% (2 reactors). It is probable that the poor sensitivity of the IFN test was mainly due to its low analytical sensitivity in a flock with a relatively high proportion of culture positive sheep (23%) and low proportion of histopathology positive sheep (4%). Despite the 9 h period between commencement of blood collection and the start of antigen stimulation, the mitogen response rate of 72% indicated that blood cells were still viable. J_B Johnin PPD was used for this trial.

**Flock C**

The IFN-γ test was evaluated in a 3-year longitudinal experiment as part of another study (University of Sydney project OJD.028 run by the Professor Richard Whittington and Helen McGregor). In the trial, of 385 sheep 19 (4.9%) were tissue culture positive, 17 (4.4%) were histopathology test positive and 23 (6.0%) were positive to either reference test. There were 13...
(3.4%) sheep that were positive to both tissue culture and histopathology. The sensitivity estimates of the IFN test were approximately 57% (CI 34.5-76.8) and 44% (CI 23.2-65.5) for the cut-points of J-N ≥ 0.05 and J-A ≥ 0.05, respectively, in comparison to the combined reference standards. The estimates were lower than those for flock A and much higher than those for flock B. In flock C, the IFN test (J-N ≥ 0.10) detected 6 of 9 faecal shedders (sensitivity 67%).

With flock A, there was a higher proportion of IFN test positive sheep with severe histopathological lesions (3a-3c) than mild (score 1 and 2) suggesting a higher test sensitivity in animals with more severe lesions. This difference was not statistically significant. However, this same trend was confirmed in the longitudinal trial (flock C) where there was a much higher proportion (80%) of IFN positive sheep with severe lesions compared to mild lesions (25%). Furthermore, in flock C, an IFN test sensitivity of 77% (reference standards interpreted in series) for J-N ≥ 0.10 was obtained in 10 of 13 sheep that had predominantly severe lesions and were culture positive. Thus some sheep, that have cured their infection, and therefore not culture positive but still have low grade lesions, may no longer be sensitised and consequently do not generate an IFN response. Sheep with more severe, active infections with tissue invasion, granuloma formation and subsequent faecal shedding (early lepromatous form) probably generate a more aggressive CMI response with release of high amounts of IFN from sensitised lymphocytes.

In the flock A sensitivity trial, using the J-N ≥ 0.05 and J-N ≥ 0.10 criteria, there were a relatively high number of apparent false positives (33% and 13%, respectively). The prevalence of infection in this flock was relatively high (37% culture or histopathology positive). For flock C, the prevalence of infection was relatively low (6% by culture or histopathology) and the false positive rate for J-N ≥ 0.05 was 10%. Raising the J-N cut-point to ≥ 0.10 reduced the sensitivity of the test (48%) but did not reduce the number of false positives (11%). Subtracting the avium background response from the Johnin PPD (J-A ≥ 0.05) reduced both the sensitivity (44%) and the number of false positives (2%). There are several explanations for the high false positive rate: (i) Infected, IFN positive sheep, were not detected by either tissue culture or histopathology. (ii) The presence of IFN positive sheep that have cured their infections would also increase the false positive rate. (iii) Exposure to environmental mycobacteria, induced non-specific IFN responses. All of these factors may have contributed, in an unknown proportion, to the positive IFN responses in the sheep that were not detected as infected. The IFN test does not discriminate between true positives and false positives.

The Johnin PPDs (J_A and J_B sourced by Pfizer Animal Health) used for the specificity trial and sensitivity trials in flock A and B were not available in sufficient quantities for some of the bleeds in the longitudinal trial (flock C). As Johnin PPD could not be sourced globally from any other manufacturer, Johnin (J_i, in-house Johnin PPD) was prepared at CLI, Geelong from a standard bovine M. avium subsp. paratuberculosis strain (CLIJ623) isolated at the CLI laboratory. Comparisons of potency were undertaken during the longitudinal trial. For J_A and J_B there was moderate to substantial agreement in potency (bleed 5) and for J_i in comparison to J_A (bleed 6), substantial agreement. For the J_i comparison, there were a larger number of positive reactors and OJD infected sheep. J_i PPD has been stored for standardising the potency of future batches.
Conclusions

- To achieve high specificity (>98%), raised cut-points were required and this reduced the overall mean sensitivity of the IFN test to below 50% thus limiting its application for early detection of infection or disease freedom certification.

- Other major limitations for the IFN test are: (a) vaccination precludes its use and (b) there is the requirement for prompt initiation of laboratory testing following blood sample collection so that test result validity is not compromised.

- In small high value stud flocks, the IFN assay may useful as a surrogate test for faecal shedding in unvaccinated OJD infected sheep and consequently for a test and cull program to reduce pasture contamination and exposure of uninfected sheep. This will need further confirmation.

Recommendations

1. A consistent supply of quality controlled, potency tested Johnin PPD from one source is required.

2. Confirm the hypothesis that the IFN test has acceptable sensitivity (>60%) in sheep for detecting OJD faecal shedders using the high cut-point criteria to increase specificity (>98%). If confirmed, test hypothesis in small flocks that a sheep culling strategy, using repeat IFN and absorbed ELISA assays can eradicate OJD from these flocks.
Manipulation of the Interferon - gamma assay to maximise responses to M. ptb antigen in sheep

Acknowledgements
The assistance of the following is greatly appreciated. Staff from NSW Department of Primary Industries, the NSW Rural Lands Protection Boards (Riverina, Dubbo and Armidale), Department of Agriculture Western Australia, Primary Industries SA and Department of Primary Industries Victoria selected the sheep properties, collected faecal samples and assisted in the bleeding of sheep for the specificity trial. Peter Morcombe coordinated the sample collection in Western Australia. Pooled faecal cultures were performed by the Elizabeth MacArthur Agricultural Institute (EMAI) Menangle, the Regional Veterinary Laboratory (RVL), Orange, the Western Australia Department of Agriculture, South Perth, IDEXX Laboratories, Adelaide and the Department of Primary Industries, Attwood. Graham Bailey and Leslie Reddacliff provided laboratory space at the RVL, Orange (Dubbo specificity trial) and EMAI, respectively (longitudinal trial bleed 6). NSW Department of Primary Industries and EMAI performed autopsies, histopathology and culture of tissues for sensitivity trial flock A (part of NSW Department of Primary Industries project OJD.024). The University of Sydney performed autopsies, histopathology and culture of tissues for sensitivity trial flock B (part of University of Sydney project OJD.002) and the longitudinal trial flock C (part of University of Sydney project OJD.028). Pfizer Animal Health provided the BOVIGAM™, avian PPD and J A and J B Johnin PPD antigens for the IFN tests and the PARACHEK™ kits for the absorbed ELISA assays. Colin Trengove (South Australia), Geoff Green (Armidale, NSW), Chris Hourigan (Victoria) and Aiden Mansfield and Chris Darcy, CSIRO Livestock Industries, assisted with sampling sheep in the specificity trial. Funding for the project (OJD.025), validation of the interferon-γ test in sheep for diagnosis of ovine Johne’s disease, was provided by Meat and Livestock Australia.

K.L. Bosward, D. Begg, K. de Silva, L. Di Fiore, D. Taylor, R. Whittington

Introduction
The Bovigam® assay (CSL, Australia) is designed to detect IFN-γ released in response to M. paratuberculosis (M. ptb) antigen in a whole blood culture system by a sandwich enzyme immunoassay. While there is published data on the optimisation of the assay conditions for use in cattle1, there is little published information on the optimal conditions for use in sheep. Investigations were therefore made into the effect of duration of incubation, anticoagulant used, blood storage temperature and time delay to incubation in order to improve detection of M. ptb infected sheep.

Materials and Methods
The experiments were performed on blood collected into tubes containing different anticoagulants from vaccinated (Gudair®) Merino sheep. The blood was incubated at 37 °C for 24, 48 or 72 hours in either media alone or containing M. ptb antigen. To examine the effects of storage temperature and delay to incubation, the plates containing the blood and media ± M. ptb infected sheep.
antigen (added either at plate setup or just prior to placing the plate in the incubator) were held for 4, 8 or 24 hours at both room temperature and 4°C.

**Results**

Maximal IFN-γ responses were obtained when blood was collected into Lithium heparin tubes and culture was allowed to proceed for at least 48 hours. When the blood was stored for 24 hours prior to incubation, storage at 4°C resulted in lower IFN-γ responses than storage at room temperature. In addition, when incubation was delayed for 24 hours, adding the antigen at the time of blood collection resulted in greater IFN-γ responses.

**Conclusions and Future Work**

While the findings of maximal IFN-γ response with Lithium heparin anticoagulant and storage of the blood at room temperature are consistent with previous studies in cattle\(^1\), in this study, maximal IFN-γ responses were obtained by harvesting at 48 hours rather than 16 to 24 hours as is recommended by the manufacturer. The results of this study also suggest that if incubation is to be delayed, collection tubes containing antigen and media supplementation may improve IFN-γ responses.

Experiments are currently being conducted on blood collected in a field setting from larger numbers of vaccinated sheep to determine if adding *M. ptb* antigen immediately after blood collection and commencing incubation at 37°C by means of a portable incubator throughout the transportation phase to the laboratory will result in increased IFN-γ responses. Opportunities also exist to improve specificity of the test by identifying then removing cell populations in blood that are responsible for false positive reactions.

**Acknowledgements**

This work is funded by Meat and Livestock Australia (MLA). We thank Natalie Schiller and Anna Waldron for their excellent technical assistance and perseverance. The portable incubator used in experiments currently underway was supplied by Cellestis Ltd. The Bovigam® assay test kit was kindly supplied by Pfizer.

**Reference**

Evaluation of a Pourquier ELISA kit in relation to agar gel immunodiffusion (AGID) test for assessment of the humoral immune response in sheep and goats with and without Mycobacterium paratuberculosis infection

Gumber S², Eamens G and Whittington RJ

²Faculty of Veterinary Science, The University of Sydney
Private Bag 3, Camden 2570, NSW Australia
Phone: 02 9351 1610; Fax: 02 9351 1693
Email: sanjeevg@camden.usyd.edu.au

Background and objectives

Johne’s disease in sheep was first reported in Australia in 1980 from an experimental farm in Victoria, while in goats it was first reported in 1977 ¹,². Diagnosis of paratuberculosis is difficult because of the prolonged incubation period and the slow progression of the disease in the animal ³. The main serological test used for flock screening for ovine Johne’s disease (OJD) in Australia is the agar gel immunodiffusion (AGID) test. Serological tests are less expensive, faster and easier to perform than faecal culture but are also influenced by the age of the animal and the level of shedding of organisms ⁴. An ELISA for sheep was developed in the 1990’s by the NSW Department of Primary Industries (DPI) but it has not been used except in research because of inadequate specificity. There is considerable concern among the goat industries in efficient diagnosis of paratuberculosis as well as in tests for use in the market assurance programme to provide confidence that herds are free of the infection ⁵. Currently there are an AGID test and two ELISA assays, the Elizabeth Macarthur Agricultural Institute (EMAI) assay and the CSL Parachek EIA approved for use in goats in Australia ⁶. As an alternative to the AGID already used, and several other ELISA assays, the development of a single, simple ELISA for use in sheep and goats would allow greater automation of testing procedures. The present study was designed to evaluate a commercial ELISA kit (Institut Pourquier) under Australian conditions and to compare its accuracy with the existing AGID test for the diagnosis of ovine and caprine paratuberculosis.

Methods

To estimate diagnostic specificity of the tests for sheep, a total of 2200 serum samples were tested both by ELISA and AGID. Samples were comprised of 1,002 sera from Western Australia collected in 2004 and 1,198 samples from different low prevalence locations in NSW, collected in 1991. For goats, 945 serum samples were tested from Western Australia collected in 1997. Individual infection status was assumed to be negative. To estimate diagnostic sensitivity serum samples were tested from 152 sheep with histopathological lesions. Serum samples from 36 goats in which M. paratuberculosis (M. ptb) infection had been confirmed by histopathological examination and tissue culture along with 88 serum samples from goats from infected properties but without individual confirmatory tests were included in the study to calculate the diagnostic sensitivity of the tests. The samples were collected from different locations in NSW. Some of the goats were likely to have been selected originally based on their reaction in the EMAI absorbed ELISA for Johne’s disease ⁵, while others were reactors in this test but were not examined in the confirmatory tests.
The ELISA was conducted according to the manufacturer’s instructions except that all controls and tests were conducted in paired wells. The method used was very similar to that described by OIE.

Results were expressed as signal of the test sample as a proportion of the positive control, corrected for the negative control (S/P) according to the formula:

\[
S/P = \frac{OD_{450} \text{ value of the sample} - OD_{450} \text{ value of the negative control}}{OD_{450} \text{ value of the positive control} - OD_{450} \text{ value of the negative control}} \times 100
\]

Any sample with S/P equal to or greater than 70% was considered to be positive. Results for plates were accepted or all samples on the plate were retested according to decision-limit criteria (mean ± 2 standard deviation) for positive and negative control sera, where the mean OD and standard deviation of positive and negative controls from 193 ELISA plates were calculated to define the decision limit criteria. The results of the retest were used for analysis. A total of 8,187 serum samples were included in the study. Individual samples with a coefficient of variation (CV) for paired replicates of more than 15.0% were also retested. Reproducibility was estimated as the coefficient of variation (CV) for paired replicates of more than 15.0% were also retested. Reproducibility was estimated as the coefficient of variation (CV) for paired replicates of more than 15.0% were also retested. Reproducibility was estimated as the coefficient of variation (CV) for paired replicates of more than 15.0% were also retested.

The AGID test was performed as described by Whittington et al.

**Results**

**Evaluation for sheep**

The sensitivity of ELISA was estimated at one cut point S/P value (70%) according to the manufacturer’s recommendations. The overall sensitivity of ELISA was 34.9% (95% confidence limits, 27.3 to 43.0) from 152 infected sheep and it varied from 19.3% to 50.0% depending on the extent of histopathological lesions. The sensitivity of ELISA was greater for animals having Perez histopathological lesion types 2 and type 3a than for animals with other lesion categories. The sensitivity on the basis of tissue culture results was 32.8%. The overall sensitivity of AGID was 13.8% (95% confidence limits, 8.8 to 20.3) and like ELISA also varied (from 6.5% to 36.8%) depending on histopathological lesion grade. The maximum sensitivity of AGID was recorded for Perez type 3b multibacillary lesions. Only 11.2% of tissue culture positive animals were detected by AGID. The sensitivity of the ELISA was significantly higher than that of the AGID (McNemar \( \chi^2 = 19.22, \text{d.f.} = 1, p < 0.001 \)). The kappa value of 0.158 (95% confidence limits, 0.024 to 0.292) suggested a slight agreement between the two tests.

Specificity was estimated individually for the two states of Australia. The specificity of AGID was 100.0% for both states. The specificity of ELISA for sheep from Western Australia and NSW was 99.4% and 98.3%, respectively. The overall specificity of ELISA irrespective of states was 98.8%. Specificity of ELISA was estimated at different S/P cut points; it was increased to 99.0% at a cut point of 80, but sensitivity was then decreased from 34.8% to 31.5%.

**Evaluation for goats**

The sensitivity of ELISA was estimated at one cut point S/P value (70%) according to the manufacturer’s recommendations. The overall sensitivities of ELISA and AGID for 124 likely infected goats were 56.4 % and 39.5 %, respectively. The sensitivity of ELISA and AGID from the 36 known-infected population was 77.8% (95% confidence limits, 60.8 to 89.9) and 58.3% (95% confidence limits, 40.8 to 74.5), respectively. The sensitivity of the ELISA was significantly lower than that of the AGID (McNemar \( \chi^2 = 19.22, \text{d.f.} = 1, p < 0.001 \)). The kappa value of 0.158 (95% confidence limits, 0.024 to 0.292) suggested a slight agreement between the two tests.
higher than that of the AGID (McNemar $\chi^2 = 9.25$, d.f. = 1, $p < 0.005$). The kappa value was 0.448 (95% confidence limits, 0.280 to 0.616), which suggested a moderate agreement between the two tests.

The specificity for both ELISA and AGID was 100.0% based on testing 945 uninfected reference goat samples. Specificity of ELISA was also calculated at different cut points. There was a considerable increase in overall sensitivity of ELISA (66.9%) after decreasing the cut point from 70 to 30 without a decrease in specificity.

Quality assurance and reproducibility of the ELISA

A total of 8,187 sera were tested on a total of 193 ELISA plates with the same kit lot and batch of reagents, over a 10-month period, by one person. The mean OD value of the positive control was 0.804 (spread 0.624 to 0.984) (mean ± 2 standard deviations). According to the decision limit criterion, 6 plates with values lying outside this range were rejected and retested. The data for the positive control were normally distributed (Anderson Darling normality test, A-squared = 0.028, $p = 1.000$). The mean OD of the negative control was 0.058, with a spread of 0.048 to 0.068 (mean ± 2 standard deviations) and 6 plates with values outside this range were rejected and retested. The negative control data were also normally distributed (Anderson Darling normality test, A-squared = 0.421, $p = 0.320$). The CV for the mean of the paired replicates of the positive and the negative controls were 11.2% and 8.6%, respectively. The CV for the ratio of mean OD of negative control and positive control (N/P) was 7.3%. Thirty percent of the total plates tested showed a CV > 15.0% for the paired replicates of the positive control, but only 3.0% of the plates showed a CV > 15.0% for paired replicates of the negative control. Approximately 12.3% of the 8,187 samples showed a CV > 15.0% for the paired well data. Only those samples whose infection status was influenced by paired well variation ($n = 14$) were retested, that is those samples where the S/P value was close to 70%. The assay had a high level of reproducibility under different washing conditions using three plate washers. There were linear relationships between the data for plate washer 1 and plate washer 2 ($p = 0.000$, $R^2 = 92.0$%), plate washer 1 and plate washer 3 ($p = 0.000$, $R^2 = 98.3$%) and plate washer 2 and plate washer 3 ($p = 0.000$, $R^2 = 92.3$%).

Discussion

The specificity of the ELISA for sheep from Western Australia (99.4%) was higher than that of NSW sheep (98.3%). Although the samples tested were from very low prevalence areas in NSW, the disease is endemic in NSW and it might be possible that the disease was present but not identified in 1991, which might have confounded the results. OJD was detected in Western Australia in 2003 and 2004 and may have been present for more than 7 years. Consequently, the true specificity of ELISA for sheep is likely to be greater than the figures reported, because it was not possible to be certain that all animals in the specificity series were free of paratuberculosis. The specificity of the AGID was higher than that of the ELISA, but sensitivity was lower. An unexpected finding was that the ELISA detected more animals with Perez type 2 lesions along with the Perez type 3a and 3b lesions. However, the results of AGID revealed higher sensitivity in multibacillary lesions. The sensitivity of the ‘Institut Pourquier’ ELISA for goats was comparable to other studies but the specificity was higher. However, there was a bias in selection of samples from infected goats, as the samples were previously screened by an alternate ELISA. Therefore, the true sensitivity for goats would be less than reported here and most likely would be similar to the values for sheep. The ELISA in the present study provided a high level of reproducibility under different washing conditions suggesting robustness of the assay. The low CV for both the negative and positive control suggested high precision of the ‘Institut Pourquier’ ELISA.
Conclusion
The results presented in this study suggest that the ‘Institut Pourquier’ ELISA can be used for national disease control programmes for goats, as it would be unlikely to produce false positive reactions. Specificity of 98.8% suggests the need for confirmatory tests in national disease control programmes in sheep. Sample sizes for flock diagnosis can be selected to meet specific levels of confidence using the data provided.

Acknowledgements
The authors are thankful to Lab Diagnostics Australia for the supply of ELISA kits and Meat and Livestock Australia for funding the field trials. Laboratory management was provided by Anna Waldron and serum samples were collected with the assistance of Helen McGregor, Om Dhungyel, Craig Kristo and staff of Agriculture Western Australia.

References
Diagnosis – New Approaches
Early cellular responses in ovine Johne’s disease

Kumudika de Silva, Douglas Begg, Lyrissa Di Fiore, Deborah Taylor, David Emery and Richard Whittington

aFaculty of Veterinary Science, University of Sydney
425 Werombi Road, Camden NSW 2570
Phone: 02 9036 7731; Fax: 02 9351 1693
Email: kdesilva@camden.usyd.edu.au

Background and objectives

Protection against mycobacterial disease is dependent on T-cell dependent immune responses. In Johne’s disease, cell-mediated immune responses predominate in subclinical disease. As disease progresses these responses diminish and can be undetectable while there is a concurrent strong humoral response, demonstrated by elevated serum anti-M.ptb (Mycobacterium paratuberculosis) antibodies.

If the infection is not eliminated by the initial T-cell response then M. ptb are able to persist and proliferate resulting in lesion formation and chronic infection. M. ptb are obligate intracellular organisms and antibodies do not play an important role in eliminating disease.

Much work has been published on cellular responses throughout the course of bovine Johne’s disease but knowledge of similar responses in sheep is lacking. The aim of this on-going study is to identify changes in the distribution of lymphocyte subsets and antigen-driven proliferation of lymphocyte subsets during the course of early OJD.

Methods

Animals

In this on-going study, Merino lambs have been orally challenged with M. ptb; two groups of lambs will be experimentally infected annually. Peripheral blood and faecal samples were taken bimonthly while tissues, including ileal, jejunal and prescapular lymph nodes were collected at the end of the study when animals were sacrificed. Faecal and tissue samples were used to detect the presence of M. ptb by culture in a radiometric system (Bactec).

Cell phenotyping

Peripheral blood mononuclear cells (PBMC) and lymph node cells were isolated by density gradient centrifugation. Expression of the following cell surface markers was determined by flow cytometric analysis: CD4, CD8αα, CD8αβ, 86D, WC1, CD5, CD6, CD14, CD11a, CD45R and WC4.

Proliferation assay

Cells were labelled with CFDA-SE (carboxyfluoroscein diacetate-succinimidy ester) and then cultured for 4 days in the presence of culture medium alone, M. ptb antigen (316v; 10 µg/ml) or concanavalin A (10 µg/ml). Cell proliferation was detected by the decrease in CFDA-SE fluorescence by flow cytometric analysis. Results are expressed as either percentage of CFDA-SE^dim cells in the PBMC population or as a Stimulation Index (SI):

\[ SI = \frac{\text{% CFDA-SE}^\text{dim} \text{ cells in stimulated culture}}{\text{% CFDA-SE}^\text{dim} \text{ cells in control culture}} \]

An SI of greater than 2 is considered to indicate proliferation.
Phenotype of proliferating cells after 4 days in culture was determined by double staining CFDA-SE-labelled cells for cell surface markers.

**Results**

Phenotyping and proliferation assays were initially carried out on 12 experimentally infected animals 15.5 weeks after infection (n=3 per group, Groups 1-3 were infected with three doses of 4x10^4, 4x10^5 or 4x10^7 respectively while Group 4 remained uninfected).

There were no significant differences in the subtypes of PBMC amongst the different groups of animals.

**PBMC phenotype in experimentally infected and control animals at 15.5 weeks post-infection**

![Graph showing PBMC phenotype](image)

Proliferation of PBMC increased in response to *M. ptb* antigen in two of the group of three animals exposed to 4x10^5 or 4x10^7 Mptb/dose (6 fold and 4.5 fold respectively when compared to the medium only control), but not in the other two groups. Proliferation of lymph node cells in response to *M. ptb* antigen was seen in all animals, irrespective of experimental exposure to *M. ptb*. These results suggest that antigen-driven proliferation of PBMC could be useful in detecting early exposure of sheep to *M. ptb*. At the termination point of this study, none of the animals had any lesions characteristic of OJD nor was *M. ptb* detected in faeces.
Proliferation of mononuclear cells from experimentally infected and control animals at 15.5 weeks post-infection

In another group of experimentally infected animals (n=23), PBMC of half of the 12 animals tested responded to *M. ptb* antigen in the proliferation assay 3 months after *M. ptb* exposure. However, 11 months after infection, only 26% of the entire group showed a similar response.

Proliferation of PBMC from experimentally infected animals 3 and 11 months post-infection

In yet another group of experimentally infected sheep (n=18), *M. ptb* antigen-driven proliferation could be detected in 66% of animals four months after infection. Prior to infection, PBMC from these animals did not respond to *M. ptb* antigen.
Disease status of both these groups of animals is being monitored.

**Conclusions to date**

The *M. ptb* antigen-driven proliferation assay using PBMC detects exposure of most sheep to *M. ptb* as early as 3-4 months after experimental infection. However, whether these animals will later succumb to infection is yet unknown. Therefore these experimentally infected animals are still being monitored. Knowledge of their infection status will enable calculation of test sensitivity and specificity at each time point. The lymphocyte proliferation assay is currently a research tool but acts as a defacto for other types of assay which might be applicable for practical diagnostic application. Results to date do not show an alteration in PBMC subsets as a result of exposure of sheep to *M. ptb*.

**Acknowledgements**

This project is being funded by Meat & Livestock Australia. We are grateful to Nicole Carter, Reena Mehta and Anna Waldron for their excellent technical support.
Apoptotic responses during the pathogenesis of ovine Johne’s disease

Sally Brownea, Kumudika de Silva, Richard Whittington, David Emery

Farm Animal Health Group
Faculty of Veterinary Science, University of Sydney
425 Werombi Rd, Camden NSW 2570
Phone: 02 9351 1610; Fax: 02 9351 1693
E-mail: sbro2018@mail.usyd.edu.au

Background and objectives

The immunological mechanisms underlying the progression of Johne’s disease from a subclinical to a clinical disease state remain unclear. More knowledge regarding the interactions between host immune cells and Mycobacterium avium subsp. paratuberculosis (M. ptb), including apoptotic responses, may allow further advance in the area of early detection and control of the disease and identification of resistant animals.

Apoptosis or programmed cell death is a tightly controlled form of cell death that typically leads to DNA degradation and sequestering of cell contents into apoptotic bodies, without inducing an inflammatory response (Thornberry and Lazebnik 1998). Macrophages infected with mycobacteria will undergo apoptosis, in which TNF-α plays a key role, both in vitro and in tissues (Gao and Abu Kwaik 2000).

Host macrophage apoptosis is an innate defense mechanism which is linked to the killing of intracellular mycobacteria (Fratazzi, Arbeit et al. 1999). This process appears to require viable mycobacteria as apoptosis has been observed in bovine monocytes when infected with live but not when infected with heat-killed M. ptb using Hoechst 33342 staining (Allen, Sotos et al. 2001).

The ability of virulent mycobacteria to evade macrophage apoptosis has been documented widely: some mechanisms used to inhibit apoptosis include increasing sTNFR2 production, neutralising pro-apoptotic activity of TNF-alpha (Balcewicz-Sablinska, Keane et al. 1998) and inactivating pro- and anti-apoptotic proteins (Maiti, Bhattacharyya et al. 2001; Sly, Hingley-Wilson et al. 2003). Recently, specific genes associated with apoptosis and cell survival (anti-apoptotic) have been identified at significantly different levels in the blood of M. ptb infected cattle compared to uninfected controls (Coussens, Pudrith et al. 2005).

Furthermore, lymphocytes can induce apoptosis of infected macrophages as part of the host immune response. Lymphocytes have also been shown to undergo apoptosis in response to mycobacterial infections both in vitro and in tissues (Roger and Bermudez 2001; Zhong, Gilbertson et al. 2003). Lymphocyte apoptosis seems to require direct contact with mycobacteria-infected macrophages (Roger and Bermudez 2001). Recently, antigen induced apoptosis has been observed at significantly higher levels in the peripheral blood lymphocytes of M. ptb infected cattle than in control animals (Susanne Grell, personal communication).

The focus of this project is to investigate the apoptotic responses of lymphocytes and macrophages throughout the course of Ovine Johnes Disease (OJD) with particular focus on evaluating T cell apoptosis as a diagnostic indicator of early subclinically infected animals. In the present study we report significant differences in antigen-induced apoptosis in vitro within a group of clinically infected sheep when compared to uninfected control animals.
Method

Merino sheep aged 3 years were purchased from a NSW property with a high prevalence status for OJD. Infection was confirmed by AGID and faecal culture tests. Out of 100 sheep that were tested, 7 with moderate to advanced subclinical disease and 4 uninfected animals were chosen for this study.

Mononuclear cells were isolated from peripheral blood (PBMCs) prior to sacrifice using density gradient centrifugation. Samples were then taken from the ileal (ILN), jejunal (JLN) and prescapular (PLN) lymph nodes. Isolated cells were incubated with medium alone, Mptb antigen (10 µg/ml) or Con A (10 µg/ml) for up to 6 days.

Apoptosis was measured using a fluorescent analog of the pan caspase inhibitor Z-VAD-FMK (Promega), which irreversibly binds to activated caspases, a central component of most apoptotic pathways. Fluorescence was detected using flow cytometry on days 0 and 6 of culture.

Results

There were no significant differences in apoptosis in mononuclear cells, regardless of infection status or sample site. PBMC apoptosis was < 10%. Apoptosis in ILN and JLN cells was generally higher in all sheep ranging from 4-14% and 5-25% respectively with the exception of one infected animal where apoptosis in the JLN was almost 60%. Generally apoptosis was higher in the JLN of infected animals than in uninfected animals (data not shown).

Antigen-induced apoptosis of PBMCs on day 6 was not significantly different from media alone in all animals except one infected sheep in which antigen induced apoptosis was significantly less than media alone.

Antigen-induced apoptosis in ILN mononuclear cells was significantly increased ($P <0.05$) in 3 of 4 infected sheep tested and significantly reduced in 1 of 4 of the tested animals compared to media alone. Importantly, $M. ptb$ antigen did not significantly alter apoptosis in ILN mononuclear cells from all three uninfected sheep tested.

$M. ptb$ antigen induced apoptosis in 4 of 7 infected animals to a significantly greater extent than medium control during culture of JLN mononuclear cells. A similar significant increase was seen in 1 of the 3 uninfected animals tested.
Percentage of apoptotic cells (caspase+) on day 6 of culture

1.1 Uninfected

Discussion and conclusions to date
Antigen-induced apoptosis in ILN and JLN mononuclear cells may be an indicator of disease in 3 year old sheep with established OJD. This work is part of an ongoing series of animal trials, looking at apoptotic responses at different stages of the disease in both naturally and experimentally infected sheep.

Further studies are also being carried out to quantify relative changes in expression of pro-apoptotic and anti-apoptotic genes in response to Mptb infection. In addition, the TUNEL assay will be used to detect apoptosis within intestinal tissues during the course of infection.

Acknowledgments
The author receives an MLA postgraduate scholarship. This project is funded by MLA. Special thanks also to Nicole Carter and Anna Waldron of the Farm Animal Health Group at Sydney University for their technical assistance during the course of this project.
References


ELISPOT – new methods for detecting IFN-γ

Douglas Begg, Katrina Bosward, Kumudika De Silva, Lyrissa Di Fiore, Deborah Taylor and Richard Whittington.

Faculty of Veterinary Science
University of Sydney,
425 Werombi Rd, Camden, NSW, 2570
Phone: 02 90367737; Fax: 02 93511693
Email: dougb@camden.usyd.edu.au

Abstract

IFN-γ is a cytokine produced as part of the early cell mediated immune response to M. paratuberculosis. Detection of IFN-γ production is being used as a diagnostic test to detect mycobacterial infected ruminants. However in sheep the IFN-γ assay has not proved as successful as initially expected, especially for the detection of subclinically M. ptb infected animals. Therefore new strategies are being investigated to improve the sensitivity of IFN-γ detection methods. These include the use of ELISPOT and Cell ELISA where the IFN-γ is detected directly from the culture of the animal’s leucocytes.

Background and objectives

Early immune responses against mycobacterial infections such as M. paratuberculosis (M. ptb) are thought to be cell mediated, resulting in production of immune cytokines such as IFN-gamma (IFN-γ). Thus, detection of IFN-γ was initially thought to improve immunodiagnostic testing for mycobacterial infections in ruminants, providing early detection of infected animals. Unfortunately in the diagnosis of Johne’s disease, the use of commercially available IFN-γ detection methods has not resulted in the anticipated levels of detection. The IFN-γ assay for detection of M. ptb infection in ruminants is based on IFN-γ production in blood samples stimulated with mycobacterial antigens. The plasma from the stimulated blood sample is then removed from the culture and tested for IFN-γ using a sandwich ELISA (Enzyme Linked Immuno Sorbent Assay). The sensitivity of the IFN-γ assay can be directly correlated to the stage of disease. One study found that 70-90% of subclinically infected and 100% of clinically infected cattle could be detected with the IFN-γ assay (Billman-Jacobe et al., 1992). Another study, also in cattle, reported a sensitivity of 50-75% with a specificity of 98% for the IFN-γ assay (Stabel and Whitlock, 2001). In sheep, similar sensitivity values of 50-75% were found using the Bovigam (Pfizer) assay (Stewart et al., 2002). The ELISPOT assay differs from the ELISA in that the cells are cultured with the antigen in the well used to detect IFN-γ and this can allow enumeration of the IFN-γ producing cells, with each individual IFN-γ producing cell shown as a spot on the bottom of the membrane. The ELISPOT (Enzyme Linked Immuno Spot) assay has been shown to be 10-200 times more sensitive in the detection of cytokines than the traditional ELISA (Tanguay & Killion, 1994). The objectives of this study are to examine alternative methods of IFN-γ detection other than the typical ELISA currently available. These methods have included ELISPOT and its cousin, the Cell-ELISA assay, which yields a colour change rather than spots.

Methods and results

The discovery of several cross reactive monoclonal antibodies that detect ovine IFN-γ led to the development of a functional ELISPOT and Cell-ELISA that was then optimised in terms of antibody dilutions, incubation times and changes in cell numbers using blood from vaccinated
and challenged animals. After the blood sample was collected from the animal, the white blood cells were purified by using a density gradient (Ficoll-Paque). In the ELISPOT assay, once the cells had been isolated and counted, serial dilutions of cells were placed in a plate coated with an antibody against IFN-γ. In the Cell-ELISA, the cells were still placed in antibody coated plates, however no serial dilutions of the cells were needed. In both assays, mycobacterial antigens were then added to the cells to stimulate IFN-γ production. After 24 or 48 hours incubation, the cells were washed, a second anti IFN-γ antibody was added, together with the appropriate substrates to complete the reaction, resulting in spots for the ELISPOT and colour changes for the Cell-ELISA. Initial experiments have shown that the ELISPOT assay works best when incubated for 24 hours, with increased background occurring when incubation is extended to 48 hours. A field trial where blood samples were transported from a farm, held at room temperature overnight, and then incubated for 24 hours showed this incubation period for the blood samples with antigen was not successful in demonstrating IFN-γ production indicating that longer incubation times may be required in samples that have had to endure delays prior to processing (See Figure 1). Initial experiments have also suggested that the ELISPOT assay may have greater sensitivity than the ELISA. A small study using naturally infected animals showed the ELISPOT from blood samples could detect 8/10 infected animals that were positive for \( M. \text{ptb} \) from intestinal tissues, whereas the Bovigam assay only detected 6/10 infected animals. Experiments carried out on experimentally infected animals have shown that even though the ELISPOT and Cell ELISA assay are essentially the same there is not always a complete correlation.

**Figure 1:** Representative ELISPOT results from (A) a blood sample from a vaccinated animal, transported to the lab then processed the next day; note the low number of spots including the positive control stimulated cells. (B) A blood sample from a vaccinated animal processed on the day of collection and incubated for 24 hours before the spots were developed. (C) A blood sample from a vaccinated animal processed on the day of collection and incubated for 48 hours before the spots were developed; note the increased background in the \( M. \text{ptb} \) antigen stimulated wells. Two dilutions of cells are shown for each animal in A, B and C; column 1 for each animal contained \( 2.5 \times 10^5 \) white blood cells per well, while column 2 contained \( 1.25 \times 10^5 \) cells per well. Serial dilutions of cells were conducted to ensure that at least one well had an optimal number of spots to enable accurate counting.
Conclusions to date

While the ELISPOT appeared to be more sensitive in initial small group studies, cut off points still need to be defined to distinguish between infected and non-infected animals and this can be explored more after testing additional groups of known infected animals. In addition, work is needed to improve the reliability of the assay and its overall robustness. At the present time, the Bovigam assay is a very simple assay to complete due to the minimal blood sample processing requirements. Simplification of the ELISPOT and Cell-ELISA assay technique, for example by simplifying the way the white blood cells are purified from a blood sample, will allow these tests to become more cost effective and enable high throughput of blood samples. Typically, cells used in the ELISPOT assay are subjected to complex Ficoll separation techniques to enhance purity, however, initial ELISPOT studies have shown that white blood cells with reduced purity will still show IFN-γ production. Further studies are needed to determine how reduced purity will affect antigen specific production of IFN-γ. The ELISPOT and Cell-ELISA are technically more difficult to perform than the traditional ELISA method but there is room to explore methods to enable automation of the processes involved. Unfortunately, unlike the Bovigam assay, repeat testing requires additional blood samples.

The use of the ELISPOT and Cell-ELISA tests in groups of naturally infected animals will assist in determining sensitivity and specificity and to define the cut-off points that will allow identification of infected and non infected animals. Testing of greater numbers of naturally infected animals will provide more information on the effectiveness of these assays as a diagnostic test.

As has been noted in the Bovigam assay, it appears that transportation and storage of blood samples will be critical in how the assays work as diagnostic tests. There is a need to overcome problems caused by transport and longer term sample storage. This may involve the use of additives such as the cytokine, IL-12, (Jungersen et al., 2005) which has been shown to extend the storage time of cattle blood to 24 hours while still allowing IFN-γ detection.

Acknowledgements

This project is funded by MLA. The authors would also like to acknowledge the help of Anna Waldron, Craig Kirsto, Nicole Carter and Natelie Schiller with this work.

References


Jungersen, G., S. N. Grell, A. Clemensen and C. J. Howard (2005). Interleukin-12 potentiation of the interferon-gamma test rescues day old blood samples for diagnosis of paratuberculosis PPD specific cellular mediated immune responses. 8th International Colloquium on Paratuberculosis, Copenhagen, Denmark.


Detection of Mycobacteria from blood

Kate Goldsmitha, Doug Begg, Lyrissa Di Fiore and Richard Whittington

aUniversity of Sydney
425 Werombi Rd, Camden, 2570
Phone: 02 9351 1610
Email: katrinag@camden.usyd.edu.au

Abstract

Johne’s disease, or paratuberculosis, is a chronic wasting disease causing intestinal thickening due to granulomatous inflammation in the ileum and jejunum of ruminants. Transmission of paratuberculosis is primarily by oral exposure to *Mycobacterium avium* subspecies *Paratuberculosis* (*M. ptb*) in young animals, with clinical signs of disease developing many years later. Between exposure and clinical disease there is a long subclinical phase in which shedding of the organism in faeces occurs intermittently.

Demonstration of the organism in blood from infected sheep and cattle by PCR has sparked recent interest in developing a diagnostic test based on culture or molecular detection of the organism from blood. Detection of *M. ptb* DNA in blood, culture of the organism from milk and extra intestinal sites, and demonstration of transmammary and intrauterine transmission, indicate that at some stage in the disease, bacteraemia occurs.

Background

In paratuberculosis infected animals there is considerable evidence that the bacteria are present in the blood at some stage in the disease process. *M. ptb* was isolated from peripheral blood mononuclear cells by bacteriologic culture on Herrold’s egg yolk medium from 1 of 7 clinically diseased cattle [1]. Direct PCR of DNA extracted from the blood of clinically affected sheep using IS900 primers in one study returned positive results in 9 out of 12 histologically positive sheep [2]. A follow-up study to examine the sensitivity of detection of *M. ptb* by PCR from blood in experimentally-inoculated, subclinically-infected sheep returned unfavourable results with only 2 positive blood samples after repeated serial testing of 7 sheep with subclinical infection that were confirmed by histology [3]. A study using nested PCR on milk and blood of cattle demonstrated positive results from both with 72% of clinically infected animals testing positive. PCR on blood was less sensitive than PCR on milk in subclinically infected cows with sensitivities of 33% and 45%, respectively [4]. Another study using PCR on blood, faeces, milk and liver in advanced subclinically infected cows demonstrated a sensitivity of 40% for PCR on blood samples [5]. Compared with ELISA testing, PCR on blood appeared to detect different forms or stages of the disease [6].

In addition to demonstrating *M. ptb* DNA in blood of Johne’s infected animals, systemic spread has been observed in tissues such as the liver [2, 3, 5], mammary tissue [7], milk [4, 5, 7], spleen [8], foetal tissues [1, 7] reproductive tissues [8-10], and extra intestinal lymph nodes [1, 8].

Culture of pathogenic mycobacteria from blood is of increasing importance to human medicine due to the tendency for these pathogens to cause disease in HIV positive patients. While the growth requirements of *M. tuberculosis* and *M. avium* complex differ from *M. ptb*, sample processing and culture techniques for these pathogens may be adaptable for culture of *M. ptb*. Concentrate from Isolator tubes (Wampole Laboratories, Cranbury, N.J., previously
manufactured by Du Pont Co., Wilmington, Del.), which contains saponin, a cell lysis agent, and SPS which neutralizes the bactericidal properties of blood, have been successfully inoculated onto a variety of solid and liquid media to isolate mycobacteria [11, 12].

Concentration of mycobacteria from clinical samples has been shown to be improved by using carboxypropylbetaine (CB-18) to counter the buoyancy and clumping associated with mycobacteria. CB -18 has been used to concentrate M. ptb from cattle faeces and tissue samples[13, 14], M. avium from tissue, bone marrow, blood and faeces[15], and M. tuberculosis from respiratory samples[16]. This method may be useful in concentrating organisms in blood samples from infected animals.

The Bactec TB 460 system, which measures radioactive CO\textsubscript{2} generation to detect growth, has successfully been used to culture ovine strains of M. ptb from faeces, tissues and milk using Bactec 12B media [7, 17]. The limitation of using Bactec 12B media for culture from blood is the small volume of sample used for inoculation; 0.2mls was used in one study [11]. Bactec 12B media has been successfully used for culture of isolator concentrate [11], however some studies have shown inhibition of growth due to the polypropylene glycol which is eliminated with a wash step prior to inoculation [18, 19].

The Bactec 13A media, which also uses the Bactec TB 460 radiometric detection system, is formulated specifically for the culture of mycobacteria directly from blood allowing larger volumes to be inoculated. This method when used to detect mycobacteraemia in HIV patients, returned a comparable number of positives to Isolator tubes [12]. Supplements required for growth of M. ptb, such as mycobactin J and egg yolk for sheep strains must be added.

Objectives

The objective of this project is to optimise detection methods for culture and molecular detection from blood. To do this different processing methods using spiked blood and samples from clinically and subclinically infected animals will be compared. This will be used to determine feasibility of detection of the organism from blood as a diagnostic test. Currently methods under evaluation are lysis centrifugation and buffy coat preparations inoculated into the Bactec 12B medium, modified as previously described [17], whole blood culture using a modified Bactec 13A medium, and conventional and real time IS\textsuperscript{900} PCR on buffy coat preparations and lysis pellets.

Furthermore, this project aims to develop a better understanding of the behaviour of the organism during the bacteraemic phase. Tracking M. ptb and infected monocytes following experimentally induced bacteraemia in unchallenged animals may allow us to better describe the spread of the organism following bacteraemia. Correlation of lesion types and stage of disease in experimentally induced and naturally infected animals may allow us to determine the stages of disease where bacteraemia occurs.

Acknowledgments

This project was funded by Meat and Livestock Australia who also provided a scholarship for the senior author. Thanks are also due to Anna Waldron, Craig Kristo and Angela Reeves for their technical assistance.
References


Proteomic analysis of sheep serum by SELDI TOF-MS: Identification of putative biomarkers of ovine Johne’s disease

Ling Zhonga, Deborah L. Taylor and Richard J. Whittington

aFaculty of Veterinary Science, University of Sydney
425 Werombi Road, Camden, NSW 2570, Australia.
Phone: 02 9351 1610; Fax: 02 9351 1693
Email: lzho9275@mail.usyd.edu.au

Introduction

*M. paratuberculosis* (*M. ptb*) is the causative agent of paratuberculosis or Ovine Johne’s Disease (OJD), a slow-developing, infectious disease characterised by chronic granulomatous enteritis, thickening of the intestinal wall and progressive weight loss resulting ultimately in the death of the animal. The current diagnostic tests for Johne’s disease require detection of the microorganism by culture or PCR, or detection of specific host reactions to infection. Most of these tests are better able to detect animals in later stages of infection rather than in earlier stages and with moderate sensitivity and specificity. Therefore, there is a current need for new tests to be developed due to the deficiencies in their sensitivity and specificity.

