



# Field Evaluation of the Tracer Weaner Model

Early Detection of natural infection of sheep with Mycobacterium avium subsp. paratuberculosis

Project number OJD.014

Report prepared for MLA by:

NSW Agriculture Elizabeth Macarthur Agricultural Institute

Meat & Livestock Australia Limited Locked Bag 991 North Sydney NSW 2059

ISBN 1 74036 201 2

# November 2002

MLA makes no representation as to the accuracy of any information or advice contained in this document and excludes all liability, whether in contract, tort (including negligence or breach of statutory duty) or otherwise as a result of reliance by any person on such information or advice.



Animal Health and Welfare

#### ABSTRACT

Experiments were conducted on two separate farms in the endemic area for OJD to determine whether tracer animals could be used to detect infective levels of S strain *M. a. paratuberculosis* on pasture. Culture from tissues was shown to be the most sensitive method for the detection of early infection in sheep after natural exposure to S strain *M. a. paratuberculosis*. The organism was detected in at least one naive introduced sheep from every potentially exposed group, 6 to 12 months post-exposure. Antemortem diagnostic tests (skin testing, IFN- $\gamma$  and faecal culture) were shown to have low sensitivity at this early stage of naturally acquired disease. The prevalence of infection early after exposure was similar in sheep first exposed as neonates, as weaners or as adults. Lambs born from an infected flock became tissue culture-positive sooner than naive lambs suckling uninfected ewes introduced to the same infected environment. These findings suggested that groups of naive sheep, used as tracer animals and tested by tissue culture at slaughter after 6 months exposure, might be useful to assess pasture infectivity in disease control programs.

#### EXECUTIVE SUMMARY

Eradication of ovine Johne's disease (OJD) requires de-stocking sheep and spelling land until *M. a. paratuberculosis* has died out. The use of tracer animals (sentinels) can provide an early warning that infectious organisms are still present. However, Johne's disease differs from most other infectious diseases of farm livestock because of the long incubation period, and a method for early detection of infection in tracer animals was needed. In a pilot study (Project TR.073), 100% of weaner sheep experimentally infected with  $>10^7$  organisms of S strain *M. a. paratuberculosis* were detected by culture of tissues two to four months later, and skin-testing with Johnin detected 66% of infected sheep with 100% specificity. If such early detection were also possible in naturally exposed sheep, and particularly if skin testing allowed targeting of likely-infected sheep for necropsy, then the use of tracer weaner sheep to determine the possible infectivity of pasture could be a practical option in the control of OJD.

Experiments were conducted on two separate farms in the endemic area for OJD. Despite significant uncontrolled differences, a general ranking of likely levels of exposure to *M. a. paratuberculosis* from very high to low was possible. The examination of the tracer concept in several different environments was considered important, if the technique was to be used in the future to examine pasture infectivity in a wide range of field situations.

In the most heavily contaminated environment, 43 to 75% of sheep sampled  $\geq$ 8 months post-exposure were culture-positive. Infection in introduced naive sheep was first detected by culture of tissues 5 - 6 months after potential exposure. In environments with lower levels of contamination, infection with *M. a. paratuberculosis* was detected by tissue culture in at least one naive introduced sheep from every potentially exposed group, 6 to 12 months post-exposure. In the lowest contamination scenario, where pasture had been grazed by infected ewes for two months only, then left ungrazed for a month before the introduction of tracers, a single infected sheep was detected 12 months after first exposure.

Antemortem testing for specific CMI responses was not useful in the field application of the tracer concept. Skin-tests and IFN- $\gamma$  tests had very low sensitivities in samplings <12 months post-exposure.

The preferred sites for tissue culture at necropsy were refined. Tonsil is not recommended as a sampling site. Its culture did not add significantly to the number of infected sheep detected, removal during necropsy was time-consuming, and tonsil would also be highly impractical to sample from lines of sheep at an abattoir, where future monitoring might take place. When freshly pooled homogenates were cultured alongside the original tissue homogenates, no differences in isolation or contamination rates were seen. Thus, culture of pooled homogenate from TI, ICV and MLN is recommended as an economical alternative to culture of individual tissues for the detection of early *M. a. paratuberculosis* infection.

This study confirmed that culture of tissues is the most sensitive method currently available for the detection of early *M. a. paratuberculosis* infection, and demonstrated tissue culture as a practical tool for the detection of early infection in sheep after natural exposure. In contrast, routine ante-mortem testing in the first 12 months post-exposure detected few of the infected sheep. Skin-tests detected 28%, IFN- $\gamma$  tests detected 10% and faecal culture detected 12%. The low sensitivities for these tests in this study reflect their use in the very early stage of the disease process and were not unexpected. Another finding of particular interest was that histological lesions, often used as the "gold standard" for *M. a. paratuberculosis* infection in sheep, were rare in sheep exposed to *M. a. paratuberculosis* for less than 12 months. Only 17% of culture-positive sheep had typical histological lesions.

These experiments also suggested that age has little effect on the early establishment of infection. Naive neonates, weaners or ewes had similar infection levels after similar periods of exposure. However, lambs born from an infected flock became tissue culture-positive sooner than naive lambs suckling uninfected ewes introduced to the same infected environment. This trial did not attempt to examine the effect of age of exposure on subsequent disease development.

Overall, this study suggests that groups of naive sheep of any age, tested at slaughter by culture of pooled tissues from individual sheep after 6-12 months exposure, could be utilised as practical and economical tracers to assess pasture infectivity in disease control programs.

#### ACKNOWLEDGMENTS

Most of the investigations reported here were performed as part of the PhD studies of Leslie Reddacliff, and comprise Chapter 4 of her thesis, "Aspects of the pathogenesis of Ovine Johne's disease", which has been available in draft form since March 2002. Special thanks are due to Richard Whittington who conceived the project, supervised the PhD program, and provided invaluable guidance; advice and practical hands-on help throughout. Particular thanks are also due to Terry and Cecily Hayes of "Hillwood" (Farm H) for their ongoing cooperation, practical on-farm assistance and support. Investigations on Farm A (Sydney University, Arthursleigh) were done in conjunction with Kym Abbott and Helen McGregor. At EMAI, Shayne Fell, Vanessa Saunders, Anna Waldron, Aparna Vadali and Lisa Smith provided skilled technical assistance. Gamma interferon EIAs were kindly facilitated by Stephen Jones of CSL. Evan Sergeant provided useful epidemiological and statistical advice.

#### **Table of Contents**

| ABSTRACT  | 1  |
|---|----|
| EXECUTIVE SUMMARY   | 2  |
| ACKNOWLEDGMENTS   | 3  |
| 1. BACKGROUND AND INDUSTRY CONTEXT                                      | 6  |
| 2. PROJECT OBJECTIVES   | 7  |
| 3. METHODS  | 8  |
| 3.1. Experimental design  | 8  |
| 3.2. Intradermal testing for delayed hypersensitivity (DTH)             |    |
| 3.3. Gamma interferon (IFN-γ) assay                                     | 10 |
| 3.4. Agar gel immunodiffusion test                                      | 10 |
| 3.5. Necropsy sampling  | 11 |
| 3.6. M. a. paratuberculosis isolation                                   | 11 |
| 3.7. Histopathology   | 11 |
| 3.8. Classification of sheep as infected                                | 12 |
| 4. RESULTS  | 13 |
| 4.1. Overview of findings on Farm H                                     | 15 |
| 4.2. Overview of findings on Farm A                                     | 16 |
| 4.3. Culture of M. a. paratuberculosis                                  | 17 |
| 4.4. Comparison of culture of individual tissues and pooled homogenates | 17 |
| 4.5. Intradermal testing for delayed hypersensitivity                   | 17 |
| 4.6. IFN-γ  |    |
| 4.7. Agar gel immunodiffusion test                                      | 18 |
| 4.8. Histopathology   | 19 |
| 5. DISCUSSION   | 20 |

| 6. RECOMMENDATIONS FOR PRACTICAL USE OF THE TRACER CONCEPT IN THE FIELD. | 24 |
|--|----|
| 7. SUCCESS IN ACHIEVING OBJECTIVES                                       | 25 |
| 8. IMPACT ON MEAT AND LIVESTOCK INDUSTRY                                 | 27 |
| REFERENCE LIST   | 28 |
|  |    |
|  | 31 |

# **1. BACKGROUND AND INDUSTRY CONTEXT**

Eradication of ovine Johne's disease (OJD) requires de-stocking sheep and spelling land until *Mycobacterium aviium* subsp. *paratuberculosis* (*M. a. paratuberculosis*) has died out. At the conclusion of a decontamination period, currently deemed to be 2 consecutive summers or 15 months, there is no simple way to assess either the residual level of contamination of the environment or the risk residual contamination poses to livestock. The sheep (S) strains of *M. a. paratuberculosis* have been shown to survive for 13 months on shaded pasture, and for 7 months in exposed situations,<sup>38</sup> but such studies will not determine whether infectious doses of the organism remain or whether these viable organisms are available to sheep. For example they might be caught in the soil profile and might pose no risk.

For the above reasons it was thought necessary to develop an infection model that uses live sheep. Such an approach is used in other disease control or monitoring programs, particularly for viral diseases, and the animals involved are known as tracers or sentinel livestock. The tracers or sentinels provide an early warning that an infectious disease is present or active. Johne's disease differs from most other infectious diseases of farm livestock because the incubation period is measured in years not days or weeks. This is a major disadvantage for use of tracer animals. There is a need to find a way of detecting the infection soon after it has occurred. A tracer weaner pilot study (Project TR.073) was undertaken in 1999-2000 for this reason.

In the pilot study, 100% of weaner sheep experimentally infected with  $>10^7$  organisms of S strain *M. a. paratuberculosis* were shown to have become infected by culture of tissues two to four months later, and skin-testing for delayed type hypersensitivity (DTH) to Johnin detected 66% with 100% specificity. If such early detection were also possible in naturally exposed sheep, and particularly if skin testing allowed targeting of likely-infected sheep for necropsy, then the use of tracer weaner sheep to determine the possible infectivity of pasture could be a practical option in the control of OJD.

There are, however, a number of potential problems in extending the tracer weaner pen model to the field situation. Firstly it is not possible to predict with accuracy when infection might be first acquired, nor the size of any infectious dose. Large bolus doses or repeated small doses might both occur. Availability of infectious organisms might vary with season and climate, with pasture conditions and with changes in grazing behaviour of the sheep. While contamination levels on spelled pasture can be expected to drop over time, "hot spots" such as around sheep camps, watering points, other wet areas, heavily shaded areas or hand feeding areas may have high residual levels. The chance of exposure of lambs to infective levels of *M. a. paratuberculosis* will vary from one particular field situation to another. With regard to the use of the skin test to target likely-infected sheep for necropsy, exposure of pastured sheep to a wide variety of potentially cross-reacting environmental organisms is possible. Other effects on the ability to mount a good cell-mediated immune (CMI) response, such as intercurrent infections or general environmental stressors may further complicate the picture.

