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DNA TYPING OF JOHNE'S DISEASE ORGANISMS

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Abstract

This study was undertaken to determine whether Johne's disease in sheep and cattle in Australia can be considered to be separate diseases subject to independent control programs. Isolates of the bacterium that causes Johne's disease, namely *Mycobacterium avium* subsp. *paratuberculosis*, were typed genetically in order to see whether those in sheep were different to those in cattle. About 350 separate isolates were evaluated from about 100 farms in New South Wales, Victoria, Tasmania and South Australia. A new test was developed to enable the bacterium to be typed quickly and accurately. Johne's disease in sheep was almost always due to sheep strains of *M. avium* subsp. *paratuberculosis* while cattle were almost always infected with cattle strains. However, it was concluded that Johne's disease has occasionally spread from sheep to cattle in New South Wales, probably under unusual circumstances. Johne's disease may also spread from sheep to goats. As a result of this project Johne's disease control programs will be assessed on an ongoing basis as more information is obtained about the way the disease may be spread between farm animals.

Executive summary

This project was undertaken to determine whether Johne's disease in sheep and cattle can continue to be considered to be different diseases, subject to separate control and market assurance programs.

The specific objectives were to genetically type the bacterium that causes Johne's disease, namely *Mycobacterium avium* subsp. *paratuberculosis*, to compare isolates of this bacterium from cattle, sheep, goats and other farm animals, to develop a rapid laboratory method for distinguishing sheep isolates from cattle isolates and to identify whether cross-species infection occurs between sheep and cattle. All of the objectives were achieved.

A total of 354 isolates of *M. avium* subsp. *paratuberculosis* was tested using restriction fragment length polymorphism (RFLP) analysis and other techniques. Isolates were obtained mainly from sheep and cattle from about 100 farms in New South Wales and Victoria, but some samples were included from Tasmania, South Australia and France.

Fourteen RFLP types were found. Type S1 was the dominant type in sheep in New South Wales, accounting for 95% of isolates, and was the only type found in sheep from Victoria. Several sheep isolates obtained in New South Wales during the 1980's together with several isolates from sheep from France, were of cattle type. These bovine types of *M. avium* subsp. *paratuberculosis* are currently not involved in the epidemiology of ovine Johne's disease in Australia and would appear to represent unusual events in the past.

Seven RFLP types were present in cattle. Types C1 and C3 were the most common, but C1 was not found at all in New South Wales and C3 was absent from beef cattle in Victoria. Type C5 was the next most common type, accounting for 7% of isolates. Several RFLP types were present in some geographic regions in Victoria, for example types C1, C3, C5, CU4 and C12 were found amongst beef and dairy cattle in Gippsland. Several types may occur in cattle on the one farm; 2 RFLP types were found on 6 farms while 3 RFLP types were found on 2 farms. In general it appeared that individual cows were infected with only one type, but one cow was infected with both C5 and CU4. Given the existence of geographical and farm enterprise restriction in RFLP type, the technique may be applied to trace the future spread of Johne's disease in the cattle industries.

A rapid typing method was developed based on the IS*1311* gene. Over 400 isolates of *M.*

avium subsp. *paratuberculosis* were examined with the new test and complete agreement was obtained with the RFLP test and host species.

The IS*1311* test was further developed and used to examine animal tissues stored in archives. Several cases of Johne's disease in cattle were found to have been due to sheep strains of *M. avium* subsp. *paratuberculosis*. In each case it appeared that young calves had been reared in association with infected sheep. The disease did not appear to have become established in cattle herds and one herd is now tested negative in the cattle market assurance program. Serological investigation of cattle was also undertaken on 3 farms with ovine Johne's disease during this study. The cattle had grazed as calves with infected sheep and were > 2 years old when sampled. All were seronegative. These findings suggest that cattle have not commonly become infected with sheep strains of *M. avium* subsp. *paratuberculosis*, despite exposure to the organism.

Ovine Johne's disease was shown to have spread from sheep to fibre goats on a farm in New South Wales during this study. There had been a high degree of contact between infected sheep and the goats on the property over a long period.

The main conclusion from this work is that sheep and cattle in Australia tend to be infected with mutually exclusive strains of *M. avium* subsp. *paratuberculosis* however, cross-species transmission has occurred occasionally. This is vital information for industry because it provides general validation of the current recommendations for control of ovine Johne's disease and market assurance program testing of cattle, i.e. that cattle can safely graze pasture after removal of infected sheep. However, it also indicates a need for industry to continue to monitor the situation and avoid the grazing of calves where exposure to high levels of contamination from sheep could occur. Similar comments could apply to goats, although their susceptibility as adults to ovine strains of *M. avium* subsp. *paratuberculosis* is uncertain.

As a result of the findings of this study, a working group was established under Veterinary Committee to make recommendations on cross-species transmission. Detailed recommendations from this group will follow further analysis of data obtained from New South Wales and Victoria.

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Main research report

Background and industry context

Johne's disease is a chronic granulomatous enteropathy of herbivores caused by infection with *Mycobacterium avium* subsp. *paratuberculosis*. In Australia the disease has been identified in beef cattle, dairy cattle, sheep, goats, alpaca, llama and a rhinoceros, but in other countries species including deer, rabbits, bison and various herbivores in zoological collections have been found with Johne's disease.

It is well-established that Johne's disease in cattle is quite distinct from the disease in sheep and where the two species graze together the infection is believed rarely to be transmitted between them. There are a number of possible explanations for these observations:

1. There are different strains of *M. avium* subsp. *paratuberculosis* which may have genuine host specificity, host preference or host adaptation. The different strains may have different routes of infection or environmental survival which, when combined with factors below, tend to favour infection of one or other host species.

2. Sheep and cattle may have different grazing behaviour which may tend to result in isolation of infection within one or other of the species.

3. Management practices may tend to reduce the chance of spread of infection between the two species.

There is now a body of literature on typing of *M. avium* subsp. *paratuberculosis* and it has been clearly established that there are strains or types that tend to infect sheep and other strains or types that tend to infect cattle (Collins et al 1990). Unfortunately the nomenclature of strains is not yet standardised and some of the literature is confusing, even to those actively involved in this type of research.

The main industry context of this project is sheep and cattle grazing and dairy farming. However, because goats, alpaca, deer and other herbivores may come into contact with sheep and cattle in Australia all relevant animal industries are considered in this project.

Project objectives

The principal aim of this project was to determine whether cross-species infection of *M. avium* subsp. *paratuberculosis* occurs between sheep and cattle in Australia. Two approaches were used: comparison of the strains of *M. avium* subsp. *paratuberculosis* present in sheep and cattle populations and a field survey using serological testing of cattle exposed to sheep with ovine Johne's disease. This was combined with the development of improved technology.

The specific objectives were to:

1. Identify whether sheep and cattle/goat/alpaca isolates of *M. avium* subsp. *paratuberculosis* are genetically different

2. Develop a rapid laboratory method for distinguishing sheep isolates of *M. avium* subsp. *paratuberculosis* from cattle isolates of *M. avium subsp. paratuberculosis.*

3. Identify whether cross-species infection of *M. avium* subsp. *paratuberculosis* occurs between sheep and cattle.

Note: this project is related to TR.007

Methodology

A) DNA typing using restriction fragment length polymorphism (RFLP) analysis

Selection of samples for evaluation. It was determined in MLA planning meetings for this project that the following should be evaluated: sheep from 20 farms from New South Wales (5 isolates per farm, total 100 isolates), 3 farms from Victoria (15 isolates) and 2 farms from Tasmania (10 isolates); 20 isolates from cattle (15 New South Wales, 5 Victoria), and; 5 isolates from goats/alpaca from Victoria and/or New South Wales.

Ovine and some caprine samples for bacterial purification from intestine. Sheep with Johne's disease were selected from farms in New South Wales and Victoria so that different farms and geographic areas were represented. Wherever possible, multiple sheep were selected from each farm. Attempts to procure samples from sheep from Flinders Island, Tasmania were unsuccessful. In New South Wales, sheep with suspected clinical Johne's disease were collected from farms and transported to Regional Veterinary Laboratories at Orange and Menangle for necropsy. In Victoria, sheep were subjected to necropsy as part of a separate project. Some samples from goats in New South Wales were obtained by direct purification from the intestinal mucosa where it was thought that an ovine strain of *M. avium* subsp. *paratuberculosis* was likely to be present.

Bulk culture of bovine samples and samples from other species. Isolates of *M. avium* subsp. *paratuberculosis* were collected from routine diagnostic and research accessions to laboratories in New South Wales (EMAI) and Victoria (VIAS). Isolates of *M. avium subsp. paratuberculosis* were obtained from faeces or tissues using double incubation centrifugation methods and culture in modified BACTEC 12B medium or on HEYM slopes (Australian Standard Diagnostic Tests for Johne's Disease, 1998 - draft).

At EMAI, isolates from lyophilised cultures were reconstituted in 250 uL sterile distilled water and the entire volume was inoculated on HEYM+MJ slopes. These were incubated at 37°C for 4-6 months. Isolated colonies were subcultured into 120 mL Watson Reid broth medium containing mycobactin J and pyruvate and incubated for 4-8 months. Isolated colonies were also subcultured from archived HEYM slopes stored at room temperature. In this case colonies were collected onto a sterile cotton swab moistened with Watson Reid broth medium. The swab was placed in 120 mL Watson Reid broth medium for 1 min then

withdrawn and the broth was incubated at 37°C for 4-8 months. If growth was not apparent the procedure was repeated by subculture to a 10 ml volume of Watson Reid broth medium and later the entire volume was added to 120 mL of this medium.

At VIAS, isolates from lyophilised cultures were reconstituted in 0.4 mL Donor Horse Serum (CSL Pty Ltd) and 0.1 mL PANTA Plus and incubated overnight at 37°C. An aliquot of 0.3 mL was inoculated into modified BACTEC 12B medium and incubated at 37°C. When the growth index was greater than 500, 2 mL from the BACTEC 12B medium was inoculated into 60 mL of modified Watson Reid medium and incubated at 37°C. Isolates were also recovered from stored HEYM slopes: colonies were suspended in 1.2 mL PBS and 0.1 mL PANTA Plus, incubated overnight at 37°C then transferred directly to flasks containing 60 mL modified Watson Reid medium. Modified Watson Reid medium cultures were incubated for 5-10 months.

Cultures were harvested by centrifugation at 15,000 g for 30 min at room temperature. Pellets were washed 3 times with sterile PBS pH 7.2 and once with sterile distilled water. Bacterial pellets were dispensed in 0.5 g aliquots and stored at -20°C.

Extraction of M. avium subsp. paratuberculosis from the intestinal lining. The RFLP method requires relatively large amounts of DNA and this material is generally obtained by culture of *M. avium* subsp. *paratuberculosis*. However, bulk culture of ovine strains of *M. avium* subsp. *paratuberculosis* is very difficult and often not technically possible. Consequently, methods were developed at EMAI in 1996 to enable extraction of *M. avium* subsp. *paratuberculosis* directly from the intestinal lining of affected sheep (Choy et al 1998) (Apendix 1, Sections A-C). Briefly, the terminal 8 m of small intestine was removed from each sheep immediately after euthanasia, cut into 2 m lengths, rinsed through with tap water to remove all intestinal contents, placed in a plastic bag and frozen at -20°C. Intestinal samples from New South Wales and Victoria were transported to EMAI by departmental vehicles rather than by commercial couriers to minimise the chances of the tissues thawing in transit. Further work was undertaken on the intestines only after the results of histopathological examination; intestines that lacked lesions consistent with ovine Johne's disease and that did not contain acid fast bacilli in the lamina propria were discarded; about half the intestinal samples collected could not be examined as they were paucibacillary cases. *M. avium* subsp. *paratuberculosis* was purified from mucosal homogenates from the intestinal tissues using differential and gradient centrifugation. The

level of contamination due to irrelevant bacteria was determined in each sample by microscopic examination of smears stained by Gram and ZN methods and by enumerating viable bacteria in a plate count (Appendix 2).

Extraction and purification of DNA from M. avium subsp. paratuberculosis. A protocol based on several from the scientific literature was optimised for extraction of DNA from *M. avium* subsp. *paratuberculosis* (Appendix 1, Section D). Cells were lysed using lysosome and mutanolysin and digested using proteinase K. DNA was extracted using CTAB and purified using chloroform/isoamyl alcohol and alcoholic precipitation. The yield and quality of genomic DNA was assessed by electrophoresis in agarose with ethidium bromide staining.

Restriction fragment length polymorphism (RFLP) analysis. DNA was digested with *Bst* EII, separated in 1% agarose at 35V for 20 hrs (Appendix 1, Section E), transferred under vacuum to positively charged nylon membrane and probed with an IS*900* fragment labelled with digoxigenin (Appendix 3). The probe was the 229 bp PCR product produced from *M. avium* subsp. *paratuberculosis* strain 316V using primers P25/P26 of Millar et al (1995) and labelling was undertaken using a random hexamer labelling kit (Boehringer Mannheim). The membrane was developed using a chemiluminescent kit (Appendix 4). The banding pattern was identified by matching with figures in the scientific literature (Collins et al 1990 and Pavlik www). Unfortunately, there are discrepancies in nomenclature between Collins et al (1990) and Pavlik (www) for several isolates. For this reason and for reasons of scientific precedent, names were first allocated to isolates according to the patterns of Collins et al (1990). Remaining isolates were named according to Pavlik (www), but if a pattern was new, S was used to indicate an isolate similar to existing S isolates, C was used for isolates similar to existing C isolates, U was used to signify a new/unknown type and lastly consecutive numbers were allocated (eg. SU1, SU2, CU1).

IS900 polymerase chain reaction. PCR to detect IS*900* was used to confirm the identity of isolates as *M. avium* subsp. *paratuberculosis* where a new/unknown IS*900* RFLP pattern was obtained, or where no RFLP pattern was obtained despite the presence of DNA. The protocol used primers P90/P91 of Millar et al (1995) and has been described in detail (Whittington et al 1998).

B) Development of a rapid laboratory test

One purifed DNA sample from an ovine isolate of *M. avium* subsp. *paratuberculosis*, one crude DNA sample from a BACTEC culture from the same sheep, two purified DNA samples from bovine isolates of *M. avium* subsp. *paratuberculosis* and an *M. Avium* complex isolate were used as standard samples for evaluation of DNA tests in preliminary experiments.

In order to fulfil the requirement for a rapid test it was felt that the most logical candidate test would be PCR-based. In addition, it was considered highly desirable to base any new test on a repetitive element because this might offer advantages in test sensitivity and possibly make the test useful on a wide range of sample types. The strategy pursued was to seek insertion sequences other than IS*900* (because this gene is identical in sheep and cattle isolates) in *M. avium* subsp. *paratuberculosis* and to trial PCR protocols to amplify the DNA between these and IS*900*. DNA regions between other known genes were also amplified, in the hope that differences in fragment sizes and restriction site differences in the fragments could be used to distinguish the different strains of *M. avium* subsp. *paratuberculosis*. The insertion sequence IS*1311*, known from *M. avium* was found in *M. avium* subsp. *paratuberculosis* and was sequenced in four strains of *M. avium* subsp. *paratuberculosis* in order to identify possible restriction site polymorphisms.

Although random primed PCR (also known as RAPD PCR) was originally proposed for investigation, it was not pursued because reports suggested that this method was difficult to standardise between laboratories.

C) Serological survey

The project plan included a survey in high risk cattle grazing on five farms where ovine Johne's disease was present.

High risk cattle herds were defined as follows:

Johne's disease was present in sheep when cattle were $\leq$ 6 months old;
Cattle were calves when run with sheep or on pasture contaminated by sheep;
Cattle are now over 2 years old.

Table 1. Source of isolates of *M. avium subsp. paratuberculosis*

Source	EMAI		VIAS		Total isolates
	No. isolates	Year	No. isolates	Year	
Intestinal scraping					
Ovine	160	1995-1998	21	1998	181
Caprine	2	1996	-		2
Cultured					
Ovine	-		6	1980-1986	6
Caprine	2	1981-1982	5	na*	7
Bovine	45	1995-1997	107	1985-1997	152
Camelid	-		3	1996-1997	3
Rhinoceros	1	1995	-		1
Human	-		2	1984	2
Total	210		144		354

* not available

Table 2. Geographical origin of isolates of *M. avium* subsp. *paratuberculosis*

Species	NSW	VIC	TAS	SA	France	Total isolates
Ovine	165	21	-	-	1	187
Caprine	4	-	-	-	5	9
Bovine	44	101	7	-	-	152
Camelid	-	2	-	1	-	3
Rhinoceros	1	-	-	-	-	1
Human	-	2	-	-	-	2
Total no. isolates	214	126	7	1	6	354
Total no. farms	61	38	na*	1	na	100

* not available

A2. RFLP types by species, farm enterprise and geographical origin

Of the 354 isolates available for typing, there was insufficient DNA for the RFLP typing of 19. These were all isolates from cattle from New South Wales which grew very poorly in liquid medium. Data for these isolates are shown as 'nt' in the tables that follow. In addition, there were 25 isolates which did not produce bands in Southern blots and are shown as 'NB' in the tables that follow. There was either insufficient DNA to enable distinct bands to develop, or the DNA was degraded. Therefore, of the 354 isolates, an RFLP type was determined for 310. Fourteen RFLP types were found: C1, C3, C5, C12, C13, CU1, CU2, CU3, CU4, S1, SU1, SU2, SU3, SU4 (Figure 1).

The RFLP type of isolates was analysed with respect to host species, farm enterprise and geographic location. State-based data are given in Tables 3 - 6. Seven RFLP types were identified from sheep from New South Wales. Type S1 was most prevalent, and in New South Wales comprised 95% of the 144 isolates for which an RFLP type could be determined (Table 3). These sheep came from 33 farms. Only type S1 was found amongst the 21 isolates from sheep from 4 farms in Victoria. The other RFLP types from sheep, namely SU1, SU2, SU3, SU4, C3, C5, CU3 were rare (Table 3). The occurrence of bovine strains in sheep will be discussed in detail later in this report. The only French isolate was type C5, which was not found in Australian sheep.

Figure 1A. Schematic diagram of RFLP types identified in this study - sheep strains lane 1, molecular size markers in kb; lane 2, S1 (#163); lane 3, SU1 (#103); lane 4, SU2 (#124); lane 5, SU3 (#80); lane 6, SU4 (#54) (# in parentheses are key list numbers from Appendix 5).

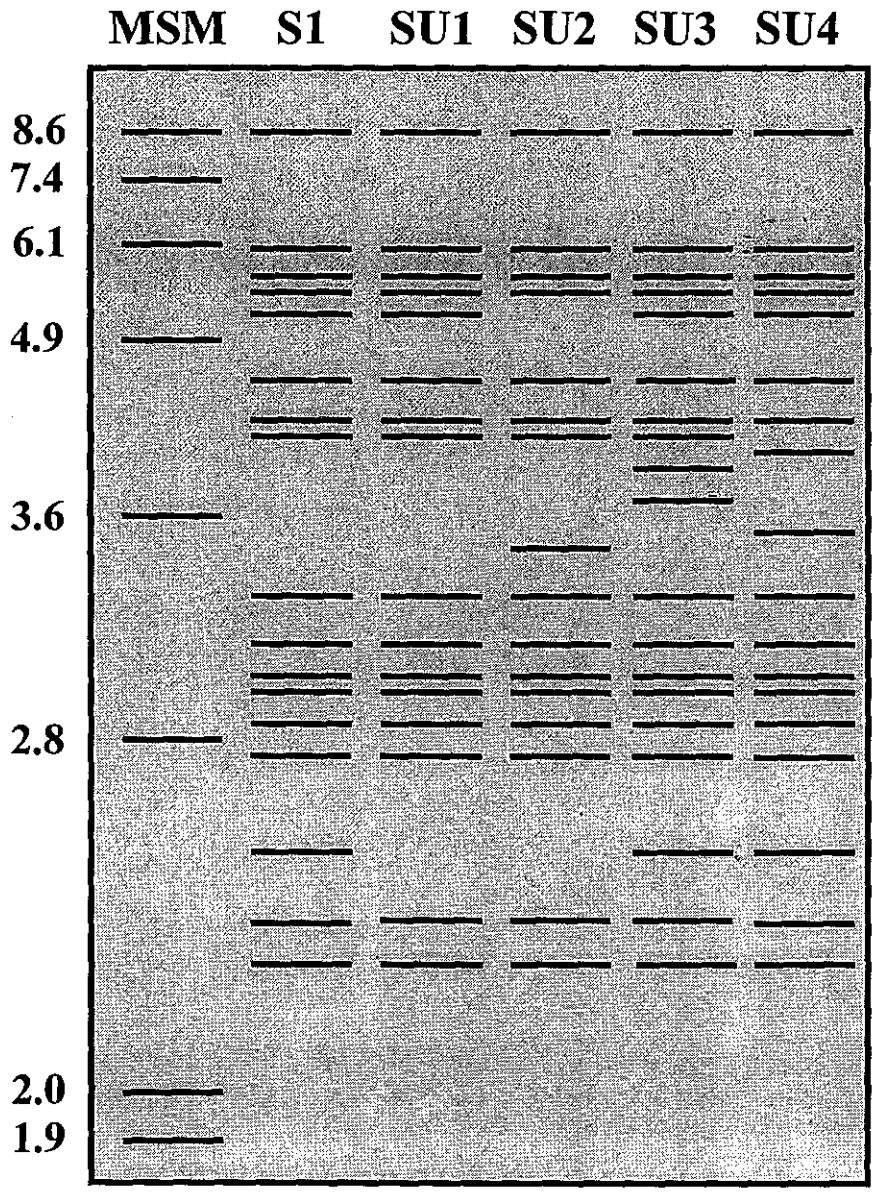

Figure 1B. Schematic diagram of RFLP types identified in this study - cattle strains lane 1, molecular size markers in kb; lane 2, C1 (#232); lane 3, C3 (#238); lane 4, C5 (#233); lane 5, C12 (#229); lane 6, C13 (#280); lane 7, CU1 (#239); lane 8, CU2 (#267); lane 9, CU3 (#273); lane 10, CU4 (#244); (# in parentheses are key list numbers from Appendix 5).

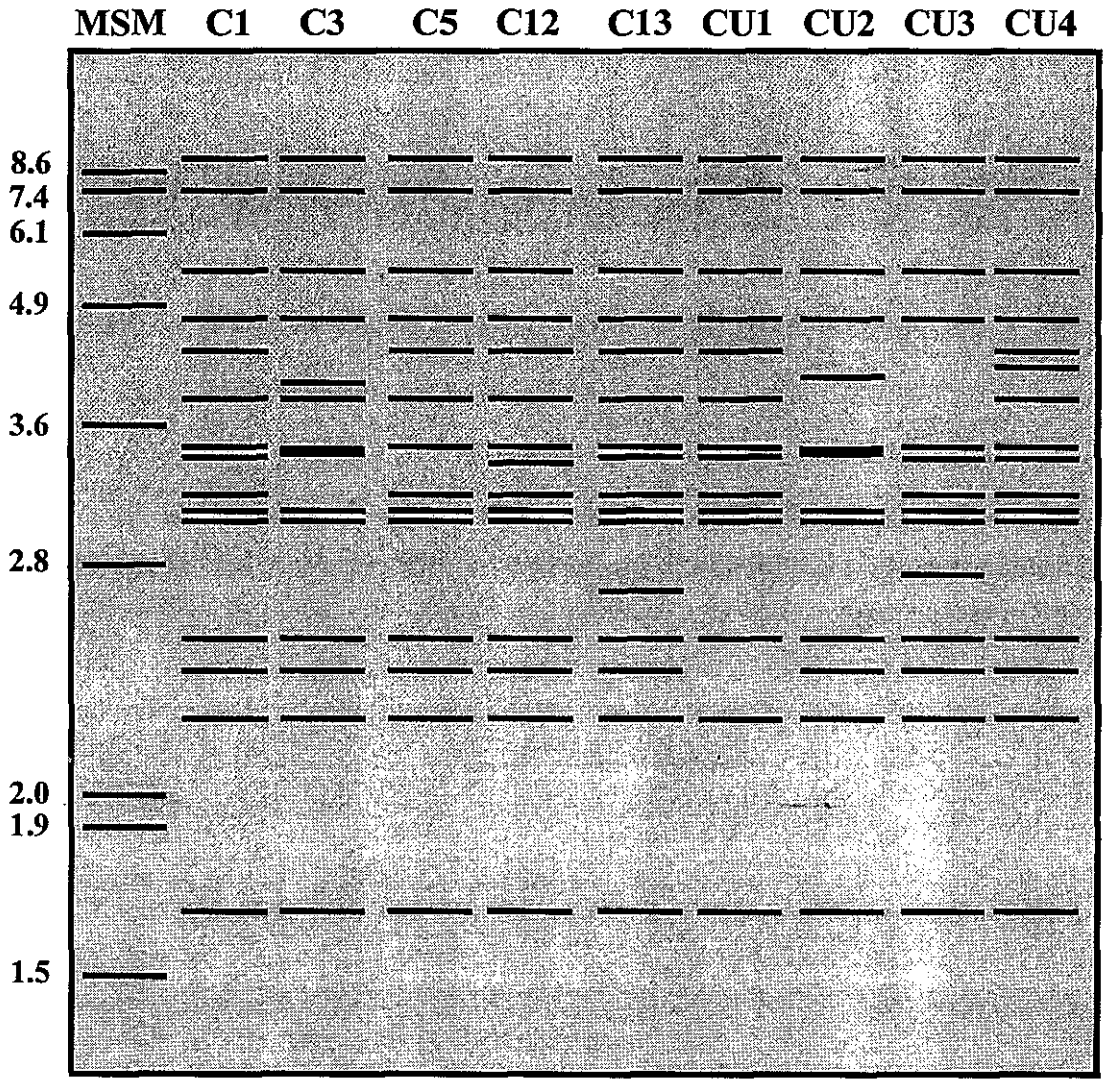

Table 3. RFLP type of isolates of *M. avium* subsp. *paratuberculosis* from sheep by geographical origin. Farms were wool enterprises (France - enterprise unknown)

RFLP	NSW	VIC	TAS	SA	France	Total isolates
S1	137	21	-	-	-	158
SU3	2	-	-	-	-	2
SU1	1	-	-	-	-	1
SU2	1	-	-	-	-	1
SU4	1	-	-	-	-	1
C3	1	-	-	-	-	1
C5	-	-	-	-	1	1
CU3	1	-	-	-	-	1
NB*	21	-	-	-	-	21
Total	165	21	-	-	1	187

* no bands obtained due to insufficient or degraded DNA

Only 9 isolates were available from goats (Table 4). RFLP types C1, C3, C13 and S1 were found. The occurrence of ovine strains in goats will be discussed in detail later in this report.

Table 4. RFLP type of isolates *M. avium* subsp. *paratuberculosis* from goats by geographical origin and farm enterprise

RFLP	NSW		France	Total isolates
	dairy	fibre	nr*	
C1	-	-	4	4
C3	2	-	-	2
S1	-	2	-	2
C13	-	-	1	1
Total	2	2	5	9

* farm enterprise not recorded

Seven RFLP types were identified in cattle, namely C3, C1, C5, CU1, C12, CU2 and CU4 in decreasing order of prevalence (Table 5). These isolates were obtained from 7 beef and 37 dairy farms in New South Wales, 34 beef and 67 dairy farms in Victoria and 7 Tasmanian farms. RFLP type C1 was not present amongst 44 isolates from cattle from New South Wales but comprised 47% of the 101 isolates from cattle from Victoria. Type C3 was the most common type found in New South Wales, occurring in both beef and dairy cattle but was not found in beef cattle in Victoria. Type C3 was the most common type in dairy cattle in Victoria. The remaining RFLP types were uncommon.

Table 5. RFLP type of isolates of *M. avium* subsp. *paratuberculosis* from cattle by geographical origin and farm enterprise

RFLP	NSW		VIC		TAS	Total isolates
	beef	dairy	beef	dairy	nr*	
C3	3	21	-	34	6	64
C1	-	-	26	21	-	47
C5	-	-	5	5	-	10
CU1	-	1	-	3	-	4
C12	-	-	2	1	-	3
CU2	-	-	-	-	1	1
CU4	-	-	1	-	-	1
not typed**	4	15	-	-	-	19
NB***	-	-	-	3	-	3
Total	7	37	34	67	7	152

* farm enterprise not recorded

** insufficient DNA and RFLP typing not undertaken

***no bands obtained due to insufficient or degraded DNA

RFLP types C1, C3 and C5 were found in the few samples examined from camelids, a rhinoceros and humans (Table 6).

Table 6. RFLP type for isolates of *M. avium* subsp. *paratuberculosis* from camelids, rhinoceros and humans by geographical origin

Species	RFLP	NSW	VIC	SA	Total isolates
Camelid	C1	-	1	-	1
	C3	-	-	1	1
	NB*	-	1	-	1
Rhinoceros	C5	1	-	-	1
Human	C1	-	1	-	1
	C5	-	1	-	1

*no bands obtained due to insufficient or degraded DNA

A3. Regional analysis of RFLP types within states

As relatively large numbers of isolates were tested from sheep and cattle, a regional analysis within states was possible. In sheep from NSW, 165 isolates were examined from 33 farms from 6 regions described by Rural Lands Protection Board (RLPB) boundaries (Table 7). RFLP type S1 was present in each RLPB; other RFLP types were found only where relatively large numbers of isolates were examined from a region. In Victoria, 21 isolates were examined from sheep from 4 farms from 2 regions, but only RFLP type S1 was found (Table 8).

Table 7. RFLP type for isolates of *M. avium* subsp. *paratuberculosis* from sheep from NSW by geographical origin (Rural Lands Protection Board - RLPB)

RLPB	RFLP								Total isolates	Total farms
	S1	SU1	SU2	SU3	SU4	CU3	C3	NB*		
Bathurst	74	1	-	-	1	-	-	8	84	11
Carcoar	33	-	1	-	-	1	1	11	49	15
Goulburn	18	-	-	2	-	-	-	2	20	4
Molong	1	-	-	-	-	-	-	-	1	1
Yass	1	-	-	-	-	-	-	-	1	1
Young	10	-	-	-	-	-	-	-	10	1
Total	137	1	1	2	1	1	1	21	165	33

*no bands obtained due to insufficient or degraded DNA

Table 8. RFLP type for isolates from sheep from Victoria by region

Region	Total isolates RFLP S1	Total farms
Gippsland	18	3
Melbourne	3	1
Total	21	4

The 44 isolates from cattle in New South Wales came from 26 farms across 9 RLPB regions (Table 9). RFLP type C3 was found in 7 regions while type CU1 was found in one non-identified region. There was insufficient DNA for RFLP analysis from the only two isolates from the remaining 2 regions (these isolates were typed as cattle isolates by IS*1311* PCR).

Table 9. RFLP type for isolates of *M. avium* subsp. *paratuberculosis* from cattle from NSW by farm enterprise and geographical origin (Rural Lands Protection Board - RLPB)

RLPB	Farm enterprise	RFLP			Total isolates	Total farms
		C3	CU1	nt*		
Albury	beef	2	-	2	4	4
Bega	dairy	3	-	2	5	5
Casino	dairy	2	-	-	2	2
Deniliquin	dairy	-	-	1	1	1
Jerilderie	dairy	1	-	-	1	1
Maitland	dairy	1	-	3	4	2
Molong	beef	-	-	1	1	1
Moss Vale	beef	-	-	1	1	1
	dairy	3	-	-	3	3
Tweed Lismore	beef	1	-	-	1	1
	dairy	11	-	9	20	4
nr**	dairy	-	1	-	1	1
Total	beef	3	-	12	7	7
Total	dairy	21	1	7	37	19
Total	all	24	1	19	44	26

*insufficient DNA for RFLP analysis ** RLPB uncertain

A total of 101 isolates from 31 farms in 6 geographic regions of Victoria were included in this study (Table 10). Type C1 was found in 5 regions, type C3 in 3 regions and type C5 in two regions. Type C3 was found only in dairy cattle. Dairy cattle in Gippsland were infected with four RFLP types (C1, C3, C5 and C12) while beef cattle in this region were infected with types C1, C5 and CU4. Three RFLP types (C1, C3 and CU1) were found in dairy cattle in both Goulburn and the Western District. Two RFLP types (C1 and C5) were present in beef cattle in the Central Highlands.

