PROJECT TR.060

DEVELOPMENT OF A RAPID COST EFFECTIVE TEST FOR OVINE JOHNE'S DISEASE BASED ON TESTING OF POOLED FAECES

FINAL REPORT

NSW AGRICULTURE
MEAT & LIVESTOCK AUSTRALIA

Ian Marsh and Richard Whittington

NSW Agriculture
Elizabeth Macarthur Agriculture Institute
PMB 8 Camden NSW 2570
Tel 02 46 406343
Fax 02 46 406384

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1. Abstract

This project was undertaken to develop and evaluate a rapid, cost-effective, flock test for *Mycobacterium paratuberculosis* in pooled faecal samples, based on hybridisation-capture polymerase chain reaction (HC-PCR). However, a simpler direct technique (DPCR) was found to be more sensitive than HC-PCR. About 67% of culture positive pooled faecal samples were positive when tested using DPCR. In a blind trial, 83% of 12 farms identified by culture of pooled faecal samples were detected using DPCR. The cost of DPCR is no greater than that of other flock detection strategies. The test is suitable for use in the National Ovine Johne's Disease Control and Evaluation Program. A constraint exists in that Veterinary Committee does not recognise the results of DNA-based tests for *M. paratuberculosis* as being definitive. The costs of follow-up testing to confirm infection are high. Recommendations are made to improve the test and reduce its cost.

2. Executive Summary

This project was undertaken to develop and evaluate a rapid cost effective flock test for the detection of *Mycobacterium paratuberculosis* in pooled faecal samples, based on the hybridisation-capture polymerase chain reaction (HC-PCR) technique. HC-PCR was developed in the United Kingdom for the detection of *M. paratuberculosis* in tissues from Crohn's disease patients and from faeces of animals with Johne's disease. The technique was modified in Australia by NSW Agriculture at the Elizabeth Macarthur Agricultural Institute (EMAI) to enable the test to be evaluated for routine use in diagnostic testing.

The specific objectives of the project were to evaluate the HC-PCR technique on 100 infected and 100 non-infected faecal samples from individual sheep and pooled faecal samples. Critical control points were to be identified and further improvements made. The test was then to be evaluated on faecal samples tested previously by the pooled faecal culture (PFC) technique. At the conclusion of this trial the appropriate documentation was to be prepared so that the technology could be transferred to other laboratories with a view to incorporating the test into the testing regime for ovine Johne's disease in Australia.

During the initial evaluation of the HC-PCR method we included a simpler PCR test which was used to identify *M. paratuberculosis* DNA directly from the faecal extract. Surprisingly, we found the simpler direct PCR (DPCR) technique to be more sensitive than HC-PCR. We identified several aspects of the HC-PCR technique that could be responsible for its apparent lack of sensitivity including the length and location of the capture probe, deterioration of the capture probe over time and deletion of Southern blotting. Each of these was addressed, but modifications to HC-PCR did not greatly improve the sensitivity of the test. Further experiments identified inefficiencies in the hybridisation and capture events that were responsible for the lack of sensitivity. The work on HC-PCR ceased as the technical improvements required were likely to have been too time consuming and costly to identify. The remainder of the project was focused on the simpler DPCR technique.

Initially, DPCR was performed with the same PCR reaction used in HC-PCR. This targets IS*900*, a gene thought to be unique to *M. paratuberculosis*. Recent studies have shown that some environmental mycobacteria posses IS*900*-like elements that react in PCR assays for *M. paratuberculosis*. As a result, restriction endonuclease analysis (REA) of the amplified product is required to confirm results as DNA consistent with *M. paratuberculosis*. However, non-specific products from sources other than *M. paratuberculosis* in the faecal sample were amplified simultaneously and this made confirmation by REA difficult. We evaluated several new PCR reactions for IS*900* and IS*1311*, another gene used to identify *M. paratuberculosis* (Collins *et al.*, 1997, Whittington *et al*., 1998, Marsh *et al.*, 1999) , on 107 pooled faecal samples that had been evaluated by culture. A PCR reaction with superior sensitivity to the original PCR reaction was identified and was suitable for REA. The new reaction confirmed DNA consistent with *M. paratuberculosis* in 66.6 % of the culture positive pooled faecal samples.

A prospective, blind trial was performed with the new DPCR reaction on 326 pooled faecal samples submitted to EMAI for routine culture. DNA consistent with *M. paratuberculosis* was found in 66.6% of the culture positive pools. When the results were analysed by farm 83% of the properties positive by culture were positive by DPCR. An attempt was made to improve the sensitivity of DPCR by increasing the amount of faeces used from 0.1 g to 2 g. This was not successful but remains an area of future opportunity.

A rapid test for the detection *M. paratuberculosis* in pooled faecal samples could be offered to industry. Results would be available within a few days of receipt of samples at a laboratory. The costs of the new test are no greater than those of other flock tests. The technology would be transferable to detection of Johne's disease in other species of livestock. Significant constraints exist to the immediate application of this technology, not the least of which is the perception by Veterinary Committee that the results of DNA-based tests alone are not definitive for *M. paratuberculosis* infection.

3. Table of Contents

4. Main research report

4.1 Background and industry context

Mycobacterium paratuberculosis is the causative agent of Johne's disease, a chronic and incurable disease affecting many ruminant species. Two broad strains of *M. paratuberculosis* are known to exist: sheep and cattle (Collins *et al*., 1990). Unlike other infectious diseases where the incubation time is short and animals become sick in days or weeks, years are required before clinical signs are observed. This makes diagnosis and control or eradication of the disease extremely difficult. The problem has been compounded by difficulties in developing reliable, sensitive and specific diagnostic tests.

Conventional culture techniques have been the gold standard for diagnosis but can take up to 6 months before a result is achieved and are only useful in identifying cattle strains. Sheep strains of *M. paratuberculosis* have not been cultivable until recently. New culture methods have been developed for both sheep and cattle strains and have reduced the time for results to 8-12 weeks (Whittington *et al*., 1998, Whittington *et al*., 1999). However, this period is still perceived to be too long. Therefore, the quest for a rapid test continues.

Hybridisation-capture PCR (HC-PCR) was developed in the United Kingdom to detect *M. paratuberculosis* in tissues from Crohn's disease patients and in faeces from animals with Johne's disease (Millar *et al*., 1995). HC-PCR can produce a result within days of sample receipt and is suitable for sheep and cattle strains of *M. paratuberculosis*. In its original format, HC-PCR was not suitable for routine testing and the protocol was modified by NSW Agriculture at the Elizabeth Macarthur Agricultural Institute (Appendix 1) to make the test more functional and robust and to enable it to be evaluated for routine use.

Validation of the HC-PCR protocol could provide the sheep industry with a test for Johne's disease that could provide results in a timely manner. This technology would be readily transferable to other animal industries where Johne's disease is a problem.

4.2 Project objectives

The principal aim of this project was to develop a rapid and cost effective test to identify *M. paratuberculosis* in pooled faecal samples from sheep.

4.2.1 Specific tasks

1. Evaluate the sensitivity and specificity of the existing HC-PCR procedure on 100 infected and 100 non-infected faecal samples from both individual animals and pooled samples that had been previously examined by pooled faecal culture (PFC) to determine their status.

2. Identify the critical points in the test with respect to sensitivity, specificity, quality control, test repeatability and ease and modify the test accordingly. This will include assessment of sample preparation, hybridisation-capture, PCR and the methods used to detect amplified product.

3. Re-evaluate the modified HC-PCR test comparing the results with PFC and other diagnostic tests.

4. Prepare the appropriate documentation to transfer the technology to other laboratories.

The above tasks were modified during the project to address specific problems and issues (Appendices 4, 6).

4.3 Introduction

Authors of recent reviews on Johne's disease have discussed the important role molecular biology can play in the diagnosis of Johne's disease (Sanftleben,1990; Clarke, 1997; Stevenson and Sharpe, 1997). Polymerase chain reaction (PCR), a DNA-based assay, has become widely used to confirm the presence of *M. paratuberculosis* in clinical samples. While PCR is theoretically capable of detecting a single bacterium in a sample, this is rarely if ever achieved. In reality multiple organisms are required to produce a positive signal and the non-specific DNA derived from the host or other microbes or PCR inhibitory substances in clinical samples can prevent amplification particularly when faeces is the sample. These limitations make detection of early, sub-clinical or paucibacillary forms of Johne's disease by PCR difficult.

Hybridisation capture-PCR (HC-PCR), a nucleic acid sequence capture method described by Millar *et al*.(1995) was successfully used for the detection of *M. paratuberculosis* in tissues from Crohn's disease patients and on a limited number of faecal samples from cattle known to have Johne's disease. HC-PCR targets IS*900*, a gene thought to be unique to *M. paratuberculosis* (Green *et al.*, 1989) and was developed to overcome some of the limitations of PCR by concentrating *M. paratuberculosis* DNA from a large sample into a small volume while simultaneously removing PCR inhibitory substances. HC-PCR represented a test that could identify *M. paratuberculosis* in clinical samples in days and was theoretically suitable for both sheep and cattle strains of *M. paratuberculosis*.

In its original format, HC-PCR was not practically suited for routine diagnostic testing. The protocol was modified by NSW agriculture at the Elizabeth Macarthur Agricultural Institute to make the test more robust (Appendix 1). The modifications have transformed the test from a 3 to 4 day test without practical application to a 2 day test that could be evaluated for diagnostic application. Unfortunately, some of the necessary modifications have come at the cost of reduced sensitivity. Southern blotting, which was used in the original format as a final detection technique to improve the sensitivity and specificity of the test was omitted for two reasons. Firstly, newly detected environmental mycobacteria possess IS*900*-like elements that amplify in IS*900* PCR, giving identical size products to *M. paratuberculosis* which are indistinguishable by Southern blotting (Cousins *et al.*, 1999). Secondly, Southern blotting would make the cost of the test too high, limiting its use in rural industries.

The aim of this project was to evaluate the HC-PCR protocol on a large number of faecal samples from individual sheep and pooled faecal samples from sheep, make modifications to improve the test and prepare the appropriate documentation to transfer the technology to other laboratories.

4.4 Methodology

4.4.1 Polymerase chain reaction (PCR)

All PCR assays for the detection of DNA consistent with *M. paratuberculosis* in this study are defined in Table 1, using primers given in Table 2. Briefly, a reaction volume of 50 ul 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 TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.8) was used. Amplification was undertaken in 200 µL tubes with individual lids. Only one PCR tube was opened at any time throughout the entire PCR procedure. A 96-place thermal cycler (Corbett Research, Sydney, Australia) was used with the following conditions: one cycle of denaturation at 94°C for 3 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. A modified hot start procedure was used for all PCRs: samples were placed in the thermal cycler after the block had reached 94°C during the initial denaturation cycle. Unless otherwise stated, PCR results were assessed by electrophoresis in 2% agarose gels stained with ethidium bromide using the molecular size marker number VIII (Roche - Boehringer Mannheim).

4.4.2 Restriction endonuclease analysis of PCR products

The endonuclease(s) and predicted band sizes following digestion of the PCR products are given in Table 1. All restriction endonucleases were used as recommended by the manufacturer (New England Biolabs Incorporated). 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's with *Mse* I) 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 agarose gel electrophoresis in 3 and 4% gels, for IS*900* and IS*1311*, respectively.

Table 1: PCR assays used in this study and the REA used for each PCR with predicted band sizes with each strain of *M. paratuberculosis*.

PCR	Name	Target	Forward Primer	Reverse Primer	Predicted Product (bp)	Enzyme(s)	Strain	Predicted and sizes (bp)
1	900M	IS*900*	P90	P91	413	*Alw* I	Cattle and sheep	147, 266
						Mse I	Cattle and sheep	130, 283
2	900V	IS*900*	M120	M121	229	Nil		
3	900MV	IS*900*	P90	M121	367	*Mse* I.	Cattle and sheep	129, 160
4	900VM	IS*900*	M120	P91	289	Nil		
5	1311S	IS*1311*	M56	M94	268	*Hinf* I	Sheep	268
							Cattle	50, 218, 268
6	1311L	IS*1311*	M56	M119	608	*Hinf* I/*Mse* I	Sheep	285, 323
							Cattle	67, 218. 285, 323
							M. avium	134, 189, 285

Table 2: Primers used in this study.

Primer	Name	Target	Sequence
1	P90	IS*900*	GAA GGG TGT TCG GGG CCG TCG CTT AGG
2	P91	IS*900*	GGC GTT GAG GTC GAT CGC CCA CGT GAC
3	M120	IS*900*	CCG CTA ATT GAG AGA TGC GAT TGG
4	M121	IS*900*	AAT CAA CTC CAG CAG CGC GGC CTC G
5	M56	IS*1311*	GCG TGA GGC TCT GTG GTG AA
6	M94	IS*1311*	CAG CGA TCG TCG ACA GTG TG
7	M119	IS*1311*	ATG ACG ACC GCT TGG GAG AC
8	P21	IS*900*	^{bio}GCG CTC GAG TAG CCG CGT TC
9	P8	IS*900*	^{bio}TGT GGC GTT TTC CTT CGG TG

4.4.3 Evaluation of the HC-PCR protocol

4.4.3.1 Selection of faecal samples

The HC-PCR procedure was performed on 126 faecal samples from individual sheep previously tested by PFC (Appendix 2). Fifty three of these were culture positive and 73 were culture negative. Each of the individual samples was tested by direct PCR (DPCR). DPCR was performed using reaction 900M directly on the purified DNA extract without prior hybridisation and capture. HC-PCR was then performed on those samples that tested negative in DPCR. HC-PCR and DPCR was performed on 155 pooled faecal samples also previously tested by PFC (each pool

contained 1 faecal pellet from each of 50 sheep), of which 93 were culture positive and 62 were culture negative (Appendix 3).

