Demystifying Johne's disease in Deer

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Abstract

There has been an incremental increase in the incidence of M. paratuberculosis in New Zealand deer herds throughout the past decade. This has been seen through the increased numbers of cases of Johne's disease (Jd) confirmed by microbiological culture or the identification of M. paratuberculosis using specific molecular probes (IS900) to characterise M. paratuberculosis infection (de Lisle et al 2002). In recent years our laboratory (DRL) has noted an ever increasing number of tuberculin reactor deer presenting with immunological profiles that are not typical of either M. bovis or M.avium. The most logical interpretation of these reactions is that they are caused by infection with M. paratuberculosis. It is ironic that the diagnosis of M. paratuberculosis is rarely the primary goal, as most diagnostic specimens are submitted for Tb diagnosis. Up to 1/3 of pathological specimens submitted for culture to date had histopathology considered as 'typical of Tb'. More than 600 animals from 300 deer herds have been diagnosed with Jd over the past 15 years (de Lisle et al 2002). Because there has been no policy to eradicate the disease, it is likely that the majority of these herds have remained infected and continue to contribute to the cumulative total of infected herds nationwide. At this time it is not possible to estimate the true prevalence of Jd in New Zealand deer herds and it is likely that, depending on ones prejudice, the disease prevalence is likely to be grossly underestimated or overestimated. Suffice to say the disease has been diagnosed in deer herds throughout all geographic regions of New Zealand, with apparent 'hot spots' in some areas. The fact that Jd is a "Non-notifiable' disease in New Zealand makes it likely that the true prevalence of Jd is being underestimated. There is little incentive for individual farmers to confirm the presence of a disease within their herd, when diagnosis introduces ethical and marketing considerations. The absence of well refined methods to diagnose the disease accurately, provides little assurance that should the disease be diagnosed, it could be controlled within, or eradicated from, a deer herd. The objective of this paper is to attempt to demystify some of the concerns about Jd diagnosis so that farmers can more easily commit to a programme for the control of Jd in New Zealand deer herds.

Introduction

Johnes disease is difficult to study in living animals for a number of reasons:

- The disease has a prolonged period of incubation that may last for up to 5 years.
- Traditional culture techniques take months, and many sheep strains of *M. paratuberculosis* were impossible to culture.
- There are no good immunodiagnostic tests capable of diagnosing Jd in live animals, except those with terminal clinical disease.

However, significant advances have been made in improving Jd diagnosis recently, and these will be outlined in this paper.

Jd in deer presents with unique features which suggest that diagnostic systems need to be developed so that deer Jd can be diagnosed and managed more efficiently.

Deer may become infected with either cattle or sheep strains of *M. paratuberculosis,* while cattle and sheep are infected only with the homologous strain.

Deer may contract acutely fatal Jd where animals die between 8-15 months of age.

Deer Jd may cause significant mortality (10-20%) in young animals.

Jd lesions in the intestinal lymphatics of deer may become necrotic and indistinguishable from *M. bovis* infection.

Jd lesions in deer may be found in the retropharyngeal lymph nodes, the site where Tb is found predominantly in deer.

Diagnostic Methods

Microbial culture

It has been possible to isolate bovine strains of ***M.paratuberculosis*** using standard Herrold's egg yolk medium, supplemented with mycobactin J. However, until recently, it was not possible to isolate ovine strain ***M. paratuberculosis*** using this medium. Whittington (1999) showed that enriched Herrold's medium with increased concentrations of egg yolk (25%), was suitable to grow ovine strain ***M. paratuberculosis.*** Our laboratory has not had problems culturing ovine strain ***M. paratuberculosis,*** providing enriched medium is used. The disadvantage of this system is that it may take 3-4 months to isolate the bacteria.