Since the early conception of the tracer studies, the emphasis for control of OJD has moved away from eradication using de-stocking, and towards control using vaccination. Many situations remain, however, where eradication on particular properties will be required, and the potential for tracer animals to provide early warning of the continued activity of *M. a. paratuberculosis* may be important. Moreover, the insights into the early pathogenesis of *M. a. paratuberculosis* infection that this project could provide will have flow-on benefits for many aspects of OJD control and policy formulation. Insights into the risk of infection of recently exposed sheep of different ages and the performance of diagnostic tests in the early stages of infection will be of particular interest to industry and regulators.

# **2. PROJECT OBJECTIVES**

- Determine whether the tracer concept will work in the field in a heavily infected environment.
- Assuming the above, will the tracer concept work in the field in environments with lower levels of infectivity?
- Determine whether antemortem testing for specific CMI responses would allow selection of likelyinfected sheep for necropsy.
- To further refine the sites for culture at necropsy, and to evaluate the effects of pooling homogenates from several sites.
- Provide further insights into the pathogenesis of OJD in the early stages post-infection, in particular the effects of age at first exposure on subsequent *M. a. paratuberculosis* infection rates, and the performance of routine diagnostic tests.

# 3. METHODS

#### 3.1. Experimental design

The examination of the tracer concept in several different environments was considered important, if the technique is to be used in the future to examine pasture infectivity in a wide range of field situations. Consequently, the experiments were conducted on two separate farms, both located in the southern highlands of NSW in the endemic area for OJD. While the same methods were applied to the experimental sheep on the two farms, and a general ranking of likely levels of exposure to *M. a. paratuberculosis* was possible, there were significant uncontrolled differences (source of sheep, pasture availability, watering methods, stocking rates, infection history) which complicate direct comparison between the two farms.

**Farm H:** The design is summarised in Table 1. Experiments were conducted over two successive years, commencing in December 1999. The first year was essentially a pilot trial that extended the pen trial to a highly contaminated field environment. If tissue culture at necropsy could detect *M. a. paratuberculosis* infected sheep within several months of exposure, then further work was warranted. If not, there would be little point in extending field trials to lightly contaminated environments. In the second year on Farm H, the effect of age at first exposure was examined, as well as comparing sheep first exposed as weaners over the two successive years.

| Year      | Group                | Source | No. of<br>sheep | No. killed<br>at each<br>sampling | Exposure<br>level | Age at first<br>exposure | Time of first<br>exposure <sup>a</sup> | Sampling<br>schedule <sup>a</sup> |
|-----------|----------------------|--------|-----------------|-----------------------------------|-------------------|--------------------------|--|-----------------------------------|
| One       | Lamb-1W              | EMAI   | 40              | 10                                | high              | weaner                   | 0                                      | 2,4,6,12                          |
| (99-2000) | Lamb-1C              | EMAI   | 10              | 2                                 | none <sup>d</sup> | na                       | na                                     | 2,4,6,12                          |
| Two       | Ewe-2L <sup>e</sup>  | EMAI   | 30              | 10                                | high              | adult                    | -3                                     | 0,3,5                             |
| (2000-01) | Lamb-2L <sup>e</sup> | EMAI   | 38              | 10                                | high              | neonate                  | -3                                     | 0,3,5,9                           |
|           | Ewe-2W <sup>f</sup>  | EMAI   | 20              | 10                                | high              | adult                    | 0                                      | 3,5                               |
|           | Lamb-2W <sup>f</sup> | EMAI   | 20              | 10                                | high              | weaner                   | 0                                      | 3,5                               |
|           | Lamb-2H              | Farm H | 60 <sup>b</sup> | 10                                | high              | prenatal?                | -8?                                    | 0,3,5,9,15,21                     |
|           | Ewe-2C <sup>c</sup>  | EMAI   | 10              | 0                                 | none <sup>d</sup> | na                       | na                                     | 3,9                               |
|           | Lamb-2C <sup>c</sup> | EMAI   | 10              | 0                                 | none <sup>d</sup> | na                       | na                                     | 3,9                               |

#### Table 1. Experimental design for field evaluation of tracer weaner concept on Farm H

<sup>a</sup> Expressed as months relative to weaning of the lamb groups

<sup>b</sup> Sheep sampled at 15 and 21 months in this group were at the same times as cohorts in concurrent biopsy project The final sampling was in September 2002.

These sheep were not necropsied, but used only as controls for skin testing and IFN-γ assay.

d Controls held at EMAI

с

<sup>e</sup> Transported immediately prior to lambing to Farm H. Group Lamb-2L are offspring of Group Ewe-2L, born on Farm H.

Transported at weaning (3 months) to Farm H. Group Lamb-2W are offspring of Group Ewe-2W.

*Exposure levels (pasture contamination):*This farm was considered to be very highly contaminated with *M. a. paratuberculosis.* Annual mortalities of up to 20% from OJD amongst older sheep had been experienced, and seropositivity in the agar gel immunodiffusion test (AGID) amongst random groups of 50 to 133 clinically normal sheep 2-3 years of age ranged from 8 to 16%. Because the sensitivity of the AGID is low, and moreover, because the majority of AGID-positive sheep have the multibacillary form of

OJD (and therefore likely to be heavy faecal shedders of *M. a. paratuberculosis*),<sup>28</sup> these figures indicate that very high levels of environmental contamination were likely. In these trials, continuous high contamination levels were further assured by running the experimental sheep together with flocks experiencing on-going mortalities due to OJD. In Year one, experimental weaners were run with a flock of infected wethers and in Year 2 experimental lambs, weaners and ewes were run with a flock of infected ewes. The sheep were stocked at levels typical of fine-wool merino enterprises in the tablelands, in large paddocks used in the routine farm enterprise. All feed was provided by the pasture and water was from streams and farm dams. As there was no pasture on Farm H considered free of contamination, control sheep were run on pasture at the Elizabeth Macarthur Agricultural Institute (EMAI). Subjective observations of prevailing pasture conditions were made at each sampling time. Pasture was classified as short, medium or long, and as green or dry. Monthly rainfall data was obtained from the owners of Farm H, an official measuring site for the Bureau of Meteorology.

Animals: In the first year, Merino weaners were from the OJD-free EMAI flock, and of the same source flock, genetic background and age as those used in the earlier pen trial. In the second year, Merino lambs from Group Lamb-2H were the offspring of the infected ewe flock on Farm H, while Merino sheep in all other groups were from the OJD-free EMAI flock, although from a different line of sheep to those in Year 1. These groups enabled comparisons between first exposure as lambs, weaners or adults, and also with lambs born from potentially infected mothers. Inclusion of Group Lamb-2W enabled a comparison of the effect of different years (different climate and pasture conditions). In addition, some sheep from Group Lamb-2H were retained for supplementary sampling at 18 and 24 months of age (ie beyond the period of concern for tracer studies).These supplementary samplings were designed to ascertain whether the pattern of disease in this cohort of sheep was typical of that experienced previously on this farm, and also to provide baseline data for the concurrent biopsy trial (Individual Animal Tests, OJD.020). Results from these additional samplings were considered separately from the main trial.

**Farm A:** The design is summarised in Table 2. Field aspects of experiments on Farm A were conducted concurrently with those in Year 1 on Farm H, but samples collected at necropsy were stored, and only processed after the first positive culture results from Farm H became available. The effects of two different levels of contamination were examined, as well as the effect of age of first exposure (weaner or neonate).

| Group     | No. of<br>sheep | No. killed<br>at each<br>sampling | Level and duration<br>of exposure              | Age at first<br>exposure | Time of first<br>exposure <sup>c</sup> | Sampling<br>schedule <sup>c</sup> |
|-----------|-----------------|-----------------------------------|--|--------------------------|--|-----------------------------------|
| A-High-W  | 40              | 10                                | high from weaning <sup>a</sup>                 | weaner                   | 0                                      | 2,4,6,12                          |
| A-Low-W   | 40              | 10                                | low from weaning                               | weaner                   | 0                                      | 2,4,6,12                          |
| A-High-LW | 32              | 8                                 | high from birth and from weaning <sup>ab</sup> | neonate                  | -3                                     | 2,4,6,12                          |
| A-High-L  | 32              | 8                                 | high from birth to weaning only <sup>b</sup>   | neonate                  | -3                                     | 2,4,6,12                          |
| A-Control | 20              | 5                                 | none   | na                       | na                                     | 2,4,6,12                          |

| Table 2. Experimental design | for field evaluation of tracer | weaner concept on Farm A |
|------------------------------|--------------------------------|--------------------------|
|------------------------------|--------------------------------|--------------------------|

<sup>a</sup> These sheep shared the same pasture after weaning.

<sup>b</sup> These sheep shared the same pasture from birth until weaning.

<sup>c</sup> Expressed as months relative to weaning

*Exposure levels (pasture contamination):* Farm A had a past history of low prevalence OJD infection and unlike Farm H, mortalities had never occurred. A series of small paddocks (5 to 10 ha) with pasture of varying contamination levels had been prepared as part of other research (OJD.002, Epidemiology of OJD) and were available for use in this trial. Occasional supplementary feeding was necessary and water was provided from troughs. **Control pasture**, considered free of *M. a. paratuberculosis* contamination, had carried no infected sheep for at least 18 months and had been grazed by cattle in the interim. **Low contamination pasture** had been grazed by infected ewes for two months only, commencing 3 months prior to weaning, so that pasture was free of infected sheep for one month prior to the introduction of experimental weaners. **High contamination pasture** had been grazed by infected ewes for six months,

commencing four months prior to weaning. Thus Group A-High-LW was running with infected ewes from birth until 2 months post-weaning, Group A-High-L from birth until weaning and Group A-High-W from weaning until 2 months post-weaning. The infected ewes used for these contaminations had been specifically purchased from another heavily infected property, and were from a flock experiencing annual OJD mortalities of up to 10% and with a seropositivity rate of 12.7%. In contrast to the situation on Farm H where pasture contamination levels were high and continuous, pasture contamination levels on Farm A were likely to be dropping over time because the infected adults were removed before or soon after weaning. In previous studies, a 2 log reduction in numbers of viable *M. a. paratuberculosis* on pasture occurred over a 10 week period.<sup>38</sup>

*Animals:* All experimental lambs and weaners were surplus offspring from a flock of OJD-free Merino ewes purchased specifically for other OJD research on Farm A. They were unlikely to be of the same genetic line as those used on Farm H.