4.4.3.2 Quality control for the HC-PCR protocol

All procedures involving magnetic beads were performed in a class 2 biological safety cabinet as this provided a confined area that could be decontaminated easily after each procedure with UV light. To avoid cross contamination of samples by aerosols only screw top 1.5 mL tubes were used and only one tube was opened at any time throughout the entire HC-PCR procedure.

4.4.3.3 DNA extraction

Faeces (0.1 to 0.2 g) was added, using a sterile wooden applicator stick, to a sterile 1.5 mL screw cap centrifuge tube containing 700 µL of 1x PBS and mixed with the stick to form a slurry. The slurry was vortexed for 2 min, incubated at 55^{o}C for 30 min in a dry heating block, vortexed again for 2 min, boiled at 105^{o}C for 30 min then centrifuged at 12,000 *g* for 5 min.

4.4.3.4 DNA purification

After centrifugation, the top of the screw capped sample tube was wiped with a tissue soaked in 70% ethanol taking care not to disturb the pellet at the base of the tube. Three hundred microlitres of the supernatant was carefully taken up into a 2.5 mL syringe containing 1 mL of 6M guanidine thiocyanate, after inserting a sterile 18 gauge needle, with attached syringe, through the lid of the tube using forceps to hold the tube to avoid needle stick injury. The needle was withdrawn, the cover was replaced over the needle and the contents of the syringe were briefly vortexed twice over 1 min. The needle was removed and discarded and a minicolumn was placed on the end of the syringe. Purification was then completed according to the manufacturer's instructions (Promega - Wizard^{TM} PCR preps DNA purification system - Cat No. A7170) using a vacuum manifold (Vac-man, Promega). Fifty microlitres of sterile water pre-heated to 80^{o}C was added to the column and allowed to stand for 2 min, followed by centrifugation at 10,000 *g* for 20 secs to elute the DNA into the attached tube. DNA samples were stored at 4^{o}C for immediate use or at -20^{o}C.

4.4.3.5 Hybridisation of the capture probe

For each sample, 40 µL of the purified faecal DNA was added to a reaction mixture comprised of 45 µL of hybridisation buffer (30 mM HEPES, 3 mM EDTA, pH7.5), 30 µL of 5 M NaCl, 25 µL of sterile water and 10µL (14.2 fmol) of the Millar capture probe. The reaction tube was boiled for 5 min in a water bath and then incubated overnight at 65^{o}C.

4.4.3.6 Capture on streptavidin coated magnetic beads

The tubes were allowed to cool to room temperature before 10 µL (6.5 x 10^{6}) of washed streptavidin-coated magnetic beads (M-280, Dynal Cat 112.06) were added and gently mixed by flicking the base of the tubes with a finger. The tubes were then layed horizontally on a platform and allowed to incubate for 4 hours at 37^{o}C with gentle rocking.

4.4.3.7 Washing the beads after capture

After capture, the beads were washed twice with bead wash buffer (BWB) (1x PBS, 0.1% w/v BSA). To do this the beads were collected at the side of the tube using a magnetic particle concentrator (MPC-M, Dynal) for 1 min, removing the supernatant and adding 1 mL of BWB gently down the side of the tube opposite the beads. The beads were gently resuspended for 2 minutes then they were allowed to re-collect on the MPC-M and 950 µL of supernatant was removed. The beads were then washed for a second time with 950 µL of BWB. The beads were collected once again and all the supernatant was removed. The magnet was removed from the MPC-M and the

beads were resuspended by gently washing the beads down the wall of the tube with in 5 µL of sterile TE. The whole 5 µL was then used in PCR.

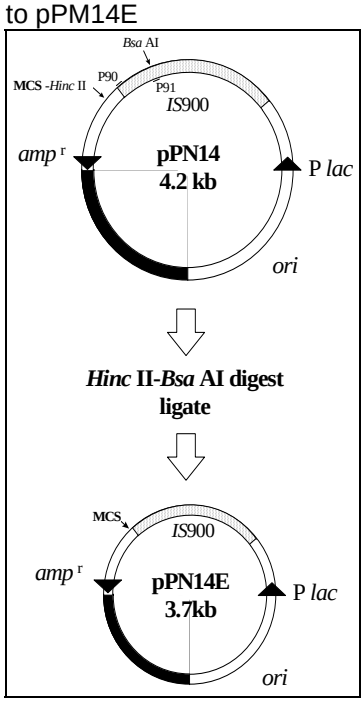
4.4.4 Modifications to the HC-PCR protocol

Based on the relatively poor performance HC-PCR compared to DPCR in the initial evaluation, several aspects of the HC-PCR protocol were checked with the view to modifying and improving the protocol . The factors examined are listed in Appendix 4.

4.4.4.1 Preparation of the pPN14E plasmid

The plasmid pPN14 (4.2 kb), which contains the full length IS*900* gene, was digested with the restriction endonucleases *Hinc* II and *Bsa* AI to excise a 488 bp fragment containing the 900M detection PCR region of IS*900* and re-ligated to produce a new plasmid, pPN14E (Figure 1). To produce large quantities of pPN14E, *E.coli* cells were transformed with pPN14E and grown on LB + AMP plates overnight. Single colonies were transferred to LB + AMP broths and grown overnight. After harvesting the *E.coli* cells the plasmid was extracted and purified using a Qiagen Miniprep spin kit (Cat .No 27104). The new plasmid pPN14E was subjected to IS*900* PCR to confirm that the detection PCR region had been removed and that the remaining IS*900* region had been retained. The new plasmid was used to produce a range of new capture probes for HC-PCR. The removal of the 488 bp fragment with the detection PCR region of IS*900* was done to ensure that capture probes could not contain template for the detection PCR reaction.

Figure 1: Diagram of the modification of pPM14 to pPM14E

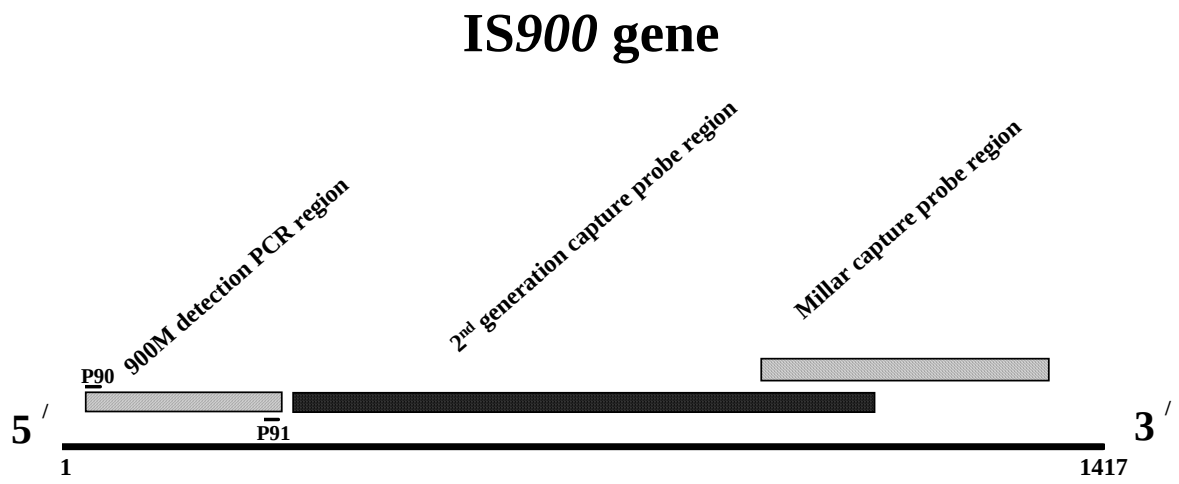


4.4.4.2 Production of the 2nd generation amplified capture probes

Biotinylated primers (M133 to M150, Table 3) were used to amplify capture probes by PCR. All primers were chosen to produce probes of varying length located on IS*900* between the detection PCR region and the Millar capture probe (Figure 2). This was done to examine the effects of probe length and location of the capture probe on test sensitivity. Primers selected using a computer programme (Primer Designer, Scientific and Educational Software) have been termed matched and those that were selected arbitrarily have been called un-matched.

Fourteen PCRs were performed as described previously with combinations of primers as shown in Table 4. After amplification the PCR product from each reaction was purified using a resin based DNA purification kit (Promega - Wizard™ PCR preps DNA purification system - Cat No. A7170) according to the manufacturer's instructions. The purified probes were diluted 1:20 in sterile water and examined after electrophoresis on a 2 % agarose gel (Figure 3). All 14 2nd generation probes and the Millar capture probe (amplified with the primers P8/P21) were diluted 1:50 in sterile water for use in HC-PCR.

Figure 2 : Schematic diagram of IS*900* and the location of the 900M detection PCR region, the region select for the 2nd generation capture probes and the position of the Millar capture probe.



4.4.4.3 Evaluation of the 2nd generation amplified capture probes

The 2nd generation probes were evaluated in HC-PCR against a dilution series of faeces from an infected sheep that was mixed with faeces from a non-infected sheep to simulate a single infected animal amongst 10, 25, 50 and 100 non-infected animals. Undiluted faeces from the infected sheep and non-infected sheep were used as the positive and negative controls, respectively. DNA extraction and purification was performed 16 times and like dilutions or controls were pooled to provide a large volume of homogenous DNA sample that could be used to evaluate each of the 2nd generation capture probes. Each of the capture probes was evaluated in HC-PCR, using four probes per experiment. DPCR was also performed on each of the faecal dilutions and controls.

4.4.4.4 Evaluation of the 2nd generation primer capture probes

Six 25 bp primer probes (M127 to M132, Table 3) were purchased to evaluate the use of very short capture probes from different locations along the IS*900* gene. The primer probe pairs M127/M128, M129/M130 and M131/M132 were evaluated as described above for the amplified capture probes.

Table 3: Primers used in this study for PCR, as capture probes (CP) and production of capture probes by PCR (CP-PCR).

Primer	Direction	Position	Sequence (5'-3')	Use
P21	F	819	^{bio}GCG CTC GAG TAG CCG CGT TC	CP-PCR
P8	R	1331(C)	^{bio}TGT GGC GTT TTC CTT CGG TG	CP-PCR
M127	F	438	^{bio}CGT TGC TGA TCG CCT TGC TCA TCG C	CP
M128	R	498(C)	^{bio}GTG CGC CCG GGA ATA TAA AGC AGC C	CP
M129	F	701	^{bio}CGC CCA GCT GCT GGA ATA CTT TCG G	CP
M130	R	751(C)	^{bio}GTT GTA GTC GAA GGC GCG TTC CAG C	CP
M131	F	1032	^{bio}ACC GCC ACG CCG AAA TCA TCC TGA G	CP
M132	R	1194(C)	^{bio}CTG ATG CGG CCG GAA TCT CGT GGT A	CP
M133	F	438	^{bio}CGT TGC TGA TCG CCT TGC TC	CP-PCR
M134	R	666(C)	^{bio}ACC AGA TCG GAA CGT CGG CT	CP-PCR
M135	F	653	^{bio}CGT TCC GAT CTG GTG GCT GA	CP-PCR
M136	R	946(C)	^{bio}CAC AGT GGC CGC CAG TTG TT	CP-PCR
M137	F	417	^{bio}ACC TCA ACG CCG GCG GCG CC	CP-PCR
M138	R	567(C)	^{bio}GCG ATG ATC GCA GCG TCT TT	CP-PCR
M139	R	717(C)	^{bio}ATT CCA GCA GCT GGG CGC GC	CP-PCR
M140	R	1017(C)	^{bio}TCC TCG ATC ATC GCG TCG GT	CP-PCR

M141	F	518	^{bio}AGT TAC CGC GGC GAA GGC AA	CP-PCR
M142	R	779(C)	^{bio}TAA GCA GGA TCA GCG CGG CA	CP-PCR
M143	F	762	^{bio}CCG CGC TGA TCC TGC TTA CT	CP-PCR
M144	R	1095(C)	^{bio}AGG AAC TCA GCG CCC AGG AT	CP-PCR
M145	F	477	^{bio}TGC TTT ATA TTC CCG GGC GC	CP-PCR
M146	R	627(C)	^{bio}ACT GCG ATG TCA TCG CCG GC	CP-PCR
M147	R	777(C)	^{bio}AGC AGG ATC AGC GCG GCA CG	CP-PCR
M148	R	1077(C)	^{bio}ATG ACG CCG AAT CCG GGC AT	CP-PCR
M149	F	717	^{bio}TAC TTT CGG CGC TGG AAC GC	CP-PCR
M150	F	777	^{bio}TTA CTG GCT ACC AAA CTC CC	CP-PCR

Table 4: Combination of biotinylated primers used to amplify the 2nd generation probes.