Table 10. RFLP type for isolates from cattle from Victoria by farm enterprise and geographical region

Region	Farm enterprise	RFLP							Total isolates	Total farms
		C1	C3	C5	CU1	CU4	C12	NB*		
Central Highlands	beef	23	-	3	-	-	-	-	26	1
Gippsland	beef	2	-	2	-	1	-	-	5	1
	dairy	16	7	5	-	-	1	1	30	15
Goulburn	dairy	3	4	-	2	-	-	-	9	4
Loddon-Campaspe	dairy	1	-	-	-	-	-	-	1	1
Western district	dairy	1	23	-	1	-	-	1	26	7
Wodonga	beef	-	-	-	-	-	2	-	2	1
nr	beef	1	-	-	-	-	-	-	1	1
	dairy	-	-	-	-	-	-	1	1	1
Total	beef	26	-	5	-	1	2	-	34	4
Total	dairy	21	34	5	3	-	1	3	67	27
Total	all	47	34	10	3	1	3	3	101	34

*no bands obtained due to insufficient or degraded DNA
**region not recorded

A4. Typing of multiple isolates from the same animal

Among the individual Victorian cattle that had been cultured, there were 12 animals from which isolate had been obtained on different occasions. There were 8 animals with 2 isolates, 1 animal with 3 isolates, 2 animals with 4 isolates and 1 animal with 5 isolates. The animals represented 6 farms. The circumstances of isolation of *M. avium* subsp. *paratuberculosis* and the results of typing are given in Table 11. There was only one example where more than one RFLP type occurred in a single animal (animal TPGQ 331). In addition to isolates from cattle, two isolates were analysed from the same human; one

was type C1 while the other was type C5.

Table 11. Typing of multiple isolates of *M. avium* subsp. *paratuberculosis* from individual cattle

Source of multiple isolates		Animal	RFLP
Samples of faeces collected on the same day	4 faeces	2588	C1
Samples of faeces and tissues collected	15 months apart	1813	C3
	10 months apart	1927	C3
	6 weeks apart	B923 B926 Y443	C1 C1 C1
Multiple tissues collected on the same day	4 tissues	B923	C1
	3 tissues	Y443	C1
	2 tissues	313 460 5064	CU1 C3 C3
Samples of faeces collected 2 days apart	2 faeces	TPGQ 331	C5/CU4

A5. Typing of multiple isolates from individual farms

Of the 100 farms that were represented in this study, 42 contributed more than one isolate (Table 12). On all but 8 farms only one RFLP type was detected. Two RFLP types were detected on 6 farms while 3 RFLP types were detected on 2 farms (Table 13).

Table 12. Number of isolates examined within farms

No. of isolates examined	No. of farms
1	58
2-5	21
6-10	13
11-15	5
16-20	2
26	1
Total	100

Table 13. RFLP types of *M. avium* subsp. *paratuberculosis* identified on farms where more than one RFLP type was found

Farm	Host species	Region	RFLP type (no. of isolates)											Total isolates
			S1	SU1	SU2	SU4	C1	C3	C5	C12	CU1	CU4	NB*	
17	Ovine	Bathurst NSW	11	1	-	-	-	-	-	-	-	-	1	13
19	Ovine	Carcoar NSW	3	-	1	-	-	-	-	-	-	-	1	5
9	Ovine	Bathurst NSW	6	-	-	1	-	-	-	-	-	-	-	7
33	Bovine dairy	Gippsland VIC	-	-	-	-	8	-	5	1	-	-	1	15
44	Bovine dairy	Goulburn	-	-	-	-	1	4	-	-	-	-	-	5
54	Bovine dairy	Western District VIC	-	-	-	-	-	2	-	-	1	-	-	3
59	Bovine beef	Gippsland VIC	-	-	-	-	2	-	2	-	-	1	-	5
60	Bovine beef	Central Highlands VIC	-	-	-	-	23	-	3	-	-	-	-	26

* no bands obtained due to insufficient or degraded DNA

A6. Occurrence of bovine strains of M. avium subsp. *paratuberculosis in sheep*

Isolates 273 and 274 (Appendix 5) were both obtained from sheep from the Carcoar region of New South Wales in 1980-1983 and both were typed as bovine strains. Isolate 274 was a C3 strain while 273 was an unknown bovine strain which we called CU3. The latter strain was typed previously in the MRC-funded project TR.007 'Typing of Johne's disease isolates' and given the strain name 'I'. Strain I was identified also in two cattle from the Bega region of New South Wales in that study. Also included in TR.007 was a third New South Wales sheep isolate of bovine type (type C = C3). This isolate came from a farm in the Bathurst region in 1988. Each of these isolates was obtained by culture of samples from affected sheep at a time when culture from sheep was almost universally unsuccessful, a factor that points also to the involvement of bovine strains. Surprisingly, and running counter to this line of reasoning, a typical S1 isolate was also obtained by culture from sheep from a farm in the Carcoar region in 1986 (RFLP type L=S1 in TR.007 Final Report, equals isolate 275, Appendix 5 in this study).

These findings indicate that bovine strains of *M. avium* subsp. *paratuberculosis* have occurred in sheep in New South Wales. However, there is no evidence from this study that bovine strains are commonly or currently involved in the pathogenesis of Johne's disease in sheep in New South Wales or Victoria.

A7. Occurrence of ovine strains of M. avium subsp. *paratuberculosis in goats*

In TR.007, isolates of *M. avium* subsp. *paratuberculosis* from Australian goats (n=3) were found to be type C3 as were 2 isolates from dairy goats in this study. These findings are consistent with the widely-held view that Johne's disease in dairy goats in Australia is caused by bovine strains of *M. avium* subsp. *paratuberculosis*. This view stems in part from the general ease with which *M. avium* subsp. *paratuberculosis* has been cultured from goats with Johne's disease in Australia.

Until recently, Johne's disease had not been seen in fibre goats in Australia. A suspicion that ovine strains of *M. avium* subsp. *paratuberculosis* may have infected fibre goats in New South Wales developed in 1996 when negative culture results were obtained from an Angora goat with Johne's disease. Subsequent field investigation resulted in the diagnosis of ovine Johne's disease in sheep on the same farm. Isolates of *M. avium* subsp. *paratuberculosis* obtained from sheep and goats on this farm were all type S1 (isolates 33-

40, Appendix 5), confirming the spread of infection from sheep to goats. The circumstances leading to transmission of Johne's disease between these species include a high degree of contact between mobs of sheep and goats over a long period. The same situation has since been confirmed on a second property in the Central Tablelands region of New South Wales. Trace forward investigations from the first property have resulted in the identification of other infected farms. However, there is as yet no information as to whether infection has been transmitted from the introduced goats to the home-bred goats.

B) Development of a rapid laboratory test

B1. Discovery of insertion sequences and intergene PCR typing tests

The experiments undertaken during this phase of the research are summarised in Appendix 6. IS*1311* was detected in *M. avium* subsp. *paratuberculosis* strain 316V. PCR reactions for the 16SrRNA gene, 23SrRNA gene and 65-kDa heat shock protein (HSP) gene were established. Outward looking primers from the 5' end of the 16SrRNA gene, the 3' end of the 23SrRNA gene and both ends of the HSP, IS*900* and IS*1311* genes were designed. PCR tests were then undertaken to amplify DNA segments located between these genes; long distance protocols and reagents were used in some cases. These tests, with few exceptions, either did not appear to separate ovine and bovine isolates of *M. avium* subsp. *paratuberculosis* or provided acceptable separation but only at certain DNA input concentrations (i.e. the identity of the isolate was DNA-concentration dependent). Perhaps the most useful reaction was an IS*900*-IS*900* intergene test using primers M27+M29 and a short distance protocol (experiments T23 and T25, Appendix 6). In this reaction, bovine-type isolates tended to test positive for a 190 bp product whereas ovine-type isolates tended to test negative for this product. However there were discrepancies and this test could not be used for any practical purpose.

B2. Sequencing of IS1311 and development of a rapid and sensitive test

After the discovery of the insertion sequence IS*1311* in *M. avium* subsp. *paratuberculosis* using PCR with existing published primers DD2/DD3, primers were designed to amplify the whole gene. The gene from 2 bovine and 2 ovine strains of *M. avium* subsp. *paratuberculosis* was sequenced and compared to the sequence known from *M. avium.* As well as a number of mutations in *M. avium* subsp. *paratuberculosis* that resulted in restriction site changes compared to *M. avium,* a polymorphism was discovered which

permitted the differentiation of ovine and bovine strains of *M. avium* subsp. *paratuberculosis*. A collection of DNA samples from *M. avium* subsp. *paratuberculosis* and *M. avium* was kindly provided by Dr Debby Cousins, Agriculture WA to enable early evaluation of this potentially important discovery. Many of the samples stemmed from Project TR.007. The polymorphism was shown to be present in all bovine isolates of *M. avium* subsp. *paratuberculosis* but in none of the ovine isolates in the collection. These data provided the theoretical and practical means to develop a rapid PCR-based test for an insertion sequence, consistent with our strategy. The data are now accepted for publication in an international scientific journal, the first step in acceptance of a new test by Veterinary Committee. The manuscript is provided as Appendix 7.

The IS*1311* PCR typing method was then subjected to optimisation by redesigning primers to provide a sensitive, specific and easily interpreted result. These data have been submitted for publication in an international scientific journal and are provided as Appendix 8. The test is known as reaction IS*1311*-1 and consists of a PCR assay followed by restriction endonuclease analysis with 2 enzymes.

Reaction IS*1311*-1 is recommended for routine typing of ovine and bovine isolates of *M. avium* subsp. *paratuberculosis*, had a sensitivity equivalent to IS*900* PCR, did not cross react with a panel of 24 mycobacterial species and provided an unequivocal outcome from a panel of 89 isolates of *M. avium* subsp. *paratuberculosis* from sheep, cattle, alpaca, goats, rhinoceros and humans from Australia, New Zealand, South Africa and North America. The DNA samples were as varied as highly purified DNA from cultured bacteria or from bacteria purified from intestinal scrapings and crude DNA from BACTEC radiometric cultures and solid media. In addition, this assay also differentiated *M. avium* subsp. *paratuberculosis* from *M. avium*. This was evaluated using DNA samples from 28 *M. avium* isolates from a range of sources (Appendix 8).

In parallel with this project a method was developed for PCR amplification of IS*900* sequence from formalin-fixed paraffin-embedded specimens, supported by the McGarvie Smith Trust. This was undertaken to assist in the diagnosis of Johne's disease in circumstances where appropriate samples for culture of *M. avium* subsp. *paratuberculosis* are not submitted to a laboratory. The procedure was successful and was submitted for publication in a scientific journal as the first step in gaining Veterinary Committee approval for use of the test in veterinary diagnostic laboratories in Australia (Appendix 9).

The IS*1311* PCR typing technique was then optimised for use on formalin-fixed paraffin-embedded samples. The details are given in Appendix 8. The recommended test is known as reaction IS*1311*-2. It has a sensitivity equivalent to IS*900* PCR, is specific for *M. avium* subsp. *paratuberculosis* across a panel of 24 mycobacterial species and was used successfully to differentiate ovine and bovine strains of *M. avium* subsp. *paratuberculosis* present in 27 formalin-fixed paraffin-embedded tissues from sheep, cattle and goats (Appendix 8).

B3. Full comparison of IS1311 PCR typing and RFLP typing

In addition to the 89 isolates of *M. avium* subsp. *paratuberculosis* tested during validation of the test (Appendix 8), all 354 isolates in part A of this study were evaluated. Of the 310 isolates for which an RFLP type was assigned in this study, all but 3 were tested by IS*1311* PCR typing and there was absolute agreement between the two tests with respect to sheep or cattle type (Table 14).

Table 14. Comparison of results of typing methods for those isolates of *M. avium* subsp. *paratuberculosis* for which an RFLP type was obtained

IS*1311* PCR	RFLP		Total
	S	C	
S	162	-	162
C	-	145	145
nt*	3	-	3
Total	165	145	310

* not tested

Isolates that could not be examined by RFLP due to lack of DNA (n=19) all were derived from cattle and all were typed as C type by IS*1311* PCR (Table 15). In addition, 9 of 25 isolates that did not yield an RFLP result because of insufficient or degraded DNA were typed successfully by IS*1311* PCR with complete agreement with host species of origin (Table 15). Only 2 isolates gave a positive IS*900* PCR result (isolate 3, Appendix 5) or a recognisable RFLP pattern (isolate 152, Appendix 5) with a negative IS*1311* PCR result. The reasons for this are uncertain but probably relate to sample preparation for PCR. We were unable to repeat these assays in time for the results to be included in this report. Thus overall, of the 354 isolates of *M. avium* subsp. *paratuberculosis* available for typing in this

study, 310 were typed successfully by RFLP while 335 were typed successfully by IS*1311* PCR.

Table 15. Comparison of RFLP and IS*1311* PCR typing methods

RFLP	Total isolates	IS*1311* PCR			IS*1311* PCR type		
		Negative	Positive	nt*	S	C	nt**
C1	53	-	53	-	-	53	-
C12	3	-	3	-	-	3	-
C13	1	-	1	-	-	1	-
C3	68	-	68	-	-	68	-
C5	13	-	13	-	-	13	-
CU1	4	-	4	-	-	4	-
CU2	1	-	1	-	-	1	-
CU3	1	-	1	-	-	1	-
CU4	1	-	1	-	-	1	-
NB	25	14	11	-	5***	4****	16
S1	160	1	158	1	157	-	3
SU1	1	-	1	-	1	-	-
SU2	1	-	1	-	1	-	-
SU3	2	-	2	-	2	-	-
SU4	1	-	1	-	1	-	-
nt	19	-	19	-	-	19	-
Total	354	15	338	1	167	168	19

* not tested because there was insufficient DNA
**not tested because IS*1311* PCR was negative or insufficient PCR product for REA
*** isolated from sheep **** isolated from cattle

B4. Practical application of IS1311 PCR typing assays

Reaction IS*1311*-1 is now used routinely at EMAI to differentiate ovine and bovine types of *M. avium* subsp. *paratuberculosis* in BACTEC cultures, cultures on solid media and soil cultures. It also provides confirmation that isolates are *M. avium* subsp. *paratuberculosis* and not *M. avium.* The method is rapid, simple, economical and is more likely to provide an outcome than an RFLP analysis because large amounts of high quality DNA are not

required. The method has considerable application in epidemiological investigations, including other projects that are currently supported by MLA.

B5. Occurrence of ovine strains of M. avium subsp. paratuberculosis in cattle

Reaction IS*1311*-2 is now used routinely at EMAI to differentiate ovine and bovine types of *M. avium* subsp. *paratuberculosis* in histological material. A survey of archival paraffin blocks was made to evaluate the type of *M. avium* subsp. *paratuberculosis* present in cattle with Johne's disease where there had been direct contact of the cattle with sheep with Johne's disease, and where cultures from tissues/faeces of the affected cattle were negative, suggesting presence of an ovine strain. Transmission of ovine strains of *M. avium* subsp. *paratuberculosis* from sheep to cattle was confirmed to have occurred in 1989, 1993 and 1995 (Appendix 10). This was the first evidence that ovine Johne's disease has occurred in cattle. The magnitude of the risk is now being assessed by a working party and is currently believed to be small. Spread of infection from sheep to cattle was associated with the rearing of calves in association with infected sheep.

B6. Technology transfer

The IS*1311* methods and methods for processing paraffin-embedded histological samples have been transferred to Animal Health Laboratories in South Perth (Agriculture WA) and to the Victorian Institute of Animal Science Attwood (VIAS, Agriculture Victoria). Training in IS*1311* PCR methods was provided at EMAI to Wendy McDonald from VIAS.

C) Serological survey

Out of a target of 5 herds in New South Wales, only 3 were identified where the owners were prepared to submit their cattle for Johne's testing, despite offering the incentive of free testing that could be used for Market Assurance Program entry purposes. In general, owners feared the repercussions that might stem from the identification of Johne's disease in their cattle.

Samples from all of the cattle yielded negative results in the bovine Johne's disease absorbed ELISA (Table 16).

Table 16. Seroepidemiological survey of cattle on farms in NSW that graze sheep with ovine Johne's disease

Farm	Location	Date sampled	Lab accession no.	No. cattle tested
1	Vittoria, Bathurst	19.03.97	ON97/1137	173
1	Vittoria, Bathurst	20.03.97	ON97/1152	105
2	Bathurst	27.05.97	ON97/1986	97
3	Limekilns, Bathurst	15.07.98	ON97/2521	51

Discussion

There was a lack of diversity of RFLP type among isolates of *M. avium* subsp. *paratuberculosis* from sheep in New South Wales and Victoria, with almost all cases being due to type S1. Occasional examples of other types (SU1, SU2, SU4) occurred but were mostly from farms where type S1 was dominant, suggesting that these types were variants of S1. The exception was type SU3 which was responsible for infection in the only two sheep examined from property 14, located at Goulburn New South Wales.

There was a similar lack of diversity amongst isolates from cattle from New South Wales, type C3 being predominant. There was only one example of another type in New South Wales. In contrast, 6 RFLP types were found in Victorian cattle, with C1, C3 and C5 being most common and CU1, CU4 and C12 being quite uncommon. C3 was confined to dairy cattle. Multiple RFLP types were found within geographical regions and even amongst cattle from the same farm. The differences observed between RFLP types in cattle in New South Wales and Victoria and between dairy and beef cattle enterprises in Victoria are interesting and have potential application in epidemiological studies. The reasons for the absence of type C1 in New South Wales cattle and C3 in Victorian beef cattle require further investigation but probably relate to trading practices in the cattle industries.

Several archival isolates of *M. avium* subsp. *paratuberculosis* from sheep from New South Wales were of a bovine type. The epidemiological circumstances that resulted in bovine strains infecting sheep in New South Wales are unknown. These circumstances appear to be rare and there is no evidence that bovine strains play any current role in the epidemiology of ovine Johne's disease in New South Wales or Victoria.

There were several biases inherent in this study. Only culturable isolates were examined from cattle, making it less likely that ovine strains would be detected in the first place. Most of the isolates from cattle were obtained in the period prior to the availability of methodologies suitable for recovery of ovine strains from faecal/tissue samples. Conversely, few culturable isolates were examined from sheep, and those that were examined tended to be from cases of multibacillary Johne's disease. The question of whether multibacillary and paucibacillary Johne's disease are caused by different RFLP types could not be answered by this study as techniques for bulk culture of *M. avium* subsp. *paratuberculosis* from sheep still are not available.

Only limited serological evaluation of cattle grazing on properties with ovine Johne's disease was possible during this study because of the reluctance of most owners to subject their cattle to testing. Nevertheless there was no evidence of transmission of ovine Johne's disease from sheep to cattle on the 3 farms studied. Further testing of this kind will occur as part of ongoing monitoring of cross-species transmission under the auspices of Veterinary Committee.

The discovery of differences in the IS*1311* gene sequence between sheep and cattle isolates of *M. avium* subsp. *paratuberculosis* was a very useful because it enabled the development of a rapid typing test. This led directly to the discovery that cattle on several farms had been infected with sheep strains of *M. avium* subsp. *paratuberculosis.* This resulted in reassessment and confirmation of recommendations for control of ovine Johne's disease and market assurance program testing for cattle. The IS*1311* test will continue to play a role in monitoring the success of control programs as it is the single most useful tool for strain typing under Australian conditions, is cheap, relatively simple and can be used in any veterinary diagnostic laboratory with PCR capability.

As a result of this project it is known that Johne's disease in sheep and cattle in Australia essentially are separate diseases, but under unusual circumstances the disease has spread from one species to the other. The disease may also spread from sheep to goats.

Success in achieving objectives

Each of the objectives was achieved.

Impact

The impact of this project is immediate in that policies for control and eradication of Johne's disease can now be based on current data obtained under Australian conditions. While cross-species transmission cannot be ignored, it is unlikely to prevent successful control of Johne's disease in Australia.

Conclusions and recommendations

The data provided are not exhaustive but provide a useful insight into the RFLP types of *M. avium* subsp. *paratuberculosis* present in sheep and cattle populations in Australia. There was no evidence for current or widespread exchange of *M. avium* subsp. *paratuberculosis* infection between sheep and cattle, even though it has occurred on several

farms in New South Wales in the past. *M. avium* subsp. *paratuberculosis* has also spread from sheep to goats on several farms in New South Wales. The circumstances responsible for these transmission events need to be determined. In addition, industry, through Veterinary Committee, should seek ongoing monitoring of the types of *M. avium* subsp. *paratuberculosis* responsible for Johne's disease in each state.

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APPENDIX 1

DNA typing from gut samples

This section covers:

A. Specimen receival and processing
B. Intestinal scraping (ver 12/10/98; file a:\gutwork)
C. Purifying *M. paratuberculosis*
D. DNA extraction
E. Restriction endonuclease digestion

A. Specimen receival and processing

A1. Upon receival of sample (gut) assign local JD accession number (sequentially in 9.25 JD accession book) in both laboratory 9.25 accession book and DNA Typing Project – Sample Record and History Worksheet. Where multiple guts apply, a single JD accession is assigned with sub numbering within that accession (eg. JD 96/x–1, JD 96/x–2 etc.).

A2. Fill in any cross referencing accession numbers on worksheet (ie. CM – EMAI microbiology, MN – RVL Menangle or ON – RVL Orange).

A3. Fill in sample description section on worksheet from accompanying specimen advice form.

A4. Fill in worksheet with respect to any alterations to sample.

A5. Label sample and store in a logical fashion, at –20 °C and record location on worksheet.

A6. Record sample details in freezer catalogue.

A7. File worksheet in the DNA Typing Project folder. Note: This will be accessed frequently throughout the procedure.

A8. Follow up histopathology and serology results on sample if these are not on the specimen advice. This will require communication with either Menangle or Orange RVL.

DNA Typing Project – Sample Record and History

Sample Accession Number(s) for Cross Referencing

Accession Number for	Prefix and Number
EMAI Laboratory 9.25	JD No.
EMAI Microbiology	CM No.
RVL Menangle	MN No.
RVL Orange	ON No.

Sample Description

Date received	
Species	
JD Status	
Description of sample	
Owner	RLPB
Alterations to sample prior to storage	
Storage location and temperature	Location No.1 at –20 °C

Intestinal Scraping

Scraping	Date	Length (m)	Total weight of scrapings (g)	Aliquots	Storage at location 1 (−20 °C)	Storage at location 2 (−20 °C)
1st						
2nd						
3rd						
4th						

Purification

Homogenisation and sucrose–KCl	Date
Miles and Misra count for contaminating organisms and gram stain of growth	organisms per ml of suspension from C25. Equals_____ bacteria per gram of final pellet. Gram stain:
Stained smears for acid fast/gram positive bacteria and/or host–derived cellular material	*Descriptions* ZN stained smear: Gram stained smear:
Weight of final pellet	grams
Storage details	*Location No.1*: at −20 °C aliquots of grams *Location No.2*: at −20 °C aliquots of grams

DNA extraction

* see DNA Extraction worksheet Group No._____

Storage details	*Location No. 1*: at 4 °C aliquots of _____µl containing _____µg each *Location No. 2*: at 4 °C aliquots of _____µl containing _____µg each

Restriction Enzyme Digest and Southern Blot

* see Restriction Enzyme Digest and Southern Blot worksheet Group No._____

B. Intestinal Scraping

NOTE : This procedure is performed in a Class II Biological Safety Cabinet (BSC). All implements (eg. scissors) should be sterile.

B1. Thaw gut(s) by placing on a stainless steel tray at 4 °C for approximately 16 hours (overnight).

B2. Wash BSC with 70% ethanol prior to turning on and UV irradiate for 15 minutes. Wash plastic cutting board with 70% ethanol and dry. Fill a plastic tray with ice and place in BSC. Place the cutting board on the ice.

B3. Place gut on the cutting board. If gut is greater than 2 metres in length, cut into 2 metre lengths use one and re-freeze the others. These will have to be labelled prior to storage. Alterations to sample needs to be recorded on the Sample Record and History Worksheet.

B4. Record the date and the length of the gut to be scraped on the Sample Record and History Worksheet.

B5. Cut gut into approximately 40 cm lengths and align evenly on cutting board (should be 5 lengths). Slit open each length of gut longitudinally and pin out flat to cutting board with mucosal surface facing up. Use a pair of sterile forceps to handle the gut where possible.

B6. Rinse the mucosal surface with sterile MQW to dislodge intestinal contents, dirt and debris. This and all other rinsing steps are to be performed using a plastic wash bottle. Collect liquid waste in a suction bottle before transferring to a steel bucket for autoclaving.

B7. Rinse the mucosal surface with sterile phosphate buffered saline, pH 7.0 (PBS) solution. Accumulate liquid waste as described in B6.

B8. Rinse the mucosal surface with sterile PBS solution containing 200 µg of ampicillin per ml of PBS. Accumulate liquid waste as described in B6.

B9. Prepare a smear of one site on each 40 cm length of gut by firmly scraping using a sterile microscope slide and transferring the material onto a clean microscope slide. Allow the smear to air dry.

B10. Fill another plastic tray with ice and place this over the existing tray containing the gut. The rationale for using this "ice lid" is to keep the gut cold to prevent unwanted bacterial proliferation whilst performing B11 and B12.

B11. Heat fix the smear and stain by the Ziehl Neelson technique.

B12. Examine the stained smear under a light microscope at x 1000 magnification to detect acid fast bacteria (AFB) which appear red/pink against a blue background of non acid fast material. Identify sections of the gut where AFB can be located and subsequently harvested.

B13. Replenish the ice in the plastic tray if necessary and place the cutting board, with the gut, over the ice. Scrape the mucosal surfaces which contained AFB with a sterile microscope slide and remove both the mucosa and the submucosa from the muscle layers of the gut.

B14. Collect the gut scrapings in a sterile beaker and mix to an even suspension before dispensing 5 gram aliquots into six 30 ml gamma–irradiated polycarbonate tubes. If the total weight of the scrapings exceed 30 grams, dispense the remaining scrapings into 20 gram aliquots. Label the tubes with the date and local JD accession number (lab 9.25).

B15. Store the aliquots of gut scrapings at –20 °C in 2 separate freezers and record storage details on Sample Record and History Worksheet.

B16. Wash the plastic cutting board, steel tray and implements used with concentrated detergent and accumulate liquid waste in the steel bucket. Cover bucket with aluminium foil, seal with autoclave tape and autoclave at 131 °C for 30 min.

B17. Dispose unwanted gut in plastic autoclave waste bags, seal with autoclave tape and autoclave as per B16.

B18. Soak the cutting board in the plastic tray filled with 1 M HCl for approximately 1 hour to lyse any bacterial cells and to denature any DNA on the surfaces. Collect the HCl and keep at room temperature in the fume cupboard for reuse.

Buffers and reagents

Solution for mucosal washing

10 x Phosphate buffered saline solution, pH 7.0
80 g NaCl, 2 g KCl, 11.5 g Na_2HPO_4, 3.1 g KH_2PO_4 made up to 1 L with MQW. Autoclave and store at room temperature. For working strength, 100 ml made up to 1 L with MQW.

Ampicillin stock solution Progen, catalogue # 200–0040 (25 g)
2 g ampicillin made up to 10 ml with sterile MQW. Dispense into 0.5 ml volumes in sterile ETs and store at –20°C. For working strength, 0.5 ml made up to 500 ml.

C. Purifying M. paratuberculosis

Note: This procedure is described for the purification of bacteria from a single gut and utilises sterile reagents. All homogenisation steps and sucrose/KCl fractionation steps are to be performed in the BSC. Keep test samples chilled on ice during the procedure wherever possible.

C1. Thaw 5 grams of gut scrapings at 4 °C for 2 hours.

C2. Transfer the gut scrapings to a 50 ml Falcon tube and suspend in 20 ml of 0.2 M sucrose.

C3. Place the Ultra Turrax in the BSC after wiping the BSC surfaces with 70% ethanol. Homogenise gut scrapings using the Ultra Turrax at maximum speed for approximately 45 seconds. Following every homogenisation, wash the tip of the probe thoroughly by submerging in a beaker of Medol disinfectant, diluted 1:20 with sterile MQW (For laboratory waste disposal of TB cultures, manufacturer recommends 1:20 dilution), and operating the Ultra Turrax at medium to high speed for 2 minutes. Detach the probe, rinse with RO water and dry thoroughly.

C4. Centrifuge the homogenate in a Beckman J-6B floor centrifuge at 4°C at 1000 rpm (250 g) for 20 minutes.

C5. Transfer the supernatant (Supernatant 1) to a sterile Falcon Tube using a sterile plastic transfer pipette and retain on ice. Resuspend the pellet in 20 ml 0.2 M sucrose and rehomogenise as per C3. Perform in the BSC.

C6. Recentrifuge the resulting homogenate as per C4.

C7. Combine the supernatant (Supernatant 2) with Supernatant 1. Discard the pellet.

C8. Dispense 12 ml volumes of 0.3 M sucrose into four 40 ml Sorvall centrifuge tubes.

C9. Vortex the combined supernatants (Supernatant 1 and Supernatant 2) and 'carefully' stratify 10 ml volumes (using a sterile 5 ml Gilson pipette) over the 0.3 M sucrose in the Sorvall centrifuge tubes. Note: The supernatant suspensions should sit neatly on top of the sucrose solutions.

C10. Centrifuge the tubes in a Sorvall RC-5B superspeed refrigerated centrifuge (using an SS34 rotor) at 6,800 rpm (6,000 *g*) for 10 minutes at 4 °C.

C11. Discard the supernatants using sterile plastic transfer pipettes and place tubes containing the bacterial pellets on ice.

C12. Resuspend each pellet in 5 ml 0.2 M sucrose and pool. Retain the suspension on ice.

C13. Dispense 12 ml volumes of 1.5 M KCl into two 40 ml Sorvall centrifuge tubes.

C14. Using sterile plastic transfer pipettes, carefully stratify between 10 ml to 12 ml volumes of the bacterial suspension over the 1.5 M KCl in the Sorvall centrifuge tubes. Note: The bacterial suspensions should sit neatly on top of the KCl solutions.

C15. Centrifuge the tubes as per C10.

C16. Remove the supernatants using sterile plastic transfer pipettes and discard supernatant. Place the tubes containing the bacterial pellets on ice.

C17. Resuspend each pellet in 5 ml 0.2 m sucrose and pool. Retain the suspension on ice.

C18. Dispense 12 ml of 1.5 M KCl into a 40 ml Sorvall centrifuge tube and carefully stratify the bacterial suspension over it.

C19. Recentrifuge the tube as per C10.

C20. Resuspend the final pellet in 10 ml sterile saline solution (0.15 M NaCl) and transfer to a sterile pre–weighed Falcon tube.

C21. Centrifuge the suspension in the Beckman J–6B floor centrifuge at 3,250 rpm (2750 g) for 10 minutes at 4°C.

C22. Discard the supernatant with a sterile plastic transfer pipette and wash the pellet again with 20 ml sterile saline, centrifuging as per C21.

C23. Wash the bacterial pellet with 20 ml sterile MQW, centrifuging as described in C21.

C24. Prepare two thin smears of the washed bacterial pellet by placing a drop of sterile MQW on the microscope slides and transferring small amounts of the bacterial pellet onto the slide with a flamed loop or sterile swab. Air dry, heat fix and stain the smears by the Ziehl Neelson and the Gram techniques before examining under a light microscope at x 1000 magnification. Record the presence or absence of AFB, Gram positive bacteria and/or host derived cellular debris on the Sample Record and History Worksheet.

C25. Make the pellet up to 20 ml with sterile MQW. Vortex at high speed for 30 seconds before transferring approximately 500 μl of the suspension to a sterile ET to be used for Miles and Misra plate counts.

C26. Recentrifuge the tube as per C21.

C27. Resuspend pellets in MQW, transfer to pre–weighed 10 ml centrifuge tubes, centrifuge as per C21.
C28. Discard the supernatant, determine the wet weight the bacterial pellet. Record this weight on the Sample History and Record Worksheet.

C29. According to the wet weight of pellet (yield of bacteria), resuspend the pellet in sterile MQW and transfer the bacteria in 0.5 gram aliquots in sterile ETs.

C30. Centrifuge the aliquoted bacterial suspensions in a benchtop microfuge at 12,000 rpm for 5 minutes. Remove the supernatant fron each tube and discard supernatant.

C31. Label the ETs and store at –20°C in two separate locations. Fill out the storage details on the Sample Record and History Worksheet.

Buffers and reagents

Solutions for purification

0.2 M Sucrose solution (5 x stock solution)
342.3 g sucrose made up to 1 L with MQW. Autoclave, store at room temperature. For working strength, 60 ml made up to 300 ml with sterile MQW (sufficient volume for the purification of 4 lots of 5 g tissue).

0.3 M sucrose solution (2 x stock solution)
205.38 g sucrose made up to 1 L with MQW. Autoclave, store at room temperature. For working strength, 100 ml made up to 200 ml with sterile MQW (sufficient volume for the purification of 4 lots of 5 g tissue).