The development of new diagnostic tests has been hindered largely by our limited knowledge of the host factors involved in the immune response to the organism, and the lack of appropriate molecular tools to dissect the host-pathogen interaction. Recent developments in tools for molecular biology has meant that global gene expression of both the host and pathogen in response to infection can now be analysed for many species. However, the availability of these genomic resources for ruminants is still a limiting factor for advancement of research in these species. A number of proteomic approaches are now available that require no pre-existing knowledge of host factors.


Hypothetically, various physiological and pathological processes could modify the proteome, generating a unique, disease-specific signature, known as proteomic signature, or disease signature (Rocken *et al.*, 2004). Proteomic profiling of any protein resource, such as serum, could facilitate the discovery of different patterns of protein expression between uninfected or infected animals. This could lead to a new diagnostic test capable for detecting infected animals at an earlier stage of disease.

Methods

SELDI TOF-MS based proteomic profiling of serum can generally be divided into four stages: optimisation of SELDI trace acquisition, pilot scale biomarker discovery, putative biomarkers verification and key protein identification and characterisation.
SELDI trace acquisition optimisation

In the SELDI optimisation experiment, a range of conditions need to be optimised. To determine the optimal EAM (energy absorbing molecule) or matrix component, two EAM molecules, CHCA and SPA, have been evaluated with two different ProteinChip® array surfaces. To examine the effects of total protein concentration on the SELDI profiles, five different dilutions of serum were evaluated: undiluted, 1:4, 1:8, 1:15 or 1:25. To determine conditions that can produce the optimal spectrum, defined as the maximum number of peaks generated and optimum spectra generation (the peak resolution), four array surfaces were evaluated in combination with multiple binding buffers. To evaluate the quality of spectra generated with SELDI-TOF MS in different conditions, a number of criteria have been suggested such as the number of peaks, peak average broadness and peak separations (Cordingley et al., 2003). In addition, the optimal intensity of peaks needs to be taken into account to evaluate the quality of trace.

Pilot scale biomarker discovery

The aim of a pilot scale biomarker discovery is to identify if putative biomarkers from a small sample size can be detected. A total of eleven sheep were selected and divided into two groups: infected and non-infected. The infection status of all sheep was confirmed by a variety of tests including histology and tissue culture. Of the infected sheep, two were in an early stage of infection with paucibacillar lesion, two were at a later stage with multibacillary lesion and three were at later stage with paucibacillary lesion. The sera from all eleven sheep were applied to three optimized ProteinArray® chip types as determined by the optimisation experiment. Protein in the mass range of 2-20 kDa were analysed. The peaks were baseline-subtracted, calibrated on mass accuracy, detected, and clustered automatically by the analysis software. In the Biomarker Wizard, the normalised peak intensity of each peak detected in sera from sheep infected with M. ptb was compared with that in the control group by nonparametric two-sample Mann-Whitney t-test to identify the peaks that were significantly increased or decreased in infected sheep. The software returns P values of each cluster and only the cluster with P value less than 0.05 will be focused.

Results

Optimised conditions

CHCA matrix was found to generate very sharp and informative traces under 15 kDa, however it hardly revealed any peaks in the larger molecular weight range. The SPA compound in contrast, generated reasonably good traces up to 20 kDa. Therefore SPA was selected as the EAM molecule for sheep serum in the following experiments. For all four ProteinChip® array types, better traces were generated with either 1-in-4 sample dilution (about 6.5mg/ml) or undiluted sample (about 26mg/ml) than 1-in-8, 1-in-15 or 1-in-25 dilutions. The optimal ProteinChip® array types in combination with optimal binding buffers are listed in Table 1. The optimised conditions in this stage will be taken forward in the pilot scale biomarker discovery experiment.
Table 1. Optimal ProteinArray® chip types in combination with optimal binding buffers.

<table>
<thead>
<tr>
<th>ProteinChip® Array</th>
<th>General description (Ciphergen)</th>
<th>Washing / Binding buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q10</td>
<td>Strong anion exchange</td>
<td>Tris. HCL pH7.0,</td>
</tr>
<tr>
<td>CM10</td>
<td>Weak cation exchange</td>
<td>Tris.HCL pH7.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Low stringency buffer</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Ciphergen)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>High stringency buffer</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(50mM HEPES, pH7.0)</td>
</tr>
<tr>
<td>IMAC 30 Cu / Ni</td>
<td>Immobilized metal affinity</td>
<td>PBS (Ciphergen)</td>
</tr>
<tr>
<td></td>
<td>capture</td>
<td></td>
</tr>
</tbody>
</table>

Putative biomarker identification

Although all three different ProteinChip® array types were tested, the IMAC 30 ProteinChip® array surface loaded with copper sulphate generated the maximum number of significantly different clusters (Figure 1). Five potential serum biomarker clusters (arrows in Figure 1 panel A) with differential expression in *M. ptb* infected sheep vs. the uninfected sheep were observed, and they were down-regulated in infected sheep in comparison with the control group.
Fig 1. Example of significantly different clusters from two groups.

A. Clusters generated by Biomarker Wizard by using IMAC 30 ProteinChip® array surface loaded with copper metal. The square represents the uninfected group and the circle represents the infected group. Only the clusters with P values less than 0.05 are shown. The five clusters with arrow were differentially expressed in infected group vs. control sheep.

B. SELDI traces from the two groups correspond to a different cluster in Biomarker Wizard view in panel A. Top 4 traces belong to the un-infected group and the bottom seven traces are from the infected group. The x-axis represents the mass/charge ration and y-axis represents the relative intensity.

Conclusions to date
Surface enhanced laser desorption ionisation (SELDI) time of flight mass spectrometry has facilitated the discovery of a disease specific protein profile from many different biological samples, including serum and tissues. Our pilot scale biomarker discovery results have raised the possibility that protein profiles may become a powerful diagnostic tool for OJD and need to be further verified with an increased sample size and continued efforts on methodological standardisation.

Acknowledgement
The author receives an MLA post graduate scholarship. This project is funded by MLA.
References


Gene expression signals from the host

Lyrissa Di Fiore\textsuperscript{a}, Deborah Taylor, Kumudika de Silva, Kate Bosward, Helen McGregor and Richard Whittington

\textsuperscript{a}University of Sydney  
425 Werombi Road, Camden NSW 2570  
Phone: 02 9036 7737; Fax: 02 9351 1693  
Email: lyrissa@camden.usyd.edu.au

Background and objectives

Mycobacterium paratuberculosis (\textit{M. ptb}) causes a chronic progressive wasting disease in sheep known as ovine Johne’s disease (OJD). A large number of organisms are typically found within the gut in chronically infected animals. Lesions develop in the gut wall and these contain epitheloid macrophages and giant cells and there is a general thickening of the mucosa. The changes occurring following infection at the molecular level have not been well characterised in sheep. Most of the data on the molecular pathogenesis of the disease has come from studies in other species such as cattle in the advanced stages of the disease, or from macrophage infection models. Molecular techniques used in these studies include microarray analysis, selective capture of transcribed sequences (SCOTS) and RNA arbitrarily primed-PCR (RAP-PCR). Few studies have looked at OJD using DD-PCR, and those have primarily focused on bacterial gene expression.

There are needs for effective ways of identifying all genes that are differentially expressed in different cell types or under altered or diseased conditions. Tools that allow us to determine when and where genes are turned on or off as these processes occur have become increasingly important. Differential display PCR (DD-PCR), representational difference analysis (RDA), serial analysis of gene expression (SAGE) and cDNA microarray analysis are well established tools for studying differentially expressed genes under normal and altered/diseased conditions. These approaches all stem from the same origin; differential screening and differential hybridisation.

The objective of this study is to examine host gene expression during the initial stages of \textit{M. ptb} infection and during the clinical stage of infection to identify known or novel genes regulating the response to infection, and to define genes that can be used to identify \textit{M. ptb} infected animals earlier than currently possible.

Methods and results

Long distance differential display PCR (DD-PCR) is a technique used to identify differences in gene expression patterns between two or more biological samples. It was employed to identify differentially expressed genes in lymph node and ileal tissues collected from uninfected, early stage infected or clinically infected Merino sheep.

Animals - Female Merino sheep of three age groups (lambs, hoggets and adults) were co-grazed with infected sheep for 14.5 weeks after which they were removed. Animals were killed 2.5 years after initial exposure and samples of terminal ileum and mesenteric lymph node were collected. Histological data was obtained and animals were classified according to the Perez scale.

Long Distance DD-PCR – The Delta Differential Display kit (Clontech) was used. RNA was extracted from tissues using the Qiagen RNeasy mini kit. DNAase treated RNA was reverse transcribed with oligo (dT) primers and AMV-RT to produce single stranded cDNA. PCR was
performed using anchored 3’ primers (T1-T9), arbitrary 5’ primers (P1 – P10) and α 35S-dATP to label cDNA products. PCR products were run on an 8% acrylamide gel, which was then dried and exposed to X-RAY film. Differentially expressed bands were excised and DNA extracted from the gel slice. Bands of interest were re-amplified using the primers in the original PCR. Amplified products were analysed on an agarose gel and cloned into a TOPO cloning vector.

Eleven bands appear to be differentially expressed between uninfected and clinically infected animals. Re-amplification of these bands produced products with sizes ranging from 400-1500bp. Two genes were found to be differentially expressed in lymph nodes and nine genes in the terminal ileum. Most of the differences observed occurred between control and Perez 3b infected animals. One gene was found to be preferentially expressed in Perez 2 and 3a animals and not in 3b or control animals. Expression of these genes will be confirmed by qPCR. Other primer combinations are being assessed on these control and infected animal samples.

Gene identification – Cloned products are currently being sequenced and the data from this will be presented.

Conclusions to date

Preliminary findings have shown 11 cDNA bands to potentially be differentially expressed between control and infected lymph nodes or ileum. The changes observed in the ileum primarily occurred in Perez 3b animals (multibacillary disease). The identification of the cloned products will provide further insight into the pathways being regulated at the host level as a consequence of *M. ptb* infection.

Long distance DD-PCR is an effective albeit expensive and labour intensive tool for identifying differentially expressed genes between control and infected animals. It allows the opportunity to discover genes that are up or down regulated and also allows the comparison of more than two samples unlike microarrays, where only two samples can be compared per experiment.

This technique has the potential to identify unique or known genes that could be used to identify infected animals early in the disease process.

Acknowledgements

This work is funded by Meat and Livestock Australia (MLA).

Thanks also to the technical staff in the Farm Animal group at Sydney University – Anna Waldron, Reena Mehta, Nicole Carter, Natalie Schiller.
Molecular approaches to studying the host-parasite interaction

Deborah Taylor\textsuperscript{a}, Sanjeev Gumber, Kumudika de Silva, Kate Goldsmith, Lyrissa Di Fiore, Doug Begg & Richard Whittington

\textsuperscript{a}University of Sydney
425 Werombi Road, Camden NSW 2570
Phone: 02 9036 7731; Fax: 02 9351 1693
Email: dtaylor@camden.usyd.edu.au

Background and objectives

Successful colonisation of the host by \textit{Mycobacterium avium} subspecies \textit{paratuberculosis} (\textit{M. ptb}), the etiologic agent of Johne’s disease, involves a highly complex series of interactions between the parasite and host. In order to facilitate their survival and growth within the host, successful pathogens have evolved a variety of specific gene products, as well as mechanisms to regulate expression of these factors in response to particular stimuli. The response to these cues is the coordinately regulated expression of multiple virulence-associated genes that facilitate the organisms’ survival in an environment that can be hostile. In response, the host has evolved a variety of defense mechanisms that aim to eliminate invading pathogens. The result is the highly coordinated regulation in both cell types that ensures that genes required at specific stages of the infection process are expressed at the appropriate time. The identification of the genetic elements that encode these virulence-associated factors and the alteration in host immune response is a major objective of molecular microbiology, as it allows better understanding of how these factors contribute to pathogenesis in the susceptible host.

Following ingestion of \textit{M. ptb}, the bacteria initially translocate through the mucosa, most probably via the M-cells overlaying the ileal peyer’s patches. From here they are internalised and persist in the underlying epithelial macrophages. A major obstacle that \textit{M. ptb} must overcome is killing by the macrophage. The bacteria are able to survive and replicate within the macrophage phagosome, possibly by preventing the production of reactive oxygen intermediates (ROI) and reactive nitrogen species (RNS) or by preventing phagosome maturation and/or lysosome fusion as is the case with \textit{Mycobacterium tuberculosis}. This is a hostile environment that presents a number of situations which the bacteria must overcome. These stresses include host sequestration of essential nutrients, the production of reactive species and the depletion in available oxygen resulting in a hypoxic environment.

Our current understanding of the biology of \textit{M. ptb} infection has been hindered largely by our limited knowledge of the host factors involved in immune responses to the organism, and the lack of appropriate molecular tools to dissect the host-pathogen interaction. Nonetheless, there is a clear need for better diagnosis, control and understanding of the pathogenesis of the organism. Recent development in tools for molecular biology has meant that global gene expression of both the host and pathogen in response to cell internalisation can now be analysed for many species. However, the availability of these technologies and genomic sequences for livestock is still a limiting factor for advancement in these species.

We are using a variety of molecular techniques to study the host and pathogen responses under various conditions including natural infection, experimental infection and dormancy induced by nutrient starvation and hypoxia. In addition, novel assays for the development of new, molecular based diagnostic tests involving detection of the organism are underway. This presentation will outline some of our current and proposed work on the organism.
Genomic approaches

Analysis of the host response to infection

To analyse the host response to experimental infection with *M. ptb* we are using a murine macrophage model of infection in preliminary investigations to characterise alterations in host gene expression, protein secretion, induction of apoptosis, macrophage survival and bacterial persistence in response to long term infection with *M. ptb*. Cellular parameters assessed were cell number, apoptosis (caspase activity determined by flow cytometry), number of infected cells and number of *M. ptb* per cell. RNA was isolated from the macrophages at each time point and differential display PCR (DD-PCR) is being used to identify novel changes in gene expression in response to infection over time. Differences in expression of known immuno-responsive genes has also been determined using quantitative PCR. Our results demonstrate that the presence of *M. ptb* reduces proliferation of RAW264.7 cells, and significantly affects the expression of a number of gene products.

Novel assays for *M. ptb* detection

Quantitative polymerase chain reaction (qPCR) has become the most sensitive technique for the detection of DNA and mRNA molecules, particularly if present in low copy number, and it is now widely used to validate microarray data, quantify differential gene expression and diagnose and quantitate microbial infection. Recently there has been an increasing application of qPCR to the study of mycobacterial pathogenesis.

Quantitative PCR assays using Taqman based chemistries have been developed to improve the sensitivity and specificity of diagnosis of *M. ptb* infection from animal samples and will be employed during an investigation of systemic bacterial infection.

In addition a number of gene expression assays have been designed to examine differential gene expression in *M. ptb* during growth in the host and in experimentally infected cell lines.

Proteomic approaches

Two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) has traditionally been used to discriminate between differentially expressed proteins and peptides. However, its low resolution of low-abundance and low mass proteins and requirement for large amounts of starting material limit its application. The development of protein chip mass spectrometry has complemented 2-D PAGE. Surface enhanced laser desorption ionisation time-of-flight mass spectrometry (SELDI-TOF MS) combines retention chromatography with TOF-MS detection. Its sensitivity allows detection of low abundance proteins and those in the low mass range with incredible resolution beyond that of 2D-PAGE. In addition, it requires very little starting material. SELDI-TOF MS is being used to analyse differential protein expression in *M. ptb* in various projects including:

- Comparison of Sheep and Cattle strains (see paper by Ian Marsh in this volume)
- Comparison of strains isolated from clinically infected ileum and those cultured in the laboratory
- Analysis of dormancy associated factors induced by nutrient starvation and hypoxic stress
Acknowledgements
This project is funded by Meat & Livestock Australia. We are grateful to Anna Waldron, Craig Kristo, Reena Mehta and Nicole Carter for their excellent technical support.
Review of Immunology, Pathology and Pathogenesis from 8th International Colloquium on Paratuberculosis

Mark Lanigan
CSIRO Livestock Industries, Australian Animal Health Laboratory, 5 Portarlington Road, Geelong VIC 3220
Phone: 03 5227 5737; Fax: 03 5227 5555
Email: mark.lanigan@csiro.au

Summary

The colloquium was opened by Prof. Rod Chiodini who gave his perspectives on the history of paratuberculosis. His central message was that while there was a period in the early part of last century when elimination of paratuberculosis was feasible, that time has now well and truly passed. Given that, with the exception of the introduction of IS900 PCR, there have been no dramatic improvements to testing technologies in the last 50 years, he emphasised that the focus should instead be on control of the disease and that we have all the tools at our disposal to achieve this. Indeed, in silico modelling data presented during Theme 1 sessions show promise for the use of programmes that encompass farm-to-farm trade restrictions in conjunction with differential milk pricing as a means of effectively controlling and reducing disease prevalence. It is, therefore, as important as ever to continue to investigate the causative agent, Mycobacterium avium subspecies paratuberculosis (M. ptb) at a molecular level so as to allow better detection-, control- and even treatment-strategies to be developed.

Oral presentations

Theme 2 sessions were preceded by a presentation by Dr Kingston Mills who set the scene for considering the interplay of various cytokines and immune cells in the progression of tuberculosis. Dr Mills gave a comprehensive review of the role of regulatory T cells (T_r) in mediating the pathogen-induced immunological response and suggested that some viruses, bacteria and parasites, by inducing IL-10 and in turn the T_r response, could be protective against auto-immune disease.

Theme 2 was opened with a keynote address by Dr Ingrid Olsen from the National Veterinary Institute, Oslo (Key note 3). Dr Olsen considered the limitations of using the well known IFN-γ test to detect M. ptb in cattle. This test is notoriously unreliable when used with young animals, either because of the exposure of young cattle to environmental mycobacteria or the activity of so-called ‘non-specific responders’. Dr Olsen investigated the role of Natural Killer (NK) cells as potential non-specific responders in the IFN-γ test using a monoclonal antibody (MAb) against a receptor protein found on NK cells.

Neutralising NK cells in peripheral blood mononuclear cells with this MAb resulted in the IFN-γ response to secreted M. ptb antigens MPP14 and ESAT-6 being eliminated. Neutralising IL-12 has some slight effect on IFN-γ in these experiments suggesting that other cytokines are likely to be involved. Dr Olsen concluded that NK cells are able to produce IFN-γ in response to specific antigens and that by paying more attention to these cells the efficacy of the IFN-γ test in young animals could be improved.

The importance of analysing cytokine response in M. ptb pathogenesis was reiterated with a presentation by Dr Paul Coussens (Short Oral 8) on the role of IL-1α and TRAF1 production by...
macrophages. The mRNA of both these cytokines is highly expressed in infected cattle and both are produced by \textit{M.\ ptb} -infected macrophages. Dr Coussens hypothesised a role for TRAF1 in \textit{M.\ ptb} survival; TRAF1 is produced, via the action of IL-1\textalpha on NF-B cells, by \textit{M.\ ptb} -infected macrophages as a means by which the life of the macrophage is prolonged. Future work will be focussed on investigating the role that TRAF1 has on macrophage recruitment during disease progression.

The second keynote address in Theme 2 was delivered by Dr Ayala Livneh (Key note 4). Dr Livneh's group is attempting to apply Koch's postulates to their study of \textit{M.\ ptb}; i.e. first identifying virulence genes by generating genetic knock-out mutants, then reintroducing the gene and thereby reintroducing virulence. Bacteriophage was used to randomly deliver a transposable element into the genome of the virulent \textit{M.\ ptb} strain, K-10 and in so doing knocking-out the gene at the site of mutation. In this fashion approximately 13,500 mutants have been generated which theoretically represent approximately 95\% of the \textit{M.\ ptb} genome. Three mutants were chosen for further \textit{in vivo} and \textit{in vitro} study and results to date indicate that these mutants showed no reduction in their ability to colonise the guts of baby goats or mice but that \textit{in vitro}, intracellular survival (in a transformed bovine macrophage cell-line) was reduced compared with K-10. The production of NO via inducible NO synthase (iNOS) was also investigated; although NO production by macrophages was observed in the presence of \textit{M.\ ptb} mutants and the use of a specific iNOS inhibitor confirmed the role of iNOS, the abrogation of NO production in macrophages did not appear to affect the survival of \textit{M.\ ptb} mutants.

The first proteomics paper for this colloquium was delivered by Dr Valerie Hughes (Short Oral 6) and focussed on a comparison of \textit{in vivo} and \textit{in vitro} \textit{M.\ ptb} proteomes. Dr Hughes gave an apt summary of the inherent difficulties in comparing such proteomes: \textit{in vitro} proteomes are ones derived from pure cultures that are grown on defined medium under controlled conditions, whereas \textit{in vivo} proteomes are derived from bacteria from a complex environment, in coexistence with other microflora under varying conditions. An additional challenge in considering the proteome of \textit{M.\ ptb} taken from field isolates is the indeterminate length of infection and how this can be referred back to a laboratory reference. These obstacles notwithstanding, comparing the proteomes of \textit{in vitro} and \textit{in vivo} grown \textit{M.\ ptb} should yield clues to proteins responsible for viability in the host over ‘house-keeping’ proteins responsible for growth and division of the bacilli under laboratory conditions. \textit{M.\ ptb} was extracted from JD-infected ovine gut tissue and either homogenised and analysed on large-format 2D gel electrophoresis (2DE) or grown first in stirred cultures to provide an \textit{in vitro} 2DE control. Using this method Dr Hughes’ group was able to identify several proteins from gels of \textit{in vivo} \textit{M.\ ptb} that were not present when the same isolate was grown in laboratory culture. These included proteins involved in: heat-shock and hypoxic response, metabolic shifts (eg. activation of propionate metabolism in nutrient-starved environment), responses to Fe- and arginine-restriction and several proteins with undefined roles in virulence.

During question time Dr Hughes was asked whether faecal \textit{M.\ ptb} would have been a better control in this study. It would indeed be useful to compare the proteomes of resident \textit{M.\ ptb} with that shed into faeces as a way of understanding not only \textit{M.\ ptb} survival in the host but also the molecular mechanisms of disease dissemination. This presentation by Dr Hughes provided a complement to work presented later in the colloquium by Egan \textit{et al}. (Merkal award 1) on a novel method for isolating \textit{M.\ ptb} from gut tissue in a manner directly compatible with subsequent proteomic analysis. As a co-author on the paper of Egan \textit{et al}. I took the opportunity to discuss Dr Hughes’ work with her in greater detail. Whereas our approach has been to use an inert density gradient to remove any non- \textit{M.\ ptb} (proteinaceous) material from extracts, Dr Hughes’ group uses a method that was originally developed specifically for the extraction of nucleic acid and one that carries the risk of contamination with protein from bovine (host) tissue.
Dr Hughes explained that this obstacle is overcome by running parallel 2D gels of bovine tissue as controls, as well as ensuring during bioinformatic analysis (when identifying proteins from gel spots) that proteins are confirmed as bacterial, rather than bovine, in origin. For both bodies of work it remains to be established whether differences observed are real differences between the two experimental conditions studied and not simply artefacts of comparing bacteria at different stages of growth.

An alternative method for undertaking comparative analyses of *M. ptb* proteins was presented by Dr John Bannantine (Short Oral 7) with his group’s work on a *M. ptb* partial-protein array. The partial sequences of over 60 proteins, representing hypothetical proteins, cell-surface- and *M. ptb*-specific proteins and previously characterised *M. ptb* antigens, were expressed and proteins purified. The protein array was used to screen sera from cows with clinical JD, experimentally-infected JD cows as well as sera from rabbits and mice infected with *M. ptb*. The array was also used to monitor the response during the course of infection: from pre-challenge, 70-days and 321 days post challenge. A range of responses were observed to several of the partial protein sequences in the array and the strongest of these show promise as potential vaccine candidates. In the best spirit of collegiality engendered by the colloquium, Dr Bannantine was keen to stress that the array is available for use by other labs for collaborative activities.

The final two oral presentations in this session concentrated on the investigation of specific vaccine candidates: Ag85, presented by Dr Kris Huygen, standing in for Dr Valerie Rosseels (Short Oral 9) and HSP70, presented by Dr Ad Koets (Short Oral 10). Ag85 and its homologues are mycolyl transferases, responsible for strong, impermeable cell walls and are present in all mycobacteria. While Ag85 is cross-reactive with other species of mycobacteria, when used in conjunction with a good diagnostic tool, a subunit vaccine of Ag85 could be effective against multiple species. Dr Koets then showed work on the use of HSP70 as a paratuberculosis candidate. HSPs are immunogenic despite conservation across several species and induce B and T cell activation. It was found that vaccination with HSP70 effected a significant reduction if faecal shedding (approximately 60% relative to control) in *M. ptb*-infected female calves. During question time it emerged that necropsy was still in the process of being performed on these animals. Because orally-challenged animals do not always become infected it could not, at that stage, be ruled out that those animals which did not display shedding were not actually infected. Follow-up experiments are underway to assess whether HSP70 can be used as a therapeutic vaccine for already infected animals.

**Poster presentations**

As espoused in the oral presentations for not only theme 2 but throughout the colloquium, an important aspect of understanding JD pathogenesis is understanding the interaction between *M. ptb* and the host cells it invades and resides in, at the molecular level. Studies from two separate laboratories covered this subject from the perspective of examining the effect of *M. ptb* on apoptosis. Browne *et al.* (Poster 14) monitored the level of the apoptotic marker enzyme, caspase, in Merino sheep orally-infected with *M. ptb*. Using flow cytometry, caspase activity was determined in lymph-node cells sampled from ileal-, jejunal- and prescapular lymph nodes, but did not appear to change with the level of disease status. While this approach may not be useful in studying early exposure of sheep to *M. ptb*, further studies using peripheral blood mononuclear cells (PBMC) may hold more promise.

Grell *et al.* (poster 19) also used flow cytometry to determine caspase activity in PBMC from paratuberculosis-infected cattle. Their results support the suggestion that the development of clinical paratuberculosis may involve the progressive loss of T cell-mediated immunological response by antigen-induced apoptosis.
This year the colloquium featured its first concurrent sessions, which were titled “Prevention and Control - Herd Level” (Theme 3a) and “Implications for Public Health” (Theme 3b). Theme 3b featured presentations that focused not only on the prevalence of *M. ptb* in the water- and food-supply chains but also the potential impact (or not) this organism might have on public health. It is therefore important to consider the ability of animal species other than cattle, sheep and goats to harbour *M. ptb* not only from a human health perspective, but also one of infection (and re-infection) of paratuberculosis-susceptible herds.

It was fitting that the poster session for Theme 2 contained several studies concerning the effect of *M. ptb* on animal species other than sheep, cattle or goats. Beran *et al.* (poster 13) used golden hamsters (*Mesocricetus auratus*) to investigate the virulence of 5 isolates of *M. ptb* sourced from syrphid fly larvae, infected cattle herds or human patients with Crohn’s Disease. Although no chronic diarrhoea or emaciation was recorded during the course of infection this work illustrates the potential for developing a new small-animal model for studying paratuberculosis.

Griffin *et al.* (poster 20) investigated disease severity and differences in pathology between sheep and red deer (*Cervus elaphus*) experimentally infected with *M. ptb*. A secondary aim of their study was to investigate histopathological differences between experimentally- and naturally-infected animals. While no such differences were observed between experimentally- and naturally-infected animals, the histopathology of samples from infected deer showed characteristics not seen in sheep. Paratuberculosis in red deer was also the focus of work by Mackintosh *et al.* (poster 29). They have developed a young-animal model of infection (3 month old animals) that is comparable with natural infection. The strength of the model is illustrated by the fact that it has subsequently been used in vaccine and epidemiology studies.

Similarly, Sivakumar *et al.* (poster 37) studied the efficacy of different diagnostic methods for paratuberculosis in water buffaloes (*Bubalus bubalis*). Gross and histological lesions were examined in intestinal tissues from over 400 animals; IS900 PCR was found to be more sensitive in detecting paratuberculosis than bacterial culture or examination of smears.

I had the fortune of being able to present two posters at the colloquium: two under this theme (Lanigan *et al.*, poster 27 and McWaters *et al.*, poster 31) and one by Wilkins *et al.* (poster 121) in Theme 4. Poster 27 summarised our laboratory’s efforts to compare the proteomes of *M. ptb* with the closely related organism *M. avium*. In particular we are focussing on analysing the subset of each organism’s proteome that contains only envelope, or surface-exposed proteins. The selective extraction of these proteins (which we refer to as the ‘membranome’) is undertaken using a non-ionic detergent, Triton X-114, that undergoes a phase separation on heating allowing the partitioning of hydrophobic and hydrophilic proteins. Over three hundred proteins have been identified from extracts of these two organisms and their usefulness as disease biomarkers is being evaluated.

The second poster from our laboratory, by McWaters *et al.*, summarised our efforts in understanding the interaction between *M. ptb* and bovine primary blood macrophages. Given that *M. ptb* is able to survive macrophage engulfment and persist in long-term infection, clues to the pathogenicity of this organism should be found in understanding its survival mechanisms at the molecular level. To this end, engulfment assays have been developed to evaluate uptake and survival of *M. ptb* in bovine primary blood macrophages.

The molecular sequelae of *M. ptb /macrophage interaction was also covered by other groups within this theme. Sommer *et al.* (poster 38) used a *M. ptb* -infected murine macrophage cell-line to examine cytokine production, gene expression and protein-DNA interactions and found that *M. ptb* infection is associated with reduced expression of the cytokine IL-6.
A murine macrophage model of infection was also used by Taylor et al. (poster 41) to examine host gene expression, protein secretion, apoptotic events (described in poster 14), macrophage survival and bacterial persistence. In addition to observing significant changes to the expression of a number of gene products it was noted that M. ptb infection reduces the proliferation of this cell line.

**Conclusion**

The closing session of the meeting was delivered by the president of the International Association for Paratuberculosis, Dr Michael Collins. He used this opportunity to summarise various issues raised during the week, paying particular attention to the putative link between M. ptb and Crohn’s Disease. Dr Collins stressed that if a link between the two is ever established the implications would be global and would utterly dwarf those currently being dealt with regarding paratuberculosis and the agricultural sector. In his words “We must not dodge the issue or allow politics to influence objectivity”.

The 8ICP was the first of these colloquia that I have attended and, I trust, not the last. My lasting impression of the meeting was that of diversity. The work presented at the colloquium, either as seminars or at poster sessions was of a very high quality and was a testament to the effort being undertaken worldwide to control this persistent disease. The depth of analysis was matched by the breadth of subjects covered over the course of the meeting and underlined the importance of the colloquium as a means of bringing together experts from a variety of disciplines for the common goal of controlling paratuberculosis.
Review of Molecular Biology, Microbiology, and Culture from 8th International Colloquium on Paratuberculosis

Ian Marsh
Elizabeth Macarthur Agricultural Institute
PMB 8 Camden, NSW 2570, Australia
Phone: 02 4640 6502; Fax: 02 4640 6384
Email: ian.marsh@agric.nsw.gov.au

Summary

Research presented at the 8th International Colloquium on Paratuberculosis (8ICP) was once again heavily influenced by current and emerging techniques in the field of molecular biology. As with the previous ICPs, the molecular biology section continues to grow in the number of studies being presented (Table 1). At the 8ICP the number of molecular biology based presentations was equal in the number of studies presented, to each of the other sections (Table 2).

Oral studies presented in the molecular biology section of the 8ICP covered a range of topics including: genomic and proteomic comparative studies of the strains of Mycobacterium avium subsp. paratuberculosis (M. ptb) and between M. ptb and the closely related Mycobacterium avium subsp. avium (M. a. avium), in vivo proteomic analysis of M. ptb, development of luminescent markers used to aid in vivo studies of M. ptb and for the first time in M. ptb or Johne’s disease research the introduction of lipidomics which is the large-scale study of non-water-soluble metabolites (lipids).

A large number of posters were also presented at the 8ICP covering topics such as: production of monoclonal antibodies, new strain typing tests, potential vaccine candidates, genomic and proteomic studies, DNA extraction, host specificity and molecular diagnosis techniques.

The aim of this paper is to clearly and concisely review the key studies presented in the molecular biology section of the 8ICP. Results from these studies should contribute to our understanding of M. ptb and Johne’s disease.

Table 1: Trends in the molecular biology based studies presented at the 6th to the 8th International Colloquium on Paratuberculosis

<table>
<thead>
<tr>
<th>Colloquium</th>
<th>Oral</th>
<th>Poster</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>12</td>
<td>10</td>
<td>22</td>
</tr>
<tr>
<td>7</td>
<td>15</td>
<td>14</td>
<td>29</td>
</tr>
<tr>
<td>8</td>
<td>7</td>
<td>33</td>
<td>40</td>
</tr>
</tbody>
</table>
Table 2: Number of presentations at the 8ICP per section.

<table>
<thead>
<tr>
<th>Section of the 8ICP</th>
<th>Oral</th>
<th>Poster</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prevention and Control</td>
<td>7</td>
<td>7</td>
<td>14</td>
</tr>
<tr>
<td>Immunology, Pathology and Pathogenesis</td>
<td>7</td>
<td>34</td>
<td>41</td>
</tr>
<tr>
<td>Prevention and Control – Herd level</td>
<td>7</td>
<td>33</td>
<td>40</td>
</tr>
<tr>
<td>Public Health</td>
<td>7</td>
<td>9</td>
<td>16</td>
</tr>
<tr>
<td>Molecular Biology</td>
<td>7</td>
<td>33</td>
<td>40</td>
</tr>
<tr>
<td>Diagnosis</td>
<td>7</td>
<td>57</td>
<td>64</td>
</tr>
<tr>
<td>Epidemiology</td>
<td>7</td>
<td>32</td>
<td>39</td>
</tr>
</tbody>
</table>

Molecular biology, microbiology and culture - oral presentations

Molecular biology based research of *M. ptb* has clearly progressed and benefited from the completion of the *M. ptb* genome and this was evident with 4 of the 7 oral presentations in the molecular biology section of the 8ICP utilising these technologies to compare between the strains of *M. ptb* or between *M. ptb* and *M. a. avium*. Interestingly, each of these studies presented many unique results but also identified a number of common results indicating that the global *M. ptb* research effort is now finding common themes that will hopefully provide a sound platform for future work and hopefully some interesting breakthroughs in understanding this pathogen.

Genomics

I was in the fortunate position of starting the molecular biology session with a review of the work I have undertaken towards my PhD. I presented an extensive study comparing the sheep (S) and cattle (C) strains of *M. ptb* using a variety of modern DNA and protein based techniques including: representational difference analysis (RDA), polymerase chain reaction (PCR) and sequencing, microarray, two dimensional electrophoresis proteomics and surface enhanced laser desorption-ionisation (SELDI) proteomics (Marsh et al., 2005, 8ICP, key note No. 9). Using these techniques a number of differences between the sheep and cattle strains of *M. ptb* were identified and these are discussed more thoroughly in my paper titled: Genomic and proteomic comparative study of the sheep and cattle strains of *Mycobacterium avium* subsp. *paratuberculosis* included in these proceedings. However, the discovery of three large deletions in the S strain of *M. ptb* using a whole genome microarray based on the *M. ptb* K10 genome was one of the key outcomes from this study. The 3 deletions were equivalent to 29,208 bp (~0.6%) of the *M. ptb* K10 genome and include 24 open reading frames (ORFs). The results from this study confirm large genomic variations between the S and C strains of *M. ptb* and have identified a number of genes that require further examination. Understanding what effect the absence of these genes has on the S strain, may be critical to understanding the host specificity of both strains of *M. ptb*.

Two further studies were presented utilising microarray technology. Paustian et al., 2005 (8ICP, key note No. 10) used a microarray based on the *M. ptb* K10 genome strain to compare between strains of *M. ptb* and reported similar observations to those presented in the Marsh et al., 2005 (8ICP, key note No. 9) study. However, this study identified a further deletion in the sheep strain thus confirming 4 major genomic differences between the S and C strain of *M. ptb*. Furthermore, this study compared *M. ptb* isolates from a range of host species, but all identified
as C strains and found to be identical to the *M. ptb* K10 genome strain. Interestingly, the copy number of a new and unique *M. ptb* insertion sequence, ISMAP04, was found to vary across different isolates and may represent a new target for molecular based diagnostic tests.

Semret *et al.*, 2005 (8ICP, short oral No. 24) used a microarray based on a combination of *M. ptb* and *M. a. avium* genes to compare between isolates from *M. a. avium* complex. This study identified a 16 kb region present in both the S strain of *M. ptb* and *M. a. avium* that was absent in the C strain of *M. ptb* and a 7 kb region present in both the C strain of *M. ptb* and *M. a. avium* that was absent from the S strain of *M. ptb*. The results from these studies clearly indicate a great deal of genomic heterogeneity amongst the S and C strain of *M. ptb* and *M. a. avium*. Making these genomic regions ideal targets for future research that might identify genes and proteins involved in host specificity, pathogenicity and other aspects of *M. ptb*.

**Proteomics**

Proteomic based studies were also reported in this section of the 8ICP. Marsh *et al.*, 2005 (8ICP, key note No.9) reported using proteomics to compare the S and C strains of *M. ptb*. Using this technique a number of proteins were identified to be uniquely or differentially expressed in the S and C strain of *M. ptb* when cultured on solid media. Similar observations to these results were reported by another study using this technique. Garcia-Sanchez *et al.* 2005 (8ICP, short oral No. 23) used proteomics to compare between S and C strains and intermediate strains, the later sharing similarities with both S and C strains. The results from this study also indicated that while S and C strains express type specific proteomes, intermediate strains do not.

**Other areas**

Egan *et al.* 2005 (8ICP, Merkal Award No.1) presented an important Australian based study on the development of a rapid technique that can isolate intact *M. ptb* directly from intestinal tissues of animals, making it possible to perform proteomic studies to identify proteins expressed under *in vivo* conditions. This may help identify antigens and virulence determinants that explain many aspects of this disease and that have not been identified under routine *in vitro* type experimental conditions. Another almost complementary study was presented by Rosseels *et al.*, 2005 (8ICP, short oral No. 21). In this study, *M. ptb* were reported to be transformed with luminescent genes allowing them to be observed more easily during *in vivo* studies in mice spleens. This technique was shown to be advantageous when monitoring the efficacy of *M. ptb* vaccines *in vivo*.

The 8ICP saw the introduction of a new and exciting research tool in *M. ptb* and Johne’s disease research in the form of lipidomics or the study of non-water-soluble metabolites (lipids) of which *M. ptb* has many. In this presentation, Eckstein *et al.* 2005 (8ICP, short oral No. 22) presented their initial results when using this technique to compare *M. ptb* and *M. a. avium*. Total lipids from whole cells and culture filtrates were compared using two-dimensional thin layer chromatography. To date, 9 lipids have been identified in *M. ptb* that are absent in *M. a. avium*. Further analysis of these 9 lipids, across all 28 serovars of *M. a. avium*, indicates that 4 are truly unique to *M. ptb*. Interestingly, all 4 lipids were only associated with the whole *M. ptb* cells not the culture filtrate.

**Molecular biology, microbiology and culture - poster presentations**

Posters presented in the Molecular biology, Microbiology and Culture section of the 8ICP were generally of an extremely high standard, very informative and covered a diverse range of research areas associated with *M. ptb* and Johne’s disease. As with the oral presentations, the majority of the 37 posters in this section could be divided into one of a number of categories and will be reviewed according to this division.
Proteomics

Three proteomic studies were presented including the development of a *M. ptb* cell disruption technique for use in further proteomic studies (Donaghy *et al.* 2005, 8ICP, poster No. 99). This was an interesting study that effectively compared 4 commercial disruption techniques. However, other techniques have been used elsewhere (Hughes *et al.* 2005, 8ICP, short oral No. 6 and Marsh *et al.*, 2005, 8ICP, key note No. 9) utilising alternate disruption techniques and these should also be considered for those wanting to undertake proteomic studies of *M. ptb*. Proteomics was also used to identify 14 secreted proteins (from culture filtrate) that may prove useful for the development of new serodiagnostic tests (Cho *et al.*, 2005, 8ICP, poster No. 95). Proteomics was also used to identify protein profiles identifying differentially expressed proteins from *M. ptb* and *M. a. avium* grown under iron limiting culture conditions (Wilkins *et al.* 2005, 8ICP, poster No. 121). The aim of this study is to identify the potential role such proteins play in cellular metabolism and pathogenicity.

Molecular based typing techniques for *M. a. paratuberculosis*

A number of posters presented in this section of the 8ICP focused on new alternatives to the current molecular based typing techniques for *M. ptb* and the application of the current typing techniques in molecular based epidemiological surveys of Johne’s disease.

The use of restriction fragment length polymorphism (RFLP) and polymerase chain reaction (PCR) based typing techniques would be considered the gold standard for *M. ptb* strain and sub-strain typing. However, both techniques have limitations that may be overcome by the introduction of new typing techniques. Three studies were presented using mycobacterial interspersed repetitive unit-variable number tandem repeat (MIRU-VNTR) based typing techniques (Biet *et al.* 2005, 8ICP, poster No. 92 and Nishimori *et al.* 2005, 8ICP, poster No. 109 and Romano *et al.* 2005, 8ICP, poster No. 113). All three studies indicated the potential of this approach to separate *M. ptb* and *M. a. avium* isolates using this simple PCR based procedure. However, the use of this technique and the advantages it offers has yet to be demonstrated in extensive epidemiological studies of *M. ptb*. Whereas, an alternate technique known as multilocus short sequence repeat (MLSSR) was reported to be capable of differentiating strains of *M. ptb* previously indistinguishable by other methods (Harris *et al.* 2005, 8ICP, poster No. 106). When applied to an epidemiological survey of dairy cattle in the USA, the MLSSR technique identified multiple strains within herds and even within individual animals.

Other studies were presented using more traditional *M. ptb* typing techniques. Johansen *et al.* 2005 (2005, 8ICP, poster No. 107) presented an interesting RFLP based study that comparing the presence or absence of ISmpa1, IS1245 and IS1311 in *M. ptb* and *M. a. avium*. The results indicate that the *M. ptb* and *M. a. avium* genomes both posses IS1311 and ISmpa1. However, the later was not present in all *M. a. avium* strains. Both insertions sequences can be used to differentiate between the species, when present. IS1245 was confirmed to be present in *M. a. avium* only.

Three epidemiological studies were presented, two from India (Sevilla *et al.* 2005, 8ICP, poster No. 115 and Singh *et al.* 2005, 8ICP, poster No. 116) and one from Spain (de Juan *et al.* 2005, 8ICP, poster No. 96), that used the more traditional RFLP and PCR-REA typing techniques. All three studies identified cross-infection of sheep and cattle with the C and S strain strains respectively, and in other species. Furthermore, the *M. ptb* bison strains were found in sheep and goats in India. The results from these studies clearly indicate that global strain variation of *M. ptb* and host susceptibility are geographically dependent. Furthermore, these typing
techniques still have an important role in the diagnostic testing regimes required to fully understand Johne’s disease in different parts of the world. As they provide important information in decision making for geographically distinct control and evaluation programmes of this disease.

Polymerase chain reaction

As with previous ICP a number of studies were presented on the use of PCR in Johne’s disease research and diagnosis. IS900-like elements in non-\textit{M. ptb} species have been reported as a problem in \textit{M. ptb} literature and at both the 6\textsuperscript{th} and 7\textsuperscript{th} ICP but are considered to be extremely rare. However, an Italian study (Taddei \textit{et al.} 2005, 8ICP, poster No. 119) identified 21 mycobactin-independent Mycobacteria that were positive in IS900 PCR. These results once again reinforce the diagnosis of \textit{M. ptb} based on IS900 PCR alone should be interpreted with extreme caution.