Reaction	Forward Primer	Forward Primer	Probe length	Primer pairing
1	M133	M134	229	Matched
2	M133	M136	509	Matched
3	M135	M136	294	Matched
4	M137	M138	151	Unmatched
5	M137	M139	301	Unmatched
6	M137	M140	601	Unmatched
7	M149	M140	301	Unmatched
8	M141	M142	262	Matched
9	M141	M144	578	Matched
10	M143	M144	334	Unmatched
11	M145	M146	151	Unmatched
12	M145	M147	301	Unmatched
13	M145	M148	601	Unmatched
14	M150	M148	301	Unmatched

4.4.4.5 Silver staining with polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE) with silver staining was evaluated as an alternate method of detection to agarose gel electrophoresis with ethidium bromide staining. This was done in an attempt to overcome the loss of sensitivity due to the omission of Southern blotting from the protocol. PAGE was performed as described by Bassam *et al.*, (1991). Briefly, 4 gels were prepared simultaneously with: 6.9 mL 40% acrylamide/bis. (19:1, Bio Rad, Cat. No. 161-0156), 11 mL 5x TBE (0.45M Tris, 0.45M boric acid, 0.01M EDTA), 36.7 mL of sterile water, 365 µL 10% APS (Bio Rad Cat. No. 161-0700) and 44 µL TEMED (Bio Rad Cat. No. 161-0800). After the gels were poured they were allowed to set for at least 1 hour before use. The unused gels were stored wrapped in moist paper towel and plastic food wrap at 4^{o}C. When running the gels, 20 µL of amplified product was loaded per lane with 4 µL of loading buffer (25% w/v bromophenol blue, 40% sucrose in sterile water). The gels were run at 20-30 mA/gel for 40-50 minutes with a recycling water cooling system. The gels were silver stained (Appendix 5) and then dried using a gel drying system (Novex Dry Ease^{TM}, Cat. No. N12387A).

4.4.4.6 Evaluation of PAGE for the detection of amplified product

Analytical sensitivity of PAGE and agarose gel electrophoresis was determined using amplified product from reaction 900M with *M. paratuberculosis* 316V DNA as target. Dilutions of 1 in 10, 1 in 20, 1 in 50 and serial 10 fold dilutions from 10^{-2} to 10^{-10} were used.

Diagnostic sensitivity was determined using amplified product from reaction 900M conducted on samples submitted for PFC. The samples were chosen based on previous agarose gel

electrophoresis results and included 2 weak, 2 medium and 2 strong PCR reactions. After PCR the amplified product was subjected to REA with *Alw* I. The results for both PCR and REA were evaluated by PAGE and agarose gel electrophoresis.

4.4.4.7 Examination of the shelf life of the Millar capture probe

To evaluate the shelf life of the Millar capture probe, an experiment was conducted using a batch of this capture probe that had been prepared in 1997 and stored at -20^{o}C. This batch of capture probe was last used for HC-PCR in 1997 on a dilution series of faeces prepared to simulate a single infected animal amongst 10, 25, 50 and 100 non-infected animals.

The 1997 experiment was repeated using the same faecal samples, which had been stored at -20^{o}C. The faecal dilution series was prepared by mixing a single pellet from the infected sheep with 9, 24, 49 and 99 pellets from a known non-infected sheep. Each dilution of pellets was then mixed thoroughly for two 30 second runs in a commercial blender (Waring® Commercial Blender). The faeces was scraped off the wall of the blender between each run with a sterile spatula. This procedure was then repeated with faeces from a second known infected sheep (also tested in the 1997 experiment). Both animals were chosen based on histological examination and represented multibacillary forms of Johne's disease. The HC-PCR, as described previously, was performed on 0.1 to 0.2 grams of the faeces from each dilution. Undiluted infected faeces from both animals was included as a positive controls while the non-infected faeces was used as the negative control.

4.4.4.8 Assessment of reasons for poor sensitivity of HC-PCR

4.4.4.8.1 **Experiment 59**

The efficiency of capture of biotinylated capture probe by streptavidin coated beads was determined by titrating the Millar capture probe (1.7µg/mL to 1.7 fg/mL final concentration in 450 µL hybridisation reaction) in hybridisation buffer without target DNA and performing a standard bead capture. PCR with the primers P8/P21, which are specific for the capture probe, was performed on the beads and the amplified product was then serially diluted (10^{-1} to 10^{-4}) to quantify the results in agarose gel electrophoresis. The capture probe was precipitated from the supernatant left after removal of beads from the hybridisation buffer, using ethanol, and then reconstituted in TE. The solution was serially diluted (10^{-1} to 10^{-4}) then subjected to PCR with primers P8/P21. Amplified product was evaluated by agarose gel electrophoresis.

4.4.4.8.2 **Experiment 60**

The efficiency of probe hybridisation to target DNA and bead capture of biotinylated probe was determined by performing a titration of *M. paratuberculosis* 316V target DNA (11.1ng/mL to 111ag/mL final concentration in 450 µL hybridisation buffer) with Millar capture probe at a final concentration of 17ng/mL. After hybridisation-capture the beads were processed as usual and subjected to PCR with reaction 900M. The amplified product was serially diluted (10^{-1} to 10^{-4}) and evaluated in agarose gel electrophoresis. The supernatant from the hybridisation reaction was retained and residual DNA, comprising target DNA and capture probe, was precipitated in ethanol and reconstituted in TE. This solution was serially diluted (10^{-1} to 10^{-4}) and evaluated by PCR in reaction 900M and with primers P8/P21 to determine the presence of residual target DNA and capture probe, respectively. The amplified product from both reactions was evaluated in agarose gel electrophoresis.

4.4.4.8.3 **Experiment 61**

The previous experiment was repeated with two concentrations of target DNA (1.11ng/mL and 111fg/mL) and a series of concentrations of Millar capture probe (170ng/mL to 170 fg/mL).

4.4.4.8.4 Experiment 61b

The previous experiment was repeated as a more extensive cross titration of target DNA (27 ng/mL to 27 fg/mL) and Millar capture probe (100 ng/mL to 6.25 ng/mL).

4.4.5 Direct PCR

Even with modifications HC-PCR had inherent problems with sensitivity that were unrelated to the length or location of the capture probe, effects of time and storage of the capture probe or choice of detection methods. Research on HC-PCR ceased and the project focused on the simpler DPCR method, after recommendation to MLA (Appendix 6).

4.4.5.1 Detection of *M. paratuberculosis* in pooled faecal samples by DPCR

The detection of *M. paratuberculosis* directly from faeces was evaluated using 4 PCR assays (Table 1, assays 1- 4) targeting IS*900*. Ten samples (Appendix 3, samples 206, 207, 209-216) were used to compare the assays. The sample set was comprised of 6 culture positive and 4 culture negative faecal pools.

4.4.5.2 Analytical sensitivity and specificity of reaction 900VM

To evaluate the analytical sensitivity of reaction 900VM, PCR was performed on a 10 fold dilution series of *M. paratuberculosis* DNA (10 pg/µL to 1 fg/µL) that had been quantified using a Pharmacia Biotech GeneQuant II RNA/DNA calculator. PCR with reaction 900M, which is currently used for routine detection of *M. paratuberculosis* in our laboratory, was also performed on the dilution series and the results were compared. A reference collection of 27 mycobacterial species (Table 5, S1-S27) were cultured on Lowenstein-Jensen agar at 37°C (except *M. marinum*, 22°C) and used to evaluate the analytical specificity. Boiled lysates of a colony of each were examined in each assay.

Table 5: Mycobacterial species used to assess the analytical 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. thermoresistibile*	ATCC 19527
S20	*M. triviale*	ATCC 23292
S21	*M. tuberculosis*	NCTC 7416
S22	*M. vaccae*	ATCC 15483
S23	*M. xenopi*	NCTC 10042
S24	*M. genevense*	EMAI 95/0808
S25	*M. paratuberculosis*	EMAI 96/360
S26	*M. shimoidei*	EMAI FDC 2338
S27	*M. bovis*	EMAI 1995 GA8C

4.4.6 Preliminary evaluation of DPCR on pooled faecal samples

4.4.6.1 Pooled faecal samples

One hundred and seven pooled faecal samples that had been tested previously by culture were evaluated. Of the 107 samples, 81 were culture positive and 26 were culture negative. Briefly, each pool was comprised of 50 faecal pellets representing 50 individual sheep. Each pool was blended to form an homogenous mixture of faeces then evaluated by PFC. The remaining faeces was stored at -80^{o}C until the completion of PFC and then placed at -20^{o}C. The PFC results for the 107 samples used in this study are given in Appendix 7.

4.4.6.2 Evaluation of DPCR using IS*900* and IS*1311* as the targets for PCR

DNA extractions were performed all 107 pooled faecal samples in batches with a maximum of 11 samples per batch. The following controls were included with each extraction batch: positive control faeces (faeces from an infected sheep), negative control faeces (faeces from a non-infected sheep) and a process control (sterile water), the later being subject to the entire DPCR procedure to evaluate the integrity of the reagents. Thus there were a maximum of 14 samples per extraction batch. DNA extracts from all 107 pooled faecal samples were tested using reactions 900M, 900VM 1311S and 1311L. The following controls were included with each PCR batch: positive control DNA (100 fg/µL *M. paratuberculosis* DNA) , negative control (sterile water) and a process control (PCR cocktail without sample). The process control was not opened until the completion of the PCR and was designed to assess the integrity of the PCR reagents. After PCR, samples that gave a positive result were subjected to REA appropriate to each PCR assay. An REA result was considered to be positive only when complete digestion of the PCR product to a recognised REA pattern, without non-specific bands, was achieved. Any positive PCR results where non-specific bands co-amplified were considered inconclusive as these non-specific bands could cause confusion when interpreting REA results. Pools that were culture positive but either negative or weakly positive in DPCR when examined by agarose gel electrophoresis were further evaluated with PAGE.

4.4.7 Blind evaluation of DPCR using pooled faecal samples

4.4.7.1 Pooled faecal samples

Three hundred and twenty six pooled faecal samples that were submitted to EMAI for routine culture (PFC) were evaluated in DPCR using reaction 900VM. Briefly, each pool was comprised of 50 faecal pellets representing 50 individual sheep. Each pool was blended to form an homogenous mixture of faeces then evaluated by culture. The remaining faeces was stored at -80^{o}C until the

completion of culture and then placed at -20^{o}C. The culture results for the 326 pools used in this trial are given in Appendix 8.

The protocol used for DPCR included use of reaction 900VM, evaluation of PCR product in agarose and REA in agarose. PAGE was not undertaken.

This was a blind prospective study as the results of PFC were unknown when DPCR tests commenced and were not made available to the researchers until all DPCR results had been finalised. The aim of this trial was to test pooled faecal samples in DPCR without knowledge of their status and match the results of the two tests at the completion of the trial. This was done to avoid any bias in the results and is a close approximation to 'real-world' use of the test

4.4.8 Direct PCR using large faecal samples

Ordinarily 0.1 to 0.2 grams of faeces are processed in DPCR to extract *M. paratuberculosis*. In an attempt to increase the sensitivity of DPCR a series of experiments were conducted to evaluate the use of larger volume of faeces, up to 2 grams. This was done to increase the number of *M. paratuberculosis* available for PCR.

4.4.8.1 Preparation of diluted faecal samples

A faecal dilution series was prepared, as previously described, by adding faeces from an infected sheep with faeces from a non-infected sheep to give final dilutions of 1 in 10, 1 in 50, 1 in 100, 1 in 500, 1 in 1000 and 1 in 5000.

4.4.8.2 Evaluation of faecal dilution series by DPCR

DPCR was performed on the faecal dilution series described above using the faeces from the infected sheep as the positive control and the faeces from the non-infected sheep as the negative control. This was done to determine the analytical sensitivity of the DPCR method using 0.1 to 0.2 grams of faeces and to select a strong positive dilution and a weak positive dilution that could be used to evaluate the large volume faecal extraction methods.

4.4.8.3 Large volume faeces experiments

Three methods (A, B, C) that differed in buffer and supernatant volumes were evaluated for isolating *M. paratuberculosis* from large volume faecal samples:

A. *EMAI based method*. Faeces suspended in 15 mL of buffer and 5 mL of supernatant used to isolate *M. paratuberculosis* (Whittington *et al.*, 1998).

B. *Whitlock based method*. Faeces suspended in 35 mL of buffer and 5 mL of supernatant used to isolate *M. paratuberculosis* (Whitlock, 1990).

C. *NADC based method*. Faeces suspended in 35 mL of buffer and 30 mL of supernatant used to isolate *M. paratuberculosis* (Stabel, 1997).

For each method, 2 grams of faeces was added to a 50 mL centrifuge tube, the required volume of buffer was added and the contents of the tube was mixed thoroughly. The contents of the tube was allowed to settle after which the supernatant was transferred to a new tube and allowed to settle for a second time. The supernatant was transferred to a new tube, centrifuged and the supernatant was discarded. The settlement times and centrifugation conditions for each experiment are given in Table 6. The pellet was re-suspended in 700 µL of PBS and purified according to the HC-PCR method (sections 4.4.4.3 to 4.4.3.4).

Two grams of faeces at 2 dilutions was tested. One strong positive sample (1 in 100) and one weak positive sample (1 in 1000) plus faeces from the non-infected sheep as the negative control were used for all experiments.

Table 6: Combinations of buffers final volumes of buffers and centrifugation speeds used to extract *M. paratuberculosis* from large volumes of faeces.