1.5 M KCl solution (2 x stock solution)
223.65 g KCl made up to 1 L with MQW. Autoclave, store at room temperature. For working strength, 75 ml made up to 150 ml with sterile MQW (sufficient volume for the purification of 4 lots of 5 g tissue).

0.15 M NaCl (Saline solution) (10 x stock solution)
43.83 g NaCl made up to 500 ml with MQW. Autoclave, store at room temperature. For working strength, 20 ml made up to 200 ml with sterile MQW (sufficient volume for the purification of 4 lots of 5 g tissue).

D. DNA Extraction

D1. Vigorously poke at cell pellets using sterile wooden applicator sticks to break up any clumps of aggregated bacteria.

D2. Suspend each bacterial pellet in 700 μl of TE buffer (Perform in BSC).

D3. Heat at 80°C (heating block) for 30 min to kill cells. Allow to cool to room temperature for 10 minutes.

D4. Prepare fresh lysozyme (200 mg/ml in 10 mM Tris). Add 120 μl lysozyme solution and 200 units of mutanolysin (20 μl of a 10,000 units per ml stock).

D5. Incubate at 37°C (micro hot room) overnight with very gentle end over end mixing on a Ratek blood mixer.

D6. Transfer to a 10 ml centrifuge tube, add 35 μl of a 10 mg/ml proteinase K solution and 210 μl of 10% SDS.

D7. Incubate at 65°C (hybridisation oven) for 20 minutes with gentle mixing (by hand) every 5 minutes.

D8. Add 195 μ1 5M NaCl, then 165 μl of prewarmed (to 65 °C) CTAB/NaCl and mix very gently (by hand) until "milky". Incubate at 65 °C (hybridisation oven) 10 minutes.

D9. Add an equal volume of 24:1 chloroform/isoamyl alcohol and mix gently (by hand) for 10 seconds

or until an emulsion forms.

D10. Centrifuge at 3,500 rpm (2,700g, Beckman GP benchtop centrifuge) for 5 minutes.

D11. Transfer aqueous phase to 2 sterile screw cap ETs (0.9 ml in one ET and approx. 0.4 ml in the other).

D12. Add 0.6 volume isopropanol (approx. 0.54 ml and 0.24 ml, respectively) and invert once gently by hand.

D13. Incubate at −20°C for 2 hrs.

D14. Centrifuge 15 mins at 11,600 rpm (12,000 *g*) in Heraeus microfuge.

D15. Very carefully decant (pour off, holding tube so that pellet remains uppermost) and discard the supernatant. Add 1 ml of ice cold 70% ethanol.

D16. Centrifuge as per D14.

D17. Pour off supernatant as above if pellet is visible. If not, aspirate carefully and very slowly using multiple aspirations with a P200 Gilson.

D18. Briefly pulse and pipette the remaining fluid with a P200 Gilsen. Note: A pellet should be visible.

D19. Dry pellet under vacuum in desiccator for 30 minutes.

D20. Resuspend each DNA pellet in 35 μl of TE buffer. Incubate at 37°C for one hour to reconstitute the DNA. Pool the two DNA solutions from the same sample together.

D21. Assess DNA by electrophoresis (refer to general gel electrophoresis protocol).

Buffers and reagents

Solutions for DNA extraction

TE buffer (10 mM Tris, 1 mM EDTA pH 8.0)
Tris 0.6055 g, EDTA 0.187 g, 480 ml sterile MQW, adjust pH to 8.0 and make up to 500 ml. Autoclave, dispense into 50 ml aliquots (Falcon tubes) and store at room temperature.

Lysozyme solution (50 mg/ml) Sigma Chemical Company, Catalogue # L6876 (1 g)
200 mg lysozyme made up to 4 ml with sterile MQW. Dispense into 250 μl aliquots and store a −20 °C. Thaw immediately before use, do not use again after thawing.

Mutanolysin Sigma Chemical Company, Catalogue # M9901 (5000 units)
Add 1 ml of TE buffer (pH 8.0) to 5,000 unit lyophilised powder in bottle as purchased. Stored at −20°C. Thaw immediately before use and replace at −20°C after use.

Proteinase K Boehringer Mannheim, Catalogue # 745 723 (100 mg)
50 mg of proteinase K made up to 5 ml with sterile MQW. Dispense into 135 μl aliquots and store at −20°C. Thaw immediately before use and replace at −20°C after use.

10% SDS
10 g SDS made up to 100 ml. Dispense into 10 ml aliquots and store at room temperature.

5 M NaCl
29.22 g NaCl made up to 100 ml with MQW. Autoclave, dispense into 10 ml aliquots and store at room temperature.

CTAB/NaCl
Dissolve 4.1 g NaCl in 80 ml MQW. While stirring, add 10 g CTAB. Heat solution to 65°C to dissolve. Adjust volume to 100 ml. Store at room temperature.
Note: CTAB = Hexadecyltrimethylammonium Bromide, Sigma Chemical Company, Catalogue # H5882 (100 g).

24:1 chloroform/isoamyl alcohol
480 ml of chloroform and 20 ml of isoamyl alcohol. Store at room temperature.

Sterile MQW
Use virology MQW, autoclave in virology, dispense into 1 ml aliquots and store at room temperature.

DNA Typing Project – DNA Extraction Worksheet

Date ____/____/____ Group No. ____

Reagents

TE buffer	# _____
Lysozyme solution	# _____
Mutanolysin	# _____
Proteinase K	# _____
10% SDS	# _____
5 M NaCl	# _____
CTAB/NaCl	# _____
24:1 chloroform/isoamyl alcohol	# _____
Isopropanol	# _____
70% ethanol	# _____
Sterile MQW	# _____

E. Restriction Endonuclease Digestion

Note: For every restriction digestion reaction a positive control must also be carried out for subsequent detection by RFLP. Positive controls may include 316V, McPaul bovine strain or an S1 sheep strain which has been characterised previously. Negative controls may also be performed and include *M. phlei* or *M. avium* (organisms which do not contain IS*900* in their genome).

E1. Keep DNA samples on ice.

E2. Determine the volume of DNA preparation to be used for each sample based on DNA concentration and add to sterile, labelled screw-capped ETs. Use 3–4 μg per digest, assuming a large gel with 10 wells per comb. (2–3 μg is sufficient for 20 well comb).

E3. Prepare the digestion mixture as follows: to the appropriate volume of DNA solution add sterile MQW to a final volume of 18 μl. Add 2 μl 10 X restriction buffer. Add 1 μl (10U) enzyme (*Bst*EII, Boehringer Mannheim, catalogue # 567 612 [5,000 units]). Mix carefully by flicking tube with a finger or briefly pulsing in a microfuge

E4. Incubate the digestion reactions at 60 °C (using hybridisation oven) for 3 hours.

E5. Prepare a 1% w/v large (14 x 12 cm approx.) agarose gel with a single 10 well comb.

E6. Following digestion of the DNA, add 4 μl loading buffer (blue goop) and load the entire reaction mixture onto the gel in lanes 2–9.

E7. Add 5μl digoxigenin-labelled MW standards to an equal volume of loading buffer and load 5ul in lanes 1 and 10. (Molecular weight marker VII, digoxigenin labelled, Boehringer Mannheim, Catalogue # 1 669 940 [10 μg/ml, 500 μl]).

E8. Electrophorese at 35V, 200 mA for 20 hrs in 1% agarose using fresh TBE running buffer. Check the parameters on the power pack before leaving the lab for the day.

E9. Examine the gel very briefly under UV and photograph to record the digestion. Do not expose gel to UV for any longer than necessary to obtain the record.

DNA Typing Project – Restriction Endonuclease Digestion Worksheet

Date ____/____/____ Group No. ____

Sample ID	JD No.	Volume of DNA (μl)	Volume of MQW (μl)	Volume of buffer (μl)	Volume of enzyme (μl)

APPENDIX 2

The Miles and Misra plate count method for the enumeration of viable organisms

(Ver 17.12.96 file :\milesmis)

Method based on Miles and Misra (1938) *J. Hyg.* (Lond.) 38: 732.

A1. Calibrate a plastic transfer pipette to measure the number of drops per ml. Fill the pipette up to the 1 ml mark with water and release the liquid drop wise. Count the drops until no drops remain (keep the pipette vertical when calibrating).

A2. Prepare ten fold dilutions of the test sample (eg. 200 μl suspension being added to 1.8 ml of sterile PBS). Fresh sterile pipettes must be used for each dilution. Dilutions ranging between 10^{-1} and 10^{-6} should be prepared. Stored at 4 °C until required.

A3. Blood agar plates can be obtained from the RVL. Three plates are required for each test sample. After drying the plates for 15 minutes in a 37 °C incubator with the lids slightly raised, label them and divide each plate into 6 equal sections. Label these sections 10^{-1} to 10^{-6}.

A4. Light the bunsen burner and work close to the flame. One drop of each dilution is dropped onto the designated section of each of 3 replicate blood agar plates. Suspensions should be dropped from a height of 2.5cm. Note: A single pipette can be used for each dilution of the same sample provided that the suspensions are dropped in reverse order (ie. from 10^{-6} to 10^{-1}). The pipette must be kept vertical.

A5. Allow the drops to dry on the plate by keeping them on the laboratory bench. Keep the plates close to the bunsen burner to minimise airborne contamination.

A6. Once drops have dried, incubate them at 37 °C overnight or longer if necessary.

A7. The areas on the plate which received the lower dilutions (10^{-1} and 10^{-2}) usually yield circular patches of growth (confluent growth can be expected when an abundance of organisms are present). The areas which received the higher dilutions (10^{-5} and 10^{-6}) yield fewer colonies. Counts are made in drop areas containing the greatest amount of colonies without signs of confluence or of gross diminution of colony size due to overcrowding.

A8. Determine the mean count of the replicate plates.

Count = mean count x drops/ml x dilution factor
= colony forming units/ml

and represents the number of viable and culturable bacteria per ml in the suspension examined.

APPENDIX 3
Southern Blotting to detect IS*900*

(ver 18.03.97; file a:\southern)

Based on methods in "The DIG System User's Guide for Filter Hybridisation" by Boehringer Mannheim, 1995 and Sambrook et al Molecular Cloning. A Laboratory Manual 2nd Ed, pages 9.31–9.55.

A. Gel electrophoresis with DIG–labelled MW standards
Perform standard agarose gel electrophoresis but include DIG–labelled MW standards to enable later detection by immunological reaction on the membrane.

Boehringer Mannheim digoxigenin–labelled Molecular weight marker VI or VII, Catalogue # 1 218 611 or 1 669 940, respectively. Use MW marker VI for the detection of PCR products (for each lane take 10 µl of undiluted marker to which is added 3 µl blue goop). Use MW marker VII for the detection of RFLPs (for each lane take 15 µl of undiluted marker to which 4 µl blue goop is added).

After electrophoresis, examine and photograph the gel under UV light.

B. Vacuum transfer of DNA (see alternative proceedure, capillary blot, if required)
Method based also on Amersham Product information for Hybond–N+; positively charged nylon membrane. If transfer of fragments >15 kb is required, depurination is suggested: soak the gel for 10 minutes in several volumes of 0.2N HCl and then rinse briefly with distilled water. Then proceed to B1. This produces fragments of about 1 kb.

B1. Rinse the gel in distilled water and place in denaturation buffer such that the gel is completely covered. Leave at room temperature for 30 minutes with constant gentle agitation on a platform rocker.

B2. Pour off the denaturation buffer and rinse the gel in distilled water. Neutralise the gel by soaking it in neutralisation buffer at room temperature for 30 minutes with constant gentle agitation, changing the buffer after 15 minutes.

B3. Set up the Hybaid "Vacuaid" vacuum transfer apparatus unit.

a) Place the support lattice and porous screen over the Vacuaid transfer unit base .

b) Pre–moisten a sheet of Whatman filter paper and place on the porous screen.

c) Place an oversized sheet of Amersham Hybond–N+ nylon transfer membrane on the filter paper ensuring that it is in line with the eventual location of the aperture in the rubber mask.

d) Place the rubber mask over the nylon membrane. Replace the top manifold and clip into place. Flood the aperture in the rubber mask with a small amount of transfer buffer.

B4. Carefully place the gel over the rubber mask such that the edges of the gel overlap the rubber on all sides. Apply firm pressure to the gel to exclude any air bubbles. Note: It is important to ensure correct alignment of the gel as quickly as possible since DNA may be transferred to the nylon membrane at first contact.

B5. Apply an 80 cm vacuum by attaching the tubings to the pump and turning the power on. Add a small amount of transfer buffer to the top of the gel until a meniscus is evident.

B6. Allow the transfer to proceed for 90 minutes, after which the gel should be examined under UV light to confirm the complete transfer of DNA. Briefly rinse the membrane with 2 x SSC to remove any adhering gel.

(Optimal transfer is achieved with vacuum pressure of 80 cm water for 60 minutes with a 5 mm 0.8% agarose gel; if the gel collapses use 40 cm pressure; pressure is set with the valve on the vacuum line. Longer times may be required for higher % gels.)

B7. After transfer, rinse the appartus thoroughly in distilled water and dry.

C.Fixing DNA to the membrane

C1. Turn the vacuum oven on (Block 6 – Equipment room 6.25) and set to 80 °C.

C2. Add 1 μl of 1:1000 diluted positive control DIG–labelled DNA (from the DIG DNA Labelling and Detection Kit, Block 8 freezer) to the top of the membrane as a spot.

C3. Place the membrane onto a glass petri dish and dry in the pre–heated 80 °C vacuum oven for 2 hours.

C4. Remove the membrane from the oven and wrap in plastic Glad wrap.

C5. At this stage it is safe to either:
a. Store the membrane in plastic film at 4°C, in an airtight container OR
b. Continue with the hybridisation procedure.

Buffers and reagents for Southern Blotting

20x SSC (3M NaCl; 0.3M Tri sodium citrate)

NaCl	87.65 g
Na_3 citrate	44.1 g

Make up to 500 ml with MQW.
Autoclave.

Denaturation Solution (1.5M NaCl, 0.5M NaOH)

NaCl	43.83 g
NaOH	10.0 g

Make up to 500ml with MQW.

Neutralisation Solution (1.5M NaCl; 0.5M Tris–HCl pH 7.2, 0.001M EDTA)

NaCl	43.83 g
Tris	30.28 g

add 450 ml MQW and adjust pH to 7.2

EDTA	0.186 g

Make up to 500ml with MQW.
Autoclave.

Stock solution 10 x blocking buffer

Blocking reagent Boehringer Mannheim		10 g
Maleic acid buffer	to	100 ml

Autoclave.
Store at 4°C. (solution remains cloudy).

Maleic acid buffer (0.1M maleic acid, 0.15M NaCl pH 7.5)

Maleic acid	11.6 g
NaCl	8.77 g

Add MWQ, adjust pH to 7.5, make up to 1000 ml
Autoclave.

Standard Prehybridisation/hybridisation Buffer
(5 x SSC; 0.1% N–lauroylsarcosine; 0.02% SDS; 1% Boehringer Mannheim blocking reagent)

20 x SSC	25 ml
N–lauroylsacosine	0.1 g
SDS	0.02 g
10 x blocking buffer	10.0 ml

Make up to 100ml with MQW.
Autoclave.
Store at 4°C.

Wash solution 1 (2 x SSC, 0.1% SDS)

20 x SSC	100 ml
SDS	1 g
MQW to	1000 ml

Wash solution 2 (0.5 x SSC, 0.1% SDS)

20 x SSC	25 ml
SDS	1 g
MQW to	1000 ml

APPENDIX 4

Immunological/Colorimetric Detection Protocol for DNA on membranes

(ver 12.10.98; file a:\digdetec)

Development of Southern Blots using the Boehringer Mannheim DIG-DNA Labelling and Detection Kit, Catalogue # 1 093 657. This method is extracted from Dig System Users Guide.

Note: Volumes of reagents used depend on the size of the membrane and the size of the plastic tray. Use volumes which allow the membrane to be fully submerged. Use clean glassware or disposable plastic ware throughout.

A1. Place the membrane in a flat bottomed plastic tray and briefly equilibrate the membrane in washing buffer for 2 to 5 minutes, agitating the solution on an orbital shaker.

A2. Decant the washing buffer, replace with blocking solution and incubate at room temperature for 30-60 minutes with constant gentle agitation.

A3. Prepare an anti-DIG-Alkaline Phosphatase conjugate by diluting the anti-DIG-AP (vial 8 from the DIG labelling and detection kit, fridge in laboratory 9.25) 1:10,000 in blocking solution (eg. 2 µl to every 20 ml of 1 x blocking solution.

This reagent is stable for at least 12 months at 4°C. Check expiry date. The working solution is stable for 12 hours at 4°C. Centrifuge the AP conjugate vial in a microfuge at 10,000 g for 15 min to remove any precipitates before using, if spotty background is a problem.

A4. Pour off the blocking solution and replace with the conjugate solution. Incubate the membrane at room temperature for 30 minutes.

A5. Wash the membrane twice in 100 ml washing buffer for 15 minutes per wash. Pour off the solution. Use a fresh tray for these washes.

A6. Equilibrate the membrane in detection buffer for 2 minutes.

A7. For chemiluminescent detection:
Keep the membrane wet.
Dilute CSPD or PPD reagent 1:100 in detection buffer. Prepare 10 ml. Incubate each membrane for 5 min in this reagent, then seal in a plastic bag. Do not allow the membranes to dry out.
Store working strength PPD reagent at 4°C for up to a week.

A8. Incubate the membrane at 37°C for 30 min.

A9. Working in the darkroom under safelight, load Xray film and the membrane into a cassette, avoiding repositioning of the membrane. Close the cassette and leave for 5-60 minutes. Develop Xray film for 1 min and fix for 2 min; wash for 5 mins then dry. The exposure can be repeated if necessary, with results obtained even after storage of membrane for 2 weeks at 4°C. Prolonged exposures, eg overnight, may be required to reveal bands in samples with very low DNA concentrations.

Store the membrane, sealed in the plastic bag, at 4°C.

The protocol ends here, unless reprobing or colorimetric detection is also desired.

A10. For reprobing the membrane:
Do not allow the membrane to dry out.
Wash the membrane in MQW for 1 min.
Wash the membrane twice for 10 min each in 0.2M NaOH, 0.1% SDS.
Wash the membrane in 2 x SSC.
Commence reprobing with desired probe after prehybridisation.

A11. For colorimetric detection:
Prepare a colour-substrate solution by adding 45 µl of NBT-solution and 35 µl BCIP (X-phosphate) solution to 10 ml of detection buffer; protect from light. Note: this colour-substrate solution must be freshly prepared.

Pour off the detection buffer, transfer the membrane to a sealable plastic glad bag and add the freshly prepared NBT colour solution. Seal the bag and incubate in the dark (in a styrofoam box, lined with foil and place inside a cupboard. Alternatively do this reaction in a sealed plastic box. Do not shake the membrane. Note: The colour will appear within minutes but will take 16 hours to mature.

Stop the reaction by washing the membrane in MQW for 5 minutes.

Dry the membrane in air and photocopy or photograph the membrane. Mount membrane in lab notes sealed under plastic. The colour fades in air.

Note: colorimetric detection can be undertaken after chemiluminescent detection. Wash the membrane for 5 min in detection buffer before proceeding with colorimetric detection.

Buffers and reagents for Southern Blotting and Dig-immunological detection

Maleic acid buffer (0.1M maleic acid, 0.15M NaCl pH 7.5)

Maleic acid	23.20 g
NaCl	17.54 g

Add MWQ to 1800 ml, adjust pH to 7.5 with 10 N NaOH or solid NaOH
Add MQW to 1000 ml
Autoclave.

10 x Blocking solution

Boehringer blocking reagent		10 g
Maleic acid buffer	to	100 ml

Heat in a microwave to dissolve. Will also require stirring.
Autoclave.
Store at 4°C or -20°C.
Solution will remain cloudy.

Blocking solution
Dilute 10 x stock solution 1:10 in maleic acid buffer

OR,

10 g blocking reagent in 1000 ml maleic acid buffer

Washing buffer

Maleic acid buffer	1000 ml
Tween 20	3 ml

Detection buffer (0.1 M Tris-HCl, 0.1M NaCl, pH 9.5)

NaCl	5.85 g
Tris-HCl	12.11 g

Add 900 ml MQW, adjust pH to 9.5, make up to 1000 ml
Autoclave.
Store 4°C.

(Addition of 50 mM $MgCl_2$ [10.2 g/L] results in a cloudy solution and is not specified in current Dig System Users Guide, nor currently recommended by BM)

DNA typing project results

append5.xls

Sample	Accession	No.	Animal ID	Origin	Region	Property code	Lab	Species	Farm	RFLP	AS900	RSA	IRI311	RSA	Replist
scrape	98/5001			NSW	Carcoar	1	EMAI	ovine	wool	S1			2	S	1
scrape	98/5002			NSW	Carcoar	1	EMAI	ovine	wool	S1			3	S	2
scrape	98/5003			NSW	Carcoar	1	EMAI	ovine	wool	NB	1	0	0	nt	3
scrape	98/5004			NSW	Carcoar	1	EMAI	ovine	wool	S1			3	S	4
scrape	98/5005			NSW	Carcoar	1	EMAI	ovine	wool	NB	0	nt	0	nt	5
scrape	98/5008			NSW	Carcoar	1	EMAI	ovine	wool	S1			3	S	6
scrape	98/50010			NSW	Carcoar	1	EMAI	ovine	wool	NB	0	nt	0	nt	7
scrape	97/3703			NSW	Carcoar	1	EMAI	ovine	wool	NB	0	nt	0	nt	8
scrape	97/3706			NSW	Carcoar	1	EMAI	ovine	wool	S1			3	S	9
scrape	96/101			NSW	Carcoar	2	EMAI	ovine	wool	S1			3	S	10
scrape	97/3601			NSW	Goulburn	3	EMAI	ovine	wool	S1			3	S	11
scrape	97/3602			NSW	Goulburn	3	EMAI	ovine	wool	S1			3	S	12
scrape	97/3604			NSW	Goulburn	3	EMAI	ovine	wool	S1			1	S	13
scrape	97/3605			NSW	Goulburn	3	EMAI	ovine	wool	S1			2	S	14
scrape	97/3606			NSW	Goulburn	3	EMAI	ovine	wool	S1			3	S	15
scrape	97/3607			NSW	Goulburn	3	EMAI	ovine	wool	S1			3	S	16
scrape	97/3608			NSW	Goulburn	3	EMAI	ovine	wool	S1			3	S	17
scrape	96/8			NSW	Carcoar	4	EMAI	ovine	wool	S1			3	S	18
scrape	96/12			NSW	Carcoar	5	EMAI	ovine	wool	S1			3	S	19
scrape	97/101			NSW	Carcoar	5	EMAI	ovine	wool	S1			3	S	20
scrape	97/102			NSW	Carcoar	5	EMAI	ovine	wool	S1			3	S	21
scrape	97/106			NSW	Carcoar	5	EMAI	ovine	wool	S1			3	S	22
scrape	97/108			NSW	Carcoar	5	EMAI	ovine	wool	S1			3	S	23
scrape	97/109			NSW	Carcoar	5	EMAI	ovine	wool	S1			3	S	24
scrape	97/1010			NSW	Carcoar	5	EMAI	ovine	wool	NB	0	nt	0	nt	25
scrape	97/1011			NSW	Carcoar	5	EMAI	ovine	wool	NB	0	nt	0	nt	26
scrape	97/1012			NSW	Carcoar	5	EMAI	ovine	wool	S1			3	S	27
scrape	97/1013			NSW	Carcoar	5	EMAI	ovine	wool	S1			3	S	28
scrape	97/1018			NSW	Carcoar	5	EMAI	ovine	wool	NB	0	nt	0	nt	29
scrape	97/1020			NSW	Carcoar	5	EMAI	ovine	wool	S1			3	S	30
scrape	97/301			NSW	Carcoar	5	EMAI	ovine	wool	S1			3	S	31
scrape	97/302			NSW	Carcoar	5	EMAI	ovine	wool	S1			3	S	32
scrape	96/1101			NSW	Carcoar	6	EMAI	ovine	wool	S1			3	S	33
scrape	96/1102			NSW	Carcoar	6	EMAI	ovine	wool	S1			3	S	34
scrape	96/6001			NSW	Carcoar	6	EMAI	ovine	wool	S1			3	S	35
scrape	96/6002			NSW	Carcoar	6	EMAI	Caprine	wool	S1			3	S	36
scrape	96/6003			NSW	Carcoar	6	EMAI	Caprine	wool	S1			3	S	37
scrape	98/3001			NSW	Carcoar	6	EMAI	ovine	wool	S1			3	S	38
scrape	98/3002			NSW	Carcoar	6	EMAI	ovine	wool	S1			Tr	nt	39
scrape	98/3003			NSW	Carcoar	6	EMAI	ovine	wool	NB	0	nt	0	nt	40
scrape	97/701			NSW	Bathurst	7	EMAI	ovine	wool	S1			3	S	41
scrape	97/702			NSW	Bathurst	7	EMAI	ovine	wool	S1			3	S	42
scrape	97/7013			NSW	Bathurst	7	EMAI	ovine	wool	S1			3	S	43
scrape	97/7014			NSW	Bathurst	7	EMAI	ovine	wool	S1			3	S	44
scrape	97/7015			NSW	Bathurst	7	EMAI	ovine	wool	S1			3	S	45
scrape	97/7016			NSW	Bathurst	7	EMAI	ovine	wool	S1			3	S	46
scrape	97/7017			NSW	Bathurst	7	EMAI	ovine	wool	NB	0	nt	0	nt	47
scrape	97/7018			NSW	Bathurst	7	EMAI	ovine	wool	S1			3	S	48
scrape	97/7019			NSW	Bathurst	7	EMAI	ovine	wool	S1			3	S	49
scrape	97/1702			NSW	Bathurst	7	EMAI	ovine	wool	S1			3	S	50
scrape	97/1703			NSW	Bathurst	7	EMAI	ovine	wool	S1			3	S	51

APPENDIX 5 - RESULTS

DNA typing project results

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Sample	Accession	No.	Animal ID	Origin	Region	Property code	Lab	Species	Farm	RFLP	18900	REA	TR1311	REA	Keylist
scrape	97/1902			NSW	Bathurst	8	EMAI	ovine	wool	S1			3	S	52
scrape	97/1905			NSW	Bathurst	8	EMAI	ovine	wool	S1			3	S	53
scrape	97/16401			NSW	Bathurst	9	EMAI	ovine	wool	SU4			3	S	54
scrape	97/16402			NSW	Bathurst	9	EMAI	ovine	wool	S1			2	S	55
scrape	97/16403			NSW	Bathurst	9	EMAI	ovine	wool	S1			3	S	56
scrape	97/16405			NSW	Bathurst	9	EMAI	ovine	wool	S1			2	S	57
scrape	97/16406			NSW	Bathurst	9	EMAI	ovine	wool	S1			2	S	58
scrape	97/1501			NSW	Bathurst	9	EMAI	ovine	wool	S1			3	S	59
scrape	97/1502			NSW	Bathurst	9	EMAI	ovine	wool	S1			3	S	60
scrape	97/4201			NSW	Goulburn	10	EMAI	ovine	wool	S1			3	S	61
scrape	97/4205			NSW	Goulburn	10	EMAI	ovine	wool	S1			3	S	62
scrape	97/4206			NSW	Goulburn	10	EMAI	ovine	wool	S1			3	S	63
scrape	98/3101			NSW	Bathurst	11	EMAI	ovine	wool	S1			3	S	64
scrape	98/3102			NSW	Bathurst	11	EMAI	ovine	wool	S1			3	S	65
scrape	98/3103			NSW	Bathurst	11	EMAI	ovine	wool	S1			3	S	66
scrape	98/3104			NSW	Bathurst	11	EMAI	ovine	wool	S1			2	S	67
scrape	98/3105			NSW	Bathurst	11	EMAI	ovine	wool	S1			3	S	68
scrape	98/3106			NSW	Bathurst	11	EMAI	ovine	wool	S1			2	S	69
scrape	97/1601			NSW	Bathurst	12	EMAI	ovine	wool	NB	0	nt	0	nt	70
scrape	97/1602			NSW	Bathurst	12	EMAI	ovine	wool	S1			3	S	71
scrape	97/1605			NSW	Bathurst	12	EMAI	ovine	wool	S1			3	S	72
scrape	97/4004			NSW	Bathurst	12	EMAI	ovine	wool	S1			3	S	73
scrape	97/4005			NSW	Bathurst	12	EMAI	ovine	wool	S1			3	S	74
scrape	98/2701			NSW	Bathurst	12	EMAI	ovine	wool	S1			2	S	75
scrape	98/2702			NSW	Bathurst	12	EMAI	ovine	wool	S1			1	S	76
scrape	98/2901			NSW	Bathurst	12	EMAI	ovine	wool	S1			3	S	77
scrape	98/2902			NSW	Bathurst	12	EMAI	ovine	wool	S1			1	S	78
scrape	95/8			NSW	Carcoar	13	EMAI	ovine	wool	S1			3	S	79
scrape	96/301			NSW	Goulburn	14	EMAI	ovine	wool	SU3			3	S	80
scrape	96/302			NSW	Goulburn	14	EMAI	ovine	wool	SU3			3	S	81
scrape	98/2303			NSW	Bathurst	15	EMAI	ovine	wool	S1			3	S	82
scrape	96/7			NSW	Bathurst	15	EMAI	ovine	wool	S1			3	S	83
scrape	97/1101			NSW	Bathurst	16	EMAI	ovine	wool	S1			3	S	84
scrape	97/1102			NSW	Bathurst	16	EMAI	ovine	wool	NB	0	nt	0	nt	85
scrape	97/1104			NSW	Bathurst	16	EMAI	ovine	wool	S1			3	S	86
scrape	97/1105			NSW	Bathurst	16	EMAI	ovine	wool	S1			3	S	87
scrape	97/1106			NSW	Bathurst	16	EMAI	ovine	wool	S1			3	S	88
scrape	97/1107			NSW	Bathurst	16	EMAI	ovine	wool	S1			2	S	89
scrape	97/1109			NSW	Bathurst	16	EMAI	ovine	wool	S1			3	S	90
scrape	97/1301			NSW	Bathurst	17	EMAI	ovine	wool	S1			3	S	91
scrape	97/1302			NSW	Bathurst	17	EMAI	ovine	wool	S1			3	S	92
scrape	97/2101			NSW	Bathurst	17	EMAI	ovine	wool	S1			3	S	93
scrape	97/5402			NSW	Bathurst	17	EMAI	ovine	wool	S1			3	S	94
scrape	97/138			NSW	Bathurst	17	EMAI	ovine	wool	S1			2	S	95
scrape	97/14801			NSW	Bathurst	17	EMAI	ovine	wool	S1			3	S	96
scrape	97/14802			NSW	Bathurst	17	EMAI	ovine	wool	S1			3	S	97
scrape	98/2601			NSW	Bathurst	17	EMAI	ovine	wool	S1			3	S	98
scrape	98/2603			NSW	Bathurst	17	EMAI	ovine	wool	S1			3	S	99
scrape	98/2604			NSW	Bathurst	17	EMAI	ovine	wool	S1			3	S	100
scrape	98/2605			NSW	Bathurst	17	EMAI	ovine	wool	S1			3	S	101
scrape	98/2606			NSW	Bathurst	17	EMAI	ovine	wool	NB	1	1	3	S	102
scrape	98/2607			NSW	Bathurst	17	EMAI	ovine	wool	SU1	2	1	2	S	103