Studies aimed at improving \textit{M. ptb} detection by PCR included: improved DNA extraction techniques from \textit{M. ptb} isolated from milk samples (Gwordz \textit{et al.} 2005, 8ICP, poster No. 104), the development of an internal PCR control to validate each PCR reaction (Brey \textit{et al.} 2005, 8ICP, poster No. 93) and new real time PCR reactions (Ravva, \textit{et al.} 2005, 8ICP, poster No. 112 and Sweeney \textit{et al.} 2005, 8ICP, poster No. 118). Further examination of each of these studies will provide valuable information with respect to the development of the direct-PCR (D-PCR) test currently being undertaken at the Elizabeth Macarthur Agricultural Institute (NSW Department of Primary Industries) funded by Meat and Livestock Australia.

Other PCR based studies included the development of a multiplex PCR designed to detect \textit{M. ptb}, \textit{M. a. avium} and \textit{M. bovis} mixed infections in red deer (\textit{Cervus elaphus}) (Godford \textit{et al.} 2005, 8ICP, poster No. 102) and an alternate PCR based technique capable of differentiating \textit{M. ptb} and \textit{M. a. avium}, based on sequence differences in the fibronectin-attachment protein genes in both subspecies.

Host genomics

One of the key areas to yet to be addressed in Johne’s disease research is the host response to \textit{M. ptb}. DiFiore \textit{et al.} 2005 (2005, 8ICP, poster No. 97) presented one of the first studies attempting to identify gene expression from the host perspective, in sheep, during early and clinical stages of disease as opposed to other studies that have looked at this during advanced stages of disease. Using a technique known as long distance differential display polymerase chain reaction (DD-PCR), uninfected and infected tissues were screened to identify differentially expressed genes in infected sheep. Using this technique, gene expression has been identified in both non-infected and infected tissues; however, differential expression has yet to be observed. A number of alternative PCR primer sets have yet to be used to evaluate this technique.

\textit{M. a. paratuberculosis} culture

As with the PCR based studies, a number of studies were presented on \textit{M. ptb} culture techniques focusing on either the improvement of current techniques or the introduction of new culture methods.

Gwordz \textit{et al.} 2005 (2005, 8ICP, poster No. 105) compared decontamination methods for the isolation of \textit{M. ptb} from effluent. The results from this study indicate that a double incubation technique at 42\textdegree C was selective for \textit{M. ptb} and suggested that this may have an important role in the isolation of \textit{M. ptb} from faeces, tissue and milk. A new culture technique was described for the isolation of \textit{M. ptb} from milk by Ruzante \textit{et al.} 2055 (2005, 8ICP, poster No. 114).
technique introduced a new procedure where *M. ptb* in the milk sample are concentrated using a solution known as CB18. The inclusion of this procedure was reported to decrease the time required to culture the organism from raw milk. Once again this may be an important outcome and of use in improving local culture and detection techniques of *M. ptb* directly from faeces.

Two studies compared *M. ptb* culture on either solid media (Wiszniewska *et al.* 2005, 8ICP, poster No. 122) or in liquid culture (van Maanen *et al.* 2005, 8ICP, poster No. 120), the latter study including the recently developed TREK ESP para-JEM Culture System II. As with previous studies of this nature the results might indicate the relative efficiencies different laboratories have with different culture techniques and therefore should be considered with ability to reproduce results in more than one laboratory. This issue was highlighted in a poster from the National Veterinary Services Laboratories from Ames Iowa. This laboratory has been requested to develop a standardised culture technique for the United States to overcome the wide range of sensitivities and variability in culture techniques currently in use in the USA for the diagnosis Johne’s disease (Payeur *et al.* 2005, 8ICP, poster No. 111).

**Other studies**

In addressing an unmet need from the 7ICP, Bannantine *et al.* 2005 (8ICP, poster No. 91) reported the development of several monoclonal antibodies for a number of *M. ptb* epitopes plus aptamers for the conserved hypothetical protein MAP0105c and single-chain antibody (ptb9) for the *M. ptb* specific protein encoded by MAP0858. The authors hope that these detection reagents will be of use in many Johne’s disease research applications. *M. ptb* specific monoclonal antibodies were also produced in the development of a new PCR based diagnostic test used to detect *M. ptb* in colostrum from cattle (Ebert *et al.* 2005, 8ICP, poster No. 100). This test will provide rapid detection of *M. ptb* in colostrum and as a result support herd-level testing regimes in dairy cattle.

Three studies were reported on the survival of *M. ptb* in bovine macrophages, further improving our understanding of how this organism survives in the host and how the host responds to the organism. Partridge *et al.* 2005 (8ICP, poster No. 110) used real time PCR and microarray analysis to identify those genes expressed by *M. ptb* during infection. Where as, examination of the organism-host interactions was reported in two studies, the first looking at the response of the host, mouse macrophages, to different strains of *M. ptb* isolated from a range of hosts (Mitchell *et al.* 2005, 8ICP, poster No. 108) and the second looking more specifically at the interaction between different strains of *M. ptb* with bovine macrophages Gollmick *et al.* 2005 (8ICP, poster No. 103). The results from these studies should provide useful information on the host immune response to *M. ptb*, the host specificity of the different strains of *M. ptb* and the potential for different hosts to act as reservoirs for non-specific host strains of *M. ptb*.

A new vaccine candidate was reported based on the development of a fusion construct, including antigens from two membrane and two secreted proteins and was shown to elicit a significant attenuation of the infective load of *M. ptb* in the spleen and liver of mice (Bull *et al.* 2005, 8ICP, poster No. 94). With these encouraging results the authors hope to continue their research in vaccination studies on larger animals with the view to providing an effective *M. ptb* vaccine for both animals and humans.

Immunomagnetic detection of *M. ptb* was re-visited using a *M. ptb* specific peptide bound to magnetic beads (Stratmann *et al.* 2005, 8ICP, poster No. 117). The test, which includes capturing the *M. ptb* on magnetic beads followed by PCR and restriction endonuclease analysis of the amplified product, was successfully used to detect S and C strain *M. ptb* in spiked
individual and bulk milk samples. It would appear that the test has yet to be evaluated with real milk samples and the results from this further evaluation are eagerly awaited.

Finally, in a more traditional microbiological study, lactic acid bacteria were used to inactivate \textit{M. ptb} that have survived pasteurisation of milk to used for the production of dairy products such as yogurt and cheese (Donaghy \textit{et al.} 2005, 8ICP, poster No. 98). The authors report that the lactic acid bacteria are capable of inhibiting other food pathogens and technique was shown to be successful in this study with \textit{M. ptb}. Although, the results seem to suggest that inhibition of \textit{M. ptb} may not be the result of lactic acid. Regardless of the mechanism of inactivation the association with the lactic acid bacteria appears to have an inhibitory effect on \textit{M. ptb} and further work is required to establish the mechanism and the potential of this approach.

**Conclusion**

As with previous ICP and each new publication on Johne’s disease or \textit{M. a. paratuberculosis}, we hope for a ground breaking or new development that will have a significant impact on dealing with this insidious disease. The reality is that like other mycobacterial diseases such as tuberculosis and leprosy, progress in the research that aims to develop or assist our understanding of the diseases and/or the organisms that cause them, is slow. However, the progress demonstrated at the 8ICP is extremely encouraging. Moreover, the widespread adoption of new and exciting molecular based research approaches should yield some interesting and informative outcomes in the not to distant future, hopefully before or at least by the next time the International Association on Paratuberculosis meets in Japan at the 9ICP.
Vaccination
INSERT PFIZER ADD HERE
Overview of OJD vaccination – international perspective

Peter Windsor
Farm Animal Health
University of Sydney
PMB 3, Camden, NSW 2570
Phone: 02 9351 1710; Fax: 02 4655 0618
E-mail: pwindsor@camden.usyd.edu.au

Abstract
The availability of vaccination against paratuberculosis from April 2002 in Australia, effectively 'rescued' the Australian sheep industry from a devastating socioeconomic calamity that had divided the industry and led to open revolt by producers against animal health authorities attempting to control the disease through regulation. At the time vaccination was being considered as an option for disease control, there were conflicting opinions on the advisability of this approach. In particular there was concern over reported problems associated with vaccination, such as adverse reactions and injection site lesions in sheep and people, plus the possibility that the vaccine would be less efficacious in Australian Merinos in the Australian environment. Analysis of published reports of vaccination trials in the international literature, demonstrates a clear positive effect from vaccination in reducing mortalities, prevalence of lesions and shedding, with negative effects in only a few vaccination trials. Now that several large field trials have been conducted in infected New South Wales Merino flocks, there is considerable evidence that supports the ongoing use of vaccine to both reduce the impact of disease and decrease the level of shedding of infectious organisms from infected Australian sheep. This work has also suggested that vaccination works particularly well when combined with management strategies to reduce infection rates in the flock and that the side effects of vaccination are well tolerated by the industry. These studies have opened a dialogue that is considering the use of vaccination to assist control of bovine paratuberculosis in Victorian dairy herds. Although the international perspective of vaccination suggests that it will prove to be efficacious in Australian dairies, conclusive demonstration that vaccinated cattle have significantly reduced faecal shedding of the infectious organisms in the Australian environment is desired. Acceptance of vaccination against paratuberculosis may yet prove to be the most significant advance in controlling the disease and reducing the potential public risk should the evidence finally conclude that paratuberculosis in livestock is a risk factor in human Crohn’s disease.

Introduction
Livestock Johne’s disease (JD) due to infection with *Mycobacterium avium* subspecies *paratuberculosis* (*M. ptb*) is an emerging complex animal health problem prompting a number of different approaches to controlling the disease, including enhancing herd and flock immunity by vaccination. Several vaccines have been used in livestock, with mostly encouraging evidence of efficacy (Juste *et al.*, 2002), particularly when the program has used a vaccine with a Freund's adjuvant-like nature to provoke cellular immunity (Windsor *et al*., 2005). Vaccination has been readily embraced for the control of paratuberculosis in sheep, with considerable success, as documented in the United Kingdom (Cranwell, 1993), Spain (Garcia Marin JF *et al*., 1999), Iceland (Fridriksdottir *et al*., 2000) and more recently Australia (Windsor *et al*., 2002). Currently a vaccine study is underway in sheep in North America using Mycopar™ cattle vaccine (Stehman, pers. comm.).
Vaccination of cattle has been used sporadically, particularly where animal health authorities have committed to a test and cull or test and management approach to controlling the disease. For example in the USA, a commercial vaccine is available following approval by a State Veterinarian on a herd by herd basis, with published evidence from a controlled clinical trial that by using a whole cell bacterin, the incidence of clinical JD is reduced (Larson et al., 1978). However less consistent results demonstrating the effect of vaccination on faecal shedding have led to variable support for vaccination programs, despite the now considerable evidence supporting a role for vaccination in modifying the progress of infection from early subclinical infection to open disease (Juste et al., 2002). In addition to the paucity of reports of large scientifically controlled trials that convincingly demonstrate the efficacy of vaccination on shedding, it is likely that several other factors have led to a cautious approach to the uptake of vaccination, despite evidence that vaccination is cost-effective due to reduction of culling of clinically affected cattle (van Schaik et al., 1996). These concerns include the prevalence of adverse reactions and lesions at injection sites or beyond and the risk of self-injection injury from using a vaccine containing a provocative adjuvant, plus problems with vaccination programs in cattle in countries where tuberculosis exists because vaccination can interfere with tuberculin and other testing programs used in control of bovine TB. This paper provides an overview of the historical work and the most recent investigations on the use of vaccination to control paratuberculosis, from an international perspective.

An overview of previous studies

Evidence for efficacy of vaccine

A meta-analysis of reports of vaccination trials against paratuberculosis was completed in 2002 and involved reviewing the results presented in 16 reports of cattle, 11 reports of sheep and 6 reports of goats following vaccination (Juste et al., 2002). The average combined reduction in incidence of clinical disease in these reports following vaccination was 96% of 280,743 cattle, 71% of 11,661 sheep and 45% of 9,951 goats. The average reduction in mycobacterial shedding in these reports following vaccination was 72% of 42,197 cattle, 50% of 1,735 sheep and 83% of 1,425 goats. The average reduction in lesions of paratuberculosis following vaccination was 58% of 4,317 cattle, 90% of 27,290 sheep and 96% of 5,724 goats. There were few reports on vaccine failure, with one report involving oral administration, another of reduced efficacy due to vaccine composition with sonicated cells, and more recently, a report with no effects on faecal shedding compared to other control measures (Juste et al., 2002). A clear conclusion from these reports is that vaccination is mostly an effective tool in reducing the incidence of clinical disease and mortalities plus the rate of shedding from infected animals and prevalence of lesions in vaccinates. However as it does not completely protect the vaccinated animal from infection it cannot by itself lead to eradication of \textit{M. ptb} from an infected flock or herd, a potential issue of concern with the increasing evidence for a role of human exposure to \textit{M. ptb} as a potential risk for Crohn's disease (Juste et al., 2002).

Impact of vaccine on lesions

The repeated observation that vaccination against paratuberculosis significantly reduces the number of clinical cases in field conditions by modifying the progress of lesions, but doesn't prevent infection, has been examined in a few studies. To confirm that vaccination modified the type of lesion produced following infection, a study from Spain that minimised the role of genetic factors in lesion development by the use of twin lamb pairs is of interest (García Marín et al., 1996). One lamb out of each one of eight pairs of twin lambs was vaccinated when it was 15 days old and all lambs were orally infected with a pathogenic ovine strain of \textit{M. ptb} at 50 days of age and killed at either 150 or 350 days post-infection and submitted to necropsy and
histological evaluation. At both sampling times after infection, vaccinated lambs had only ‘regressive-type’ granulomata limited to Peyer's patches and ileocecal valve lymphoid tissues, with unvaccinated animals displaying spread of granulomatous lesions to other areas of the intestinal wall.

The age at which vaccine is applied is also of particular interest as although animals are mostly vaccinated when they are younger than 6 months, in the face of significant economic losses producers will request vaccination of adult sheep, many of which are likely to be infected and some expected to have developed lesions of paratuberculosis. Immunisation of 70% of the adult dairy sheep in two flocks of about 250 adult animals in each, retaining 30% of the sheep as controls, was undertaken (García-Pariente et al., 2002). During the 12 months after vaccination, a total of 56 sheep (40 vaccinates and 16 controls) were culled and necropsied, including 17 sheep in the first 6 months (9 having diffuse lesions) and 11 between 6-12 months post vaccination (3 having diffuse lesions). It was concluded that as vaccination of the adult sheep induced strong humoral and cellular immune responses and there was a reduction in the number of clinical cases of paratuberculosis with a decrease in culls from the vaccinated group and the number of animals having diffuse lesions, the study provides some evidence that vaccination may assist in limiting the disease to focal lesions.

Is eradication by vaccination possible?

There is mounting evidence that control rather than eradication of paratuberculosis is a more realistic short to medium goal of paratuberculosis control programs, particularly where vaccination is the key tool used to reduce the prevalence of infection. The experience of OJD in Iceland has a number of parallels to that in Australia, with the importation of infected sheep from abroad, rapid spread of infection to the main sheep breeding areas and unsuccessful attempts to eradicate the disease by regulatory approaches including quarantine, widespread immunological testing, extensive culling of sheep in endemic areas and restocking with healthy sheep (Fridriksdottir et al., 2002). As vaccination experiments demonstrated that mortality could be reduced by 94%, since 1966, vaccination of sheep has been compulsory in endemic areas, with considerable reduction in losses. However in 2001, paratuberculosis suddenly appeared on a farm in an area which had been declared free from paratuberculosis and where vaccination had ceased in the whole quarantine area 3 years earlier. Sheep from all farms in the area were then tested serologically and 50 of 400 sheep on the affected farm were seropositive and/or had clinical symptoms, with one seropositive sheep found on another farm. All positive animals were culled and the rest vaccinated with compulsory vaccination continuing again in the whole quarantine area. The reasons for the outbreak and the question of whether eradication of paratuberculosis by vaccination is possible, remain undetermined (Fridriksdottir, pers. comm.).

Most recent developments

Australian efficacy studies in sheep

Several studies have been recently completed evaluating the use of Gudair™ to control OJD. These have presented convincing data that supports vaccination as a key tool to induce cellular immunity in vaccinates and rapidly control OJD at the flock level (Windsor et al., 2002; Eppleston et al., 2004, 2005). Over the productive life of Australian Merino sheep in 3 high prevalence flocks, vaccination reduced mortalities by 90% and delayed and reduced faecal shedding of M. ptb by 90%, although all of the few vaccinates that developed OJD had multibacillary disease and excreted infectious doses of the organism (7 of 600 vaccinates). These results led to registration of the vaccine and have encouraged a significant voluntary adoption of the vaccine in endemically infected and adjacent areas. Ongoing monitoring of the performance of the vaccine in flocks of different prevalence levels is continuing (Windsor, 2005).
and a gross margin to show breakeven points for vaccination to inform affected producers of the length of time required for a return on an investment in vaccination to be achieved has been developed (Bush et al., 2005).

Due to considerable producer interest in vaccination of all ages of sheep in the flock when faced by sudden increases in overwhelming losses, a study in a single large flock with over 8,000 sheep and a very high mortality rate estimated at 25% per annum, involved annual vaccination of all sheep with the exception of up to 150 controls in each age cohort. Vaccination was accompanied by significant change to the flock profile, initially by the culling of clinical cases, sale of ‘at risk’ stock, replacement of much of the ewe flock with wethers and reduction in stocking rates by intensive de-stocking due to severe drought, resulting in a decline in the ‘sheep years at risk’ to 5,571 over the 4 years of the trial, accompanied by a significant decline in annual mortality rate from 24% to 2.7%. Further, despite observations of a decline from a high to a moderate prevalence of shedders following vaccination of 2 year-old sheep, mortalities due to OJD and shedding of M. ptb continued, particularly in wethers. Vaccination at 2 years of age in this high M. ptb prevalence environment had limited impact on mortality rate due to OJD, whilst vaccination at age 3 months and 8 months was of similar efficacy, with lower prevalence of M. ptb shedders than from unvaccinated controls. A decline in the prevalence of shedders in the unvaccinated sheep during the 4 years of the project was also observed (McGregor et al., 2005; Windsor, 2005).

Adverse reactions

Vaccination site lesions in the Australian studies occur in about 50% of vaccinates but declined to between 20-25% during the course of the 5 year study (Eppleston et al., 2004, 2005). The potential costs of the vaccination site lesions were found to be negligible following investigations of 20 lines of sheep at slaughter, where 65% of lamb carcases and 18% of mutton had vaccine lesions (Eppleston, 2004). A study of adverse reactions in producers vaccinating sheep identified by survey, that exposure to the vaccine was not uncommon at greater than 1 in 10,000 doses (Windsor et al., 2005). However whilst the risk of self-inoculation with the vaccine appeared to be very low at about 1 in 500,000 doses, most self-injection lesions resulted in untoward consequences, with granulomatous to necrotising lesions leading to fistulation requiring extensive surgical intervention in some cases to enable drainage of the injected material. More recently, amputation of a finger was necessary to resolve a chronic lesion that failed to respond to surgery.

A new vaccine for cattle

A new vaccine has recently been produced and targeted for use in cattle. SILIRUM™ vaccine, also produced by CZ Veterinaria of Spain, the manufacturers of Gudair™, is also a killed vaccine but uses a different adjuvant, presumably in an attempt to reduce vaccination site lesions. A field study is being conducted in Spain in a Friesian herd of 468 animals with 12% annual clinical cases of BJD. The trial involves vaccination of 75% of all cattle with 25% retained as controls for 30 months post-vaccination (Garcia-Pariente et al., 2005). More than 90% of vaccinates produced persistent cellular and humoral immune responses to the vaccine and no new cases of BJD occurred from 6 months post-vaccination. The percentage of the 192 animals culled during the trial with lesions of BJD was significantly higher in the controls than vaccinates, with 92.3% of controls having lesions compared to 56.6% of vaccinates, indicating a significant reduction in BJD in the herd following vaccination. In addition, a pilot field study with SILIRUM™ was also conducted in a herd of 113 cattle with a clinical incidence of BJD of 8% and faecal culture prevalence of 5.5% (Garrido JM et al., 2005). After one year of control, no new clinical cases of BJD have occurred, the faecal culture prevalence is 3.6% and no interference with intradermal testing for tuberculosis was identified, suggesting expansion of the trial to a
maximum of 50 herds is likely. SILIRUM™ was also evaluated in 10 of 18 calves that were challenged orally with $6.9 \times 10^{10}$ cfu of *M. ptb* following vaccination without adverse reactions, although the author of the poster revealed that all vaccinates had injection site lesions up to 15cm (Munoz et al., 2005). Cellular and humoral responses were evaluated with interferon-gamma results detected at 30 days and still high at 330 days post-vaccination. The calves were killed at 180 and 330 days post-vaccination and only one vaccinate had a diffuse lesion of BJD, although several had lesions mainly in lymphoid tissues. Isolation of *M. ptb* was reduced in vaccinates, suggesting this vaccine will reduce bacterial load and severity and numbers of lesions in challenged calves.

**Conclusion**

There is an emerging consensus that eradication of endemic *M. ptb* by test and cull procedures is frequently not possible nor profitable in many herd and flock situations. Until it becomes essential, as may occur if a more positive link from animal *M. ptb* to human Crohn's disease is established, control strategies that limit losses will be most appropriate for the majority of herds and flocks. There is now considerable evidence that this can be readily achieved by vaccination with the additional advantage that at least in sheep, there is a considerable reduction in faecal shedding of infectious organisms. In infected Australian sheep flocks, vaccination is likely to remain a voluntary long-term *M. ptb* control strategy, although how long producers will continue to use the vaccine when losses are no longer observed remains an interesting question for the future. The experience from Iceland suggests the disease will re-emerge following cessation of vaccination. Fortunately concerns that adverse reactions and injection site lesions, or the occasional occurrences of self-injection may compromise the use of vaccine appear to be unfounded.

In conclusion, vaccination is a key tool to enable rapid reduction in the within-herd prevalence of infection in sheep and goat flocks, with a potential for use in dairy and possibly beef cattle operations to minimise economic losses to cattle producers. However further information from clinical trials and research is required to fine-tune effective vaccination programs in cattle. If *M. ptb* is conclusively shown to be a public health risk, effective control of paratuberculosis and elimination of clinical cases will be necessary to protect human health and satisfy public demands for food safety. Examination of vaccination as a means to eliminate clinical BJD and potentially to reduce shedding from infected dairy cattle should be undertaken as soon as possible in Australia. If shown to be efficacious, as is likely when one considers the international perspective, vaccination is likely to prove a most important component of paratuberculosis control strategies in herds where clinical cases are occurring.

**Acknowledgements**

The assistance of Dr Ramon Juste of NEIKER, Bizkaia Spain is gratefully acknowledged, as is advice from Drs V Fridriksdottir of Iceland and S Stehman of USA. The Australian studies have been a collaborative effort involving many individuals, including Helen McGregor, Russell Bush, Jeff Epplestone, Leslie Reddacliff, Richard Whittington, Evan Sergeant and others. Projects were funded by the NOJDP through Meat and Livestock Australia and conducted by researchers and support staff at the RLPB Central Tablelands, NSW Department of Primary Industries at EMAI and OAI, and the Faculty of Veterinary Science, at the University of Sydney.
References


Efficacy of a killed *Mycobacterium paratuberculosis* vaccine for the control of OJD in Australian sheep flocks

L Reddacliff, J Eppleston, P Windsor, R Whittington, S Jones

*NSW Department of Primary Industries*
PMB 8, Camden, NSW, 2570
Phone: 02 4640 6314; Fax: 02 4640 6384
Email: leslie.reddacliff@dpi.nsw.gov.au

Abstract

A major field trial was undertaken from 1999 until 2004 to determine the efficacy of a killed *M. a. paratuberculosis* (*M. ptb*) vaccine, Gudair™, for the control of ovine Johne's disease (OJD) in Australian merinos run under Australian pastoral conditions. The vaccine stimulated cell-mediated and humoral immune responses. Gudair™ reduced mortalities due to OJD by 90% and delayed faecal shedding for the first year post-vaccination (pv). Thereafter, the prevalence of shedders among vaccinates was reduced by 90%. The numbers of *M. ptb* excreted by the vaccinated groups were also reduced by at least 90% at most sampling times. However, high levels of excretion by vaccinates occurred on some occasions, and although only 7 of 600 vaccinates died from OJD, all 7 had multibacillary disease. Thus there remains a risk that some vaccinated sheep could transmit OJD. Small reductions in liveweight gain were found in vaccinated lambs in the first year pv, but there was little effect on wool production. Vaccine injection site lesions were detected in almost 50% of sheep 2 months pv, and these persisted for at least 4 years in 20-25% of vaccinates. Data from this trial enabled the registration of Gudair™ in Australia in 2002 and underpins the pivotal role of vaccination in the current management of OJD.

Introduction

Vaccination against OJD had been used in several overseas countries, with encouraging results. However, there were few scientifically controlled reports demonstrating its efficacy. A major field trial was undertaken from 1999 until 2004 to determine the efficacy of a killed *M. a. paratuberculosis* (*M. ptb*) vaccine, Gudair™, for the control of OJD in Australian Merino sheep run under Australian pastoral conditions. The main objectives were to determine the effect of Gudair™ vaccination on OJD-related mortality and on the excretion of *M. ptb* in the faeces (shedding). Secondary objectives were to investigate the cellular and humoral immune response following vaccination with Gudair™, to assess the field safety of Gudair™, and to determine changes in productivity parameters associated with either vaccination or subclinical disease.

Methods

On each of three farms experiencing significant OJD-related mortality (5 to 15% per annum), 200 Merino lambs (age 1-4 months) were vaccinated with Gudair™ and 200 control lambs were left unvaccinated. Animal assessments and sample collections were conducted twice yearly until 4 or 5 years of age. The impact of vaccination on mortality, faecal shedding of *M. ptb* (by pooled and individual faecal culture), cellular (BOVIGAM™) and humoral (PARACHEK™) immunity, vaccine injection site lesions, liveweight, condition score and wool productivity were examined. A standard set of tissue samples was collected from each animal autopsied and examined histologically, with any lesions observed classified according to pre-determined criteria.
Results

Mortality due to OJD (severe clinical OJD)

Mortalities due to OJD are shown in Figure 1 for each age class, across all farms for the duration of the trial. Vaccination was associated with both a delay and a reduction in OJD-related mortality. OJD mortalities in control sheep began at 23, 14 and 19 months of age compared to 41, 23 and 43 months in vaccinates on farms 1, 2 and 3 respectively. This represents a mean delay of about 17 months. The total mortality due to OJD was significantly reduced on all three farms by vaccination. On P1, 10 controls and one vaccinate, on P2, 39 controls and 5 vaccinates and on P3, 31 controls and one vaccinate died of OJD. Across all three farms, the magnitude of reduction in OJD mortalities due to vaccination was about 90% (a total of 80 controls and 7 vaccinates). Of the vaccinates that died due to OJD, all had diffuse multibacillary lesions. Of the controls, 70 (88%) had multibacillary lesions – not significantly different from the vaccinates.

Subclinical OJD

The prevalence of subclinical infection was assessed in a sample of sheep at abattoir slaughter at 2 years of age, and again at 4-5 years of age by histopathology. At both ages, significantly fewer vaccinated than control sheep had paratuberculous lesions. At 2 years there was about a 65% reduction in the proportion of vaccinates with subclinical OJD compared to controls (6 of 35 and 21 of 43 with lesions, for vaccinates and controls, respectively). At 4-5 years there was about a 50% reduction in the proportion of vaccinates with subclinical OJD compared to controls (24 of 163 and 41 of 144 for vaccinates and controls, respectively). Among sheep with lesions there was no significant difference between vaccinates or controls with multibacillary lesions. Abattoir surveillance for gross lesions of OJD in the older sheep detected 20 of 21 sheep with diffuse lesions, but only two of 44 sheep with focal or multifocal lesions. Two other sheep considered to have gross lesions at abattoir inspection had no histological changes consistent with OJD. No gross lesions were recorded in the 2 year old sheep.
Figure 2. Prevalence of sheep excreting *M. paratuberculosis* in faeces, based on PFC results, assuming 100% sensitivity and specificity using method 2 from the Pooled Prevalence Calculator. Error bars show the 95% binomial confidence limits.

Property 1.

![Figure 1](image1.png)

**Months post-vaccination**
- Vaccinated
- Control

Property 2.

![Figure 2](image2.png)

**Months post-vaccination**
- Vaccinated
- Control

Property 3.

![Figure 3](image3.png)

**Months post-vaccination**
- Vaccinated
- Control
Figure 3. Estimated total excretion of *M. a. paratuberculosis* by vaccinated and control groups on each farm.

**Property 1.**

![Graph showing total excretion (log10) by vaccinated and control groups over months post-vaccination.]

**Property 2.**

![Graph showing total excretion (log10) by vaccinated and control groups over months post-vaccination.]

**Property 3.**

![Graph showing total excretion (log10) by vaccinated and control groups over months post-vaccination.]

Indicates the maximum excretion that could go undetected because of the analytical sensitivity of the culture technique.
Figure 4. Proportion of animals showing positive gamma interferon responses

Property 1.

Property 2.

Property 3.
Figure 5. Proportion of animals showing ELISA responses

Property 1.

Property 2.

Property 3.
Excretion of *M. a. paratuberculosis*

Figure 2 shows the estimated prevalence of shedders on each farm at each sampling time obtained from the pooled prevalence calculator (http://www.ausvet.com.au/pprev/). There were obvious differences between the three properties. P1 had the lowest prevalence of sheep excreting *M. ptb*. P2, had the highest prevalence and showed a distinct peak, reaching 26% in control sheep at 24 mths pv. By 48 mths, the prevalence in these control sheep had dropped to less than 2%, presumably because many of the infected sheep had died. Despite these differences, the proportion of positive PFC results in the vaccinated sheep was less than that of the controls at every sampling time after 2 months pv on each farm.

The absence or reduction of faecal shedding in the vaccinated groups compared to the controls at each sampling time was very clear on farms P1 and P3. On these farms, there was no detectable excretion of *M. ptb* by the vaccinated groups until 18 months pv (about 21 months of age), whereas excretion, was detected in the controls as early as 6 or 8 months pv (9 -11 months of age). The reduction in prevalence of excreters averaged about 90%, and was maintained throughout the trial. The results from these two farms thus clearly demonstrate a delay and reduction in faecal excretion of *M. ptb*, with maintenance of the protection for the economic life of the sheep. The results for P2 differed in that there was a smaller delay in the start of shedding, and at the final 2 samplings at 42 and 48 mths pv, there was little difference in prevalence between the vaccinated and control groups. The peak prevalence for excreters over the life of the sheep was, however, very much lower, and delayed, in the vaccinated group.

Figure 3 shows the total daily amount of *M. ptb* excreted by each group at each sampling time. This is a measure of the potential for environmental contamination with the organism, and thus the risk to subsequent generations of sheep. At each sampling time throughout the trial on P1, and up to 30 months pv on the other farms, the numbers of *M. ptb* excreted by the vaccinated group were at least one log lower than the controls. Note, however, that the actual amount of *M. ptb* excreted can be very much higher than indicated by prevalence data alone. This may be a reflection of the large contribution of a single (or a few) multibacillary infected sheep.

Immunological responses

Figures 4 and 5 show the proportions of sheep with positive IFN-γ responses and ELISA antibody at each sampling time on each farm. The stimulation of both the cell mediated and humoral immune systems by vaccination is evident.

*Cell-mediated (IFN-γ) responses (Figure 4)* - In controls, positive IFN-γ responses were detected by 8-12 months pv and were likely to be due to environmental exposure to *M. ptb*. The proportion of vaccinates with positive IFN-γ responses was maximum at the first pv test on each farm, and then declined. These proportions remained higher than in the controls at every subsequent sampling time. Positive pv IFN-γ response was negatively associated with shedding and with infection. No significant association with OJD-mortality was found, but there were very few vaccinated sheep that died of OJD.

*Humoral responses* - The pattern of ELISA results (Figure 5) was similar on P1 & P3, with high levels of seroconversion in vaccinates, which declined slowly for about 18 months, and then levelled out. On P2, the most severely affected property, antibodies to *M. ptb* were present in 6-7% of lambs at the time of vaccination. This indicates the presence of passive maternal antibody, which may have affected the ELISA responses to vaccination. On this property fewer sheep responded initially, and by 8 months pv the percentage of reactors peaked at only 63%, in comparison with levels above 80% on the other two farms. At the 24 months pv sampling there was a large increase in the percentage of vaccinates with positive ELISA results, which
may indicate an immune memory effect as vaccinates are exposed to increasing levels of infection. Among control sheep, seroconversion occurred from about 12 months and the proportion of ELISA positives was also greatest at 24 months pv. These seroconversions likely reflect the development of advancing disease. Positive pv ELISA response was negatively associated with shedding, with infection and with OJD-mortality.

The effect of detectable maternal antibodies on the subsequent development of ELISA and IFN-γ responses in vaccinated sheep was assessed by comparing the post-vaccinal immunological results for lambs with and without maternal antibody on P2. The presence of detectable antibodies was negatively correlated with CMI responsiveness following treatment. A similar trend was observed with regard to ELISA results. To ascertain whether the association of maternal antibodies with reduced post-vaccinal CMI responses was of practical significance, we examined the association of detectable maternal antibodies on the subsequent OJD status in both vaccinated and unvaccinated sheep, and no significant effect was found.

Vaccination site lesions

Injection site lesions were not identified in any control sheep, but 42% of vaccinated lambs had lesions at 2 months post vaccination. The prevalence of lesions decreased to a minimum 12 months pv, and then remained steady at approximately 20% of vaccinates having palpable lesions until 3-4 years of age. The low percentage of palpable lesions at 12 months pv was most likely associated with the sheep carrying lots of wool, so that lesions were harder to detect. No association was found between the presence of a palpable post-vaccinal lesion with excretion of *M. ptb*, infection status, nor with OJD-mortality. Thus the presence or absence of a pv lesion was of little use to predict efficacy of vaccination in individual sheep. At both the hogget cull and final slaughter, post-vaccinal lesions did not result in significant losses on the slaughter floor.

Comparison of IFN-γ, ELISA and post-vaccinal lesions as indicators of immune response - Among vaccinated sheep, the presence of a post-vaccinal lesion (2mths pv), IFN-γ responses and ELISA responses (2 mths pv, P2,3 and 12 mths pv, P1) were compared using the kappa statistic. Although all three may be considered as indicators of vaccine response, there was no significant correlation between them, except for IFN-γ and ELISA on P3.

Production measurements

Data used were from surviving sheep at each sampling time, so that loss of production due to OJD mortalities is not included in this analysis.

Liveweight and condition score – GLM analysis for liveweight, with weight at the time of vaccination included as a covariate, revealed significantly higher liveweight gain for control sheep compared to vaccinates at 2, 6 and 12 months pv. The magnitude of the differences was small, however (0.22, 0.34 and 0.75 kg at each time period respectively). In older sheep there were no significant differences, except at 30 months pv, when the controls were again heavier than the vaccinates. There were highly significant differences in weight gains between farms, but farm and vaccination interactions were not significant, except at 30 months pv. GLM analyses were also used to compare liveweight of infected sheep with those of sheep not detectably infected. This examines the effect of subclinical OJD at the time of measurement. Infected sheep were significantly lighter, 1-2 kg at 24, 30 and 42 months pv, but there were no differences at other times. GLM analyses for condition score, with respect to vaccination or infection status, yielded similar findings to liveweight, except at 30 months pv, when the vaccinates scored higher than the controls.

Fleece characteristics - There were no differences between controls and vaccinates in average greasy fleece weight or fibre diameter in 2001, nor in 2002. At the 2003 shearing, when sheep
were 3-4 years old, greasy fleece weight for vaccinates was slightly higher (0.123 kg) than controls. There were no differences in fibre diameter at any sampling time. With respect to infection status, there were no significant differences at any sampling time.

**Discussion**

This trial has been the first detailed, objective and controlled examination of the efficacy of vaccination of sheep against paratuberculosis on commercial farms. In most overseas work to date, properly controlled studies have not been done, and it has not been possible to isolate the effects of vaccination from other aspects of control programs. Preliminary results from this trial facilitated the registration of Gudair™ in Australia in 2002.

By project end there had been only seven confirmed OJD mortalities in vaccinates compared to 80 from the controls (a reduction of about 90%), and OJD mortalities in the vaccinated sheep were delayed by a mean of 17 months. This alone would justify the use of vaccination and will provide immediate benefit to producers who are experiencing significant OJD-related mortality in their flocks.

The PFC results demonstrated a delay and reduction in faecal excretion of *M. a. paratuberculosis* in vaccinated sheep, with maintenance of the protection for the economic life of the sheep. The reduction in prevalence of shedders was about 90%, and the total amount of *M. a. paratuberculosis* organisms shed onto pasture was also reduced by more than 90% at most sampling times. Reduced environmental contamination may lead to reduction in OJD prevalence in subsequent generations of sheep. Vaccination also increased the age at which shedding commenced by about 12 months, thus providing a much larger window of safety for management of pasture contamination. These findings indicate that the estimates used in modelling studies that show vaccination should over time reduce within flock OJD prevalence were reasonable, and underscore the incorporation of vaccination into assurance programs.

Development of immune responses was demonstrated following vaccination, and both pv IFN-γ and ELISA responses of individual sheep were negatively correlated with subsequent faecal shedding, OJD mortality and infection status. Post-vaccinal ELISA response was at least as good as pv IFN-γ response in predicting the subsequent OJD status of vaccinated sheep. Humoral responses are not considered in themselves to be protective, but the results from this trial indicate that ELISA response may be a suitable marker for ‘vaccination take’.

There was no indication of any short term adverse clinical effect of vaccination. Vaccination site lesions were persistent, but were not a major concern to processors. The presence of a pv lesion was of little use to predict efficacy of vaccination in individual sheep, but the persistence of lesions in a proportion of vaccinates might act as a marker for flock vaccination.

A small reduction in the weight gains of vaccinates over the first 12 months pv was seen. The mechanism for this is unclear. Post-vaccinal lesions may be painful and/or reduce the animals’ enthusiasm for grazing, but when vaccinated sheep with lesions were compared to those without lesions no significant differences were found. Presumably a systemic reaction to vaccination, and/or some grossly unobservable local reaction was responsible for the lower weight gains. The lower weight gain in young sheep is unlikely to be an economic disincentive to vaccination in a fine-wool merino enterprise, but could be of concern in fat lamb operations. Vaccinates cut slightly more wool than controls at 3-4 years of age, but as no differences were seen at other times, increased fleece yields could not be used to support vaccination. However, these measurements of production parameters only include surviving sheep at each sampling time. Thus they ignore the considerable effects of vaccination in reducing OJD mortality.
There were differences between the three farms in the pattern of OJD expression during the trial. Farm 2 appeared to have had a very high level of *M. ptb* challenge, with the highest proportion of OJD mortalities, and the highest proportion of subclinical OJD in culled hoggets (87% among controls). The proportion of IFN-\(\gamma\) reactors in controls peaked at 58% at 18 months pv, and prevalence of shedders reached 26% at 24 months. Farm 3 was less severely infected, and the peaks in mortality, subclinical infection and IFN-\(\gamma\) reactors occurred later. Farm 1 had the lowest numbers of mortalities, of shedders, and of IFN-\(\gamma\) reactors, and on this farm, the vaccinated group had no detectable shedders at several of the later sampling times.

Several aspects of the design of this trial should be considered when translating the findings into management and control recommendations for the wider industry.

Firstly, we investigated the effect of vaccination only against a very heavy challenge, because of the difficulty in detecting and quantifying *M. ptb* infection in low prevalence flocks, where few or no OJD mortalities are recognised. Moreover, in the current trial, unvaccinated control sheep were run with the vaccinates, providing an on-going high level of challenge. Under normal field conditions, the entire cohort of an age group or mob would be vaccinated, so that this additional challenge from cohorts would be reduced. Thus, the current trial was a “worst-case scenario” from the perspective of disease challenge. Intuitively, one may expect that vaccination should be more effective when other management procedures are used concurrently to reduce *M. ptb* challenge, but this cannot be inferred from the current trial.

Secondly, in these experiments, we examined a single cohort of sheep over 4 to 5 years. In the real world situation, a new crop of lambs would be raised and vaccinated each year. The amount of *M. ptb* exposure would be expected to be successively reduced, with a reduction in the opportunity for transmission of the disease to future generations. Any effects of vaccination on successive generations of lambs over time is not reflected in the results from the current study. Some useful insights on the likely long term effects of vaccination can be gained from the experience in Iceland, where vaccination of sheep was introduced only after more traditional control measures, such as quarantine, and test and cull programs, had failed. There, vaccination has been an effective control measure and was shown to reduce mortality by 94%, but despite long term mandatory vaccination, OJD infection remains widespread.

There are also a number of potential concerns for the use of vaccination in control programs for OJD highlighted by the current trial.

All of the vaccinates that died had severe multibacillary infection, and would have been excreting enormous numbers of *M. ptb* leading up to their death. While there were very few of these sheep, the contribution of just a single sheep with multibacillary infection to environmental contamination is large. Just one, or a few, sheep with multibacillary lesions may have also been responsible for the relatively large amounts of *M. ptb* excreted by vaccinated groups at some sampling times. If this occurred at a critical time (eg in a ewe in the lambing paddock when pasture was short and many young lambs were beginning to graze) a whole cohort of young susceptible animals may be exposed to large numbers of *M. ptb*. Another important observation was that the effect of vaccination on subclinical disease, although significant, was less than the effect on clinical disease, and vaccination did not decrease the proportion of subclinically affected sheep that had multibacillary lesions. These findings have particular relevance when vaccinated sheep from infected farms are moved to areas of low prevalence for OJD, and imply that sustained vaccine use will be necessary to avoid recrudescence in infected flocks.

In this trial, vaccination was seen to provide life-long protection. However, because of the ongoing high challenge with *M. ptb*, a natural boosting effect may have been operating. The increase in the proportion of vaccinated sheep with positive IFN-\(\gamma\) responses at 18 mths pv on
P2, and 24 mths pv on P3, is indicative of such an effect. Whether vaccinated sheep in a low or zero challenge environment would still be protected if suddenly exposed after several years to high levels of *M. ptb* is unknown.

**Conclusions**

In flocks with a high prevalence of OJD, vaccination with Gudair™ stimulated specific immune responses and significantly reduced and delayed both OJD-related mortality and the excretion of *M. ptb*. Protection was maintained for the economic life of the sheep. Thus vaccination will provide immediate benefit to producers who are experiencing significant OJD-related mortality, and should provide benefit to subsequent generations of sheep. However, ignoring the considerable OJD-related mortality, there were only small effects of either vaccination or subclinical OJD on production parameters.

High levels of *M. ptb* excretion by vaccinates on some occasions, multibacillary lesions in the few vaccinates that died from OJD, and persistence of subclinical OJD in a significant proportion of vaccinates, indicate risk that some vaccinated sheep could transmit the disease. This has implications for translocation of vaccinated sheep to very low prevalence areas, and implies that sustained vaccine use will be necessary to avoid recrudescence in heavily infected flocks.

**Acknowledgements**

We thank Ian Lugton for initial establishment of the trial, Kevin Thornberry for assistance during sample collection, Idris Barchia for biometrical advice, and staff from the Orange Agricultural Institute, CSL Animal Health and from EMAI for the detailed laboratory investigations. Special thanks are due to Shayne Fell for his diligence with the OJD cultures. Research funding was provided through Meat and Livestock Australia as part of the NOJDP.

**References**


4. Garcia Marin JF, Tellechea J, Gutierrez M, Corpa JM, Perez V. Evaluation of two vaccines (killed and attenuated) against small ruminant paratuberculosis. 6th International Colloquium on Paratuberculosis, Melbourne, Australia 1999; 234-41.