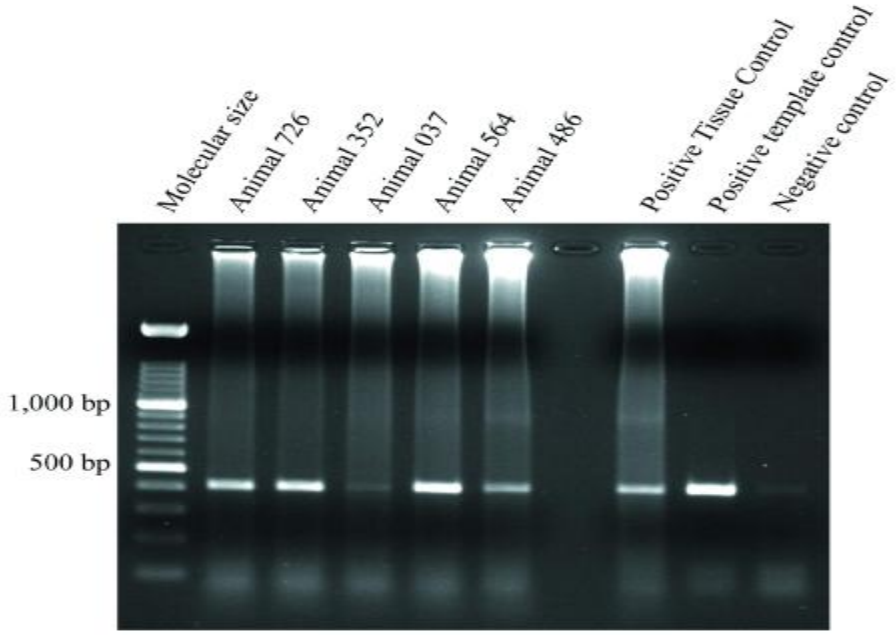
The radiometric ***Bactec*** system which uses detection of ^{14}C Radiolabelled CO_2, produced by actively metabolising mycobacteria. It was developed for ***M. tuberculosis*** isolation from human specimens. In modern Tb diagnosis it has become superceded by new detection MGIT methods which use expression of fluorescent markers to detect mycobacterial growth. Whittington (1999) has shown that the Bactec system, using enriched media, is suitable for isolating ***M.paratuberculosis,*** even the difficult to culture ovine strains. de Lisle's laboratory at Wallaceville (de Lisle 2002) now use the Bactec system to isolate both ***M. bovis*** and ***M. paratuberculosis*** . We are attempting to develop the MGIT system to isolate ***M. paratuberculosis***. The complicating factor is that the increased level of egg yolk used to supplement the medium causes quenching of the fluorescent probe. The advantage of the Bactec system is that it can produce a positive diagnosis of Jd within 3-5 weeks. The MGIT system is potentially even quicker should it be possible to adapt the technology for ***M. paratuberculosis*** isolation.

Molecular techniques to characterise M. paratuberculosis

The closely related subspecies of the ***M. avium*** complex group of mycobacteria may be distinguished at the DNA level based on the presence of specific genomic elements. Such genetic markers include Insertion Sequences (IS); a family of mobile genetic elements which occur frequently in mycobacteria. Certain of these insertion sequences have been found to be associated ***exclusively*** with individual members of the ***M. avium*** complex. ***M. avium*** subs. ***avium, paratuberculosis*** and ***silvaticum*** may each be identified by the presence is IS***901***, IS***900*** and IS***902***, respectively (Harris and Barletta, 2001).

We have applied Polymerase Chain Reaction (PCR) DNA amplification techniques to directly amplify copies of IS***900*** from the genomic DNA of ***M. paratuberculosis*** organisms present within the gut lymphatic tissues of suspect Jd infected animals. Following agarose gel electrophoresis of the PCR reaction, the presence of an amplification product of 400 base pairs (bp) is indicative of ***M. paratuberculosis*** infection. The results obtained from a series of ileocaecal lymph nodes, from affected animals, are given in Figure 1.

Figure 1. PCR products obtained directly from lymphatic tissues of 'suspect' Jd infected deer.



Diagnosis:

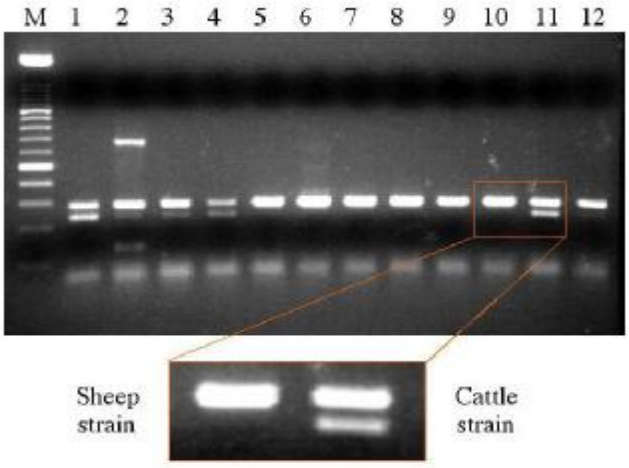
M. paratuberculosis confirmed directly from lymphatic tissues of affected animals; Animal Nos.726, 352, 564 & 486 - Positive.

Animal No. 037 – Negative

Positive tissue control – a DNA preparation from a known JD infected animal; positive template control – a DNA preparation from cultured *M. paratuberculosis*; negative control – reagent control containing no DNA template.