#### 3.2. Intradermal testing for delayed hypersensitivity (DTH)

All surviving sheep were tested at each sampling. In addition, the ewes from Year 2 were tested about 3 months before and again immediately prior to lambing. Sheep were injected intradermally on the wool-free inner thigh with 0.1 mL of Avian tuberculin purified protein derivative (PPD) (25,000 IU/mL, CSL, Parkville, Victoria). Skin fold thickness was measured with vernier callipers before injection and 72 hours later. At the 6 month sampling sheep from Groups Lamb-1W and Lamb-1C were tested concurrently on the opposite hind leg with 0.1 mL of Johnin PPD (0.5 mg/mL, CSL, Parkville, Victoria). Positive-negative cut-off points were established using all available age-matched unexposed pastured sheep from both farms and both years of the trial. The results (mean plus 2 standard deviations, giving a theoretical specificity of 97.5%) for tests on unexposed sheep of ages 0-6 months (n = 201), 7-12 months (n = 58), >12-18 months (n = 18) and  $\geq$ 24 months (n = 117) respectively were pooled to provide the cut-off values. Sheep were classified as reacting positively in the skin test if they had a positive result on at least one occasion.

#### 3.3. Gamma interferon (IFN-γ) assay

This was performed on samples from Year 2 only. Blood was collected from the jugular vein into lithium heparin vacutainers, and held at room temperature for less than 12 hours prior to processing using a commercial test kit (Bovigam, Bovine gamma interferon test, CSL, Parkville, Victoria). For each sample three 1.5 mL aliquots of well mixed blood were incubated for 18 hours at 37 °C with  $100\mu$ L of Johnin PPD ( $300 \mu$ g/mL), Avian PPD ( $300 \mu$ g/mL) or phosphate buffered saline (PBS) in polystyrene cell culture plates (Costar, Corning International, New York). Plasma was collected after centrifugation at 500 g for 10 minutes, and transported overnight at 4 °C to CSL for the enzyme immunoassay (EIA). Results were assessed using the manufacturer's recommended criteria. A response to Avian PPD was recorded if optical density (OD) (Avian PPD) was > 0.05 above that for PBS (equivalent to results reported in Project TR.073). Similarly a response to Johnin was recorded when OD (Johnin) was > 0.05 above that for PBS. Only when OD (Johnin) was > 0.05 above both OD (PBS) and OD (Avian PPD) was the result was recorded as positive. This latter criterion was recommended by the manufacturer in an attempt to avoid false positive results. Sheep were classified as reacting positively in the IFN- $\gamma$  test if they had a positive result on at least one occasion.

#### 3.4. Agar gel immunodiffusion test

This was performed only on sheep from Group Lamb-2H, at samplings from 12, 18 and 24 months of age. Blood was collected from the jugular vein into plain vacutainers, and samples were allowed to clot and retract at room temperature, with subsequent storage at 4 °C. Serum was removed within 48 hours for testing in an AGID test. Results were recorded as negative, inconclusive, or positive (1+, 2+ or 3+).

#### 3.5. Necropsy sampling

At each sampling period, sheep were selected for necropsy on the basis of the skin test results obtained immediately prior to necropsy. Half of the sheep selected from each group had the largest increases in skin thickness and half had the smallest increases. Sheep were euthanased with intravenous barbiturate, and necropsy performed in the field. The abdomen was opened using a left flank incision. The terminal ileum (TI), caecum and jejunum were exteriorised without opening the gut, and carefully examined for the presence of gross lesions. Enlarged mesenteric lymph nodes (MLN), thickening of TI, or mesenteric lymphangitis were recorded as possible gross lesions of OJD. Intestinal and lymph node samples were then collected, before the tonsils were exposed via a ventral paramedian incision between the mandibles, which was extended from the oral cavity caudally into the oesophagus.

A range of tissues (ileocaecal valve (ICV), TI (5 g pool of 3 samples from last 300 mm of ileum), MLN (5 g pool from three nodes) and tonsil (pool from both sides) were collected into sterile containers and transported at 4 °C for *M. a. paratuberculosis* isolation. Duplicate samples from adjacent tissue were collected into 10% neutral buffered formalin for histopathology. Faeces were also collected for *M. a. paratuberculosis* isolation from selected samplings in Year 1 and from all necropsied sheep in Year 2. Faeces were collected from the supplementary sheep not only at necropsy, but also at each of the 9, 15 and 21 month samplings.

To avoid cross contamination between sheep, instruments and chopping boards were disinfected between each sheep by scrubbing in a 1:50 solution of Phensol (Whitely Chemicals), followed by rinsing in clean water. To avoid cross-sample contamination within sheep, MLN samples were collected before the intestine was opened and separate instruments were used to collect tonsil.

#### 3.6. M. a. paratuberculosis isolation

Tissue samples from Farm H, Year 1 (2, 4 and 6 month samples) were processed within 24 hours of collection by standard tissue culture techniques.<sup>37</sup> Tissue samples for all other groups were frozen at -80 °C within 12 hours of collection for up to 12 months until processed using a final centrifugation step as previously described.<sup>30</sup> All faecal samples were frozen at -80 °C for up to 12 months until processed routinely.<sup>37</sup> Bactec vials were incubated for 12 weeks, and the identity of any isolates confirmed by IS*900* PCR and REA.<sup>7 37</sup> Gram-stained smears were prepared from selected growth index (GI) positive Bactec vials which were also subcultured onto modified 7H10 Middlebrook agar,<sup>36</sup> with and without Mycobactin J to characterise contaminating organisms.

*Culture of pooled homogenates:* During the processing of tissue samples 1 mL aliquots of tissue homogenates from each of TI, ICV and MLN were removed, pooled and frozen at -80 °C. Selected pooled homogenates were later thawed and processed using the centrifugation method. Pooled homogenates from the 9, 15 and 21 month samplings in Year 2 were not frozen, but processed concurrently with the individual tissue samples. The ability to detect infected sheep by culture of individual tissues or by culture of pooled homogenate was compared using McNemar's Chi-square test for paired observations.<sup>24</sup> Culture using individual tissues was considered positive in this analysis if any of TI, ICV or MLN was positive. The contamination rates for cultures of individual tissues and pooled samples were compared using the Chi-square test on 2 x 2 contingency tables. A tissue sample was considered contaminated if it had a positive growth index in Bactec culture but was negative for *M. a. paratuberculosis* by IS*900* PCR. Contamination which did not prevent *M. a. paratuberculosis* identification was ignored in this analysis.

#### 3.7. Histopathology

Tissues were processed routinely for histopathology, sectioned at 5 µM, stained with haematoxylin and eosin (H&E) and a Ziehl-Neelsen (ZN) technique, and examined by light microscopy. H&E stained sections were examined routinely for lesions, scanning first under lower powers, then focusing on suspicious areas under higher magnification up to 400 X. For ZN stained sections particular attention was focused on any suspicious areas identified in H&E sections. These were examined thoroughly under 400 X magnification and any fields containing material suspicious of being acid-fast bacilli (AFB) were examined under oil-immersion. Sheep were classified as histologically positive for Johne's disease when typical focal, multifocal or diffuse lesions consistent with OJD were present in the H&E sections. The least

pathology recorded as positive was the finding of at least two clumps of macrophages with typical epithelioid morphology and no other cause apparent in a usual predilection site, whether or not AFB were present.<sup>6 27</sup> An equivocal result was recorded when no AFB were detected in ZN stained sections, and isolated clumps of cells with morphology suggestive, but not completely typical of the macrophages usually associated with OJD, or granulomas suggestive of OJD, but more consistent with another aetiology, were present in H&E stained sections. Macrophages were considered atypical if they contained refractive or pigmented material, and granulomas were considered more consistent with another aetiology if they had mineralised contents, contained material suggestive of vegetable matter or the possible remnants of parasites (such as helminths or coccidia), or if there were large numbers of eosinophils within the granuloma.

#### 3.8. Classification of sheep as infected

Sheep were considered to be infected if at necropsy they had a positive tissue-culture, a positive faecal culture, or had unequivocal histological lesions (whether or not acid-fast organisms were detected). This classification was the standard against which the various test sensitivities and specificities were expressed.

#### 4. RESULTS

A summary of the group results for all trials is given in Table 3 (Farm H) and Table 4 (Farm A). Pasture and rainfall data is shown in Figure 1. Detailed results for each sheep are summarised in Appendix 1 (Farm H, Year one), Appendix 2 (Farm H, Year 2) and Appendix 3 (Farm A).

Figure 1: Rainfall and pasture conditions during Years 1 and 2 of field exposure on Farm H