8. West DM. Johne's disease in New Zealand: history from first cases to current situation.
On-farm impacts of vaccination with Gudair™

Jeff Eppleston
Central Tablelands Rural Lands Protection Board
PO Box 20, Bathurst NSW 2795
Phone: 02 6331 1377; Fax: 02 6331 6915
Email: jeff.eppleston@ctrlpb.org.au

Abstract
In addition to the major vaccination trial conducted in three high prevalence flocks (Trial OJD.009), two field studies and several surveys and strategic samplings have been undertaken in other vaccinating flocks. These studies were conducted to better assess the full on-farm impact of vaccination in a range of commercial situations. Both a survey of 50 early vaccine users and a whole farm vaccination study confirm the major reduction in clinical disease measured in Trial OJD.009. A preliminary study in seven high prevalence flocks in which a vaccination program has commenced, suggests a 50% reduction in shedding after three years of vaccination. Results from a longer term monitoring program in 12 flocks with variable initial disease prevalence will be available in 2-3 years time. Vaccination site lesions have produced infrequent adverse effects on-farm, but a survey of a small number of abattoir consignments of vaccinates suggests they will be of little consequence at slaughter. A survey of vaccinators indicated an exposure rate to Gudair of around 1 in 7,000 doses applied and a number of cases of self-injection injury have been reported.

Introduction
Gudair™ was first released in Australia under permit in late 1999 for use in a major trial, (known as the ‘Intensive vaccination trial’ - MLA project OJD.009 1) funded under the National Ovine Johne’s Disease Program (NOJDP), aimed at determining the efficacy of vaccinating Merino lambs against OJD. Soon afterwards the vaccine was also supplied, again under permit, to owners of heavily infected flocks that had been experiencing large OJD-related losses (>5%). Following encouraging results from Trial OJD.009, Gudair™ was registered in April 2002 for wider use by the sheep industry and since the completion of the NOJDP, vaccination against OJD has been actively encouraged as a means of significantly reducing the on-farm impact of the disease and the risk associated with the purchase of re-stocker sheep.

The detailed outcomes from Trial 009 are being presented at this conference by Leslie Reddacliff. In brief the trial showed that in flocks with a high prevalence of OJD, vaccination with Gudair™ stimulated specific immune responses and significantly reduced and delayed both OJD-related mortality and the excretion of M. a. paratuberculosis (M. ptb). Protection was maintained for the economic life of the sheep.

Trial 009 was conducted in commercial flocks. However the application of the vaccination program was not reflecting commonly used management practices and in fact could be considered ‘worst-case’ scenario. The trial was conducted in one generation of sheep only (the first to be vaccinated on each property) and under extreme challenge from M. ptb. The trial flocks had a very high initial prevalence of OJD, the vaccinates were run with unvaccinated controls and OJD clinical sheep from other mobs were grazed with the trial sheep at a time when they were young and susceptible to infection. Under normal farming practices all lambs in the cohort would be vaccinated and other management procedures would be used concurrently to reduce M. ptb challenge, i.e. they would graze pastures with the lowest levels of contamination and not co-graze with older unvaccinated sheep. In addition many flocks would
also commence vaccination at lower disease prevalence and as the program proceeds an increasing number of previous generations will have been vaccinated, resulting in decreasing levels of pasture contamination. Therefore it is important that the outcomes from Trial 009 be confirmed in a larger number of commercial flocks.

Further, while Trial 009 has demonstrated clear benefits from vaccination, it has also identified two potentially negative outcomes: i) that shedding continues at a level that is likely to be infective, and ii) that persistent vaccination site lesions occur in around 25% of vaccinated sheep. These issues resulted in the establishment of two additional field trials funded by the NOJDP outlined previously.

In addition to these two field studies a number of other surveys and samplings aimed at a better understanding of vaccination have been conducted in commercial flocks without the limitations imposed by the experimental design used in Trial 009. This paper will outline the results of these studies and discuss them in relation to the on-farm impact of vaccination on the incidence of clinical and subclinical disease, the level of shedding, vaccination site lesions and vaccine safety issues.

Reduced clinical and subclinical disease

The most prominent and practically beneficial outcome from Trial 009 was the observation that vaccination reduced mortalities due to OJD by 90% in these high prevalence flocks. Another high prevalence flock Trial OJD.015 (Whole flock vaccination) estimated a similar reduction in mortalities in the two years following the implementation of whole flock vaccination. Anecdotally most producers and animal health staff in high prevalence areas confirm that the incidence of OJD-mortalities decreases rapidly following the commencement of a vaccination program.

Confirmation of reduced mortalities is also provided by a survey conducted in early 2003, of the first 50 high prevalence flocks allowed to vaccinate under permit, concurrent with Trial 009. Thirty four of the 37 respondents (74% response rate) ran a self-replacing Merino enterprise and because all had commenced vaccination in 2000 most (21 - 62%) had all their sheep vaccinated, including four properties where the whole flock had been vaccinated before 16 weeks of age. Of the 13 flocks still containing non-vaccinates, nine had only the oldest drop left unvaccinated, while the remainder still contained two or three of the oldest drops as unvaccinated sheep.

Flock owners were asked to estimate the proportion of sheep dying with OJD-consistent (wasting) symptoms for each age group. While these data are subjective, all the flock owners had previously observed significant mortalities in their flocks and were experienced in identifying clinical sheep. In general these estimates agreed with the significant reduction in clinical disease observed in Trial 009. The incidence of mortality increased with age at vaccination. The proportion of flocks reporting mortalities in cohorts vaccinated before 4 months of age, was less than in cohorts vaccinated after 4 months of age (13 of 33 – 39% vs. 43 of 49 – 88%; P<0.01). Accurate data on the magnitude of reductions in losses was not obtained, however most reported reductions of more than 75% in sheep vaccinated as lambs compared to previous losses in unvaccinated sheep of the same age.

In terms of subclinical effects, Trial 009 estimated a 70% reduction in the level of subclinical infection amongst vaccinates compared with controls, which is less than the 90% reduction observed in clinical disease. In Trial 009 there was no significant production loss associated with subclinical infection which contrasts with the well documented reduction in milk production in subclinically infected dairy cattle, and a report from New Zealand which estimated significantly lower lamb and wool production in subclinically infected sheep. Again owners of
high prevalence flocks often report anecdotal improvements in production in vaccinates compared to previously unvaccinated generations.

**Changes in shedding**

Trial 009 showed a delay and a 90% reduction in both the prevalence and total excretion of *M. ptb* in vaccinates. However, in these high prevalence flocks, the level of excretion detected would be likely to result in infection if sheep were moved to an uninfected flock. Modelling has suggested that the rate of shedding would fall rapidly with successive generations of vaccinates but this needed to be confirmed in the field. As a result, a long-term prospective trial has begun to monitor the rate of reduction in shedding from successive generations of vaccinated Merino sheep in flocks with varying initial levels of infection (Monitoring shedding in high and low prevalence flocks - MLA 0332), but final results will not be available for another 3 years.

Monitoring shedding in high and low prevalence flocks - Trial OJD.033

In Trial 009 the impact of vaccination was only assessed in the vaccinated generation and where vaccinates had been exposed to extremely high levels of *M. ptb*. The potential value in vaccination is likely to become evident over an extended period following vaccination of a number of successive lamb crops. In addition, the period required to reduce the risk of disease transfer in traded vaccinated sheep is likely to be dependent on the disease prevalence at the time of commencing a vaccination control program. Many producers are likely to commence a vaccination program when within flock disease prevalence is lower and the impact of vaccination in these flocks is still unclear. The objective of this trial is to document the rate of decline in OJD risk following the introduction of a vaccination strategy in flocks of varying disease prevalence.

Twelve self-replacing Merino flocks were identified and classified as high, medium or low prevalence (4 flocks per category) based on previous test results and owner estimates of mortality. In each flock, the age groups were identified into 3 cohorts (1 and 2, 3 and 4, and 5 and 6 year olds), with the 2 older groups being sampled every 2 years to estimate disease (shedding) prevalence. At each consecutive sampling, another age group will have been vaccinated as lambs so that the following comparisons can be made.

**Sampling 1**

3/4 yo non-vaccinates vs. 5/6 yo non-vaccinates

**Sampling 2**

3/4 yo vaccinates vs. 5/6 yo non-vaccinates

**Sampling 3**

3/4 yo vaccinates vs. 5/6 yo vaccinates

Comparisons between the same age groups at different samplings will provide an estimate of disease prevalence in each flock at the commencement of vaccination, and will estimate any change in prevalence that occurs in subsequent generations.
Results from the first sampling have established benchmark prevalence levels in the monitored flocks (Figure 1).

**Figure 1.** The estimated prevalence of shedding in 3 to 6 year old sheep in the 12 monitor flocks.

Monitoring shedding in seven ‘extensive’ flocks

Because of the long term nature of Trial 033, 7 of the 50 flocks permitted to use vaccine in the ‘extensive trial’ were sampled opportunistically to determine any reduction in shedding over the first 3 years of a vaccination program.  

Faecal samples were collected in pools of 50 sheep from the 1999-drop and 2001-drop sheep when they were approximately 2 years of age. The mean flock prevalence of *M. ptb* excretion at 2 years of age as assessed by the proportion of culture positive pools in each flock varied between flocks but was significantly lower in the 2001-drop compared to the 1999-drop vaccinates (28 ± 5% vs. 55 ± 6%, respectively; P < 0.01). The amount of *M. ptb* excreted was also lower in the 2001-drop (P < 0.01) but on some farms there was similar levels of total excretion in both drops, a reflection of the contribution of just one, or a few pools with very high levels of bacteria.

Preliminarily, these data indicate a reduction in the prevalence of shedding of around 50% after 3 years of a vaccination program and support modelling work suggesting that the prevalence of *M. ptb* infection should decrease rapidly after the commencement of a vaccination program in heavily infected flocks. However results should be interpreted cautiously pending the results of the more comprehensive study (Trial 033).

The impact of vaccination site lesions

In Trial 009 around 50% of vaccinates developed lesions at the injection site within 2 months of vaccination. The prevalence of lesions decreased to around 20% by 2 years after vaccination, and remained at that level until the end of the trial when the animals were 4 years of age. The lesions were large and ranged in size up to a diameter of 45 mm. The presence of injection site lesions can have adverse consequences in the live animal and potentially in the carcase at slaughter.
Adverse consequences in live vaccinates

Several reports from producers of adverse reactions in live sheep resulting from the presence of lesions have been collated:

- One producer had to apply a preventative flystrike treatment to his Merino weaners in December, 10 weeks post vaccination, after 30 were struck near fistulating injection sites.

- Several producers reported problems associated with injection site lesions when the vaccine was applied at a site other than the recommended neck area. A ram developed a large lesion near the brisket, likely to result in mating difficulties. Another producer reported that vaccinating lambs on the cheek resulted in the development of lesions on the face that could impair grazing efficacy.

- In 4 flocks 5-6% of lambs or weaners died after developing paralysis following apparent deep injection near the vertebrae resulting in focal chronic inflammation presumed to have caused compression of the spinal cord.

Producers therefore should be encouraged to follow the recommended site of vaccination and to ensure that sheep are adequately restrained prior to treatment.

The impact of lesions at slaughter – Trial OJD.032

Because of the prevalence of lesions observed in Trial 009 and reports particularly from New Zealand of discounts being applied to vaccinated carcases, a survey of 20 consignments of vaccinates was conducted at slaughter to determine the variation in the prevalence of lesions and their impact on carcase value.

The prevalence of lesions observed in the slaughter survey was 18% for mutton and 65% for lamb carcases. However there was considerable variation in the prevalence of lesions observed amongst consignments of mutton sheep (Figure 2; range 1 – 41%), but this was not related to age at vaccination or to the interval between vaccination and slaughter.

**Figure 2.** The prevalence of injection site lesions by consignment.
The value of the trim removed on the slaughter chain was insignificant and the labour cost of its removal was nil. No carcase was downgraded to a lower value grade. This study was conducted at a time of low sheep supply, in carcases vaccinated at the recommended site high on the neck. In these circumstances OJD vaccination site lesions are not a significant cost to producers or to the processing industry and will represent only a very small proportion of the total cost of OJD control by vaccination.

Self-injection

While a more detailed examination of vaccine safety will be presented separately at this conference, a discussion of the on-farm impacts of vaccination cannot ignore the risks of vaccine exposure and self-injection. As part of the survey of the first 50 flocks approved to use vaccine described previously, data on the incidence of vaccine exposure was also collected. Respondents reported they had vaccinated a total of 155,523 sheep, which included one contractor who had vaccinated 63,000 sheep. There were five reports of incidents where the operator had splashed themselves with vaccine, and 16 instances of needle stick injury without injection. There were no reports of vaccine self-injection. This represents an exposure rate of 1 in 7,406 and a needle stick rate of 1 in 9,720. If the contractor is deleted from the data set, the exposure rate and needle stick rate amongst farmer vaccinators increased to 1 in 4,406 and 1 in 5,783 respectively. There were no reports of detrimental outcomes to exposed users in this data.

Reports of six cases of accidental self-injection resulting in medical intervention including surgical removal of injected material have been collated elsewhere.

These data suggest that accidental exposure is not rare in Australia and steps such as adequate restraint of lambs, minimising the number of activities undertaken when vaccinating, improved vaccination technique and seeking immediate medical attention following exposure should be stressed to vaccine users.

Implications for industry

The benefits at a flock level of vaccinating against OJD in high prevalence flocks are obvious; rates of infections and shedding are rapidly reduced to levels that are insignificant to the owner of the infected flock. However, in these flocks the continued existence of low numbers of vaccinates with multibacillary disease means that the risk of disease spread remains in the flock should vaccination be discontinued or in other flocks should the vaccinates be traded.

The value of vaccination in low prevalence flocks, whether low prevalence at the beginning of a vaccination program or because of an ongoing vaccination program, is less evident. Intuitively the cumulative effects of vaccination on shedding over generations should mean the continued reduction in risk associated with vaccinated sheep. Indeed the reduction in shedding measured in the seven high prevalence ‘extensive trial flocks’ is encouraging but the final results from the 12 flocks with varying levels of infection in Trial 033 will be more informative. In this regard it is important that the prevalence of gross lesions be monitored by abattoir surveillance as this is likely to provide useful information on changes in regional within-flock prevalence as district vaccination proceeds.

While the prevalence of vaccination site lesions is significant, the cost of these to the sheep industry appears to be of little concern provided the recommended site of injection is used. Two important extension messages for producers should be i) the importance of vaccinating high on the neck, and ii) strategies available for reducing the risks and impacts of vaccine exposure to the vaccinator.
Acknowledgments
These studies were the result of much collaboration between Sydney University, NSW DPI and RLPB's. We acknowledge the assistance of the many OJD-affected producers in these on-farm studies.

References
Public Health and National Programs
Review of National Level Prevention and Control from 8th International Colloquium on Paratuberculosis

Luzia Rast
RMB 417
GUNDAGAI NSW 2722
Phone: 02 69 441 588
Fax: 02 69 441 876
E-mail: lrast.gundagai@bigpond.com

Background

Papers and posters were presented from Holland(3), Switzerland(1), Australia(2), USA(2), Italy(1), Sweden(2), France/Holland(1), Israel(1) and the Czech Republic(1). Most work was focused on the dairy cattle industry. Australia and Sweden also reported work done on the beef cattle and sheep industries.

It was recognized that the potential link between Crohn’s disease in humans and paratuberculosis remains unresolved and that once this question is answered, future approaches to JD control in livestock may change.

Generally in countries with large livestock numbers and endemic JD it was accepted that eradication of paratuberculosis on a national level was technically not possible, necessary or economic. Therefore the main focus of paratuberculosis control needs to be to reduce the economic losses due to the disease. Any national program needs to actively involve industry in the decision making and needs a communication and education strategy for the program to be widely accepted. Until the potential public health issue is resolved the main effort needs to be to reduce \( M. \text{ptb} \) levels in foods that could transmit \( M. \text{ptb} \) to humans if contained in high concentrations, mainly dairy products.

An exception to this was work from Sweden and Switzerland. Sweden has a very low prevalence of JD in cattle and sheep and hence their control programs are aimed at protecting this status by strict import controls and a ‘stamping out’ policy should JD be detected.

Switzerland recognized that there is a need to better define the prevalence of JD and its geographical distribution and to conduct a cost benefit analysis, prior to making any decision for a national JD control program.

Dutch Studies

Two modeling studies were presented:

1. Development of a milk quality assurance program for paratuberculosis: stochastic simulation of within-herd infection dynamics and economics by Weber M. F. \textit{et al.}; and

2. Development of a milk quality assurance program for paratuberculosis: from within-and between herd dynamics to economic decision analysis by van Roermund H.J.W., \textit{et al.}.

These studies recognized that due to the as yet unresolved issue of \( M. \text{ptb} \) role in Crohn’s disease in humans, \( M. \text{ptb} \) infection in cattle is of concern to the dairy industry and a milk quality assurance program for paratuberculosis may reduce the potential risk of transmission to humans through consumption of milk products. Importantly the goal of the milk quality assurance program is to reduce the concentration of \( M. \text{ptb} \) in milk rather than to eradicate \( M. \text{ptb} \). A milk quality assurance program is more cost effective than certification, surveillance and
control programs aimed at low risk trade of cattle and elimination of \textit{M. ptb} at the herd level. Herds were categorised into green (\textit{M. ptb} levels below a certain level) and red herds (infection confirmed resulting in \textit{M. ptb} levels in milk above a certain level) depending on test results. (Entry and maintenance/surveillance)

These modeling studies concluded that:

1. Herd examination by ELISA for entry into the quality assurance program and continued surveillance by ELISA effectively ensures the quality of bulk milk by ensuring the concentration of \textit{M. ptb} did not exceed a maximum acceptable concentration ($<10^3 \text{ M. ptb } /L$ milk) in closed herds.
2. Faecal culture test was more efficient in identifying cattle for culling (red herds)
3. Additional preventative measures reduced within herd spread and increased the proportion of herds that could be certified as low \textit{M. ptb} in bulk milk.
4. Low \textit{M. ptb} bulk milk certified herds need to exclude animal trade to retain that status.
5. Management measures are less important when trade is restricted but are always important in herds that are above minimum level (red herds)
6. Price difference between low level \textit{M. ptb} milk (green herds) and higher level \textit{M. ptb} milk (red herds) is important to encourage farmers to have an incentive to join the program.

The results of each modeling study depended on some assumptions. They included amount of bacteria in milk and effect of management on the within herd transmission of infection. However these assumptions were studied in a sensitivity analysis.

A third paper presented an overview of paratuberculosis control in the Netherlands (Paratuberculosis control in the Netherlands; the target and an overview of the activities. Franken P.)

Initially a control program was focused on preventative management and certification of infection free herds to prevent within and between herd transmission of paratuberculosis. A new approach is based on limiting \textit{M. ptb} infection in dairy products, mainly milk, since it is economically the most important product. The program needs to be cost effective and farmer friendly, simple to manage and easy to communicate.

It is based on a three step program:

- A first test for classification of the herd
- A second test for surveillance of the previously negative tested herds
- A third test for control of herds that have tested positive.

Test used is ELISA of all animals older than 3 years (blood or milk)

Combined with control measures (such as careful movement of stock, culling of infected cows and their lastborn calves) the numbers of negative testing herds are maintained.
Evaluation of intervention strategies for control of Johne’s disease in Switzerland (Antognoli M.C. et al.)

The prevalence of JD in Switzerland is not well known despite several small scale studies and mandatory reporting of clinical cases. It is suspected that disease is under reported and that many farmers and veterinarians have limited knowledge on JD.

The hypothetical link between BJD and human Crohn’s disease, the economic losses due to JD in the dairy industry and potential non-tariff trade barriers stimulated research into the cost, benefit and disadvantages of initiating a program for control of JD in Swiss cattle.

The animal health population institute at Colorado State University and the Swiss Federal Veterinary Office collaborated in a study to provide scientifically sound recommendations for the selection of intervention strategies for the control of JD in Switzerland.

The study evaluated various testing strategies (herd sample test with ELISA and follow up of reactors with faecal culture, whole herd ELISA testing, whole herd faecal culture testing, pooled faecal culture testing) and found that classification of herd status was not feasible with any of these strategies if within herd prevalence was <5% due to poor sensitivity of the tests and small herd sizes.

Management aimed at reducing exposure of susceptible calves to contaminated manure would be more effective in controlling disease.

The study recommended the need for more complete and updated information on the prevalence and geographic distribution of JD in Switzerland before making a decision on any national control strategies. An abattoir survey, culturing abdominal lymph nodes was proposed to achieve this.

US studies

A national Johne’s disease veterinary certification program: Key to success by McDonald J., et al.

In 2002 the US Department of Agriculture created a national JD control program and mandated that veterinarians in each state must be educated and certified to implement the control program. The US needed a program that would provide a uniform base of knowledge and deliver a constant message on JD diagnosis and control. An on-line certificate program for veterinarians was developed with JD experts from across the country. The program developed is in modular format with four basic information modules and two modules with national program information. While the certificate program was developed nationally its implementation was the responsibility of states and allowed for adaptation to state specific practices, policies and procedures. The program was launched in February 2004 and since has been adopted by 35 states and has trained and certified 752 veterinarians.

A poster titled ‘Laboratory proficiency in testing for antibodies to Mycobacterium avium subspecies paratuberculosis: a new approach for the United States’, by Lombard J.E. et al. was presented

A new laboratory proficiency testing for M. ptb antibodies has been adopted and is based on a scoring system that uses qualitative (positive/negative) and quantitative (Z-scores) results. It makes allowance for the expected variability near the cutoff of test results. Z-scoring uses the median and interquartile range values from participating labs’ results in the analysis. This score can then be used to evaluate inter and intra lab variation in ELISA readings and allows more scrutiny of sample values and can be used to provide labs with feedback on consistency.
Australian studies

‘Increasing involvement of herd owners in controlling paratuberculosis through assurance based trading’ by Kennedy D. *et al* and ‘The Victorian OJD management and control program’, by Tobin F. *et al* were the two papers presented from Australia.

The first paper outlined the significant change in approach to JD control on a national level from voluntary herd/flock certification to provide low risk replacements, regulatory control of identified infected or high risk herds/flocks and movement restrictions, to an assurance based control program that is driven by all (affected and non-affected) producers and therefore is more equitable and encourages disease prevention and management.

An assurance based credit system has been developed for the sheep industry and is based on a quantitative risk assessment at the flock level. Scores based on background prevalence in the region, test results and vaccination history are allocated to each flock or consignment of sheep and declared in writing at the point of sale.

In the dairy cattle industry a herd scoring system (dairy assurance score) based on results of herd testing and prevalence in the geographic region of origin is being developed.

JD is endemic in both these industries in Australia. The systems also provide pathways for improving the scores by controlling the disease.

Surveillance in the beef cattle industry has detected very little JD in beef herds that had no contact with dairy herds. The low risk nature in the beef sector has been recognised by a new assurance category for beef only herds that is based on biosecurity alone.

The alpaca industry appears to have successfully controlled disease in the 1990’s and subsequently no or very little infection has been confirmed. Therefore this industry is in a good position to concentrate on protecting itself. A voluntary quality assurance system is achieved by individual animal identification, movement records and herd biosecurity.

Success of these schemes will require a continued communication and auditing system to detect any problems, to educate users and to confirm the confidence in the schemes.

The second paper by Tobin *et al*. outlined the progress in OJD management and control in Victoria since 2002. It outlined how OJD management had moved from a largely government sponsored effort at eradication, heavily reliant on regulatory bureaucratic processes with little industry support, to an industry sponsored management program supported by government.

Key factors in the success of the OJD management program in Victoria were:

- Appointment of an industry representative OJD advisory committee
- Development of a financial assistance package to provide incentive to owners of infected flocks to actively manage the disease
- Extensive and ongoing communication process
- Collaboration with all stakeholders
- Social support mechanism for affected producers
- Flexibility of program and ability to promptly adjust program based on feedback of stakeholders
French/Dutch modelling study

A modelling study was conducted with the aim to evaluate if truly JD free herds acquired JD certification and kept their status over time by Ezanno P et al. The Dutch program was used to construct a deterministic model of the evolution of the number of herds per health status over 25 years.

Results showed that certification programs that use tests with perfect specificity, or confirm positive results with a test with perfect specificity, to reduce false positive results maintained numbers of truly free certified herds over the 25 year modeling time, while at the same time detecting or clearing infected herds. Test and cull strategies alone will not lead to a rapid increase in certified and truly *M. ptb* free herds but restrictions on purchase of animals from herds at the highest level of certification and a decrease in within herd prevalence through herd management is also needed to increase the number of truly JD free certified herds over the 25 year modeling period.

Swedish studies

Two posters were presented:

1. Control of paratuberculosis in live cattle and in semen imported into Sweden 1995 to 2004 by, Holmstrom A. and Stenlund S.; and


Because Sweden has a very low prevalence of JD in its cattle population their main effort is to protect this status by minimising the risk of introducing infection from other countries. When Sweden joined the EU in 1995 they applied additional assurances and control measures. A voluntary import control program advises farmers to import semen or embryos instead of live animals. It further recommends that semen or embryos originate from tested herds. In the dairy industry it is prohibited to purchase and introduce live animals from other countries.

These animal programs have successfully limited the number of live animals being imported. Since 1995 only 278 animals have been imported to Sweden and they all tested negative by faecal culture and serology. Most herds of origin were also tested negative by serology. Since 1998 there have been no dairy cattle introduced into Sweden.

JD is a notifiable disease in Sweden and has never been detected in Swedish sheep. One imported ram was detected as JD infected while in quarantine in 1999.

Since 1993 annual screening of the sheep population for JD has occurred. Serology (AGID) was used until 2004 and since then faecal culture is being used. In addition all suspect clinical cases are investigated. No infection has been detected to date.

Johne’s disease control program in the Israeli dairy herds (Koren O., et al)

Israel has initiated a voluntary control program for JD in 2003 that aims at detecting infected herds and provides management solutions to reduce and prevent herd infection.

More than half of the dairy herds in Israel are infected with JD but the within herd sero-prevalence is generally low.

The control program consists of: obligation of herd to follow a management program, whole herd testing by ELISA (serum or milk) with faecal culture follow up of positive sero-reactors and risk assessment. The herd is then classified on a risk scale (1-8) and a management program aimed at reducing this risk is initiated.
**Application of a pilot control program against paratuberculosis in an alpine region in northern Italy** (Liandris E. et al)

Eighty-one dairy herds in this region were tested by ELISA and only 8 herds had no reactors. Prevalence amongst adult cattle was estimated at 19%.

On the basis of these results, a voluntary control program was implemented aimed at decreasing the disease prevalence within the herds. The program was aimed at being economic (cost and labour).

A risk assessment was completed first and then farmers implemented management strategies to reduce disease transmission. Cattle are tested prior to parturition with ELISA. Faeces of any cow with a doubtful pre-parturition serology result is tested after parturition with real time PCR. All positive animals are recommended to be culled.

**Paratuberculosis in cattle in the Czech Republic: current situation results of national control program and the main risk factors** (Pavlik I. et al.)

The Czech Republic has 1.7 million cattle on 5,405 farms (1998 figures). Around 3,500 herds are considered as economically significant for milk production. Paratuberculosis was introduced through importation of cattle and by 2004 paratuberculosis was diagnosed in just under 20% of these imported cattle.

Since 1998 a nationally subsidised control program is being implemented. It is based on faecal culture testing of all animals older than 18 months twice a year. Paratuberculosis is controlled in about 25-30 herds.

The main risk factors for JD spread are:
- Widely used practice of feeding calves with mixed colostrums and milk
- Use of progeny of infected cows for breeding
- Purchase of cattle with unknown status

The control program is hoping to encourage farmers to address these risk factors.
Review of Prevention and Control at a Herd Level from 8th International Colloquium on Paratuberculosis

Peter Windsor\textsuperscript{a} and Jeff Eppleston

\textsuperscript{a} Farm Animal Health
University of Sydney
PMB 3, Camden, NSW 2570

Introduction

Theme 3a was one of the largest themes of the colloquium with 37 papers submitted for either oral or poster presentation. These were accompanied by 2 review papers presented at the end of the Theme 3a session. In addition one of the workshops held after the conference was on vaccination and additional information was presented and discussed in some detail which assisted integration of some of the new information into current thinking. For example, the success of the Australian vaccine research has increased the likelihood of re-examination of vaccination in the control of bovine paratuberculosis in the USA. Twenty four of the papers dealt with control of paratuberculosis in dairy cattle herds, mostly in Europe or the US. Six papers were on control in goat herds and there were 7 papers on control in sheep flocks, including 3 papers presented on Australian vaccine work conducted under the National OJD Control program (NOJDCP).

The major questions addressed in this theme were;

i) Are JD control programs cost-effective?
ii) What are the main risk factors in the spread of the disease?
iii) Which tests should be used in ‘test and cull’ programs?
iv) Can animal treatments reduce the risk of disease transmission?
v) Can treatment of faeces reduce the risk of disease transmission?
vi) Does vaccination effectively control paratuberculosis?

Summary of findings from papers and posters

(i) Are JD control programs cost-effective?

The likelihood of producers adopting voluntary control &/or eradication programs for paratuberculosis will depend on the cost effectiveness of the control program adopted. Five papers presented data on the cost, either in production or financial terms of running infected herds. All presented data documenting significant costs associated with infection. For example in the US dairy industry one paper documented a 4 fold reduction in lifetime milk production in ELISA positive cows and an increased risk of other diseases during their lifetime; another measured significantly reduced milk production in heavy shedders. In his review paper Scott Wells of the USA concluded that in heavily infected herds the production costs do warrant the cost of effective control programs.

However there are varying levels of intervention possible to control paratuberculosis in cattle and another 3 papers modelled control options to determine the ‘best’ program for producers in an environment where control is voluntary. In general these investigations concluded that often programs involving improved calf hygiene and minimal testing were the most cost effective for producers, rather than the strict application of an intensive test and cull program. Russell Bush
of Australia presented the only paper on sheep. He developed an economic model aimed at determining the on-farm financial effects of different control options.

It was noted however that the outcomes from modelling were likely to change under mandatory control programs should, in the future, human health concerns dictate this type of approach.

(ii) What are the main risk factors in the spread of the disease?

Four papers examined dairy industry survey data for associations between herd management factors and measures of within herd JD prevalence. Most confirmed previously identified risk factors such as herd size and purchasing replacement cows. Herd hygiene, particularly those activities aimed at reducing the exposure of calves to contamination from the dairy environment and from pooled colostrum or milk, was identified as important for minimising herd prevalence. In this regard a survey of Irish dairy practices identified poor calf biosecurity as a major limitation to controlling the on-farm impact of JD.

The importance of segregating offspring from their dams at birth was demonstrated in a Spanish dairy goat herd where the infection rate of kids not separated from their mothers was 73% compared to 26% in separated kids Two papers reported a significant reduction in JD prevalence in dairy goat herds following the implementation of a program of post partum kid separation and feeding with artificial colostrum and milk.

In a survey of Italian dairy farms the level of disease was positively associated with the iron content of the drinking water suggesting that further investigation into this relationship could be worthwhile.

(iii) Which tests should be used in ‘test and cull’ programs?

Ten papers or posters addressed the issue of tests involved in herd control programs. Broadly they could be divided into studies examining the difficulties of using current testing regimes for controlling paratuberculosis at the herd level, or in a number of cases, early herd prevalence data in countries where control programs have only recently or are yet to commence.

One study examined the interferon gamma test. It was evaluated in goats in Norway with tests performed a day after collection, comparing results from unvaccinated herds without paratuberculosis, vaccinated herds without paratuberculosis and vaccinated herds with a history of paratuberculosis. Despite re-sampling being required when 5% of animals in control samples were found to be positive, the study concluded that the low response rate of 1% of positive animals in 1,200 animals from herds free of paratuberculosis, suggests a role for this test in monitoring unvaccinated herds.

Several studies examined the ELISA combined with faecal culture or PCR to investigate the risk of milk from herds of differing paratuberculosis status, or to more rapidly predict the status of a tested animal. Classification of 668 dairy cows as low, medium or high risk based on four annual herd screens using the ELISA and one annual herd screen by faecal culture, found that 4 of 386 cows classified as low risk (never positive) had PCR positive milk, posing a serious risk of disease transmission. Another study used a combined screening test used on milk using bulk milk serology (Svanovir™) followed by capture-PCR to detect *M. ptb.* DNA. This approach was considered a viable option to protect the consumer by excluding *M. ptb.* containing milk from the manufacturing process. In a study using the mean ELISA adjusted optical density measures as a cut-off for groups of cattle with negative, low and high faecal shedding culture results, the *M. ptb.* shedding status in 2,578 adult cattle from six herds was predicted. The mean ELISA cut-off for a positive test for negative, medium or high shedders was 3.2, 9.7 and 1,600 respectively. This suggests the ELISA adjusted OD measures can provide a more rapid prediction of the probability of a cow shedding *M. ptb.* than the 8-16 weeks required for culture.
One study examined the sensitivity of pooling of faecal samples for culture from cattle at slaughter, using pools of 3, 5, 8, 10 and 15 samples per pool. The study found that pools of 10 were of adequate sensitivity (59%) but pools of 5 were optimum (67%).

A survey of ruminants in several districts of northern India for incidence of *M. ptb.* using ELISA (Bison type antigen) found prevalence of antibody as high as 40.3%, 42.6% and 50.0% in buffalo, cattle and goats respectively across the region, with higher rates of infection found in bovids when tested by faecal (62.5%) or milk culture (70.3%). Further, examination of goat kids between 0 and 6 months using culture and PCR on faeces and tissues at slaughter, plus ELISA on serum, identified high rates of infection with Bison type *M. ptb.* up to 56%. In Portugal, a preliminary study of paratuberculosis in cheese producing sheep identified that 5.6% of 1,220 animals were positive by ELISA, with 14 of 145 animals positive by AGID, all of which had gross lesions of bowel thickening but only 7 having histological lesions of paratuberculosis. Six isolates of *M. ptb.* were obtained from these 14 sheep, with molecular identification to follow.

(iv) Can animal treatments reduce the risk of disease transmission?

A paper by Whitlock *et al.* presented results of a randomised double blind trial in 12 calves dosed with *M. ptb.* between days 7 and 9 then treated twice daily with 35mg Monensin in their milk replacer until euthanasia between days 65 and 67. The study suggested that calves fed Monensin had a 55-72% reduction in both passive shedding and tissue culture of *M. ptb.* At 70mg/day, this higher rate of Monensin content than is normally added to calf starter may have a role in reducing the bioburden of *M. ptb.* in neonatal calves, probably through direct action on the mycobacterial cell.

(v) Can treatment of faeces reduce the risk of disease transmission?

As *M. ptb.* is known to survive in faeces for extended periods in the environment, separate experiments on survival of the organism in compost and following calcium hydroxide treatment was examined. In a short paper by Gobec *et al.* from Slovenia, survival of *M. ptb.* in sheep manure following composting in a bioreactor vessel was considerably shortened, with positive PCR results for *M. ptb.* up to 7 days only in the composting system versus the full 21 days of the experiment when faeces was left in open storage. Treatment with calcium hydroxide (to raise the pH to 12.7) of sterile faecal slurry spiked with *M. ptb.* (10⁹ cfu in 300ml) from Irish dairy herds resulted in a reduction but not elimination of the organism from the slurry up to 72hrs after treatment. These observations confirm the survival of *M. ptb.* in environmental samples, suggesting such treatments are impractical methods of inactivating the organism.

(vi) Does vaccination effectively control paratuberculosis?

The Australian paper by Eppleston *et al.* which evaluated use of Gudair™ to control OJD presents convincing data that supports vaccination as a key tool for the control of OJD at the flock level. Over the productive life of Australian merino sheep in 3 high prevalence flocks, vaccination reduced mortalities by 90% and delayed and reduced faecal shedding of *M. ptb.* by 90%, although the few vaccinates that developed OJD had multibacillary disease and excreted infectious doses of the organism. Vaccination site lesions occurred in about 50% of vaccinates but declined to between 20-25% during the course of the 5 year study. The potential costs of the vaccination site lesions were found to be negligible following investigations of 20 lines of sheep at slaughter, where 65% of lamb carcases and 18% of mutton had vaccine lesions.

Three posters presented data on the new vaccine SILIRUM™ in cattle. The vaccine is also produced by CZ Veterinaria of Spain, and is a killed vaccine but uses a different adjuvant to that in Gudair™ in an attempt to reduce vaccination site lesions. A field study was conducted in Spain in a Friesian herd of 468 animals with 12% annual clinical cases of BJD, with 75% of all
cattle vaccinated and 26% retained as controls for 30 months post-vaccination. More than 90% of vaccinates produced persistent cellular and humoral immune responses to the vaccine and no new cases of BJD occurred from 6 months post-vaccination. The percentage of the 192 animals culled during the trial with lesions of BJD was significantly higher in the controls than vaccinates, with 92.3% of controls having lesions compared to 56.6% of vaccinates, indicating a significant reduction in BJD in the herd following vaccination. A pilot field study with SILIRUM was also conducted in a herd of 113 cattle with a clinical incidence of BJD of 8% and faecal culture prevalence of 5.5%. After one year of control, no new clinical cases of BJD have occurred, the faecal culture prevalence is 3.6% and no interference with intradermal testing for tuberculosis has occurred, suggesting expansion of the trial to a maximum of 50 herds is likely. SILIRUM™ was also evaluated in 10 of 18 calves that were challenged orally with 6.9 x 10^10 cfu of *M. ptb* following vaccination without adverse reactions (although my questioning of the author of the poster revealed that all vaccinates had injection site lesions up to 15cm). Cellular and humoral responses were evaluated with interferon-gamma results detected at 30 days and still high at 330 days post-vaccination. The calves were killed at 180 and 330 days post-vaccination and only one vaccinate had a diffuse lesion of BJD, although several had lesions mainly in lymphoid tissues. Isolation of *M. ptb* was reduced in vaccinates, suggesting this vaccine will reduce bacterial load and severity and numbers of lesions in challenged calves.

What can we conclude from current research into herd level control?

The review and perspectives session on herd control provided papers by Scott Wells (scientists view) and George Caldow (consultants view), focused on the reasons for controlling paratuberculosis and prospects for herd level control, particularly in dairy herds. The US dairy herd prevalence of 20-40% from 1996 NAHM survey is considered to be only half the true prevalence and although the within herd prevalence is mostly below 5%, some have up to 50%. Expensive test and cull programs appear to have been largely unsuccessful due to failure to recognise and test for the disease and inadequate compliance with control procedures, plus the perceived costs of joining the control program. At current milk and beef prices, there is no incentive to join a control program unless there is a high prevalence in the herd.

The cost of paratuberculosis control in positive herds with prevalence below 10% is estimated as $97/cow, compared to costs of $245/cow where the prevalence exceeds 10%, although these figures may underestimate the true costs due to difficulty of measuring subclinical losses. Key findings on transmission of the disease include risks from faeces and pooled colostrum. In infected herds, survival of *M. ptb* has been recorded in bovine slurry at 5°C for over 8 months. Further, in infected herds, the farm environment is culture-positive in 95% of herds (with at least one positive pool of cow faecal samples) with cow alleyways 77% positive, manure storage areas 68% positive, calving areas 21% positive and cow sick pens 18% culture positive, are evidence of the risk of environmental transmission. However the risk of colostrum and raw waste milk is significant, with 27% of subclinically infected cows found to have culture positive supramammary lymph nodes and 12% having culture-positive raw milk.

So what are the best control strategies for infected dairies? Firstly, define the status. Currently there is debate as to the best method to do this, with ELISA tests compared to PFC and the prospect that environmental sampling may provide the cheapest screening test. Secondly, encourage infected herds to enter a Voluntary Johne’s Disease Herd Status Program (VJDHSP) or equivalent, to:

1. Test/cull: problem being that can currently only detect animals when they are infectious
2. Vaccinate: possible problem being there is limited proof that vaccination reduces shedding in cattle
3. Reduce exposure of herd to *M. ptb*. Options include the possibility of neonatal calf treatment with monensin to decrease risk of infection and to test and remove positives (especially the 'supershedders') although is this cost effective?

However the most important strategies are implementing calf management, including avoiding use of pooled colostrums, segregated heifer rearing and closing the herd to introductions of possible undetected infected carriers. This suggests that test and management is a more appropriate approach to control, except in high prevalence herds where test and cull may result in more rapid control. However further work on vaccination in cattle is required to determine if the results showing rapid reduction of shedding in sheep after vaccination can be applied to cattle control.

In conclusion, the tools are available to reduce the within-herd prevalence of infection in sheep flocks and in dairy and beef cattle operations to minimise economic loss to cattle producers. However we need further information from clinical trials and research to fine-tune effective management programs in cattle as it is currently unclear whether we are able to provide cost-effective eradication of Johne's disease from cattle herds. Effective control of paratuberculosis and elimination of clinical cases will be necessary if *M. paratuberculosis* is conclusively shown to be a public health risk to protect human health and satisfy public demands for food safety.
Sally Spence
Locked Bag 21
ORANGE NSW 2800
Ph: 02 6391 3630; Fax: 02 6361 9976
Email: sally.spence@dpi.nsw.gov.au

Background

The possibility that *Mycobacterium avium* subsp. *paratuberculosis* (MAP) causes human disease is the principle driver for implementing control and eradication programs in animal populations in many countries.

Papers and posters presented under the theme ‘Implications for public health’ at the 8th International Colloquium on Paratuberculosis addressed a number of issues. These were:

- the presence of MAP organisms in patients with Crohn’s disease and other diseases
- the presence of MAP organisms in the environment and food products
- the impact of pasteurisation on the survival of MAP organisms in milk.

Summary of papers and posters

a) Human disease and MAP

Both Crohn’s disease and paratuberculosis are increasing in prevalence throughout the world. Crohn’s disease is more prevalent in developed countries (6 cases per 100,000 in the USA and Northern Europe) than in the third world (1 case per 100,000).

Many Colloquium attendees appeared to accept that MAP is, at the very least, an antigen provoking an autoimmune process in genetically susceptible people. Little discussion was heard suggesting that MAP is a primary infectious agent causing human disease.

CT Dow of the USA postulated that MAP acts as a ‘superantigen’ in individuals with the CARD15 mutation which confers susceptibility to Crohn’s disease. Mutations on the same chromosome are associated with Blau syndrome, a rare familial, juvenile, systemic granulomatosis with primary clinical findings of uveitis, arthritis and dermatitis. Dr Dow found that all 6 granulomatous tissue samples examined from 5 individuals with Blau syndrome were IS900 positive.

Dr Dow also postulated that MAP acts as an immune antigen to trigger Type 1 diabetes. He linked this to the finding that early exposure to cows milk increases the risk of acquiring Type 1 diabetes in the genetically at risk.

Some papers demonstrated an association between the detection of MAP and Crohn’s disease while others found that there was no association between MAP and Crohn’s disease.

Analysing blood samples using a nested PCR Elquezabal *et al* in Spain did not find any association between Inflammatory Bowel Disease and MAP. They found a high frequency of MAP carriers in the healthy population: 41.5% of Inflammatory Bowel Disease patients and 42.5% of normal controls were IS900 positive.
An Italian study by Taddei et al failed to culture MAP in biopsy samples from 30 patients with inflammatory bowel disease (including 8 Crohn’s disease patients) and 80 healthy patients.

A Mexican study by Favila-Humara et al found that a greater proportion of Crohn’s disease patients with granulomas were MAP IS900 positive than those without granulomas. Nine of 14 patients were positive.

Singh et al in India cultured MAP from the stools of 2 people suffering clinical bowel disorders and was unable to culture MAP from the stools of 6 healthy individuals.

The review by Dr Ole Thomsen argued against a link between MAP and Crohn’s disease. He mentioned that a wide range of chemotherapies have been trialled on Crohn’s disease patients without success. He also pointed out that Crohn’s disease patients respond to immunosuppressive therapy while TB is reactivated by such therapy. He mentioned that inoculating animals with material from Crohn’s patients does not result in disease in the animals.

b) MAP in the environment and food products

A study by Pickup et al found that 32.3% of water samples from the Taff river in Wales were IS900 positive over a 12 month period. There was a significant relationship between positive samples and rainfall on the preceding 6 days. They were able to culture MAP from 38.7% of the positive samples. Following DNA examination, they concluded that a different strain of MAP was present in the positive water samples from which they had been unable to culture MAP. MAP was also isolated from sediment taken from reservoirs on the Taff river and from 1 of 70 house water tanks sampled. They linked the distribution of Crohn’s patients in Cardiff to wind direction and postulated that aerosols carrying MAP from the river Taff was responsible for that distribution.