Cattle and sheep 'strains' of *M. paratuberculosis* may be distinguished based upon a point-mutation polymorphism which has been reported within IS*1311* (Marsh *et al.* 1999; Whittington *et al.* 1998). Following IS*1311* PCR amplification and subsequent restriction enzyme digestion of the PCR product, sheep strains of *M. paratuberculosis* generate a single DNA fragment (268 bp) whereas cattle strains exhibit two distinct fragments (268 bp and 218 bp), thus allowing these two variants to be readily distinguished. Data given in Figure 2 demonstrates that Samples 1, 3, 4 and 11 appear to be cattle strain, whereas the remaining isolates are sheep strain. Deer have been observed to be infected with either of the two strains.

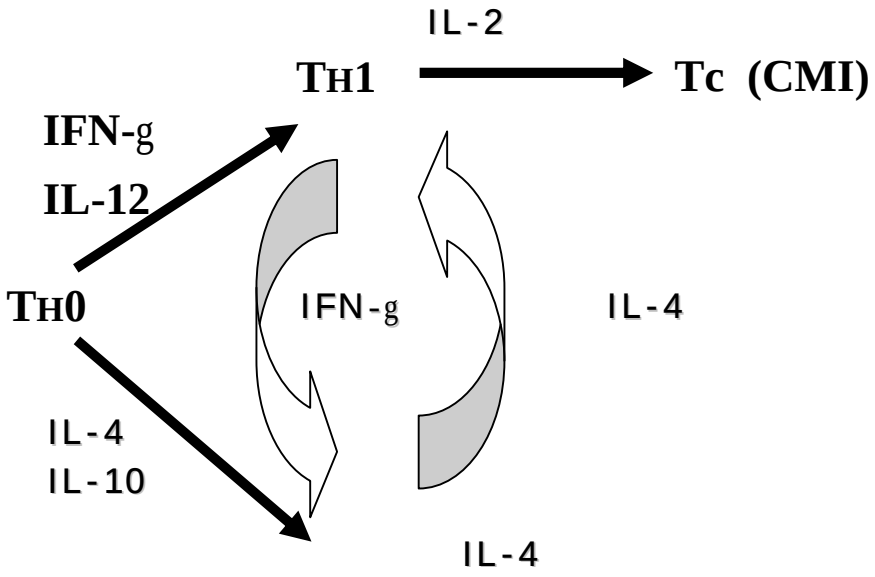
Figure 2. Typing of 'Bovine' and 'Ovine' strains of *M paratuberculosis* from deer isolates 1-12.



Immunodiagnosis of Johne's disease

Immunological techniques are used widely to diagnose infections where the infectious agent is difficult to isolate. *M. paratuberculosis* fits this category where intracellular organisms cannot be isolated except in cases where the animal is actively excreting bacteria in their faeces. Bulk faecal culture has been used at the flock/herd level to diagnose infected groups of animals but it cannot diagnose infection at the individual animal level. While this is possible in clinically affected animals it is not appropriate to diagnose subclinical infection. Jd produces a polarised response where early infected or immune animals (paucibacillary) tend to produce **TH1** type responses (Figure 3). At the other extreme clinically affected animals tend to produce **TH2** type responses. The goal is to develop immunodiagnostic techniques to cover all aspects of immunity to *M. paratuberculosis.* We have chosen to measure the levels of cytokines to monitor the relative amounts of **TH1** or **TH2** reactivity. Interleukin – 2 (IL-2) and Interferon- gamma (IFN-γ) is used to monitor **TH1** reactivity, while Interleukin – 4 & 10 (IL-4, IL-10) is used to monitor **TH2** responses.