| Group   | Age at first<br>exposure | Time  | e of necr                    | opsy                                | Cultur   | e +ve <sup>a</sup>         | Histo +ve <sup>b</sup>                             | Skin test +ve <sup>c</sup>                         | IFN-g +ve <sup>d</sup>                             |
|---------|--------------------------|---|------------------------------|-------------------------------------|--|----------------------------|--|--|--|
|         | -                        | age at<br>necropsy                                      | mths after<br>weaning        | mths after<br>exposure              | no. of sł<br>Tissues                                     | neep (%)<br>Faeces         | no. of sheep<br>(culture +ve)                      | no. of sheep<br>( culture +ve)                     | no. of sheep<br>(culture +ve)                      |
|         |                          |   |                              |                                     |  | Year 1                     |  |  |  |
| Lamb-1W | weaner                   | 5<br>7<br>9<br>15                                       | 2<br>4<br>6<br>12            | 2<br>4<br>6<br>12                   | 0 (0)<br>0 (0)<br>2 (20)<br>6 (75)                       | ns<br>0<br>0<br>0          | 0 (0)<br>0 (0)<br>0 (0)<br>2 (2)                   | 5 (0)<br>2 (0)<br>0 (0)<br>2 (1)                   | ns<br>ns<br>ns<br>ns                               |
| Lamb-1C | na                       | 5<br>7<br>9<br>15                                       | 2<br>4<br>6<br>12            | na<br>na<br>na<br>na                | 0 (0)<br>0 (0)<br>0 (0)<br>0 (0)                         | ns<br>ns<br>ns<br>0        | 0 (0)<br>0 (0)<br>0 (0)<br>0 (0)                   | 0 (0)<br>1 (0)<br>1 (0)<br>0 (0)                   | ns<br>ns<br>ns<br>ns                               |
|         |                          |   |                              |                                     |  | Year 2                     |  |  |  |
| Ewe-2L  | adult                    | adult<br>adult<br>adult                                 | 0<br>3<br>5                  | 3<br>6<br>8                         | 0 (0)<br>1 (10)<br>6 (60)                                | 0<br>0<br>1                | 0 (0)<br>0 (0)<br>1 (1)                            | 2 (0)<br>0 (0)<br>0 (0)                            | 0 (0)<br>0 (0)<br>2 (1)                            |
| Lamb-2L | neonate                  | 3<br>6<br>8<br>12                                       | 0<br>3<br>5<br>9             | 3<br>6<br>8<br>12                   | 0 (0)<br>1 (10)<br>1 (10)<br>3 (43)                      | 0<br>0<br>0<br>0           | 0 (0)<br>0 (0)<br>0 (0)<br>2 (2)                   | 3 (0)<br>5 (0)<br>2 (0)<br>4 (3)                   | 0 (0)<br>0 (0)<br>0 (0)<br>1 (1)                   |
| Ewe-2W  | adult                    | adult<br>adult  | 3<br>5                       | 3<br>5                              | 0 (0)<br>2 (20)  | 0<br>0                     | 0 (0)<br>0 (0)                                     | 1 (0)<br>0 (0)                                     | 3 (0)<br>0 (0)                                     |
| Lamb-2W | weaner                   | 6<br>8  | 3<br>5                       | 3<br>5                              | 0 (0)<br>2 (20)  | 0<br>0                     | 0 (0)<br>0 (0)                                     | 4 (0)<br>2 (0)                                     | 0 (0)<br>0 (0)                                     |
| Lamb-2H | prenatal?                | 3<br>6<br>8<br>12<br>18 <sup>f</sup><br>24 <sup>f</sup> | 0<br>3<br>5<br>9<br>15<br>21 | >3<br>>6<br>>8<br>>12<br>>18<br>>24 | 3 (30)<br>2 (20)<br>3 (30)<br>6 (60)<br>5 (50)<br>6 (67) | 0<br>0<br>2<br>2<br>3<br>3 | 0 (0)<br>0 (0)<br>0 (0)<br>3 (3)<br>2 (2)<br>5 (4) | 3 (1)<br>3 (1)<br>4 (2)<br>5 (5)<br>5 (4)<br>4 (4) | 0 (0)<br>0 (0)<br>0 (0)<br>1 (1)<br>3 (3)<br>2 (2) |
| Ewe-2C  | na                       | na  | 3, 9 <sup>e</sup>            | na                                  | ns   | 0 <sup>e</sup>             | ns   | 4  | 0  |
| Lamb-2C | na                       | na  | 3, 9 <sup>e</sup>            | na                                  | ns   | 0 <sup>e</sup>             | ns   | 1  | 0  |

| Table 5. Tracel weather their evaluation on Failling - Summary resum | Table 3. | Tracer | weaner | field | evaluation | on | Farm | Н- | summary | y resul | ts |
|--|----------|--------|--------|-------|------------|----|------|----|---------|---------|----|
|--|----------|--------|--------|-------|------------|----|------|----|---------|---------|----|

<sup>a</sup> +ve in any of up to 5 separate tissue cultures (MLN, TI, ICV, tonsil, +/- pool)

<sup>b</sup> Sheep with typical lesions (+/- AFB) are listed as +ve

c Number of sheep +ve on at least one occasion prior to necropsy

<sup>d</sup> Number of sheep +ve on at least one occasion prior to necropsy; (OD (Johnin) exceeds both OD (control) and OD (Avian PPD) by > 0.05)
<sup>e</sup> Time when complete these sheep was not accurately for any use and control and outputs for the whole group.

Time when sampled; these sheep were not necropsied; faecal culture results are from pooled cultures for the whole group.

f These sheep considered as supplementary samples, and discussed separately

ns Not sampled

-

na Not applicable

#### 4.1. Overview of findings on Farm H

Sheep first exposed as weaners: In Year 1 there were no positive cultures from weaners (Group Lamb-1W) necropsied 2 or 4 months after exposure. At 6 months 20% were tissue culture-positive, and at 12 months 75 % were tissue culture-positive. In Year 2, results for comparable weaners (Group Lamb-2W) were similar, with no culture-positive sheep 3 months after exposure but 20% culture-positive at 5 months.

Sheep first exposed as neonates (Group Lamb-2L): Results were similar to the above with respect to time after first exposure, with no culture-positive sheep among those necropsied 3 months post-exposure (ie at weaning, or 3 months old). A single culture-positive lamb was first detected after 6 months exposure, and another at 8 months. By 12 months 43 % were culture-positive.

Sheep first exposed as adults: The results were also broadly similar to those for weaners and neonates. No culture-positive sheep were detected until 5-6 months after first exposure. A single ewe from Group Ewe-2L was culture-positive in February (six months post-exposure), and two ewes from Group Ewe-2W were culture-positive in May (five months post-exposure). Exceptionally, at that same May sampling 60% of ewes from Group Ewe-2L were culture-positive, eight months post-exposure. One of these ewes was also faecal culture-positive.

Lambs born from potentially infected ewes (Group Lamb-2H): These results followed a different pattern from those of the introduced naive sheep above. 20 to 30 % of necropsied lambs were culture-positive at each of the samplings at 3, 6 and 8 months of age, and 60% were culture-positive at 12 months. Four of the tissue culture-positive lambs were also faecal culture-positive.

Lambs born from potentially infected ewes (Group Lamb-2H), supplementary samplings at 18 and 24 months of age: 50 to 67% of these lambs were culture-positive, and 5/11 tissue culture-positive sheep plus one additional sheep were faecal culture-positive.

Results for histopathology, skin testing and IFN- $\gamma$  are discussed in detail in the relevant sections below. Briefly, some sheep from all age classes had histological lesions of OJD, but never before 8 months of potential exposure to *M. a. paratuberculosis*. False positive skin test results were frequent at the early samplings. Few sheep had positive IFN- $\gamma$  results. IFN- $\gamma$ -positive lambs were also culture-positive, but IFN- $\gamma$ -positive ewes were often culture-negative.

| Group     | Exposure            | Necı<br>mths after<br>weaning | opsy<br>mths after<br>exposure | Cultur<br>no. of sh<br>Tissues | e +ve <sup>a</sup><br>neep (%)<br>Faeces | Histologically +ve <sup>b</sup><br>no. of sheep<br>(also culture +ve) | Skin test +ve <sup>c</sup><br>no. of sheep<br>(also culture +ve) |
|-----------|---------------------|-------------------------------|--------------------------------|--------------------------------|--|---|--|
| A-High-W  | High from weaning   | 2                             | 2                              | 0 (0)                          | ns                                       | 0 (0)   | 1 (0)  |
|           |                     | 4                             | 4                              | 0 (0)                          | ns                                       | 0 (0)   | 1 (0)  |
|           |                     | 6                             | 6                              | 2 (20)                         | ns                                       | 0 (0)   | 0 (0)  |
|           |                     | 12                            | 12                             | 1 (11)                         | 0  | 0 (0)   | 0 (0)  |
| A-Low-W   | Low from weaning    | 2                             | 2                              | 0 (0)                          | ns                                       | 0 (0)   | 0 (0)  |
|           | -                   | 4                             | 4                              | 0 (0)                          | ns                                       | 0 (0)   | 0 (0)  |
|           |                     | 6                             | 6                              | 0 (0)                          | ns                                       | 0 (0)   | 0 (0)  |
|           |                     | 12                            | 12                             | 1 (11)                         | 0  | 0 (0)   | 0 (0)  |
| A-High-LW | High from birth and | 2                             | 5                              | 0 (0)                          | ns                                       | 0 (0)   | 1 (0)  |
| 0         | high from weaning   | 4                             | 7                              | 0 (0)                          | ns                                       | 0 (0)   | 0 (0)  |
|           | 0 0                 | 6                             | 9                              | 2 (25)                         | ns                                       | 0 (0)   | 0 (0)  |
|           |                     | 12                            | 15                             | 1 (14.3)                       | 0  | 0 (0)   | 1 (0)  |
| A-Hiah-L  | High from birth     | 2                             | 5                              | 0 (0)                          | ns                                       | 0 (0)   | 1 (0)  |
| 5         | until weaning       | 4                             | 7                              | 2 (25)                         | ns                                       | 0 (0)   | 0 (0)  |
|           | 6                   | 6                             | 9                              | 0 (0)                          | ns                                       | 0 (0)   | 1 (0)  |
|           |                     | 12                            | 15                             | 0 (0)                          | 0  | 0 (0)   | 0 (0)  |
| A-control | none                | 2                             | na                             | 0 (0)                          | ns                                       | 0 (0)   | 0 (0)  |
|           |                     | 4                             | na                             | 0 (0)                          | ns                                       | 0 (0)   | 0 (0)  |
|           |                     | 6                             | na                             | 0 (0)                          | ns                                       | 0 (0)   | 0 (0)  |
|           |                     | 12                            | na                             | 0 (0)                          | 0  | 0 (0)   | 1 (0)  |

#### Table 4. Tracer weaner field evaluation on Farm A - summary results

<sup>a</sup> +ve in any of up to 5 separate tissue cultures (MLN, TI, ICV, tonsil, +/- pool)

<sup>D</sup> Sheep with typical lesions (+/- AFB) are listed as +ve

Number of sheep +ve on at least one occasion prior to necropsy

<sup>a</sup> Number of sheep +ve on at least one occasion prior to necropsy; (OD (Johnin) exceeds both OD (control) and OD (Avian PPD) by > 0.05)

ns Not sampled

na Not applicable

#### 4.2. Overview of findings on Farm A

Sheep first exposed as weaners: There were no positive tissue cultures from sheep necropsied 2 or 4 months after first exposure. At 6 months 20% of the high exposure group (A-High-W) were tissue culture-positive, and 11% were positive at 12 months. For the weaners on low contamination pasture, there was a single culture-positive result at the 12 month sampling.

Sheep first exposed as neonates: Again, these results were similar to those of sheep first exposed as weaners when interpreted with respect to length of exposure. The first positive tissue culture results were found 7 months post-exposure (7 months of age, 4 months post-weaning) when 25% of sheep necropsied from Group A-High-L (exposed from birth until weaning only) were positive, but there were no further culture-positive sheep detected in this group at subsequent samplings. In Group A-High-LW (exposed from birth through weaning) there were no culture-positive sheep detected at 5 or 7 months post-exposure, but 25% and 12.5% respectively of sheep necropsied after 9 and 15 months exposure (6 and 12 months post-weaning) were tissue culture-positive.