Experiment	Method	Buffer	Mix (min)	Settle (min)	Settle (min)	Centrifuge	RPM	g force
1	A	PBS	30	30	30	Beckman BT	1145	300
		PBS	30	30	30	Beckman BT	1984	900
		PBS	30	30	30	Beckman BT	3437	2700
	B	PBS	30	30	30	Beckman BT	1145	300
		PBS	30	30	30	Beckman BT	1984	900
		PBS	30	30	30	Beckman BT	3437	2700
	C	PBS	30	30	30	Beckman BT	1145	300
		PBS	30	30	30	Beckman BT	1984	900
		PBS	30	30	30	Beckman BT	3437	2700
2	A	Saline	30	30	30	Beckman BT	1984	900
		Saline	30	30	30	Beckman BT	3437	2700
	A	Saline/Tween 80	30	30	30	Beckman BT	1984	900
		Saline/Tween 80	30	30	30	Beckman BT	3437	2700
3	A	PBS	30	30	30	Beckman BT	1984	900
		PBS	30	30	30	Beckman BT	3437	2700
		PBS	30	30	30	Beckman HS	4200	5000
4	A	PBS	30	30	30	Sorvall	8000	5000
		PBS	30	30	30	Sorvall	11,500	10,000
		PBS	30	30	30	Sorvall	14,000	15,000
	C	PBS	30	30	30	Sorvall	8000	5000
		PBS	30	30	30	Sorvall	11,500	10,000
		PBS	30	30	30	Sorvall	14,000	15,000
5	C	PBS	120	O/N	30	Sorvall	14,000	15,000
		PBS	O/N	120	30	Sorvall	14,000	15,000
		PBS	60	30	30	Sorvall	14,000	15,000

4.4.9 SYBR Green detection of DNA with PAGE

Although silver staining of polyacrylamide gels was found to be more sensitive than agarose gel electrophoresis, the silver staining technique itself was found to be difficult and time consuming. Recent developments in DNA detection has led to the use of a DNA stain known as SYBR Green as replacement for silver staining. This new stain has been reported to have detection capabilities equal to silver staining but requires only 30 min post staining of polyacrylamide gels as opposed to the 2 hours required for silver staining. SYBR Green stained gels can be rapidly recorded using computer based photographic imaging systems that provide permanent electronic records of gels within minutes of completing the staining procedure. Whilst these images can be obtained with silver stained gels they require more time consuming scanning procedures.

To enhance the suitability of PAGE for routine use in conjunction with PCR for diagnostic testing for Johne's disease we evaluated the SYBR Green stain as a replacement for silver staining. The analytical sensitivity of SYBR Green staining versus silver staining of PAGE was determined by

evaluating a dilution series of amplified product from reaction 900VM, derived from *M. paratuberculosis* DNA (dilutions of 1 in 10, 1 in 20, 1 in 50 and serial 10 fold dilutions from 10^{-2} to 10^{-10}).

4.5 Results

4.5.1 Evaluation of the HC-PCR protocol

Individual faecal samples from 126 sheep were tested. Of the 53 culture positive faeces, 39 (74%) were positive by DPCR and of the 14 remaining samples, 3 were positive using HC-PCR, 10 remained negative after HC-PCR and 1 was not tested due to insufficient faeces (Table 7). The cases detected included 8 with unknown histology, 3 with suggestive histology (no acid fast bacilli), 1 paucibacilliary case and 27 multibacilliary cases, 3 of which had very mild lesions. The 10 culture positive samples that were not detected by DPCR/HC-PCR included 1 multibacilliary case, 3 paucibacilliary cases, 4 cases that lacked histological lesions and 2 cases that were not assessed by histopathology.

Of 73 culture negative faeces, 6 were positive in DPCR and a further 7 were positive in HC-PCR. Three of the 6 DPCR positive samples were from 2 infected flocks while three were from 3 suspect flocks. The seven HC-PCR positive samples were from 5 infected flocks.

The sensitivity of DPCR was compared to HC-PCR using pooled faeces. Relative to culture, the sensitivity of DPCR was 49.5% while that for HC-PCR was 45.2% (Table 8). Both tests identified positive samples from among the 73 culture negative pools. Three of the 4 DPCR positive/culture negative pools came from known-infected flocks and were from submissions that also included culture positive pools. The remaining pool was from a suspect flock. All 8 pools that were culture negative/HC-PCR positive came from known-infected flocks (6 pools were from one flock) and from among culture positive pools. Therefore the results of both assays were meaningful.

The relatively poor sensitivity of HC-PCR stimulated research to identify the cause of the problem and find a solution.

Table 7: Comparison of culture and PCR tests on faeces from individual sheep. HC-PCR was undertaken only on those samples that tested negative in DPCR.

Culture		DPCR		HC-PCR	
Positive	n = 53	Positive	n = 39	not tested	
		Negative	n = 14	Positive	n = 3
				Negative	n = 10
				not tested	n = 1
Negative	n = 73	Positive	n = 6	not tested	
		Negative	n = 67	Positive	n = 7
				Negative	n = 60

Table 8: Comparison of culture and PCR tests on pooled faeces (pooling rate= 50 sheep/sample).

Culture		DPCR		HC-PCR	
Positive	n = 93	Positive	n = 46	Positive .	n = 34
				Negative	n = 12
		Negative	n = 47	Positive	n = 8
				Negative	n = 39
Negative	n = 73	Positive	n = 4	Positive	n = 1

		Negative	n = 3
Negative	n = 58	Positive	n = 7
		Negative	n = 51

4.5.2 Modifications to the HC-PCR protocol

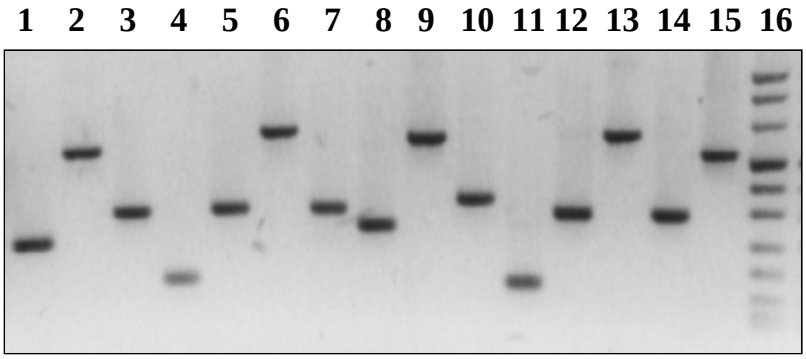
4.5.2.1 Preparation of the pPN14E plasmid

The detection PCR region of IS*900* was successfully removed from the pPN14 plasmid as indicated by negative results in 900M PCR. Positive results from PCR using primers M141/M144 indicated that the region downstream from the detection PCR region had been retained in the pPN14E plasmid making it suitable for the production of the 2nd generation capture probes.

4.5.2.2 Production of the 2nd generation amplified capture probes

All 14 PCR reactions from Table 4 resulted in amplified product of the predicted size from plasmid pPN14E (Figure 3). After PCR, the amplified product was purified and diluted 1 in 20. Twenty microlitres of each of the diluted products was run on a 2 % agarose gel with DNA molecular weight marker VI (Roche) to confirm the efficiency of the purification procedure.

Figure 3: Amplified product from each of the PCR reactions used to prepare the 14 2nd generation capture probes. Lanes 1 to 14 correspond with reactions 1 to 14 in table 4, lane 15 is the original 513 bp capture probe and lane 16 is a molecular size marker (Roche DNA molecular weight marker VIII).



4.5.2.3 Evaluation of the 2nd generation amplified capture probes

As a control and reference point, HC-PCR with the Millar capture probe was performed on two occasions with the faecal dilution series samples. Diminishing product yield was observed from HC-PCR as the faecal dilutions increased, with almost identical results in both experiments. There was a similar pattern with DPCR on the same samples but the intensities of the DPCR products were markedly stronger than those n HC-PCR. This confirmed again that DPCR was more sensitive than HC-PCR with the Millar capture probe.

Each of the 2nd second generation amplified capture probes were functional in HC-PCR as amplified product was obtained for most of the faecal dilutions. Many of the 2nd generation capture probes (2, 3, 4, 5, 6, 7, 8, 11 and 12) produced superior results compared to the Millar capture probe while the remainder (1, 9, 10, 13 and 14) performed equally. However, when compared to DPCR, almost all HC-PCR assays with 2nd generation probes lacked sensitivity. Probe 3 produced HC-PCR results that were greater than or equal to DPCR.

4.5.2.4 Evaluation of the 2nd generation primer capture probes

The 3 sets of primer probes were functional in HC-PCR. However, the reactions were less sensitive than DPCR.

4.5.2.5 PAGE for the detection of amplified product

Amplified product from 900M PCR with *M. paratuberculosis* DNA as target was serially diluted and evaluated in both agarose gel electrophoresis and in PAGE in order to determine the analytical sensitivity of each detection method. Amplified product was observed at a dilution of 10^{-2} in agarose gel electrophoresis but at a dilution of 10^{-4} in PAGE. Use of PAGE represented a 100 to 1000 fold increase in the detectable limit of amplified product.

Six samples that had been previously examined by agarose gel electrophoresis were re-examined. Amplified product from 5 of the 6 samples could be observed in both agarose gel electrophoresis and PAGE. However, the amplified product was much more intense in PAGE. Amplified product from the last sample could be detected only by PAGE. When REA was performed on the amplified products, only 5 samples could be confirmed as DNA consistent with *M. paratuberculosis* in agarose gel electrophoresis, whereas all 6 samples produced an REA pattern consistent with *M. paratuberculosis* with PAGE. Clearly PAGE is useful for the detection of amplified product from samples that have negative or weak results in agarose gel electrophoresis. Use of PAGE would increase the sensitivity of HC-PCR and DPCR.

4.5.2.6 Examination of the shelf life of the 513 bp capture probe

Samples from a faecal dilution series were retested after an interval of 2 years in order to evaluate the shelf life of capture probe. The amplified product from each sample was graded (0, 1+ to 4 +) relative to the controls to compensate for any variances between the two experiments.

Amplified product was observed with the undiluted faeces from both infected sheep and at all dilutions of faeces in both experiments. Whilst the 1997 PCR results were generally more intense than the 1999 results, so too were the results for positive PCR controls suggesting that variation between the two sets of results was attributable to run-ton-run variation in PCR and not as a result of the hybridisation-capture component or the Millar capture probe. In summary, the Millar capture probe had not deteriorated over the two year period it had been stored at -20^{o}C.

Table 9: Evaluation of the shelf life of the 513 bp capture probe. The PCR result for each sample was graded; 1+ to 4+, relative to the HC-PCR and PCR positive controls.

Sample	1997 result	1999 result
Neat (sample A)	3+	4+
1 in 10	3+	3+
1 in 25	3+	3+
1 in 50	3+	3+
1 in 100	3+	2+
Neat (sample B)	4+	3+
1 in 10	3+	2+
1 in 25	3+	1+
1 in 50	3+	2+
1 in 100	3+	3+
HC-PCR positive control faeces	4+	3+
HC-PCR negative control faeces	-	-
HC-PCR process control	-	-
Hybridisation control	-	-
Capture control	-	-
PCR positive control (DNA)	3+	2+
PCR negative control (MQW)	-	-
PCR process control	-	-

4.5.3 Assessment of reasons for poor sensitivity of HC-PCR

4.5.3.1 Experiment 59

To determine if the biotinylated capture probe could be depleted from the hybridisation reaction by binding to streptavidin magnetic beads an experiment was performed to detect capture probe bound to the beads and in the resulting supernatant of the hybridisation reaction. Amplified product derived from capture probe bound to the beads was obtained only when the final concentration of probe in the 450 µL volume of hybridisation buffer was greater than or equal to 1.7 ng/mL. However, residual probe was present in the hybridisation buffer supernatant at all dilutions of the capture probe tested. These results indicate that even where streptavidin binding sites on the beads were unsaturated the binding of biotinylated probe was highly inefficient (Table 10).

Table 10: PCR with primers P8/P21to detect capture probe bound to beads or in the supernatant from the hybridisation buffer.

Probe concentration	PCR results	
	Beads	Supernatant
1.7 µg/mL	4+	3+
170 ng/mL	Tr	3+
17 ng/mL	-	3+
1.7 ng/mL	-	2+
170 fg/mL	-	1+
17 fg/mL	-	1+
1.7 fg/mL	-	1+

4.5.3.2 Experiment 60

The efficiency of removal of target DNA from hybridisation buffer by the combined effects of hybridisation to biotinylated probe and capture of probe by magnetic beads was evaluated. Target DNA was detected on beads when the hybridisation buffer was seeded with target DNA at a concentration of greater than 111 fg/mL. However, there was residual DNA in the hybridisation reaction supernatant when the hybridisation buffer was seeded with target DNA at final concentrations as low as 111 fg/mL, i.e. detection of target DNA on beads was less sensitive than detection of target DNA in the waste hybridisation supernatant. The amount of target DNA remaining in solution was proportional to the amount of target DNA added to the hybridisation reaction, which is consistent with inefficient hybridisation by probe or capture of probe-target DNA complex by the magnetic beads. As in the previous experiment, residual capture probe was detected in the supernatant, and was not affected by the concentration of target DNA in the hybridisation buffer (Table 11).

4.5.3.3 Experiment 61

The detection of target DNA on beads was no more effective than its detection from hybridisation supernatant. Probe concentrations less than 17ng/mL were limiting in that target DNA was then less effectively detected on beads. Furthermore the amount of residual target DNA in the supernatant was not influenced by probe concentration (Table 12).