Figure 3: TH1 vs TH2 Based Regulation of Immunity

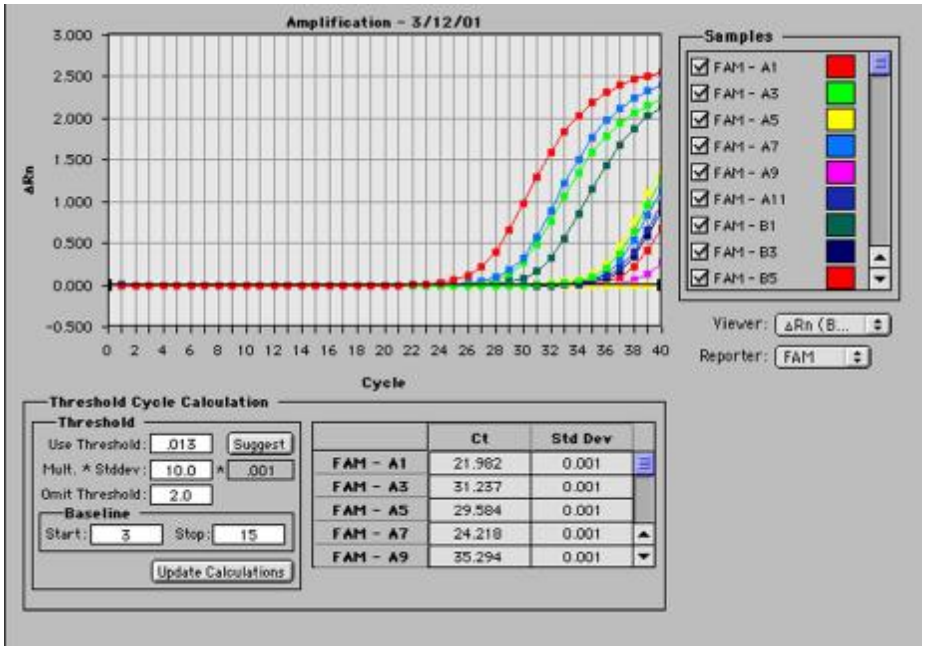


TH2 → B cell (IgG1)

Traditionally, immunological techniques have been used to measure cytokine levels produced by blood or lymphatic lymphocytes. These are difficult to develop for exotic species as they require specialised monoclonal antibodies to assay each cytokine. Some cytokines are produced at such low levels and are absorbed onto the cells that produce them.

A potentially more flexible technique is to use molecular probes to detect mRNA for individual cytokines. A recently developed Real Time PCR (RT-PCR) allows for the relative quantification of cytokine mRNA. Extracted RNA is transcribed into cDNA and amplified by PCR using sequence specific primers for each cytokine. Cells producing high levels of mRNA will have relatively large amounts of cDNA which will in turn be detected early in a Real Time PCR reaction. The technique is shown graphically in Figure 4. The cytokines present at the highest levels are detected first in the PCR reaction. By determining the number of PCR cycles necessary to produce detectable levels of PCR product, it is possible to estimate the relative amounts of cytokine mRNA produced by immune cells of different animals.

We have developed RTPCR techniques capable of detecting all of the cytokines necessary to characterise **TH1** or **TH2** reactivity. These will be applied to blood samples from *M. paratuberculosis* infected deer, stimulated with tuberculins (A, B & J), to obtain maximal



expression of cytokine genes

Figure 4: Real Time PCR to detect cytokine production

PCR reactions are actively monitored throughout each cycle to ascertain relative quantities of cytokine mRNA.

IgG1 ELISA and Jd diagnosis

As is shown in Figure 4 above, it is possible to measure the **TH2** response indirectly by monitoring levels of IgG1 antibody specific for *M. paratuberculosis* antigens. We have developed an IgG1 ELISA test which can measure responses in animals infected with *M. paratuberculosis.* Results from a group of animals with confirmed Jd lesions is given in Table 1. These show that when tuberculins from *M. bovis* (PPD-A), *M. avium* (PPD-A) and

M. paratuberculosis (PPD-J) are used in the IgG1 ELISA it is possible to identify most animals as positive for J (i.e. J >> B). A small group of non-lesioned animals are included for comparison.

Table 1. IgG1 ELISA values in necropsied deer considered 'at risk' from Johne's disease

Animal Number	IgG1 ELISA (E units)			Lesion status*
	PPD-A	PPD-B	PPD-J	
59	177	152	183	3
123	173	114	172	3
129	182	87	171	3
444	161	69	174	3+
5650	195	149	194	4
336	175	149	179	3
351	168	114	170	3+
398	180	138	183	3+
459	186	156	181	3
792	165	98	180	3+
919	179	97	183	3
954	162	158	175	3
CHW360	193	160	195	3
103	180	119	188	3
447	41	0	27	0
1156	29	0	9	0
151	33	52	59	0
1996	74	7	36	0
1423	13	1	0	0
2392	20	14	0	0

* Lesions were classified on a scale of 1-5, where 1 indicates minimal granulomatous reactivity with no acid fast bacilli and 5 indicates extensive necrotic lesions with multiple acid fast organisms. All lesioned animals had *Mycobacterium paratuberculosis* confirmed by IS900 (+) PCR reactivity. All lesion free animals (0) were PCR (-)

Whereas the immune profile seen in all the lesion (+) animals showed J>B reactivity, there was a close correlation between the level of antibody to A *vs.* J. We need to further refine the assay to get greater discrimination between A and J antigens. We are about to necropsy 50 animals with differing immune profiles and we will carry out detailed necropsies and follow-up histopathology and microbiological analysis. These will be correlated retrospectively with the immune profiles to determine if the ELISA can detect subclinically affected animals.

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