MAP was detected by IS900 based PCR or by culture from 8% of raw water samples entering water treatment plants in Northern Ireland by Rowe et al. No significant correlation was found between numbers of coliforms or faecal coliforms and the presence of MAP. They found that MAP was readily engulfed and concentrated by several types of protozoa. They also found that chlorine caused a larger drop in MAP numbers when the organism is free than when it is engulfed by protozoa. They suggested that the protozoa may help MAP survive in water and protect it from chlorination.

Hruska et al from the Czech Republic cultured MAP from 2% of powdered milk samples and detected IS900 in 49% of powdered milk samples. The powdered milk sampled had originated in 7 different EU countries. They had also sampled the milk from a small number of cows shedding MAP in the faeces. Most of these cows were shedding MAP in their milk but in all the cases they tested only one quarter was shedding MAP.

Irene Grant discussed the presence of MAP organisms in food in her review presentation. She said that MAP had not been detected in sheep milk but it had been detected in goats milk. She pointed out that cattle infected with MAP can have disseminated MAP organisms in lymph nodes, muscle and blood consequently localised infection can be spread through a whole batch of beef by mincing.

c) MAP and pasteurisation

A number of papers addressed the issue of the impact of pasteurisation on survival of MAP. Many studies demonstrated that some MAP organisms survive commercial pasteurisation but 5 to 7 log reductions in the numbers of organisms does occur.

One study found that homogenisation of milk prior to pasteurisation did not contribute to the inactivation of MAP organisms.
High hydrostatic pressure milk treatment was found to be at least as effective as pasteurisation for MAP inactivation.

Microfiltration can only be used with skim milk but does remove 98% of organisms.

Herman et al demonstrated that clumping of MAP organisms in milk or faeces was not the reason some MAP organisms survive pasteurisation. They were able to demonstrate that heat activation of MAP growth does occur. They found that heating milk results in a shorter time to MAP growth being detected but does not increase the total number of positive samples if culturing continues for 12 months.

Conclusions

The question of whether MAP causes disease in humans was not answered at the Colloquium.

It was postulated that MAP acts as an antigen which can trigger Crohn’s disease and other autoimmune diseases such as Blau syndrome and Type 1 diabetes in genetically susceptible individuals. MAP was demonstrated to be present in granulomatous lesions sampled in Blau disease patients.

Of the papers examining an association between Crohn’s disease and the presence of MAP in humans some demonstrated an association while others did not.

Several papers demonstrated that MAP was readily found in water ways in England and Ireland and that some organisms survive chlorine treatment.

MAP IS900 was detected in 49% of Czeck powdered milk and MAP was cultured from 2% of the samples.

Several studies demonstrated that some MAP organisms survive commercial pasteurisation but pasteurisation results in a 5 to 7 log reduction in the number of MAP organisms present in milk.
An infectious cause for Crohn's disease has been sought since the disease was first described in 1932. Recent attention has focussed on Mycobacterial avium subspecies paratuberculosis (M. ptb) since its isolation from 3 of 11 patients with Crohn's disease in 1984. Numerous reports have appeared since then describing detection of the organism in tissue by PCR and culture. It has also been detected in blood and even breast milk. However, while some workers have confirmed these findings other have not found differences between Crohn's disease and controls. Epidemiological and other factors also provide contradictions if M. ptb is the cause of Crohn's disease and hence the role of the organism remains controversial.

Conventional anti-tuberculous antibiotic regimens are generally ineffective in patients with Crohn's disease. However, M. ptb appears to exist in a cell wall deficient form and this, combined with its intracellular location and slow growth, make it resistant to these antibiotics. Anecdotal open-label studies using antibiotics that are effective against MAP have reported favourable results. However, there have been no large controlled trials until now. The Australian trial of anti-paratuberculosis therapy (APT) randomised 213 patients to either clarithromycin, rifabutin and clofazimine or matching placebos in addition to 16 weeks of a reducing dose of oral prednisolone. Patients in remission after that time continued antibiotics or placebos for up to two years. They were then followed for a further one year off trial medications. Although a short term benefit was found for the antibiotic combination at 16 weeks this was not maintained at one or two years of treatment. One year after the trial agents were ceased, only 13.7% of those who were on active treatment and 9.0% of those on placebo were in remission, a non-significant difference. These findings argue against a role of active infection with MAP as a significant cause in the majority of patients with Crohn's disease.
Epidemiology, Economics
&
Grazing Management
Epidemiology of ovine Johne’s disease cross species transmission and survival of the organism in the environment

Richard Whittington, Barbara Moloney and Catherine Taragel

Faculty of Veterinary Science, The University of Sydney
425 Werombi Rd, Camden NSW 2570
Phone: 02 9351 1619; Fax: 02 9351 1618
E-mail: richardw@camden.usyd.edu.au

Background

In 1995 gaps in knowledge about the behaviour of ovine Johne’s disease (OJD) in populations of sheep precluded tight recommendations being made to limit spread of the disease. Therefore when the national control program commenced, research was commissioned by Meat & Livestock Australia (MLA) to reduce the number of unknown factors. This paper covers aspects of the epidemiological research program that are not presented elsewhere in this volume.

Some important questions in 1995 related to the:

- Differences between Johne’s disease in cattle and sheep
  - Are the diseases in sheep and cattle caused by different organisms?
  - Can OJD and bovine Johne’s disease (BJD) be managed separately in Australia?
- Possible reservoirs of OJD in cattle and goats
  - Are cattle/goats susceptible to OJD?
  - Are cattle/goats suitable reservoir hosts for OJD?
  - Can OJD be controlled in the presence of cattle/goats?
- Possible reservoirs of OJD in the environment
  - How long does the organism survive in the environment?
  - Do climatic factors explain the lack of OJD in western NSW and QLD?
  - Does soil type influence the occurrence of OJD?

Sheep and cattle

The success of control based on depopulation and decontamination of farms with multiple grazing species depended on the segregation of infection within individual species. In Australia, where sheep and cattle are the predominant grazing species and where M. avium subspecies paratuberculosis (M. ptb) was believed not to be spread commonly between these species, a management option was to graze cattle on pastures during the decontamination period after depopulation of OJD infected sheep.

The first projects commissioned by MLA were laboratory based, molecular, epidemiological studies to confirm that Johne’s disease (JD) in sheep and cattle in Australia were due to different organisms and had quite separate epidemiological pathways. A significant aspect about this work was that it was conducted before the sheep strain could be cultured routinely. In a comprehensive study, molecular fingerprinting using RFLP (Restriction Fragment Length Polymorphism) analysis, which required large amounts of DNA, was mostly undertaken on...
organisms extracted laboriously from the lamina propria of sheep intestines that had been screened for infection using histopathology (1). This research confirmed that sheep were infected with a dominant strain known as the S strain while cattle were infected with a variety of cattle (or C) strains (3, 10). In this regard the pattern of OJD and BJD in Australia appeared to resemble that in New Zealand (2), where S and C strains were first described, and was dissimilar to the situation in Europe where sheep, cattle and other ruminants were most often infected with various C strains (8). An explanation for this difference may be the relatively recent introduction of OJD (S strain) into Australia and the concentration of BJD in Australia in the dairy industry which has had very little contact with the sheep industry. These findings enabled greater confidence in the management of both OJD and BJD in Australia.

Rapid molecular tests based on DNA sequence polymorphisms in the IS\textsubscript{1311} gene were soon developed to facilitate routine differentiation of S and C strains, including retrospective testing of formalin-fixed paraffin-embedded tissues in laboratory archives (6, 9, 14). Further studies have been undertaken to identify the extent of genomic differences between S and C strains in an effort to better define the microbial factors underlying the observed epidemiological differences (see Ian Marsh, this volume).

During the 1980’s and early 1990’s there were several anomalous cases of JD in cattle in NSW in which tissue or faecal cultures were negative. These were investigated using the IS\textsubscript{1311} method on archival tissues and S strain infection was confirmed from three NSW properties. Consequently, MLA commissioned a further project to determine the prevalence of disease in cattle exposed to sheep infected with OJD and the likely risk factors (7). In this study, 1,808 cattle from 12 properties were tested by ELISA (for the presence of M. \textit{ptb} antibodies) and faecal culture. All properties had a known history of JD in sheep and were mostly selected from participants in a trial being conducted within the Australian National Ovine Johne’s Disease Control program. All properties had young cattle likely to be susceptible to JD. All animals gave negative results on serology; only one animal from a herd of 349 gave a single positive faecal culture result, with histopathology being negative, which suggested passive excretion of M. \textit{ptb} organisms. However, two properties undergoing testing for the cattle Market Assurance Program each had two serological reactor animals and all four animals were faecal culture positive (S strain). Three were subsequently diagnosed as histologically positive for JD. One of these properties did not have sheep present on it, but OJD was present on a neighbouring farm adjacent to the calving paddock. The other property was located at Berry on the NSW South Coast, which is outside the known OJD infected areas, but had raised heifers from weaning to springing on a known OJD infected property in the Central Tablelands. These observations confirmed that the risk of transmission was low except where young cattle were reared in contact with infected sheep. Overall the risk of transmission of OJD from sheep to cattle was assessed as low, but could not be precisely determined. The prevalence of OJD in cattle exposed as calves to infected sheep could not be accurately determined but one estimate was that up to 0.4% to 0.6% of cattle running on OJD infected properties may be infected with M. \textit{ptb} (S strain), but the upper confidence limit was as high as 30%.

**Sheep and goats**

JD was first reported in goats in Australia in 1977 and the infection was confined mainly to dairy herds (4). These infections were due to C strains. In 1995, OJD was suspected as the cause of JD in a cashmere buck but it was not until 1996 that infection of a fibre goat with the S-strain of M. \textit{ptb} was confirmed. The results of existing field and laboratory investigations were summarised and cross-sectional or prospective surveys were conducted on three extensively grazed farms where sufficient time (> 2 years) was likely to have elapsed for spread to have occurred. Samples were collected for ELISA and/or gel test and faecal culture from animals
greater than 2 years of age. Estimated losses due to OJD in sheep 3-to-8-years old were 1.5 to 6% per annum whereas in goats the owners did not see clinical signs of JD and estimated losses overall were < 1% per annum. The seroprevalence of JD was higher in sheep than goats (Table 1). Trace forward investigations suggested that \textit{M. ptb} infection was transmitted from introduced goats to homebred goats and homebred sheep. In all cases there was close contact of goats with sheep, both directly and indirectly, including supplementary ground feeding during periods of low pasture growth. Stocking rates on the properties would be considered high.

The infected goats were detected using serological tests for caprine JD, implying that the strain of \textit{M. ptb} causing the infection is irrelevant for diagnosis. As JD due to \textit{M. ptb} S strain in goats appears to be a milder infection than in sheep it is reasonable to expect diagnostic tests such as serology and faecal culture to be less sensitive in goats than in sheep. The reason is that these tests are most effective in animals that have proceeded through the early stages of the disease; if fewer animals have progressed through the disease, the tests are less able to detect infection in the herd.

Goats and sheep need to be considered separately when herds and flocks are sampled for market assurance testing because of differences in the prevalence of infection and the likely performance of diagnostic tests between the two species.

Possible reasons for the apparent relative resistance of goats to OJD include their browsing behaviour which may lead to ingestion of lower doses of the organism than in sheep, greater inherent natural resistance to infection compared to sheep or because the S strain of \textit{M. ptb} may not be sufficiently adapted to goats to easily or quickly cause clinical disease.

<table>
<thead>
<tr>
<th>Table 1: Comparison of OJD prevalence in goats and sheep on three farms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Farm</td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td>B</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>C</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>D</td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>
Survival of the organism in the environment

While research was being conducted on the feasibility of eradication of OJD by whole flock depopulation, resting of pasture and restocking with healthy sheep (see Pat Taylor, this volume), research was also conducted on the survival of the organism. Data on the resistance of the organism were reported in 1944 (5) for bovine faeces exposed in a bowl in London. The organism survived for about nine months. Other reports suggest a trend for prolonged survival except where urine is also present, or in silage with low pH or high ammonia levels (reviewed in (13)). These data come from the northern hemisphere where livestock are commonly housed indoors during winter on straw bedding and from experimental models without field validation.

In this MLA project, the survival of *M. ptb* was studied by culture of faecal material sampled at intervals for up to 117 weeks from soil and grass in pasture plots and boxes (13). Survival for up to 55 weeks was observed in a dry shaded environment, with much shorter survival in unshaded locations. Moisture and application of lime to soil did not affect survival. The organism survived for up to 24 weeks on grass that germinated through infected faecal material applied to the soil surface in shaded boxes and for up to nine weeks on grass in 70% shade. Survival of *M. ptb* in the environment is finite, consistent with its taxonomic description as an obligate parasite of animals. As a rough guide, about 90% of the organisms deposited on a paddock in sheep faeces die off each month.

In a related experiment, the survival of the organism was studied in dam water and sediment in large water troughs which were placed in either a semi-exposed location or in a shaded location, and compared to survival in faecal material and soil in the shaded location (11). Survival, in water and/or sediment in the shade, was observed for 48 weeks compared to 36 weeks in the semi-exposed location. Survival in sediment was 12 to 26 weeks longer than survival in the water column. Survival in soil and faecal material in the shaded terrestrial environment was only 12 weeks. The results suggest that water may be a significant reservoir of *M. ptb* infection. Further research on the biology of the organism in aquatic environments is warranted. Animal health authorities will need to provide appropriate advice to farmers to minimise exposure of livestock to potentially infected water sources. Survival of the organism in water destined for human consumption will need to be addressed if the organism is found to be involved in the aetiology of Crohn’s disease.

The aim of a further experiment was to determine whether *M. ptb* could be isolated from soil-pasture, faecal, water and sediment samples on farms before and after removal of OJD infected sheep (12). The organism was recovered from approximately 20% of such samples from six properties. The positive samples were from a range of locations; however, low lying areas tended to have larger numbers of organisms. When the same sites were sampled again about five months later, only one was culture positive, and none were culture positive > 12 months later. The organism was rarely recovered from water samples on infected farms. Of 96 water samples, 90 sediment samples and 93 soil samples from farms that had been destocked of infected sheep/goats for 9-24 months, one sediment sample from a farm in Victoria (destocked for 12 months) and two sediment samples from a farm in New South Wales (destocked for 10 and 19 months) were culture positive. Recontamination from cattle or water could not be excluded as a cause of the positive cultures from the second farm. Improved culture methods are needed for critical surveys and to study the movement and fate of the organism in the environment.
Conclusions
These studies enabled closure of previous gaps in knowledge regarding OJD and animal health authorities were able to develop recommendations for control of OJD with greater confidence. In particular, the important role that cattle can play in rotational grazing with sheep was of low risk of interference with OJD control whereas goats were established as a threat to OJD control. The survival of the organism in the environment was lengthy, but finite. Useful degrees of pasture decontamination could be achieved in only a few months, but complete sterilisation would require more than a year. This knowledge, when combined with data from actual OJD transmission studies (see McGregor, this volume), has led to the development of rational recommendations for grazing management to reduce the impact of OJD.

References
9. Whittington, R., I. Marsh, E. Choy, and D. Cousins. 1998. Polymorphisms in IS1311, an insertion sequence common to Mycobacterium avium and M. avium subsp. paratuberculosis, can be used to distinguish between and within these species. Molecular and Cellular Probes 12:349-358.


Development of grazing management strategies for the control of ovine Johne’s disease

H McGregor\textsuperscript{a}, K Abbott and O Dhungyel, R Whittington

\textsuperscript{a}Sydney University, Faculty of Veterinary Science, 425 Werombi Rd Camden, NSW
Phone: 02 9351 1792; Fax: 02 9351 1618
Email: helenm@camden.usyd.edu.au

Introduction

On high prevalence, OJD infected properties in NSW annual mortality rates up to 21% have been estimated, with over 70% of these mortalities due to OJD.\cite{1,2} The annual costs from OJD related mortalities have been estimated across 12 infected properties in NSW and are considerable, with a reduction in gross margin on average of 6.4\%\cite{1} In heavily infected flocks it is common for animals to start to die from OJD as early as 18 months of age, well before the peak of their productivity. Practical options for control of OJD are necessary to reduce this economic loss. Based on recent studies on farms where OJD is well established, the use of vaccine has proven to be a financially viable control option as it reduces mortalities, shedding of infectious organisms and lost income due to OJD.\cite{3} However, there is evidence that the use of vaccine alone may not be the optimal approach for control. Where OJD prevalence is low, the cost of vaccination makes its widespread use less economically attractive. Control of OJD through grazing management will provide additional and complementary options to vaccine use to reduce the levels of exposure experienced by young sheep, and thus the severity of infection.

Two extensive field-based, epidemiological studies were conducted between 1999 and 2004 to facilitate the development and validation of grazing management protocols for the control of OJD. In the first study (MLA OJD.002a) the role of the ewe as a source of infection for lambs and the relative benefits of the provision of low contamination pastures in the pre-weaning and post-weaning periods were evaluated. The second study, (MLA OJD.028) which commenced in 2002, was conducted to determine whether age of sheep at exposure and level of pasture contamination affects the development of OJD.

Methods

Experimental site

The experimental site was at the University of Sydney farm, Arthursleigh, in the southern tablelands of NSW. The site comprised secure paddocks and a laneway system which enabled two replicates for each treatment. Erosion gullies were fenced to exclude livestock and these and the laneways were incorporated in ways which assisted in the separation of paddocks with different levels of OJD contamination. The pasture on the experimental site was unimproved, consisting of native grasses and volunteer-introduced species and weeds. There were few shade trees on the site and, generally, all or most parts of all paddocks did not have shade. A reticulated water system was installed to service experimental paddocks. Water was originally supplied from a tank serviced by a fenced dam within the site and then later due to increasing drought conditions from the Wollondilly river.

Pasture contamination history
Experimental pastures had been grazed by cattle only, for 18 months prior to the start of experiment 002a. The cattle were tested on 2 occasions 2 years apart, and were negative in the Johne’s disease absorbed ELISA. The OJD seroprevalence of the sheep grazing the pasture prior to this 18-month period was 0.25%. Prior to trial 002a which commenced in September 1999, pastures were considered to have negligible levels of \textit{M. ptb} infection. At the start of each trial paddocks to be infected were grazed by OJD infected sheep. These sheep were identified from high prevalence infected properties and tested for active shedding, initially by ZN staining of faecal smears or AGID serology and retrospectively by individual faecal culture. For trial 002a, infected ewes grazed designated paddocks prior to and during the lambing and pre-weaning period. For trial 028 infected donor sheep were co-grazed with trial sheep for the initial 14.5 weeks of the trial. The number and the faecal culture status of OJD infected animals grazing a paddock for a designated time determined the pasture contamination rate. The contamination history of each paddock was calculated from the numbers of ‘shedders’ grazing the area for a designated period of time. Paddocks required to be largely free from infection were not grazed at all or were grazed only by the clean trial sheep.

\textbf{Experimental Design}

\textit{MLA OJD.002a (Table 1)}

Pregnant Merino ewes were sourced for this experiment from two flocks with similar bloodlines in NSW, one testing negative over serial tests for OJD (flock U) and the other a known high prevalence flock (flock I). The principle experimental sheep were the lambs born onto the experimental site to infected (flock I) and uninfected (flock U) ewes. Lambs were raised to weaning age on pastures of 3 levels of contamination, low, medium and high, determined by dam infection status and grazing history. At weaning lambs were stratified on the basis of weight, sex and pre-weaning exposure history and allocated to post weaning paddocks with high or low levels of contamination. Treatment groups were based on history of dam infection status, pre-weaning and post-weaning exposure. The 7 treatment groups were replicated, each replicate containing 36 animals. Once allocated to a treatment group, animals remained within that group and replicate for the duration of the trial. Blood and faecal samples were collected 3 monthly and then 6 weekly from lamb marking for the duration of the trial to monitor the onset of shedding. Lambs were euthanased at 3 years of age and samples collected for histopathology and tissue culture to determine prevalence and severity of infection.

\textit{MLA OJD.028}

A total of 840 female Merino sheep of three age groups, lambs, hoggets and adults were obtained from an uninfected property. Sheep were stratified on bodyweight and age and randomly allocated to a treatment group so that there were 35 of each age. Each mixed age group mob of 105 sheep was grazed in a 20 hectare paddock within the experimental site (treatment group). The infected donor sheep were co-grazed with each mob at predetermined stocking rates to provide four levels of contamination, control (very low), low, medium and high (0, 0.1, 0.35 and 0.7 infected sheep per hectare respectively). Each of the contamination treatments was replicated once. Pooled faecal culture and agar gel immunodiffusion assays were conducted on all sheep at regular intervals during the trial, while individual faecal culture, ELISA and gamma-interferon assays were applied to subgroups at some time points. The animals remained in the experiment for 2.5 years and were then euthanased and sampled for histopathology and tissue culture to establish the prevalence of infection with \textit{M. ptb}.

\textbf{Results}

\textit{MLA OJD.002a (Table 1)}
Bacteriology

Shedding of *M. ptb* organisms was confirmed in all treatment groups apart from ULL and ULH at 12 months of age with PFC. At 18 months of age faecal samples were collected from 481 remaining animals for individual faecal culture. Twenty-seven samples (5.6%) were positive for *M. ptb*. Prevalence varied between groups and was highest in groups with continuing exposure throughout pre- and post-weaning periods (IHH and UHH ≥ 9.9%) and the lowest in groups exposed to the lowest levels of infection on pasture (ULH and ULL ≤ 2.8%)

Death rate/survival to 3 years of age

Between 18 months and 3 years of age there were 35 deaths. Nineteen (70%) of the 27 sheep faecal culture positive at 18 months of age died before 3 years of age. Of those which were negative (454), 16 died. This difference in mortality rate was highly significant (P < 0.001). Deaths from OJD commenced at 18 months of age and the rate of mortality peaked at approximately 30 months. Most sheep which developed patent infections died within the subsequent 18 months.

There was a direct relationship between the level of exposure and the incidence of OJD infection and death rate. Continuing exposure in both the pre-weaning and post-weaning phase resulted in high levels of infection. Sheep beyond weaning age remained susceptible to infection.

Table 1. Results of death rate, histopathology and tissue culture for OJD infection at 3 years and faecal culture status at 18 months of age.

<table>
<thead>
<tr>
<th>Exposure History</th>
<th>Infected dam flock</th>
<th>Pre-weaning exposure</th>
<th>Post-weaning exposure</th>
<th>Average exposure (E/D)*</th>
<th>OJD infection rate (%)</th>
<th>OJD death rate (%)</th>
<th>Faecal culture positive rate at 18 months of age (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IHH</td>
<td>+</td>
<td>High</td>
<td>High</td>
<td>494</td>
<td>36.6</td>
<td>8.3</td>
<td>10.0</td>
</tr>
<tr>
<td>IHL</td>
<td>+</td>
<td>High</td>
<td>Low</td>
<td>476</td>
<td>38.8</td>
<td>6.0</td>
<td>7.7</td>
</tr>
<tr>
<td>ILL</td>
<td>+</td>
<td>Low</td>
<td>High</td>
<td>311</td>
<td>20.3</td>
<td>2.9</td>
<td>2.8</td>
</tr>
<tr>
<td>UHH</td>
<td>+</td>
<td>Low</td>
<td>Low</td>
<td>309</td>
<td>28.6</td>
<td>8.6</td>
<td>9.9</td>
</tr>
<tr>
<td>UHL</td>
<td>+</td>
<td>Low</td>
<td>High</td>
<td>50</td>
<td>17.4</td>
<td>1.4</td>
<td>4.5</td>
</tr>
<tr>
<td>ULH</td>
<td>+</td>
<td>Low</td>
<td>High</td>
<td>301</td>
<td>17.6</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>ULL</td>
<td>+</td>
<td>Low</td>
<td>Low</td>
<td>0</td>
<td>18.3</td>
<td>2.8</td>
<td>2.8</td>
</tr>
</tbody>
</table>

* E/D represents the measurement of availability of *M. ptb* infection on pasture, calculated from the number of faecal culture positive ewes per hectare per day allowing for a 3-logarithm reduction in contamination per month due to the natural decay of organisms.
Bacteriology

The pooled faecal culture results suggested a higher prevalence of infection and faecal shedding in lambs than hoggets and ewes, and in high contamination groups compared to other groups, with the greatest numbers of positive pools in the second half of the trial (periods 6-10). (Table 2) These results were confirmed by the results of individual faecal culture.

Table 2. Aggregated pooled faecal culture results by age and treatment group. Data are the total number of positive pools in each age and group over the trial.

<table>
<thead>
<tr>
<th>Group</th>
<th>Average exposure*</th>
<th>Lambs</th>
<th>Hoggets</th>
<th>Ewes</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>8</td>
<td>6</td>
<td>1</td>
<td>15</td>
</tr>
<tr>
<td>Low</td>
<td>102</td>
<td>6</td>
<td>1</td>
<td>5</td>
<td>12</td>
</tr>
<tr>
<td>Medium</td>
<td>301</td>
<td>3</td>
<td>8</td>
<td>0</td>
<td>11</td>
</tr>
<tr>
<td>High</td>
<td>709</td>
<td>28</td>
<td>17</td>
<td>4</td>
<td>49</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>45</td>
<td>32</td>
<td>10</td>
<td>87</td>
</tr>
</tbody>
</table>

* Average exposure is expressed as: No. of patent donor sheep days = sum across donors of the number of days each donor with patent infection was present within the 14.5 week co-grazing period.

A total of 26 lambs were IFC positive on at least one occasion compared to 19 hoggets and 4 ewes. Corresponding data for 2 or more occasions positive were 14, 8 and 2. More animals shedding \textit{M. ptb} were in the high contamination group than in other groups. Faecal shedding occurred for the first time in lambs in September 2002, 6 months after first exposure to infected pasture. The median age of lambs at this time was 11 months. Faecal shedding was more frequent in lambs in the high contamination treatment at this time and later. This is comparable to the results of the pooled faecal culture detecting shedding in lambs at 12 months of age in trial 002a. Faecal shedding in hoggets commenced in September 2003, 18 months after first exposure, while in ewes it occurred in March 2003, 11 months after first exposure.
Mortalities due to OJD

There were more deaths due to OJD in lambs compared to hoggets and ewes. (Table 3) All of the deaths due to OJD occurred in the second half of the trial (periods 6-10) and most occurred in the high contamination group.

Table 3. Total mortality during the trial for animals in each age class and treatment group. Death due to OJD was determined by post mortem examination and histopathology (lesions >3a), or ascribed to animals that died after at least two positive ante-mortem tests (AGID and/or individual faecal culture).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Lambs</th>
<th>Hoggets</th>
<th>Ewes</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Low</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Medium</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>High</td>
<td>9</td>
<td>5</td>
<td>0</td>
<td>14</td>
</tr>
<tr>
<td>Total</td>
<td>10</td>
<td>5</td>
<td>1</td>
<td>16</td>
</tr>
<tr>
<td>Total period 1-5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total period 6-10</td>
<td>10</td>
<td>5</td>
<td>1</td>
<td>16</td>
</tr>
</tbody>
</table>

Histopathology

Histopathological lesions consistent with OJD were more common in lambs (21) compared to hoggets (15) and ewes (7), with lesions present in animals in each contamination group. (Table 4) Severe lesions were also more common in lambs (17) than hoggets (11) or ewes (1). In lambs, most of the severe lesions occurred in animals in the high contamination group. Ignoring age, histopathological lesions occurred with similar frequency in sheep in each contamination group, although severe lesions were more frequent in the high contamination group.

Table 4. Severe histopathological lesions in each age and treatment group. Severe lesions of OJD were grades 3a and 3b according to Perez.
Serology
There was a similar number of AGID reactors among lambs and hoggets, and a lower number among ewes. Ignoring age, the highest number of AGID reactors occurred among sheep in the high contamination group, but the trend did not apply to lambs or ewes. Most of the positive results occurred in the second half of the trial in periods 6-10.

Conclusions
The results from both experiments confirm that post weaning lambs remain highly susceptible to infection. Lambs are highly susceptible to infection compared to adult sheep, evidenced by greater prevalence of severe histological lesions and mortality in this age group, particularly when exposed to high levels of infection. Similarly faecal shedding of the organism was more common when animals were first exposed as lambs. The results from MLA OJD.028 suggest that transmission of *M. ptb* occurred very quickly after first exposure, with patent infection established in the lambs within 6 months of first exposure. This work confirms that there is a strong relationship between the level of natural exposure to *M. ptb*, age and the subsequent incidence of OJD infection. It should therefore be possible to reduce the incidence or severity of OJD by reducing the levels of exposure in young sheep in the pre-weaning and immediate post-weaning periods. Recommendations for flock testing to detect infection are dependant on the age of the animals. However, the use of faecal culture to detect infection may also be a valid tool to predict future disease and mortalities, particularly in young animals. These results only allow an estimation of how long lambs may remain susceptible but a reasonable hypothesis would be that susceptibility will start to decline from around 12 months of age. The use of infected, actively shedding, co-grazer sheep to successfully create an infected environment in both experiments demonstrates that even relatively low numbers of clinically affected sheep per hectare (0.1-0.7 in OJD.028) will lead to transmission of infection to susceptible sheep. This finding illustrates the importance of monitoring for clinical sheep frequently and culling them from all mobs to reduce the levels of *M. ptb* on pasture. The occurrence of OJD in control groups in both experiments demonstrated that lateral spread of the disease is a serious issue and it is not necessary for infected sheep to graze the same immediate area for transmission to occur if infected sheep are in neighboring paddocks. It is also important to note that all age groups of sheep may become infected even following exposure to relatively low levels of infection on pasture. This finding suggests that highly susceptible individuals probably exist within all age groups, therefore transmission of disease through shedding is a possibility even in sheep exposed to relatively low levels of infection as adults.

Recommendations
It is possible to recommend several approaches to control OJD based on the data from this study:

- Minimise exposure of lambs and hoggets to heavily contaminated pasture
- As adult sheep are more resistant to infection and disease impacts of OJD, if clean pastures are scarce, they, rather than younger animals, should be grazed on contaminated pasture
- Cull adult sheep with clinical signs of OJD, or conduct PFC to obtain evidence of patent OJD infection and manage or cull these mobs to reduce the level of pasture contamination and future mortalities.
Manage pastures by spelling, grazing with adult cattle or grazing with approved vaccinates in order to reduce the contamination level for lambs, weaners and young sheep.

In all infected flocks including those vaccinated, regularly observe all classes of sheep and identify and remove individuals that develop signs of weight loss to reduce the enormous contribution of ‘multibacillary shedders’ to the level of contamination.

Maintain boundary fencing to reduce the likelihood of lateral spread from an infected neighbour. As a single fence line does not prevent lateral spread, consider double fencing with wide laneways or segregation of critical pastures using a tree belt, gully or other natural feature. Maintain high value lambs in paddocks well away from paddocks known to contain infected sheep.

Endoparasite control on prepared, low infectivity pastures needs to be considered to avoid a conflicting effect from OJD control and management practices. Extra vigilance with endoparasite control regimes may be necessary where weaner animals are at risk of exposure to potentially high levels of endoparasite infection on prepared pastures. The use of young sheep for the preparation of low infectivity pastures for *M. ptb* may lead to increased risk from high levels of contamination with endoparasites. The use of adult sheep (vaccinated as lambs) to reduce *M. ptb* levels on pasture will produce an environment with a lower risk for endoparasite infection but may be a higher risk option for *M. ptb* control. Spelling, crops or grazing adult cattle may be the safest approach for preparation of low contamination pastures for OJD control.

Acknowledgements
Trials OJD.002a and OJD.028 were both funded through Meat and Livestock Australia.

The cooperation, support and expertise of staff at Sydney University is gratefully acknowledged, especially Anna Waldron and Craig Kristo. The support of “Arthursleigh” farm staff through the field trial phases of these projects was critical for their successful completion.

References
Within flock spread of OJD - a case study from a sheep flock located in southeastern New South Wales

L Rasta, RJ Whittington
aGundagai Rural Lands Protection Board
PO Box 21, Gundagai NSW 2722
Phone: +61 2 6944 1588; Fax: +61 2 6944 1867
Email: luzia.rast@rlpb.org.au

Abstract

On farm investigation and monitoring for ovine Johne’s disease (OJD) was performed in a recently infected flock of around 3,000 sheep, between 1997 and 2002 to better understand OJD prevalence, distribution and spread on this farm and to plan practical disease control and intervention strategies.

Tests used were serology (AGID), pooled faecal culture (PFC) and histopathology, including partial and then whole flock testing using PFC annually three times. Faecal shedding of *Mycobacterium paratuberculosis* (*M. ptb*) commenced in home bred sheep around six to seven years after a single introduction of a mob of 410 infected sheep in 1993. For at least seven years there was clustering of infection and shedding within one or two age groups only. Sheep in these age groups appeared to have been exposed to mycobacterial contamination at an early age (<12 months) and commenced shedding at five years of age or older. Groups that were exposed to contamination as adults did not shed detectable levels of *M. ptb* during the study period.

These results provided indirect evidence of the finite duration of survival of *M. ptb* on pasture and the influence of age on susceptibility of sheep to develop patent *M. ptb* infection.

A further feature of the epidemiology in this flock was the slow transmission and spread of *M. ptb*, related to the long incubation period (exposure to shedding interval) of five years and the absence of clinical signs throughout the study period.

The findings suggest that partial flock culling, selective grazing management and vaccination could lead to a reduction in mycobacteria contamination on-farm, possibly to a level at which patent *M. ptb* infection no longer occurs. Better understanding of disease spread within flocks over time through flock profiling using PFC will help in devising surveillance strategies (including testing protocols for market assurance testing) that account for clustering of infection as well as very slow transmission and spread of infection through a flock.

Introduction

OJD is a chronic wasting disease caused by *Mycobacterium paratuberculosis* (*M. ptb*). The major mode of transmission of bacteria is by the faecal-oral route. Once ingested, it usually takes at least 12 months before a patent infection establishes and detectable levels of bacteria are shed via faeces (Chaitaweesub *et al*, 1999). Appearance of clinical signs of chronic wasting usually takes many years (Whittington *et al*, 2001). The organism remains viable in the environment for up to about 12 months but infectious doses may be available for a lesser period (Whittington, 2001). The principle means of spread of OJD between flocks is through movement of infected sheep. Less is known about transmission and spread of *M. ptb* in sheep than cattle, but calves are reported to be more susceptible to infection than mature cattle and the same might apply to sheep (Whittington *et al*, 2001).
There is little objective information regarding the spread of *Mptb* within an infected flock. It is assumed that most sheep are exposed via pasture, and that all animals in a flock have an equal chance of becoming exposed. Surveillance programs to detect infection entail sampling based on this assumption and ignore potential clustering of infection within mobs or incubation periods of more than three years. Negative test results following random sampling across a flock will have lower than stated confidence if the infection is confined to some mobs only, rather than evenly distributed throughout a flock. This will reduce the accuracy of testing to detect OJD infection in flocks but also testing to show absence of disease for market assurance purposes. In some areas, recommendations for control of OJD are based on whole flock destocking or quarantine and other trading restrictions to reduce further spread. These measures make no allowance for the possibility that infection is confined to subgroups or mobs within a flock. The availability of pooled faecal culture (PFC) (Whittington et al., 2000) has enabled relatively inexpensive testing of whole flocks, to better determine the source and distribution of infection.

The aim of this study was to apply whole flock testing (flock profiling) over time to determine the prevalence, distribution and spread of infection in a recently infected flock, with a view to planning practical on-farm intervention strategies for disease control.

**Material and methods**

**Management and flock structure**

The farm consisted of 1,739 hectares of improved pasture and was located in southeastern NSW, a 600 mm average annual rainfall area. The main enterprise was sheep breeding, but around 700 beef cattle were also grazed and 250-300 hectares were cropped annually. The sheep flock consisted of a commercial Bond breed flock of approximately 3,000 breeding ewes and a Bond stud of about 300 ewes. Lambing was in April/May. Wether lambs were sold to slaughter as fat lambs from December onwards each year. Culls were sold to slaughter annually in September/October and usually included the entire oldest age group (six year olds). Prior to their first joining, the ewes were classed and around 50 were selected each year to enter the stud. Sheep were segregated based on age and run in separate mobs, up to six years old. The commercial and stud ewes were run separately and mobs were grazed in a rotational pattern, fitting in with pasture availability and the cropping program. Other than the introduction of some Merino sheep (described below) and a once off purchase of five Dorset rams in 1997 no sheep were routinely introduced.

**History of OJD acquisition**

In 1993 the owner purchased 410 mixed age fine wool Merino ewes from farm X, located in what is now recognised as the high prevalence OJD area in NSW. On the study farm the Merino sheep had no direct contact with the Bond flocks. OJD was confirmed on farm X in late 1996.

In early 1996 the owner of the study farm sold 287 of the Merino ewes to another farm (farm F) and the remainder to slaughter. Any remaining progeny from these Merino ewes were sold to slaughter in December 1996.

In October 1996, 237 merino ewes on farm F were tested with AGID and there were nine reactors. Three reactors were examined at post mortem and histology on intestinal tissue showed lesions consistent with multibacillary Johne’s disease in all three sheep. The acid fast organisms in the formalin-fixed paraffin-embedded tissues of each sheep were identified as S strain *M. ptb*.
Eventually, in March 2000, OJD was confirmed on the study farm in homebred 5-year old Bond ewes (see results). Whole flock testing of adult sheep by PFC commenced later that year and was repeated annually three times in total. Methods for testing for OJD and further details are described in Rast and Whittington, 2005.

Results
The patterns of testing and infection on the study farm are provided in Figure 1 and Table 1.

Testing prior to 2000
Testing commenced on the study farm in 1997, as potentially infected sheep had been introduced from farm X in 1993.

- In 1997, 50 homebred 1992-born Bond sheep, which may have had contact with contaminated land, were tested using the AGID test, with negative results.
- In June 1998, 460 homebred Bond sheep, born in 1993 and 1994, were tested using the AGID and PFC, with negative results.
- In March 2000, OJD was confirmed in 1995-born homebred Bond ewes by histopathology following serology (AGID) tests of 450 sheep (Table 1, Figure 1).

Testing was required on three occasions over a four-year period to confirm infection in homebred sheep. Formalin-fixed paraffin-embedded tissues from one sheep with multibacillary lesions were examined by PCR and were confirmed to contain S strain of *M. ptb*.

Testing in 2000
Sheep born from 1994 to 2000 were present and all sheep older than two years were tested with PFC in June 2000. Shedding of *M. ptb* was confirmed in the 1995-born commercial ewes (prevalence 2.5%). This was the age group known to be infected based on AGID tests and subsequent post mortem examination and histopathology testing, performed earlier in the same year. All results from other age groups were negative (Figure 1 and Table 1).

Testing in 2001
Sheep born from 1996 to 2001 were present and all sheep older than 12 months were sampled for PFC in September 2001. Shedding of *M. ptb* was detected in the 1996-born commercial Bond ewes (prevalence 0.8%) and 1999-born stud ewes (prevalence 1.4%) (Figure 1 and Table 1).

Testing in 2002
Sheep born from 1996 to 2001 were present and all sheep older than 12 months were tested using PFC in September-October 2002. Shedding was confirmed again in the 1996-born commercial ewes and prevalence had increased from 0.8% to 2.5%. The 1997-born commercial ewes were also positive (prevalence 0.7%). All other results were negative, including the 1999-born stud ewes that had tested positive the previous year (Table 1 and Figure 1).

Disease management on the study farm
After notification of infection on Farm X (source of putative infected Merino sheep) but prior to OJD confirmation on the study farm

The owner identified the land used to graze the putative infected introduced Merino sheep and their progeny and ceased sheep grazing on this land in 1996. He applied lime
at a rate of 2.5 tonnes per hectare and used the land for cattle grazing or cropping instead.

After OJD confirmation on the study farm in 2000

The first whole flock test by PFC in 2000 established that only the 1995-born sheep were shedding detectable levels of *M. ptb* at the time of sampling. Faecal samples were collected in June, however the serology and histopathology results of multibacillary OJD from a limited number of sheep in that age group in March 2000 made it likely that they were shedding by then. Therefore lambs born to these ewes in April/May 2000 were at high risk of exposure and infection. A recommendation was made in 2000 to cull the entire 1995-born age group, including the lambs born to them. The owner sold all remaining 1995 born ewes (207 ewes) to slaughter in October 2000, and the lambs as they reached market weight between October and December 2000.

At that stage it was considered that the 1999-born sheep were potentially at high risk of infection through contamination from *M. ptb* shed by the infected 1995-born sheep in 2000 or earlier. The 1996 and 1997 born sheep were also considered at risk by exposure to contamination from the introduced Merino sheep (Figure 1). However culling of other age groups considered at risk of infection was not an economically viable option.

It was recommended to use the land on which the confirmed infected and shedding 1995-born age group had run, for purposes other than sheep grazing. The owner was able to identify and use the paddock these sheep had run in from the beginning of 1999 to late 2000 for cropping and cattle grazing in the following years.

During 2001

In 2001, use of Gudair® vaccine was approved on the study farm and a vaccination program began. All ewe lambs born in 2001 and the 1999-born commercial and stud ewes were vaccinated at the time of faecal sampling. Other age groups were not vaccinated due to the doubtful efficacy of vaccine administered post exposure to adults and the cost.

During 2002

As the test results from the 2001 sampling showed an apparent increase in shedding, all sheep (including adults) considered at risk but not already shedding were vaccinated. These were the 1998-, 2000- and 2002-born ewes and all rams.

Discussion

Observations and test results support the following scenario:

1) Infection was introduced onto the study farm with Merino sheep purchased in 1993 and this source of contamination was removed in 1996

2) Shedding in infected merino sheep occurred prior to their detection, and infective concentrations of *M. ptb* originating from the Merino sheep were present in the environment on the study farm from 1995 to 1997. This is based on a 12-month survival period on pasture after removal of the infected sheep (Whittington *et al*, 2004)

3) Only sheep less than 12 months old when exposed to *M. ptb* developed patent infections, that is progressed to shed *M. ptb* during the study period

4) Shedding commenced when sheep were five years or older, a significantly longer time period than the shortest reported 12 months incubation period in sheep (Chaitaweesub *et al*, 1999).
The source of infection on the study farm was most likely environmental contamination with *M. ptb* from some of the 410 Merino sheep and their progeny introduced from farm X in 1993 and sold in 1996. No other sources of infection are known or likely. Although the Merino sheep were never tested while present on the study farm, these introduced sheep were likely to be shedding *M. ptb* because 8 months after their sale to farm F the prevalence of sero-positive sheep was 3.8%. Estimation of the true prevalence of infection from these results is problematical because of the reported extreme variation in sensitivity of the AGID (Sergeant *et al.*, 2003; Sacks *et al* 1989). Shedding as early as 1995/1996 by these sheep can be assumed because the seropositive sheep examined on farm F had multibacillary intestinal lesions, which are highly correlated with shedding of *M. ptb* in faeces (Whittington *et al.*, 1999). Negative test results in the home-bred Bond sheep born in 1994 or earlier (therefore four to six year old when tested) strengthens the assumption that the Merino sheep introduced from farm X did not shed sufficient numbers of *M. ptb* prior to 1994 to contaminate the environment and transmit infection to lambs present on the study farm at that time.

Whole flock testing using PFC of all adult sheep in 2000 confirmed that shedding was limited to the 1995-born age group. It is possible that contamination originating from the Merino sheep was very localised and only the 1995-born commercial Bond ewes were exposed (i.e. by chance). Alternatively - and more likely considering subsequent test results - age susceptibility to infection meant that only lambs exposed to contamination developed patent infection. If this is the case and if the Merino sheep produced sufficient contamination with *M. ptb* from 1995 and lasting until 12 months after their departure from the study farm in 1996, then sheep born in 1995, 1996 and 1997 were at risk of infection, but only the 1995-born sheep were old enough to shed at the time of sampling in 2000.