Table 11: Titration of *M. paratuberculosis* target DNA to detect capture probe-target DNA bound to beads, with reaction 900M and residual target DNA and capture probe in the supernatant from the hybridisation

buffer with 900M and primers P8/P21, respectively.

Target DNA concentration	PCR results		
	900M on beads	900M on supernatant	P8/P21 on supernatant
11.1 ng/mL	4+	4+	4+
1.11 ng/mL	2+	2+	4+
111 fg/mL	Tr	1+	4+
11.1fg/mL	Tr	Tr	4+
1.11 fg/mL	-	Tr	4+
111 ag/mL	-	Tr	4+

Table 12: Further analysis of *M. paratuberculosis* target DNA bound to beads, with reaction 900M and residual target DNA and capture probe in the supernatant from the hybridisation buffer with 900M and primers P8/P21, respectively

Probe concentration	PCR results at 2 dilutions of target DNA					
	900M on beads		900M on supernatant		P8/P21 on supernatant	
	1.11ng/mL	111 fg/mL	1.11ng/mL	111 fg/mL	1.11ng/mL	111 fg/mL
170 ng/mL	2+	1+	2+	Tr	4+	4+
17 ng/mL	2+	Tr	2+	Tr	4+	4+
1.7 ng/mL	1+	-	2+	Tr	4+	4+
170 fg/mL	Tr	-	2+	Tr	4+	4+

4.5.3.4 Experiment 61b

The detection of target DNA was less effective from magnetic beads (Table 13) than from the supernatant of the hybridisation buffer (Table 14), the difference being two $\log_{10}$ dilutions of target DNA. Target DNA was detected on beads in all treatments where the hybridisation reaction contained 27 or 2.7 ng/mL *M. paratuberculosis* DNA and there was no effect of concentration of the capture probe over the range tested. Both target DNA and capture probe were detected in the supernatant remaining after removal of beads, indicating incomplete probe-target hybridisation and/or capture of probe by magnetic beads (Tables 14 and 15).

Table 13: Reaction 900M on the beads, results are grade 4+ (highest) to 1+ and trace (Tr, lowest).

Capture probe (ng per mL)	Target DNA (ng per mL)				Negative control
	27	2.7	0.27	0.027	
100	2+	Tr	-	-	-
50	2+	Tr	-	-	-
25	1+	Tr	-	-	-
12.5	2+	Tr	Tr	-	-
6.25	2+	Tr	-	-	-

Table 14: Reaction 900M on supernatant, results are grade 4+ (highest) to 1+ and trace (Tr, lowest).

Capture probe (ng per mL)	Target DNA (ng per mL)				Negative control
	27	2.7	0.27	0.027	
100	3+	2+	1+	-	-

50	-	-	-	-	-
25	3+	-	-	-	-
12.5	3+	1+	1+	-	-
6.25	2+	2+	1+	Tr	-

Table 15: PCR with primers P8/P21on supernatant, results are grade 4+ (highest) to 1+ and trace (Tr, lowest).

Capture probe	Target DNA (ng per mL)				Negative
(ng per mL)	27	2.7	0.27	0.027	control
100	-	3+	4+	4+	4+
50	4+	3+	-	-	2+
25	3+	3+	3+	4+	3+
12.5	-	-	3+	4+	4+
6.25	4+	4+	4+	-	-

4.5.4 Direct PCR

4.5.4.1 Preliminary comparison of four IS*900* PCR assays for the detection of M. paratuberculosis in pooled faecal samples

Four IS*900* PCR assays (1 to 4, Table 1) were evaluated for the detection of *M. paratuberculosis* in DNA purified directly from pooled faecal samples from sheep (DPCR). Reactions 900M and 900V detected 5 of 6 and 6 of 6 culture positive pools, respectively. However, both assays produced varying degrees of non-specific PCR product or DNA smearing from faecal samples regardless of their culture status. Reaction 900VM correctly identified 6 of 6 culture positive pools with greater yields of PCR product than reaction 900M and with negligible non-specific PCR product or smearing. All 6 PCR positive samples and the PCR positive controls from reaction 900VM were confirmed as being DNA consistent with *M. paratuberculosis* after REA with *Mse* I. Amplified product was observed only in the PCR positive control in reaction 900MV

4.5.4.2 Analytical sensitivity and specificity

Reactions 900M and 900VM had similar analytical sensitivities when applied to purified DNA. Both assays were capable of detecting *M. paratuberculosis* DNA at a concentration of 1 fg/µL. However, reaction 900VM appeared to offer results with less non-specific banding than reaction 900M which sometimes has produced a double band even from purified DNA.

DNA from 27 mycobacterial species (Table 5) were examined with reaction 900VM to determine the analytical specificity of the reaction. *M. paratuberculosis* and one other species produced amplified product. REA with *Mse* I was used to differentiate between the two species. *M. paratuberculosis* produced an REA pattern consistent with DNA from this species while a single band was observed from the other species after REA indicating absence of a cut site. REA is required to confirm the specificity of IS900 PCR and this is already part of protocols in the relevant laboratories in Australia.

4.5.5 Evaluation of direct PCR on pooled faecal samples

4.5.5.1 Preliminary evaluation of DPCR on pooled faecal samples

Reactions 900M, 900VM, 1311S and 1311L were used to screen 107 pooled faecal samples with known culture status (81 pools culture positive) to determine whether the IS*900* gene or the IS*1311* gene or both were appropriate targets for DPCR. REA was undertaken on PCR products from reactions 900VM, 1311S and 1311L but could not be done for products of reaction 900M which produced non-specific banding.

Reaction 900M was the least sensitive; only 38.3% of 81 culture positive pools were DPCR positive (but without REA) (Table 16). Reaction IS1311L was also insensitive (27.2%). Reaction IS1311S was more sensitive (63.0%). Reaction 900VM was also relatively sensitive (66.6%).

The PCR reaction product from culture positive pools that were initially negative in DPCR with reaction 900 VM because there was either insufficient PCR product for REA in agarose or no PCR product detected in agarose were further tested by PAGE. An additional 14 pools then yielded positive results, giving a sensitivity estimate for reactions 900VM of 84.0%. Reaction products from 1311S and 1311L were not further evaluated by PAGE.

Of the 26 culture negative pools, 1 was positive in DPCR in reaction 900VM and 1311S. Additional pools yielded PCR product but there was insufficient for REA in agarose and PAGE was not undertaken. This DPCR positive pool was 1 of only 3 culture negative pools from a submission of 23 pools from an infected flock.

The incubation time (in weeks) required for growth index of BACTEC cultures to reach 999 appeared to influence DPCR results as negative results were often associated with lengthy incubation times (Table 17). Time to reach growth index 999 is a surrogate measure of the number of organisms present in the faecal sample. With reaction 900VM most of the culture+ve/DPCR-ve outcomes were for samples that took 5 weeks or longer to reach 999 whereas most of the culture+ve/DPCR+ve outcomes were for samples that took 5 weeks or less to reach 999. There was a similar pattern for reactions 1311S and 1311L.

Table 16. Comparison of culture and DPCR for 107 pooled faecal samples.

DPCR		Culture result		
Name	Result	Positive	Negative	Total
900M	Positive	31	0	31
	Negative	50	26	76
900VM	Positive	68	1	69
	Negative	13	25	38
1311S	Positive	51	1	52
	Negative	30	25	55
1311L	Positive	22	0	22
	Negative	59	26	85
	Total	81	26	107

Table 17: The trends in the DPCR results with reference to the time the culture's took to reach a growth index of 999.

Week to 999	PCR assay and Culture vs DPCR result					
	900VM		1311S		1311L	
	Culture (+) DPCR (+)	Culture (+) DPCR (-)	Culture (+) DPCR (+)	Culture (+) DPCR (-)	Culture (+) DPCR (+)	Culture (+) DPCR (-)
3		1		1		1
4	22		19	3	12	10
5	26	2	22	6	6	22
6	16	3	9	10	4	15
7	3	1	1	3		4

8	1	5		6		6
10		1		1		1
Total	68	13	51	30	22	59

4.5.5.2 Blind evaluation of DPCR using pooled faecal samples

Of the 326 pooled faecal samples that were examined by DPCR with reaction 900VM, 30 were culture positive and 296 were culture negative (Appendix 8). Of the 30 culture positive pools, 20 (66.7%) were DPCR positive and all of these were confirmed as DNA consistent with *M. paratuberculosis* after REA in agarose (Table 18). There were no trace reactions in agarose so PAGE was not required. Of the 10 culture positive pools not identified by DPCR, 8 were from submissions from flocks where at least one other pool was culture and DPCR positive. Overall, 10 of the 12 properties identified by culture were also identified with DPCR.

Of the 296 culture negative pools, 6 were DPCR positive. Three of the 6 DPCR +ve pools came from submissions from farms where there was at least one other DPCR +ve/culture +ve pool. The remaining 3 DPCR +ve pools were not accompanied by culture positive pools; 2 were from submissions for properties undergoing surveillance testing and 1 was part of a market assurance programme test.

There was a correlation between DPCR results and the incubation time (in weeks) required for the culture positive pools to reach a growth index of 999 (Table 19). DPCR negative results were associated with samples that required 5 weeks or more to develop a growth index of 999 whereas positive results were mostly for samples that required 5 weeks or less.

Table 18: Blind comparison of DPCR and culture on 326 pooled faecal samples.

Culture		DPCR (900VM)		REA	
Positive	n = 30	Positive	n = 20	Positive	n = 20
				Negative	n = 0
		Negative	n = 10		
Negative	n = 296	Positive	n = 7	Positive	n = 6
				Not tested	n = 1
		Negative	n = 289		

Table 19: Trends in the DPCR results with reference to the time required to reach a growth index of 999.

Week to 999	Culture (+) DPCR (+)	Culture (+) DPCR (-)
4	8	
5	8	2
6	4	6
8		1
12		1
Total	20	10

4.5.6 Direct PCR using larger amounts of faeces

4.5.6.1 Evaluation of faecal dilution series by DPCR

DPCR with reaction 900VM was capable of detecting *M. paratuberculosis* DNA in all faecal dilutions up to and including 1 in 1000. All DPCR experiments on large volume faecal samples were done with the 1 in 100 (strong positive) and 1 in 1000 (weak positive) dilutions and faeces from the non-infected sheep as the negative control.

4.5.6.2 Experiments with larger volumes of faeces

Three isolation methods were attempted. Each resulted in identification of DNA consistent with *M. paratuberculosis* in both the 1 in 100 and 1 in 1000 faecal dilutions. The results from all 3 methods using 2 g of faeces, regardless of the combination of buffer, volumes and centrifugation speeds, were at best only equivalent to the standard isolation method using 0.1 g of faeces. However, centrifugation was the most critical parameter as higher *g* forces generally produced the best results.

4.5.7 SYBR Green detection of DNA with PAGE

The analytical sensitivity of SYBR Green staining was found to be equivalent to silver staining. Both staining techniques resulted in detection of amplified product at a dilution of 10^{-4}. However, the SYBR Green stained gel produced more intense bands when the amount of amplified product was limiting.

4.5.8 Cost of DPCR

The direct cost of material used in DPCR is approximately $9.20 per sample and approximately 45 minutes total operator time per sample is required to complete the assay. These estimates include the time and costs of homogenisation of a pooled faecal sample. Using a typical algorithm for laboratory infrastructure and management overheads the estimated net cost to producers for a DPCR test would be $75 excluding GST.

4.6 Discussion

4.6.1 HC-PCR protocol development and application

This project was established in order to validate HC-PCR for the detection of *M. paratuberculosis* in pooled faecal samples from sheep. It followed successful pilot studies with pooled faeces (Appendix 1)**.** Modifications were required to the protocol to ensure its specificity and these were expected to cause a reduction in the sensitivity, particularly the exclusion of Southern blotting. However, the reduction in sensitivity was greater than envisaged by these changes alone. This is explained largely by the advent of faecal culture as the 'gold standard' test against which other tests need to be compared. Faecal culture is considerably more sensitive than previous methods of assessing the infection status of sheep - principally histopathology of intestinal tissues, which was the best available method at the time this project was initiated. Consequently, data presented in this report indicate imperfect sensitivity for DNA-based faecal tests. Despite 'raising the bar', aspects of the HC-PCR protocol were investigated with a view to improving sensitivity and to

understanding the limitations of the technology (Appendix 4). A simpler method of testing faeces was developed, referred to in this report as DPCR.

In its original format (Millar et al 1995), HC-PCR was used to detect both *Mycobacterium avium* subsp. *paratuberculosis* and *Mycobacterium avium* subsp. *silvaticum*, a closely related sub-species in the *M. avium* complex. Another insertion sequence, IS*902*, was used to detect and differentiate *Mycobacterium avium* subsp. *silvaticum* from *Mycobacterium avium* subsp. *paratuberculosis*. IS*902* is closely related to IS*900* and the two share a high degree of homology, especially at the 3 prime end of the genes. However, the 5 prime end of each gene is sufficiently dissimilar to enable differentiation by PCR. The aim of the original HC-PCR method was to use the same capture probe for both species but to use different PCR assays for detection. There was a gap on the IS*900* gene of approximately 400 bp separating the sites of binding of the capture probe and the detection primers for PCR. Any gene captured with the Millar capture probe that did not contain the detection PCR region of IS*900* or in which the detection region had been sheared off during processing would not react in the detection PCR. This wide separation between capture and detection regions was thought to be a weak point in the test and in order to improve the sensitivity of the test new capture probes were produced and assessed. Before the new capture probes could be produced, the plasmid pPN14 which contains the entire IS*900* gene needed to be modified to remove the detection PCR region to produce a new plasmid, pPN14E. This was done to ensure that newly-made probes would not cause false positive results in HC-PCR.

