EXPERIMENTAL *MYCOBACTERIUM BOVIS* INFECTION IN RED DEER WEANERS - PRELIMINARY FINDINGS

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INTRODUCTION

Under the current Deer Tuberculosis Control Programme in New Zealand all Tb infected deer must be slaughtered. In order to conduct detailed studies on the epidemiology and immunology of *Mycobacterium bovis* (Tb) in deer it is essential to have a deer farm where Tb infected animals can be held long term and managed appropriately.

Special permission was granted by the Chief Veterinary Officer and the Animal Health Board to conduct long term Tb research on a deer farm in a Tb endemic area near Milton, south of Dunedin. Specific requirements for quarterly wildlife control in a 3 km radius around the "Infected Deer Farm" (IDF) were imposed in order to ensure the safety of the neighbouring farms. The perimeter fence of the IDF was "possum-proofed" with a combination of wiremesh and electrified outriggers. MAFQual independently monitor possum populations on the IDF and surrounding 1 km zone twice-yearly to ensure possum numbers remain low.

The original deer herd had around 5% Tb prevalence, but because it was impossible to define when and how they became infected it was decided that the farm should be depopulated and restocked with Tb-free animals and start with"a clean slate".

Development of an experimental Tb model

The most efficient system for testing epidemiological and immunological parameters is to use an experimental infection system which repeatably reproduces naturally acquired Tb. The most important variables in such a Tb infection model are: age of the deer, route of administration and dose rate. Other variables include the health and nutritional status of the animals and the strain of Tb. A trial was undertaken on the IDF to develop an experimental Tb infection system in young red deer farmed under typical NZ conditions, using a field strain of *M. bovis* isolated from a clinically diseased red deer, but with two different dose rates and three different routes of inoculation.

MATERIALS AND METHODS

Fifty red deer weaners were purchased in June 1993 from a farm in a Tb-free accredited herd in a Tb-free area, were moved onto the IDF and were subjected to a blood test for Tb (BTB, ie, lymphocyte transformation test plus ELISA). All were negative to the BTB and they were randomly allocated to 5 groups of 6 inoculated animals plus 2 groups of 10 and 1 group of 5 uninoculated animals (see Table 1).

TABLE 1. Allocation of animals to treatment groups.

Group No.	No.	Route of inoculation	Estimated* dose of *M bovis* (c.f u.)
1	5	Intra-tracheal	10^2
2	5	"	10^4
3	5	Intra-tonsil	10^2
4	5	"	10^4
5	5	Intra-nasal	10^2
6	5	"	10^4
7	10	Long term (35 week) in-contact controls	Nil
8	10	Short term (9 week) in-contact controls	Nil
9	5	Remote controls	Nil

c.f.u. = colony forming units
* actual dose = two times estimated dose

Organism - A strain of *M. bovis* which had been isolated from a clinical case of Tb in a deer from another district in NZ was used. This had a restriction endonuclease pattern different from the *M. bovis* isolated in the Milton area

Inoculation - All the inoculated weaners were heavily sedated with an intravenous injection of xylazine 0.8 mg/kg.

Intra-tracheal - A 10x10 cm square of hair was clipped in the mid line of the neck over the trachea. A 16 g x 1" needle was pushed through the skin into the lumen of the trachea and a 21 g x 1½" needle was passed down it. A 1 ml dose of inoculum (estimated 10^2 or 10^4 organisms) was injected and flushed in with 2 ml of saline.

Intra-tonsil - With the animal in sternal recumbency and the mouth held open by an assistant the tonsils could be visualised. A 1 ml tuberculin syringe with 21 g x 1½" needle was carefully directed through the mouth and the tip of the needle inserted 10 mm inside the left tonsillar crypt and 0.2 ml injected (estimated 10^2 or 10^4 organisms).

Intra-nasal - A 3" long needle, with the bevelled tip bent over the orifice to create a spray when injected, was introduced up the left nostril and 4 ml (estimated 10^2 or 10^4 organisms) squirted in under pressure as the animal inspired.

Actual dose - Subsequent culture showed that the two dose rates of inoculum contained $2x10^2$ or $2x10^4$ colony forming units.

Recovery - The sedation was reversed with an intravenous injection of yohimbine (0.25 mg/kg).

Ten uninoculated control animals were run in-contact with the group for the entire 35 weeks. A second group was introduced after 24 weeks and run together for the last 9 weeks.

Five uninoculated control animals were run in paddocks never grazed by inoculated deer and always with one paddock separating them from infected animals.