Results from testing in 2001 and 2002 support this hypothesis, and showed that in 2001 shedding was occurring in the 1996-born sheep and in 2002 in 1997-born sheep. Contamination left by the Merino sheep was probably quite low, leading to lower ingested doses of *M. ptb*, which would explain the late onset of shedding (5 year old) in the 1995-, 1996- and 1997-born age groups (Whittington *et al.*, 2001). Also these sheep were in good body condition and perhaps other environmental or genetic effects contributed to late onset of shedding. It is also possible that the 1996- and 1997-born sheep became infected as adults from contamination by homebred Bond ewes that were confirmed to shed at the 2000 and 2001 testing (Figure1). However we consider this unlikely due to insufficient time for development of lesions and shedding and the low prevalence of shedding (Table 1).

At the 2001 testing, shedding was confirmed in the 1999-born stud ewes but not in the commercial ewes of the same age (Table 1). The source of infection of this age group is uncertain. Infection could have been transmitted by chance exposure to high contamination (produced by homebred sheep shedding in 2000, 2001 or earlier). This is unlikely because the prevalence in homebred sheep was low (see Table 1). Intrauterine infection may have been the source as their dams could have been the 1995- or 1996-born infected ewes. However intrauterine transmission is improbable unless sheep are clinically infected, and there were no clinically infected sheep observed on the study farm (Lambeth *et al.*, 2004). The same mob, when retested in 2002 was apparently no longer shedding bacteria. Intermittent shedding may reflect either stage of disease (Whittington *et al.*, 2001), passive shedding of ingested organisms acquired from contaminated pasture or perhaps the effect of vaccination (Eppleston *et al.*, 2003). Alternatively, the sheep being responsible for shedding could have died during the interval between the two tests.
Conclusion

The history and detailed and extensive testing available on this farm, allowed identification of the time period over which mycobacterial contamination occurred by the introduced infected Merino sheep and highlights the slow spread of infection within the home bred flock. OJD infection was clustered, remaining limited to one or two age groups for at least seven years after the infection was introduced.

Opportunities for disease control and risks to effective surveillance are apparent retrospectively. On this farm, it may have been possible to stop transmission of OJD through timely implementation of disease management such as selective culling, strategic grazing and vaccination. If these disease control strategies had been implemented earlier than was possible on this farm, disease transmission may have been stopped.

In retrospect, action that could have been taken in 1996/97 that may have led to elimination of the infection from the study farm would include:

- Implementation of an infected flock profile immediately upon identification of the infected introduced sheep
- Culling of these sheep and their progeny
- Culling of home bred sheep born during the preceding and following 12 months, corresponding to the period of contamination, and prior to reaching 12 months of age
- Implementation of a vaccination program of all remaining sheep and annually of lambs at marking time
- Whole flock testing using PFC (infected flock profile) about 5 years after commencement of the program to monitor flock infection status

Vaccination was used on the study farm and the owner plans to vaccinate ewe lambs annually at marking or weaning. Based on recent research results with Gudair® vaccine under Australian conditions, vaccination on this farm is likely reduce the levels of \textit{M. ptb} contamination as well as delaying the onset of shedding (Eppleston \textit{et al}, 2003). It may be that in very low challenge situations such as this, vaccination eventually prevents shedding of detectable levels of \textit{M. ptb}. Further testing would be needed to confirm this.

This study also demonstrates how difficult effective OJD surveillance may be unless exact history is known and the correct age groups or mobs are tested or unless testing occurs repeatedly over a long period of time. Clustering of infection reduces the confidence in a negative test outcome if sampling is random. This has significant implications for market assurance program testing (Whittington \textit{et al}, 2001) and surveillance testing with a view to assess the OJD risk of a flock or mob of sheep intended for sale.

Acknowledgments

This study was supported by Meat & Livestock Australia. Gordon and Jill Forsyth, who lost their stud business through OJD quarantine and despite their emotional and financial hardship, cooperated with investigations over many years and always made us welcome. Peter Windsor and Rob Walker are thanked for useful discussions, while Evan Sergeant provided a spreadsheet for calculation of prevalence from pooled samples. Georgina Marshall, Ben McDonald, Col Elphick, and Tim Jones assisted in the field, while the efforts of Deb Walker, Shayne Fell and Anna Waldron with pooled faecal culture and Leslie Reddacliff and Vanessa Saunders with strain typing from paraffin blocks was greatly appreciated.
References


**Figure 1.** Representation of *M. ptb* spread on the study farm. Sheep mobs are indicated by horizontal bars where the left edge of each bar indicates when sheep were born and the right edge when sheep were tested. Dark bars indicate mobs with positive results in tests for OJD, while white bars indicate mobs that had negative test results. The dark block represents the likely interval of environmental *M. ptb* contamination from introduced merino sheep, while light dotted block represents the likely period of environmental *M. ptb* contamination from homebred Bond sheep.

Table 1. The prevalence of OJD infection based on AGID and PFC test results, for groups of samples where either test was positive

<table>
<thead>
<tr>
<th>Test date</th>
<th>Year of birth</th>
<th>Sample size</th>
<th>Breed</th>
<th>No. sheep/pool</th>
<th>Test</th>
<th>No. positive</th>
<th>Prevalence of sero-positive or faecal culture positive sheep % (95% C.L.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oct 1997</td>
<td>1990/91</td>
<td>239</td>
<td>Merino</td>
<td>na*</td>
<td>AGID</td>
<td>9^b</td>
<td>3.8 (1.7 – 7.0)</td>
</tr>
<tr>
<td>Mar 2000</td>
<td>1995</td>
<td>95</td>
<td>Bond</td>
<td>na</td>
<td>AGID</td>
<td>4^b</td>
<td>4.2 (1.7 - 11.9)</td>
</tr>
<tr>
<td>Jun 2000</td>
<td>1995</td>
<td>202 (10 pools)</td>
<td>Bond</td>
<td>20-22</td>
<td>PFC</td>
<td>4</td>
<td>2.5 (0.1 - 5.0)</td>
</tr>
<tr>
<td>Sep 2001</td>
<td>1999</td>
<td>100 (2 pools)</td>
<td>Bond</td>
<td>50</td>
<td>PFC</td>
<td>1</td>
<td>1.4 ( 0 - 4.1)</td>
</tr>
<tr>
<td>Oct 2001</td>
<td>1996</td>
<td>306 (6 pools)</td>
<td>Bond</td>
<td>50-56</td>
<td>PFC</td>
<td>2</td>
<td>0.8 ( 0 - 1.9)</td>
</tr>
<tr>
<td>Oct 2002</td>
<td>1996</td>
<td>350 (7 pools)</td>
<td>Bond</td>
<td>50</td>
<td>PFC</td>
<td>5</td>
<td>2.5 (0.2 – 4.8)</td>
</tr>
<tr>
<td>Oct 2002</td>
<td>1997</td>
<td>325 (7 pools)</td>
<td>Bond</td>
<td>26(1 pool), 49-50</td>
<td>PFC</td>
<td>1</td>
<td>0.3 ( 0 - 0.9)</td>
</tr>
</tbody>
</table>

AGID  Agar gel immunodiffusion  
PFC  Pooled faecal culture  
^a  not applicable; all sheep were tested individually  
^b number of positive individuals

Risk factors for OJD prevalence in infected flocks

Dhand NK\textsuperscript{a}, Eppleston J, Whittington RJ and Toribio J-ALML

\textsuperscript{a}Faculty of Veterinary Science, The University of Sydney, 425 Werombi Road, Camden NSW 2570 Australia
Phone: 02 9351 1610; Fax: 02 9351 1693
Email: navneetd@camden.usyd.edu.au

Introduction

The level of clinical disease experienced due to ovine Johne’s disease (OJD) appears to vary considerably between infected sheep flocks in Australia, even in those having apparently similar characteristics. Some inter-flock variation may be due to differences in time since OJD infection and past sheep purchasing history. However, there appear to be other factors capable of affecting the clinical expression of disease in the flock. Only a small number of studies have been conducted to investigate these factors (Lugton, 2004; Mainar-Jaime and Vazquez-Boland, 1998; Reviriego et al., 2000). The one risk factor study conducted in Australia (Lugton, 2004) could not make conclusive recommendations due to limitations of the study design as outcome variables in this study were based on the subjective information provided by the producers. Investigation of risk factors in a 12-farm mortality study (MLA OJD.023) identified some potential risk factors for increased prevalence, but interpretation was constrained by the small sample size (Toribio et al., 2004). In MLA project OJD.028, high levels of pasture contamination and exposure of young sheep were the main risk factors identified but this study was not designed to investigate a range of factors (Whittington and McGregor, 2005). In the absence of concrete scientific information, there is speculation among farmers about the potential effect of some factors like sheep management practices, soil pH, micronutrient levels and soil type. On the other hand, though vaccination appears to be an effective control strategy, it is relatively expensive and may not be economically viable for all flocks. Thus there is a strong need to investigate risk factors for clinical expression of disease that may help development of recommendations for on-farm control of OJD in the absence of vaccination, or as an adjunct to vaccination.

Methods

The project consisted of a cross-sectional study on 92 infected properties in Australia that met specific selection criteria:

1. Self-replacing Merino flocks
2. Located in all identified OJD infected areas/regions in Australia including NSW, Victoria, Kangaroo Island, Tasmania (including Flinders Island) and Western Australia
3. Flock infected for 3 or more years
4. Non-vaccinated 3-year old, 3 & 4-year old (6-8 tooth) or 4 & 5-year old sheep present in flock
5. $\geq 210$ sheep in the 3-year old, 3 & 4-year old or 4 & 5-year old cohort (as 7 pools of 30 sheep were used to classify flocks as high or low prevalence)

A cohort represented the sheep of a specific age and sex group in the flock. Usually only one cohort was selected from every flock, however if on the day of faecal sampling, 210 sheep of same age and sex were not present in the flock, then samples from another age or sex group, representing additional cohorts, were also selected.
Mycobacterium avium subsp. paratuberculosis (\(M. \text{ptb}\)) in faeces was detected by pooled faecal culture (PFC) (Whittington et al., 2000a) and confirmed by polymerase chain reaction and restriction endonuclease analysis. Animal level prevalence for each sheep cohort, based on PFC results, was calculated by a method for variable pool size (Williams and Moffitt, 2001) using the Pooled Prevalence Calculator available online at http://www.ausvet.com.au/pprev (Sergeant, 2004). Based on OJD prevalence, the cohorts were categorised into low (<2%), medium (2-10%) and high (>10%) prevalence categories to create outcome variables.

Information about potential risk factors and confounders was collected by face-to-face interview with each producer using a pretested questionnaire and from 3 soil samples collected from paddocks grazed by the cohort sheep. Univariable analyses using the logistic regression SAS LOGISTIC procedure (Stokes et al., 2000) were performed to investigate the unconditional association between outcome variable and each explanatory variable. The variables significant in the univariable analyses \((P<0.25)\) were then tested in the multivariable model first by forward selection and then by backward removal (retaining only variables with \(P<0.10\)) to investigate their relationship with OJD prevalence level after allowing for other variables and confounders. Separate final models were built for husbandry and soil variables.

**Results**

In total, 233 known OJD-infected flocks were investigated to identify eligible flocks that met the specific selection criteria and 92 flocks were enrolled in the study by 31 July, 2004. Visits to each enrolled flock for faecal and soil sample collection, and producer interviews were completed by 21st December, 2004.

For better data consistency and reliability, a sub dataset was prepared after excluding cohorts comprised of 3 or lesser faecal pools as well as those of 5 year old age group. This dataset consisted of records of 98 sheep cohorts representing 87 flocks and was used in the present analyses. Average OJD prevalence based on PFC among these 98 sheep cohorts was 14.8% (median 4.5%, range 0 to 58.9%) and the number of sheep cohorts categorised as low (<2%), medium (2-10%) and high (>10%) prevalence were 24, 52 and 22, respectively.

In the final model, there was a strong relationship between the OJD prevalence and the level of OJD mortality as well as with sex (Table 1). Stocking rate of the lambing paddock and number of Gudair® vaccinated sheep drops present in the flock were the main husbandry risk factors significantly associated with OJD prevalence level. In addition, presence of wildlife aside from kangaroos and rabbits, and application of fertilizer other than single super, molybdenum super and lime were also associated with cohort OJD prevalence. Among the soil risk factors investigated (Table 4), OJD prevalence was higher in flocks raised on soils with higher phosphorus buffering index (\(\geq 70\)) and higher clay percentage (\(>15\%\)).
Table 1: Final ordinal logistic regression model for cohort OJD prevalence categorised as low (<2%), medium (2-10%) and high (>10%), based on 98 sheep cohorts. OR LCL and OR UCL represent the profile likelihood lower and upper confidence limits for odds ratios, respectively. P values are based on likelihood ratio test of significance.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Categories</th>
<th>b</th>
<th>Odds ratios</th>
<th>OR LCL</th>
<th>OR UCL</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept-1</td>
<td></td>
<td>0.65</td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Intercept-2</td>
<td></td>
<td>-3.57</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Current OJD mortality(^a)</td>
<td>No mortalities</td>
<td>2.35</td>
<td>10.5</td>
<td>2.9</td>
<td>42.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>&lt;2% mortalities</td>
<td>2.35</td>
<td>10.5</td>
<td>2.9</td>
<td>42.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>≥ 2% mortalities</td>
<td>3.91</td>
<td>49.7</td>
<td>11.2</td>
<td>276.4</td>
<td></td>
</tr>
<tr>
<td>Cohort sex</td>
<td>Ewes</td>
<td>1.92</td>
<td>6.8</td>
<td>2.2</td>
<td>23.4</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>Wethers</td>
<td>1.92</td>
<td>6.8</td>
<td>2.2</td>
<td>23.4</td>
<td></td>
</tr>
<tr>
<td>Cohort age</td>
<td>3 years</td>
<td>0.22</td>
<td>1.2</td>
<td>0.4</td>
<td>3.8</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>4 years</td>
<td>0.22</td>
<td>1.2</td>
<td>0.4</td>
<td>3.8</td>
<td></td>
</tr>
<tr>
<td>Stocking rate in the lambing paddock(^b)</td>
<td>&lt; 14 dse/hectare</td>
<td>2.03</td>
<td>7.6</td>
<td>2.7</td>
<td>23.9</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>≥ 14 dse/hectare</td>
<td>2.03</td>
<td>7.6</td>
<td>2.7</td>
<td>23.9</td>
<td></td>
</tr>
<tr>
<td>Number of drops in the flock vaccinated with Gudair®</td>
<td>1 or 2 drops vaccinated</td>
<td>-2.74</td>
<td>0.07</td>
<td>0.01</td>
<td>0.25</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>No drops vaccinated</td>
<td>-2.56</td>
<td>0.08</td>
<td>0.01</td>
<td>0.39</td>
<td></td>
</tr>
<tr>
<td>Presence of wildlife other than kangaroos and rabbits</td>
<td>No</td>
<td>-0.94</td>
<td>0.39</td>
<td>0.14</td>
<td>1.05</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>-2.27</td>
<td>0.10</td>
<td>0.02</td>
<td>0.50</td>
<td></td>
</tr>
<tr>
<td>Application of fertilizers other than super and lime</td>
<td>No</td>
<td>-2.27</td>
<td>0.10</td>
<td>0.02</td>
<td>0.50</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>-2.27</td>
<td>0.10</td>
<td>0.02</td>
<td>0.50</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)Owner reported OJD mortality for previous 12 months

\(^b\)Where 1 dam = 2.45 dse

Discussion

This cross-sectional study was conducted to investigate risk factors for OJD so as to aid better on-farm control of the disease. Within the limitations imposed by this study type, we made considerable efforts to maximise the strength of the study. For example, infection-level was measured objectively based on PFC which has better sensitivity compared to serology (Sergeant et al., 2001) and almost perfect specificity. The study questionnaire was completed via face-to-face interviews with producers and focus was maintained on a specified age cohort of sheep on each property to reduce owner recall bias. Also, information about potential confounders was collected from producers and included in multivariable analyses to minimise confounding bias.

Final model for Husbandry variables

Out of a total of 29 variables included in the multivariable model, four were significantly associated with cohort OJD prevalence in the final model after allowing for confounders. Amongst confounders, cohort age was non-significant while both current mortality and cohort sex were significantly associated with OJD prevalence level (Table 1). This finding provides strong evidence in support of the anecdotal observation of higher losses in wether mobs. This is generally attributed to differences in management of wether and ewe mobs, however it requires further investigation. Strong association of level of OJD mortality with OJD prevalence upholds the assumption that prevalence based on PFC can be used as an indicator of clinical expression of OJD.
Vaccination with Gudair®

The numbers of sheep drops vaccinated with Gudair® in the flock were significantly associated with OJD prevalence\(^4\). The flocks with >2 vaccinated drops had significantly lower cohort OJD prevalence as compared to those with only 1-2 vaccinated drops (Table 1). It may be an indication of possible reduction in infection level on the property with several drops of vaccinated sheep shedding lower *M. ptb*. Similar observations were also made in a vaccination trial as a significant reduction in mortality risk was reported in the 3\(^{rd}\) and 4\(^{th}\) years after commencement of vaccination (McGregor, 2004). In contrast, the OJD prevalence in flocks not vaccinating at all was lower than in the reference group (1-2 drops vaccinated). This could be due to the possibility that producers in non-vaccinating flocks may not be going for vaccination as their flocks were not experiencing high losses. Only 21.4% (3 of 14) of cohorts of non-vaccinating flocks reported high current mortalities in comparison to 38.1% cohorts (32 of the 84) of vaccinating flocks. Overall, vaccination of sheep with Gudair® appears to reduce the prevalence of OJD over time.

Stocking Rate of the Lambing paddock

High stocking rate in the lambing paddock/s (≥14 dse /hectare) was associated with higher cohort OJD prevalence. This is a very important finding and highlights the importance of lambs being the most susceptible age group in a sheep flock. Dams may be at increased stress due to high stocking rate coupled with parturition and lactation. Heightened stress may lead to higher level of *M. ptb* faecal shedding, which may in turn lead to higher *M. ptb* pasture and environmental contamination in the lambing paddock. Consequently, higher doses at an age when sheep are more susceptible to infection increase both the probability of infection and the probability of establishment of infection leading to a greater likelihood of the clinical expression of the disease. Our findings complement the results from the MLA OJD.028 project that concluded that lambs were the most susceptible age group and if exposed to high levels of contamination, may develop severe infection leading to death (Whittington and McGregor, 2005). Although stocking rate has been investigated in previous cross-sectional studies (Daniels *et al.*, 2002; Lugton, 2004; Reviriego *et al.*, 2000), this is the first study to identify an association between Johne’s disease and higher stocking rate. Toribio *et al.* (2004) reported a tentative link between OJD mortality and stocking rate but conclusions were guarded due to small sample size.

The stocking rate in the weaning, hogget and adult paddock, and overall stocking rate of the flock were also significant risk factors by themselves in the univariable analysis. However, these variables were not significant in the multivariable model due to multicollinearity with lambing paddock stocking rate. Interestingly, if lambing paddock stocking rate was temporarily removed from the model then each of the stocking rate variables added by turn to the final model, was significant after allowing for other confounders and variables. It indicates that stocking rate is a major risk factor for OJD and hence should be considered in formulating any disease control strategy.

\(^4\) All sheep cohorts selected for the study were required to be non-vaccinated and the results reported here are about the number of drops vaccinated *in the flock* in question.
Table 2: Association of stocking rates with OJD prevalence when the stocking rate variables are forced into the final model one by one after temporarily excluding lambing paddock stocking rate

<table>
<thead>
<tr>
<th>Stocking rates at various stages of life (high versus low)</th>
<th>b</th>
<th>Adjusted odds ratios</th>
<th>OR LCL</th>
<th>OR UCL</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weaning paddock</td>
<td>0.93</td>
<td>2.5</td>
<td>1.0</td>
<td>6.7</td>
<td>0.05</td>
</tr>
<tr>
<td>Hogget paddock</td>
<td>1.16</td>
<td>3.2</td>
<td>1.2</td>
<td>8.8</td>
<td>0.02</td>
</tr>
<tr>
<td>Adult paddock</td>
<td>0.98</td>
<td>2.7</td>
<td>1.0</td>
<td>7.5</td>
<td>0.05</td>
</tr>
<tr>
<td>Overall flock</td>
<td>1.00</td>
<td>2.7</td>
<td>1.1</td>
<td>7.0</td>
<td>0.03</td>
</tr>
</tbody>
</table>

Stocking rates are categorised into low and high based on their respective median value.

Odds ratios are adjusted for all variables present in the final model (except for lambing paddock stocking rate) namely: current mortality, cohort age, cohort sex, number of drops vaccinated with Gudair®, application of fertilizer other than super and lime, and presence of wildlife other than kangaroos and rabbits.

Application of fertilizers other than single super, molybdenum super and lime

A history of applying fertilizers such as bio-soil, pasture gold, organic manure, reactive phosphorus rock, mono-ammonium phosphate (MAP), di-ammonium phosphate (DAP), sewage ash, super potash and pasture special on the property was associated with lower cohort OJD prevalence. Use of such fertilizers, other than single super, molybdenum super or lime was reported for 11 cohorts. Such strong protective association was opposite to the effect of application of single and molybdenum super fertilizers which was detrimental in the univariable model. The alkaline nature of most of these fertilizers was worthy of note, but soil pH was not significantly associated with disease prevalence in this study and neither was the soil pH of these properties significantly different from the rest. The present results could either be an aberration of the data or a new finding that requires further investigation to identify the influential components of these fertilizers or other factors closely linked to the application of these less common fertilizers.

Presence of wildlife other than kangaroos and rabbits

The presence of wildlife other than kangaroos and rabbits on the study property was associated with lower cohort OJD prevalence. The presence of other wildlife species was reported by the owners/managers for 32 cohorts and included feral pigs, feral goats and wombats beside others. This protective association was an unexpected finding and requires further investigation. Although goats and pigs can develop paratuberculosis and shed *M. ptb*, goats are widely known to be more susceptible to the C strain than the S (Thoresen and Olsaker, 1994; Whittington et al., 2000b).

Final model for soil variables

In addition to the confounders, 10 variables were included in the final soil multivariable model. Parent soil type, though significant at the univariable level, was not significant after accounting for other variables and confounders in the multivariable model. Finally, only 2 soil variables, phosphorus buffer index (PBI) and clay% were significant after allowing for cohort age and sex, current mortality and parent soil type (Table 4).
Clay percent in the soil

OJD prevalence was higher in flocks raised on soils with a higher clay percentage (>15%). It has been reported previously that *M. avium* binds to clay particles and a similar phenomenon has been inferred for *M. ptb* (Brooks et al., 1984; Whittington et al., 2003). Attachment of *M. ptb* to clay particles could increase the availability of the organism to sheep by maintaining it in the upper soil layers rather than allowing it to be leached to deeper layers. However, there is no experimental evidence to support this hypothesis and further research is needed to substantiate it.

Table 4: Final ordinal logistic regression model for cohort OJD prevalence categorised as low (<2%), medium (2-10%) and high (>10%) for soil variables (average of 3 soil samples) based on 98 cohorts. OR LCL and OR UCL represent the profile likelihood lower and upper confidence limits for odds ratios, respectively. P values are based on likelihood ratio test of significance.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Categories</th>
<th>b</th>
<th>Adjusted odds ratios</th>
<th>OR LCL</th>
<th>OR UCL</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphorus buffer index</td>
<td>&lt; 70</td>
<td>1</td>
<td>1</td>
<td>0.96</td>
<td>2.60</td>
<td>0.96 7.35 0.06</td>
</tr>
<tr>
<td></td>
<td>≥ 70</td>
<td>0.96</td>
<td>1</td>
<td>0.96</td>
<td>7.35</td>
<td>1.28 9.06 0.01</td>
</tr>
<tr>
<td>Clay % of the soil</td>
<td>≤ 15%</td>
<td></td>
<td>1</td>
<td>0.96</td>
<td>2.60</td>
<td>0.96 7.35 0.06</td>
</tr>
<tr>
<td></td>
<td>&gt; 15%</td>
<td>1.20</td>
<td>1</td>
<td>0.96</td>
<td>7.35</td>
<td>1.28 9.06 0.01</td>
</tr>
</tbody>
</table>

a. Odds ratios are adjusted for current OJD mortality, cohort sex, cohort age and parent soil type.

Phosphorus buffer index

A higher phosphorus buffer index (PBI) is an indicator of better fertility of the soil and was associated with increased OJD prevalence in the cohorts (Table 4). Better fertility may be acting as a confounder for some flock management practices like stocking rate. With an increase in fertility of the soil there may be better availability of pasture and the producers may be tempted to increase stocking rate. In this study, cohorts raised on soils with high PBI (≥70) were consistently maintained at higher stocking rates as compared to their counterparts raised on soils with low PBI, but the association was not significant across all stages (Table 4). Improved pasture may also result in greater shading that may increase survivability of *M. ptb*. However, further studies are required to fully elucidate these relationships. This is apparently contrary to the findings of Lugton (2004) where more OJD was found in flocks raised on light texture soils, but the comparability of the soil descriptors in the two studies is not yet clear.

Table 4: Relationship of stocking rates with soil phosphorus buffer index: Overall flock stocking rate was calculated by dividing number of adult sheep >2 year old with total grazing area of the property; other stocking rates are in dse/hectare where dam = 2.45 dse, weaner = 1dse, hogget = 1 dse, ewe=1.5 dse and wether =1dse. All stocking rates are categorised into low and high based on their respective median value.

<table>
<thead>
<tr>
<th>Phosphorus buffer index</th>
<th>Lambing paddock</th>
<th>Weaning paddock</th>
<th>Hogget paddock</th>
<th>Adult paddock</th>
<th>Overall flock</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low</td>
<td>High</td>
<td>Low</td>
<td>High</td>
<td>Low</td>
</tr>
<tr>
<td>&lt; 70</td>
<td>32</td>
<td>26</td>
<td>29</td>
<td>28</td>
<td>33</td>
</tr>
<tr>
<td>≥ 70</td>
<td>14</td>
<td>23</td>
<td>12</td>
<td>24</td>
<td>14</td>
</tr>
<tr>
<td>p</td>
<td>0.10</td>
<td></td>
<td>0.10</td>
<td></td>
<td>0.05</td>
</tr>
</tbody>
</table>
Conclusions and recommendations

The strong association of current mortality with OJD prevalence based on PFC indicates that the latter can be used to objectively measure the clinical expression of OJD. This is the first study to confirm the anecdotal observation of higher OJD levels in wethers than ewes. The high stocking rates, particularly in the lambing paddock, are associated with higher OJD prevalence and producers should be advised to take necessary measures to reduce the stocking rates to lessen the OJD prevalence. Also, vaccination appears to reduce the prevalence over time and continued vaccination programme should be recommended. The association of disease prevalence with fertile soils is a new finding and needs further investigation.

References


Economic modelling of the impact of OJD

Russell Bush\textsuperscript{a}, Jenny-Ann Toribio, Peter Windsor and Stewart Webster

\textsuperscript{a}Faculty of Veterinary Science, University of Sydney
PMB 3 Camden, NSW 2570
Phone: 02 9351 1785; Fax: 02 9351 1693
E-mail: rbush@camden.usyd.edu.au

Introduction

To date the work conducted to estimate the financial impact of OJD in Australia has been based on both farm-level (NSW Farmers, 1997; Webster & Hall, 2000; Webster, 2002) and industry-level studies (Hassall and Associates, 2000; Hassall and Associates, 2003; Sergeant, 2002; Sergeant, 2003). To assist sheep producers to better manage OJD it is more important that they understand the on-farm financial impact of the disease and associated implications, rather than the industry-wide financial impact.

As an infected flock cannot return to an uninfected status without prohibitive cost (Taylor & Webster, 2005), the difference between infected and uninfected enterprise profit has little meaning. Of greater value is the proportion of the total cost of OJD that is avoidable through disease control strategies such as vaccination and management (McInerney \textit{et al.}, 1992; Webster & Mullen, 2000).

Gross margin (GM) calculations are commonly used by Australian sheep producers when budgeting and planning. They are a simple and quick method of providing a direct comparison of the relative profitability of similar enterprises (Dijkhuizen & Morris, 1997) and can be used to analyse actual enterprise performance under different disease scenarios.

A GM model was developed to predict the on-farm financial impact of OJD for Merino and first or second cross prime lamb enterprises, comparing non-infected, infected (status quo) and infected (vaccination) disease scenarios within Australia. The model estimates changes in profit due to increased mortalities as a result of OJD, and the benefits and costs of control through the use of Gudair\textsuperscript{TM} vaccination, including a breakeven point.

Methods

Spreadsheets for the GM model were constructed in MS Excel 2000 with the more complex worksheet functions programmed using MS Excel Visual Basic 6.0. The model allows potential users to enter information that is either specific to their own farm enterprise or of interest when contemplating a change in enterprise mix.

The model user enters information describing the farm carrying capacity, enterprise mix, income and costs of production as well as OJD status. Choices of individual GM budgets include Merino fine (19$\mu$m), medium (21$\mu$m) and strong (23$\mu$m) wool enterprises as well as 1\textsuperscript{st} and 2\textsuperscript{nd} cross lamb production. Merino sheep are further separated into ewe and wether enterprises. The user determines the size of each enterprise by entering the number of breeding ewes (Merino, 1\textsuperscript{st} or 2\textsuperscript{nd} cross) or wethers (Merino). Flock numbers and mortalities are simulated throughout the year.

Relevant production and husbandry information is entered to gain the income and expenditure for each enterprise. The user is able to enter specific information or can opt to use preset values based on information current in 2005. Drenching regimes for internal parasite control are preset and are specific for the likely location of each enterprise (NSW Agriculture, 2002).
The disease scenario is modelled over a 20 year period. Disease scenarios include non-infected, infected (status quo) (Figure 1) and infected (vaccination) (Figure 2). The infected scenario allows users to choose between low (<3%), medium (3 to < 7%) and high (≥ 7%) OJD mortalities. An ‘at risk’ category is for flocks with a positive OJD diagnosis which are vaccinating with Gudair™ despite not yet encountering noticeable OJD mortalities. The status quo scenario assumes no control and the vaccination scenario simulates a reduction in OJD mortalities, based on Australian mortality and vaccine research (Sergeant, 2002; Eppleston et al., 2003; Eppleston et al., 2004).

**Figure 1:** Infected (status quo) disease scenarios depicting flock-average OJD mortality rates for low, medium and high levels of disease over a 20-year period

![Figure 1: Infected (status quo) disease scenarios](image1)

**Figure 2:** Infected (vaccinated) disease scenarios depicting flock-average OJD mortality rates for low, medium and high levels of disease, as well as early vaccination with Gudair™, over a 20-year period

![Figure 2: Infected (vaccinated) disease scenarios](image2)

The model output is presented as cumulative gross margin per dry sheep equivalent expressed in net present value terms (GM (NPV)/DSE) at 5, 10, 15 and 20-year intervals to take into account a range of producer investment horizons. A discount value is applied to account for capital effects and
to facilitate comparison over differing time periods. This discount value has a default value of 8%, though it can be set by the user.

The model estimates the gross margin for each disease category, including no infection. The total cost of OJD is comprised of ‘avoidable’ and ‘unavoidable’ components. The unavoidable cost of OJD for any given enterprise is considered to be the difference in gross margin between an uninfected state and that for the most cost-effective OJD control strategy (vaccination). Hence, the avoidable cost of OJD for a given enterprise is the difference in gross margin between the infected (status quo) and vaccinated states. Breakeven points for vaccination are reported at varying disease levels for each enterprise.

Results

Model outputs were obtained for an example 1,000 head flock for four sheep enterprises considering four disease categories (high, medium, low and at-risk) within three disease scenarios (non-infected, infected (status quo) and infected (vaccination)). These outputs are contained in Table 1.

Table 1: Cumulative gross margins (GM (NPV)/DSE) for four sheep enterprises

<table>
<thead>
<tr>
<th>Enterprise</th>
<th>Disease category</th>
<th>5 years ($)</th>
<th>10 years ($)</th>
<th>15 years ($)</th>
<th>20 years ($)</th>
</tr>
</thead>
<tbody>
<tr>
<td>19µm Merino ewes</td>
<td>No infection</td>
<td>116.16</td>
<td>231.65</td>
<td>346.80</td>
<td>461.84</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>87.63</td>
<td>157.25</td>
<td>219.73</td>
<td>279.94</td>
</tr>
<tr>
<td></td>
<td>Medium</td>
<td>98.99</td>
<td>184.00</td>
<td>262.58</td>
<td>335.34</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>109.82</td>
<td>213.98</td>
<td>312.82</td>
<td>406.40</td>
</tr>
<tr>
<td></td>
<td>At-risk</td>
<td>115.57</td>
<td>230.46</td>
<td>345.02</td>
<td>459.46</td>
</tr>
<tr>
<td>19µm Merino wethers</td>
<td>No infection</td>
<td>130.89</td>
<td>261.78</td>
<td>392.67</td>
<td>523.55</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>104.42</td>
<td>200.60</td>
<td>293.97</td>
<td>386.96</td>
</tr>
<tr>
<td></td>
<td>Medium</td>
<td>112.71</td>
<td>213.03</td>
<td>311.03</td>
<td>409.88</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>123.50</td>
<td>241.84</td>
<td>355.33</td>
<td>464.58</td>
</tr>
<tr>
<td></td>
<td>At-risk</td>
<td>130.09</td>
<td>260.18</td>
<td>390.27</td>
<td>520.36</td>
</tr>
<tr>
<td>1st Cross</td>
<td>No infection</td>
<td>169.38</td>
<td>339.34</td>
<td>509.65</td>
<td>680.12</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>144.72</td>
<td>279.75</td>
<td>411.19</td>
<td>541.85</td>
</tr>
<tr>
<td></td>
<td>Medium</td>
<td>153.24</td>
<td>295.87</td>
<td>435.54</td>
<td>573.92</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>163.46</td>
<td>322.33</td>
<td>477.10</td>
<td>627.95</td>
</tr>
<tr>
<td></td>
<td>At-risk</td>
<td>168.62</td>
<td>337.83</td>
<td>507.38</td>
<td>677.09</td>
</tr>
<tr>
<td>2nd Cross</td>
<td>No infection</td>
<td>184.07</td>
<td>368.14</td>
<td>552.20</td>
<td>736.27</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>159.47</td>
<td>308.18</td>
<td>452.13</td>
<td>595.03</td>
</tr>
</tbody>
</table>
The year in which each enterprise reaches a vaccination breakeven point is reported in Table 2 and suggests a vaccination breakeven point takes several years to achieve for the breeding enterprises, such as Merino ewe, 1st cross and 2nd cross, compared to a Merino wether enterprise where a breakeven point is reached in year one. In the absence of OJD mortalities with the at-risk disease category, a vaccination breakeven point is never reached.

**Table 2:** Vaccination breakeven points (in years) for eight sheep enterprise types at four disease categories

<table>
<thead>
<tr>
<th>Enterprise</th>
<th>Disease category</th>
<th>19µm Merino ewes (years)</th>
<th>19µm Merino wethers (years)</th>
<th>1st Cross (years)</th>
<th>2nd Cross (years)</th>
</tr>
</thead>
<tbody>
<tr>
<td>High</td>
<td></td>
<td>3</td>
<td>1</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Medium</td>
<td></td>
<td>4</td>
<td>1</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Low</td>
<td></td>
<td>7</td>
<td>1</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>At-risk</td>
<td></td>
<td>not reached</td>
<td>not reached</td>
<td>not reached</td>
<td>not reached</td>
</tr>
</tbody>
</table>

**Discussion**

To date there has been limited research into predicting the financial impact of OJD on Australian sheep farms. A modelling approach has been used to simulate the financial impact of OJD on flocks in Australia over time (Sergeant, 2002; Sergeant, 2003) however this was developed prior to the availability of detailed epidemiological and vaccine effectiveness data.

This GM model was developed to better represent the on-farm financial impact of OJD across a range of wool and sheep-meat enterprises and disease scenarios within Australia. A GM approach was taken as it could easily be used by Australian sheep producers when comparing non-infected with infected and control versus no control. This will enable sheep producers to analyse actual enterprise performance and allow informed decision making with regards to enterprise mix and disease control.

The GM model is designed to process one enterprise type at a time so that business enterprises can be monitored individually to enable financial planning and control (Dijkhuizen & Morris, 1997). The model was restricted to a comparison of sheep enterprises only as the infrastructure required for sheep was assumed to be already in place, whereas a move to an alternate enterprise such as cattle or cropping required a potential shift in infrastructure, management and labour skill requirements.
The model suggests a vaccination break-even point is achieved in two to three years for breeding enterprises if the level of OJD is high. If the level of OJD is low a vaccination break-even point is achieved in three years for either a 1\textsuperscript{st} cross or 2\textsuperscript{nd} cross enterprise and in seven years for a Merino ewe enterprise. Although all breeding enterprises vaccinate lambs, the Merino ewe enterprise takes the longest time to reach a vaccination break-even point as a greater number of young sheep are retained annually. With Merino wethers a vaccination break-even point is reached in year one for all disease categories due to introduced vaccinated replacement hoggets providing an immediate response in reducing OJD mortalities. In the absence of OJD mortalities in the at-risk disease category, a vaccination break-even point is not reached within the model’s 20-year time frame for any of the enterprises.

The total cost of OJD for the high disease level scenario across all enterprises is due to a high number of OJD mortalities. The avoidable cost of OJD, resulting from the use of vaccination, reduced OJD mortalities and improved the cumulative GM for an infected (vaccination) flock compared to an infected (status quo) flock. At a medium and low disease level the total cost of OJD and the avoidable costs of OJD associated with vaccination are reduced for all enterprises due to lower OJD mortality rates. In the at-risk disease category there is a minimal reduction in cumulative GM associated with the total cost of OJD due to the assumption that an annual OJD mortality rate of 0.2\% is possibly being experienced. There is also a negative return to vaccination due to the cost of vaccination not being compensated by a reduced OJD mortality rate. Benefits from vaccination are likely to be associated with the market advantage these at-risk vaccinated animals could command, however this benefit was not calculated.

In addition to the costs associated with OJD mortality rates and control through vaccination and management it is suggested that sub-clinical losses should also be considered when establishing all the productivity losses associated with a disease (Bennett, 2003). However, these may be difficult to establish as there continues to be some debate regarding the existence and financial impact of sub-clinical losses. Reductions in bodyweight have been recorded around 12 months prior to death in one study (MLA.OJD.002A), leading to infected sheep being 32\% (12kg) lighter at death and producing 6\% less wool annually than similar animals free of the disease. However, this may not be a consistent finding. In a separate study investigating the efficacy of vaccination with Gudair\textsuperscript{TM}(MLA.OJD.009), small reductions in live weight gain were found in vaccinated lambs in the first year following vaccination. Even so, over the course of the 5-year trial there was little difference in weight or condition score, nor in fleece parameters, between vaccinates and non-vaccinates. These conflicting observations suggest that the extent of sub-clinical losses is variable between flocks. Regardless of these findings, as subclinical losses of 6\% less wool has been identified in at least one study, it is probable that economic losses reported in this model may be an underestimate of the actual losses.

The extent of trading losses for individual farms primarily depends on the disease status, enterprise mix, production system and market forces/demands. The financial impact of trading losses for a farm primarily selling sheep for slaughter will be minimal compared to a farm on-selling animals to other producers. With assurance based trading (Australian Animal Health Council, 2004) there is an opportunity for Australian sheep producers with infected flocks to improve their trading position through the use of vaccination as part of their on-farm management of OJD.
Application for industry

This GM model provides Australian sheep producers and their advisors with an accurate on-farm estimate of the total and avoidable cost of OJD for Merino as well as first or second cross prime lamb enterprises. Breakeven points for vaccination inform affected producers of the length of time required for a return on their investment in vaccination, providing a useful decision making tool when developing on-farm strategies for the control of OJD at different disease levels.

References


NSW Farmers (1997), The financial and emotional affects of ovine Johne's disease (OJD) on affected sheep breeders: Results from case studies with OJD affected sheep breeders. NSW Farmers' Association, Sydney.


Genomic and proteomic comparative study of the sheep and cattle strains of *Mycobacterium avium* subsp. *paratuberculosis*

Ian Marsh, John Bannantine, Mark Tizard and Richard Whittington

Abstract

In Australia and other countries the distinction between ovine Johne’s disease (OJD) and bovine Johne’s disease (BJD) at the microbiological level plays an important role in the development and implementation of control and evaluation programmes for both forms of this insidious disease. However, the differences between the sheep and cattle strains of *Mycobacterium avium* subsp. *paratuberculosis* (*M. ptb*) at the DNA level that result in the different host specificities are still extremely poorly understood. To date the DNA techniques that exist to differentiate these strains provide excellent tools for epidemiological investigations and diagnosis of Johne’s disease but have yielded little if any insight into the relationship between genotype and phenotype. In this study an intensive comparison was made of the sheep and cattle strains of *M. ptb* using a variety of modern DNA and protein based techniques including: representational difference analysis (RDA), polymerase chain reaction (PCR) and sequencing, microarray, two dimensional electrophoresis proteomics and surface enhanced laser desorption-ionisation (SELDI) proteomics. The majority of these techniques were used in conjunction with the recently completed *M. ptb* K10 genome sequence in order to identify those genes or proteins with unique characteristics in either strain. Using these techniques 12 single nucleotide polymorphisms, 3 large polymorphisms and a number of proteomic differences were observed between the sheep and cattle strains of *M. ptb*. These results confirm earlier studies that differentiate the sheep and cattle strains of *M. ptb* and continue to support management strategies that rely on this distinction.

Introduction and background

After more than 100 years of research on *M. ptb* and Johne’s disease, much still remains a mystery about this elusive organism and the disease it causes. However, what is clear is that Johne’s disease presents itself differently in cattle and sheep with respect to clinical, pathological and epidemiological features [34]. Early epidemiological studies identified that cases of Johne’s disease in sheep and cattle were caused by different strains of *M. ptb* [27] [28]. The strains isolated from sheep were difficult to grow on primary culture [27] [18] and were readily identifiable by their orange pigmentation [28]. Problems associated with primary culture of sheep strains persisted [9] but have been overcome more recently [33]. DNA based studies of *M. ptb* isolates from a range of hosts, using restriction fragment length polymorphism of genomic DNA, confirmed the existence of two distinct groups of *M. ptb* [9]. These are now commonly referred to as either sheep strains (S) or cattle strains (C). However, the differences observed between the S and C strains to date do not provide genotypic explanation of their altered phenotypes and divergent host specificity [9] [7] [31] [8] [14].

To protect disease free flocks and herds within infected districts and the non-infected regions, control programs have been developed in Australia including the National Ovine Johne’s disease Control and Evaluation Program (NOJDP). The success of control programs is dependent on the ability to make sound decisions regarding on-farm management practices and the movement of
animals between districts. At present these decisions are being made based on an incomplete understanding of *M. ptb* and the disease. For example, policies regarding mixed farming of cattle and sheep have been based on current knowledge of the apparent host specificity of *M. ptb*. Similarly, de-stocking and spelling polices that aim to eliminate *M. ptb* from pastures assume equivalence of strains. Consequently such polices may be flawed and the expected outcomes not achievable.

The aim of this study was to compare the S and C strains of *M. ptb* using genomic and proteomic techniques to identify differences in the host specificity and pathogenicity of this organism and provide scientific data to support the current management strategies of this disease.

**Materials and methods**

*M. a. paratuberculosis* isolates

*M. ptb* isolates used in this study are described in Table 1. Genomic and proteomic techniques were used to compare one S strain (Telford 9.2) and one C strain (CM00/416). A second C strain (316v) was used as a positive control when required. The S strain (Telford 9.2) and C strain (CM00/416) were grown on modified 7H10 plus mycobactin J slopes at 37°C for up to 3-4 months [33], while C strain 316v was grown in modified Watson-Reid medium with mycobactin J [22] at 37°C for 1-2 months. The cultures were harvested, washed 3 times with sterile PBS and stored at -70°C until required. DNA from the *M. ptb* K10 isolate was obtained from the National Animal Disease Centre (NADC), Ames Iowa. The remaining 32 DNA samples were from *M. ptb* isolates used in a previous epidemiological study of Johne’s disease in Australia and included: 16 S isolates and 16 C isolates [32].