DNA typing project results

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Sample	Accession	No.	Animal ID	Origin	Region	Property code	Lab	Species	Farm	RFLP	IS900	REA	TR1.111	REA	Keylist
scrape	97/1001			NSW	Bathurst	18	EMAI	ovine	wool	S1			3	S	104
scrape	97/1002			NSW	Bathurst	18	EMAI	ovine	wool	S1			3	S	105
scrape	97/1004			NSW	Bathurst	18	EMAI	ovine	wool	S1			2	S	106
scrape	97/1006			NSW	Bathurst	18	EMAI	ovine	wool	S1			3	S	107
scrape	97/1007			NSW	Bathurst	18	EMAI	ovine	wool	S1			3	S	108
scrape	97/1008			NSW	Bathurst	18	EMAI	ovine	wool	NB	0	nt	0	nt	109
scrape	98/2201			NSW	Bathurst	18	EMAI	ovine	wool	S1			3	S	110
scrape	98/2202			NSW	Bathurst	18	EMAI	ovine	wool	NB	0	nt	no DNA	nt	111
scrape	98/2204			NSW	Bathurst	18	EMAI	ovine	wool	S1			3	S	112
scrape	98/2205			NSW	Bathurst	18	EMAI	ovine	wool	NB	1	1	3	S	113
scrape	98/2206			NSW	Bathurst	18	EMAI	ovine	wool	NB	1	1	3	S	114
scrape	98/2207			NSW	Bathurst	18	EMAI	ovine	wool	S1			no DNA	nt	115
scrape	98/2208			NSW	Bathurst	18	EMAI	ovine	wool	S1			3	S	116
scrape	98/2209			NSW	Bathurst	18	EMAI	ovine	wool	S1			2	S	117
scrape	97/14701			NSW	Bathurst	18	EMAI	ovine	wool	S1			2	S	118
scrape	97/14705			NSW	Bathurst	18	EMAI	ovine	wool	S1			2	S	119
scrape	97/2301			NSW	Bathurst	18	EMAI	ovine	wool	S1			3	S	120
scrape	97/2302			NSW	Bathurst	18	EMAI	ovine	wool	S1			3	S	121
scrape	97/16501			NSW	Carcoar	19	EMAI	ovine	wool	S1			2	S	122
scrape	97/16503			NSW	Carcoar	19	EMAI	ovine	wool	S1			3	S	123
scrape	97/16504			NSW	Carcoar	19	EMAI	ovine	wool	SU2			2	S	124
scrape	97/16506			NSW	Carcoar	19	EMAI	ovine	wool	S1	3	1	3	S	125
scrape	97/16507			NSW	Carcoar	19	EMAI	ovine	wool	NB	1	1	no DNA	nt	126
scrape	98/2801			NSW	Bathurst	20	EMAI	ovine	wool	S1			3	S	127
scrape	96/21			NSW	Yass	21	EMAI	ovine	wool	S1			3	S	128
scrape	97/2201			NSW	Carcoar	22	EMAI	ovine	wool	S1			3	S	129
scrape	97/2202			NSW	Carcoar	22	EMAI	ovine	wool	S1			3	S	130
scrape	97/2203			NSW	Carcoar	22	EMAI	ovine	wool	NB	1	1	3	S	131
scrape	97/3801			NSW	Carcoar	23	EMAI	ovine	wool	NB	1	1	3	S	132
scrape	96/9			NSW	Carcoar	24	EMAI	ovine	wool	S1			3	S	133
scrape	97/2001			NSW	Carcoar	23	EMAI	ovine	wool	S1			3	S	134
scrape	97/2002			NSW	Carcoar	23	EMAI	ovine	wool	S1			3	S	135
scrape	98/20023			VIC	Melbourne	25	VIAS	ovine	wool	S1			3	S	136
scrape	98/20024			VIC	Melbourne	25	VIAS	ovine	wool	S1			3	S	137
scrape	98/20027			VIC	Melbourne	25	VIAS	ovine	wool	S1			3	S	138
scrape	98/20013			VIC	Gippsland	26	VIAS	ovine	wool	S1			3	S	139
scrape	98/20019			VIC	Gippsland	26	VIAS	ovine	wool	S1			3	S	140
scrape	98/2002			VIC	Gippsland	26	VIAS	ovine	wool	S1			3	S	141
scrape	98/20022			VIC	Gippsland	26	VIAS	ovine	wool	S1			3	S	142
scrape	98/20010			VIC	Gippsland	27	VIAS	ovine	wool	S1			3	S	143
scrape	98/20011			VIC	Gippsland	27	VIAS	ovine	wool	S1			2	S	144
scrape	98/20015			VIC	Gippsland	27	VIAS	ovine	wool	S1			3	S	145
scrape	98/20016			VIC	Gippsland	27	VIAS	ovine	wool	S1			3	S	146
scrape	98/20017			VIC	Gippsland	27	VIAS	ovine	wool	S1			2	S	147
scrape	98/20018			VIC	Gippsland	27	VIAS	ovine	wool	S1			3	S	148
scrape	98/2004			VIC	Gippsland	27	VIAS	ovine	wool	S1			3	S	149
scrape	98/2005			VIC	Gippsland	27	VIAS	ovine	wool	S1			3	S	150
scrape	98/2006			VIC	Gippsland	27	VIAS	ovine	wool	S1			2	S	151
scrape	98/2008			VIC	Gippsland	27	VIAS	ovine	wool	S1			0	nt	152
scrape	98/2001			VIC	Gippsland	28	VIAS	ovine	wool	S1			3	S	153
scrape	98/20012			VIC	Gippsland	28	VIAS	ovine	wool	S1			2	S	154
scrape	98/20020			VIC	Gippsland	28	VIAS	ovine	wool	S1			3	S	155

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Sample	Accession	No.	Animal ID	Origin	Region	Property code	Lab	Species	Farm	RFLP	IS900	REA	TR1311	REA	Keylist
scrape	98/2003			VIC	Gippsland	28	VIAS	ovine	wool	S1			3	S	156
scrape	97/1401			NSW	Bathurst	29	EMAI	ovine	wool	S1			3	S	157
scrape	97/1403			NSW	Bathurst	29	EMAI	ovine	wool	S1			3	S	158
scrape	97/1404			NSW	Bathurst	29	EMAI	ovine	wool	S1			3	S	159
scrape	97/1406			NSW	Bathurst	29	EMAI	ovine	wool	S1			3	S	160
scrape	97/1408			NSW	Bathurst	29	EMAI	ovine	wool	S1			3	S	161
scrape	97/13604			NSW	Bathurst	29	EMAI	ovine	wool	S1			3	S	162
scrape	97/13605			NSW	Bathurst	29	EMAI	ovine	wool	S1			1	S	163
scrape	96/201			NSW	Bathurst	29	EMAI	ovine	wool	S1			3	S	164
scrape	97/35			NSW	Goulburn	30	EMAI	ovine	wool	S1			3	S	165
scrape	98/3202			NSW	Goulburn	30	EMAI	ovine	wool	S1			3	S	166
scrape	98/3205			NSW	Goulburn	30	EMAI	ovine	wool	S1			3	S	167
scrape	97/4101			NSW	Goulburn	30	EMAI	ovine	wool	S1			3	S	168
scrape	97/4102			NSW	Goulburn	30	EMAI	ovine	wool	S1			3	S	169
scrape	97/4103			NSW	Goulburn	30	EMAI	ovine	wool	S1			3	S	170
scrape	97/4107			NSW	Goulburn	30	EMAI	ovine	wool	S1			3	S	171
scrape	97/4109			NSW	Goulburn	30	EMAI	ovine	wool	S1			3	S	172
scrape	97/901			NSW	Young	31	EMAI	ovine	wool	S1			3	S	173
scrape	97/902			NSW	Young	31	EMAI	ovine	wool	S1			3	S	174
scrape	97/903			NSW	Young	31	EMAI	ovine	wool	S1			3	S	175
scrape	97/905			NSW	Young	31	EMAI	ovine	wool	S1			3	S	176
scrape	97/2401			NSW	Young	31	EMAI	ovine	wool	S1			3	S	177
scrape	97/2403			NSW	Young	31	EMAI	ovine	wool	S1			3	S	178
scrape	97/2404			NSW	Young	31	EMAI	ovine	wool	S1			3	S	179
scrape	97/2405			NSW	Young	31	EMAI	ovine	wool	S1			3	S	180
scrape	97/2409			NSW	Young	31	EMAI	ovine	wool	S1			3	S	181
scrape	97/24010			NSW	Young	31	EMAI	ovine	wool	S1			2	S	182
scrape	95/1002			NSW	Molong	32	EMAI	ovine	wool	S1			3	S	183
culture	953652	4	1588	VIC	Gippsland	33	VIAS	bovine	dairy	C1			2	C	184
culture	930898	9	H89	VIC	n.r.	34	VIAS	bovine	beef	C1			2	C	185
culture	935712	14	51	VIC	Western district	35	VIAS	bovine	dairy	C3			2	C	186
culture	935713	15	312	VIC	Western district	36	VIAS	bovine	dairy	C1			3	C	187
culture	930000	16	466	VIC	Gippsland	37	VIAS	bovine	dairy	C1			3	C	188
culture	935615	18	Noid	VIC	Gippsland	38	VIAS	bovine	dairy	C1			2	C	189
culture	935762	21	Noid	VIC	Western district	39	VIAS	bovine	dairy	C3			2	C	190
culture	935538	23	Y61	VIC	Gippsland	40	VIAS	bovine	dairy	C1			2	C	191
culture	930001	24	469	VIC	LoddonOCampaspe	41	VIAS	bovine	dairy	C1			2	C	192
culture	935901	28	664	VIC	Western district	42	VIAS	bovine	dairy	C3			3	C	193
culture	935903	29	284	VIC	Gippsland	43	VIAS	bovine	dairy	C3			1	C	194
culture	935933	32	437	VIC	Goulburn	44	VIAS	bovine	dairy	C1			3	C	195
culture	936030	34	67	VIC	Gippsland	45	VIAS	bovine	dairy	C3			3	C	196
culture	936033	37	249	VIC	Western district	35	VIAS	bovine	dairy	C3			3	C	197
culture	936069	38	637	VIC	Goulburn	46	VIAS	bovine	dairy	C1			3	C	198
culture	936105	39	28	VIC	Gippsland	47	VIAS	bovine	dairy	C3			1	C	199
culture	936119	40	328	VIC	Gippsland	48	VIAS	bovine	dairy	C1			2	C	200
culture	936120	41	32	VIC	Gippsland	49	VIAS	bovine	dairy	C3			2	C	201
culture	936140	42	80	VIC	Gippsland	50	VIAS	bovine	dairy	C1			3	C	202
culture	936210	44	346	VIC	Gippsland	37	VIAS	bovine	dairy	C1			3	C	203
culture	936292	46	132	VIC	Goulburn	51	VIAS	bovine	dairy	C1			3	C	204
culture	936311	47	Red12	VIC	Gippsland	52	VIAS	bovine	dairy	C3			1	C	205
culture	936313	49	380	VIC	Gippsland	53	VIAS	bovine	dairy	C3			3	C	206
culture	936315	50	456	VIC	Western district	54	VIAS	bovine	dairy	C3			2	C	207

Sample	Accession	No	Animal ID	Origin	Region	Property code	Lab	Species	Farm	RFLP	IS900	REA	IS1311	REA	Keylist
culture	936315	51	474	VIC	Western district	54	VIAS	bovine	dairy	C3			3	C	208
culture	936315	53	451	VIC	Western district	54	VIAS	bovine	dairy	CU1	3	1	3	C	209
culture	936323	54	166/167	VIC	Gippsland	55	VIAS	bovine	dairy	C1			1	C	210
culture	936324	55	326	VIC	Gippsland	56	VIAS	bovine	dairy	C1			3	C	211
culture	936428	61	Ted	VIC	Gippsland	57	VIAS	bovine	dairy	C3			2	C	212
culture	932281	64	823	VIC	Western district	58	VIAS	bovine	dairy	C3			2	C	213
culture	962467	77	2315	VIC	Western district	58	VIAS	bovine	dairy	C3			3	C	214
culture	962467	78	2109	VIC	Western district	58	VIAS	bovine	dairy	C3			3	C	215
culture	962467	80	1912	VIC	Western district	58	VIAS	bovine	dairy	C3			3	C	216
culture	952938	82	2011	VIC	Western district	58	VIAS	bovine	dairy	C3			2	C	217
culture	952938	83	1927	VIC	Western district	58	VIAS	bovine	dairy	C3			1	C	218
culture	952938	84	1943	VIC	Western district	58	VIAS	bovine	dairy	NB	1	1	1	C	219
culture	952938	85	2017	VIC	Western district	58	VIAS	bovine	dairy	C3			1	C	220
culture	952938	86	1912	VIC	Western district	58	VIAS	bovine	dairy	C3			1	C	221
culture	952938	87	1813	VIC	Western district	58	VIAS	bovine	dairy	C3			1	C	222
culture	952938	88	1702	VIC	Western district	58	VIAS	bovine	dairy	C3			3	C	223
culture	953652	94	1923	VIC	Gippsland	33	VIAS	bovine	dairy	C5			2	C	224
culture	953652	95	1811	VIC	Gippsland	33	VIAS	bovine	dairy	C5			1	C	225
culture	953652	96	1724	VIC	Gippsland	33	VIAS	bovine	dairy	C5			2	C	226
culture	953652	97	2451	VIC	Gippsland	33	VIAS	bovine	dairy	C5			2	C	227
culture	962251	115	1855	VIC	Gippsland	33	VIAS	bovine	dairy	NB	3	1	2	C	228
culture	962251	117	1918	VIC	Gippsland	33	VIAS	bovine	dairy	C12			3	C	229
culture	962251	118	1737	VIC	Gippsland	33	VIAS	bovine	dairy	C1			3	C	230
culture	962251	128	1855	VIC	Gippsland	33	VIAS	bovine	dairy	C1			3	C	231
culture	962251	131	1799 (DI47)	VIC	Gippsland	33	VIAS	bovine	dairy	C1			3	C	232
culture	962853	133	TPGQ 331	VIC	Gippsland	59	VIAS	bovine	beef	C5			3	C	233
culture	963854	135	TPG 029	VIC	Gippsland	59	VIAS	bovine	beef	C5			3	C	234
culture	961400	137	91 B	VIC	Central Highlands	60	VIAS	bovine	beef	C5			3	C	235
culture	961400	138	95 B	VIC	Central Highlands	60	VIAS	bovine	beef	C5			3	C	236
culture	961400	139	30 R	VIC	Central Highlands	60	VIAS	bovine	beef	C5			3	C	237
culture	964668	140	144 (3756)	VIC	Goulburn	44	VIAS	bovine	dairy	C3			3	C	238
culture	964651	141	313 (LN)	VIC	Goulburn	61	VIAS	bovine	dairy	CU1	3	1	3	C	239
culture	964651	142	313 (TISS)	VIC	Goulburn	61	VIAS	bovine	dairy	CU1	3	1	3	C	240
culture	964765	143	144 (3756)	VIC	Gippsland	62	VIAS	camelid	hobby	C1			2	C	241
culture	964659	145	2018	NSW	NSW	nr	VIAS	bovine	dairy	CU1'	3	1	3	C	242
culture	962251	155	588	VIC	Gippsland	33	VIAS	bovine	dairy	C5			3	C	243
culture	962852	158	TPGQ 331	VIC	Gippsland	59	VIAS	bovine	beef	CU4			3	C	244
culture	960995	161	5064(LN)	VIC	Western district	64	VIAS	bovine	dairy	C3??,FAINT	3	1	3	C	245
culture	960995	162	5064(TISS)	VIC	Western district	64	VIAS	bovine	dairy	C3			3	C	246
culture	960995	163	460(LN)	VIC	Western district	64	VIAS	bovine	dairy	C3			3	C	247
culture	960995	164	460(TISS)	VIC	Western district	64	VIAS	bovine	dairy	C3			3	C	248
culture	965163	170	2588	VIC	Gippsland	33	VIAS	bovine	dairy	C1			3	C	249
culture	965163	172	2588	VIC	Gippsland	33	VIAS	bovine	dairy	C1			3	C	250
culture	965163	174	2588	VIC	Gippsland	33	VIAS	bovine	dairy	C1			3	C	251
culture	965141	176	1943	VIC	Western district	58	VIAS	bovine	dairy	C3			3	C	252
culture	965141	177	1813	VIC	Western district	58	VIAS	bovine	dairy	C3			2	C	253
culture	965141	178	1927	VIC	Western district	58	VIAS	bovine	dairy	C3			3	C	254
culture	965843	181	287	VIC	Goulburn	44	VIAS	bovine	dairy	C3			3	C	255
culture	964771	182	444	VIC	Melbourne	65	VIAS	camelid	hobby	NB	3	1	3	C	256
culture	965275	183	TPG 013	VIC	Gippsland	59	VIAS	bovine	beef	C1			3	C	257
culture	965275	184	Calf	VIC	Gippsland	59	VIAS	bovine	beef	C1, FAINT	3	1	3	C	258
culture	965163	185	2588	VIC	Gippsland	33	VIAS	bovine	dairy	C1			3	C	259

DNA typing project results

append5.xls

Sample	Accession	No.	Animal ID	Origin	Region	Property code	Lab	Species	Farm	RFLP	IS900	RSA	IS1311	PRA	Keylist
culture	967585	186	268	VIC	Goulburn	44	VIAS	bovine	dairy	C3			3	C	260
culture	845493	195		VIC	Melbourne	66	VIAS	human	na	C5			3	C	261
culture	x	196	M21	VIC	n.r.	67	VIAS	avian	nr	NB	0	nt	3	A	262
culture	852483	197	x	TAS	TAS	nr	VIAS	bovine	nr	C3			3	C	263
culture	853382	198	x	TAS	TAS	nr	VIAS	bovine	nr	C3			3	C	264
culture	854197	199	x	TAS	TAS	nr	VIAS	bovine	nr	C3			3	C	265
culture	853471	200	x	TAS	TAS	nr	VIAS	bovine	nr	C3			3	C	266
culture	851776	201	x	TAS	TAS	nr	VIAS	bovine	nr	CU2	3	1	3	C	267
culture	853770	202	x	TAS	TAS	nr	VIAS	bovine	nr	C3			2	C	268
culture	871088	203	x	TAS	TAS	nr	VIAS	bovine	nr	C3			3	C	269
culture	845493	204		VIC	n.r.	66	VIAS	human	nr	C1, FAINT	3	1	3	C	270
culture	nr	206	10770582	France	France	nr	VIAS	ovine	nr	C5			3	C	271
culture	CM86/3017	207	Sharwood FD1104	NSW	Carcoar	68	VIAS	ovine	wool	NB	1	1	1	C	272
culture	CM83/717	208	Meak FD1066	NSW	Carcoar	69	VIAS	ovine	wool	CU3	3	1	2	C	273
culture	CM80/39337	210	Dowsett FD1061	NSW	Carcoar	70	VIAS	ovine	wool	C3			3	C	274
culture	CM86/1337	211	Van FD1046/1065	NSW	Carcoar	71	VIAS	ovine	wool	S1, FAINT	3	1	3	S	275
culture	CM82/5517	212	Wrigley FD1068	NSW	Carcoar	72	VIAS	ovine	wool	NB	0	nt	0	nt	276
culture	nr	214	Rampon902	France	France	nr	VIAS	caprine	nr	C1			3	C	277
culture	nr	215	Rampon504	France	France	nr	VIAS	caprine	nr	C1			3	C	278
culture	nr	216	8642	France	France	nr	VIAS	caprine	nr	C1			3	C	279
culture	nr	217	147089	France	France	nr	VIAS	caprine	nr	C13??	3	1	1	C	280
culture	nr	218	Grange17	France	France	nr	VIAS	caprine	nr	C1			3	C	281
culture	977487	219	Mystique12708	SA	SA	73	VIAS	camelid	hobby	C3			3	C	282
culture	955449	221	1447	VIC	Goulburn	44	VIAS	bovine	dairy	C3			3	C	283
culture	955664	222	NoID	VIC	n.r.	33	VIAS	bovine	dairy	NB	0	nt	0	nt	284
culture	973319	223	Y443	VIC	Central Highlands	60	VIAS	bovine	beef	C1			3	C	285
culture	973319	224	Y443	VIC	Central Highlands	60	VIAS	bovine	beef	C1			3	C	286
culture	973319	225	Y443	VIC	Central Highlands	60	VIAS	bovine	beef	C1			3	C	287
culture	973319	227	B926	VIC	Central Highlands	60	VIAS	bovine	beef	C1			3	C	288
culture	973319	228	B926	VIC	Central Highlands	60	VIAS	bovine	beef	C1			3	C	289
culture	973319	229	B920	VIC	Central Highlands	60	VIAS	bovine	beef	C1			3	C	290
culture	973319	230	B920	VIC	Central Highlands	60	VIAS	bovine	beef	C1			3	C	291
culture	973319	232	B923	VIC	Central Highlands	60	VIAS	bovine	beef	C1			3	C	292
culture	973319	233	B923	VIC	Central Highlands	60	VIAS	bovine	beef	C1			3	C	293
culture	973319	234	B923	VIC	Central Highlands	60	VIAS	bovine	beef	C1			3	C	294
culture	973319	235	B923	VIC	Central Highlands	60	VIAS	bovine	beef	C1			3	C	295
culture	972044	236	G13	VIC	Central Highlands	60	VIAS	bovine	beef	C1			3	C	296
culture	972044	238	P217	VIC	Central Highlands	60	VIAS	bovine	beef	C1			3	C	297
culture	972044	239	P21	VIC	Central Highlands	60	VIAS	bovine	beef	C1			3	C	298
culture	972044	240	W80	VIC	Central Highlands	60	VIAS	bovine	beef	C1			3	C	299
culture	972044	242	W21	VIC	Central Highlands	60	VIAS	bovine	beef	C1			3	C	300
culture	972044	244	P207	VIC	Central Highlands	60	VIAS	bovine	beef	C1			3	C	301
culture	972044	246	G118	VIC	Central Highlands	60	VIAS	bovine	beef	C1			3	C	302
culture	972044	247	P215	VIC	Central Highlands	60	VIAS	bovine	beef	C1			3	C	303
culture	972044	248	W24	VIC	Central Highlands	60	VIAS	bovine	beef	C1			3	C	304
culture	972044	257	Y443	VIC	Central Highlands	60	VIAS	bovine	beef	C1			3	C	305
culture	972044	259	B923	VIC	Central Highlands	60	VIAS	bovine	beef	C1			3	C	306
culture	972044	261	B926	VIC	Central Highlands	60	VIAS	bovine	beef	C1			3	C	307
culture	972044	262	Y42	VIC	Central Highlands	60	VIAS	bovine	beef	not Mptb	2	0			308
culture	WN 95 /4016	J183/5		NSW	Tweed Lismore	74	EMAI	bovine	dairy	C3			2	C	309
culture	CM 96 / 734	J332/5	177	NSW	Bega	75	EMAI	bovine	dairy	C3			2	C	310
culture	W 96 / 0529 F	J232/2	541	NSW	Casino	76	EMAI	bovine	dairy	C3			2	C	311

Sample	Accession	No.	Animal ID	Origin	Region	Property code	Lab	Species	Farm	RFLP	IS900	REA	IS1311	REA	Keylist
culture	CM 96 / 816	J348/4		NSW	Tweed Lismore	77	EMAI	bovine	beef	C3			1	C	312
culture	CM 96 /1048	J375/5		NSW	Moss Vale	78	EMAI	bovine	dairy	C3			1	C	313
culture	M96 / 3039	J244/2		NSW	Maitland	79	EMAI	bovine	dairy	C3			2	C	314
culture	CM 96 / 811	J346/4		NSW	Jerilderie	80	EMAI	bovine	dairy	C3			2	C	315
culture	CM 96 /181	Purnell	R 81	NSW	Tweed Lismore	81	EMAI	bovine	dairy	C3			2	C	316
culture	CM 96 /181	37	R 79	NSW	Tweed Lismore	81	EMAI	bovine	dairy	C3			2	C	317
culture	CM 96 /181	135	R 66	NSW	Tweed Lismore	81	EMAI	bovine	dairy	C3			2	C	318
culture	CM 96 /181	145	Y 76	NSW	Tweed Lismore	81	EMAI	bovine	dairy	C3			2	C	319
culture	CM 96 /181	115	Y 14	NSW	Tweed Lismore	81	EMAI	bovine	dairy	C3			2	C	320
culture	CM 96 / 111	J220/5		NSW	Bega	82	EMAI	bovine	dairy	C3			2	C	321
culture	CM 95 / 842	J842/10	N55	VIC	Wodonga	83	EMAI	bovine	beef	C12			2	C	322
culture	CM 95 / 5374	J178/1	244	NSW	Albury	84	EMAI	bovine	beef	C3			2	C	323
culture	CM 95 / 842	J842/13	M36	VIC	Wodonga	83	EMAI	bovine	beef	C12			2	C	324
culture	CM95/447	J48/1		NSW	Casino	85	EMAI	bovine	dairy	C3			2	C	325
culture	CM95?	Rhino 2498		NSW	bbo West.Plains Z	86	EMAI	Rhino	zoo	C5			3	C	326
culture	CM82/028	FD 1026		NSW	Moss Vale	87	EMAI	caprine	dairy	C3			3	C	327
culture	CM81/660	FD 1028		NSW	Moss Vale	88	EMAI	caprine	dairy	C3			3	C	328
culture	CM95/797	J16005		NSW	Albury	89	EMAI	bovine	beef	C3			3	C	329
culture	CM97/188	J48703	Y65	NSW	Tweed Lismore	81	EMAI	bovine	dairy	C3			3	C	330
culture	CM97/188	J48902	Y76	NSW	Tweed Lismore	81	EMAI	bovine	dairy	C3			3	C	331
culture	CM96/1138	J39901	Y29	NSW	Tweed Lismore	81	EMAI	bovine	dairy	C3			3	C	332
culture	CM96/566	J31405		NSW	Moss Vale	90	EMAI	bovine	dairy	C3			3	C	333
culture	CM97/0236	J49905		NSW	Tweed Lismore	91	EMAI	bovine	dairy	C3			3	C	334
culture	CM97/188	J49002	R72	NSW	Tweed Lismore	81	EMAI	bovine	dairy	C3			3	C	335
culture	CM96/1151	J39405		NSW	Moss Vale	92	EMAI	bovine	dairy	C3			2	C	336
culture	CM95?	McPAUL		NSW	Bega	93	EMAI	bovine	dairy	C3			2	C	337
culture	CM 97 / 452	J452079	G 3	NSW	Moss Vale	90	EMAI	bovine	beef	nt	3	1	3	C	338
culture	CM 97 / 1515	J64102		NSW	Bega	94	EMAI	bovine	dairy	nt	3	1	3	C	339
culture	CM 97 / 1043	W85		NSW	Tweed Lismore	95	EMAI	bovine	dairy	nt	3	1	3	C	340
culture	CM 97 / 0818	J56003	505	NSW	Maitland	96	EMAI	bovine	dairy	nt	3	1	3	C	341
culture	CM 97 / 958	J57503	333	NSW	Tweed Lismore	95	EMAI	bovine	dairy	nt	3	1	3	C	342
culture	CM 97 / 1043	W97		NSW	Tweed Lismore	95	EMAI	bovine	dairy	nt	3	1	3	C	343
culture	CM 97 / 0648	J54402		NSW	Bega	97	EMAI	bovine	dairy	nt	3	1	3	C	344
culture	CM 97 / 0897	J57105	C 15	NSW	Molong	98	EMAI	bovine	beef	nt	3	1	3	C	345
culture	CM 97 / 1043	W69		NSW	Tweed Lismore	95	EMAI	bovine	dairy	nt	3	1	3	C	346
culture	CM 97 / 1043	W57		NSW	Tweed Lismore	95	EMAI	bovine	dairy	nt	3	1	3	C	347
culture	CM 97 / 0818	J56004	548	NSW	Maitland	96	EMAI	bovine	dairy	nt	3	1	3	C	348
culture	CM 97 / 1043	W59		NSW	Tweed Lismore	95	EMAI	bovine	dairy	nt	3	1	3	C	349
culture	CM 97 / 1043	W52		NSW	Tweed Lismore	95	EMAI	bovine	dairy	nt	3	1	3	C	350
culture	CM 97 / 1043	W26		NSW	Tweed Lismore	95	EMAI	bovine	dairy	nt	3	1	3	C	351
culture	CM 97 / 1043	W103		NSW	Tweed Lismore	95	EMAI	bovine	dairy	nt	3	1	3	C	352
culture	CM 97 / 0844	J56402		NSW	Albury	99	EMAI	bovine	beef	nt	3	1	3	C	353
culture	CM 97 / 1228	J62004		NSW	Deniliquin	100	EMAI	bovine	dairy	nt	3	1	3	C	354
culture	CM 97 / 0818	J56005	67	NSW	Maitland	96	EMAI	bovine	dairy	nt	3	1	3	C	355
culture	CM97/0982	J58503	170	NSW	Albury	101	EMAI	bovine	beef	nt	3	1	3	C	356

APPENDIX 6

Table A6.1. Summary of insertion sequence discovery and intergene PCR typing experiments

EXPT	Name	Primers	DNA	Result (approx size in bp)	Comment
BACTEC-PCR EXPT 20	65K HSP M.tb	TB13, 65-1400	316V MAC	-ve -ve	
	IS1245-IS1311	PA, PB	316V MAC	-ve -ve	
	16SrRNA	MYCOGEN - F, R	316V MAC	1100 1100	
	65K HSP M.tb	TB11, TB12	316V MAC	500 500	
	IS1311 M.av	DD2, DD3	316V MAC	190 190	
	IS1245 M.av	P1, P2	316V MAC	-ve 400	weak
	65K HSP M.tb	M1, M2	316V MAC	-ve -ve	
T1 (about 10 ng DNA per reaction)	IS900-16S	m29, m30	316V McPaul 96/60-1 ex gut 96/60-1 ex BT	404, 320 404, 320 320 -ve	
	IS900-16S	m29, m31	316V McPaul 96/60-1 ex gut 96/60-1 ex BT	900, 404, 320 404, 320, 242 900, 320 -ve	
	16S-IS900	m32, m27	316V McPaul 96/60-1 ex gut 96/60-1 ex BT	320, 147 320, 147 320, 147 320, 147	v. strong v. strong strong strong
	16S-IS900	m32, m28	316V McPaul 96/60-1 ex gut 96/60-1 ex BT	many 404-100 many 404-100 404 404	
	IS900-HSP	m29, m33	316V McPaul 96/60-1 ex gut 96/60-1 ex BT	many 320-100 many 320-100 501, 320 -ve	weak
	HSP-IS900	m34, m27	316V McPaul 96/60-1 ex gut 96/60-1 ex BT	242 242 -ve 242	weak

EXPT	Name	Primers	DNA	Result (approx size in bp)	Comment
	HSP-IS900	m34, m28	316V McPaul 96/60-1 ex gut 96/60-1 ex BT	-ve 404, 320 -ve -ve	weak
	IS900-IS900	m29, m27	316V McPaul 96/60-1 ex gut 96/60-1 ex BT	190 190 -ve -ve	strong strong
	IS900-IS900	m29, m28	316V McPaul 96/60-1 ex gut 96/60-1 ex BT	320, 67 320, 67 320, 67 -ve	strong strong less strong
	HSP-16S	m34, m30	316V McPaul 96/60-1 ex gut 96/60-1 ex BT	-ve -ve -ve -ve	
	HSP-16S	m34, m31	316V McPaul 96/60-1 ex gut 96/60-1 ex BT	-ve -ve -ve -ve	
	16S-HSP	m32, m33	316V McPaul 96/60-1 ex gut 96/60-1 ex BT	-ve smear -ve -ve	
T2	IS900-16S	m29, m31	316V McPaul 96/60-1 ex gut 96/60-1 ex BT	-ve 404, 320 -ve -ve	weak
	16S-IS900	m32, m28	316V McPaul 96/60-1 ex gut 96/60-1 ex BT	many 404-100 many 404-100 404 -ve	
	IS900-HSP	m29, m33	316V McPaul 96/60-1 ex gut 96/60-1 ex BT	692, 501, 320-100 550, 320-100 570 -ve	weak
T3	vary Mg, buffers	m29, m31	316V McPaul 96/60-1 ex gut 96/60-1 ex BT	responded to higher Mg with more bands; glycerol, DMSO no good	

EXPT	Name	Primers	DNA	Result (approx size in bp)	Comment
T4	vary dNTP and primer inputs	m29, m31 m32, m28 m29, m33	316V, 96-60-1 ex gut	no effect higher signal little signal anyway	
T5	vary target DNA conc.	m29, m31 m32, m28 m29, m33	316V McPaul 96/60-1 ex gut	poor signal for all responsive to DNA responsive to DNA	banding pattern depends on DNA conc.
T6	Annealing temp	m29, m31 m32, m28 m29, m33	316V 96/60-1	poor signal for all responsive responsive	
T7	IS900-IS1311	m29, m50 m29, m51 m29, m52 m28, m51 m53, m52 m28, m52 m27, m52 m51, m53 m27, m51 m27, m50 (F) m28, m50 (F) m50, m53 (F)	316V	190 -ve 242 500 147+ 489, 242 404 smear 489 320, 242 404 242	 weak weak weak weak
T8	IS900-HSP	m29, m33 m29, m34 (F) m34, m28 m33, m28 (F) m34, m53 m33, m53 (F) m34, m27 m33, m27 (F)	316V	-ve -ve -ve -ve -ve -ve 242 242	
	IS900-16S	m29, m30 m29, m32 (F) m29, m31 m32, m27 m30, m27 (F) m31 m27 (F) m32, m28 m30, m28 (F) m31, m28 (F) m32, m53 m30, m53 (F) m31, m53 (F)	316V	-ve -ve -ve -ve -ve -ve -ve -ve -ve -ve -ve -ve	