Monitoring infection - All the animals were weighed, examined and blood sampled every 2 to 4 weeks for the following 35 weeks. The lymphocyte transformation test (LT) plus ELISA (Griffin *et al*, 1991), also known as the blood test for Tb (BTB) was performed on all blood samples. Acute inflammatory proteins (AIP) and haematology were measured on weeks 18, 22, 29, 31 and 33 using methods described elsewhere (Cross, 1987).

On four occasions (10, 18, 22 and 29 weeks after inoculation) two animals from each group were sedated with xylazine (0 8 mg/kg) and swabs of the nasal passages and the throat were taken, followed by 50 ml of saline passed into the lungs via a 16 g x 2" veni-catheter passed directly into the trachea (as per inoculation) and the "lavage" liquid was then sucked up by syringe attached to a 30 cm fine catheter passed down the veni-catheter. The swabs and lavage liquid were cultured for *M. bovis*.

Skin test - A comparative cervical test (CCT) using avian and bovine PPD at two closely clipped sites on the neck, was carried out after 33 weeks. The test was regarded as positive if the bovine site increased by ≥2 mm and was ≥ the avian site increase, when the double skin thickness was measured before and 72 hours after injection

Lesion severity prediction - Prior to slaughter the animals were ranked according to the likelihood of having Tb lesions and the degree of severity of any lesions, based on LT, ELISA, haematology and AIP profiles throughout the trial.

Group A: Unlikely to have lesions (negative LT, negative ELISA, negative AIP.
Group B: Some pathology but unlikely to be Tb (negative LT, negative ELISA, low to moderate AIP).
Group C: Low number of lesions: positive LT, negative ELISA, negative to low AIP.
Group D: Moderate number of lesions: elevated LT, ELISA positive latterly, low to moderate AIP
Group E: Severe lesions: high LT, consistently elevated ELISA, moderate to high AIP.

Slaughter - At 35 weeks after inoculation all 55 animals were transported to the Invermay Deer Slaughter Plant and slaughtered. All the thoracic and visceral organs were removed and examined The tonsils and all major lymph nodes (LNs) were removed, finely sliced and examined for Tb.

Histopathological examination - Samples of all gross lesions were examined histologically and confirmed as typical or suspicious of tuberculosis. Estimates of acid fast organisms (AFOs) numbers were made for each Tb lesion.

Culture - All lesions were cultured for Tb. In all cases where lesions were not typical of Tb or there were no visible lesions, four pools of lymph nodes (LN) were cultured; (a) left and right retropharyngeal LN, (b) left and right bronchial LNs, (c) anterior and posterior mediastinals, (d) mesenteric chain.

RESULTS

The following is a brief summary of the results which will be published in more detail elsewhere.

Clinical assessment - One animal in the 10^4 intra-trachea group died 13 weeks after inoculation having lost 4 kg in the previous two weeks at a time when the rest of the group lost 1-2 kg due to a late cold spring and feed limitations. The remaining animals all gained weight for the rest of the trial and the growth rates and final weights were not correlated with final Tb outcome.

During the trial no obvious signs of disease were seen in any of the animals apart from an abscess 10x10 cm on the face of a weaner over the masseter muscle. Samples were taken and they were ZN smear and culture negative and the lesion healed up within 2 weeks

Lymphocyte transformation test (LT) - All deer that were culture positive for *M. bovis* at slaughter had consistent LT (bovine) values (see Table 2). The majority of these infected animals became LT positive 4-6 weeks after inoculation, with a few as early as 2 weeks. Only one animal (Y6) had consistently high LT values and was culture negative. It had only one gross lesion in an ileocaecal LN which was classified as suspicious on histological examination (see later) . All other animals were NVL, culture negative and had low LT values which were very variable and oscillated between avian, negative and low bovine reactivity.

ELISA - In general, the animals with the most severe lesions (see scale in Table 3) had the highest LT (Bov) responses and the most prolonged antibody levels, whereas animals with few small lesions had low or negative ELISA values. There were two exceptions: one animal (Y28), with lesions in the lung, head and abdominal lymph nodes and high LT, was ELISA negative, and one animal (Y6), with a single lesion and high LT (Bov), had a high ELISA titre. This latter animal, together with five others, had negative ELISA values, except in the last blood sample taken at week 35, ten days after the CCT.

The inoculated groups (1-6) had 3, 5, 2, 5, 1 and 3 animals respectively with antibody at some stage during the trial, and of these 2, 5, 0, 0, 0 and 2 respectively were consistently elevated, ie, 7/10 of the animals inoculated by the intra-tracheal route and 2/5 given 10^4 intra-nasally.

CCT - The CCT results are presented in Table 2. All the CCT +ve animals yielded *M. bovis* isolates and were LT +ve except for Y6 which was LT positive (B9) and ELISA positive (B3) but culture negative. All the CCT negative animals were culture negative and negative or low LT positive and ELISA negative.