None of the experimental sheep from Farm A had detectable histological lesions at necropsy. False positive skin test results were also a feature on this farm, mainly from the highly exposed groups, and no culture-positive sheep were detected by the skin test. No sheep was faecal culture-positive at the 12 month sampling.

#### 4.3. Culture of M. a. paratuberculosis

In total, 47 of 324 (14.5%) sheep with possible exposure to *M. a. paratuberculosis* for up to 15 months were confirmed infected after necropsy and culture of tissues. No additional sheep were detected to be infected by faecal culture, nor by histopathology (observed sensitivity for tissue culture of 100%). 29 control sheep were also cultured and all were culture-negative. Five of 207 of these exposed sheep were positive by faecal culture. All were from Farm H; one a ewe 8 months after first exposure, and four lambs from Group Lamb-H at 8 or 12 months of age. All faecal culture-positive sheep were also tissue culture-positive (sensitivity of 5/41 (12.2%) with 100% specificity).

Among the 19 supplementary sheep (18 and 24 months of age/exposure), 11 were confirmed infected by tissue culture. A further sheep was confirmed infected by positive faecal culture only, and another by histopathology only, giving 13/19 infected sheep. Sensitivity for tissue culture in these sheep was 11/13 or 84.6%. Sensitivity of faecal culture was 6/13 or 46.2%.

# 4.4. Comparison of culture of individual tissues and pooled homogenates

Samples from 144 sheep from which the pooled homogenate had been frozen prior to culture were examined. Significantly more sheep were detected when individual tissues were cultured compared to the use of pooled homogenate. Of 22 culture-positive sheep, 10 were detected by both methods, 10 were detected only by culture of individual tissues, compared to just two which were detected only by culture of pooled homogenate (Chi-square = 5.53, P < 0.05). Also, significantly more cultures of pooled homogenate were contaminated. 44/144 (30.6%) pooled cultures were contaminated, compared to 65/432 (15.0%) of individual tissue cultures (Chi-square = 16.93, P < 0.001). Samples from 38 sheep (these included 2 necropsies from the IAT project) for which the pooled homogenate was not frozen were also examined, and in this case, there were no significant differences in detection rates or contamination rates for the individual compared to pooled cultures. A total of 22/38 sheep were infected. 19 were detected by both methods, one only by individual tissues culture, and two only by culture of pooled tissues. 9/114 (7.9%) of the individual tissue cultures and 2/38 (5.3%) of the pooled cultures were contaminated.

#### 4.5. Intradermal testing for delayed hypersensitivity

Results for unexposed sheep are summarised in Figure 2, illustrating a trend for greater skin thickness increases after intradermal Avian PPD with increasing age. The cut-off points established for young sheep were 0.54 mm (0-6 months), 0.98 mm (7-12 months) and 1.9 mm (>12-18 months). For adult sheep the corresponding cut-off was 4.23 mm, but the generally accepted value of 4.0 mm was used.<sup>29</sup> Using these age-related cut-off values, and considering sheep up to 15 months post-exposure, 13 of 47 infected sheep were detected by the skin test (observed sensitivity 13/47 or 28%). 263 of 306 uninfected sheep were skin test negative (observed specificity 263/306 or 85.9%). No difference was seen between the responses to Avian PPD or Johnin in the 23 lambs from Groups Lamb-1W and Lamb-1C tested six months after weaning, but there were no positive skin tests from this group at this sampling for either preparation.

Among the 19 supplementary sheep (18 and 24 months of age/exposure), 9 had a positive skin test on at least one occasion and 8 of these were infected (sensitivity 8/13 or 61.5% with 5/6 or 83.3% specificity).



Figure 2. Skin-fold thickness increase 72 hours after intradermal Avian PPD in pastured sheep unexposed to *Mycobacterium avium* subsp. *paratuberculosis* 

#### **4.6. IFN-**γ

Of the samples taken 3 days prior to necropsy from sheep with up to 15 months exposure, only 3 were positive using the manufacturer's revised specific criteria, and these sheep were also culture-positive (all after at least 8 months of potential exposure). Another 4 ewes gave a positive IFN- $\gamma$  result 2-3 months prior to necropsy. All were culture negative and 3 of those positive IFN- $\gamma$  results were obtained before any known exposure to *M. a. paratuberculosis*. As there were 30 culture-positive sheep from a total of 147 necropsied in Year 2, the observed sensitivity of IFN- $\gamma$  testing was 10% (3/30), and the observed specificity was 97% (113/117). When considering reactions to Avian PPD in isolation (equivalent to the IFN- $\gamma$  testing used in the pilot study), 13 ewes and 2 lambs showed reactivity on at least one occasion, 9 of these in the absence of any known *M. a. paratuberculosis* exposure. Sensitivity and specificity were 7% (2/30) and 89% (104/117) respectively. Considering reactions to Johnin alone, 18 ewes and 5 lambs reacted on at least one occasion, 10 in the absence of any exposure, with sensitivity and specificity of 27% (8/30) and 87% (102/117) respectively.

Among the 19 supplementary sheep (18 and 24 months of age/exposure), five had at least one positive IFN- $\gamma$  test, and all five were infected (sensitivity 5/13 or 38.5% with 100% specificity).

#### 4.7. Agar gel immunodiffusion test

Gel testing was not used in the main part of the tracer trial, because of the very low likelihood of positive results in sheep under 12 months of age/exposure. Of the 29 remaining sheep from Group Lamb-2H, none were positive at 12 months of age. One of 19 was positive at 18 months (2+) and it was still positive (3+) when euthanased with clinical OJD at 22 months of age. No other sheep reacted in the gel test, yielding an observed sensitivity among the supplementary sheep of 1/13 or 7.7% with 100% specificity.

#### 4.8. Histopathology

When considering sheep with <15 months of exposure, only eight of a total of 324 potentially exposed sheep had definite histological lesions of OJD, and all were confirmed as infected by culture of *M. a. paratuberculosis* from tissues (observed sensitivity of 8/47 or 17%, with 100% specificity). All were from Farm H. Seven were lambs, 12 to 15 months old, potentially exposed to *M. a. paratuberculosis* for at least 12 months, and one was a ewe exposed for eight months. Five sheep (4 lambs and one ewe) had focal lesions only in MLN, two with small numbers of AFB. Two lambs had focal or multifocal lesions only in the TI (both AFB negative). A single lamb had diffuse locally extensive lesions in one only of three TI sections with very large numbers of AFB. This lamb also had focal lesions, but only 7 of these (12.5%) were confirmed as infected by culture at necropsy, about the same number as would be expected by chance (overall, 47/353 (13.3%) of necropsied sheep were culture-positive).

Among the 19 supplementary sheep, necropsied at 18 and 24 months of age, seven had histological lesions of OJD, giving an observed sensitivity of 7/13 or 53.8%. One of these was culled as a clinical case at 22 months of age, and had typical diffuse multibacillary pathology. Another (24 months) had typical diffuse paucibacillary lesions, and the remainder had focal lesions. All except one 24 month animal with focal lesions were also culture-positive.

#### 5. DISCUSSION

In this series of experiments, conducted on two independent farms, infection with *M. a. paratuberculosis* was detected by tissue culture in at least one naive introduced sheep from every potentially exposed group, 5 to 15 months after first exposure. In the heavily contaminated environment on Farm H, 43 to 75% of sheep sampled after  $\geq$ 8 months of exposure were culture-positive, whereas in the low contamination paddock on Farm A, a single infected sheep was detected 12 months after first exposure.

The cultural findings in this study reinforce those from the pen trial study, and from other reported studies in experimentally infected sheep,<sup>4</sup> that culture of tissues is the most sensitive method currently available for the detection of early *M. a. paratuberculosis* infection. The current study is, in addition, the first demonstration of tissue culture as a practical tool for the detection of early infection in sheep after natural exposure to S strain *M. a. paratuberculosis*. However, indications from the earlier pen trial that culture two or four months post-exposure would detect infected sheep were not borne out in the current field trials. It appears that in the paddock situation about six months exposure is necessary before tissue culture will detect infection. Only on severely contaminated pasture were any positive results obtained earlier (at five months).

Ante-mortem testing in the first 12 months post-exposure detected few of the infected sheep. Skin testing for DTH detected only 28% of infected sheep with 86% specificity, IFN- $\gamma$  testing detected only 10% with 97% specificity, and faecal culture detected only 12% with 100% specificity. The relatively low sensitivities for these tests found in this study reflect their use in the very early stage of the disease process and were not unexpected. All of these tests performed better when applied to sheep exposed for more than 12 months, with observed sensitivities among the supplementary sheep of 61.5% (skin test), 38.57% (IFN- $\gamma$ ) and 46.2% (faecal culture).

Nor were histological lesions, often used as the "gold standard" for M. a. paratuberculosis infection in sheep, frequent. Only 17% of culture-positive sheep had typical histological lesions, and all sheep with such lesions were infected. Unequivocal lesions were not seen in sheep with less than 8 months of exposure. This lack of lesions in the early months after first infection reinforces the findings for weaners experimentally infected with S strain M. a. paratuberculosis in the earlier pen trials. This is in contrast to results commonly reported for experimental infection in sheep with high doses of cattle (C) strain M. a. paratuberculosis, which describe lesions in the first months after infection in some sheep. 31725 Such highdose. C strain infection scenarios may not be representative of the usual infection of sheep in natural situations, so that data generated from such trials, eq. the performance of diagnostic tests, should to be viewed with caution. The positive histopathological findings from the present trial, although few, also provide some additional insight into the development of lesions in sheep. Most of the lesions were focal in distribution, with few or no AFB present – typical of the early lesions described by others.<sup>17 25 27</sup> However, one 15 month old lamb, potentially exposed for 12 months, had lesions which were typical of the diffuse multibacillary form of OJD in only one of three ileal sections. Such pathology is usually associated with sheep having advanced disease, and is usually expected to be widespread in the intestine. This lamb had no gross lesions, was clinically normal, and was negative on the skin test and faecal culture. The histopathological findings in this lamb suggest that in some sheep, focal lesions may progress directly to the multibacillary form, rather than gradually spread to be multifocal, and only later become multibacillary in character.