EXPT	Name	Primers	DNA	Result (approx size in bp)	Comment
T9	IS1311	m24, m23 m54, m55 m56, m57 m24, m55 m24, m57 m54, m23 m54, m57 m56, m23 m56, m55	316V	200 850 >900 270 500 692 900 692 850	180 (expected) 968 1259 228 468 909 1197 971 1019
T10 (5 ng DNA per reaction)	IS900-IS1311	m29, m50 m29, m52 (F) m28, m51 m50, m53 (F)	316V McPaul 96/60-1 96/2	190, <124 (w) 242, <124 (w) 550, (404) (242)	 404 in cattle 242 in cattle
T11	IS900-HSP	m34, m27 m33, m27 m34, m49 m33, m49	316V	-ve -ve -ve -ve	
	IS900-16S	m32, m49 m30, m49 m31, m49	316V	242 -ve -ve	
	IS900-23S	m47, m49 m47, m28 m47, m53 m47, m27 m47, m29(F) m48, m29(F) m48, m49 m48, m28 m48, m53 m48, m27	316V	>900 >900 >900 >900 -ve -ve >900 -ve >900 >900	
T12 (5ng and 0.5 ng DNA per reaction)	IS900-IS1311	m28, m51 m50, m53(F)	316V McPaul 96/60-1 96/2 96/60-3 316V McPaul 96/60-1 96/2 96/60-3	550, 500 550, 500 550 550 550 242 242 -ve -ve 242	500 not present at lower DNA weak weak weak

EXPT	Name	Primers	DNA	Result (approx size in bp)	Comment
T13 (5 ng DNA per reaction)	IS900-IS1311	m28, m51 m50, m53	316V McPaul 96/60-1 96/2 96/60-3 316V 96/60-1	similar bands cattle and sheep	
	IS900-23S	m48, m49		sheep band slightly heavier than cattle band (no. Stds.)	Cattle bands stronger than sheep bands
	IS900-HSP	m34, m27		-ve	
	IS900-16S	m32, m49		similar bands cattle and sheep	Cattle bands stronger than sheep bands
T15 LD (250 ng DNA per reaction)	IS900-IS1311	m28, m51	316V McPaul 96/60-1 96/60-3	8 kb 8 kb 8 kb -ve	very strong bands
		m50, m53	316V McPaul 96/60-1 96/60-3	-ve -ve -ve -ve	
T16 LD (50-0.5 ng DNA per reaction)	IS900-IS1311	m 28, m51	316V McPaul 96/60-1 96/60-3	7, 2(w), 1(w) 7, 2(w), 1(w) 7, 2(w), 1(w) 7	DNA conc dependent
T21 LD (20 ng DNA)	IS900-HSP	m29, m33 m34, m49 m33, m49(F) m29, m34(F)	316V	-ve -ve -ve -ve	(std XV only so size estimates are very poor in T21)
	IS900-23S	m48, m49 m29, m48(F)		?4 -ve	
	IS900-16S	m29, m30 m32, m49 m29, m32(F) m30, m49(F)		-ve ?12(w), 10, 9, ?1, ?0.3 -ve -ve	
	IS900-IS900	m28, m29		smear>10, ?0.3	
	IS1311-IS1311	m50, m51		?6, ?5, ?0.9	
	IS1311-16S	m51, m30 m32, m50 m30, m50(F) m32, m51(F)		-ve ?0.9, ?0.3 ?0.9 ?0.5	
	IS1311-23S	m48, m50 m51, m48(F)		?0.9 Smear>9, ?1	

EXPT	Name	Primers	DNA	Result (approx size in bp)	Comment
	IS1311-HSP	m51, m33 m34, m50 m51, m34(F) m33, m50(F)		-ve -ve ?0.3 -ve	
T22	IS1311-IS1311	m50, m51	316V 96/2	-ve	
	IS1311-16S	m51, m30 m32, m50 m30, m50(F) m32, m51(F)		-ve -ve -ve -ve	
	IS1311-23S	m48, m50 m51, m48(F)		-ve -ve	
	IS1311-HSP	m51, m33 m34, m50 m51, m34(F) m33, m50(F)		-ve -ve -ve -ve	
	IS900-IS1311	m28, m51 m50, m53(F)		600 240 (w)	sheep weak sheep -ve
T23 (1:10 DNA, 0.4 taq, 2 min extension)	IS900-IS900	m27, m29	36 DNA samples from WA	190 6/10 bovine +ve 3/3 alpaca +ve 3/3 caprine +ve 1/1 rhino +ve 2/13 ovine +ve 2/2 rabbit UK +ve 3/4 ref +ve	many +ves were weak
T24 LD (20 ng DNA)	IS900-23S	m48, m49	316V 90/60-1	?3 kb ?3 kb	
	IS900-16S	m32, m49	316V 90/60-1	?15(w), ?12(w), 9, 7(w), 1.2, 0.9, 0.32 kb 9, 1.0(w), 0.9(w), 0.7(w), 0.3	
	IS900-IS900	m28, m29	316V 90/60-1	?4, 0.24 kb 0.24 kb	all strong bands
	IS1311-IS1311	m50, m51	316V 90/60-1	?8(w),?4, 1.1, 0.9, 0.4(w), 0.32(w) smear, ?4(w), others(w)	
	IS1311-16S	m32, m50	316V 90/60-1	?3(w), 0.4(w), 0.3 0.3(w)	

EXPT	Name	Primers	DNA	Result (approx size in bp)	Comment
	IS1311-16S	m30, m50(F)	316V 90/60-1	-ve -ve	
	IS1311-16S	m32, m51(F)	316V 90/60-1	0.9(w), 0.5, 0.3(w) 0.9(w), 0.5(w), 0.3(w)	
	IS1311-23S	m48, m50	316V 90/60-1	0.9(w) 0.5(w)	
	IS1311-23S	m51, m48(F)	316V 90/60-1	1.2(w) 0.5(w)	
	IS900-IS900	m27, m29	316V 90/60-1	?15(w), ?12, 10, 9(w), ?3, 1.1(w), 0.6, 0.24 1.1(w)	m27 primer incorrect conc.
T25 (neat DAN, 0.2 taq, 1 min extension)	IS900-IS900	m27, m29	66 DNA samples WA	190 bp 18/26 bovine +ve 4/5 alpaca +ve 1/3 caprine +ve 1/1 rhino +ve 5/13 ovine +ve 5/6 rabbit UK +ve 5/6 ref +ve	many reactions weak or equivocal

316V bovine strain
McPaul bovine strain
96/60-1 ovine strain
w weak reaction

APPENDIX 7

Paper in press - Molecular and Cellular Probes

Polymorphisms in IS*1311*, an insertion sequence common to *Mycobacterium avium* and *M.avium* subsp. *paratuberculosis*, can be used to distinguish between and within these species

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IS*1311* is an insertion sequence from *Mycobacterium avium* and *M. avium* subsp. paratuberculosis. Using a 180 bp fragment of IS*1311* as a probe, 7-10 copies of IS*1311* were revealed in strains of *M. avium* subsp. *paratuberculosis*. With a given restriction enzyme, the restriction fragment length polymorphism patterns obtained from isolates of *M. avium* subsp. *paratuberculosis* from cattle were all identical, but they differed from those of isolates from sheep, which could be separated into 2 types. A 1259 bp fragment of IS*1311* produced by polymerase chain reaction (PCR) from two isolates of *M. avium* subsp. *paratuberculosis* from cattle and two isolates from sheep was sequenced and compared to the sequence known from *M. avium*. Apart from five point differences the sequences were identical. Restriction endonuclease analysis (REA) of the PCR product was used to distinguish isolates of *M. avium* subsp. *paratuberculosis* from *M. avium*, in addition to the conventional test for IS*900*. In isolates of *M. avium* subsp. *paratuberculosis* from cattle the IS*1311* gene was polymorphic at position 223, which enabled isolates from sheep and cattle to be distinguished by PCR-REA. These simple tests will be used to enhance disease control programs for Johne's disease in ruminants. The findings of this study raise interesting questions about the evolution of *M. avium* subsp. *paratuberculosis*.

INTRODUCTION

Insertion sequences have been identified in many mycobacterial species[1,2]. *Mycobacterium avium* subsp. *paratuberculosis*, the causative agent of Johne's disease of ruminants, is apparently unique in its possession of IS*900*. Detection of this element by polymerase chain reaction (PCR) is now considered to be the definitive test for the identification of this organism. As about 15-18 copies of the gene are present in each genome, PCR assays for IS*900* are highly sensitive and can be used to simultaneously detect and identify *M. avium* subsp. *paratuberculosis* in primary cultures during early stages of growth[3,4]. However, for epidemiological purposes it is also desirable to differentiate among isolates of *M. avium* subsp. *paratuberculosis*. For example, isolates of *M. avium* subsp. *paratuberculosis* from sheep have different biological and epidemiological properties compared to isolates from cattle. Currently sheep and cattle isolates can be distinguished only by restriction fragment length polymorphism (RFLP) analysis using IS*900* probes[5]. This method is complex, expensive, requires large amounts of genomic DNA and therefore can be undertaken only after large-scale culture of isolates, which is itself costly and very time consuming. Simpler and cheaper methods are therefore being sought.

Several insertion sequences are known from *M. avium*. These include elements related to IS*900*: IS*901*[6], IS*902*[7] and IS*1110*[8]. Two members of the IS*256* family of insertion sequences, IS1245[9] and IS*1311* (GenBank accession number U16276) are also known from *M. avium*. This paper is concerned with IS*1311*. IS*1245* and IS*1311* share 85% sequence homology and cross hybridize[10]. Up to 27 copies of IS*1245* were found in *M. avium* [9] and this element was detected by PCR and Southern hybridization in the only isolate of *M. avium* subsp. *paratuberculosis* included in that study. IS*1245* and IS*1311* coexist in isolates of *M. avium*[11]. In a recent study IS*1311* but not IS*1245* were identified in 10 isolates of *M. avium* subsp. *paratuberculosis* by PCR [12].

During studies to find a rapid means of differentiating among strains of *M. avium* subsp. *paratuberculosis* we sought evidence for the presence of IS*1245* and IS*1311* in this species. We confirm the widespread occurrence of IS*1311* among isolates of *M. avium* subsp. *paratuberculosis* and polymorphisms in the gene within the subspecies and between it and *M. avium*. The existence of these apparently stable polymorphisms permits the rapid differentiation of isolates of *M. avium* and *M. avium* subsp. *paratuberculosis* from different ruminant hosts by PCR and restriction endonuclease analysis.

MATERIALS AND METHODS

Bacterial isolates, culture and DNA extraction
A reference strain of *M. avium* subsp. *paratuberculosis*, and other isolates of the organism from cattle, sheep, goats, alpaca, and rhinoceros represented a wide range of geographic locations including Australia, New Zealand, South Africa and Canada (Table 1). Field isolates were obtained on Herrold's egg yolk medium (HEYM) or in modified BACTEC 12B medium from cases of Johne's disease. Most isolates were then subcultured on up to 6 HEYM slopes, or grown in 75 mL of modified Middlebrook 7H9 medium in a 150 cm^2 tissue culture flask [13]. Ovine strains that failed to grow on HEYM or in the liquid medium were inoculated into 4 BACTEC12B (Becton Dickinson, Sparks, MD, USA) vials without egg yolk, and incubated until the growth index approached 999. In addition, isolates 316V and McPaul were grown in Watson Reid medium and several strains from sheep were purified directly from the intestinal mucosa[14]. Bacteria were washed in sterile water and the organisms were heat treated at 75°C for 40 min. DNA was then prepared from 50 - 500 mg of cells as described[14,15]. A reference collection of other mycobacterial species was also used (Table 1). These were cultured on Lowenstein-Jensen agar at 37°C (except *M. marinum* - 22°C). A crude suspension of DNA for use in PCR was obtained from these species by suspending a colony in distilled water, washing 3 times, suspending the cells in 100 μl water and boiling the washed cells for 20 mins.

Polymerase chain reaction (PCR) and restriction endonuclease analysis of PCR product Primer sequences are given in Table 2. A reaction volume of 50 μl containing 5 μl of the DNA sample, 250 ng of each primer, 200 μM of each of the nucleotides dATP, dTTP, dGTP and dCTP, 66.8 mM Tris–HCl, 16.6 mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 1.65 mg/ml bovine serum albumin, 10 mM beta–mercaptoethanol, 2 U *Taq* polymerase, in buffer (10 mM Tris–HCl, 0.1 mM EDTA, pH 8.8) was used. Amplification was undertaken in 200 μl tubes in a 96–place thermal cycler (Corbett Research, Sydney, Australia) using the following conditions: one cycle of denaturation at 94°C for 2 min followed by 37 cycles of denaturation at 94°C for 30 sec, annealing at 62°C for 15 sec and extension at 72°C for 1 min. PCR amplification products were evaluated by electrophoresis at 90V in 2% agarose gels stained with ethidium bromide, using appropriate size markers (Boehringer Mannheim, GmbH, Mannheim, Germany). PCR product (6 μl) and 2 U of one of the restriction enzymes *Alw* I, *Bsr* DI, *Fnu* 4HI, *Hinf* I, *Bst* XI, *Drd* I, *Mse* I, *Tse* I or *Hinc* II (New England Biolabs Inc., Beverly, MA, USA) were included in digestion reactions according to the manufacturer's recommendations. Reactions were assessed by electrophoresis in 2-4% agarose gels stained with ethidium bromide.

Restriction fragment length polymorphism analysis
Four enzymes were tested for their ability to detect polymorphisms due to IS*1311* in *M. avium* subsp. *paratuberculosis* isolates from a variety of animal species and geographic regions (Table 1). Approximately 2 μg of extracted DNA was digested overnight with *Pvu* II, *Bst* EII, *Bam* HI or *Pst* I (Boehringer Mannheim) and subjected to electrophoresis at 45V for 16 hours in 1% agarose in a refrigerated (14°C) buffer recirculation system. DNA was transferred to positively charged nylon membrane (Boehringer Mannheim) using 0.4 M NaOH for 1 hr at 40 mm pressure on a variable vacuum pump blotting apparatus (Pharmacia Biotech, Uppsala, Sweden). A 180 bp IS*1311* probe was prepared by PCR of *M. avium* subsp. *paratuberculosis* 316V using primers DD2/DD3 and labelled with digoxigenin using a random primed hexamer labelling kit (Boehringer Mannheim). The nucleotide sequence of the probe was determined and found to be 100% homologous to the relevant region of IS*1311* from *M. avium*. Membranes were hybridised at 68°C overnight with 25 $ng.ml^{-1}$ labelled probe. Post hybridisation washes were carried out in 50 ml of 2 x SSC (0.3 M NaCl, 30 mM Tri-sodium citrate) containing 0.1% SDS for 2 X 5 min at room temp and 50 ml of either 0.1 or 0.5 x SSC (75 mM NaCl, 7.5 mM Tri-sodium citrate) containing 0.1% SDS for 2 x 15 min at 68°C. The membrane was then

developed with a commercial digoxigenin chemiluminescent kit (Boehringer Mannheim) and exposed to X-ray film for 1-3 hrs.

DNA sequencing
PCR amplification of the IS*1311* gene of four isolates of *M. avium* subsp. *paratuberculosis* (316V, McPaul, 96/60-1 and 97/1-2) was undertaken using primers M56 and M57 to produce 1259 bp products which were purified using 6 M guanidine thiocyanate and silica columns according to the manufacturer's instructions (Wizard PCR Preps DNA Purification System; Promega Corporation, Madison, USA) with elution of purified DNA in 50 μl of sterile distilled water. Dye terminator sequencing reactions were undertaken with 300 ng DNA and 4.8 pmol of primer using Terminator Ready Reaction Mix on a robotic workstation (Catalyst 800 Molecular Biology Labstation, Perkin Elmer Applied Biosystems, Foster City, CA, USA) and were analyzed using a 377 DNA Sequencer (Perkin Elmer). The forward and reverse strands of the M56/M57 PCR product of each isolate were sequenced using primers M56,M62,M74 and M57, M63, M75, respectively (Fig. 1). Sequences were edited and aligned using the program ESEE (Eric Cabot, University of Rochester, NY).

RESULTS

PCR amplification of IS*900*, IS*1245* and IS*1311*
The reference mycobacterial isolates *M. avium* TMC 715 and *M. avium* subsp. *paratuberculosis* 316V were evaluated initially in PCR for the presence of the IS*900*, IS*1245* and IS*1311* genes using primers P90/P91, P1/P2 and DD2/DD3, respectively. Based on the finding of PCR products of predicted size (Table 2), IS*900* was present in *M. avium* subsp. *paratuberculosis* only, IS*1245* was present in *M. avium* only, while IS*1311* was present in both species (data not shown). IS*1245* was not evaluated further.

PCR products of the predicted size were obtained subsequently for both IS*900* and IS*1311* from each *M. avium* subsp. *paratuberculosis* isolate in Table 1, confirming that both insertion sequences are present in *M. avium* subsp. *paratuberculosis* (Fig. 2).

Selected isolates of *M. avium* subsp. *paratuberculosis* were also evaluated by PCR using primers M56/M57, which encompass nearly the full length gene, and combinations of internal primers from IS*1311* with positive results for each organism in each reaction, suggesting a high degree of sequence homology of the gene in *M. avium* subsp. *paratuberculosis* with that in *M. avium*. All the other mycobacterial species in Table 1 were analysed by PCR using primers M56/M57 and none yielded products, suggesting that the full length gene was absent from species other than *M. avium* subsp. *paratuberculosis* and *M. avium*. However, products were obtained from some species using primers DD2/DD3. *M. chitae, M. microti, M. neoaurum, M. thermoresistable* and *M. tuberculosis* yielded strong products of the predicted size while *M. flavescens* and *M. marinum* yielded non-specific products of approximately 900 bp and 600 bp, respectively (Fig. 3). These data indicate that elements related to an internal region of IS*1311* exist in species other than *M. avium* subsp. *paratuberculosis* and *M. avium*.

Restriction fragment length polymorphism analysis
Between 7 and 10 copies of IS*1311* were found in *M. avium* subsp. *paratuberculosis*. Up to four RFLP patterns were found with each enzyme among the isolates listed in Table 1. The lowest degree of diversity was obtained with *Pvu* II (2 patterns); *Bst* EII, *Bam* H1 and *Pst* I each produced 4 patterns (Fig. 4, Table 1). With any of the latter enzymes, isolates of *M. avium* subsp. *paratuberculosis* from cattle all had the same pattern, designated 01, while those from sheep had 1 or 2 different patterns, designated 02 or 04. The 04 pattern was found only in the ovine isolate from South Africa. Isolates from a goat and an alpaca had the 01 pattern, while an isolate from a rhinoceros had a distinct pattern, designated 03.

DNA sequence of IS*1311*
Complete sequence (EMBL accession numbers AJ223974, AJ223975) was obtained for the four isolates of *M. avium* subsp. *paratuberculosis* for the positions 23-1241 of the *M. avium* IS*1311* gene, numbered according to the deposited sequence (GenBank accession number U16276). There was almost complete homology between

the IS*1311* sequence for *M. avium* and the sequences determined for the IS*1311* gene of each of the four isolates of *M. avium* subsp. *paratuberculosis*. Five differences in individual bases were observed (Table 3). Interestingly, four of the five differences involved changes from T to C in the *M. avium* subsp. *paratuberculosis* isolates while the last difference was a change from G to A in *M. avium* subsp. *paratuberculosis*. Both isolates of *M. avium* subsp. *paratuberculosis* that were derived from cattle had a C/T polymorphism at bp 223, while both sheep isolates and *M. avium* subsp. *paratuberculosis* were homologous for C at this location.

The DD2/DD3 PCR products obtained from *M. tuberculosis* and *M. thermoresistable* were also sequenced (EMBL accession numbers AJ223976, AJ223977). The sequence of the 142 bp *M. tuberculosis* product (i.e. without the primer sequences) had 58% homology with IS*1311* and 100% homology with cosmid Y77 of *M. tuberculosis* (EMBL accesion number MTCY77; coding position 3300, minus strand). The sequence of the 142 bp *M. thermoresistable* product (also without the primer sequences) had 56% homology with IS*1311* and 86% homology with the same segment of cosmid Y77 of *M. tuberculosis*. The flanking regions of this sequence in cosmid Y77 had little homology with the primers DD2/DD3.

PCR-restriction endonuclease analysis

A 909 bp PCR product was obtained from the *M. avium* subsp. *paratuberculosis* isolates 316V, McPaul, 96/60-1 and 97/2-1 using primers DD2 and M56. Restriction enzymes were selected for specific recognition of the sites of each of the sequence differences described above, and where suitable enzymes existed, an attempt was made to separately confirm the sequence at these positions for *M. avium* TMC 715 and *M.avium* subsp. *paratuberculosis*. The activity of the enzymes was confirmed by restriction of lambda DNA (data not shown). Using *Alw* I, a T was shown to be present at position 68 in *M. avium* TMC 715 but because the 93 bp band was not completely digested a C/T polymorphism was suspected at this position. A T was absent in *M.avium* subsp. *paratuberculosis* at position 68 (Fig. 5a). The C/T polymorphism at position 223 in both bovine isolates of *M. avium* subsp. *paratuberculosis* (316V and McPaul) was confirmed using *Hinf* I (Fig. 5b). The T at position 236 could not be shown in *M. avium* TMC 715 with *Bst* XI or *Bsr* DI suggesting that this strain may possess a C at this position. At position 422, the C in *M.avium* subsp. *paratuberculosis* and a T in *M.avium* TMC 715 were confirmed with *Drd* I (Fig. 5c) and *Mse* I (Fig. 5d), respectively. At position 527, a G in *M.avium* was confirmed with both *Tse* I (Fig. 5e) and *Fnu* 4HI (Fig. 5f). The C at position 628 in *M.avium* subsp. *paratuberculosis* was confirmed also with *Fnu* 4HI (Fig. 5f), but a T at this position could not be confirmed in *M.avium* TMC 715 using *Hinc* II. Note that small restriction fragments are not resolved in Fig. 5.

As the polymorphism at position 223 was of particular interest, a range of isolates of *M. avium* subsp. *paratuberculosis* from sheep, cattle, alpaca, goat and rhinoceros were subjected to PCR with primers M56 and DD2 and restriction endonuclease analysis with *Hinf* I (Fig. 6). The predicted band sizes after restriction were 379, 287 and 243 bp for isolates without the polymorphism and 379, 287, 243, 220 and 67 bp for isolates with the polymorphism. The polymorphism was absent in all of the isolates from sheep but present in all of the isolates from the other species. These results perfectly matched those of *Bst* EII IS*900* RFLP analysis where isolates from sheep were of ovine type while those from the other species were of bovine type (Table 1).

DISCUSSION

This study has confirmed that IS*1311* occurs in *M. avium* subsp. *paratuberculosis* as well as in *M. avium* and extends the findings of a recent study in New Zealand[12]. All isolates of *M. avium* subsp. *paratuberculosis* examined possessed both IS*900* and IS*1311*. There were 7 - 10 copies of IS*1311* in the isolates examined by RFLP with an IS*1311* probe, compared with 15-18 copies of IS*900*.

Five point mutations were identified in the sequence for IS*1311* between *M. avium* subsp. *paratuberculosis* and the known sequence for *M. avium*, as well as an additional point mutation (position 223) in isolates of *M. avium* subsp. *paratuberculosis* from cattle that was not present in isolates from sheep. The mutation at bp 223 was discovered only after careful manual examination of the electropherograms derived from sequencing reaction analyses where it appeared as two overlying peaks in an otherwise clean reaction. This indicated a true polymorphism where not all copies of IS*1311* in the organism possessed the mutation. We have

yet to determine whether the number of copies of the mutant IS*1311* is constant between isolates of *M. avium* subsp. *paratuberculosis* from cattle. If it was shown to vary, for example by densitometric analysis of restriction digests, there may be additional useful epidemiological information to be gained.

The deposited sequence for IS*1311* of *M. avium* (GenBank accession number U16276) is from an unspecified isolate and it possible that it may be different to the reference isolate *M. avium* TMC 715 used in this study for PCR-RE analysis. Thus perhaps it was not surprising that 2 of the 5 sequence differences between the deposited *M. avium* sequence and the sequence determined for *M. avium* subsp. *paratuberculosis* in this study (positions 236, 628) were not confirmed by PCR-RE analysis of *M. avium* TMC 715. It remains to be determined whether the other differences are highly conserved within *M. avium*. If they are, PCR-RE targeting these sites could be used to routinely distinguish between *M. avium* and *M. avium* subsp. *paratuberculosis*. As IS*1311* was present in numerous copies in each genome it is an appealing target for PCR-RE assays to distinguish *M. avium* from *M. avium* subsp. *paratuberculosis*. PCR-RE assays for this purpose that are based on IS*1311* are likely to be considerably more sensitive than those based on single-copy genes such as rRNA [16] or *hsp65*[17], and should have sensitivity similar to that of IS*900*-based PCR assays, but this requires experimental confirmation. Amplification of the 5' region of IS*1311* with primers M56 and DD2 appeared to be specific as only *M. avium* subsp. *paratuberculosis* and *M. avium* from a collection of 24 mycobacterial species yielded a PCR product under the conditions used.

The close homology of the sequence for each of the *M. avium* subsp. *paratuberculosis* isolates with that of the deposited *M. avium* sequence suggests a very low rate of genetic drift in the IS*1311* element in both organisms. It was particularly remarkable that the divergence in sequence at a single site in only some copies of IS*1311* between isolates of *M. avium* subsp. *paratuberculosis* from cattle and sheep was highly stable, and was conserved across isolates from different geographic regions. These results are consistent with other genomic data that suggest that *M. avium* subsp. *paratuberculosis* tend to be clonal and biologically isolated[16]. As the sequence at position 223 in sheep isolates was identical to that from *M. avium*, it is possible that isolates of *M. avium* subsp. *paratuberculosis* from cattle diverged from *M. avium* more recently than did those from sheep. However, there were five other differences in IS*1311* between *M. avium* and *M. avium* subsp. *paratuberculosis* and these were common to both sheep and cattle strains, suggesting that an intermediate strain was involved in the evolutionary process. The sheep strain may have been the only intermediate strain, that is the cattle strain may have evolved from a sheep strain. Regardless of the mechanism, the cattle and sheep strains appear to have remained biologically isolated.

Elements closely related to IS*1311* occur in mycobacteria other than members of the *M. avium* complex. Although we could not find full length IS*1311* elements in species other than *M. avium* subsp. *paratuberculosis* and *M. avium*, parts of a related gene were certainly present in other species. Sequence obtained from the DD2/DD3 PCR product from *M. tuberculosis* NCTC 7416 had 58% homology with IS*1311* and 100% homology with a sequence from *M. tuberculosis* known to have some homology with an *M. avium* transposase (cosmid Y77, EMBL MTCY77). However, the flanking regions in cosmid Y77 had little homology with the IS*1311* primers we used so the mechanism of amplification is unknown. Given the high stringency of the PCR reactions used in this study we suspect that *M. tuberculosis* has an element other than the one known from cosmid Y77 that more closely resembles IS*1311*, and that it was from this proposed element that the PCR product was obtained. The DD2/DD3 PCR product from *M. thermoresistable* also had significant (86%) homology with the same region of cosmid Y77, and 56% homology with IS*1311*. Our data suggest that related sequences exist in *M. chitae*, *M. microti* and *M. neoaurum*. Guerrero et al [9] used PCR analysis to show that IS*1245* was confined to *M. avium* and related organisms within the mycobacteria, but noted that it had significant homologies with IS*1081* from *M. bovis*. Given the close sequence relationship of IS*1245* and IS*1311* and the wide distribution of other elements in the IS*256* family[18], the results we obtained on the distribution of putative IS*1311*-like elements are not unexpected.

Johne's disease in cattle and sheep has different clinical, pathological and epidemiological features, and even where the two species of ruminant graze together, transfer of the infection between them is believed to be very rare. The reasons for this restriction are unknown, but are reflected at the genome level by differences in IS*900* RFLP patterns [5,19,20]. The results of IS*900* RFLP were supported here by IS*1311* RFLP patterns and PCR

restriction endonuclease analysis at position 223 of IS*1311*.

Using RFLP with IS*1311* as a probe it was possible to differentiate ovine and bovine strains of *M. avium* subsp. *paratuberculosis* in the same manner as the *Bst* EII IS*900* RFLP method of Collins et al[5]. However, in the same group of isolates, IS*900* RFLP resulted in approximately twice as many different types as IS*1311* RFLP (Cousins, unpublished). This is most likely due to the higher copy number of IS*900*. It is unlikely that the IS*1311* probe would provide any additional information in epidemiological studies over that provided by IS*900* RFLP, a conclusion reached also in an independent study[12].

The results of this study provide the theoretical basis and practical means for a rapid PCR-based test to distinguish between isolates of *M. avium* subsp. *paratuberculosis* from sheep and cattle. Such a test will have immediate application in Johne's disease control and eradication programmes. In addition, a new approach to distinguish *M. avium* subsp. *paratuberculosis* from *M. avium* and other mycobacteria, based on presence of and sequence differences within the IS*1311* gene may be a useful alternative to analysis for the IS*900* gene.

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Captions to figs.

Fig. 1. Sequencing strategy for IS*1311*. A 1259bp PCR product generated with primers M56 and M57 was sequenced with primers M56, M62 and M74 in the forward direction and with M57, M63 and M75 in the reverse direction. Overlapping fragments were aligned, complementarity was confirmed between forward and reverse strands and M56 and M57 primer sequences were removed to yield a 1219 bp consensus sequence for each isolate.

Fig. 2. PCR analysis of *M. avium* subsp. *paratuberculosis* isolates using primers P90/P91 for IS*900* (Panel A) and primers DD2/DD2 for IS*1311* (Panel B). Lanes at left, molecular size markers; lanes 1-18 correspond to isolates shown in Table 1; +ve and -ve, PCR controls.

Fig. 3. PCR analysis of mycobacterial species for IS*1311* using primers DD2/DD3. Data are shown only for those mycobacterial species in Table 1 that yielded a PCR product. Lane 1, molecular size markers; lane 2, *M. avium*; lane 3, *M. chitae*; lane 4, *M. flavescens*; lane 5, *M. marinum*; lane 6, *M. microti*; lane 7, *M. neoaurum*; lane 8, *M. thermoresistable*; lane 9, *M. tuberculosis*; lane 10, *M. avium* subsp. *paratuberculosis* 316V.

Fig. 4. IS*1311* restriction fragment length polymorphism types of *M. avium* subsp. *paratuberculosis.* The distribution of types amoung isolates is given in Table 1. Genomic DNA was digested with restriction enzyme and probed with digoxigenin-labelled IS*1311* PCR product produced from *M. avium* subsp. *paratuberculosis* 316V with primers DD2/DD3. Molecular size markers are shown at left. Panel A, *Pvu* II; Panel B *Bst* EII; Panel 3, *Bam* HI; Panel D, *Pst* I.

Fig. 5. Restriction endonuclease analysis of the PCR product from primers DD2/M56 produced using *M. avium*. TMC 715 (lane 2) and *M. avium* subsp. *paratuberculosis* isolates 316V (lane 3), McPaul (lane 4), 96/60-1 (lane 5) and 97/1-2 (lane 6). Molecular size markers are shown in lane 1. Panel A, *Alw* I; panel B, *Hinf* I; panel C, *Drd* I; panel D, *Mse* I; panel E, *Tse* I; panel F, *Fnu* 4HI.

Fig. 6. Restriction endonuclease analysis with *Hinf* I of the PCR product produced with primers DD2/M56 using isolates of *M. avium* subsp. *paratuberculosis* which are numbered as shown in Table 1. Host species: A, alpaca; B, bovine; C, caprine; O, ovine; R, rhinoceros. Molecular size markers are shown at left and right.