<table>
<thead>
<tr>
<th><em>M. a. paratuberculosis</em> isolate</th>
<th>Species of origin</th>
<th>Number</th>
<th>Strain</th>
<th>Isolate or DNA</th>
<th>IS1311 PCR/REA</th>
<th>IS900 RFLP</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Telford 9.2</td>
<td>ovine</td>
<td>1</td>
<td>S</td>
<td>Isolate</td>
<td>S</td>
<td>S1</td>
<td>This study</td>
</tr>
<tr>
<td>CM00/416</td>
<td>bovine</td>
<td>1</td>
<td>C</td>
<td>Isolate</td>
<td>C</td>
<td>C3</td>
<td>This study</td>
</tr>
<tr>
<td>316v</td>
<td>laboratory</td>
<td>1</td>
<td>C</td>
<td>Isolate</td>
<td>C</td>
<td>C1</td>
<td>[23]</td>
</tr>
<tr>
<td>K10</td>
<td>bovine</td>
<td>1</td>
<td>C</td>
<td>DNA</td>
<td>n/a</td>
<td>n/a</td>
<td>NADC</td>
</tr>
<tr>
<td>Field isolates</td>
<td>ovine</td>
<td>11</td>
<td>S</td>
<td>DNA</td>
<td>S</td>
<td>S1</td>
<td>[24]</td>
</tr>
<tr>
<td></td>
<td>ovine</td>
<td>1</td>
<td>S</td>
<td>DNA</td>
<td>S</td>
<td>SU1</td>
<td>[24]</td>
</tr>
<tr>
<td></td>
<td>ovine</td>
<td>1</td>
<td>S</td>
<td>DNA</td>
<td>S</td>
<td>SU1</td>
<td>[24]</td>
</tr>
<tr>
<td></td>
<td>ovine</td>
<td>1</td>
<td>S</td>
<td>DNA</td>
<td>S</td>
<td>SU3</td>
<td>[24]</td>
</tr>
<tr>
<td></td>
<td>caprine</td>
<td>2</td>
<td>S</td>
<td>DNA</td>
<td>S</td>
<td>S1</td>
<td>[24]</td>
</tr>
<tr>
<td></td>
<td>bovine</td>
<td>3</td>
<td>C</td>
<td>DNA</td>
<td>C</td>
<td>C1</td>
<td>[24]</td>
</tr>
<tr>
<td></td>
<td>bovine</td>
<td>4</td>
<td>C</td>
<td>DNA</td>
<td>C</td>
<td>C3</td>
<td>[24]</td>
</tr>
<tr>
<td></td>
<td>bovine</td>
<td>3</td>
<td>C</td>
<td>DNA</td>
<td>C</td>
<td>C5</td>
<td>[24]</td>
</tr>
<tr>
<td></td>
<td>bovine</td>
<td>2</td>
<td>C</td>
<td>DNA</td>
<td>C</td>
<td>C12</td>
<td>[24]</td>
</tr>
<tr>
<td></td>
<td>bovine</td>
<td>2</td>
<td>C</td>
<td>DNA</td>
<td>C</td>
<td>CU1</td>
<td>[24]</td>
</tr>
<tr>
<td></td>
<td>bovine</td>
<td>1</td>
<td>C</td>
<td>DNA</td>
<td>C</td>
<td>CU2</td>
<td>[24]</td>
</tr>
<tr>
<td></td>
<td>bovine</td>
<td>1</td>
<td>C</td>
<td>DNA</td>
<td>C</td>
<td>CU3</td>
<td>[24]</td>
</tr>
</tbody>
</table>

n/a - not applicable, NADC - National Animal Disease Centre, USDA, Ames, Iowa
DNA extraction and strain confirmation of *M. a. paratuberculosis* strain types

DNA extraction was performed as described [21]. The concentration of the DNA was calculated by spectrophotometry using the formula (µg/mL = A<sub>260</sub> x D.F. x 50) where D.F. is the dilution factor. DNA samples were stored at 4°C. Strain identity of the Telford 9.2 and CM00/416 isolates was confirmed by IS<sup>1311</sup> PCR/REA [20] and IS<sup>900</sup> RFLP [5].

Representational difference analysis (RDA)

An RDA protocol was developed to compare the S (Telford 9.2) and C (CM00/416) strains of *M. ptb* [21]. Four rounds of RDA were undertaken using the S strain as the (tester) and the C strain as the (driver) at tester to driver ratios of 1:40, 1:400, 1:4000 and 1:40,000. RDA products were cloned, sequenced and used to query the *M. ptb* K10 database (formerly located at http://www.tigr.com) and *M. a. avium* 104 database (http://www.tigr.com) using the BLASTn algorithm [1] (National Centre for Biotechnology Information, NCBI) for identification. An extensive PCR and sequencing based strategy was used to confirm or refute the RDA sequences as divergent loci in the S and C strains [21]. Confirmation of divergent regions was achieved by BstE II RFLP analysis with a dig-labelled probe produced from the C strain and corresponding to the deleted region in the S strain [21]. Just prior to the completion of this study the entire *M. ptb* K10 genome was made publicly available (GenBank accession, AE016958) and BLASTn and BLASTx searches of the K10 genome and GenBank were performed to identify the genes included in the S strain deletion.

Identification of single nucleotide polymorphisms (SNPs) by polymerase chain reaction (PCR) and sequencing of selected genes

Thirty loci across 29 genes (Table 2) were used in a PCR and sequencing based comparison of the S (Telford 9.2) and C (CM00/416) strains of *M. ptb*, based on an experimental design used for the *M. tuberculosis* complex [26]. Genes selected were either the full or partial gene sequences available for *M. ptb* in GenBank at the time this study commenced or were derived from other mycobacterial species for which homologous loci were identified in the incomplete *M. ptb* K10 genome. The *desA1* gene in the C strain has been reported to be disrupted by a copy of IS<sup>900</sup> [3] and therefore was examined by PCR on its own (*desA1*) and in conjunction with IS<sup>900</sup> (*desA1+IS<sup>900</sup>) to determine if *desA1* in the S strain has also been disrupted by IS<sup>900</sup>. 


## Table 2: Genes analysed by PCR and sequencing for SNPs.

<table>
<thead>
<tr>
<th>Loci</th>
<th>Gene</th>
<th>Function</th>
<th>Species</th>
<th>GenBank Accession/Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>rpoB</td>
<td>RNA polymerase beta-subunit (β)</td>
<td>M. a. p.</td>
<td>AF057479</td>
</tr>
<tr>
<td>2</td>
<td>hsp65</td>
<td>heat shock protein</td>
<td>M. a. p.</td>
<td>U15989</td>
</tr>
<tr>
<td>3</td>
<td>hsp70</td>
<td>heat shock protein</td>
<td>M. a. p.</td>
<td>AF254578</td>
</tr>
<tr>
<td>4</td>
<td>groES</td>
<td>10-kDa chaperonin</td>
<td>M. a. a.</td>
<td>AF071829</td>
</tr>
<tr>
<td>5</td>
<td>sodA</td>
<td>superoxide dismutase</td>
<td>M. a. p.</td>
<td>AF180816</td>
</tr>
<tr>
<td>6</td>
<td>katG</td>
<td>catalase peroxidase</td>
<td>M. tb</td>
<td>X68081</td>
</tr>
<tr>
<td>7</td>
<td>85A</td>
<td>fibronectin- binding antigen</td>
<td>M. a. p.</td>
<td>AF280067</td>
</tr>
<tr>
<td>8</td>
<td>85B</td>
<td>fibronectin- binding antigen</td>
<td>M. a. p.</td>
<td>AF219121</td>
</tr>
<tr>
<td>9</td>
<td>85C</td>
<td>fibronectin- binding antigen</td>
<td>M. a. p.</td>
<td>AF280068</td>
</tr>
<tr>
<td>10</td>
<td>mce1a</td>
<td>mammalian cell entry</td>
<td>M.sp</td>
<td>[17]</td>
</tr>
<tr>
<td>11</td>
<td>mce2a</td>
<td>mammalian cell entry</td>
<td>M.sp</td>
<td>[17]</td>
</tr>
<tr>
<td>12</td>
<td>mce3a</td>
<td>mammalian cell entry</td>
<td>M.sp</td>
<td>[17]</td>
</tr>
<tr>
<td>13</td>
<td>mce4a</td>
<td>mammalian cell entry</td>
<td>M.sp</td>
<td>[17]</td>
</tr>
<tr>
<td>14</td>
<td>dnaA</td>
<td>chromosomal replication initiator protein</td>
<td>M. a. p.</td>
<td>AF222789</td>
</tr>
<tr>
<td>15</td>
<td>dnaN</td>
<td>DNA polymerase III subunit</td>
<td>M. a. p.</td>
<td>AF222789</td>
</tr>
<tr>
<td>16</td>
<td>recF</td>
<td>recombination gene</td>
<td>M. a. p.</td>
<td>AF222789</td>
</tr>
<tr>
<td>17</td>
<td>gyrB</td>
<td>DNA gyrase</td>
<td>M. a. p.</td>
<td>AF222789</td>
</tr>
<tr>
<td>18</td>
<td>ald</td>
<td>L-alanine dehydrogenase</td>
<td>M. a. p.</td>
<td>AF322643</td>
</tr>
<tr>
<td>19</td>
<td>fapP</td>
<td>fibronectin-attachment protein</td>
<td>M. a. p.</td>
<td>AY007557</td>
</tr>
<tr>
<td>20</td>
<td>inhA</td>
<td>enoyl reductase</td>
<td>M. a. p.</td>
<td>AJ439339</td>
</tr>
<tr>
<td>21</td>
<td>invA</td>
<td>putative invasion protein</td>
<td>M. a. p.</td>
<td>AJ439337</td>
</tr>
<tr>
<td>22</td>
<td>invB</td>
<td>putative invasion protein</td>
<td>M. a. p.</td>
<td>AJ439338</td>
</tr>
<tr>
<td>23</td>
<td>pks7</td>
<td>polyketide synthase</td>
<td>M. a. p.</td>
<td>AF232752</td>
</tr>
<tr>
<td>24</td>
<td>pks8</td>
<td>polyketide synthase</td>
<td>M. a. p.</td>
<td>AF232752</td>
</tr>
<tr>
<td>25</td>
<td>purH</td>
<td>formyltransferase</td>
<td>M. a. p.</td>
<td>AF232750</td>
</tr>
<tr>
<td>26</td>
<td>ahpC</td>
<td>alkylhydroperoxidase C</td>
<td>M. a. p.</td>
<td>AF334163</td>
</tr>
<tr>
<td>27</td>
<td>desA1</td>
<td>steroyl-ACP-desaturase</td>
<td>M. a. p.</td>
<td>AF305073</td>
</tr>
<tr>
<td>28</td>
<td>desA1 + S900</td>
<td>steroyl-ACP-desaturase</td>
<td>M. a. p.</td>
<td>AF305073</td>
</tr>
<tr>
<td>29</td>
<td>F57</td>
<td>NO similar gene, No function</td>
<td>M. a. p.</td>
<td>XT0277</td>
</tr>
<tr>
<td>30</td>
<td>Dps</td>
<td>DNA binding dormancy protein</td>
<td>M. smegmatis</td>
<td>AY065628</td>
</tr>
</tbody>
</table>

n/a – not applicable, M. a. p. – M. a. paratuberculosis, M. a. a. – M. a. avium  M. tb – M. tuberculosis  
M. sp – M. avium subsp. avium and M. bovis and M. smegmatis

### Microarray

Telford 9.2, CM00/416 and 316v were compared with the K10 isolate in Cy3 and Cy5 dye swap hybridisations, using a whole-genome array representing >95% of the M. ptb coding sequences [23]. Arrays were scanned using an ArrayWorx optical scanner (Applied Precision). Scanned images were adjusted with local background subtraction and LOWESS normalization [6]. The resulting data were analysed using SoftWorx Tracker image analysis software. Any spot where the non-K10 sample was greater than 2 fold lower than the K10 sample were selected as genes absent on the non-K10 genome. Finally, open reading frames (ORFs) that were not identified by both
hybridisations or that were not represented by at least 2 spot replicates were censored and not included in further analysis.

Each of the ORFs identified by microarray analysis as deleted from the S strain were evaluated by PCR to confirm their presence or absence in the S and C strains. PCR was also performed on a number of other ORFs not identified by the microarray but residing within or immediately flanking the regions that were identified. The latter was undertaken to determine the limits of each of the S strain deletions. The amplified products from the S strain were sequenced and used in BLASTn searches of the \textit{M. ptb} K10 genome to identify the terminal ends of each deletion. Thirty two \textit{M. ptb} field isolates (16 S strain and 16 C strain field isolates, Table 1) were evaluated using the S strain deletion PCR assays. Finally, the 3 DNA sequences derived from the \textit{M. ptb} K10 genome sequence corresponding to the regions deleted in the S strains were used in BLASTn searches to identify the presence or absence of these sequences in the \textit{M. a. avium} 104 genome (http://www.tigr.com).

**Two dimensional electrophoresis proteomics**

For 1D and 2D comparisons proteins were extracted using a method based on [30]. Briefly, proteins were extracted using 0.1 µm zirconium beads in conjunction with 3 x 45 sec pulses at maximum speed (6.5) in a Bio 101 Fastprep. For 1D experiments protein samples were extracted directly into sterile purified water and diluted 1:1 in standard SDS-PAGE buffer. For 2D experiments, proteins were extracted directly into standard 2D buffers. All samples were centrifuged at 103,320 g for 1 hr at 15°C prior to 2D electrophoresis. Protein concentrations were determined using a Bradford assay. Electrophoresis was performed in 15 cm x 16 cm (W x H) 12% polyacrylamide gels in a Hoefer SE 600 vertical slab gel electrophoresis unit for both 1D and 2D electrophoresis. IEF was achieved with 11 cm IPG strips pH 4-7 passively re-hydrated. IEF was undertaken in a BioRad Protean IEF unit. Two-dimensional experiments were run in triplicate to determine the reproducibility of this protocol. Eight protein spots (2 sheep and 6 cattle) chosen based on being either unique or demonstrating differential expression together with 3 reference proteins from each strain were analysed by mass spectrometry (QSTAR2, Bioanalytical Mass Spectrometry facility at the University of New South Wales).

**Surface enhanced laser desorption-ionisation (SELDI) proteomics**

Proteins were extracted directly into extraction/reducing buffer (8M urea, 1% CHAPS in PBS) using the same protocol as for the 2D experiments. S and C strain protein samples were diluted in extraction/reducing buffer and run in duplicate at two concentrations, 1.5 µg per spot and 10 µg per spot on 4 ProteinChips including: CM10 (weak cation exchange, at pH 4 and pH 7), H50 (hydrophobic), IMAC30-Cu (copper affinity) and Q10 (strong anion exchange, at pH 7 and pH 9). All ProteinChips were prepared using the manufacturers’ recommended protocols and scanned with a ProteinChip reader (Ciphergen Model PBS IIC) that had been pre-calibrated according to the manufacturer’s recommendations. Laser intensity and deflector sensitivity were determined empirically, after which spot and chip protocols were prepared to scan each ProteinChip. The resulting data was then analysed using Ciphergen ProteinChip Software version 3.2. Spectra were examined and only those protein peaks with peak intensity equal or greater than 1 were considered.

**Results**

**Representational difference analysis**

Three bands identified as theoretically divergent DNA regions were cloned and sequenced (RDA1, RDA3 and RDA4)[21]. RDA1 (229 bp) contained a single G\(\rightarrow\)T base difference in the S strain relative to the C strain, producing a serine\(\rightarrow\)alanine amino acid substitution in the MAP1381
conserved hypothetical gene in the S strain. This single base difference resulted in an extra Sau3A I site in the C strain and this was confirmed with a Sau3A I restriction endonuclease digest of the PCR product. RDA4 (163 bp) was an artefact. RDA3 (206 bp) was similar to sequences in the incomplete genome sequences of M. ptb K10 and M. avium subsp. avium 104 which demonstrated homology with mmpL genes in M. tuberculosis. A PCR-based analysis of these regions led to the identification of a deletion within the mmpL5 gene in the S strain. This deletion was confirmed by BsuE II RFLP analysis of genomic DNA from the S and C strains using (1) a dig-labelled probe for IS900 (Figure 1A) and (2) a 200 bp probe for the S strain deletion (Figure 1B). Further PCR analysis confirmed the deletion of a 11,584 bp region that included 10 genes (MAP1734 to MAP1743c) (see Table 5, in microarray section for more detail).

Figure 1: RFLP probed with a 229 bp IS900 probe (A) and then stripped and reprobed with a 200 bp probe used to confirm the S strain deletion (B). For each panel Lane 1, molecular size marker; lane 2, M. a. paratuberculosis S strain (Telford 9.2) genomic DNA and lane 3, C strain (CM00/416) genomic DNA.

Identification of single nucleotide polymorphisms (SNPs) by polymerase chain reaction (PCR) and sequencing of selected genes

Of the 30 PCR assays used in this study, 4 failed to amplify with either strain and were omitted from the study. From the remaining 26 assays, 12,117 base pairs of sequence were obtained and compared, of which 18 loci were found to be identical in both strains and the remaining 8 (Table 3) contained a total of 11 SNPs in the S strain compared to the C strain. Sequences from both strains were used to query the K10 genome in a BLASTn search. The C strain was identical and the 11 SNPs were confirmed in the S strain. Both desA1 regions (plus and minus IS900) were found to be identical in the S and C strains. However, a BLASTn examination of the M. ptb K10 genome using the DNA sequences derived from these PCR, found the region flanking IS900 at this loci to be erroneously [3] identified as the desA1 gene and was in fact the ORFs MAP2202c and MAP2204c. The desA1 gene was identified in the annotated M. ptb K10 genome as MAP0658c and when its amino acid sequence was compared to MAP2204c they were ~76% homologous.

The S and C strain DNA sequences were then used for in silico comparison with the incomplete M. a. avium 104 genome using 11,133 bp of sequence data (regions including IS900 and F57
sequence data were omitted as these are unique to *M. ptb*). Eighty seven SNPs (~0.8% of the sequence data compared) were identified that could be used to differentiate *M. a. avium* 104 isolate from the S and C strains of *M. ptb* (Table 4). This produced a marked difference in the ratio of synonymous to non-synonymous substitutions compared to those differentiating the S and C strains of *M. ptb*. However, more conserved amino acid substitutions were observed when comparing *M. a. avium* with *M. ptb* than between the S and C strains of *M. ptb* (Table 4). Of the 11 SNPs that differentiate the S and C strains of *M. ptb*, 6 loci from the S strain and 5 loci from C strain were identical with the *M. a. avium* 104 genome.

**Table 3**: The 11 SNPs differentiating the S and C strains of *M. a. paratuberculosis*, the resulting amino acids for each strain and the effect of the substitution.

<table>
<thead>
<tr>
<th>Gene</th>
<th>S strain versus C strain</th>
<th>S strain aa</th>
<th>C strain aa</th>
<th>Change</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>hsp65</em></td>
<td>T_C</td>
<td>Thr</td>
<td>Thr</td>
<td>conserved</td>
</tr>
<tr>
<td></td>
<td>A_G</td>
<td>Glu</td>
<td>Glu</td>
<td>conserved</td>
</tr>
<tr>
<td><em>sodA</em></td>
<td>C_G</td>
<td>Leu</td>
<td>Val</td>
<td>neutral-neutral</td>
</tr>
<tr>
<td><em>dnaA</em></td>
<td>T_C</td>
<td>Leu</td>
<td>Pro</td>
<td>neutral-neutral</td>
</tr>
<tr>
<td><em>dnaN</em></td>
<td>T_G</td>
<td>Ala</td>
<td>Ala</td>
<td>conserved</td>
</tr>
<tr>
<td><em>recF</em></td>
<td>C_T</td>
<td>Arg</td>
<td>Trp</td>
<td>basic-neutral</td>
</tr>
<tr>
<td><em>gyrB</em></td>
<td>C_T</td>
<td>Ser</td>
<td>Ser</td>
<td>conserved</td>
</tr>
<tr>
<td></td>
<td>T_G</td>
<td>Ile</td>
<td>Met</td>
<td>neutral-polar</td>
</tr>
<tr>
<td></td>
<td>T_C</td>
<td>Val</td>
<td>Ala</td>
<td>neutral-neutral</td>
</tr>
<tr>
<td><em>inhA</em></td>
<td>G_A</td>
<td>Gin</td>
<td>Gin</td>
<td>conserved</td>
</tr>
<tr>
<td><em>pks8</em></td>
<td>G_A</td>
<td>Pro</td>
<td>Pro</td>
<td>conserved</td>
</tr>
</tbody>
</table>

**Table 4**: The number of SNPs identified when comparing the S and C strain of *M. a. paratuberculosis* and *M. a. avium* including the type of substitution and the effect on the resulting amino acids.

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Number of bases compared</th>
<th>Total number of SNPs</th>
<th>Synonymous vs non-synonymous substitutions</th>
<th>Substitutions resulting from transitions vs transversions</th>
<th>Conserved vs non-conserved amino acid substitutions</th>
</tr>
</thead>
<tbody>
<tr>
<td>S strain vs C strain</td>
<td>12,117</td>
<td>11</td>
<td>6/5 (55%/45%)</td>
<td>8/3 (73%/27%)</td>
<td>6/5 (55%/45%)</td>
</tr>
<tr>
<td><em>M. a. paratuberculosis</em> vs <em>M. a. avium</em></td>
<td>11,133</td>
<td>86</td>
<td>72/14 (84%/16%)</td>
<td>64/23 (74%/26%)</td>
<td>71/15 (83%/17%)</td>
</tr>
</tbody>
</table>

**Microarray**

The C strain and 316v (laboratory strain) were identical to the *M. ptb* K10 strain; whereas, three regions including multiple ORFs were found to be deleted from the S strain including: Deletion 1
(8049 bp), Deletion 2 (19,930 bp) and Deletion 3 (1229 bp) (Table 5). In total, the 3 deletions were equivalent to 29,208 bp (~0.6%) of the *M. ptb* K10 genome and included 24 open reading frames (ORFs) (Table 5). The largest of the 3 deletions included the *mmpL5* gene, which we had identified previously using RDA. Of the remaining 24 ORFs, several have homology with genes in the *M. tuberculosis* H37Rv strain that have been shown to be associated with *in vitro* cultural requirements, intracellular survival and virulence. In a PCR-based study of 32 well well-characterised *M. ptb* field isolates (16 S strains and 16 C strains), all 3 deletion regions were absent in all S strains and present in all C strains indicating conservation of the genomic differences.

The genomic relationship between the S and C strains of *M. ptb* and *M. a. avium* was evaluated by using the *M. ptb* K10 sequences corresponding to each of the S strain deletions in BLASTn searches of the *M. a. avium* 104 genome. The regions corresponding to Deletions 1 and 3 were found to be complete and intact within the *M. a. avium* 104 genome but the region corresponding to Deletion 2 was incomplete. Only 17,384 bp (87.2%) of the Deletion 2 was found in the *M. a. avium* 104 genome in 2 clusters separated by 203,000 bp and both in the opposite orientation compared to the *M. ptb* K10 genome. Therefore, ~2500 bp of this region was found to be unique to the C strain of *M. ptb*. 
Table 5: S strain deletions identified by RDA and microarray including: base positions with respect to the *M. a. paratuberculosis* K10 genome, the partial (par) ORFs and complete ORFs. The shaded area corresponds to the region identified in the RDA study.

<table>
<thead>
<tr>
<th>S strain deletion</th>
<th>Start of deletion, base position</th>
<th>ORFs included</th>
<th>M. tb equivalent</th>
<th>M. ptb Gene name</th>
<th>Function</th>
<th>End of deletion, base position</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deletion 1</td>
<td>1625179</td>
<td>par, MAP1484c</td>
<td>Rv3181c</td>
<td>putative dioxygenases</td>
<td>MAP1485c</td>
<td>Rv0214 acyl-CoA synthase</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MAP1486c</td>
<td>Rv0456c</td>
<td>enoyl-CoA hydratase/isomerase superfamily</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>MAP1487c</td>
<td>Rv2496c</td>
<td>pyruvate dehydrogenase E1 component [beta] subunit</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>MAP1488c</td>
<td>Rv2497c</td>
<td>pyruvate dehydrogenase E1 component [alpha] subunit</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>MAP1489c</td>
<td>Rv2750</td>
<td>putative dehydrogenase</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>MAP1490</td>
<td>alpha-methylacyl-coA racemase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>par MAP1491</td>
<td>alpha-methylacyl-coA racemase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Deletion 2</td>
<td>1888735</td>
<td>MAP1728c</td>
<td>yfnB</td>
<td>2-haloalkanoic acid dehalogenase</td>
<td>MAP1729c</td>
<td>Rv2605c thioesterase II</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MAP1730c</td>
<td>putative ATP/GTP-binding protein</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>MAP1731c</td>
<td>hypothetical protein</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>MAP1732c</td>
<td>Rv0302</td>
<td>transcriptional regulator (TetR/AcrR family)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>MAP1733</td>
<td>proline rich protein precursor</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>MAP1734</td>
<td>Rv2123</td>
<td>PPE-family protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>MAP1735</td>
<td>Rv0217c</td>
<td>probable esterase</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>MAP1736</td>
<td>putative tetR-family transcriptional regulator</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>MAP1737</td>
<td>Rv0677c</td>
<td>conserved small membrane protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>MAP1738</td>
<td>Rv0676c</td>
<td>conserved large membrane protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>MAP1739c</td>
<td>Rv2002</td>
<td>3-oxoacyl-[2] reductase</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>MAP1740c</td>
<td>Rv3132c</td>
<td>sensor histidine kinase</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>MAP1741c</td>
<td>Rv2005c</td>
<td>conserved hypothetical protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>MAP1742c</td>
<td>Rv2026c</td>
<td>conserved hypothetical protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>MAP1743c</td>
<td>Rv2032</td>
<td>conserved hypothetical protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>MAP1744</td>
<td>hypothetical protein</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Deletion 3</td>
<td>2608297</td>
<td>MAP2325</td>
<td>Rv2416c</td>
<td>conserved hypothetical protein</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Two dimensional electrophoresis proteomics

The S and C strains were compared by 1D (Figure 2, panel A) and 2D (Figure 2, panels B and C) electrophoresis. The 1D comparison identified several bands in both strains with differential expression and at least one major unique band in the S strain. Further analysis of the protein samples by 2D electrophoresis confirmed the unique expression of this protein (Figure 2, panel B,
S1 and S2) in the sheep strain and identified several proteins in the C strain (Figure 2, panel C, C3-C5 and C12-C14).

All 8 spots were excised from the gel along with 3 reference spots, from both strains and identified by mass spectrometry. The reference proteins were identified in both strains as: MAP1885c (C6 and S7), 70 kDa HSP (C8 and S9) and MAP3909c (C10 and S11). The strain specific proteins were identified as: MAP2821 (S1 and S2, 2 spots due to post translation modifications), fadE3_2 and MoxR (C3, C4 and C5, 3 spots due to post translation modifications), Wag31 (C12), MAP0494 (C13) and MAP3838c (C14). MAP0494 and MAP3838c were erroneously identified based on their isoelectric points (pI). At 6.24 and 10.79, respectively, these were either too high for the location of the spot or outside the pH range of the IEF strips.

**Figure 2:** Proteomic comparison of the S and C strains of *M. a. paratuberculosis* using 1D electrophoresis (panel A; Lane 1, molecular size marker; lane 2 *M. a. paratuberculosis* S strain and lane 3, *M. a. paratuberculosis* C strain) and 2D electrophoresis (panel B, S strain and panel C, C strain). The latter shows the locations of the proteins analysed by QSTAR2 mass spectrometry.
Surface enhanced laser desorption-ionisation (SELDI) proteomics

SELDI spectra were obtained with all 4 ProteinChips at both 1.5 µg per spot and 10.0 µg per spot loading concentrations. However, protein peaks were only observed in the 5,000 to 20,000 kDa range. Numerous peaks were observed that were common to both samples but closer examination of the spectra identified many unique peaks in the S strain and a few in the C strain (Table 6, U). Evidence was also observed for differential expression of a number of proteins (Table 6, E). Several unique protein peaks, of similar molecular weight, had been identified in the S strain with 2 or more ProteinChips including a 5.2 kDa protein peak using the CM10 and IMAC30 ProteinChips and a 13.9 kDa protein peak using the H50, IMAC30 and Q10 ProteinChips. A 9.6kDa protein peak was identified as unique in the S strain by the CM10 and Q10 ProteinChips, but as differentially expressed by the IMAC30 ProteinChip. Finally, a differentially expressed 17.7 kDa protein was identified by the IMAC30 and Q10 ProteinChips. No unique or differentially expressed protein peaks were identified in the C strain with more than 1 ProteinChip.

Table 6: Summary of the SELDI results. The expanded spectra for each ProteinChip were used to identify protein peaks in the S and C strain of M. a. paratuberculosis as either unique (U) or representing a marked difference in expression (E). The size of each peak identified is given in kDa.

<table>
<thead>
<tr>
<th>ProteinChip</th>
<th>1.5 µg of protein</th>
<th>10.0 µg of protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Telford 9.2</td>
<td>CM00/416</td>
<td>Telford 9.2</td>
</tr>
<tr>
<td>kDa</td>
<td>kDa</td>
<td>kDa</td>
</tr>
<tr>
<td>CM10 (pH 4)</td>
<td>13.9 U</td>
<td>5.2, 6.0, 14.4 U</td>
</tr>
<tr>
<td></td>
<td>8.7, 9.0, 9.3, 10.2, 14.7 E</td>
<td></td>
</tr>
<tr>
<td>CM10 (pH 7)</td>
<td>5.2, 6.0, 9.6 U</td>
<td>8.37 E</td>
</tr>
<tr>
<td></td>
<td>14.7</td>
<td></td>
</tr>
<tr>
<td>H50</td>
<td>13.9 U</td>
<td>16.3 U</td>
</tr>
<tr>
<td></td>
<td>13.9(k) E</td>
<td></td>
</tr>
<tr>
<td>IMAC30 (Cu)</td>
<td>5.2, 5.3, 5.5(k), 13.9(k) U</td>
<td>5.2, 5.3, 5.9, 13.9, 14.3 U</td>
</tr>
<tr>
<td></td>
<td>9.6, 17.7, 17.9 E</td>
<td>9.6, 17.7(k) E</td>
</tr>
<tr>
<td>Q10 (pH 7)</td>
<td>6.9, 13.9 U</td>
<td>7.36 E</td>
</tr>
<tr>
<td></td>
<td>17.7</td>
<td></td>
</tr>
<tr>
<td>Q10 (pH 9)</td>
<td>8.9, 9.0, 9.6, 13.9 E</td>
<td>13.9</td>
</tr>
<tr>
<td></td>
<td>9.5(k) U</td>
<td></td>
</tr>
</tbody>
</table>

(a) Protein peak was < 1 in peak intensity but readily identifiable

Discussion

Previously unidentified differences between the S and C strains of M. ptb were identified using a combination of modern DNA and protein based techniques. RDA has already been used to identify unique regions present in the S strain but absent in the C strain [14]. In this study two further differences were discovered in the S strain using RDA including a single base difference in MAP1381 and a large-scale deletion that included the mmpL 5 gene. The latter was fully characterised as Deletion 2 by microarray analysis. Deletion 2 along with two others identified by microarray, are the first large-scale genomic differences to be reported that differentiate the S and C strains of M. ptb. In total, the 3 deletions were equivalent to 29,208 bp (~0.6%) of the M. ptb K10
genome and included 24 open reading frames (ORFs) (Table 5). PCR analysis of 32 field isolates confirmed these deletions to be highly conserved in the S strain. Homologues of many of the ORFs in Deletions 1-3 have been identified in *M. tuberculosis* and shown to play important roles in pathogenicity, host cell invasion and intracellular survival as well as *in vitro* culture. The latter may be important given the marked differences in the cultural characteristics of these strains.

The *pdhABC* genes in *M. tuberculosis*, for which the ORFs MAP1487c and MAP1488c (Deletion 1) bear homology, encode subunits of pyruvate dehydrogenase, which converts pyruvate to acetyl-coenzyme A (CoA) and carbon dioxide, and have been shown to be up regulated during nutrient starvation [2]. Similarly the ORFs MAP1740c and MAP1742c (Deletion 2) appear to be homologues of *M. tuberculosis* genes Rv3132c and Rv2026c, respectively, which were considered to be essential for optimal *in vitro* growth [24]. Interestingly, homologues in *M. tuberculosis* of many of the ORFs in Deletion 2 have been studied in association with the DevR-DevS two-component regulatory system, thought to be involved in intracellular survival. This observation of *M. tuberculosis* and the clustering of MAP1740c to MAP1743c would indicate that these genes may form part of an operon involved in the intracellular response to the host environment and that the loss of this operon may explain the shift in host specificity and possibly the *in vitro* growth requirements of the S strain of *M. ptb*. Of particular interest was the absence of the *mmpS5* (MAP1737) and *mmpL5* genes (MAP1738) as part of Deletion 2 in the S strain. MmpL proteins in *M. tuberculosis* have been shown to be essential for intracellular survival and growth, are involved in pathogenicity and contribute to host-pathogen interactions [4] [12] [11] [16] [19] [15]. Furthermore, attenuated strains of *M. bovis* that lack the *mmpL 4* gene were found to exhibit equivalent protection to *M. bovis* BCG in guinea pigs challenged with *M. tuberculosis*[10].

The smallest deletion identified in this study, Deletion 3, included only one ORF, MAP2325, which shares homology with Rv2416c in *M. tuberculosis*. Rv2416 has been described as an enhanced intracellular survival (*eis*) protein thought to be an important *M. tuberculosis* immunogen [13] present only in pathogenic mycobacteria [29] but as yet not confirmed in *M. ptb*.

In an *in silico* analysis, DNA sequences from the C strain corresponding to the S strain deletions were used to compare *M. ptb* with *M. a. avium*. Sequence for deletions 1 and 3 was complete and intact within the *M. a. avium* 104 genome; whereas, Deletion 2 was found to be only 87.2 % (17,384 bp) complete. The missing 2546 bp of this region that includes the ORFs corresponding to MAP1730 to MAP1732 are therefore unique to the C strain. This makes this deletion a very interesting target for further research on the divergence of *M. ptb* and *M. a. avium* and on the strains of *M. ptb*.

PCR and sequencing revealed eleven SNPs that can be used to differentiate the S and C strains. Five of these produced non-conserved amino acid substitutions in the S strain; however further work is required to identify what effect each of these may have on the S strain phenotype. Interestingly, a ten fold increase in the number of SNPs differentiating *M. ptb* from *M. a. avium* suggests that the divergence between the S and C strains of *M. ptb* is evolutionarily recent compared with the divergence between *M. a. avium* and *M. ptb*. More work is required in this area to confirm this hypothesis.

Proteomic techniques demonstrated their usefulness in identifying the protein expression characteristics of the S and C strains under routine culture conditions. The results indicated the usefulness of combining techniques given that two dimensional electrophoresis is more suited to proteins >20 kDa and SELDI is more useful for proteins <20 kDa when whole cell protein samples are examined. However, both techniques would benefit from fractionation techniques to simplify the protein samples. Interestingly, the protein identified by 2D electrophoresis in the S strain, MAP2821
was also evident in both the 1D electrophoresis (Figure 2, panel A) and SELDI (protein peak at ~14.0 kDa) results, which demonstrates the reproducibility of these techniques.

The results from this study have clearly demonstrated that many genomic differences including deletions equivalent to ~0.6% of the genome exist between the S and C strains of M. ptb. These results confirm and support earlier studies that differentiate the S and C strains of M. ptb and can be used to reassure animal industries that rely on this strain difference for the recommended management practices used to combat Johne’s disease. Furthermore, a number of important genomic loci have been identified that can be used to differentiate between the S and C strains. Differences in a number of important genes require further examination to understand what role they may play in pathogenicity, intracellular and environmental survival and specific host-pathogen interactions.

Acknowledgements

This project was funded by Meat & Livestock Australia. Development of the microarray was funded by the USDA-CSREES JDIP grant subcontracted to John P. Bannantine and Michael L. Paustian. The authors would like to thank: Leslie Reddacliff, Alison Collins, Shayne Fell, Sue Austin and the microbiology staff at the NSW Department of Primary Industries, Elizabeth Macarthur Agricultural Institute), Anna Waldron, Deb Taylor and Lyrissa Diforei (University of Sydney), Michael Paustian (National Animal Disease Centre, USDA-ARS, Ames, IA, USA) and Kathy Grainger (CSIRO Livestock Industries, Australian Animal Health Laboratory, Geelong, Victoria Australia).

References


I.B. Marsh, R.J. Whittington. Deletion of an mmpL gene and multiple associated genes from the genome of the S strain of Mycobacterium avium subsp. paratuberculosis identified by representational difference analysis and in silico analysis, Mol Cell Probes, Accepted, (2005).


C.M. Sassetti, D.H. Boyd, E.J. Rubin. Genes required for mycobacterial growth defined by


[31] R. Whittington, I. Marsh, E. Choy, D. Cousins. Polymorphisms in IS1311, an insertion sequence common to *Mycobacterium avium* and *M. avium* subsp. *paratuberculosis*, can be used to distinguish between and within these species, Mol Cell Probes, 12, (1998) 349-58.


A survey of potential wildlife reservoirs for *Mycobacterium paratuberculosis*

Patrick Kluver
University of Melbourne, Mackinnon Project
250 Princes Freeway
Werribee
Phone: 03 9731 2343; Fax: 03 9731 2388
E-mail: p.kluver@unimelb.edu.au

1.1 Introduction

In any disease control program eradication of disease depends upon the protection of disease free animals from any potential source of infection. Sylvatic hosts have the potential to thwart eradication campaigns. Wild bovids and ungulates are a source of bovine tuberculosis for North American herds. Eradication of bovine tuberculosis has been hampered by two non ruminant wildlife reservoirs, possums in New Zealand and badgers in England. A survey of potential wildlife reservoirs in Australia was conducted between 1997 and 1999, when eradication of ovine Johne’s disease (OJD) was seriously contemplated.

The presence of a common wildlife reservoir of Johne’s disease (JD) could render the eradication of disease impossible. In Scotland, rabbits have been demonstrated to be infected with *Mycobacterium paratuberculosis* (Grieg et al., 1997). In order to assess the risk posed to JD control due to wildlife, the survey described in this paper examined rabbits and another herbivore commonly found on Australian pastoral lands, the eastern grey kangaroo.

1.2 Aim

To survey European rabbits (*Oryctolagus cuniculus*) and eastern grey kangaroos (*Macropus giganteus*) in areas with endemic JD for the presence of *M. paratuberculosis* (*M. ptb*).

1.3 Method

Rabbits

In 3 areas where JD was present in livestock, rabbits were collected and examined for the presence of *M. ptb*. Property A was a series of three contiguous farms in the Ensay valley of Victoria. These properties had a purported long history of OJD and significant mortalities due to clinical OJD. A study examining sheep from these properties revealed JD prevalence of 21-37% (Hope et al., 1999). These properties were destocked in January 1997 and rabbits were sampled in March of that year. Property B was a commercial beef operation of 200 breeders between Geelong and Ballarat, with a history of JD of at least three years. In 1996 this property had 9 clinical cases of JD. The farm also ran a commercial merino flock and had a small flock of 9-10 feral goats. The final property, Property C, was a commercial dairy farm of 131 milkers in the Peterborough region of Victoria. The first case of JD on the farm was diagnosed in 1996. A whole herd investigation in 1999 conducted at the same time as the rabbit survey resulted in a faecal culture positive rate of 10%.

Farms were chosen on the basis of a high prevalence of JD in sheep or cattle, a high reported rabbit density in close contact with stock and the willingness of the manager to assist in the survey.
Table 1: Summary of rabbit sampling.

<table>
<thead>
<tr>
<th>Property</th>
<th>Enterprise</th>
<th>Location</th>
<th>Number of specimens</th>
<th>No of collections</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Merino</td>
<td>Ensay Valley</td>
<td>100 rabbits, 1 hare.</td>
<td>3 nights, 18/3/97-20/3/97.</td>
</tr>
<tr>
<td>C</td>
<td>Dairy Cattle</td>
<td>Peterborough</td>
<td>106 rabbits, 2 hares.</td>
<td>3 nights 3/06/99, 21/07/99, 21/06/99.</td>
</tr>
</tbody>
</table>

Rabbits were shot at night and necropsied within 12 hours of death. A minimum of 100 animals were examined from each area. Weight, sex, and any gross pathological changes were recorded. Twenty cm sections of ileum, ileo-caecal junction, ileum and mesenteric lymph nodes were taken and halved for histology and culture; faeces, when present, were stored for culture. Fresh tissue samples were pooled, stomached and processed as one sample. Samples were processed according to the relevant Australian Standard Diagnostic Technique (Tennant et al., 1996) and inoculated in BACTEC 12B (Becton Dickinson) media supplemented with Mycobactin J (Allied Monitor), 17% eggyolk and PANTA plus (Becton Dickinson). Histological sections and faeces were stored for future reference.

Kangaroos

Eastern grey kangaroos, which were being culled for pest management purposes in a central region of Victoria, were necropsied and sampled for the presence of JD from April 1997 to November 1998. This central Victorian region had a history of endemic JD.

The farm where the kangaroos were being culled was approximately 1000 hectares, in 2 portions, consisting of volcanic plains and sedimentary hills. Improved pastures and dams, for water, provided for the self-replacing Merino flock and some breeding cattle. OJD had been confirmed by necropsy after the owner had reported losses due to wasting in adult sheep. Following detection of infection on the property, six neighbouring properties were confirmed as also infected, along with numbers of close-by farms. One neighbouring farm had a bovine JD infection confirmed in 1992, and was then destocked of cattle until 1997. On another neighbouring property clinical cases in sheep were common, with gross intestinal lesions characteristic of JD detected in more than 18% of ewes and 10% of wethers at slaughter.

The sample farm, adjoining farms and roadsides had heavily treed areas providing an ideal habitat for large mobs of kangaroos that readily moved between adjoining properties. The shot samples were from adult and sub-adult kangaroos. Juvenile animals under 1 year of age were excluded for the purpose of this survey.

Sex, location, estimation of age, and any gross pathological changes were recorded. Sections of ileum, ileo-caecal valve, proximal colon, caecum and mesenteric lymph nodes were taken for histology, and fresh sections and faeces for culture. Initially all sections were cultured individually but for the last 35 animals sampled, lymph nodes and gut sections were pooled separately and
processed as two cultures. The culture technique used was in accordance with the Australian Standard Diagnostic Test recommendations (Tennant et al., 1996).

A minimum of 100 animals (kangaroos or rabbits) were examined from each site. The sample size was selected to detect a 3% infection level with 95% confidence limits.

### 1.4 Results

Rabbits

In total 310 rabbits, 4 hares and 3 feral goats were examined by culture. 

*M. ptb* was not detected in any of the samples, giving a point estimate of prevalence of 0 (95% CI 0-1.2%). Eight of 310 samples demonstrated growth of organisms other than *M. ptb*

#### Table 2: Prevalence of Johne’s disease in rabbits.

<table>
<thead>
<tr>
<th>Property</th>
<th>Number of specimens</th>
<th>Prevalence (CI 95% Wilson)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>100 rabbits, 1 hare.</td>
<td>0 (0-3.7%).</td>
</tr>
<tr>
<td>B</td>
<td>104 rabbits, 1 hare 3 goats.</td>
<td>0 (0-3.6%).</td>
</tr>
<tr>
<td>C</td>
<td>106 rabbits, 2 hares.</td>
<td>0 (0-3.5%)</td>
</tr>
<tr>
<td>Total</td>
<td>310 rabbits</td>
<td>0 (0-1.2%)</td>
</tr>
</tbody>
</table>

Eastern grey kangaroos

Thirty-seven female and sixty-three males were sampled. Five faecal culture samples had some evidence of bacterial growth in BACTEC, which was shown to be faecal contaminants; all other faecal samples and tissue samples were found to be negative.