Fourteen new probes of different lengths and located in different positions in relation to the detection PCR region of IS*900,* but all closer to the detection region than the original Millar capture probe, were amplified using the plasmid pPN14E as the target in PCR. Three sets of very short probes (primer capture probes) were also assessed along with the amplified probes. Unfortunately only one probe (amplified capture probe number 3) produced results better than the Millar capture probe. However, HC-PCR with this new capture probe was no better than the simpler method of DPCR.

The effects of long term storage on the activity of the Millar capture probe were also examined but the results indicated that storage was not detrimental. The apparent lack of sensitivity in HC-PCR could not be attributed to deterioration of the capture probe. Further experiments were performed with the Millar capture probe to evaluate the efficiency of the hybridisation and capture steps. The results indicated that capture of target DNA was relatively inefficient and furthermore that capture of biotinylated capture probe-DNA target complex by streptavidin-coated magnetic beads was highly inefficient, contrary to the advice provided by the manufacturer of the product. The end result was that not all the specific target DNA was being removed from the sample during hybridisation and capture. This is the main reason for the low sensitivity of this procedure. The authors of a recent publication suggest that the stoichiometry of the hybridisation and capture steps is quite complicated (Stevens *et al*., 1999). The authors described a biomolecular all-or-none model for hybridisation reactions in solution which addresses the thermodynamic issues of hybridisation in solution and includes a equilibrium dissociation constant formula that can be used to ascertain the optimal concentrations of capture probe to maximise the sensitivity of the test. However, fairly extreme efforts are required to achieve an optimised hybridisation reaction and maintaining quality control with such complex reactions might be difficult.

Other steps were taken to try to improve the sensitivity of the HC-PCR assay. A silver staining method was investigated for more sensitive detection of DNA in both agarose and polyacrylamide gels. Silver staining of agarose gels was unsuccessful but the combination of polyacrylamide gel electrophoresis (PAGE) and silver staining resulted in a 10 to 100 fold increase in analytical sensitivity compared to ethidium bromide staining of agarose gels. The usefulness of PAGE was further enhanced by replacing silver staining with SYBR Green staining. The two staining techniques had equivalent analytical sensitivity, however, when the amount of amplified product was limiting, SYBR Green stained gels produced more intense bands. SYBR Green technique produces a result within 40 min as opposed to silver staining which requires around 2 hours. SYBR Green staining requires little reagent preparation, but reagents are costly, whereas silver staining reagents must be prepared freshly and this can take half a day. For routine use we found that agarose gel electrophoresis was most appropriate provided that PCR reaction products yielding trace results were subjected to REA in PAGE with SYBR Green staining.

4.6.2 DPCR protocol development and evaluation

DPCR is performed on DNA purified from faeces, but the extract consists of a wide range of DNA-species including host DNA and irrelevant microbial DNA. Preliminary investigations into the use of DPCR using PCR reaction 900M indicated that amplification of non-specific PCR product would be a problem with many samples. Non-specific PCR product had not been observed over many years of use of this PCR assay with other kinds of samples: purified *M. paratuberculosis* DNA, colonies of *M. paratuberculosis* from solid media or DNA purified from BACTEC medium. To overcome this problem several PCR assays using IS*900* as the target DNA but different primer combinations were evaluated and one was found with acceptable characteristics. Reaction 900VM has equivalent analytical sensitivity to 900M but produces greater yields of amplified product especially at low concentrations of *M. paratuberculosis* DNA. Analytical specificity was high when PCR was combined with the now-standard procedure of REA. One species of mycobacteria other than *M. paratuberculosis* reacted in both 900M and 900VM reactions but was distinguished by REA.

Another insertion sequence gene, IS*1311,* was used in DPCR. Where it is desired to obtain information on the type of strain of *M. paratuberculosis* present, reaction IS*1311*S would be useful to combine with reaction 900VM. PCR reaction 1311L was relatively insensitive. It produces the longest amplified product (608 base pairs) of all the reactions examined in this study. PCR assays that amplify long DNA fragments may be less sensitive in some applications than those that amplify short fragments due to fragmentation of the DNA during the extraction and purification process leaving fewer intact copies as templates for PCR.

DPCR (reaction 900VM) had reasonable sensitivity when used to evaluate pooled faecal samples. It detected 66.6% of culture positive pools in each of two trials. In the second trial, which was a blind study, 83% of 12 infected flocks were detected. The confidence limits for the latter estimate are wide because of the small sample size of culture positive pools.

The sensitivity of DPCR is related to the number of mycobacteria in the faecal sample. The test would perform poorly as a flock test in situations where the majority of infected sheep were in early stages of the disease. While this is also true for culture it may be more of a problem for DPCR. Although both tests utilise the same type of PCR reaction for identification of the bacteria, the advantage of culture is that multiplication of the target bacteria occurs before PCR is attempted.

An attempt was made to improve the sensitivity of DPCR by increasing the amount of faeces used in the test. Up to 2 g of faeces is used in culture compared to < 0.2 g in DPCR. Three methods of isolating *M. paratuberculosis* from large volume faecal samples were evaluated. The efficiency of all 3 methods was dependent on the centrifugation step with the best results being achieved when centrifugation was performed at the highest *g* forces. Unfortunately the results were no better than those achieved with the simpler isolation method from a small amount of faeces. Larger volumes of faeces might result in too much non-specific DNA. Alternatively the DNA purification resin-column system may have become saturated, mitigating against any benefit from increased faecal volume. Further work is required in this area.

4.6.3 Constraints to immediate application of DPCR

DPCR is a relatively sensitive alternative to faecal culture for detection of *M. paratuberculosis* in faecal samples from sheep with the advantage of providing results within a few days instead of months. However there are certain constraints to its use. In decreasing order of difficulty to overcome these constraints are:

4.6.3.1 Perception and acceptability to animal health authorities

DPCR is a DNA-based test. The results of a positive test would be worded “DNA consistent with *M. paratuberculosis* detected”. This wording is common to that of a positive culture test result where PCR has been used to identify growth in BACTEC medium or on solid media.

Despite the use of DNA-based tests in human medicine, forensic science and in detection of many other diseases of livestock, Australian animal health authorities have been reluctant to accept the results of DNA-based tests for Johne’s disease unless there is other evidence of infection, usually

post mortem examination and histopathology. Furthermore, even culture results based on naked eye observation of colonies of the organism on solid culture media are not regarded as definitive evidence. This is a most unusual situation for a microbial disease where the causative agent is defined as an obligate pathogen and parasite of animals and where accepted methods of identification of microbes are used. One argument used to defend this view is the chance of sample misidentification or sample-to-sample cross contamination at a laboratory. For this reason it has been deemed that two consecutive positive culture tests are required from a flock before infection is deemed to be present. Under current Veterinary Committee guidelines, at least one of these rounds of testing must yield colonies of *M. paratuberculosis* on solid media or the status of the flock will be suspect rather than infected. In current test interpretation the benefit of doubt is given to the flock, the results of DNA-based tests are discounted and biological issues such as intermittent faecal excretion are overlooked.

New tests for Johne's disease have received a far greater level of scrutiny and attention to quality control than many other tests used in animal health laboratories, including tests for international export, interstate movement, entry to artificial breeding centres and certification. Unless the factors constraining acceptance of new tests for Johne's disease are clearly understood it is difficult to see how any new tests for Johne's disease can gain widespread acceptance under the current Australian animal health laboratory test approval system. None of these constraints were apparent when the present research was commissioned.

4.6.3.2 Confirmation of infection

Based on current information from Veterinary Committee, DCPR would not be considered a definitive test. Follow-up study would be required in each flock detected by DPCR. The cost of follow-up is high as it would be based on similar protocols to those for follow-up of pooled faecal culture positive flocks. It would be necessary either to obtain a second positive flock test result by examining a second set of pooled faecal samples (by culture or DPCR) or to demonstrate pathology due to Johne's disease in one or more sheep from the flock.

The incentive to use DPCR is significantly diminished by not regarding the demonstration of DNA consistent with *M. paratuberculosis* as a definitive result.

4.6.3.3 Cost of DPCR

Each DPCR test has a significant laboratory-level cost, approximately $75 (excluding GST) or $1.50 per head assuming a pooling rate of 50 samples. While this cost is less than the cost of pooled faecal culture the sensitivity is also lower so more sheep need to be tested. Overall, the costs of the two tests on a flock basis would be similar. The cost of follow-up or confirmation of infection would be similar for both tests.

The laboratory-level costs of DPCR can be significantly reduced by improving the method of DNA extraction from faeces but this requires further bench research. In addition, the method of detection of PCR product can be improved. Real-time PCR using fluorescent probe technology would enable PCR to be conducted without electrophoresis and would overcome the need for REA. Currently the equipment required for this technique is very costly but it is expected to fall within a few years as the technology is more widely adopted. Developmental work would be required to adapt the assay to the new detection method.

4.6.4 DPCR in surveillance and market assurance tests

Sensitivity estimates for DPCR relative to culture ranged from 74% for individual faecal samples (using reaction 900M) with lower estimates for pooled faecal samples (49.5% using reaction 900M; 66.6% using reaction 900 VM). Sensitivity estimates for DPCR on pooled samples will depend on the stage of disease of animals present in infected pools, or in other words on the number of organisms being shed by the infected sheep in the pool.

The absolute value of sensitivity of culture of pooled faecal samples is uncertain, but it is more sensitive than serology for detection of infected flocks when equal numbers of sheep are tested by each method. As with pooled faecal culture it will be possible to prescribe a sample size for DPCR

so as to be able to detect a prescribed prevalence of infection (usually 2%) with a certain level of confidence (usually 95%). A rough estimate is that the same number of sheep would need to be tested by DPCR (in pools of 50) as would need to be tested individually using the gel test. These issues require more detailed consideration by a panel of epidemiologists.

There is an argument for using DPCR and culture in series on the same pooled samples. DPCR could be used first to screen pooled samples submitted for culture. Culture would not be required on those samples testing positive in DPCR, and arguably not on any samples from a flock where one or more pools tested positive. Results for flock infection status could be reported within days of receipt of samples at a laboratory. This approach is logical where positive culture results are expected. However, it would almost double the costs of testing samples from true negative flocks because both DPCR and culture would have to be applied to all samples from the flock. In situations where negative culture results are expected no time would be saved.

4.7 Success in achieving objectives

The objectives of this project were achieved, subject to modifications agreed upon submission of milestone reports. It was not possible to submit an application for approval of the test to SCAHLS within the time frame of this project. However, the sensitivity and specificity of HC-PCR was evaluated, critical points in the assay were identified and the test was found to be inadequate for industry needs. A simpler test was developed and evaluated for use with pooled samples and may find application.

4.8 Impact on meat and livestock industry

A rapid method for identification of *M. paratuberculosis* in pooled faecal samples from sheep has been developed. If approved by Veterinary Committee, the test may be used to detect infection at a flock level and to do so within a few days of collection of samples. This is in comparison with pooled faecal culture where results are not available for months, causing significant inconvenience to producers. The new test is no more expensive than other methods of flock diagnosis. However, there are constraints to approval of this test and it is not possible to forecast when it may be used routinely.

The technology developed in this project is applicable to detection of *M. paratuberculosis* in the faeces of species other than sheep but the sensitivity of detection would require assessment in each species. The test would also be a useful research tool and may offer some advantages over culture.

4.9 Conclusions and recommendations

The results of this study indicate that a simple direct PCR test can be applied to detect *M. paratuberculosis* in DNA extracts prepared from pooled faecal samples from sheep. The sensitivity of the test is adequate for flock diagnosis and the costs no greater than other flock diagnostic strategies. Results can be available within a few days of receipt of samples at a laboratory. However, it is doubtful whether the test would be approved for use in Australian animal health laboratories in the current climate. This is because existing DNA-based tests for identification of *M. paratuberculosis* in cultures are not regarded as definitive tests.

The principal recommendation of this study is that Veterinary Committee define minimum standards for approval of DNA-based tests for detection of animal diseases in general and Johne's disease in particular, before further industry funds are expended in the development of new tests. A related recommendation is that an application for approval of DPCR for detection of *M. paratuberculosis* be made to Veterinary Committee when criteria for approval of DNA-based tests are available.

A secondary recommendation is that further developmental work be conducted to improve faecal DNA extraction methods and to replace the electrophoresis and restriction endonuclease analysis steps in the protocol with the primary aim of reducing test costs.

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HC-PCRFinal Report.doc jdwpd3

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4.12 Appendices

Appendix 1 : Manuscript in press

Quality control and optimised procedure of hybridisation capture-PCR for the identification of *Mycobacterium avium* subsp. *paratuberculosis* in faeces.