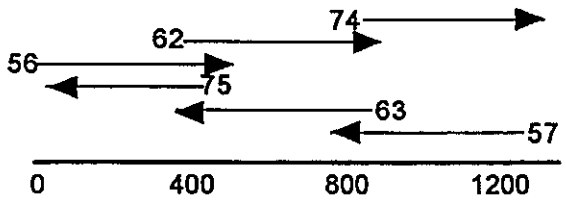
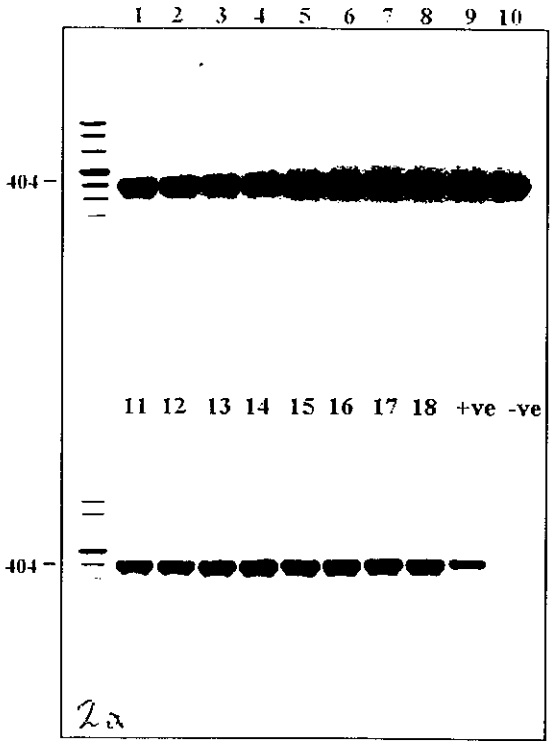


Fig 1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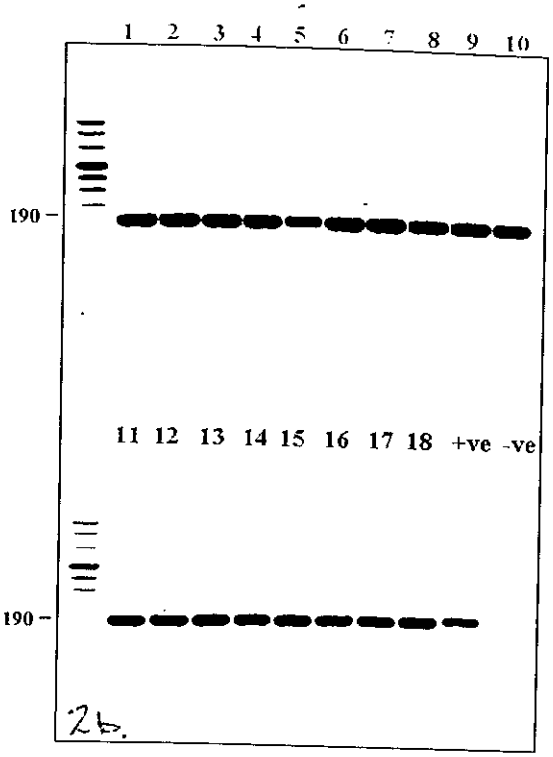
404
11 12 13 14 15 16 17 18 +ve -ve
404
2a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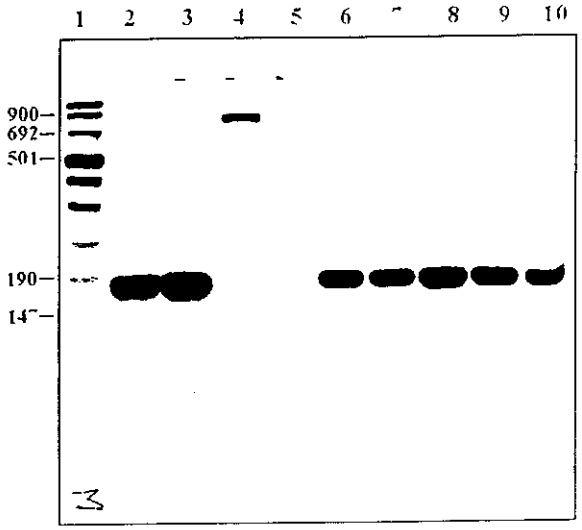
11 12 13 14 15 16 17 18 +ve -ve
190
2b.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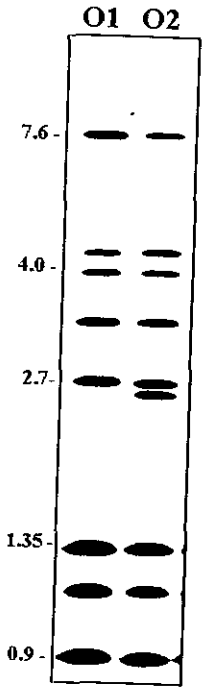
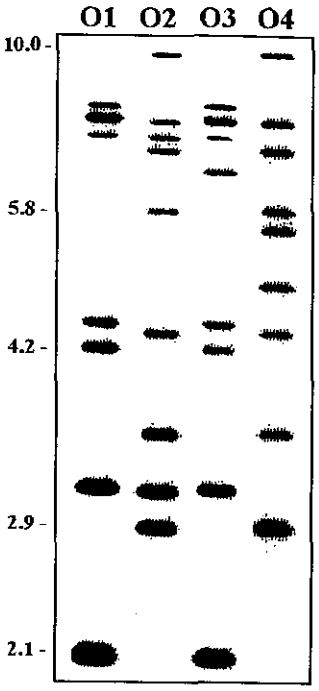
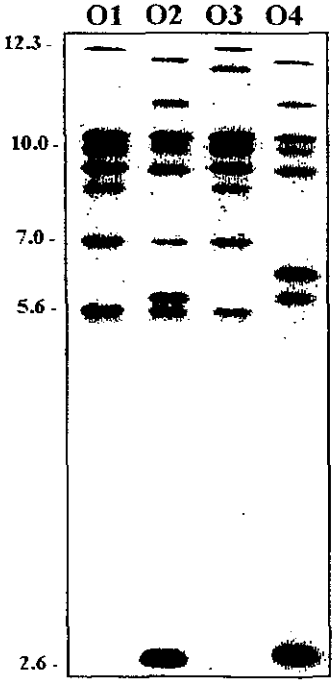
692
501
190
147
3


O1
O2
7.6
4.0
2.7
1.35
0.9

4a


O1
O2
O3
O4
10.0
5.8
4.2
2.9
2.1

4b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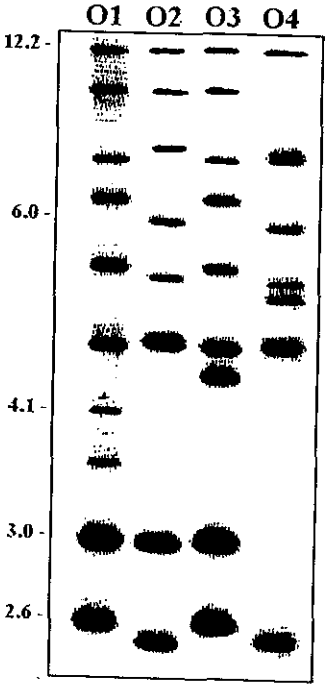
O2
O3
O4
12.3
10.0
7.0
5.6
2.6

4c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O4
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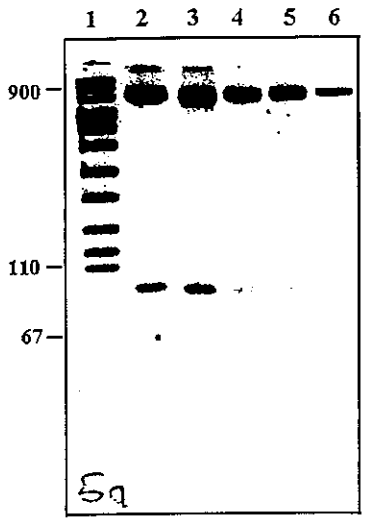
3.0
2.6

4d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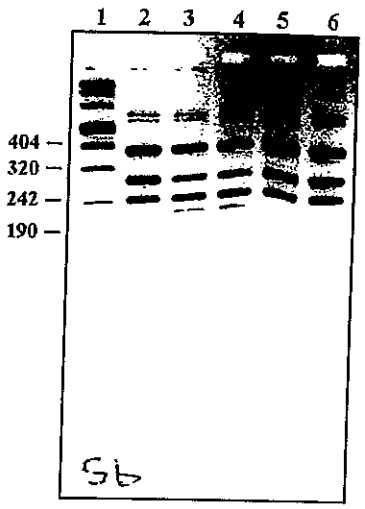
67
5a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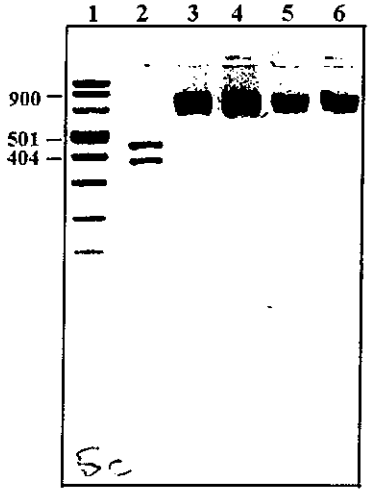
320
242
190
5b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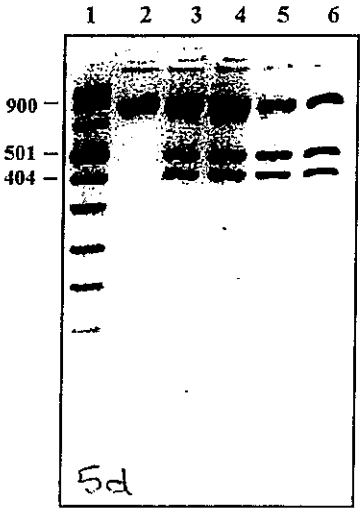
900
501
404
5c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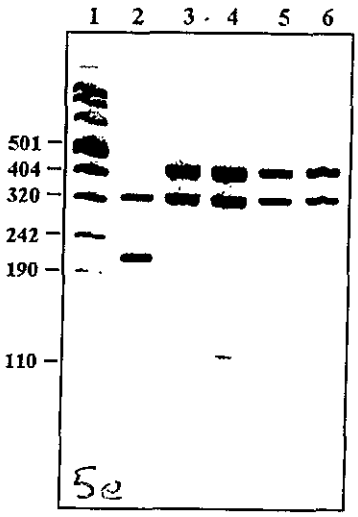
900
501
404
5d


1 2 3 4 5 6
501
404
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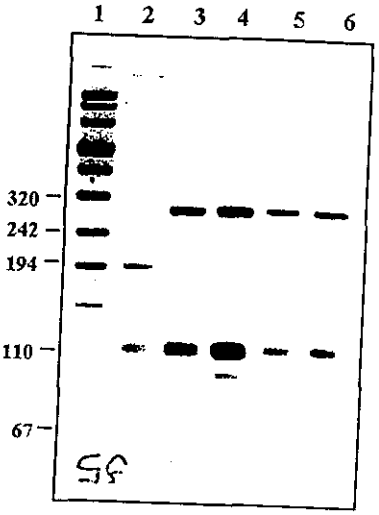
242
190
110
5e


1 2 3 4 5 6
320
242
194
110
67
5f

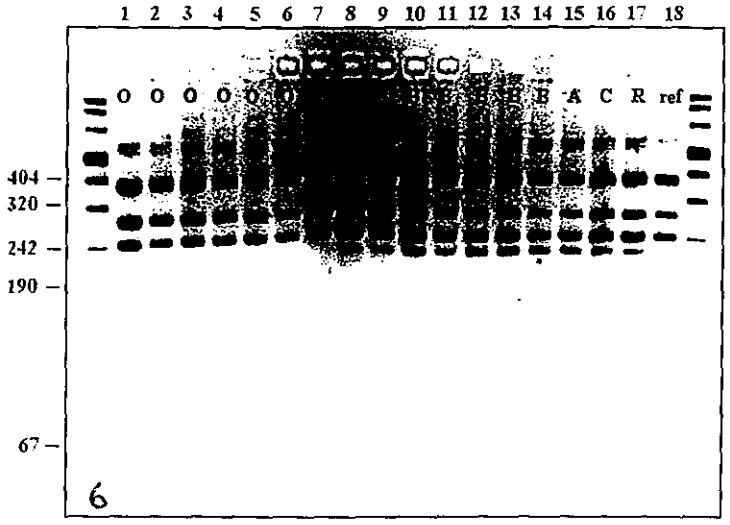

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
O O O O O O
B A C R ref
404
320
242
190
67
6

Table 1. Isolates/strains of mycobacteria used in this study and results of IS*1311* RFLP and IS*1311* PCR-restriction endonuclease analysis . All isolates/strains of *M. avium* subsp. *paratuberculosis* were positive in PCR for both IS*1311* and IS*900*. nt, not tested. IS*900 Bst* EII RFLP data is from unpublished work of the authors. nt, not tested. * indicates that purified DNA was used for all analyses. **S, sheep type; C, cattle type

Number	Organism	Source	Sample obtained	IS*900 Bst* EII RFLP type**	IS*1311* RFLP type				IS*1311* PCR-RE
					Bst EII	*Bam*HI	*Pvu* II	*Pst* I	
	Field isolates of *M. avium* subsp. *paratuberculosis*								
1	96/60-1	Ovine, New South Wales, Australia	Bacteria purified from gut*	S1	02	nt	nt	nt	S
2	97/1-2	Ovine, New South Wales, Australia	Bacteria purified from gut*	S1	02	nt	nt	nt	S
3	21503	Ovine, New South Wales, Australia	Purified DNA	S1	02	02	02	02	S
4	21633	Ovine, Victoria, Australia	Purified DNA	S1	02	02	02	02	S
5	22121	Ovine, Tasmania, Australia	Purified DNA	S1	02	02	02	02	S
6	21570	Ovine, New Zealand	Purified DNA	S1	02	02	02	02	S
7	21572	Ovine, South Africa	Purified DNA	S(int)	04	04	02	04	S
8	McPaul	Bovine, New South Wales, Australia	Cultured bacteria*	C3	01	nt	nt	nt	C
9	21619	Bovine, South Australia, Australia	Purified DNA	C3	nt	01	01	nt	C
10	21013	Bovine, Victoria, Australia	Purified DNA	C3	01	01	01	01	C
11	21191	Bovine, New South Wales, Australia	Purified DNA	C3	01	01	01	01	C
12	21573	Bovine, New Zealand	Purified DNA	C1	01	01	01	01	C
13	22032	Bovine, Tasmania, Australia	Purified DNA	C3	01	01	01	01	C

14	21197	Bovine, New South Wales, Australia, isolated from cow after importation from Canada	Purified DNA	C1	01	01	01	01	C
15	21348	Alpaca, Victoria, Australia	Purified DNA	C1	01	01	01	01	C
16	21196	Goat, New South Wales, Australia	Purified DNA	C3	01	01	01	01	C
17	21198	Rhinoceros, New South Wales, Australia	Purified DNA	C5	03	03	01	03	C
	Reference mycobacterial isolates								
18	*M. avium subsp. paratuberculosis*	316V	Cultured bacteria*	C1	01	01	01	nt	C
	M. aurum	ATCC 23366	Cultured bacteria						
	M. avium	TMC 715	Cultured bacteria						
	M. chelonae subsp.	ATCC 19977	Cultured bacteria						
	M. chitae	ATCC 19627	Cultured bacteria						
	M. duvalii	NCTC 358	Cultured bacteria						
	M. flavescens	ATCC 14474	Cultured bacteria						
	M. fortuitum	NCTC 3631	Cultured bacteria						
	M. gordonae	ATCC 14470	Cultured bacteria						
	M. intracellulare	ATCC 13950	Cultured bacteria						
	M. kansasii	ATCC 12478	Cultured bacteria						
	M. marinum	ATCC 927	Cultured bacteria						
	M. microti	NCTC 8710	Cultured bacteria						
	M. neoaurum	ATCC 25795	Cultured bacteria						

M. nonchromogenicum	ATCC 19530	Cultured bacteria
M. parafortuitum	ATCC 19686	Cultured bacteria
M. phlei	ATCC 11758	Cultured bacteria
M. scrofulaceum	ATCC 19981	Cultured bacteria
M. terrae	ATCC 15755	Cultured bacteria
M. thermoresistable	ATCC 19527	Cultured bacteria
M. triviale	ATCC 23292	Cultured bacteria
M. tuberculosis	NCTC 7416	Cultured bacteria
M. vaccae	ATCC 15483	Cultured bacteria
M. xenopi	NCTC 10042	Cultured bacteria

Table 2. Primers used for PCR amplification experiments and DNA sequencing reactions

Primer name	Gene	Sequence 5' - 3'	Orientation	Position in relevant gene	Predicted PCR product size (bp)	Ref.
P90	IS*900*	GAA GGG TGT TCG GGG CCG TCG CTT AGG	F	15	}413	21
P91	IS*900*	GGC GTT GAG GTC GAT CGC CCA CGT GAC	R	427	}	
DD3(M24)	IS*1311*	GTG CAG CTG GTG ATC TCT GA	F	732	}180	10
DD2 (M23)	IS*1311*	GTC GGG TTG GGC GAA GAT	R	911	}	
P1	IS*1245*	GCC GCC GAA ACG ATC TAC	F	197	}427	9
P2	IS*1245*	AGG TGG CGT CGA GGA AGA C	R	623	}	
M56 F	IS*1311*	GCG TGA GGC TCT GTG GTG AA	F	3	}1259	
M57 R	IS*1311*	GAT TGG TCG GCT GAA TCG GA	R	1261	}	
M62 F	IS*1311*	GCC TAT TTG CAC GGC ACC TC	F	378		
M63 R	IS*1311*	GAT CCC TTG GGC ACC TGG GC	R	862		
M74 F	IS*1311*	CAG GTG CCC AAG GGA TCA GC	F	846		
M75 R	IS*1311*	CGC GCC GAG GGC TTT AAC CA	R	437		

Table 3. DNA sequence differences identified within the IS*1311* gene of *M. avium* and *M. avium* subsp. *paratuberculosis*. Complete sequence was available for each of the *M. avium* subsp. *paratuberculosis* isolates for bp 23-1241 of the 1317 bp sequence of the *M. avium* gene.

Position in IS1311 sequence	*M. avium* Gen Bank Acc. No. U16276	*M. avium* subsp. *paratuberculosis*			
		316V	McPaul	96/60-1	97/2-1
68	T	*C*	*C*	*C*	*C*
223	*C*	*C or T*	*C or T*	*C*	*C*
236	*T*	*C*	*C*	*C*	*C*
422	*T*	*C*	*C*	*C*	*C*
527	*G*	*A*	*A*	*A*	*A*
628	*T*	*C*	*C*	*C*	*C*

APPENDIX 8

Paper in press - Molecular and Cellular Probes

PCR-restriction endonuclease analysis for identification and strain typing of *Mycobacterium avium* subsp. *paratuberculosis* and *Mycobacterium avium* subsp. *avium* based on polymorphisms in IS*1311*.

Ian Marsh[1]*, Richard Whittington[1] and Debby Cousins[2]

Elizabeth Macarthur Agricultural Institute, NSW Agriculture, Menangle, New South Wales, Australia[1], and Animal Health Laboratories, Agriculture Western Australia, Perth, Western Australia, Australia[2]

INTRODUCTION

Mycobacterium paratuberculosis is the causative agent of Johne's disease, a chronic and incurable disease affecting many ruminants. Two broad groups of *M. paratuberculosis* are known to exist based on restriction fragment length polymorphism analysis (RFLP) and cultural characteristics: sheep and cattle strains[1]. The sheep strain appears to infect sheep and goats, whereas the cattle strain is known to infect many species including cattle, goats, sheep and man[1,2]. In Australia, the cattle strain is known to infect cattle, goats and alpaca while the sheep strain is known to infect only sheep (unpublished data). Infection with *M. paratuberculosis*, as for other slowly growing mycobacteria, is difficult to diagnose due to the long incubation times required to culture and identify the organism[3]. Strain identification is currently performed by RFLP analysis on DNA extracted from the cultured organisms[1], an expensive and time consuming process. The ability to rapidly differentiate the sheep and cattle strains would be of great benefit in voluntary eradication and control programs for Johne's disease where mixed grazing is practiced. Current management practices assume that cattle are not susceptible to infection with the sheep strain and can safely graze on pasture after the removal of sheep. A rapid, sensitive test that confirms *M. paratuberculosis* and differentiates between the sheep and cattle strains would help to ensure that management practices are scientifically sound.

Mobile genetic elements known as insertion sequences (IS) are popular targets for polymerase chain reaction (PCR) tests for many mycobacterial diseases[4,5]. As IS are generally present in multiple copies in the genome of an organism they make useful targets to develop highly sensitive PCR assays. At present the IS*900* gene, a member of the IS*116* family, is the only IS known to be unique to *M. paratuberculosis*[6]. Insertion sequences from *M. avium*, IS*901*[7], IS*902*[8] and IS*1110*[9], are also members of the IS*116* family and are closely related to IS*900*. Also found in *M. avium* are IS*1311* and IS*1245*[10,11], both of which belong to the IS*256* family. Recently, the IS*1311* element was identified in *M. paratuberculosis* and was subsequently characterised from that species[12,13]. IS*1245* is present in *M. avium*, but apparently not in *M. paratuberculosis*[12].

PCR tests that target IS*900* have been developed to identify *M. paratuberculosis*[6,14,15]. However, both sheep and cattle strains of *M. paratuberculosis* yield identical results in these tests. Although IS*1311* has been demonstrated in both *M. paratuberculosis* and *M. avium*, 5 point mutations differentiate the IS*1311* sequences of the two species[13]. These point mutations can be targeted by restriction endonuclease analysis (REA) to differentiate *M. paratuberculosis* from *M. avium*. In addition, some copies of IS*1311* in the cattle strain of *M. paratuberculosis* contain an additional point mutation that can be used to differentiate it from the sheep strain[13].

The aim of this study was to develop, standardise and evaluate a test that could be used in a diagnostic laboratory to rapidly differentiate *M. paratuberculosis* from *M. avium* and discriminate between sheep and

cattle strains of *M. paratuberculosis*. We also assessed another IS*1311* PCR for use on formalin-fixed paraffin-embedded tissues specifically to discriminate between the sheep and cattle strains of *M. paratuberculosis*. We recommend a standard procedure for use on primary cultures or purified DNA and an alternate procedure for use on formalin-fixed paraffin-embedded material.

MATERIALS AND METHODS

Primer Selection. The IS*1311* primer sequences used in this study are described in Table 1. They were chosen so that PCR product would include (i) the T for C base change at base position 223 that is present in some copies of IS*1311* in the cattle strain of *M. paratuberculosis*, giving a *Hinf* I restriction site and (ii) the point mutation at base position 422 in the *M. avium* IS*1311* sequence, giving a single *Mse* I restriction site[13]. The *Hinf* I restriction site differentiates sheep and cattle strains of *M. paratuberculosis* and the *Mse* I restriction site in *M. avium* distinguishes *M. avium* from *M. paratuberculosis*. The proximity of the *Hinf* I site to the 5′ end of IS*1311* limited the choice of forward primers. Therefore, reverse primers in regions of IS*1311* that would theoretically give readable results from restriction endonuclease digests were matched to a single forward primer (M56). Reverse primers were selected using a computer programme (Primer Designer, Scientific and Educational Software) or were selected arbitrarily.

Polymerase Chain Reaction. The forward and reverse primers and the predicted product size of the reactions used in this study are given in Table 2. Reaction IS*1311*-1 was designed to detect and discriminate between the sheep and cattle strains of *M. paratuberculosis* and *M. avium*. Reaction IS*1311*/IS*1245* was a multiplex reaction designed to amplify IS*1311* from both *M. paratuberculosis* and *M. avium* and IS*1245* from *M. avium*. Reaction IS*1311*-2 was designed to discriminate only between the sheep and cattle strains of *M. paratuberculosis* from formalin-fixed paraffin-embedded tissues. Reactions IS*900*M and IS*900*V were used to confirm the identify of *M. paratuberculosis* isolates and for formalin-fixed paraffin-embedded tissues, respectively. Reaction IS*1245*C which amplifies a 198 bp region of IS*1245*, was used to identify *M. avium*.

All PCR experiments were performed as previously described[13]. Briefly, a reaction volume of 50 :l containing 5 :l of the DNA sample, 250 ng of each primer, 200 :M of each of the nucleotides dATP, dTTP, dGTP and dCTP, 66.8 mM Tris-HCl, 16.6 mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 1.65 mg/ml bovine serum albumin, 10 mM beta-mercaptoethanol, 2 U *Taq* polymerase, in buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.8) was used. Amplification was undertaken in 200 :l tubes in a 96-place thermal cycler (Corbett Research, Sydney, Australia) using the following conditions: one cycle of denaturation at 94EC for 3 min followed by 37 cycles of denaturation at 94EC for 30 sec, annealing at 62EC for 15 sec and extension at 72EC for 1 min. A modified hot start procedure was used for all PCRs: samples were placed in the thermal cycler after it had reached 94EC during the initial denaturation cycle. PCR results were assessed by electrophoresis in 2% agarose gels stained with ethidium bromide. The amount of amplified DNA required for REA was estimated from the intensity of the PCR product after electrophoresis.

Restriction endonuclease analysis of PCR products. The endonuclease(s) and predicted band sizes following digestion of the specific PCR products are given in Table 2. All restriction endonucleases were obtained from New England Biolabs Incorporated and were used as recommended by the manufacturer. REA reactions were prepared by adding 4-12 µl of PCR product, 2 U of the appropriate restriction endonuclease(s), 1.6 µl of buffer (supplied with restriction endonuclease), 1.6 µl of 100 µg/ml bovine serum albumin (for REA of reaction IS*1311*-1 product only) and made up to 16 µl with sterile purified water. Restriction digests were incubated for 2 hours at the recommended temperatures and were assessed by electrophoresis in 4% agarose gels stained with ethidium bromide.

Estimation of PCR sensitivity. A 10 fold dilution series (50 nanograms to 50 attograms) of *M. paratuberculosis* DNA (Table 3 isolate S25) was prepared in 10 mM Tris-HCl, 0.1 mM EDTA, pH 8.8 and used to evaluate the analytical sensitivity of all IS*1311* and IS*900* reactions in this study.

Estimation of PCR specificity. A reference collection of 24 mycobacterial species (Table 3, S1-S24) was

cultured on Lowenstein-Jensen agar at 37°C (except *M. marinum* - 22°C) and used to evaluate the specificity of PCR.

Mycobacteria used in this study. *M. paratuberculosis* (n=50) (Table 4, isolates 1-50), from a range of animal species and that had been typed previously by *Bst* EII RFLP (unpublished data) and *M. avium* isolates (n=28) (Table 4, isolates 51- 78), also from a range of sources, were used in preliminary evaluation of reactions IS*1311*-1, IS*1311*/IS*1245* and IS*1311*-2.

Reaction IS*1311*-1 was further evaluated with *M. paratuberculosis* isolates: extracted from intestinal scrapings from sheep (n=5); DNA extracted from 3 American Type Culture Collection (ATCC) strains from Crohn's disease patients that were previously typed by RFLP analysis, Ben ATCC 43544 (RFLP C1, Pavlik personal communication), Linda ATCC 43015 (RFLP C5,[1]) and Dominic ATCC 43545 (RFLP C,[17]) and; isolates grown in radiometric culture from soil (n=5), sheep faeces (n= 18) and cattle faeces (n=8).

Seventy five formalin-fixed paraffin-embedded tissues from sheep (n=47), cattle (n=20) and goats (n=8) were used to evaluate reaction IS*1311*-2.

Sample preparation. *M. paratuberculosis* from intestinal scrapings from sheep were prepared according to Choy *et al.*[16]. Briefly, mucosal homogenates were prepared by scraping the distal 8m of the small intestine. Smears prepared from the homogenates were examined for acid fast bacilli (AFB) after staining using the Ziehl Neelsen technique. AFB were then purified from the homogenates by differential and density gradient centrifugation using sucrose and potassium chloride as separation media. For faecal samples, *M. paratuberculosis* was cultured in radiometric medium and prepared for PCR as described by Whittington *et al.* [18].

Purified DNA was prepared for analysis of *M. paratuberculosis* (Table 3, S25; Table 4 all isolates of *M. paratuberculosis*) as described[13]. Purified DNA from *M. paratuberculosis* from intestinal scrapings of sheep was prepared as previously described [16]. Otherwise DNA was prepared from the mycobacterial reference collection (Table 3, S1-S24), *M. avium* (Table 4), *M. paratuberculosis* from humans and *M. paratuberculosis* from radiometric culture[18] by boiling cells for 20 mins in a volume of 100 µl of water[13].

Sections of 5 µm were prepared from formalin-fixed paraffin-embedded tissues stained with haematoxylin and eosin and by a Ziehl Neelsen method[19,20] and were classified by species of origin and nature of the histological lesion. Crude DNA samples were prepared for PCR from the sections by a simple boiling/freezing method[19].

Experimental Design. The critical issues for determination for each PCR were: analytical sensitivity, analytical specificity, practicality and ease of interpretation of the REA results as well as diagnostic sensitivity when applied to DNA samples from a range of sources. Analytical sensitivity was assessed in relation to an existing optimised PCR for IS*900* (reaction IS*900*M, which is used routinely to identify *M. paratuberculosis*). PCR products from a representative group of *M. paratuberculosis* (sheep and cattle strains) and *M. avium* strains were then evaluated in REA for (i) the expected digestion pattern as determined by restriction endonuclease mapping of the IS*1311* sequence and (ii) the ease of interpretation of these digestion patterns. Finally, the reactions that showed the greatest potential were further evaluated using DNA from *M. paratuberculosis* from sources appropriate to the intended use of each PCR.

RESULTS

Nine IS*1311* reactions were assessed but 7 were rejected because of lack of analytical sensitivity and/or analytical specificity. Results are reported only for those PCR that satisfied the criteria of analytical sensitivity and specificity.

Analytical sensitivity. Fifty femtograms of *M. paratuberculosis* 316V DNA was detected by reaction IS*900*M when 20 µl of PCR product was analysed by gel electrophoresis. Reactions IS*1311*-1,

IS*1311*/IS*1245* and IS*1311*-2 had similar end points to reaction IS*900*M and produced equivalent or greater yields of product at low concentrations of test DNA (Fig. 1).

Analytical specificity. In reaction IS*1311*-1, a single faint band was observed from *M. aurum* and a slight smear from *M. tuberculosis* (Fig. 2). The multiplex PCR (reaction IS*1311*/IS*1245*) amplified specific IS*1311* product of 268 bp from *M. paratuberculosis* and *M. avium*, however *M. avium* could be identified by the presence of the 198 bp product from the IS*1245* reaction. Non-specific product was detected from 17 of the 24 mycobacterial species tested with reaction IS*1311*/IS*1245*, often as multiple bands of varying yield, but both *M. paratuberculosis* and *M. avium* could be distinguished easily from these species. Reaction IS*1311*-2 produced faint non-specific product from *M. chelonae, M. duvalii, M. flavescens, M. fortuitum, M. scrofulaceum* and *M. vaccae* (Fig. 2).

Evaluation of reactions IS*1311*-1, IS*1311*/IS*1245* and IS*1311*-2. Fifty *M. paratuberculosis* DNA samples and 28 *M. avium* DNA samples (Table 4 isolates 1-78) were used to assess reactions IS*1311*-1 and IS*1311*/IS*1245*. Reaction IS*900*M detected all 50 *M. paratuberculosis* samples and reaction IS*1245*C detected all 28 *M. avium* samples. Reaction IS*1311*-1 had the same sensitivity as reactions IS*900*M and IS*1245*C, detecting all 50 *M. paratuberculosis* and all 28 *M. avium* while REA (Fig. 3) correctly typed all 78 samples as sheep or cattle strains with respect to IS*900* RFLP or species of origin.

REA on reaction IS*1311*-1 PCR product from cattle strains of *M. paratuberculosis* confirmed the presence of the polymorphism at base position 223 in this strain. This was demonstrated by the presence of an extra band (218 bp) in the cattle strain digestion pattern compared to the sheep strain digestion pattern. The extra *Hinf* I site also gives a 67 bp band that is difficult to see by electrophoresis in 4% agarose gels. The 218 bp band is consistent with the presence of a *Hinf* I site in some copies of IS*1311* in the cattle strain. The 285 bp band in the cattle strain digestion pattern is also present in the pattern from the sheep strain.

Reaction IS*1311*/IS*1245* (the multiplex reaction) had equivalent sensitivity to reactions IS*900*M and IS*1245*C detecting all 50 *M. paratuberculosis* and all 28 *M. avium*. Product from *M. avium* did not require REA. Two *M. paratuberculosis* isolates which were cultured from a single alpaca (Table 4, isolates 29 and 30), were provisionally identified as *M. avium* due to the presence of the 198 bp product from the IS*1245* reaction. The multiplex PCR products from the remaining 48 *M. paratuberculosis* isolates were typed correctly by REA as sheep or cattle strains.

In order to check the results of reaction IS*1311*/IS*1245*, all 50 *M. paratuberculosis* samples were tested in the individual IS*1311* and IS*1245* PCR that comprise the multiplex reaction, namely IS*1311*-2 and IS*1245*C, respectively. All isolates were detected in reaction IS*1311*-2 and were correctly identified after REA, including the two alpaca isolates that were erroneously typed as *M. avium* in the multiplex reaction. In reaction IS*1245*C, the two alpaca isolates yielded the specific 198 base pair fragment confirming the presence of IS*1245* in these samples. Non-specific product of approximately 380 base pairs was observed from all 50 samples in reaction IS*1245*C but was not observed in the multiplex reaction. Due to the potential complications with the interpretation of the results that was highlighted by the two alpaca isolates, the multiplex PCR was considered to be less useful than reaction IS*1311*-1.