Gross lesions - At slaughter the distribution and severity of lesions (confirmed as Tb by histopathological examination and culture) correlated with both the route of administration and the size of the inoculum ($p<0.05$) (see Table 3). The intra-tracheal route generally resulted in the most severe lesions, involving the lungs, thoracic LNs and in three cases abdominal LNs. The intra-tonsil route resulted in only retropharyngeal LN lesions apart from one abscessed tonsil and one abscessed ileocaecal LN. The intra-nasal route resulted in head (5 animals), thoracic (5 animals) and abdominal (2) LN lesions There were 1, 0, 1, 1, 4 and 0 animals respectively with no visible lesions (NVL) in the six inoculated groups respectively.

One long term in-contact animal (Y37) had two lesions (1x1x1 mm, 13x5x5 mm) in the left retropharyngeal LN.

TABLE 2. Lymphocyte transformation (LT), ELISA, Comparative Cervical Test (CCT) and culture results (*M. bovis*) for the 6 inoculated groups, 2 in-contact groups and a remote control group.

TAG No	TREATMENT GROUP ROUTE & DOSE	LT BOVINE POSITIVE weeks from inoculation when first positive	ELISA BOVINE POSITIVE weeks from inoculation when first positive	CCT RESULT	CULTURE POSITIVE
1	INTRA-TRACHEAL 10#2	4	6	POS	M b
2		4	10	POS	M b
3		4		POS	M b
4					
5		6	8	POS	M b
6	INTRA-TRACHEAL 10#4	4	4	POS	
7		2	4	POS	M b
8		4	8	POS	M b
9		2	6	ND	M b
10		6	6	POS	M b
11	INTRA-TONSIL 10#2	6		POS	M b
12					
13		4	35	POS	M b
14		6	6	POS	M b
15		4		POS	M b
16	INTRA-TONSIL 10#4	2		POS	M b
17		6	35	POS	M b
18		10		POS	M b
19		6	35	POS	M b
20		4	35	POS	M b
21	INTRA-NASAL 10#2				
22					
23					
24		6	35	POS	M b
25					
26	INTRA-NASAL 10#4	2	35	POS	M b
27		6		POS	M b
28		2		POS	M b
29		4	4	POS	M b
30		6	6	POS	M b
31	8 MTH INCONTACTS				
32					
33					
34					
35					
36					
37		10		POS	M b
38					
39					
40					
42	3 MTH INCONTACTS				
44					
46					
48					
50					
O40					
O42					
O43					
O45					
O46					
41	REMOTE CONTROLS				
43					
45					
47					
49					

TABLE 3. Summary of lesion severity distributions in the six groups of weaners inoculated with *M bovis* by three different routes and two dose rates (10^2 and 10^4).

Group (n)	NVL	Tb lesion severity and distribution			
		Small LN lesions	Moderate LN lesions	Moderate lung & LN lesions	Severe lung & LN lesions
Intra-tracheal					
1. Low dose (5)	1	1	0	1	2
2. Medium dose (5)	0	1	0	1	3 (1 died)*
Intra-tonsil					
3. Low dose (5)	1	4	0	0	0
4. Medium dose (5)	1	2	2	0	0
Intra-nasal					
5. Low dose (5)	4	0	1	0	0
6. Medium dose (5)	0	0	2	0	3
In contact controls					
7. Long term (10)	9	1	0	0	0
8. Short term (10)	10	0	0	0	0
Remote controls (5)	5	0	0	0	0

* Animal (Y9) died 13 weeks after inoculation

Culture - *M. bovis* was isolated from all lesions with histologically typical or suspicious of Tb except for one animal (Y6) in the intra-tracheal 10^4 group (see Table 2). This animal had consistently elevated LT and ELISA and was CCT positive. All the samples are currently being recultured. Apart from this animal there was 100% agreement between culture, BTB (LT and ELISA) and CCT results.

M. bovis was isolated from one long term in-contact animal. There were no *M. bovis* isolates from any of the 7 NVL inoculated animals, or the other 9 long term in-contact animals or the 10 short term in-contact animals.

Lesion severity prediction - The animals were killed in order of predicted lesion severity starting with no lesions (Group A) and ending with most severe (Group E) (see Table 4).

It was predicted that 27 animals would have Tb lesions (Groups C + D + E) of varying severity and 27 would be NVL (A + B). In fact, gross autopsy with histological and culture confirmation showed that 24 animals had lesions and 30 were NVL. There were three false positive predictions (90% specificity), but all the lesion positive animals were picked to have lesions (100% sensitivity)

Of the animals that had Tb, there was significant Spearman Correlation between the predicted and actual lesion severity (r=0.544; t=3.044; P<0.05). The Spearman Correlation between actual and predicted lesion severity based only on LT and ELISA results was also significant (r=0.42; t=2.15; $p<0.05$) showing that acute inflammatory protein profiles increase the precision of predicting lesion severity.