The difference in the pattern of culture results between sheep born from an infected ewe flock, in which 20-30% of sampled lambs were tissue culture-positive at 3, 6 and 9 months of age, and introduced naive sheep, suggests the possibility of transmission of infection from infected ewes to their offspring. This trial was not designed to specifically examine the details of such transmission. Congenital infection is a possibility, and has been demonstrated frequently in cattle,<sup>19</sup> <sup>22</sup> <sup>34</sup> and twice in sheep.<sup>1</sup> <sup>35</sup> Similarly, infection via the milk, and particularly colostrum is possible.<sup>10</sup> <sup>15</sup> <sup>32</sup> <sup>33</sup> Faecal-oral transmission is also likely to be enhanced (eg. by exposure to faecally contaminated udders), and lambs of infected ewes may be exposed to higher numbers of *M. a. paratuberculosis*. Whatever the mechanism, it appears that lambs born from an infected flock become tissue culture-positive sooner than naive lambs suckling uninfected ewes introduced to the same infected environment. This could be important in the design of disease control programs.

These experiments also suggest that age has little effect on the early establishment of infection, since

neonates, weaners and ewes on Farm H had similar infection levels after similar periods of exposure. Group Ewe-2W and their lambs (Group Lamb-2W) had exactly the same exposure history, having been moved to Farm H at weaning in December, so are directly comparable. Their infection rates were identical. Similarly Group Ewe-2L and their lambs (Group Lamb-2L) were both exposed from the time of lambing on Farm H in September. Infection rates for these two groups were broadly similar to previous ones when considered relative to length of possible exposure, despite the three-month asynchrony in terms of season. However, Group Ewe-2L was remarkable in that 60% of ewes examined 8 months postexposure were infected, compared to only 10% of their lambs after an identical exposure history. This difference may reflect the greater likelihood of grazing ewes to pick up an infectious dose off pasture as compared to their suckling lambs, and/or the effect of lactational stress on the ewes' resistance to the establishment of infection. Weaners and neonates on Farm A also had similar infection rates. Together, these findings reinforce early experimental studies with bovine strains which demonstrated similar levels of tissue infection 2½ months post infection in sheep from 3 weeks to 20 months of age.<sup>14</sup> Published studies suggest that infection of older animals, however, is less likely to progress to clinical disease.20 26 The short duration of the current work does not allow any such assessment. But some speculation concerning the likely outcome of such infections in young sheep is possible. In the highly contaminated environment of Farm H a total of 21/35 (60%) of sheep examined 8 to 12 months after first exposure were found to be infected, a figure well in excess of the proportion of adult sheep which later would succumb to OJD in the normal course of events on this farm. The rate of infection remained similar in supplementary sheep at 18 and 24 months of age. However, in related studies on Farm H, only 32% of 145 three year old ewes were culture-positive at necropsy,<sup>1</sup> and mortalities never exceeded 20%. This suggests that recovery or latent infection may be occurring in many infected sheep.

The results from these trials also suggest that the establishment of infection in sheep may be an on-going process, rather than primarily infection as neonates which then gradually progresses with age. Contrast the findings for sheep in the continuously highly contaminated environment of Farm H with those on Farm A where exposure levels were likely to be high initially but dropping over time. At the 12 month post-exposure samplings from Farm H, higher proportions of sheep were culture-positive than at previous examinations, whereas on Farm A levels were similar or decreasing.

On Farm H, the highest numbers of sheep were culture-positive at samplings in September and December, which suggests the possibility of a climatic effect on the likelihood to pick up an infectious dose following exposure to short green pastures in winter/spring. However these experiments were not designed to look specifically at seasonal effects and such suppositions are confounded by both age and length of exposure. The rainfall and pasture conditions were also markedly different across the two years of the trial. In Year 1 pasture was long and dry at weaning (December) and dry conditions prevailed over summer. In Year 2 pasture was short and green at weaning, and continued green and lush throughout a wet summer. Despite these differences the culture results for the comparable groups first exposed at weaning (Lamb-1W and Lamb-2W) were very similar. An alternate explanation may be that immunosupression following dietary stress in late winter might facilitate the establishment of initial infection or possibly unmask previously undetected latent infection, but again this cannot be assessed from these trials. However, consider again the exceptional results for Group Ewe-2L for which 60% of sheep examined in May, 8 months post-exposure, were culture-positive. These sheep were transported to Farm H at lambing, and so were initially exposed to short green spring pasture at the same time as under lactational stress.

The timing of necropsy in this study was aimed at maximising the numbers of sheep from which the organism might be cultured. A number of previous studies suggest that there is a "window period", several months after first infection, when many exposed sheep are likely to be positive by intestinal or MLN culture. *M. a. paratuberculosis* was cultured from MLN of 3/4 infected lambs 20 to 41 days post infection, but from 0/4 cultured within 18 days of infection,<sup>3</sup> and a subsequent study using similar infection protocols yielded no positive cultures at 28 days.<sup>2</sup> *M. a. paratuberculosis* was recovered from intestinal mucosa of sheep, 12 and 36 hours after oral infection but could not be isolated from sheep killed after 7 days. However, infections were again demonstrated in the intestinal mucosa and MLN after 28-56 days in most sheep.<sup>4 13</sup> In the only documented study using experimental infection of sheep with low doses of *M. a. paratuberculosis* (10<sup>3</sup> and 10<sup>6</sup> CFU) the isolation rates from tissues at necropsy were highest 3<sup>1/2</sup> months after first infection, with much lower rates at 5 or 9 months.<sup>4</sup> Taken together these findings suggest that *M. a. paratuberculosis* is rarely isolated from the tissues of infected sheep in the first few weeks to a month after infection, whereas cultures from one to several months post infection are more likely to be positive. In sheep exposed to low doses (equivalent to natural exposure?) the numbers of

sheep positive on tissue culture then decline. This "window period" may correspond to the latter part of the classical stage 2 of tuberculosis during which logarithmic growth of mycobacteria occurs within macrophages which are not sufficiently activated to destroy them.<sup>8</sup> Organisms may be inhibited by host innate immune responses soon after infection. In specific experimental studies many organisms appear degraded or lose their acid-fast staining qualities in the immediate post-infection period.<sup>9 23</sup> It may take a month or two before organisms surviving within macrophages build up to sufficient numbers to be detected by culture. Later (3 to 4 months) developing cell-mediated immunity (CMI) may significantly limit and even reduce numbers of organisms in a large percentage of sheep. Of course, in some sheep infection will progress, and larger numbers of organisms will be present facilitating diagnosis in those particular sheep by culture or other means.

In the field situation, it is impossible to predict accurately when infection might be acquired. Hence, a range of sampling times up to one year after first possible exposure was used for the main tracer studies in this series of experiments. The selected times were based on experience from the pen trials in which positive cultures were obtained 2 and 4 months after first exposure to *M. a. paratuberculosis*. In planning these field trials infection was thought more likely to occur soon after potential exposure rather than later, especially on Farm A where pasture contamination was likely to decline over time since infected sheep had been removed. Thus sampling was biased towards the first six months after exposure. The final 9 to 12 month sampling was added to make some allowance for possible seasonal variations (sheep would have experienced a full year in the contaminated environment) and to possibly provide time for the progression of lesions in some sheep. On highly contaminated pasture there was also the possibility that many sheep could have been culture-positive at early samplings and a final sampling after 12 months would then have indicated the persistence or otherwise of such early infections.

A brief discussion of the way sheep were selected for necropsy is also warranted. Within each group at each sampling time, sheep were selected on the basis of having the largest or smallest DTH reactions in the test immediately prior to slaughter. This non-random selection technique was chosen in an attempt to determine whether the skin test would be useful as a predictor of tissue culture status, as suggested by the results of the earlier pen trials. Especially on the pastures with possibly low levels of *M. a. paratuberculosis* contamination, few culture positive sheep were expected, and to make best use of the "window period", sheep needed to be cultured as soon as possible after a positive skin test. But the selection technique may have biased the results. If indeed DTH is a useful predictor of culture status, then the successive removal of DTH positive sheep at each sampling could bias culture-positive results towards the earlier samplings. Conversely, sheep with the highest DTH levels may be the more resistant animals,<sup>11</sup> and more culture-positive sheep might then be expected at later samplings.

Previous studies of DTH responses to Johnin in both cattle and sheep have indicated that repeated testing at one to 3 month intervals did not result in sensitisation of the animals.<sup>11 12 21</sup> On the other hand. local desensitisation soon after intradermal testing has been described in cattle but was almost eliminated by one month and no longer apparent by 2 months,<sup>21</sup> so a minimum 2 month interval between testing was chosen for these trials. Johnin PPD was not available in sufficient quantity for use throughout these trials so Avian PPD was used instead. Johnin and Avian PPD are reported to differ in that Johnin contains cellular components due to bacterial lysis in one month old cultures in addition to secreted proteins.<sup>31</sup> However, reports suggest that there is little difference in sensitivity when the preparations are used to elicit DTH in the field. Similar numbers of sheep responded to Johnin or Avian PPD following experimental infection with M. a. paratuberculosis, although the intensity of reaction was greater to Avian PPD in many sheep.<sup>18</sup> Johnin may, however, be more specific than Avian PPD, with only 2/6 M. aviuminfected animals showing DTH to Johnin whereas 5/6 reacted to Avian PPD.<sup>18</sup> The small amount of Johnin available in the present trials was used for a limited comparison with Avian PPD on Farm H at the 6 month sampling. Unfortunately all tests using either preparation were negative (including 2 culturepositive sheep) so no conclusions could be drawn. Most skin test positive sheep from samplings 2 to 4 months after first exposure were culture-negative for M. a. paratuberculosis. This is in marked contrast to results obtained in the pen trials when no false positive DTH responses were found (100% specificity using the same cut-off values). The reasons for false positive results in the field trials might include the lower specificity of Avian PPD as discussed above, combined with possible cross-sensitisation by other environmental antigens. Other mycobacteria were indeed detected in some contaminated cultures of intestine and MLN over several time periods on all farms, although such isolations were not correlated with positive DTH in individual sheep; (in the pen trials no tissue cultures were contaminated). Interestingly, however, most of the positive DTH results from lambs in these field trials were from the most heavily exposed groups, suggesting that many of the observed early DTH responses could indeed

be specific responses to *M. a. paratuberculosis* exposure. Exposure to *M. a. paratuberculosis* in numbers below those in the earlier pen trials, but above some threshold level for DTH responses might have resulted in levels of infection in the tissues below the detection limit of the culture system, or focal infection in unsampled tissues may have been missed. Another possibility is that CMI responses in pastured sheep may be more effective (perhaps via concurrent immunostimulation by other organisms) and early infection was controlled. At the later sampling times false positive reactions were less frequent with 75% of skintest positive sheep necropsied after 12 or 15 months of potential exposure confirmed infected by tissue culture. Overall the sensitivity of the skin test in detection of these early infections was also low (28%), again in some contrast to the results from the experimentally infected housed lambs. Possible reasons for these false negative results might include the possibility of lower specific DTH responses in pastured sheep due to various intercurrent immunosuppressive effects (eq concurrent infections, climatic or dietary stresses). It may also be a matter of timing. In experimentally infected sheep, DTH has been shown to develop about 2-3 months after exposure, with levels declining after some months in many sheep.<sup>11 12 18</sup> Some trial sheep may have had the potential for DTH responses at some time after the 6 month testing which then declined to undetectable levels by the 12 month testing, and there was no test done in between. It is also possible that some sheep had infection at a level sufficient for detection by culture but insufficient to stimulate a DTH response. Previous experimental studies have shown that some sheep with the highest levels of infection may not show positive DTH reactions, and that the largest DTH reactions were seen in sheep with the least levels of subsequent infection.<sup>11</sup> Similar observations have been recorded for other measures of CMI such as IFN-y. Experimentally sheep with the highest IFN- $\gamma$  responses were less likely to be infected at later necropsy than those with lower responses.