Reaction IS*1311*-2 was evaluated only with the 50 *M. paratuberculosis* DNA samples (Table 4 isolates 1-50) because this PCR is unable to differentiate *M. paratuberculosis* from *M. avium* as it does not include the *Mse* I site used to identify *M. avium*. All 50 isolates were correctly identified by REA as either sheep or cattle strains of *M. paratuberculosis* (Fig. 3)

Further evaluation of reaction IS*1311*-1. The 39 *M. paratuberculosis* isolates from sheep intestinal scrapings, Crohn's patients and radiometric cultures were all PCR positive in reaction IS*900*M and reaction IS*1311*-1. The amplified DNA from the latter was correctly identified by REA as *M. paratuberculosis* according to ruminant species of origin or RFLP type.

Further evaluation of reaction IS*1311*-2 with formalin-fixed paraffin-embedded tissues. Reaction

IS*900*V identified *M. paratuberculosis* in 31 of the 75 formalin-fixed paraffin-embedded tissues. Reaction IS*1311*-2 detected *M. paratuberculosis* in 33 of the 75 formalin-fixed paraffin-embedded tissues and there was sufficient amplified product for REA from 27 of these. All 27 were correctly identified with respect to the host species of origin, 19 as sheep isolates and 8 as cattle isolates. Reaction IS*1311*-1 was positive for only 5 samples and none could be identified by REA as insufficient PCR product was obtained. The positive PCR results were correlated with the presence of acid fast bacilli in tissue sections (Table 5). Three sheep samples, all from farms with Johne's disease, were classified as acid fast bacilli negative but were positive in PCR. One sample was positive in reaction IS*900*V and reaction IS*1311*-2 while two samples were positive only in reaction IS*1311*-2.

DISCUSSION

Two new and useful IS*1311* PCR-based tests for *M. paratuberculosis* were developed and evaluated in this study, extending preliminary findings of the presence of IS*1311* in *M. paratuberculosis* and the sequence differences between *M. paratuberculosis* and *M. avium*[12,13].

The PCR/REA test using reaction IS*1311*-1 was highly sensitive and specific for differentiating *M. paratuberculosis* from *M. avium* without the need for IS*900* PCR. Easily interpretable REA patterns made this test suitable for diagnostic samples. Unlike tests used to differentiate *M. paratuberculosis* and *M. avium* that are based on amplification of a single copy of a target gene such as rRNA[21] or *hsp65*[22], reaction IS*1311*-1 is based on a multicopy gene and has sensitivity equivalent to IS*900* PCR tests. When evaluated on an extensive range of samples, reaction IS*1311*-1 and REA correctly identified all 89 *M. paratuberculosis* and all 28 *M. avium* samples, be they purified DNA or crude DNA. These results confirm that reaction IS*1311*-1 is a very useful test for samples originating from a range of sources including primary radiometric cultures, purified DNA and crude DNA lysates from organisms grown on solid media.

The finding of two isolates of *M. paratuberculosis* that appeared to contain both IS*1311* and IS*1245* was unusual. Both isolates were from the same alpaca, one from faeces and one from lymph node. The multiplex PCR identified the isolates as *M. avium* yet confirmatory testing using PCR specific for each IS confirmed the presence of both IS*1311* and IS*1245* in these isolates. It is possible that this strain, confirmed as *M. paratuberculosis* by RFLP typing and IS*900* PCR, did contain both IS even though IS*1245* is considered to be specific for *M. avium*[12]. Guerrero *et al.*[11] found IS*1245* to be present in a single strain of *M. paratuberculosis* by Southern blotting. However, as IS*1245* has 85% homology with IS*1311* at the DNA level, Roiz *et al.*[10] suggest that cross-hybridisation of probes for IS*1245* and IS*1311* may occur during Southern blotting if the conditions used are not stringent enough. Another possibility is that the cultures from the alpaca were mixed and contained both *M. paratuberculosis* and *M. avium*. We consider this to be unlikely as both isolates were from different sites in the same animal and yet produced the same result. It remains to be seen whether other isolates are found as the use of these testing regimes increases. However, because of the possibility of misidentification using the multiplex PCR, it cannot be recommended.

Diagnosis of Johne's disease is sometimes required from archival material or in cases where only formalin-fixed samples are submitted. Other studies on PCR from formalin-fixed paraffin-embedded tissues report degradation of the target DNA during processing and storage, which favours the detection of short fragments of DNA[23-26]. This phenomenon was noted with IS*900* PCR, hence the use of reaction IS*900*V on formalin-fixed paraffin-embedded samples[19], and occurred also with IS*1311* PCR. Reaction IS*1311*-1, which amplifies a 608 bp fragment, detected *M. paratuberculosis* in less than 7% of formalin-fixed paraffin-embedded samples whereas reaction IS*1311*-2, which targeted a much shorter sequence (268 bp) classified 44% of the 33 samples as infected with *M. paratuberculosis*. The REA correctly identified all of these as sheep or cattle strains where there was sufficient PCR product to digest. The results confirm that reaction IS*1311*-2 is a valuable tool for typing sheep and cattle strains of *M. paratuberculosis*. It is necessary however, that formalin-fixed paraffin-embedded samples first be tested with reaction IS*900*V to differentiate *M. paratuberculosis* from *M. avium*.

PCR from formalin-fixed paraffin-embedded tissues produced positive results from three tissue sections that apparently lacked acid fast bacilli (Table 5). As these tissues had been collected from sheep from known-infected flocks, the results suggest that PCR on formalin-fixed paraffin-embedded tissues maybe more sensitive than histological examination in some cases.

Primer selection was confined to areas of the IS*1311* gene flanking the two restriction endonuclease sites required for REA. Due to the close proximity of the key polymorphism to the 5' end of the IS*1311* sequence in the cattle strain of *M. paratuberculosis*, the choice of forward primers was limited. Many IS*1311* PCRs were evaluated in this study using reverse primers chosen with the assistance of a computer programme using conventional matching criteria, followed by standard PCR optimisation experiments. However, only 2 of these reactions meet the required levels of sensitivity and specificity. A recent study on PCR optimisation suggested that extensive testing of randomly chosen primer combinations might be the key to improved assays rather than meticulous primer pairing and optimisation of reaction conditions[27]. This was certainly the case in the development of reaction IS*1311*-1.

Candidate primer pairs were initially screened for analytical sensitivity using a dilution series of *M. paratuberculosis* DNA with the aim of defining reactions with analytical sensitivity similar to that of an IS*900* PCR (reaction IS*900*M). A single *M. paratuberculosis* genome is equivalent to 5 femtograms of DNA[28]. Reaction IS*900*M detected 50 femtograms of target DNA or 10 organisms in 20 µl of amplified product indicating a similar analytical sensitivity to other IS*900* PCR[28]. Reactions IS*1311*-1, IS*1311*/IS*1245* and IS*1311*-2 were as sensitive as reaction IS*900*M and had sufficient analytical specificity, based on the examination of crude DNA lysates from a collection of mycobacterial species.

These studies have confirmed the benefits of two new PCR-REA tests for *M. paratuberculosis* (Fig. 4). Reaction IS*1311*-1 combined with REA as described in this paper provides information similar to IS*900* PCR in that it can distinguish *M. paratuberculosis* from *M. avium* but it also distinguishes strains within *M. paratuberculosis*. It has been clearly shown to be useful on a range of crude and purified DNA preparations from a variety of sources. Reaction IS*1311*-2 is a valuable tool for evaluation of histological specimens. Rapid differentiation of the cattle and sheep strains of *M. paratuberculosis* using these PCR-based methods can provide information of the sub-types of *M. paratuberculosis* to managers of disease and control programs months earlier than is available by conventional RFLP methods. This will aid control and eradication programs for Johne's disease in Australia and in other countries.

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Table 1: Primers used in PCR experiments.

Primer	Position	Sequence (5' - 3')	Reference
M56	3[a]	GCG TGA GGC TCT GTG GTG AA	13
M94	270[a]	CAG CGA TCG TCG ACA GTG TG	this report
M119	610[a]	ATG ACG ACC GCT TGG GAG AC	this report
M114	152	CTC GAC GCA CTG CGC ACC	12
M115	349	CCG CAG TTC CAG GTC CCC T	12
P90B	15	GAA GGG TGT TCG GGG CCG TCG CTT AGG	15
P91B	427	GGC GTT GAG GTC GAT CGC CCA CGT GAC	15
M120	139	CCG CTA ATT GAG AGA TGC GAT TGG	14
M121	367	AAT CAA CTC CAG CAG CGC GGC CTC G	14

[a] Primer designed using *M. avium* Genebank accession U16276

Table 2: Primer sets used for each PCR, the predicted product size from each reaction and REA parameters.

PCR Reaction	Target	Forward Primer	Reverse Primer	Predicted Product (bp)	Enzyme(s)	Species	Predicted band sizes (bp)	Reference
IS1311-1	IS*1311*	M56	M119	608	*HinfI*/ *Mse* I	*M. paratuberculosis* (ovine)	285, 323	this report
						M. paratuberculosis (bovine)	67, 218. 285, 323	
						M. avium	134, 189, 285	
IS*1311*/IS*1245*	IS*1311*	M56	M94	268	*HinfI*	*M. paratuberculosis* (ovine)	268	this report
(multiplex)	IS*1245*	M114	M115	198		*M. paratuberculosis* (bovine)	50, 218, 268	
						M. avium[A]	198	
IS*1311*-2	IS*1311*	M56	M94	268	*HinfI*	*M. paratuberculosis* (ovine)	268	this report
						M. paratuberculosis (bovine)	50, 218, 268	
IS*900*M	IS*900*	P90B	P91B	413	n.a.			15
IS*900*V	IS*900*	M120	M121	229	n.a.			14
IS*1245*C	IS*1245*	M114	M115	198	n.a.			12

(A) The 198 base pair product from the IS*1245* PCR is used to identify *M. avium* prior to REA of the multiplex PCR product and is therefore not subject to REA.
n.a - not applicable

Table 3: Mycobacteria from culture collections that were used to assess the specificity of PCR.

Isolate	Organism	Reference
S1	*M. aurum*	ATCC 23366
S2	*M. avium*	TMC 715
S3	*M. chelonae* subsp.	ATCC 19977
S4	*M. chitae*	ATCC 19627
S5	*M. duvalii*	NCTC 358
S6	*M. flavescens*	ATCC 14474
S7	*M. fortuitum*	NCTC 3631
S8	*M. gordonae*	ATCC 14470
S9	*M. intracellulare*	ATCC 13950
S10	*M. kansasii*	ATCC 12478
S11	*M. marinum*	ATCC 927
S12	*M. microti*	NCTC 8710
S13	*M. neoaurum*	ATCC 25795
S14	*M. nonchromogenicum*	ATCC 19530
S15	*M. parafortuitum*	ATCC 19686
S16	*M. phlei*	ATCC 11758
S17	*M. scrofulaceum*	ATCC 19981
S18	*M. terrae*	ATCC 15755
S19	*M. thermoresistible*	ATCC 19527
S20	*M. triviale*	ATCC 23292
S21	*M. tuberculosis*	NCTC 7416
S22	*M. vaccae*	ATCC 15483
S23	*M. xenopi*	NCTC 10042
S24	*M. shimoidei*	EMAI FDC 2338
S25	*M. paratuberculosis*	316V

Table 4. DNA samples from cultures of *M. paratuberculosis* and *M. avium* used in this study. *Bst* EII IS*900* RFLP typing was based on the method and nomenclature described by Collins at al. [1].

Isolate	Organism	Source	Origin	IS*900* RFLP
	M. paratuberculosis			
1	6167N	bovine	WA Australia	C1
2	Lillie	caprine	Vic Australia	C3
3	94/2373#37	bovine	Vic Australia	C1
4	93/0590	alpaca	Vic Australia	C1
5	VRS 1654	ovine	NSW Australia	C3
6	VRS 1059	bovine	NSW Australia	C3
7	VRS 1047	bovine	NSW Australia	C(A)
8	VRS 1022	bovine	NSW Australia	C(A)
9	VRS 1030	bovine	NSW Australia	C(A)
10	VRS 1028	caprine	NSW Australia	C3
11	VRS 1026	caprine	NSW Australia	C3
12	VRS 2496	bovine	NSW Australia	C1
13	VRS 2498	rhinoceros	NSW Australia	C5
14	94/6230	bovine	Vic Australia	C3
15	94/4544 - a	bovine	Vic Australia	C3
16	94/4544 - b	bovine	Vic Australia	C3
17	94/4544 - c	bovine	Vic Australia	C3
18	94/6805	bovine	Vic Australia	C3
19	94/6239	bovine	Vic Australia	C(A)
20	94/6239	bovine	Vic Australia	C3
21	94/4629	bovine	Vic Australia	C1
22	94/6952	bovine	Vic Australia	C1
23	94/7316	alpaca	Vic Australia	C1
24	94/6988 - a	alpaca	Vic Australia	C1
25	94/6988 - b	alpaca	Vic Australia	C1
26	95/227	bovine	Vic Australia	C3
27	94/5903	bovine	Vic Australia	C3
28	11884	bovine	SA Australia	C3
29	5163	alpaca (faeces)	Qld Australia	C1
30	5168/5169	alpaca (LN)	Qld Australia	C1
31	2,4	alpaca	WA Australia	C1
32	VRS 1068	ovine	NSW Australia	S1
33	VRS 1065	ovine	NSW Australia	S1
34	VRS 1066	ovine	NSW Australia	S1
35	95/4125	bovine	Vic Australia	C1
36	6365	bovine	New Zealand	C1
37	6770A	bovine	New Zealand	C1
38	6770B	ovine	New Zealand	S1
39	6758B	ovine	New Zealand	S1
40	575A	ovine	South Africa	S (int.)
41	506C	ovine	South Africa	S (int.)
42	96/1870	bovine	Tas Australia	C3
43	96/2267	bovine	Tas Australia	C3
44	1963	bovine	Tas Australia	C3
45	95/9708	ovine	Vic Australia	S1
46	96/10044	ovine	Vic Australia	S1
47	EO10827-12	alpaca	Vic Australia	C1
48	EO112-2	bovine	Vic Australia	C3
49	96/3745	ovine	Tas Australia	S1
50	96/3798	ovine	Tas Australia	S1

M. avium				
51	S86/534	human	Qld Australia	n.a.
52	S88/291	human	Qld Australia	n.a.
53	S88/532	human	Qld Australia	n.a.
54	S89/115	human	Qld Australia	n.a.
55	S89/131	human	Qld Australia	n.a.
56	S90/007	human	Qld Australia	n.a.
57	S90/067	human	Qld Australia	n.a.
58	S90/094	human	Qld Australia	n.a.
59	17000	porcine	WA Australia	n.a.
60	17001	porcine	WA Australia	n.a.
61	17002	porcine	WA Australia	n.a.
62	16017	porcine	WA Australia	n.a.
63	16905	porcine	WA Australia	n.a.
64	16893	porcine	WA Australia	n.a.
65	16943	porcine	WA Australia	n.a.
66	7265G	bovine	WA Australia	n.a.
67	7266E	bovine	WA Australia	n.a.
68	5225F	porcine	WA Australia	n.a.
69	2194F	avian	WA Australia	n.a.
70	2786D	avian	WA Australia	n.a.
71	58844D	avian	WA Australia	n.a.
72	5845E	avian	WA Australia	n.a.
73	5846F	avian	WA Australia	n.a.
74	ST23	human	Qld Australia	n.a.
75	ST26	human	Qld Australia	n.a.
76	ST28	human	Qld Australia	n.a.
77	ST29	human	Qld Australia	n.a.
78	ST31	human	Qld Australia	n.a.

(A) *Bst* EII RFLP did not match C1-C7 but was of C type
n.a - not applicable

Table 5: Analysis of crude DNA extracts from 75 formalin-fixed paraffin-embedded tissues. Data are the presence (+) or absence (-) of acid fast bacilli (AFB) in microscopic sections and the number of positive PCR and REA results.

Host species	AFB	IS*900*V[a]	IS*1311*-1	IS*1311*-2	REA type (IS*1311*-2)
Sheep (n=47)	+ (n=20)	18	3	19	sheep (19)
	- (n=27)	1	0	3[b]	
Cattle (n=20)	+ (n=10)	8	0	8[b]	cattle (5)
	- (n=10)	0	0	0	
Goat (n=8)	+ (n=4)	4	2	3	cattle (3)
	- (n=4)	0	0	0	
	Total	31	5	33	27

[a] Data from Whittington *et al.*[19]

[b] insufficient PCR product for REA from 3 reactions in each group. The 3 sheep were from infected flocks.

Captions to Figs.

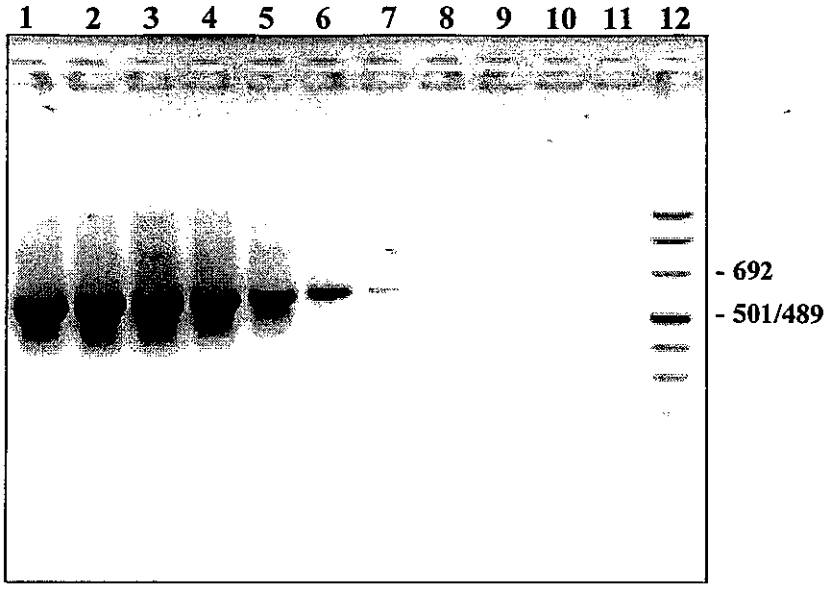
Fig. 1. Analytical sensitivity of reactions IS*1311*-1 (1a) and IS*1311*-2 (1b) compared to reaction IS*900*M (1c). For each panel: lanes 1-10, dilution series of *M. paratuberculosis* 316V DNA (50 nanograms to 50 attograms per reaction); lane 11, negative PCR control; lane 12, molecular weight marker.

Fig. 2. Analytical specificity of reactions IS*1311*-1 (2a) and IS*1311*-2 (2b) using a mycobacterial reference collection. Lanes 1-13 and 15-26 correspond to samples S1-S13 and S14-S25, respectively in Table 3. Lane 27, negative PCR control. Lanes 14 and 28, molecular weight markers.

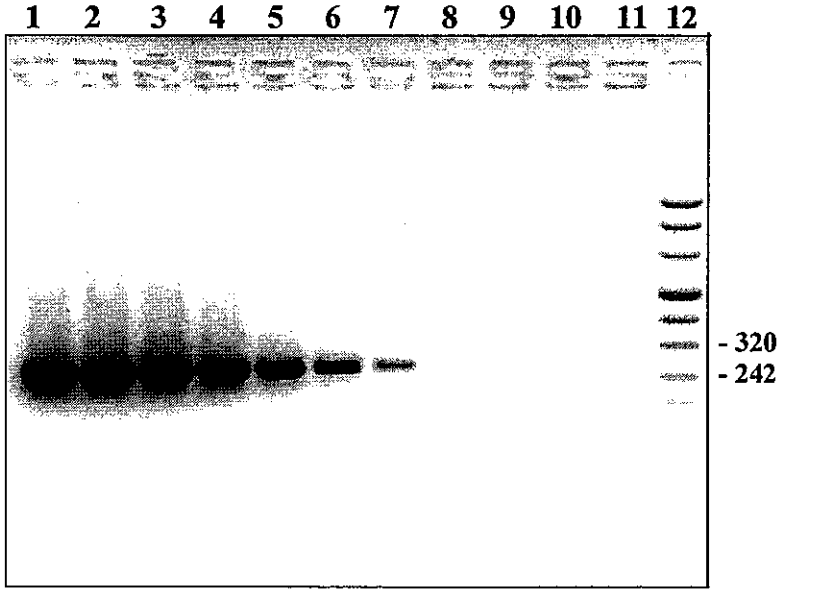
Fig. 3. *Hinf* I and *Mse* I restriction endonuclease analysis of reaction IS*1311*-1 (3a): lane 2, PCR product from *M. avium*; lane 3, *M. paratuberculosis* cattle strain and lane 4, *M. paratuberculosis* sheep strain. *Hinf* I restriction endonuclease analysis of reaction IS*1311*-2 (3b): lane 2, PCR product from *M. paratuberculosis* cattle strain and lane 3, *M. paratuberculosis* sheep strain. Lane 1, molecular weight marker.

Fig. 4. Schematic diagram of IS*1311* showing the primers for reaction IS*1311*-1 (M56/M119) and reaction IS*1311*-2 (M56/M94) and the restriction endonuclease sites for *M. avium* and cattle and sheep strains of *M. paratuberculosis*. The base position of each site is given in parentheses.

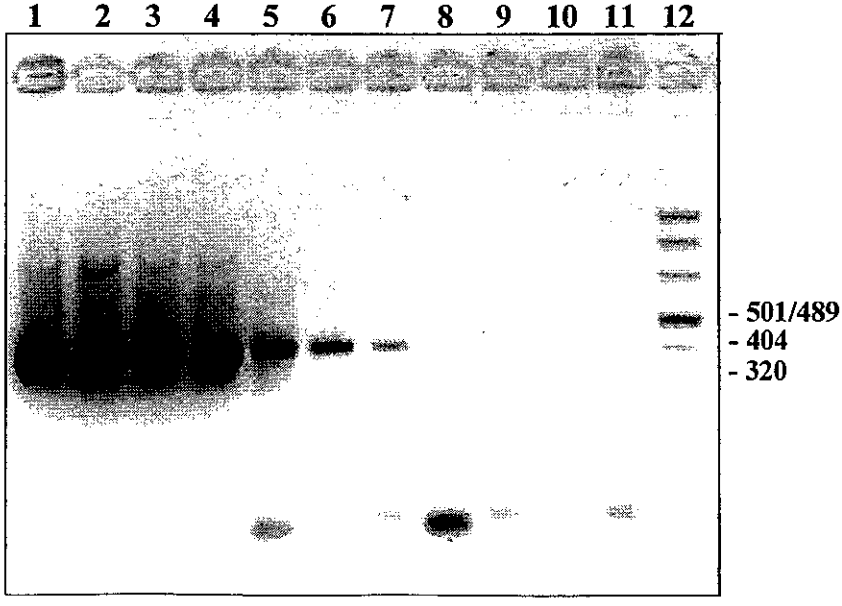
1a



1b

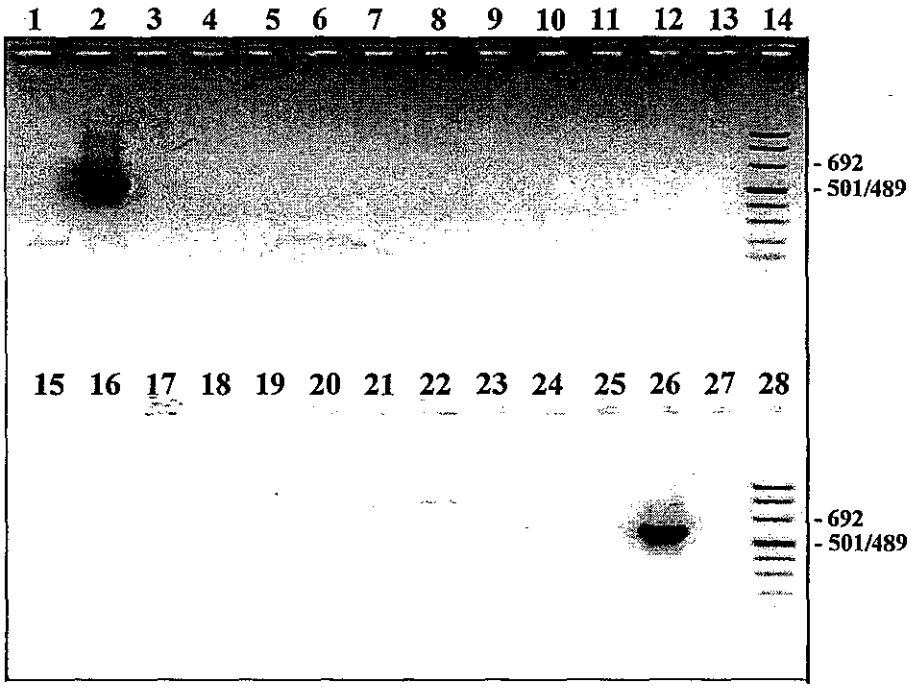


1c

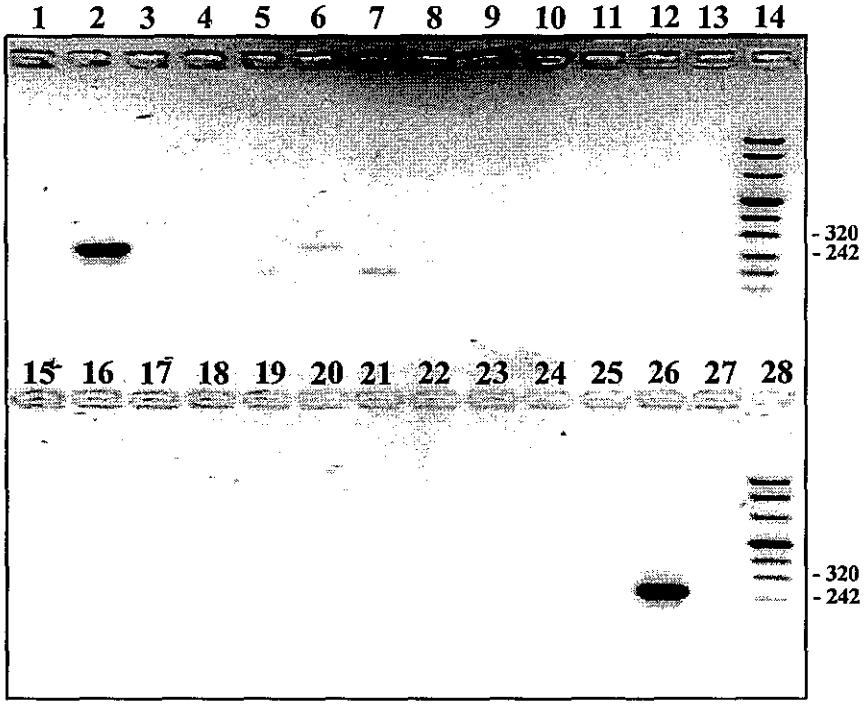


IS*1311* typing paper 1998, Figure 1

2a

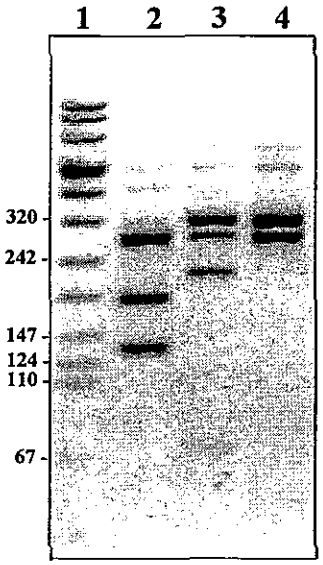


2b

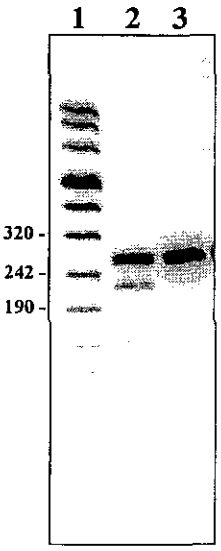


IS*1311* typing paper 1998, Figure 2

3a

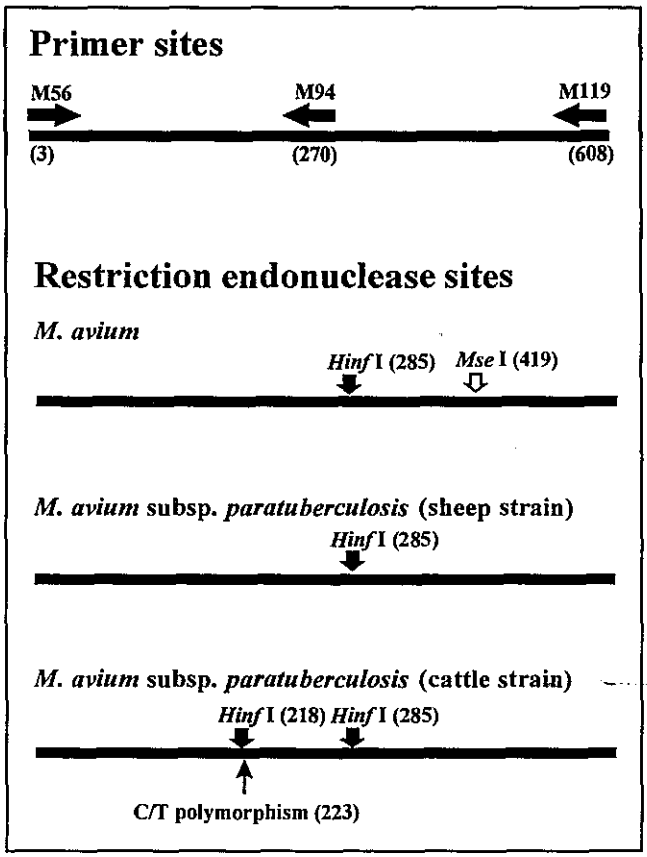


3b



IS*1311* typing paper 1998, Figure 3

IS*1311* typing paper 1998, Figure 4



APPENDIX 9
Manuscript submitted to the Australian Veterinary Journal

Detection of *Mycobacterium avium* subsp *paratuberculosis* in formalin-fixed paraffin-embedded intestinal tissue by IS*900* polymerase chain reaction

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Objectives To evaluate and compare methods for DNA extraction from formalin-fixed, paraffin-embedded tissues and methods for detection of *Mycobacterium avium* subsp *paratuberculosis* by IS*900* PCR for confirmation of Johne's disease in ruminants.

Design A laboratory study.

Procedure Three methods of DNA extraction of differing complexity and two PCR protocols using different pairs of IS*900* primers were compared. Sensitivity and specificity were assessed using samples from ruminants with and without histological evidence of Johne's disease.

Results The simplest method of DNA extraction, which involved two cycles of boiling and freezing followed by centrifugation, gave more consistent results than two methods that required solvent extraction of paraffin, proteinase digestion and DNA purification. The sensitivity of detection of *M avium* subsp *paratuberculosis* in paraffin blocks stored for 1 to 6 years from 34 cases of Johne's disease in sheep, cattle and goats was 88% for a 229 bp IS*900* PCR assay and 71% for a 413 bp assay, using the detection of acid-fast bacilli by Ziehl Neelsen staining of histological sections from the same blocks as the gold standard test. PCR results correlated with the abundance of acid fast organisms in the tissues. No false positive reactions were detected.

Conclusion PCR for identification of *M avium* subsp *paratuberculosis* in formalin-fixed, paraffin-embedded intestinal tissues from ruminants is a rapid and useful method. A simple method of sample preparation is effective. Amplification of short fragments of IS*900* is more effective than amplification of longer fragments.

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Key words: *Mycobacterium avium* subsp. *paratuberculosis*, sheep, cattle, goats, Johne's disease, polymerase chain reaction, histopathology, diagnosis, IS*900*

PCR polymerase chain reaction
bp base pair

Johne's disease is a chronic enteropathy affecting herbivores and is caused by *Mycobacterium avium* subsp *paratuberculosis*. In advanced cases the lamina propria of the intestine is infiltrated by epithelioid macrophages which contain masses of the causative acid-fast bacilli. Similar lesions may also develop in regional lymph nodes. These lesions are associated with loss of body condition and eventual death. In Australia Johne's disease has been diagnosed in cattle, sheep, goats, alpaca and a black rhinoceros and is considered to be economically important. The ruminant industries are developing quality assurance programs for progressive disease control and certification. These programs depend on accurate laboratory diagnosis.