Ian Marsh[1]*, Richard Whittington[1] and Douglas Millar[2]
[1]*Elizabeth Macarthur Agricultural Institute, NSW Agriculture, Menangle, New South Wales, Australia*, [2] *Commonwealth Scientific and Industrial Research Organisation, Molecular Science Unit, North Ryde, New South Wales, Australia*

Correspondent footnote

*Author to whom all correspondence should be addressed. Mailing address: NSW Agriculture, Elizabeth Macarthur Agricultural Institute, PMB 8, Camden NSW 2570, Australia. Phone 61 46 406502. Fax 61 46 406384. Electronic mail address ian.marsh@agric.nsw.gov.au

ABSTRACT
Nucleic acid sequence capture techniques are used to improve both the sensitivity and specificity of PCR for the diagnosis of plant, animal and human diseases. Hybridisation capture-PCR (HC-PCR) was first reported as a method for the detection of *Mycobacterium avium* subsp. *paratuberculosis* in 1995 and was successfully trialed on a small number of faecal samples from cattle with Johne's disease. A locally optimised HC-PCR method was evaluated on faeces from infected and non-infected animals. However, sample to sample cross contamination during the DNA purification step highlighted that the original format of the test was unsuitable for routine diagnostic use. In this paper we report modifications and optimisation of HC-PCR, particularly with respect to DNA purification from faeces, hybridisation and capture steps. We also identified procedurally sensitive critical points in the test during capture and washing of magnetic beads. Southern blotting was omitted from the protocol to preserve specificity but this resulted in analytical sensitivity of 5000 organisms per 200 mg faecal sample. Nevertheless HC-PCR detected *M. paratuberculosis* in pellets from infected sheep diluted at rates of up to 1 in 100 in normal faeces, suggesting that the technique should be evaluated further for low-cost diagnosis in flocks/herds using pooled samples.

KEYWORDS: *Mycobacterium avium* subsp. *paratuberculosis*, hybridisation, IS*900*, Johne's disease, diagnosis, sequence capture.

INTRODUCTION

Mycobacterium avium subsp. *paratuberculosis* (*M. paratuberculosis*) is the causative agent of Johne's disease, a chronic and incurable condition affecting many ruminant species. Infection with *M. paratuberculosis*, as for other slow growing mycobacteria, can be difficult to confirm due to the long incubation times required to culture and identify the organism. Two broad groups of *M. paratuberculosis* are known to exist based on IS*900* restriction fragment length polymorphism analysis (RFLP), IS*1311* polymorphisms and cultural characteristics: sheep (S) and cattle (C) strains[1-4]. The C strains takes up to 6 months to culture, using conventional methods and solid media while the S strains was thought to be non-culturable. However, in a recent study the BACTEC radiometric culture system was used successfully to culture S strains of *M. paratuberculosis*[5,6]. Nevertheless, the wait for cultural results is perceived to be too long and this has implications for the success of control and eradication programmes for Johne's disease. Therefore a rapid, sensitive and specific test that confirms *M. paratuberculosis* in clinical samples is still required.

Polymerase chain reaction (PCR) has been used to improve the diagnosis of plant, animal and human diseases especially where conventional microbiological detection methods have performed poorly[7-11]. Recent reviews on Johne's disease have discussed the important role of molecular biology in diagnosis[12-14] and for this reason PCR has been widely used in this role[3,15-19]. However, PCR has limitations and while theoretically capable of detecting a single genome in a sample, this is rarely if ever achieved. In reality multiple organisms are required to produce a positive signal. The effects of excessive non-specific DNA derived from the host or other microbes, or PCR inhibitory substances in clinical samples can prevent amplification[20-28] particularly when faeces is the sample[29,30]. Although these problems can be probably be overcome, the detection of early, sub-clinical or paucibacillary forms of diseases such as tuberculosis and Johne's disease will always be difficult, due to the relative scarcity of target organisms in clinical samples.

Numerous studies have been undertaken with the aim to increase both the sensitivity and the specificity of PCR through improved sample preparation, DNA extraction techniques, chemical PCR enhancers and the incorporation of highly sensitive detection techniques for amplified product[22, 25, 26, 28]. Nucleic acid sequence capture is another means of improving sensitivity. This technique has been used in conjunction with PCR in the diagnosis of viral and bacterial diseases in an attempt to extract specific target DNA or RNA and remove it from highly heterogenous samples, thus eliminating non-specific DNA or RNA and PCR inhibitory substances[29,31-34]. Nucleic acid sequence capture produces highly pure templates by first binding the target DNA or RNA to a biotinylated DNA probe then capturing this on streptavidin-coated magnetic polystyrene microparticles (beads). The beads with the DNA probe and target DNA or RNA attached are washed and then subjected to PCR.

Hybridisation capture-PCR (HC-PCR), a nucleic acid sequence capture method described by Millar *et al*.,[32], was successfully used for the detection of *M. paratuberculosis* in tissues from Crohn's disease patients and on a limited number of faecal samples from cattle known to have Johne's disease. In its initial format, IS*900* was the target sequence for PCR after which Southern blotting was performed to maximise sensitivity. Whilst IS*900* has been considered to be unique to *M. paratuberculosis*[35], IS*900*-like elements have been found in other mycobacteria[36-38] and these genes may amplify in PCR using primers for IS*900*. Unfortunately IS*900* probes used for Southern blotting cannot differentiate between *M. paratuberculosis* and mycobacterial species containing IS*900*-like genes due to the high degree of homology between IS*900* and the IS*900*-like elements[36], precluding the use of Southern blotting as a detection method in HC-PCR on grounds of specificity. Thus modifications to the protocol of Millar *et al.*[32] are required if HC-PCR is to be used for the detection of *M. paratuberculosis* and in particular, efforts are required to obtain greater sensitivity without use of Southern blotting.

The aims of this study were to optimise the HC-PCR method of Millar *et al.*[32] especially hybridisation and capture steps to improve sensitivity without use of Southern blotting and to identify procedurally sensitive and critical quality control points in the methodology.

MATERIALS AND METHODS

Faecal samples

A group of 13 faecal samples from alpaca (Johne's disease negative, n = 4), healthy sheep (Johne's disease negative, n = 3) and affected sheep (Johne's disease positive, n = 6) were used to evaluate the HC-PCR method as optimised at our laboratory. The status of each sample was based on farm history, the results from BACTEC radiometric culture of alpaca faeces and a combination of microscopic examination including Ziehl-Neelsen staining of tissue samples and agar gel immunodiffusion tests of serum from the sheep.

To evaluate and compare resin and glass bead purification procedures, HC-PCR was performed on sheep faeces spiked with whole *M. paratuberculosis* cells. Ten fold serial dilutions of whole *M. paratuberculosis* cells were prepared from a 10^9 organisms per mL suspension that had been quantified using a Helber counting chamber. The dilutions (10^8 to 10 organisms per mL) were prepared in 1x PBS after which 50 µL of each dilution was added to faeces to produce samples with bacterial loads of between $5x10^6$ to 0.5 organisms per 200 mg of faeces.

Two dilution series of faeces were prepared to simulate a single infected sheep pooled amongst 10, 25, 50 or 100 non-infected sheep. A single pellet from a known *M. paratuberculosis* infected sheep was mixed with 9, 24, 49 or 99 pellets from non-infected sheep which were housed at the Elizabeth Macarthur Agricultural Institute. Each dilution of pellets was then mixed thoroughly for two 30 second runs in a commercial blender (Waring® Commercial Blendor). This procedure was then repeated using a new blender, to avoid cross contamination of samples, on faeces from a second known-infected sheep. Both infected animals were chosen based on histological examination of the ileum and represented multibacillary forms of Johne's disease.

Overview of HC-PCR using faecal samples

All procedures involving magnetic beads were performed in a class 2 biological safety cabinet as this provided a confined area that could be de-contaminated easily after each procedure with UV light. To avoid cross contamination of samples by aerosols, only screw top 1.5 mL tubes were used and only one tube was opened at any time throughout the entire HC-PCR procedure.

A purified DNA extract was prepared from a heated faecal suspension. This was added to a hybridisation buffer containing a biotinylated DNA probe. The reaction was heated to separate double stranded DNA then incubated to facilitate hybridisation of the probe to IS*900*. Streptavidin-coated magnetic beads were added to capture the probe-target DNA complex, washed to remove irrelevant DNA, PCR inhibitors and buffer components. The bead-probe-target DNA complex was subjected to PCR for IS*900*.

Extraction and purification of DNA from faecal samples

Extraction was performed on 0.1 to 0.2 g of faeces which was added, using a sterile wooden applicator stick, to a sterile 1.5 mL screw cap centrifuge tube containing 700 µL of 1x PBS and mixed with the stick to form a slurry. The slurry was vortexed for 2 min, incubated at 55 ^{o}C for 30 min in a dry heating block, vortexed again for 2 min, boiled at 100^{o}C for 30 min then centrifuged at 12,000 *g* for 5 min. DNA was purified from the boiled faecal extract using a variety of methods. Initially a glass bead procedure (Bresatec - Bresa-Clean nucleic acid purification kit - Cat No. BRC-1) was used as described by Millar *et al*.[32]. In later experiments a resin based method was used (see below).

Polymerase chain reaction

All IS*900* PCR assays were performed with a locally-optimised protocol as previously described[5] using the primers P90 5'- GAAGGGTGTTCGGGGCCGTCGCTTAGG-3'and P91 5'- GGCGTTGAGGTCGATCGCCCACGTGAC-3'[32] that amplify a 413 bp fragment. Briefly, 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 TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.8) was used. Amplification was undertaken in 200 µl tubes with individual lids where only one PCR tube was opened at any time throughout the entire PCR procedure. A 96-place thermal cycler (Corbett Research, Sydney, Australia) was used with the following conditions: one cycle of denaturation at 94^{o}C for 3 min followed by 37 cycles of denaturation at 94^{o}C for 30 sec, annealing at 62^{o}C for 15 sec and extension at 72^{o}C for 1 min. A modified hot start procedure was used for all PCRs: samples were placed in the thermal cycler after the block had reached 94^{o}C during the initial denaturation cycle. PCR results were assessed by electrophoresis in 2% agarose gels stained with ethidium bromide. Unless otherwise stated, all gels were run with the molecular size marker number VIII (Roche - Boehringer Mannheim).

Production of a 513 bp IS*900* capture probe

A 513 bp capture probe was prepared as described[32] with the following conditions as optimised in our laboratory using the plasmid pPN14M as the target for PCR. The PCR was performed in 50 µL reactions with: 5 µL of the pPN14M, 300 ng of each biotinylated primer P8 5'-TGTGGCGTTTTCCTTCGGTG-3' and P21 5'-GCGCTCGAGTAGCCGCGTTC-3', 200 µM of each of the nucleotides dATP, dTTP, dGTP and dCTP, 66.8 mM Tris-HCl, 16.6 mM $(NH_4)_2SO_4$, 1.5 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). Amplification was performed using the following conditions: one cycle of denaturation at 94^{o}C for 2 min followed by 35 cycles of denaturation at 93^{o}C for 30 sec, annealing at 60^{o}C for 15 sec, extension at 72^{o}C for 30 sec and a final cycle of 1 min at 4^{o}C. The probe was then purified using a DNA purification system (Promega - Wizard™ PCR preps DNA purification system - Cat No. A7170) as described by Millar *et al*.[32].

Washing the beads after capture and prior to PCR

For all HC-PCR experiments the magnetic beads were washed twice with 1x PBS with 0.5% w/v BSA (BWB). The beads were collected at the side of the tube using a magnetic particle concentrator (MPC-M, Dynal®) for 1 min, removing the supernatant and adding 1 mL of BWB gently down the side of the tube opposite the beads. The beads were gently resuspended for 2 minutes then they were allowed to re-collect on the MPC-M and 950 µL of supernatant was removed. The beads were then washed for a second time with 950 µL of BWB. The beads were collected once again and all the supernatant was removed. The tubes were taken out of the MPC-M and the beads were resuspended in 5 µL of sterile TE gently washing the beads down the wall of the tube with the TE.

Evaluation of hybridisation and wash buffers

Four hybridisation buffers and five wash buffers (Tables 1 and 2) were evaluated in all possible combinations. All reactions were performed in sterile 1.5 mL screw cap tubes. The reactions with hybridisation buffers 1 to 3 and 4 were done in 450 µL and 337.5 µL of buffer, respectively. To all reactions 10 µL of capture probe (14.2 fmol) and 10 µL of *M. paratuberculosis* genomic DNA (0.5 ng/µL) was added after which the tubes were boiled in a water bath at 100 ^{o}C for 5 min. After boiling, 112.5 µL of 5M NaCl was added to the tubes containing buffer 4 to achieve a final NaCl concentration of 1M. All reactions were incubated overnight at 65^{o}C. The reactions were allowed to cool to room temperature before 10 µL (6.5 x 10^{6} beads) of streptavidin coated magnetic beads (M-280, Dynal®), washed according to the manufacturers recommendations, were added to each tube. All reactions were incubated for 2 hours at room temperature with gentle end over end mixing (Ratek RSM6 blood mixer). The beads were washed twice as described in the preceding section with the appropriate wash buffer, resuspended in 5 µL of TE then added directly to the PCR reaction. At the completion of PCR serial ten-fold dilutions of the amplified product were evaluated on a 2% agarose gel.

Hybridisation reaction volume
Hybridisation buffers 1 and 4 (Table 1) were evaluated at a range of final reaction volumes: 25, 50, 100, 150, 250, 350 and 450 µL. All reactions were performed in sterile 1.5 mL screw cap tubes and prepared so that the concentration of the buffers was the same in each tube. Ten microlitres of capture probe (14.2 fmol) and 10 µL of *M. paratuberculosis* genomic DNA (0.5 ng/µL) were added to each tube. The tubes were placed in a water bath at 100 ^{o}C for 5 min after which 5M NaCl was added to the reactions with buffer 4 to achieve a final NaCl concentration of 1M. The reactions were completed as described in the preceding section.