The tissues selected for culture in these field trials were a subset of those used in the earlier pen trials, and were chosen so that had the selected subset been used on the housed sheep, no infected sheep would have been missed. In these field trials, cultures of MLN and TI were each done from samples pooled from 3 sites of the particular tissue to limit the numbers of cultures. Care was taken that a full 5 g sample of the pooled tissue was collected for culture. This was considered a reasonable alternative to 3 separate cultures for each tissue (as done in the pen trials) because in the pen trials most tissue samples were less than 2 g in weight. Of the 54 sheep detected by separate culture of (pooled) MLN, (pooled) TI, ICV and tonsil in the field trials, only a single sheep would have been missed if tonsil was excluded. Because of the additional time taken to remove tonsils during necropsy, and the minimal extra detection value, tonsil is unlikely to be considered for sampling in further trials. Tonsil would also be highly impractical to sample from lines of sheep at an abattoir, where future monitoring might take place.

The culture of frozen pooled homogenate from the MLN, TI and ICV detected fewer infected sheep than culture of individual pooled tissues, and contamination rates were also higher for the pooled homogenate cultures. However, these results are likely to have been influenced by the extra freeze/thaw endured by the pooled homogenate samples. Among 38 sheep for which pooled homogenates were cultured alongside the original tissue homogenates, no differences in isolation or contamination rates were seen. A separate study (data not shown) comparing Bactec culture results for freshly prepared and frozen/thawed homogenates demonstrated significant decreases in *M. a. paratuberculosis* isolation rates and significant increases in contamination rates for frozen/thawed samples. Thus, culture of freshly pooled homogenate from TI, ICV and MLN may be an economical alternative to culture of individual tissues for the detection of early *M. a. paratuberculosis* infection.

A very limited investigation of the use of the tracer weaner concept in the field has been undertaken as part of OJD Project 1.1., and full details will be reported with that project. However, a brief summary/discussion of results to date is warranted in this report. 20 weaners 6 –12 months old (born to restocker ewes) from each of four properties which had been restocked for about 18 months were examined using TI, ICV and MLN cultures from each animal. Positive cultures were obtained (6 of 20 weaners) from only one property, which was a property owned by a trial 1.1 participant, and known to be OJD-infected. Of the three properties with negative tracer findings, 2 were still negative at the 24 months post-restock test, and one was positive. The failure of tracers to detect infection on this property may be due to small sample size (see below), or because the weaners were exposed to potentially infected pasture at a later time than their mothers, and the pasture may no longer have been infective.

### 6. RECOMMENDATIONS FOR PRACTICAL USE OF THE TRACER CONCEPT IN THE FIELD

To investigate the infectivity of pasture, a flock of clean sheep (of any age) would be grazed on that pasture for 6 - 12 months, at a typical stocking rate for such pasture, and tracers selected at random from that flock. Each tracer would be examined at necropsy by a single pooled tissue culture. Note that running only a small group of tracers on a large area of pasture (eg otherwise grazed by cattle, left fallow, cropped etc) may not result in the expected exposure of those tracers to surviving M. a. paratuberculosis, because of physical differences in the pasture.

The expected prevalence of infection in a tracer flock in environments of differing contamination levels can be estimated from the current trial by combining results across groups post-exposure as shown in Table 5. Severe contamination data are from Farm H over both years of the trial and represent the findings from 95 sheep (all ages), moderate contamination data include the Farm A groups exposed to high levels of contamination pre and/or post-weaning (51 sheep, lambs and weaners), and the low contamination data are those from the single group A-Low-W, exposed to low contamination pasture from weaning (19 sheep, weaners). The differences in numbers of sheep used to obtain prevalence estimates are reflected in the relatively wider confidence limits for the estimate from the low contamination pastures.

| Level of environmental contamination with M. a. paratuberculosis |          |          |  |  |  |  |
|--|----------|----------|--|--|--|--|
| Severe   | Moderate | Light    |  |  |  |  |
| 33   | 14       | 5        |  |  |  |  |
| [24 - 43]  | [6 - 27] | [0 - 25] |  |  |  |  |

#### Table 5. Prevalence (%) of infection in tracer sheep 6-12 months post-exposure

Values in brackets are the 95% confidence limits for the prevalence estimates.

Table 6 then gives the numbers of tracers required to be 95% confident of detecting infection for different expected prevalences.

| Expected prevalence of infection in tracer flock (%) | Number to be examined for 95% confidence of detecting infection |
|--|---|
| 50   | 5   |
| 40   | 6   |
| 30   | 9   |
| 20   | 14  |
| 15   | 19  |
| 10   | 29  |
| 5  | 59  |
| 2  | 149   |
| 1  | 299   |

# Table C. Numbers of theory also in a second distant infections

Adapted from Cannon and Roe (1982)<sup>5</sup>

It is apparent from these figures that examination of even 10-15 sheep would be adequate to confidently detect infection after 6-12 months when exposure levels are high. In moderately contaminated environments examination of about 20 sheep would be required. About 60 sheep would be necessary to detect a level of infection of 5% in the tracer sheep, which is approximately the level found on the lowest contamination pasture from the tracer weaner trial. In very low contamination areas, where the expected level of infection in tracers was <5% uneconomically high numbers of tracers would have to be examined to have reasonable confidence in a negative result.

The concept might reasonably be investigated further by opportunistic abattoir slaughter and sampling of clean sheep, which have been running on suspect pastures for 6-12 months.

# 7. SUCCESS IN ACHIEVING OBJECTIVES

This study successfully achieved its objectives, summarised below:

• Determine whether the tracer concept will work in the field in a heavily infected environment:

In the most heavily contaminated environment on Farm H, 43 to 75% of sheep sampled after 8 months of exposure were culture-positive. Infection in introduced naive sheep was first detected by culture of tissues 5 - 6 months after potential exposure. Examination of 10-15 tracers would be sufficient to confidently detect infection in such environments 6-12 months post-exposure.

 Determine whether the tracer concept will work in the field in environments with lower levels of infectivity.

Infection with *M. a. paratuberculosis* was detected by tissue culture in at least one naive introduced sheep from every potentially exposed group, 5 to 15 months after first exposure. In the lowest contamination paddock on Farm A, a single infected sheep was detected 12 months after first exposure. Larger numbers of tracers may be necessary to detect infection in lightly contaminated environments.

 Determine whether antemortem testing for specific CMI responses would allow selection of likelyinfected sheep for necropsy.

This did not appear to be a useful strategy in the field application of the tracer concept for individual sheep. Skin-test and IFN- $\gamma$  tests had very low sensitivities in samplings less than 12 months post-exposure. Both tests performed better when applied to sheep 12 –24 months post-exposure.

• To further refine the sites for culture at necropsy, and to evaluate the effects of pooling homogenates from several sites.

Tonsil is not recommended as a sampling site in any further investigations. The culture of tonsil was not found to add significantly to the number of infected sheep detected, additional time was taken to remove tonsils during necropsy and tonsil would also be highly impractical to sample from lines of sheep at an abattoir, where future monitoring might take place.

When pooled homogenates were cultured alongside the original tissue homogenates, no differences in isolation or contamination rates were seen. Thus, culture of freshly pooled homogenate from TI, ICV and MLN is recommended as an economical alternative to culture of individual tissues for the detection of early *M. a. paratuberculosis* infection in individual sheep.

• Provide further insights into the pathogenesis of OJD in the early stages post-infection, in particular the effects of age at first exposure on subsequent *M. a. paratuberculosis* infection rates, and the performance of routine diagnostic tests.

These experiments suggested that age has little effect on the early establishment of infection, since naive introduced neonates, weaners or ewes had similar infection levels after similar periods of exposure. Please note again that this trial did not attempt to examine the effect of age of exposure on subsequent disease development.

This study confirmed that culture of tissues is the most sensitive method currently available for the detection of early *M. a. paratuberculosis* infection, and demonstrated tissue culture as a practical tool for the detection of early infection in sheep after natural exposure. In contrast, routine ante-mortem testing in the first 12 months post-exposure detected few of the infected sheep. Skin-testing detected only 28%, IFN- $\gamma$  testing detected only 10% and faecal culture detected only 12%. The relatively low sensitivities for these tests found in this study reflect their use in the very early stage of the disease process and were not unexpected.

Another finding of particular interest was that histological lesions, often used as the "gold standard" for *M. a. paratuberculosis* infection in sheep, were rare in sheep exposed to *M. a. paratuberculosis* for less than 12 months. Only 17% of culture-positive sheep had typical histological lesions.

### 8. IMPACT ON MEAT AND LIVESTOCK INDUSTRY

Overall, the findings from this study suggest that the tracer concept could have practical application in assessing infectivity of pasture. Culture of tissues for *M. a. paratuberculosis* successfully detected infected sheep from about 6 to 12 months post-exposure in a range of infected environments, and a single pooled tissue culture from each sheep promises to be a cost-efficient method of examination. Because larger numbers of sheep are likely to be tissue culture positive in the early stages than may later show evidence of disease, manageable sample sizes are possible.