There was no evidence of JD in the kangaroos examined (95% CI 0-3.7%).

### 1.5 Discussion

Several reviews have described the occurrence of Johne's disease in wildlife and zoological collections (Chiodini et al., 1984, Morgan 1999). Wild populations of Bighorn Sheep; Rocky mountain goat; and axis, fallow, white tailed, red, sika, roe deer have been reported as being infected with *M. ptb* (Williams et al., 1979, Riemann et al., 1979, Chiodini et al., 1983, Hillermark 1966, Reid et al., 1996).

Rabbits have been experimentally infected with JD. In two similar studies when 1 day old rabbits were orally dosed with ~10^8 organisms 3 of 16 (19%) and 9 of 21 (43%) had histological evidence of paratuberculosis at necropsy 9-12 months after inoculation (Mokresh et al., 1989, Mokresh et al., 1990). These studies demonstrate that rabbits are susceptible to *M. ptb* but provide little information of the natural ecology of the disease due to the large infective dose and age of the animals infected.
There are several reports of JD in wild populations of rabbits in Scotland. A rabbit with intestinal lesions resembling JD was described on a Scottish deer farm with endemic JD in 1990 (Angus 1990). No cultural confirmation was performed but the lesions closely resembled those seen with infection in cattle. A total of 32 rabbits were examined on the farm with only one animal having any evidence of JD. The JD exposure of these rabbits was due to a ‘major outbreak’ of JD in red deer (Cervus elaphus) on an experimental farm where rabbits were in ‘plague proportions’ requiring regular culling.

The first confirmed report of wild rabbits being naturally infected with M. ptb was in the summer of 1995 (Anon 1995). Researchers at the SAC Perth investigated rabbits on four farms in the Tayside region of Scotland. The rabbits were collected on 3 properties where JD was known and a fourth property where no clinical signs of the disease had been observed (Grieg et al., 1997). A total of 33 were sampled with 23 of these being culture positive for M. ptb (one of which was faecal culture positive only); 19 had histological evidence of JD and 13 were both culturally and histologically positive.

A wider survey was carried out 1996 in Scotland (Grieg et al., 1999). This survey involved shot samples collected from farms with and without a history of JD. It involved surveys in 7 counties and again included the Tayside region. The survey revealed little infection in areas outside the original survey area of Tayside. Of the 169 rabbits collected from counties other than Tayside, there was only one confirmed as M. ptb positive, and this rabbit was from a farm with no known history of JD. Eighty-seven of the rabbits from outside Tayside were from 10 farms with known JD; M. ptb was not isolated from any of these rabbits. Two of the 87 rabbits from 2 of these10 farms had histological changes with acid fast bacilli (AFB) present. Eighty rabbits from 8 farms with no known history of JD were also tested, with one culturally positive animal and 2 rabbits showing histological changes with the presence of AFB (it is not stated whether the animal is the same). RFLP strain typing revealed that all isolates tested except one were type B_C17, the predominant RFLP type in cattle in the UK. HPLC typing revealed some overlap of strains between species on one farm and none on the other tested.

It does appear from the wider Scottish survey that JD in rabbits outside the Tayside region is uncommon. The Tayside county appears to be a hot spot of lagomorph JD. Evidence of JD has also been detected in this area in stoats (Mustela erminea) (3 of 5 culture positive) and red fox (Vulpes vulpes) (8 of 9 culture positive) (Beard et al., 1990). This is in the area of high prevalence of JD in rabbits, which is presumably the source of infection for the carnivores.

The prevalence and occurrence of JD in rabbits in the Tayside region does appear to be unusual. The significance is yet to be determined and the reason this area has such a high prevalence of JD is unknown. The farms were originally surveyed because of the concordance of high levels of JD and the heavy density of rabbits. The association of lagomorph JD in this area may be due to a number of reasons: a close interaction between a large rabbit and an infected ruminant populations with heavy exposure of rabbits to M. ptb, the age structure of the rabbit populations, a highly pathogenic strain of M. ptb or rabbit adapted strain, a highly susceptible strain of rabbit in this area, or an unspecified environmental factor in this region which renders ruminants and rabbits highly susceptible to JD.

Major differences will exist between the ecology of wild populations of rabbits between different areas. In Australia, the proportion of animals that survive over 2 years of age is highly variable and can range from 7-17% with an annual survival of adults ranging from 40-60% (Williams et al., 1995). The dynamics of a population will greatly affect the expression of a chronic disease such as JD with populations with high turnover and very few adults over 2 years of age being very unlikely to show...
expression of disease. No information about the ecology and population dynamics of the rabbits in Scotland was available, although Abbott in his review suggests that rabbit densities are higher in the Tayside region than normally found in Australia (Abbott 2000).

There is no evidence that strains of *M. ptb* differ in their pathogenicity. It is suggested that some strains, which are more commonly isolated from a particular species, demonstrate a host preference for different species, but this may be the result of an opportunity to disseminate within a species, as opposed to a strain being more pathogenic to a particular host.

A similar argument exists for host susceptibility. Breed susceptibility has been suggested in the past but it is difficult to separate breed susceptibility from greater dissemination within a breed (Chiodini *et al.*, 1983).

A similar study in rabbits on OJD infected properties was conducted by the University of Sydney (Abbott 2000). The survey included 300 rabbits collected from 8 properties. None of the 300 rabbits had any evidence of infection with *M. ptb*.

### Table 3: Combined results of Victorian and NSW surveys for Johne’s disease in rabbits.

<table>
<thead>
<tr>
<th></th>
<th>Total Sampled</th>
<th>Prevalence %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(95% CI)</td>
</tr>
<tr>
<td>Ovine Johne’s disease</td>
<td>400</td>
<td>0 (0-0.95)</td>
</tr>
<tr>
<td>Bovine Johne’s disease</td>
<td>210</td>
<td>0 (0-1.8)</td>
</tr>
<tr>
<td>Johne’s disease</td>
<td>610</td>
<td>0 (0-0.63)</td>
</tr>
</tbody>
</table>

When the results of our study are combined with those from the University of Sydney study, the estimated prevalence of *M. ptb* in rabbits in Australia is 0 with an upper 95% confidence limit of 0.63% samples (Table 3). It is very unlikely that *M. ptb* exists in rabbits in Australia to any significant degree.

Paratuberculosis has been recorded in a wide range of species; no published cases have been recorded in marsupials.

There have been recorded cases of marsupials, including Tree Kangaroos (*Dendrolagus matschei*) and Tammar wallabies (*Macropus eugenii*) infected with bacteria from the *M avium* complex and *M genavense*, (M Lynch pers com, Montali *et al.*, 1998, Peet *et al.*, 1982) in zoological collections and *M bovis* in brush tailed possums (*Trichosurus vulpecula*) in New Zealand (Davidson 1991). In general marsupials are thought to be more susceptible to mycobacteria than eutherian mammals. So it is logical to look at marsupials as a possible reservoir or carrier species for *M. ptb*.

The eastern grey kangaroo is predominantly a grazing animal with specific food preferences restricted to grasses and forbs. Their dietary habits do overlap those of sheep and they are often in close association with sheep, sharing grazing land.
Eastern greys tends to range from 4-5km for family groups and up to 20km for solitary males. All the animals sampled would have had some exposure to pasture co-grazed with OJD infected animals.

The sampling regime was one of convenience and was based on shot samples. Shot samples of kangaroos often can be biased towards males and younger animals. To avoid a bias towards younger animals, which would have been less likely to have shown evidence of JD, this age group was deliberately excluded from the study.

The sample size was determined using Freecalc. A sample size of 100 animals will detect a disease prevalence of 3% or more at 95% confidence limits. Estimates were based on the assumption of the test having a perfect sensitivity and specificity. This gives a Type 1 error of 0.05 or in other words a 5% chance that the survey would misclassify a population as free if there was in fact disease present at 3% or more.

The other potential Type 1 error is with the assumption of prevalence. Three per cent could be an over estimate of the prevalence of infection in an exposed population of animals of unknown susceptibility. The level of JD within an infected cattle herd or sheep flock is likely to be from one animal within a herd to 50% depending on the time the herd has been infected and the opportunity for infection to spread. To declare populations free in this situation where the minimum expected prevalence may be very low is very difficult without doing a complete census of the population with a test that has perfect accuracy.

Other studies have looked at eastern grey kangaroo populations for evidence of JD. Abbott looked at 300 rabbits and 300 kangaroos from farms with endemic JD. This study found no evidence of disease (0-1.3% 95% CI) with one kangaroo having a positive faecal culture. It is possible that this animal was passively passing *M. ptb* and was not actively infected. The level of *M. ptb* in the faeces was thought to be low.

Table 4: The combined results of two Kangaroo surveys in NSW and Victoria.

<table>
<thead>
<tr>
<th></th>
<th>Numbers</th>
<th>Prevalence% (95% CI*)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSW</td>
<td>300</td>
<td>0 (0-1.3)</td>
</tr>
<tr>
<td>Vic</td>
<td>100</td>
<td>0 (0-3.7)</td>
</tr>
<tr>
<td>NSW &amp; Vic</td>
<td>400</td>
<td>0 (0-0.95)</td>
</tr>
</tbody>
</table>

*Wilson

The two surveys demonstrate that it is reasonably likely that if OJD exists in eastern grey kangaroos it is at a very low prevalence of less than 1%. This is even with an expected high exposure through cograzing on heavily contaminated pasture.

A survey of macropods on Kangaroo Island examined 427 wallabies and 55 kangaroos found some evidence of *M. ptb*. Two animals (one wallaby and a kangaroo) were tissue culture positive and had
histological evidence of acid fast bacilli in lymph nodes. Six animals were tissue culture positive but
had no histological evidence of acid fast bacilli and 6 'histo positive' animals were tissue culture
negative, although 4 had lesions which were described as either severe granulomatous enteritis or
had moderate to numerous acid fast bacilli. There was no evidence of M. ptb in the faeces.

These results are difficult to interpret. Certainly there appears to be no evidence that these animals
are shedding the bacteria, so pose little if any risk as far as spreading JD. In sheep and cattle it is
not unusual to find animals which have no histological evidence of disease but from which M. ptb
can be isolated. These animals are either in the early stages of the disease with focal
histopathological lesions not detected due to sampling, are infected but controlling the disease, or it
is contamination from the gut lumen although in this survey the gut contents were washed out.

The culturally negative animals with advanced or proliferating lesions are unusual in sheep and
cattle. One animal had evidence of Mycobacterium genavense on PCR which is a difficult organism
to culture. It may be that all these animals which had proliferative lesions were in fact infected with
other fastidious non paratuberculosis Mycobacteria.

1.6 Implications

With 400 eastern grey kangaroos and 610 rabbits now sampled on the mainland of Australia, without
any evidence of the presence of disease, it seems highly unlikely that these animals play any
significant part in the epidemiology of the disease in Australia.

Although no animals were found infected with M. ptb in this survey it does not rule out that eastern
grey kangaroos or rabbits could become infected with paratuberculosis in Australia. It does appear
from the Scottish surveys that the disease in rabbits at least can be highly clustered. This survey
was not designed to look for any clustering of disease in areas; instead it was biased towards
properties with reasonably high exposure and looked at the likely prevalence under normal
conditions.

1.7 References

TR.050.

Anon. (1995). High profile for animal welfare at the 1995 conference of the AVTRW. Veterinary
Record. 136, 23: 575-578.

Angus, K.W. (1990). Intestinal lesions resembling paratuberculosis in a wild rabbit (Oryctolagus

vulpes) and stoat (Mustela erminea). Veterinary Record. 145, 21:612-613.

Chiodini, R.J., van Kruiningen, H.J. and Merkal, R.S. (1984). Ruminant paratuberculosis (Johne’s

Greig, A., Stevenson, K., Henderson, D., Perez, V., Hughes, V., Pavlik, I., Hines, M.E. II.,


Review of Epidemiology
from 8th International Colloquium on Paratuberculosis

Paul Freeman
PO Box 158
CASINO NSW 2470
Phone: 02 6662 1544; Fax: 02 6662 6012
Email: dv.casino@rlpb.com.au

Background
This report only examines the 39 papers and posters which were included in the epidemiology stream by the conference organisers. A large number of studies reported on in other streams had some epidemiological content but they will not be included in this report.

There was broad range of topics covered in the epidemiology theme, and this report collates similar study topics into arbitrary groupings. The nature of epidemiology as an integrative discipline precludes summarising an overall theme for the studies included in this stream as all the themes covered in the colloquium contained epidemiological elements and any summary would be too lengthy to be useful.

Transmission studies
Results from three studies presented at the colloquium suggest that shedding by calves may be more epidemiologically significant than previously thought.

In an experimental study in Holland, (Van Roemund et al) two groups of five week old calves housed with faecal culture positive cows for 3 months, all became infected and 50% of the infected calves were shedding during this period. In one group, 60% of shedding calves continued to shed M. ptb during a subsequent second 3 month period after the adult cows had been removed and then continued to shed until they were slaughtered at 2 years of age.

This study also demonstrated successful horizontal transmission in calves. Four shedding calves infected 2 out of 4 naïve week old calves housed with them during the second three month period when the adult shedders had been removed.

Similarly an American field study (Bolton et al) looking at faecal shedding in calves in seven infected dairy herds found M. ptb in faeces in 2% of samples from all four age groups (0-3months, 4-6months, 7-14months, 15-24months ) of calves in the study herds. This study is currently 40% complete and to date 83% of the positive faecal samples were from calves less than 14months of age and 73% were from faecal culture positive dams.

In another Dutch study (Weber et al) based on individual faecal culture results from 37,151 head of cattle in 373 dairy herds and using survival analysis techniques an estimated 5-14% of cattle in high prevalence herds (Apparent prevalence >10%) shed before 2 years of age. The shedding level was lower in lower prevalence herds.
These experimental findings are reinforced by an US modelling study (Mitchell et al) which examined the determinants for the maintenance of infection within infected dairy cattle herds, especially low prevalence herds. This model showed that aggressive test and cull strategies will not eradicate infection in the short term and that inclusion of calf faecal shedding and variable adult shedding rates (including super-shedders) in the model made the outputs more realistic.

These findings are consistent with field experience where infection is still present in some low prevalence herds that have been on test and cull programs and stringent calf management for many years as discussed by Whitlock et al (below).

Whitlock et al presented a poster study supporting the modelling work of Mitchell et al described above. Using an extensive dataset collected over 20 years from an infected low prevalence dairy herd that had been using an aggressive test and cull program coupled with stringent management practices, these authors suggested that calf to calf transmission may be a significant factor in the maintenance of infection in this herd and made quantitative estimates of the transmission efficiency of cow-calf and calf-calf transmission.

Age susceptibility in sheep has been less studied than in cattle but an Australian study (Rast et al) provides evidence that age susceptibility does occur in sheep and that management practices (eg vaccination, strategic grazing or strategic culling) targeting subgroups within the flock, may be a more appropriate approach to management of OJD infected flocks where records are sufficiently accurate to define which groups have been exposed.

Role of non ruminant hosts in the epidemiology of M. ptb infections

1. Studies on the host range of Mycobacterium avium subsp paratuberculosis

Five studies looked at the host range of M. ptb and showed that the organism is common among a wide variety of vertebrates and invertebrates but does not appear to cause clinical disease in most. The host range may just reflect the organism’s ubiquity.

One comment made by Elizabeth Manning in her study was that “Contamination of the farm environment on infected farms by infected wildlife is negligible when compared to the volume of contaminated manure produced by infected domestic livestock. They may have some epidemiological significance in M. ptb free farms located in the geographic vicinity of infected farms.”

M. ptb has been demonstrated in a wide range of hosts in localities where infected livestock reside but evidence for maintenance of infection in wildlife populations in the absence of livestock is absent, except possibly in rabbits in Scotland. All these studies reported at the colloquium have been carried out in areas where high levels of infection occur or are suspected. Much lower isolation rates would be expected in areas where infection is less common or environmental conditions not favourable for survival of the organism in the environment.
<table>
<thead>
<tr>
<th>Location of study</th>
<th>Number wild animals sampled</th>
<th>M. <em>ptb</em> positive animals</th>
<th>Isolation rate of M. <em>ptb</em></th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spain (Alvarez et al)</td>
<td>455</td>
<td>2</td>
<td>0.4% (2/455)</td>
<td>Sampling on properties with history of M. <em>ptb</em> infection</td>
</tr>
<tr>
<td>Greece (Florou et al)</td>
<td>327</td>
<td>6</td>
<td>1.8% (6/327)</td>
<td>Sampling on properties with a history of M. <em>ptb</em> infection in goats and sheep flocks.</td>
</tr>
<tr>
<td>Czech republic (Kopecna et al)</td>
<td>3078 vertebrates</td>
<td>7</td>
<td>0.22% (7/3078)</td>
<td>No details on where samples obtained. RFLP analysis indicated commonality with livestock strains.</td>
</tr>
<tr>
<td></td>
<td>8489 invertebrates</td>
<td>5</td>
<td>0.06% (5/8489)</td>
<td></td>
</tr>
<tr>
<td>USA (Manning et al)</td>
<td>774 animals on 9 dairy and beef farms in Georgia and Wisconsin</td>
<td>30</td>
<td>3.87%</td>
<td>Shedding of M. <em>ptb</em> in faeces found in 7 animals (0.9%) – raccoon, cat, opossum, and armadillos.</td>
</tr>
</tbody>
</table>

2. *M. ptb* infection in deer

High herd prevalence of infection and clinical disease are common in deer particularly farmed deer. In NZ 90% of *M. ptb* isolates picked up through abattoir surveillance of farmed deer are the bovine strain (De Lisle). Abattoir surveillance is an important tool used to monitor tuberculosis in deer. In deer tuberculosis can be difficult to distinguish from *M. ptb* infection on gross pathology and there has been a large increase in the number of infected deer herds detected in the last five years.

Intrauterine transmission is also highly efficient in clinically affected deer. Of nine female deer (hinds) showing clinical signs of *M. ptb* infection, 9 of the 10 foetuses from these hinds were culture positive and most foetal tissues showed some pathology consistent with JD (van Kooten *et al*).

In another experimental study in NZ red deer, weaners showed a strong dose response histopathologically to oral dosing of the bovine strain but the ovine strain in this experimental study appeared less pathogenic in red deer weaners (Mackintosh *et al*).

In northern Italy in an area where co-grazing of red deer with livestock was common 50.3% of the 399 red deer found dead or killed were *M. ptb* positive on tissue culture (Fraquelli *et al*).

In another farmed deer study in the Czech republic on one farm the infection rate in 167 animals at slaughter was 53.2% (tissue culture) while on another farm 34.6% of animals were faecal culture positive (Kopecna *et al*).
Case reports of the pathology of *M. ptb* infection were also reported in Mouflon sheep and hog deer.

3. *M. ptb* infection in rabbits.

Judge *et al* reported on a high prevalence area in Scotland where the site infection prevalence levels in wild rabbit populations was 39.7% and 23% respectively. These sites had previously had infection demonstrated in both wild rabbits and in cattle and sheep.

The same authors also demonstrated vertical, horizontal and pseudo-vertical transmission in the wild rabbit populations and isolated *M. ptb* from a wide variety of rabbit tissues including the reproductive tract, foetus, placenta as well as milk and faeces. However clinical disease was not recorded in any of the rabbits studied.

The authors calculated the probability of vertical transmission at 0.458 and believe that infection can be maintained in the wild rabbit population in the absence of livestock but did not provide any experimental evidence to support this view.

In a separate study by the same authors, calves and lambs were grazed on pastures contaminated by varied concentrations of rabbit faeces. While sheep appeared to avoid ingesting rabbit faeces during grazing, calves did not and a single rabbit faecal pellet could constitute an infective dose for a calf.

**Risk factor and descriptive studies**

Progress in the diagnosis and eradication of BJD in a subtropical area in Australia over a 30 year study period was one of the oral presentations featured in the epidemiology stream. (Freeman *et al*)

Of the 78 herds in the study with a history of an infected herd status at some time more than half had successfully eradicated the disease from the herd. Most were beef herds and either partial or total de-stocking were essential elements of the eradication programs in the study herds. Lateral transmission of infection between herds in this study only occurred through introduction of infected animals or by sharing contaminated facilities. Water or feed contamination, straying and roadside walking were not found to be risk factors for infection in the 78 herds.

Evidence was also presented showing that infection was being detected earlier and eradicated more quickly than had been the case in the past due to greater awareness, more flexible programs and financial assistance packages.

Another Australian study by Dhand *et al* looked at the risk factors for OJD prevalence level in 92 infected flocks and using producer interviews evaluated the importance of various flock and management practices for the risk of low medium or high within-flock prevalence.

In a report by Aly *et al* from the USA, ELISA results for 625 dairy dam-daughter pairs were compared and it was found that daughters of positive dams had a 6.6 times greater risk of being seropositive reinforcing the importance of culling the progeny of seropositive animals.

Sampling of alpine water supplies in Italy from areas grazed during summer by cattle originating from a high prevalence area did not indicate that water was a significant risk factor for the spread of *M. ptb* infection (Pozzato *et al*).

**Prevalence studies**

A structured survey of Austrian cattle in 2002-2003 ((Khol *et al*) showed a 19% seroprevalence in individual cattle using an ELISA which was an increase on the seroprevalence detected in a 1999 survey. Microbiological testing detected 4 culture positive animals.
In a regional survey in Italy 35% of 100 dairy/beef cattle herds were seropositive to the ELISA while a survey of 400 cattle at an abattoir in Iran found 2.25% (9/400) were tissue culture positive. In India, of 50 buffaloes sampled at slaughter 48% (24/50) were tissue culture positive while 46.7% of 167 serum samples from buffalo were seropositive to the ELISA.

These studies do illustrate that \textit{M. ptb} infection is present in most countries who take the time to look for it and that it appears to be on the increase. The occurrence of infection in buffaloes in the tropics does suggest that the organism is adapted to a wide range of environmental conditions.

**Molecular epidemiology**

Studies in the USA, Mexico, Germany, the Czech Republic, Greece and Italy all reported on the use of molecular techniques such as Restriction Fragment Length Polymorphism (RFLP) in order to characterise the heterogeneity of \textit{M. ptb} strains and where multiple \textit{M. ptb} hosts are involved the likely source of infection for those hosts.

A number of new RFLP types were reported on, and in one cattle herd in Germany with multiple introductions, 15 \textit{M. ptb} strains were found representing 8 RFLP types. The German study also frequently encountered multiple strains in infected individuals.

RFLP is also useful to monitor the spread of \textit{M. ptb} between regions and provide evidence for the source of infection. It would assist tracing investigations particularly where new infections are detected in low prevalence areas.

**Economic and production studies**

Aspects of the financial costs associated with OJD infection in sheep flocks were examined in two Australian studies.

One study (Bush et al) looked at the costs associated with sheep mortalities due to OJD in 12 sheep flocks in NSW and found that on average OJD accounted for about two thirds of the total annual financial costs due to sheep deaths, and the gross margin for infected flocks was $10.90/ha less than that for uninfected flocks.

Also significant was the variation in mortality rates due to OJD.

<table>
<thead>
<tr>
<th>Year</th>
<th>Average Mortality rate</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>2002</td>
<td>6.2%</td>
<td>2.1%-17.5%</td>
</tr>
<tr>
<td>2003</td>
<td>7.8%</td>
<td>1.8%-14.6%</td>
</tr>
</tbody>
</table>

The second a case control study (McGregor et al) examined the impact of OJD infection on production parameters (body weight, reproduction, wool growth and quality) and showed that OJD has a significant production impact that increases as infection progresses from sub-clinical to clinical.

**Diagnostic test evaluation**

Evaluation of JD diagnostic tests such as faecal culture and ELISAs is limited by a lack of knowledge of the true infection status of individuals in a population (gold standard- i.e. tissue culture at slaughter).
Test evaluation using non-gold standard methods (comparing test results in 2 or more populations using conditionally independent tests) was discussed in several studies (Gardener et al). While these methodologies are reasonably precise when the prevalence in the populations being compared differs significantly, much larger sample sizes are needed when the prevalences are more similar.

A recently developed computer program to assist in estimates of the within flock prevalence based on pooled faecal culture results was also featured. This program uses seven different methodologies to create prevalence estimates and confidence intervals and the limitations and appropriateness of the different methodologies in field research were discussed (Toribio et al).
Epidemiology of Johne’s disease: Recent developments and future trends

Evan SG Sergeant
AusVet Animal Health Services
69 Turner Crescent Orange NSW 2800 Australia
Phone: 02 6362 1598; Fax: 02 6369 1473
Email: evan@ausvet.com.au

Abstract

Due to the prolonged incubation period and associated difficulties in studying Johne’s disease (JD), the epidemiology of this condition is still not well understood, despite the efforts of many researchers over the years. However, with the advent of new technologies and methods, progress in understanding the epidemiology of this complex disease is still being made.

Recent studies have focused on factors affecting transmission of infection, particularly levels of excretion, intrauterine transmission and transmission via semen and milk in sheep, and the role of recently infected young cattle in transmission. Risk factors for herd and animal level infection are also of considerable interest, although no clear pattern of identifiable risk factors have emerged, other than herd size and numbers of introductions. Further studies on transmission and risk factors will hopefully progressively clarify some of these issues over the next few years.

The currently available screening tests for JD are limited by the chronic nature of the disease, and their relatively poor sensitivity, particularly in the early stages of infection. Recent developments have led to refinement of methodologies and sampling strategies to improve the efficiency and cost-effectiveness of the current tests, particularly for herd and flock screening. However, new technologies and techniques are required before any substantial improvement in test performance at the individual animal level can be expected. These advances will hopefully arise over the next few years from ongoing studies using genomic and proteomic techniques to identify suitable testing targets indicative of early infection.

Simulation modelling has also been used extensively in recent years to help understand the epidemiology of JD and for the evaluation of management and certification options. As better data on epidemiological parameters becomes available the models are becoming more sophisticated and should become increasingly more reliable.

Introduction

Johne’s disease, or paratuberculosis, is a syndrome characterised by chronic, granulomatous enteritis affecting mainly ruminants, and is caused by Mycobacterium avium subsp. paratuberculosis (MAP). The disease is characterised by infection of young animals early in life and a long incubation period, so that clinical disease usually only occurs in adults. JD is now recognised as an increasingly important source of economic loss to producers and has become the focus of national and on-farm control programs in the last 10-20 years (Kennedy and Benedictus, 2001).

Because of the prolonged incubation period and associated difficulties in studying JD, the epidemiology of this condition is still not well understood, despite the efforts of many researchers over the years. However, with the advent of new technologies and methods, progress in understanding of the epidemiology of this complex disease is still being made, and this paper
summarises some of the advances made over the last few years, including some of the findings presented at this colloquium.

This paper focuses primarily on transmission of infection, risk factors for infection, developments in testing technologies and strategies and modelling of JD.

**Transmission**

Spread of paratuberculosis is primarily via the faeco-oral route, with clinically affected animals excreting large numbers of organisms and causing significant environmental contamination. Young animals are exposed to faecal contamination of the udder, fodder and the environment, providing ample opportunity for exposure. Although the faeco-oral route appears to be the main method of transmission, other methods can also be important in some situations.

Important factors affecting the successful transmission of MAP include the level of excretion by infected animals, the route of excretion, the source of exposure and the susceptibility of exposed animals.

1. **Excretion levels**

Recent research has attempted to quantify patterns of excretion of MAP from infected animals. The level and frequency of MAP excretion depend on the stage of disease and the type of lesions. For example, sheep with multibacillary lesions were found to be excreting MAP organisms continuously, whereas companion animals from the same flock, but which did not have detectable lesions, excreted MAP organisms only intermittently, possibly due to undetectable infection or due to passive passaging of organisms through the gut (Whittington *et al.*, 2000b). In the same research, the mean excretion rate for five sheep (4 with multibacillary lesions and 1 with no detectable lesions) was in excess of $10^8$ organisms per gram of faeces ($>8 \times 10^{10}$ per sheep per day), considerably higher than previous estimates of $10^5 – 10^6$ per gram of faeces in cattle (Jorgensen, 1982).

2. **Route of excretion**

As discussed above, the primary route of excretion is via faeces. However, MAP excretion via milk and semen and intra-uterine infection of the foetus have been demonstrated previously in cattle (Stehman, 1996; Sweeney, 1996) and also more recently in sheep (Eppleston and Whittington, 2001; Lambeth *et al.*, 2004). MAP excretion via these routes is usually more frequent in animals with more advanced disease or that are showing clinical signs (Sweeney, 1996; Lambeth *et al.*, 2004).

3. **Source of infection**

Until recently, the main source of infection has generally been assumed to be older animals in the clinical or pre-clinical phase of disease. Clinical cases of JD often excrete very large numbers of organisms (see above), and therefore represent the main source of exposure. Similarly, adult animals are more likely to be in more advanced stages of disease, and are therefore also a higher risk for intrauterine and trans-mammary transmission.

However, recent research has shown that calves and younger age cattle may excrete MAP organisms soon after infection (Bolton *et al.*, 2005; van Roermund and de Jong, 2005; Weber *et al.*, 2005a). Modelling of MAP transmission has also supported the hypothesis that infected calves provide an important contribution to the spread of infection in infected cattle herds (van Roermund *et al.*, 2002; Mitchell *et al.*, 2005). Although this source of exposure may be of lesser significance in heavily infected herds, it suggests that young animals from infected herds or flocks are an important
factor in disease transmission and may pose a greater risk of spreading infection than was previously considered likely.

Studies of environmental samples from infected dairies have also identified high-risk areas for environmental contamination with MAP, and that levels of MAP contamination were correlated with percentage of culture-positive faecal-pools in the dairy herd (Raizman et al., 2004). Areas with high proportions of positive samples included cow alleyways and manure storage areas, while lower (but still important) proportions of positive samples were observed for the calving area and sick cow pens. Proportions of positive samples were lowest in water run-off and post-weaned calf areas.

4. Susceptibility of exposed animals

In cattle, young calves have generally been regarded as highly susceptible to MAP infection, with susceptibility declining with age (Sweeney, 1996). There has been a general assumption that a similar situation exists for sheep, although this has been based on extrapolation from cattle rather than on any sheep-based research. However, recent research is starting to shed more light on the situation in sheep. In one study, there was no difference in infection rates of lambs, weaners or adults following similar levels of exposure to MAP under natural conditions of heavy contamination (Reddacliff et al., 2004). This finding suggests that any age-resistance effect in sheep might not be as clearly defined as in cattle, and that under conditions of heavy challenge adult sheep can be just as susceptible to infection as young sheep. In contrast, observations on one recently infected property in Australia found that infection was strongly clustered in sheep exposed as lambs and that infection was not detectable in sheep exposed as adults, suggesting that some age-related resistance does occur but may be dependent on level of exposure. Additional research is still required to further clarify this situation, but age-related resistance to infection in sheep appears to be less-pronounced than is assumed to be the case in cattle.

Risk factors for Johne’s disease infection

The identification of risk factors for JD presents significant challenges to researchers, particularly because of the chronic nature of the disease, and the lack of reliable diagnostic tests for detection of infection. In addition, introduction of infected animals is generally recognised as the main risk factor for introduction of infection, so that most studies have concentrated on factors associated with prevalence or severity of disease in infected herds or flocks, rather than the presence or absence of infection.

1. Cattle

A number of recent studies have been undertaken to investigate possible risk factors for JD in cattle. Because of the limitations of test performance, these studies generally focused on risk factors for seropositivity for JD (at either cow or herd level), rather than for confirmed infection, and were mainly in dairy cattle. Risk factors that were identified included:

- herd size (Hirst et al., 2004; Muskens et al., 203);
- purchasing of replacements and rate of importation of replacements (Chi et al., 202; Hirst et al., 2004); and
- previous occurrence of clinical signs of JD (Hirst et al., 2004).

Increased risk of infection with increasing herd size could be related to the larger numbers of introductions into the herd (Hirst et al., 2004) or to increased cattle density resulting in greater environmental challenges (Muskens et al., 203). Purchasing of replacement animals is a logical risk factor for herd-infection with JD and the greater the number of animals introduced the greater the
likelihood that an infected animal will be introduced. In contrast, the occurrence of clinical signs of JD is probably a reflection of the presence of disease and perhaps of a higher prevalence of disease, rather than a risk factor for infection.

In addition, in one study in beef cattle herds in Texas, USA (Roussel et al., 2005), risk factors for seropositive animals included:

- species of cattle (seroprevalence was higher in *Bos indicus* than *Bos taurus*)
- geographic location in the State of Texas; and
- water source (seroprevalence was higher for cattle watered on a running stream or river).

However, only 7% of the seropositive cattle were positive on follow-up faecal culture, so that many of the serological reactions are likely to be false-positive results. Therefore, the risk factors identified are not necessarily associated with the occurrence of JD, and could be associated with some other factor leading to false positive serological reactions, highlighting the problems of basing JD studies on the results of serology.

One study, undertaken in Scotland, investigated risk factors for the suspected occurrence of JD on individual farms based on official records and owner knowledge of farm history to identify case and non-case farms, rather than using serology (Daniels et al., 2002). Risk factors identified for case farms included:

- herd size (total number of sheep and cattle);
- frequency of application of manure to pasture;
- number of rabbits present;
- wildlife access to stored feed; and
- type of water supply for cattle (piped or piped and open vs open only).

The effect of some of these factors was unexpected, and may have been due to confounding by farmers on infected farms taking active control measures. For example, application of manure to pasture ‘often’ was associated with a lower risk of JD than application sometimes or never. Similarly, use of an open water source was associated with a reduced risk compared to use of piped water, despite the use of open water sources being potentially subject to contamination and hence posing a higher theoretical risk.

Finally, one Australian study investigated the occurrence of clinical cases in infected dairy herds (Ridge et al., 2005). This study found no association between the continued occurrence of clinical cases or serological reactors and three recommended control measures for JD in dairy cattle. The control measures considered were: early removal of calf from dam; separating unweaned calves from adult cattle and effluent from adult cattle; and not grazing weaned calves on pastures grazed by adult cattle or that have been treated with effluent from adult cattle. The lack of significant effect of these measures might have been due to the relatively small sample size in the study. The study did identify that feeding of antibiotic contaminated waste milk and providing water to calves were associated with an increased occurrence of cases, while allowing cows to calve in paddocks was associated with decreased occurrence of cases. The reasons for these associations are unclear, but it is interesting to note that all of these factors are associated with the early neonatal period, supporting previous hypotheses that this is the most critical period for infection of calves.
Although an association between soil type (particularly acid soils) and occurrence of JD has been suggested (Johnson-Ifeareulundu and Kaneene, 1997) and application of lime to pasture was found to be protective in one previous study (Johnson-Ifeareulundu and Kaneene, 1998), the results of recent studies have been mixed. Two of the recent studies discussed above which specifically considered (acid) soil type as a potential risk factor found no evidence of association (Daniels et al., 2002; Muskens et al., 2003). However, one other study of the spatial distribution of infected herds in Indiana found that there was significant clustering of infected herds according to soil type (Ward and Perez, 2004), with infected herds more likely to occur on low-silt, sandy-loam or loam soils (soil types that are likely to be more acid). These findings were similar to the findings of a previous study on association between soil type and JD in sheep in Spain (Reviriego et al., 2000).

2. Sheep

One study in Australia has investigated risk factors for JD infection in sheep flocks (Lugton, 2004). This study used a postal questionnaire to owners of known-infected sheep flocks to evaluate risk factors for the level of disease observed on the farms. In this study, a large number of potential risk factors were considered, as well as several different measures of the occurrence of JD on the study farms. Because of the design limitations, further investigation is required to confirm the potential risk factors identified. In addition, there were no identified factors that were consistently associated with the occurrence of JD across multiple analyses.

Risk factors with a statistically significant association with occurrence of JD in the various analyses are summarised in Table 1.

Table 1: Summary of risk factors for occurrence of Johne’s disease in known infected flocks in Australia (Lugton, 2004).

<table>
<thead>
<tr>
<th>Outcome measure</th>
<th>Factor</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Farmer observed cases of Johne’s disease</td>
<td>Estimated time the flock had been infected</td>
<td>&gt; 10 yrs compared to 2-10 yrs and &lt; 2 yrs.</td>
</tr>
<tr>
<td>Altitude</td>
<td></td>
<td>&gt; 800m. The reason for this difference is unclear.</td>
</tr>
<tr>
<td>Predominant breed of sheep</td>
<td>Fine wool merinos vs other breeds. This might be an inherent difference in susceptibility or could be due to management factors that were not captured elsewhere.</td>
<td></td>
</tr>
<tr>
<td>Treatment of illthrifty sheep</td>
<td>Removal from flock vs drenching or no treatment. This probably reflects a response to more severe disease than a causal factor.</td>
<td></td>
</tr>
<tr>
<td>Estimated incidence of deaths due to Johne’s disease</td>
<td>Lamb marking percentage</td>
<td>This is most likely a result of Johne’s disease rather than a cause.</td>
</tr>
<tr>
<td>Age at culling</td>
<td>Association between incidence of Johne’s disease and an older age at culling is probably a result of the need to retain sheep for longer in</td>
<td></td>
</tr>
</tbody>
</table>
Proportion of quality pasture | Flocks with a higher proportion of quality pasture had a higher reported incidence, but the reason for this association is unclear.

Weeks of hand feeding | Flocks that hand fed for longer had a higher incidence, possibly associated with greater exposure to faecal contamination of fodder on the ground.

The youngest age at which sheep die from Johne’s disease | Incidence of disease | This was the one factor that accounted for most of the variation in age of youngest cases.

Soil texture | Cases occurred at a younger age in flocks on sandy soils. This could be associated with soil acidity, although soil pH and fertilizer application that affect soil pH were not important in any of the models.

Proportion of quality pasture | Flocks with a higher proportion of quality pasture had cases at a younger age. Again the reason for this association is unclear.

Breeding replacement sheep | Introduction of replacements reduced the age of youngest cases, possible by reducing the overall level of contamination on the farm.

Treatment of illthrifty sheep | Removal from flock vs drenching or no treatment. This probably reflects a response to more severe disease than a causal factor.

Age at weaning | Weaning at > 5 months of age. This may be a result of a longer period of exposure to high levels of contamination as highly susceptible lambs.

Additional analyses were undertaken with the seasonality of occurrence of cases and the occurrence of scouring as a clinical sign for OJD. The interpretation of these analyses was unclear and they are not considered further here.

### 3. Summary

To summarise, there are still no clearly identified risk factors for MAP infection and clinical disease at either the animal or herd/flock levels, other than herd size and introduction of potentially infected animals. Additional evidence supporting the role of soil type and acid soils is inconsistent, but this area perhaps warrants further research. Additional research focused on farm-level risk factors for infection and disease, such as that currently being undertaken in sheep in Australia will hopefully provide more insight into this complex area.
Limitations of current testing for Johne’s disease

One of the greatest difficulties in understanding the epidemiology of JD is the limitations of the available screening tests. These limitations are mainly associated with the chronic nature of the disease, and the fact that current tests have very poor sensitivity in the early stages of infection. Currently available tests are mainly based on serology (eg ELISA or AGID), faecal culture in solid or liquid media and PCR testing on faeces, milk or tissue samples for the detection of the unique DNA sequence IS900.

Recent research on screening tests for Johne’s disease has focused mainly on:

- optimising sampling strategies to achieve acceptable sensitivity at reduced cost;
- adapting existing tests to new samples or approaches; or
- refining and improving existing tests and technologies.

Pooling of faecal samples for culture is being investigated or applied in a number of countries and for both sheep and cattle (Whittington et al., 2000a; Tavornpanich et al., 2004; Fyock et al., 2005a; Weber et al., 2004; van Schaik et al., 2003b; Antognoli et al., 2005). Pooled culture is primarily used as a tool for identification of infected herds or flocks or conversely for certification of low-risk herds and flocks. It has also been evaluated for identification of individual infected animals for culling, but has limited value for this purpose depending on prevalence of infection (van Schaik et al., 2003b). Environmental sampling has also been used as an alternative to faecal sampling for the identification of high-risk areas on dairy farms and for evaluating the effectiveness of control programmes (Fyock et al., 2005a; Raizman et al., 2004; Lombard et al., 2005).

Johne’s disease ELISA’s have also been adapted, particularly for use with milk instead of blood samples. These have been used or proposed for both individual cow testing and for bulk milk testing. Although the milk ELISA shows promise as an alternative individual animal test to serology (Nielsen et al., 2002; Hendrick et al., 2005; Collins et al., 2005), it is likely to be of limited value as a bulk milk test for surveillance purposes (Nielsen et al., 2000; Weber et al., 2005b). Despite this, simulation studies suggest that bulk milk screening might be a useful tool to support milk quality assurance programmes (Weber et al., 2005b). Similarly, culture of bulk milk samples has also been evaluated, but was found to be less sensitive than bulk-milk PCR (Stabel et al., 2002).

Some existing tests have also been adapted or improved by uptake of newer technologies. For example, liquid culture media have the advantage of supporting more rapid growth of MAP and hence shorter culture periods for confirmation of infection (Fyock et al., 2005b). Early liquid culture systems relied on radioactivity in the media for detection of growth. Newer technologies have now allowed the use non-radioactive media, providing a more useful and efficient culture process with the potential to replace existing solid and liquid media systems (van Schaik et al., 2003a; Fyock et al., 2005b).

Despite these advances that have been made in broadening the application and efficiency of testing systems, there have been no fundamental changes to the approaches used, so that the available tests are still limited in their ability to detect early infection in individual animals.
Role of Modeling

Considering the difficulties and cost involved in conducting large studies on JD, modeling of the disease has become increasingly popular in recent years. Most of these models are based on core aspects of our understanding of the epidemiology of JD (for example age-related resistance to infection, the effect of calf-management practices and the long pre-clinical period) to predict the course and dynamics of infection in infected herds or flocks. Most models also incorporate stochastic elements to reflect uncertainty about some of the epidemiological parameters. The models have been used for a number of different purposes, including:

- to further investigate the epidemiology and dynamics of the disease in infected herds (Pouillot et al., 2004; Mitchell et al., 2005);
- to evaluate alternative strategies for management and control of JD in infected herds (Groenendaal et al., 2002; van Schaik et al., 2003b; Dorshorst and Collins, 2005); and
- to evaluate national or industry-wide strategies for herd-certification and disease management (Weber et al., 2004; Weber et al., 2005b; Tavornpanich and Gardner, 2005).

Although such models have proved very useful in supporting recommendations for on-farm control or for development of national control and certification programmes, they are still limited by the underlying assumptions and the lack of detailed knowledge of some aspects of JD epidemiology. As our understanding of the epidemiology of this complex disease improves so will the reliability and utility of the various models being used.

Future perspectives

What can we expect (or hope for) from the future for our understanding of JD epidemiology?

1. Further research and understanding of transmission mechanisms and risk factors for infection, including age-susceptibility and infectious dose, the role of environmental factors such as soil type and the identification of other risk factors that can be manipulated to help control the disease.

2. More basic research into the epidemiology and pathogenesis of JD utilising new technologies such as genomics and proteomics.

3. Development of new tests and testing technologies based on the outcomes of the fundamental research and other new technologies being developed in the bio-medical field. New tests are likely to focus particularly on the early detection of infection.

4. Sophisticated models based on sound epidemiological data to predict disease transmission and progression and the impact of a complex mix of control measures on occurrence of both infection and clinical disease.

Perhaps the day is coming when the farmer can use a pen-side test to identify at-risk animals before their first joining and log onto the internet and answer some simple questions about his/her farm to receive customised advice (based on a sophisticated model running remotely) on how best to manage the disease in the current farm environment, for both individual infected animals and the herd as a whole.
Acknowledgment

I wish to acknowledge the financial support of Meat and Livestock Australia and AusVet Animal Health Services who supported my attendance at the Colloquium and to the Colloquium Organising Committee for their invitation to present this paper.

References