A morphological diagnosis of Johne's disease can be made on gross and histological findings but aetiological diagnosis requires identification of the causative organism using a microbiological test. Culture of *M avium* subsp *paratuberculosis* requires specialised media and lengthy incubations followed by identification

of isolates based on phenotypic and genotypic characteristics and is considered to be the definitive test. PCR amplification of IS*900*, a gene believed to be unique to *M avium* subsp *paratuberculosis*,[1] is the most useful means of identifying *M avium* subsp *paratuberculosis* in culture, as phenotypic characteristics such as mycobactin dependency require an additional prolonged incubation. However, culture of *M avium* subsp *paratuberculosis* and hence mycobactin dependency testing has not been successful from cases of Johne's disease sheep in most countries.

Sometimes culture cannot be undertaken to confirm a diagnosis of Johne's disease, for example when only material for histopathological examination is submitted to a veterinary diagnostic laboratory. Options for identification of bacteria in histological sections include immunohistochemistry and in-situ PCR. The former method is not applicable for *M avium* subsp *paratuberculosis* as all currently available polyclonal antisera cross-react with other mycobacterial species and specific monoclonal antibodies have not been evaluated while the latter method requires highly specialised equipment which is not yet readily available in veterinary diagnostic laboratories. In recent years PCR methods have been developed for direct analysis of nucleic acid extracted from formalin-fixed paraffin-embedded tissues and there are several reports of the use of this technology to diagnose Johne's disease.[2,3] However, data on sensitivity and specificity are incomplete and there have been no attempts to define optimum protocols. The nature of the tissue-fixative is a critical determinant of the success of PCR amplification of DNA from paraffin-embedded tissues but fortunately the most commonly used fixative for histopathological analysis, buffered formalin, is one of the least deleterious fixatives.[4,5] In addition, the period of fixation, duration of storage of paraffin blocks, DNA extraction method and the length of the target DNA sequence influence the outcome of PCR from histological specimens.[4-7]

The aims of this study were to evaluate three methods of DNA extraction from formalin-fixed paraffin-embedded tissues, to compare the efficacy of PCR based on amplification of long (413 bp) or short (229 bp) fragments of the *M avium* subsp *paratuberculosis* IS*900* gene and to determine the sensitivity and specificity of the most appropriate method for confirmation of Johne's disease from paraffin blocks of various ages.

Materials and methods

Paraffin blocks and their preparation for PCR

Small intestinal samples from sheep, cattle and goats from farms in New South Wales of known status with respect to Johne's disease were fixed in 10% v/v buffered neutral formalin for 1 to 7 days and embedded in paraffin using routine histological processing methods. Sections of 5 μm were stained with haematoxylin and eosin and by a Ziehl Neelsen method[8] and were classified by species of origin and nature of the histological lesion. Lesions were classified as multibacillary or paucibacillary.[9] In multibacillary lesions the lamina propria was intensely infiltrated by epithelioid cells containing variable numbers of acid fast bacilli. In paucibacillary lesions the lamina propria was infiltrated by epithelioid cells to variable degrees. Paucibacillary cases were selected for this study only where acid fast bacilli were not observed, even though small numbers of acid-fast bacilli are permitted in a paucibacillary classification.[9]

Paraffin blocks were selected for PCR from archives where they had been stored for 1 to 6 years at room temperature. The immediate work area in the histology laboratory was thoroughly cleaned and non-essential apparatus were removed. The surfaces of the microtome and the immediate area of the laboratory bench were wiped with cloths soaked in 100% ethanol to remove adherent flakes of wax. Each histological block was wiped with 100% ethanol, inserted into the microtome carriage, trimmed and the waste was carefully discarded. The microtome blade and the operator's gloves were then changed. Two 5 μm sections were then cut and transferred to a sterile 1.5 mL screw-capped polypropylene centrifuge tube using a sterile disposable applicator stick. The applicator stick, microtome blade and the operator's gloves were then discarded and the microtome surfaces were cleaned with 100% ethanol before commencing the next block. A negative control block (intestine) was processed in the same way after every five blocks from cases of Johne's disease. Three methods of extracting DNA from sections were compared. These methods were representative of those in common use for PCR-based detection of intracellular bacteria in paraffin-embedded tissues.

Method A was based on those of Miller et al[3] and Coates et al. [10] Briefly, the paraffin sections in the centrifuge tube were pelleted at 16,000 *g* for 1 min and 200 μL sterile distilled water containing 0.5% v/v

Tween 20 was added. The tube was placed in a heating block at 100°C for 10 min then snap frozen in liquid nitrogen. The cycle of boiling and freezing was repeated once and the tube was boiled again for 10 min then centrifuged at 3000 *g* for 20 min. The supernatant was transferred to a fresh tube, stored at -20°C and 5 μl was used in each PCR.

Method B was based on that of Cooper et al.[11] Briefly, 1 mL of xylene was added to the centrifuge tube to deparaffinize the tissue and the tube was centrifuged at 14,000 *g* for 1 min. The supernatant was discarded, the pellet was rinsed twice in 100% ethanol and the tube was centrifuged again. The pellet was dried at room temperature in a vacuum desiccator then suspended in 1 mL 0.3 mg/mL proteinase K, 100 mM NaCl, 10 mM Tris-HCl pH 8.0, 25 mM EDTA and 0.5% w/v sodium dodecyl sulphate. The suspension was incubated at 56°C for 3 days with the addition of 0.2 mg proteinase K after 24 and 48 h. DNA was purified from the solution using a commercial silica/guanidine thiocyanate kit (Wizard PCR Preps DNA Purification System, Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions. The resulting eluate was stored at -20°C and 5 μL was used in each PCR.

Method C was based on that of Plante et al.[2] Briefly, 1 mL of xylene was added to each tube and the tube was incubated at 37°C for 20 min to deparaffinize the tissue. The tube was centrifuged at 10,000 *g* for 5 min and the xylene-supernatant was discarded. The xylene extraction was then repeated. A solution of 95% ethanol and 5% methanol (1 mL) was added and the tube was centrifuged as above. The supernatant was discarded and the alcoholic extraction was repeated. The pellet was dried at 37°C for 16 h, suspended in 90 μL of 50 mM Tris-HCl pH 7.8, 1 mM $CaCl_2$ lysis buffer and subjected to three cycles of freezing and thawing in liquid nitrogen and at 60°C for 2 min each. Proteinase K was added to achieve a final concentration of 200 μg/mL in a final volume of 100 μL. The tube was incubated at 56°C for 2.5 h then 95°C for 10 min to inactivate proteinase K. EDTA was added to achieve a final concentration of 2 mM. The sample was stored at -20°C and 5 μL was used in each PCR.

Several additional treatments were applied to the DNA extracts in some experiments. Dilution in sterile distilled water at rates of 1:10 and 1:100 was undertaken while DNA purification was performed using Instagene Matrix (BIO-RAD, Hercules, California, USA) or the Wizard (see above) kit according to the manufacturers' instructions.

PCR

Primers were chosen to amplify the 5' region of IS*900*. The primer pair P90 (5'GAA GGG TGT TCG GGG CCG TCG CTT AGG) and P91 (5'GGC GTT GAG GTC GAT CGC CCA CGT GAC) spans a 413 bp region, [12] while the primer pair 150C (5' CCG CTA ATT GAG AGA TGC GAT TGG) and 921 (5'AAT CAA CTC CAG CAG CGC GGC CTC G) spans a 229 bp region[13] within the P90 to P91 region. A reaction volume of 50 µL containing 5 µL of the DNA sample, 250 ng of each of the primers P90 and P91 or 50 pmoles of primers 150C and 921, 200 µM of each of the nucleotides dATP, dTTP, dGTP and dCTP, 66.8 mM Tris-HCl, 16.6 mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 1.65 mg/mL bovine serum albumin, 10 mM beta-mercaptoethanol, 2 U Taq polymerase (Boehringer Mannheim Australia, Castle Hill), in buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.8) was used. Amplification was undertaken in 200 µL tubes in a 96-place thermal cycler (Corbett Research, Sydney) using the following conditions: one cycle of denaturation at 94°C for 3 min with the tubes removed from an ice-bath and placed in the machine after the block had reached this temperature, followed by 37 cycles of denaturation at 94°C for 30 s, annealing at 62°C for 15 s and extension at 72°C for 1 min. Products were evaluated by electrophoresis at 94V for 45 min in 2% agarose gels stained with ethidium bromide. The intensity of the bands was graded subjectively by visual examination as negative, +, ++, +++ and ++++. Positive and negative PCR controls were included in each experiment. The analytical sensitivity of the 413 bp and 229 bp PCR was assessed using a dilution series of purified DNA from *M avium* subsp *paratuberculosis* strain 316V.

Study design

Each DNA extract in each experiment was tested in both the 413 bp and the 229 bp PCR reactions. In experiment 1 the three methods of extraction of DNA and the additional treatment of 1:10 dilution of the extract were compared using two blocks from cases of multibacillary ovine Johne's disease and one control negative block.

In the second experiment the consistency of PCR detection in consecutive sections from the same block was evaluated. Four sets of consecutive sections, each set in a separate tube, from each of six blocks (four sheep with multibacillary Johne's disease and two controls) were prepared and extracted using method A.

In the third experiment selected DNA extracts from experiment 2 were retested after the various additional treatments. Five extracts were chosen because they had yielded apparently false negative results in one or both PCR assays in experiment 2. Four extracts were chosen as positive controls because they had yielded positive results in both PCR assays in experiment 2.

In the fourth experiment the sensitivity and specificity of PCR after processing by method A was evaluated by testing blocks from 47 sheep, 20 cattle and 8 goats.

Results

Analytical sensitivity of the 413 bp and 229 bp PCR protocols
Both PCR assays had similar analytical sensitivity and detected 5-50 femtograms of purified DNA from *M avium* subsp *paratuberculosis* strain 316V (Figure 1). The reaction for the 413 bp product typically resulted in a double band when the concentration of target DNA was relatively high (Figure 1), a feature observed also with crude DNA samples.

Experiment 1
Method A was simple and quick to perform. Method B was more complex than method A while method C was very labourious. DNA extracts prepared with method A gave consistent results in both PCR assays, while some apparent false negative results were obtained with DNA extracts prepared using methods B and C (Table 1). Dilution of the DNA samples after extraction using method B enabled successful amplification, but dilution was associated with reduced PCR signal from extracts prepared with methods A and C. No PCR products were obtained from extracts from the control negative block. Method A was chosen for further evaluation as it was the simplest and appeared to be the most effective.

Experiment 2
PCR results from DNA extracts prepared using method A from sequential sections off the same block were not consistent. For the 229 bp reaction there was a false negative rate of 12% across 16 samples from 4 blocks, compared to a false negative rate of 31% for the 413 bp reaction (Chi-square 1.65, P = 0.20). In addition, the intensity of the PCR product from consecutive sections off the same block was variable (Table 2). The least consistent results were obtained from sections from the block with the lowest number of acid-fast bacilli (Table 2). All sections from the two control blocks were negative in both PCR assays.

Experiment 3
The effects of additional treatment of selected DNA extracts that were prepared during experiment 2 were variable (Table 3). The results for the 229 bp reaction from untreated extracts (8 of 9 positive) were almost as good as those after DNA purification using the Wizard kit (9 of 9 positive). The intensity of the PCR product in the 229 bp reaction generally was increased by 1:10 dilution of the DNA extract and by DNA purification. Dilution of the DNA extracts at a rate of 1:10 provided the best outcome for the 413 bp reaction (6 of 9 positive). The 229 bp reaction appeared to be more sensitive than the 413 bp reaction for all treatments (Table 3).

Experiment 4
Method A (without additional treatment) was used to prepare DNA extracts from paraffin-embedded intestinal tissues from sheep, cattle and goats (Table 4). Of 34 blocks with visible acid-fast bacilli, 88.2% were positive for the 229 bp product compared to 70.6% for the 413 bp product (Chi-square 3.24, P=0.07). The greatest distinction between the 229 bp and 413 bp reactions was apparent for paraffin blocks from cattle and goats, which had been stored for longer than those from sheep (Table 4). Only 50% of the cattle and goat blocks that contained acid-fast bacilli were positive for the 413 bp product compared to 80% (cattle) - 100% (goats) for the 229 bp product. Over 85% of the blocks from cases of multibacillary ovine Johne's disease cases were PCR positive, whereas only 12.5% of blocks from cases of paucibacillary ovine Johne's disease were positive. As in

previous experiments a range of intensities of PCR products was recorded. DNA extracts were retested for the 413 bp product after dilution at a rate of 1:10 but no additional positive results were obtained and many samples that had previously given a positive result then gave a negative result. None of the 33 samples from histologically negative sheep, cattle or goats had a positive PCR result.

Discussion

The sensitivity of detection of *M avium* subsp *paratuberculosis* in paraffin-embedded tissues by PCR amplification of the 229 bp product of IS*900* in DNA extracts prepared by method A was 88.2% over 34 blocks, but ranged from 80 to 100% among blocks from sheep, cattle and goats. The sensitivity of PCR was very low (12.5%) in blocks from sheep with paucibacillary Johne's disease which did not contain demonstrable acid fast bacilli. It is likely that the sensitivity of this method for detection of *M avium* subsp *paratuberculosis* is determined to a large extent by the number of acid-fast bacilli in the lesions. With current methodologies there would appear to be little point in submitting paraffin-embedded tissues to IS*900* PCR examination unless acid-fast bacilli were visible.

Across experiments 1 to 4 the 229 bp reaction subjectively was more sensitive than the 413 bp assay, but the differences within experiments were not statistically significant. However, meta-analysis across these experiments confirmed a significant difference. Of the 80 reactions on untreated or 1:10 diluted DNA extracts detailed in Tables 1 to 4, 52 were positive in the 413 bp reaction while 69 were positive in the 229 bp reaction (Chi-square 9.80, P = 0.0017), confirming that the 229 bp reaction was more sensitive.

The diagnostic sensitivity of PCR-detection of mycobacteria in formalin-fixed paraffin-embedded tissues has not been studied extensively. A meaningful estimate of diagnostic sensitivity could not be made in some studies, for example where there was no independent test to classify samples or where the results from serial tests or different assays on the same paraffin block were aggregated.[14,15] However, there were several studies where a sensitivity estimate could be made for a single PCR test conducted on each sample (Table 5). The sensitivity that can be expected from a PCR test for mycobacterial DNA in formalin-fixed paraffin-embedded tissue is imperfect and the results of the present study are consistent with those of other studies.

The factors that may affect the sensitivity of PCR on formalin-fixed, paraffin-embedded tissues include tissue type, length of the product to be amplified, copy number of the gene to be amplified, concentrations of target DNA, concentrations of non-target DNA and other inhibitors remaining in the DNA extracts, and duration of fixation.[4-6, 16] The reason for evaluating post-treatment dilution in this study was to attempt to dilute potential inhibitors and excess non-target DNA, but the effect was variable. Although a beneficial effect of dilution was indicated by the results of experiment 3, this was not substantiated in experiment 4, for unknown reasons. DNA purification, which aims to remove inhibitors of the PCR assay gave only an equivocal benefit for the 229 bp assay, but appeared to be potentially useful for the 413 bp assay (experiment 3).

We could not specifically evaluate fixation time in formalin during this study but routine practices in the laboratory meant that it was less than 7 days, and generally about 24 h. Data on the effect of fixation on mycobacterial DNA targets are not available although a fragment of 123 bp was amplified successfully from *M tuberculosis* after fixation of tissues in 10% formalin for up to a week.[17] In controlled studies to amplify human genomic DNA, significant reductions in the efficacy of PCR were noted as fixation time in 10% formalin was extended; fragments of about 270 bp were amplified after fixation for 30 days, those of 500 to 1000 bp were amplified after 3 days but not 8 days while those of about 1300 bp could not be amplified after fixation longer than 24 h.[4] Fixation caused fragmentation of DNA, reducing the probability that lengthy DNA fragments could be amplified.[5] Cross-linking of DNA to proteins is also thought to occur during fixation.[16] The length of storage of paraffin-embedded tissues is another factor to consider. While short (270 bp) fragments of the human beta-globin gene could be amplified after storage of paraffin blocks for about 15 years, there was a rapid decline in the success of PCR for fragments greater than 500 bp after 1 to 2 years.[7] In the present study the 229 bp product of IS*900* was amplified successfully after storage of blocks for at least 6 years, although seemingly better results were obtained from ovine tissues compared to bovine and caprine tissues in the 413 bp assay, possibly because the ovine tissues had been stored for the shortest time. Some or all of the factors mentioned above probably account for the greater sensitivity of the 229 bp reaction compared to the 413 bp reaction in this study.

An important finding in this study was the potential lack of consistency in PCR result between consecutive sets of sections cut from the same paraffin block. The reasons for this are unclear but may relate to uneven distribution of acid-fast bacilli within lesions, particularly where their intensity overall was relatively low. A logical approach to this problem in the event of an apparent false negative result would be to test multiple sets of sections from each block in separate PCR tests.

Apparent false negative results in IS*900* PCR from paraffin-embedded tissues could also occur if the acid-fast bacilli observed in histological sections were organisms other than *M avium* subsp *paratuberculosis.* However, of the many hundreds of cases of ovine, bovine and caprine Johne's disease that have been observed to date in Australia and that have been subjected to tissue and/or faecal culture or molecular analysis, all have been due to *M avium* subsp *paratuberculosis*.

The specificity of PCR from paraffin-embedded tissues appeared to be extremely high as no false positive results were obtained during this study. A stringent protocol was established for the histology laboratory to ensure that block-to-block cross contamination did not occur. This was combined with routine PCR quality control procedures.

Dewaxing of tissue sections with an organic solvent is the most frequently recommended first step in preparation of paraffin-embedded material for PCR[7] but may not always be necessary.[3,10] Proteinase K digestion is often used [15] together with purification of DNA by phenol-chloroform extraction or other methods but again this may be unnecessary in some applications.[5] Of the three methods for extraction of DNA that were compared in this study, method A was the most effective. It was rapid and simple because it did not involve solvent extraction of paraffin, proteinase digestion or purification of DNA. Method A was based on methods developed for detection of human DNA, viruses[10] and *M. bovis.*[3] Method C, which was used in an earlier study to detect *M avium* subsp *paratuberculosis,* was inordinately time consuming and complex and was less sensitive than method A.

The results of this study confirm that PCR-based examination of histological material is a practical adjunct in the confirmation of Johne's disease in sheep, cattle and goats. We now routinely use method A to extract DNA from paraffin-embedded tissues and the 229 bp PCR on undiluted and 1:10 diluted extracts to identify *M avium* subsp *paratuberculosis*. Restriction digests of the PCR product with *Mse* I and *Hae* III are used to confirm the specificity of the PCR product because false positive results have recently been detected in IS*900* PCR in PCR on microbial samples derived from animals (R Whittington and D Cousins, unpublished data). Further work in our laboratory includes the optimisation of protocols for detection of the IS*1311* gene in paraffin-embedded material from cases of Johne's disease. Polymorphisms in this gene permit the differentiation of strains of *M avium* subsp *paratuberculosis* from cattle and sheep,[18,19] thus providing additional useful information to assist in the development of disease control programs.

Acknowledgments

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Captions to figures

Figure 1. Analytical sensitivity of the 229 bp (upper panel) and 413 bp (lower panel) IS*900* PCR assays using a 10-fold dilution series of DNA purified from *M avium* subsp *paratuberculosis* strain 316V, commencing with 50 ng in lane 1. Lanes 11, 23, negative control. Lanes 12, 24, molecular size markers.

Histo paper 1998, Figure 1

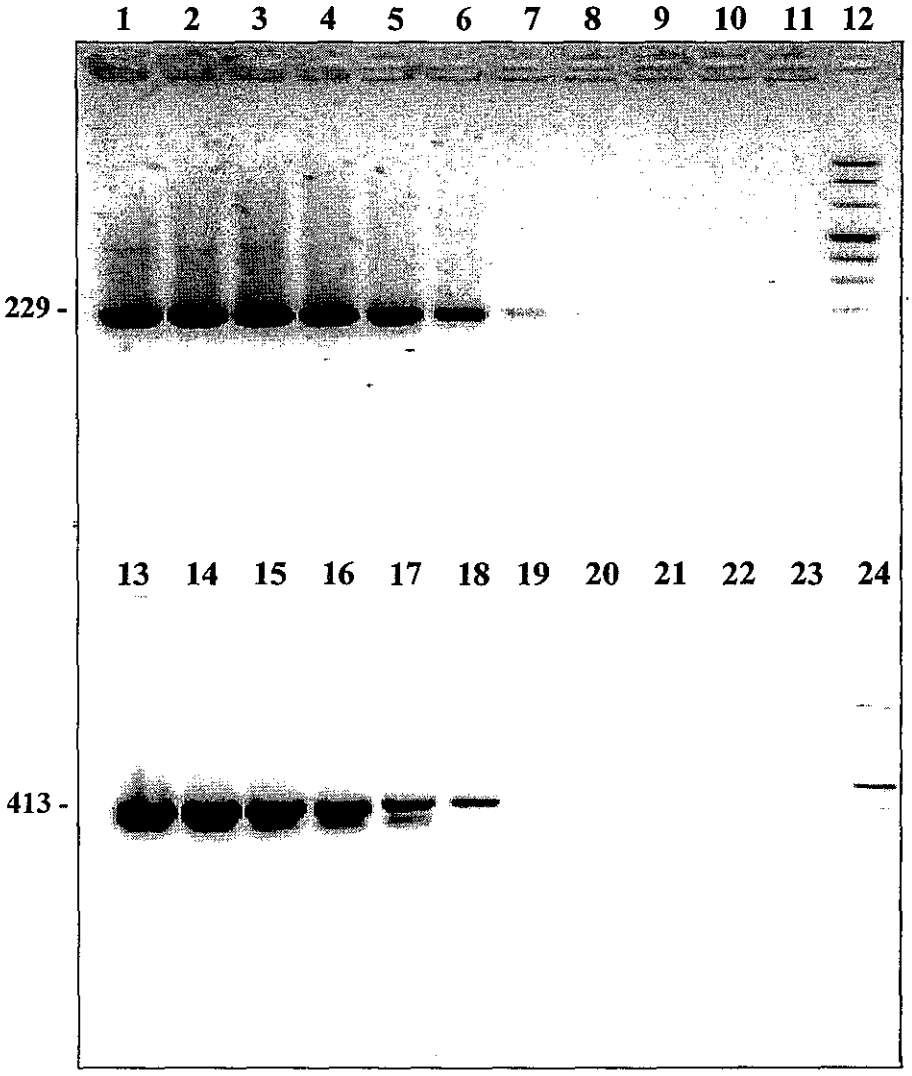

Table 1. Comparison of DNA extraction methods and PCR for different size products of IS*900* using paraffin-embedded tissues from two sheep with multibacillary Johne's disease.

Method	n	413 bp PCR product		229 bp PCR product	
		No. Positive	Intensity	No. Positive	Intensity
A undiluted extract	2	2	+++/++++	2	+++/+++
A diluted extract	2	2	+/+++	2	++/+++
B undiluted extract	2	0	-	0	-
B diluted extract	2	2	++/+++	2	++/++
C undiluted extract	2	1	+++	2	+/++
C diluted extract	2	0	-	2	+/++

Table 2. PCR results from sequential sections from paraffin blocks from four cases of multibacillary ovine Johne's disease after extraction of DNA using method A.

Block	Relative no. of acid-fast bacilli	n	413 bp PCR		229bp PCR	
			No. positive	Intensity (range)	No. positive	Intensity (range)
96/8461-1	++++	4	2	++++/+++	3	+/+++
96/1628-1	++	4	1	++	3	+
96/1628-2	++++	4	4	+/+++	4	+/+++
96/8461-3	+++	4	4	++++	4	+/+++
Total		16	11		14	

Table 3. Comparison of additional treatments of DNA extracts prepared by Method A. Data are the intensities of the PCR product from the 413 bp and 229 bp IS*900* PCR assays. AFB, relative number of acid fast bacilli.

Block	AFB	No treatment		Dilution of extract 1:10		Dilution of extract 1:100		DNA purification from extract Instagene		DNA purification from extract Wizard	
		413 bp	229 bp	413 bp	229 bp	413 bp	229 bp	413 bp	229 bp	413 bp	229 bp
96/8461-1	++++	+	+++	+++	+++	+++	++	++++	+++	-	+++
96/8461-1	++++	+	+	++	+++	+	+	++	++	-	++
96/1628-2	++++	+	+	-	++	-	+	-	++	-	++
96/1628-2	++++	+	+	++	+++	-	++	-	+++	-	+++
96/8461-1	++++	-	-	-	-	-	-	-	-	-	++
96/1628-1	++	-	+	++	++	+	+	-	-	+	++
96/1628-1	++	-	+	+	+	+	+	-	++	-	++
96/1628-1	++	-	+	+	+	+	-	-	+	+	+
96/8461-1	++++	-	+	-	-	-	-	-	+	-	+
Total positive		4	8	6	7	5	6	2	7	2	9

Table 4. Sensitivity and specificity of PCR conducted on DNA extracts from formalin-fixed, paraffin-embedded intestinal tissues prepared by method A without additional treatment.

Category	Duration of storage of blocks	Relative no. of acid-fast bacilli (range)	n	413 bp		229 bp	
				No. positive	% positive	No. positive	% positive
Sheep, Johne's disease infected flocks							
Multibacillary	1 yr	+/++++	20	17	85	18	90
Paucibacillary	1 yr	-	8	1	12.5	1	12.5
No lesions	1 yr	-	9	0	0	0	0
Sheep, Johne's disease free flocks							
No lesions	1 yr	-	10	0	0	0	0
Cattle, Johne's disease infected herds							
Multibacillary	2-5 yr	++/+++	10	5	50	8	80
Cattle, no farm history of Johne's disease							
No lesions	3-6 yr	-	10	0	0	0	0
Goats, Johne's disease infected herd							
Multibacillary	3-6 yr	++/+++	4	2	50	4	100
Goats, no farm history of Johne's disease							
No lesions	2-3 yr	-	4	0	0	0	0
Total, acid fast bacilli present			34	24	70.6	30	88.2
Total, no lesions, no acid fast bacilli present			33	0		0	

Table 5. Sensitivity of PCR for detection of mycobacteria in formalin-fixed, paraffin-embedded tissues. Data are for a single assay on each sample.

Target	No. blocks tested	% positive	Reference
M avium subsp *paratuberculosis*, IS*900*, 278 bp	21	90	2
M avium subsp *paratuberculosis*, IS*900*, 229 bp	15	80	3
M avium subsp *paratuberculosis*, IS*900*, 229 bp	34	88	this study
M avium subsp *paratuberculosis*, IS*900*, 413 bp	34	71	this study
M avium, IS*1245*, 427 bp	12	75	3
M tuberculosis, IS*6110*, 662/106 bp, nested	15	67-80[a]	6
M tuberculosis, IS*6110*, 220/123 bp, nested	15	67-87[a]	6
M bovis, IS*6110*, 123 bp	70	70	3
M leprae, repetitive sequence, 372 bp	39	80	20
M leprae, 18kDa protein, 360 bp	8	100[b]	21
Mycobacterium sp, 65-kD antigen, 234/142 bp, nested	25	64	22

[a] range among three concentrations of target DNA
[b] result may have depended on a Southern blot

APPENDIX 10

Report to Veterinary Committee & Australian Animal Health Council

Confirmation of ovine Johne's disease in cattle

file scahls2.wpd jd wp disk 4

Report prepared by:
R. Whittington, S. Ottaway, E. Sergeant
NSW Agriculture

17th August 1998

Summary
The ovine strain of *Mycobacterium paratuberculosis* has been detected retrospectively in archival laboratory samples submitted from cattle with Johne's disease between 1989 and 1995. Three farms in the central west of New South Wales were involved. In each case the cattle were exposed as calves to sheep with ovine Johne's disease. In each case disease was first detected in cattle using the bovine Johne's disease absorbed ELISA. There is no evidence that ovine Johne's disease has established in cattle herds in the central west of New South Wales, although thwere was evidence that spread may have occurred between cattle.

Recommendation

1. That the information be noted. Further investigation of the subject farms will be undertaken.

Detailed report

A. Background information

1. Since ovine Johne's disease was first detected in NSW in the early 1980s an impression was gained by experienced animal health professionals that the disease did not spread to in-contact cattle.

2. Culture of ovine strains of *Mycobacterium paratuberculosis* was not developed until 1997. Attempts at culture of the organism from sheep prior to this were almost always unsuccessful. In contrast, culture of the organism from cattle with confirmed pathological signs of Johne's disease were almost always successful.

3. Typing of *Mycobacterium paratuberculosis* to determine whether an isolate was of ovine or bovine type was developed in the 1990s and required culture of the organism followed by a DNA test known as IS900 RFLP. This test was not available in Australia until about 1996. Even then typing of *Mycobacterium paratuberculosis* from sheep was generally not possible as it was not possible to obtain isolates in culture.

4. A rapid typing test was developed in 1997 by NSW Agriculture with financial support from MRC/IWS. The test was further developed in 1998 and optimised for use on archival laboratory specimens, specifically paraffin-embedded tissues. The test is based on PCR amplification of the IS1311 gene followed by restriction endonuclease analysis (REA). Details have been published in an international scientific journal [Whittington R, Marsh I, Choy E and Cousins D (1998). Molecular and Cellular Probes (in press)].

B. Current information

1. A search of laboratory records revealed cases of Johne's disease in cattle in which there was direct or indirect contact of the infected cattle with sheep with Johne's disease AND in which the results of culture of intestinal tissues/faeces from the cattle were negative. These cases were thought to be possible examples where ovine strains of *Mycobacterium paratuberculosis* had infected cattle.

2. Paraffin embedded tissues were subjected to the IS1311 PCR-REA. In each case the suspect cases were confirmed to have been infected with an ovine strain of *Mycobacterium paratuberculosis*.

3. A summary of the history of each case is given in Table 1, with a summary of lab results in Table 2.

4. All affected cattle had been in direct or indirect contact with sheep with ovine Johne's disease as calves.

5. Multiple bovine cases may have been present on farm B1, but appropriate confirmatory tests were not undertaken and typing of *Mycobacterium paratuberculosis* was not possible on the single confirmed case as the tissues had been discarded. These cases may have been genuine cases of cattle Johne's disease, or false positive reactors, or may indicate possible spread of the ovine strain of *M. paratuberculosis* between cattle.

6. Eight histologically negative reactors were detected on farm B2. These reactors were either false positive reactors to the ELISA (unlikely, given the number) or were covertly infected animals, suggesting a significant level of spread from sheep to cattle in this instance.

7. The infection did not appear to establish in cattle on farms C1 or C2 (Table 1). One of these herds has achieved TN2 status under the NJDMAP.

Table 1. Summary of history.

Farm and year	No. Cases	Clinical signs of bovine Johne's disease	Contact with sheep known to be infected with OJD	Cases grazed as calves on a suspect property	Further testing of cattle on farm
A. 1989	1	Yes	Yes. OJD diagnosed in 1982.	Yes	Herd of 24 head destocked without further testing.
B.1 1993	1*	Yes	Probable. This bull acquired from farm B2 in 1989 when 12-18 mths of age.	Yes	Whole herd testing (approx 270 sera) in 1993-1994 with 7 reactors (2 positive & 5 inconclusive) consigned to slaughter - no further laboratory tests on reactors.
B.2 1993	1	No	Probable. Sheep on farm were not tested, but two neighbouring farms were confirmed with OJD (1984).	Yes	Whole herd testing by ELISA 1993 identified 9 positive reactors, of which 1 was confirmed by histopathology (approx 450 sera). Whole herd testing by ELISA in 1995 with negative results (460 sera).
C.1. 1995	1	Yes	Yes#. Purchased this bull 4 years previously from farm C.2.	Yes	Whole herd testing by ELISA in 1995 with negative results (160 sera).
C.2. 1995	0	No	Yes. OJD diagnosed in 1996.	n.a.	780 head tested by ELISA in 1995 with negative results. Herd currently TN2 under NJDMAP after testing in 1996 and 1997.

*probable case, not confirmed by PCR-REA
exposure on farm of origin

Table 2. Summary of laboratory findings.

Farm and year	No. Cases	Bovine Johne's disease absorbed ELISA	Tissue and or faecal culture	Histopathology	PCR-REA typing
A. 1989	1	7.78	Negative	Positive	ovine type
B.1. 1993	1*	9.47	Negative	Positive	tissues not available
B.2. 1993	1	2.15-11.46 (9 reactors)	Negative	Positive (1 of 9)	ovine type
C. 1995	1	2.46-2.89	Negative	Positive	ovine type

* originated 4 years earlier from farm B2