TABLE 4. Predicted lymphocyte transformation, ELISA and lesion status of animals from the IDF based on inflammation data.

Group	Likelihood of lesions	Animal tag number (see Table 2)
A	Unlikely to have lesions	4, 22, 23, 31, 32, 33, 34, 36, 39, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 040, 042, 043, 045
B	Some pathology, unlikely to be Tb	12, 21, 31, 38, 046
C	Low number of lesions	3, 5, 11, 13, 15, 16, 18*, 19, 20, 24,, 25, 35, 40
D	Moderate number of lesions	6, 14, 17, 26, 27, 28, 29, 37
E	High number of lesions	1, 2, 7, 8, 10, 30

* possibly a contained lesion

DISCUSSION

The objective of the trial was to develop an experimental Tb infection model for red deer weaners which resulted in the most "natural" disease, in terms of lesion distribution, severity, and speed of development, and also in terms of the LT, ELISA and acute inflammatory protein profiles.

Tb in deer most commonly affects the lymphatics in the head area. In one study of 119 tuberculous deer on four properties (Beatson, 1985 citing Livingstone, 1980) over half the animals (53%) had head lesions only, 11% head and abdomen, 9% thorax only, 6% head and thorax, 6% head, thorax and abdomen and 7% abdomen only. Altogether, 76% had head lesions alone or with other lesions. In another report on 71 confirmed cases of Tb in deer sent for slaughter (Wilcockson, 1986) 27% were head only, 31% were abdominal only and 4% thoracic only.

Exposure to Tb can also have a wide range of outcomes from inapparent infection, through mild progressive disease, to serious disseminated disease involving a number of areas of the body, and some animals develop open sinuses draining onto the skin surface. Typically, however, Tb in deer is a subacute or chronic disease (Clifton-Hadley and Wilesmith, 1991) and on farms where infection is detected early, the lesions are small (<20 mm) and tend to be confined to a small number of lymph nodes, usually in the head or abdomen, as shown by deer slaughter plant cases. This is in contrast to herds where cases have gone undetected for some time and a large proportion of animals become seriously diseased. However, even in these herds a proportion of animals remain uninfected, demonstrating a degree of "natural resistance". For example, a recent serious outbreak resulted in a herd depopulation and despite 21% of animals having generalised Tb, 55% were still NVL (Griffin, unpub. data).

In the present study, the intra-tonsil route resulted in very typical lesions at slaughter 8 months after inoculation. The lower dose rate (200 c.f.u.) produced 1 NVL and 4 mild LN lesions confined to the retropharyngeals. The higher dose rate (20,000 c.f.u) produced 1 NVL, 2 mild LN lesions and 2 moderate LN lesions. The LT, ELISA and AIP profiles closely mimicked early natural infection This route of inoculation at a dose rate of 200 c.f.u. should produce a sensitive model for use in future experimental infections, immunological and epidemiological studies, and investigations of natural and acquired resistance, including vaccine challenge trials.

By contrast the intra-tracheal route tended to result in severe lung lesions, very high persistence LT responses and early high antibody responses and one early death. Although severe lesions are seen in natural Tb in deer, they are much less common than cases involving the head and abdominal lymph nodes. However, it is unusual to see such high LT and ELISA values so early in infection and this probably reflects the unusually rapid development of serious lesions, especially in the animals receiving the higher dose ($2x10^4$ c.f.u) This presumably overwhelmed the defences of the animal and drove it towards a B cell response (antibody) which is non-protective The intra-nasal route produced very variable results. The lower dose resulted in only one confirmed case of Tb while the higher dose produced 5, all of them severe or moderately severe, with 2 cases involving head, thoracic and abdominal LNs.

Tb in deer is probably acquired by both the respiratory and oral routes (Clifton-Hadley & Wilesmith, 1991), therefore it is most appropriate to use one of these routes in an experimental infection model. Our studies showed that the intra-nasal route, as well as being risky to the operators, produced a variable and unsatisfactory result The intra-tracheal route, although more natural than intravenous or subcutaneous (de Lisle *et al*, 1983), produces very severe, atypical lesions and immune responses. Of all the routes so far investigated the intra-tonsil route, which is relatively safe and easy to accomplish, results in typical retropharyngeal lesions and the "normal" range of immunological responses and fulfils the requirements of a good experimental challenge system for deer.

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