There are a number of possible advantages in using a tracer system based on culture of tissues for the detection of potentially infectious pastures. Culture of tissues is by far the most sensitive test to detect early infection, so that positive results are possible within 6-12 months of exposure. No other test gives reliable results so early, and an early positive result on a property would allow a rethink of approach to eradication. Also, clean sheep could be run on potentially infected pasture, tracers tested and the flock removed (say to slaughter or to a known infected property) before any likelihood of their excreting significant numbers of *M. a. paratuberculosis* in faeces. Thus, one could get an idea of the infection level on a property over time without the risk of compounding any infection. And because the age of sheep does not affect the likelihood of detecting early infection, any sheep grazed on potentially infected pasture could be opportunistically used as tracers.

The demonstration in these studies that age has little effect on the early establishment of infection will also be of general interest to industry. While this trial did not examine the effect of age of exposure on subsequent disease development, the fact that older sheep are as readily infected as young lambs is important. Intercurrent stressors may confound assumptions that older sheep are less likely to develop or transmit disease. In this trial, one group of ewes (subject to lactational and climatic stress) had a 60% infection-rate after 8 months of exposure, and one of those animals was already faecal culture-positive.

The overwhelming superiority of tissue culture over antemortem testing or histopathology for the detection of early *M. a. paratuberculosis* infection is very significant. Ante-mortem testing in the first 12 months post-exposure detected few infected sheep. Skin-testing detected only 28%, IFN- $\gamma$  testing detected only 10% and faecal culture detected only 12%. Histological lesions, often used as the "gold standard" for *M. a. paratuberculosis* infection in sheep, were also rare, detected in only 17% of infected sheep. These findings should be considered in any program which attempts to assess the possible infection status or risk of animals recently exposed to *M. a. paratuberculosis*.

#### **REFERENCE LIST**

- Anon. Ewe-lamb transmission of OJD and sensitivity samples for gamma interferon test evaluation. Final Report, Project OJD.024, National Ovine Johne's Disease Control and Evaluation Program, Meat and Livestock, Australia 2002.
- Beard PM, Rhind SM, Sinclair MC et al. Modulation of gammadelta T cells and CD1 in Mycobacterium avium subsp. paratuberculosis infection. Vet Immunol Immunopathol 2000; 77(3-4):311-9.
- 3. Begara-McGorum I, Wildblood LA, Clarke CJ *et al.* Early immunopathological events in experimental ovine paratuberculosis. Vet Immunol Immunopathol 1998; 63. (3.):265-87.
- Brotherston JG, Gilmour NJL, Samuel JMcA. Quantitative studies of *Mycobacterium johnei* in the tissues of sheep. 1 Routes of infection and assay of viable *M. johnei*. J Comp Pathol 1961; 71:286-98.
- 5. Cannon R. M., Roe R. T. Livestock disease surveys: a field manual for veterinarians. Australian Bureau of Animal Health. Australian Government Publishing Service. Canberra. 1982.
- Clarke CJ. The pathology and pathogenesis of paratuberculosis in ruminants and other species. J Comp Pathol 1997; 116(3):217-61.
- Cousins DV, Whittington R, Marsh I, Masters A, Evans RJ, Kluver P. Mycobacteria distenct from Mycobacterium avium subsp. paratuberculosis isolated from the faeces of ruminants possess IS900-like sequences detectable IS900 polymerase chain reaction: implications for diagnosis. Mol Cell Probes 1999; 13(6):431-42.
- 8. Dannenberg AM Jr. Delayed-type hypersensitivity and cell-mediated immunity in the pathogenesis of tuberculosis. Immunol Today 1991; 12(7):228-33.
- Garcia Marin JF, Benazzi S, Perez V, Badiola JJ. Study of the entrance of *M. paratuberculosis* in the lambs intestinal mucosa using immunohistochemical methods for antigen detection. 3rd Inter Colloq Paratuberc, Providence, RI, USA 1992; 370-7.
- 10. Giese SB, Ahrens P. Detection of *Mycobacterium avium* subsp. *paratuberculosis* in milk from clinically affected cows by PCR and culture. Vet Microbiol 2000; 77(3-4):291-7.
- Gilmour NJ, Brotherston JG. Further studies on immunity to *Mycobacterium johnei* in sheep. Relationship between hypersensitivity and host response to infection. J Comp Pathol 1966; 76(4):341-9.
- 12. Gilmour NJL, Angus KW, Mitchell B. Intestinal infection and host response to oral administration of *Mycobacterium johnei* in sheep. Veterinary Microbiology. 1978; 2. 223-35.
- 13. Gilmour NJL, Brotherston JG. Quantitative studies of *M Johnei* in the tissues of sheep. IV. The distribution of *M Johnei* shortly after oral dosing . J Comp Pathol 1962; 72:165-9.
- 14. Gilmour NJL, Halhead WA, Brotherson JG. Studies of immunity to *Mycobacterium johnei* in sheep. J Comp Pathol 1965; 75:165-73.

- Grant IR, Pope CM, O'Riordan LM, Ball HJ, Rowe MT. Improved detection of *Mycobacterium avium* subsp. paratuberculosis In milk by immunomagnetic PCR. Vet Microbiol 2000; 77(3-4):369-78.
- Gwozdz JM, Thompson KG, Manktelow BW, Murray A, West DM. Vaccination against paratuberculosis of lambs already infected experimentally with *Mycobacterium avium* subspspecies *paratuberculosis*. Aust Vet J 2000; 78(8):560-6.
- 17. Juste RA, Garcia Marin JF, Peris B, Saez de Ocariz CS, Badiola JJ. Experimental infection of vaccinated and non-vaccinated lambs with *Mycobacterium paratuberculosis*. J Comp Pathol 1994; 110(2):185-94.
- Karpinski T, Zorawski C . Experimental paratuberculosis in sheep. I. Clinical, allergical, bacteriological and post-mortem examinations . Bulletin of the Veterinary Institute in Pulawy. 1975; 19(3-4):59-63.
- 19. Kopecky KE, Larsen AB, Merkal RS. Uterine infection in bovine paratuberculosis. Am J Vet Res 1967; 28(125):1043-5.
- 20. Larsen AB, Merkal RS, Cutlip RC. Age of cattle as related to resistance to infection with *Mycobacterium paratuberculosis.* Am J Vet Res 1975; 36(3):255-7.
- 21. Larsen AB, Johnson HW . Studies on johnin.VII. Frequency of intradermal testing as related to local desensitisation. Am J Vet Res 1949; 10:344-6.
- 22. McQueen DS, Russell EG. Culture of *Mycobacterium paratuberculosis* from bovine foetuses [letter]. 1979; 55(4):203-4.
- Momotani E, Whipple DL, Thiermann AB, Cheville NF. Role of M cells and macrophages in the entrance of *Mycobacterium paratuberculosis* into domes of ileal Peyer's patches in calves. Vet Pathol 1988; 25(2):131-7.
- 24. Motulsky H. Intuitive biostatistics. New York: Oxford University Press, 1995: 250-1.
- 25. Nisbet DI, Gilmour NJL, Brotherson JG. Quantitative studies of *Mycobacterium johnei* in tissues of sheep. III. Intestinal histopathology. J Comp Pathol 1962; 72:80-91.
- 26. Payne JM, Rankin JD. A comparison of the pathogenesis of experimental Johne's disease in calves and cows. Res Vet Sci 1961; 2. 175-9.
- Perez V, Garcia Marin JF, Badiola JJ. Description and classification of different types of lesion associated with natural paratuberculosis infection in sheep. J Comp Pathol 1996; 114(2):107-22.
- Perez V, Tellechea J, Badiola JJ, Gutierrez M, Marin JFG. Relation between serologic response and pathologic findings in sheep with naturally acquired paratuberculosis. Am J Vet Res 1997. 58. 799-803.
- Perez V, Tellechea J, Corpa JM, Gutierrez M, Garcia Marin JF. Relation between pathologic findings and cellular immune responses in sheep with naturally acquired paratuberculosis. Am J Vet Res 1999; 60(1):123-7.

- Reddacliff LA. Aspects of the pathogenesis of ovine Johne's disease. PhD Thesis, University of Sydney 2002.
- Storset AK, Berntsen G, Larsen HJ. Kinetics of IL-2 receptor expression on lymphocyte subsets from goats infected with *Mycobacterium avium* subsp. *paratuberculosis* after specific in vitro stimulation. Vet Immunol Immunopathol 2000; 77(1-2):43-54.
- Streeter RN, Hoffsis GF, Bech-Nielsen S, Shulaw WP, Rings DM. Isolation of *Mycobacterium* paratuberculosis from colostrum and milk of subclinically infected cows. Am J Vet Res 1995; 56(10):1322-4.
- Sweeney RW, Whitlock RH, Rosenberger AE. *Mycobacterium paratuberculosis* cultured from milk and supramammary lymph nodes of infected asymptomatic cows. J Clin Microbiol 1992; 30(1):166-71.
- Sweeney RW, Whitlock RH, Rosenberger AE. Mycobacterium paratuberculosis isolated from fetuses of infected cows not manifesting signs of the disease. Am J Vet Res 1992; 53(4):477-80.
- 35. Tamarin R, Landau M. Congenital and uterine infection with *Mycobacterium johnei* in sheep. Refuah Veterinarith. 1961. 18. 43-4.
- Whittington RJ, Marsh I, McAllister S, Turner MJ, Marshall DJ, Fraser CA. Evaluation of Modified BACTEC 12B Radiometric Medium and Solid Media for Culture of *Mycobacterium avium* subsp. *paratuberculosis* from Sheep. J Clin Microbiol 1999; 37(4):1077-83.
- Whittington RJ, Marsh I, Turner MJ *et al.* Rapid detection of *Mycobacterium paratuberculosis* in clinical samples from ruminants and in spiked environmental samples by modified BACTEC 12B radiometric culture and direct confirmation by IS900 PCR. J Clin Microbiol 1998; 36(3):701-7.
- 38. Whittington RJ. Survival of Johne's disease in the environment. North Sydney: Meat and Livestock Australia, 2001.

#### **APPENDICES**

#### Appendix 1: Farm H, Year 1

Appendix 1a. Farm H, Year 1, Overview Appendix 1b. Farm H, Year 1, Cultures Appendix 1c. Farm H, Year 1, Pathology Appendix 1d. Farm H, Year 1, Skin test results

#### Appendix 2: Farm H, Year 2

Appendix 2a. Farm H, Year 2, Overview Appendix 2b. Farm H, Year 2, Cultures Appendix 2c. Farm H, Year 2, Pathology Appendix 2d. Farm H, Year 2, Skin test results Appendix 2e. Farm H, Year 2, IFN-γ results

#### Appendix 3: Farm A

Appendix 3a. Farm A, Overview Appendix 3b. Farm A, Cultures Appendix 3c. Farm A, Pathology Appendix 3d. Farm A, Skin test results