Temperature and duration of the magnetic bead capture of the biotinylated probe
Forty microlitres of *M. paratuberculosis* genomic DNA (0.5 ng/µL) was added to 100 µL of buffer 4 and 10 µL of capture probe (14.2 fmol) in 1.5 mL sterile screw cap tubes. The tubes were placed in a water bath at 100 ^{o}C for 5 min after which 37.5 µL of 5M NaCl was added to each reaction. All reactions were incubated overnight at 65^{o}C and then cooled to room temperature. Streptavidin coated magnetic beads were washed and 10 µL were added to each tube as described in preceding sections. The reactions were incubated at either room temperature or 37^{o}C for 2, 4 or 8 hours with gentle end over end mixing. The reactions were completed as described above.

Method of mixing the magnetic beads during capture of the biotinylated probe
A series of identical reactions using hybridisation buffer 4 in a final volume 150 µL were prepared with 10 µL of the capture probe (14.2 fmol) but without target DNA. Each reaction was performed in duplicate and incubated for 4 hours at 37 ^{o}C using various forms of mixing or no mixing. Mixing was achieved using: a rotary blood mixer for fast and slow end over end rotation at both steep and shallow angles (Ratek RSM6), a platform rocker (Bio-line) where the tubes were laid flat and a microtitre plate shaker (IKA-Schüttler MTS 4) with the tubes standing upright. The beads were washed and subjected to PCR using primers P8 and P21, which are designed to amplify the 513 bp capture probe sequence.

Re-suspension of the magnetic beads after washing
In order to confirm suspicions that the magnetic bead-probe-DNA target complex was susceptible to disruption during post-capture washing of the magnetic beads, HC-PCR as described above, was performed on duplicate 10 fold dilution series of purified *M. paratuberculosis* DNA. The effects of a post-washing delay of 30 minutes prior to re-suspension in TE was compared with immediate re-suspension in TE. Tubes in the delayed treatment were washed, set aside in a class II biological safety cabinet with the motor running and re-suspended in TE after 30 minutes.

Evaluation of the optimised HC-PCR method
The HC-PCR method optimised in the experiments described above was evaluated by testing the group of 13 faecal samples on each of five separate occasions. The controls used for each HC-PCR experiment and their point of introduction during the procedure are given in Table 3. For these experiments and all further HC-PCR experiments the 5 M NaCl was added prior to boiling as this was found to have no adverse effect on the reaction and reduced the opportunity for cross contamination of the samples.
For each sample, 40 µL of the purified faecal DNA (glass bead procedure) was added to a reaction mixture comprised of 45 µL of buffer 4 (Table 1), 30 µL of 5 M NaCl, 25 µL of sterile water and 10µL (14.2 fmol) of the 513 bp biotinylated capture probe. The reaction tube was boiled for 5 min in a water bath and then incubated overnight at 65 ^{o}C. The tubes were then allowed to cool to room temperature before 10 µL of washed streptavidin-coated magnetic beads were added and gently mixed by flicking the base of the tubes with a finger. The tubes were then layed horizontally on a platform and allowed to incubate for 4 hours at 37 ^{o}C with gentle rocking. After capture the beads were washed with buffer A (Table 2) and subjected to PCR. The results were determined by electrophoresis only. All work prior to PCR was carried out in a class II biological safety cabinet with the motor on.

Modification of the DNA purification method
The glass bead procedure was performed and compared with several resin based methods (Promega - Wizard® PCR Minipreps DNA purification system - Cat No. A7100, Promega; Wizard® PCR preps DNA purification system - Cat No. A7170, and; Promega - Wizard® PCR preps DNA purification system - Cat No. A7280). Resin methods were evaluated using the procedures recommended by the manufacturer. The method described below using the Wizard® PCR preps DNA purification system was found to be the most appropriate.

After releasing the DNA from *M. paratuberculosis* within the faecal samples by heating and centrifugation as described previously, the top of the screw capped sample tube was wiped with a tissue soaked in 70% ethanol taking care not to disturb the pellet at the base of the tube. Three hundred microlitres of the supernatant was carefully taken up into a 2.5 mL syringe containing 1 mL of 6M guanadine thiocynate, after inserting a sterile 18 gauge needle, with attached syringe, through the lid of the tube using forceps to hold the tube to avoid needle stick injury. The needle was withdrawn, the cover was replaced over the needle and the contents of the syringe were briefly vortexed twice over 1 min. The needle was removed and discarded and a minicolumn was placed on the end of the syringe. Purification was then completed according to the manufacturer's instructions using a vacuum manifold (Promega Vac-man®). Fifty microlitres of sterile water pre-heated to 80 ^{o}C was added to the column and allowed to sit for 2 min, followed by centrifugation at 10, 000 *g* for 20 secs to elute the DNA into the attached tube. DNA samples were stored at 4 ^{o}C for immediate use or at -20 ^{o}C.

Evaluation of optimised faecal DNA purification and HC-PCR methods
The resin purification procedure and optimised HC-PCR was evaluated with the group of 13 faecal samples on two separate occasions. HC-PCR, including the resin purification method, was performed on 0.1 to 0.2 grams of each of the simulated pooled faecal samples. Undiluted faeces from each of the infected sheep were used as positive controls while faeces from the non-infected sheep was used as the negative control.

RESULTS

Hybridisation and wash buffers
Amplification of IS*900* was observed with each of the hybridisation buffers examined (Table 1) when used in conjunction with wash buffer A (Table 2). However, no amplification of IS*900* was observed when any of the hybridisation buffers were used in conjunction with wash buffers B, C, D and E. Hybridisation buffers 1 and 4 gave the greatest yield of PCR product based on visual examination of the 10 fold dilution series of the amplified product in 2% agarose gels stained with ethidium bromide and these buffers were evaluated further.

Hybridisation reaction volume
Hybridisation in the original HC-PCR format of Millar et al[32], was performed in a final volume of 500 µL including up to 450 µL of the crude DNA sample. When buffers 1 and 4 were examined over a range of final volumes the amount of amplified product increased as the final volume of the reaction increased, up to and including 150 µL. At volumes greater than 150 µL the efficiency of the reaction deteriorated dramatically. Overall, buffer 4 resulted in the highest yields of amplified product and was used for all remaining experiments in a final reaction volume of 150 µL with wash buffer A.

Temperature and duration of magnetic bead capture
Positive results were obtained when the magnetic bead capture step was performed for 2, 4 or 8 hours at room temperature or 37^{o}C. However, the yields of amplified product were greatest when capture was performed for 4 or 8 hours at 37^{o}C. For convenience 4 hours at 37^{o}C was used for all remaining experiments.

Method of mixing the magnetic beads during capture of the biotinylated probe
At the conclusion of the 4 hour incubation period at 37^{o}C, all tubes were visually examined to determine if the method of mixing had kept the beads in solution or allowed them to settle. End over end rotation on a rotary blood mixer when performed slowly at either steep or shallow angles kept the beads well mixed. However, when this method of mixing was performed with fast rotation the beads collected at the bottom of the tube. The remaining methods of mixing resulted in the beads settling to the bottom or side of the tube.

End over end mixing at a steep angle with fast or slow rotation prevented amplification of the specific IS*900* product. Amplification was observed from one of the replicates from fast end over end rotation at a shallow angle and the microtitre plate shaker and both replicates from the slow end over end rotation at a shallow angle and the platform rocker. Although amplification was observed from both replicates that were not mixed, a reduction in the intensity of the amplified product suggested that absence of agitation had a limiting effect on the procedure (Figure 1). Although end over end rotation at a shallow angle gave results as good as the platform rocker, the later was chosen for all further experiments.

Re-suspension of the magnetic beads after washing
When the beads were resuspended without delay in TE after washing, a graduated response in the amount of amplified product was observed, consistent with the DNA titration series (Figure 2, panel A). When the beads were allowed to sit in the tubes (on the MPC-M) for 30 minutes in the class II biological safety cabinet post washing, no amplification was observed (Figure 2, panel B) demonstrating that immediate re-suspension of the beads was critical to the success of the assay .

Evaluation of the optimised HC-PCR method

Five replicate tests were performed on the group of 13 faecal samples from sheep and alpaca which were prepared using the glass bead purification method. After the second test the results indicated that sample to sample cross contamination had occurred (Table 4). Whilst many of the positive and negative samples were giving the expected results, the faecal extraction process control (Table 3) was positive on 3 occasions out of 5 as were many of the negative faecal samples. To ascertain at which point in the procedure the cross contamination was likely to be occurring, two extra negative controls were included subsequently. A negative control was introduced at the hybridisation step and a further negative control at the capture step. In the next 2 tests cross contamination was found to have occurred during the faecal extraction and DNA purification steps rather than the hybridisation or capture steps, as the two additional controls were both negative. In the fifth test all the controls produced an appropriate result as did each of the 4 negative alpaca faeces and the 2 negative sheep faeces.

Modification of the DNA purification method

When HC-PCR was performed on the DNA purified from faecal samples spiked with whole cells of *M. paratuberculosis*, trace amounts of amplified product was observed from samples with a bacterial load of 500 organisms, regardless of the purification method. However, for practical purposes using visual examination of agarose gels the detection limits of HC-PCR with both purification methods was 5000 organisms in 200 mg of faeces (Figure 3). Whilst the negative control faeces for both purification procedures were negative, the faecal extraction process control for the glass bead method was positive indicating that cross contamination between this control and the other samples had occurred. In contrast the faecal extraction process control from the resin method was negative indicating that the closed system was less prone to cross contamination. The glass bead method was replaced with the resin method in later experiments.

Evaluation of the optimised faecal extraction, DNA purification and HC-PCR methods

The group of 13 faecal samples was re-evaluated, on two occasions, by the optimised HC-PCR method after faecal extraction and DNA purification using the resin method. Sample to sample cross contamination was not observed as all negative controls were negative (Table 4). The faeces from 4 of the 6 infected sheep were positive and based on the amount of amplified product. Three were strong positives and 1 (sheep 13) was weakly positive (Test 6, Table 4). Using the same criteria in test 7, 4 out of 6 of the infected sheep were strong positives and sheep 13 was a mild positive. Sheep 9 was negative in both tests and negative results were obtained from the faeces from each of the 7 non-infected animals in both tests (Table 4).

Each of the simulated pooled faecal samples , which consisted of dilutions prepared from the faeces of two infected sheep was positive (Figure 5) and each of the controls produced the appropriate result. The results indicate that the optimised HC-PCR was sufficiently sensitive to identify a single infected animal with multibacillary Johne's disease in a pooled sample from at least 100 sheep.

DISCUSSION

The HC-PCR method described by Millar *et al*.[32] was a novel and sensitive approach to the diagnosis of Johne's disease, but was not practically suited for routine diagnostic testing. In this study we modified the procedure to make the test more functional and robust for routine use and identified vulnerable points in the test. The modifications have transformed the test from a 3 to 4 day expensive test without practical application to a 2 day test which can now be evaluated for diagnostic application. Unfortunately, some of the necessary modifications have come at the cost of reduced sensitivity. The use of Southern blotting was omitted from the procedure for two reasons. Firstly, there exist yet to be classified environmental mycobacteria with IS*900*-like elements that amplify in IS*900* PCR giving identical size products to *M. paratuberculosis*[36] and which are indistinguishable by Southern blotting. The original HC-PCR method relied on the assumption that IS*900*, especially the five prime end of the gene, was unique to *M. paratuberculosis* and PCR followed by Southern blotting would be sufficient evidence to identify any PCR positive results as being due to *M. paratuberculosis*. Sequence information indicates that the IS*900*-like elements can have high degrees of homology with IS*900* from *M. paratuberculosis* in regions commonly used for PCR priming[36]. Restriction endonuclease analysis, which is more practical and less costly, has been suggested as the preferred method of confirming IS*900* PCR positives as *M. paratuberculosis*[36] as no primers for IS*900* including those used in this study have been found to be specific for *M. paratuberculosis*. Secondly, Southern blotting would make the cost of the test too high.

Several combinations of hybridisation buffers and wash buffers were evaluated during the course of this study. Each of the hybridisation buffers had been successful in other hybridisation studies[32, 39, 40]. All of the buffer combinations were evaluated by visualisation of 10 fold dilution series of the amplified product but surprisingly the combination of only two hybridisation buffers (1 and 4) and one wash buffer (A) proved to be most successful. Hybridisation buffer 1 was already known to be successful as it was based on the original HC-PCR buffer[32] but optimised for our laboratory. The polyethylene glycol included in hybridisation buffer 2 and Denhardts reagent in hybridisation buffer 3 were used in an attempt to increase the rate of hybridisation and minimise non-specific hybridisation, respectively. Both buffers prevented any hybridisation for reasons that are unknown. However, hybridisation buffer 3 which is used routinely for hybridisation reactions with nucleic acids immobilised to membranes failed in hybridisation of nucleic acids in solution suggesting that the suitability of hybridisation buffers may be dependent on their application. Hybridisation buffer 4 included HEPES in order to maintain a neutral pH during the overnight incubation at 65 ^{o}C. Straus and Ausubel[40]

suggest that a neutral pH is required to minimise depurination of DNA during lengthy incubations at high temperatures. This may explain why hybridisation buffer 4 was the most successful in this study. However, the effects of time and temperature on the pH of the buffers used for hybridisation were not investigated as part of this study.

Hybridisation buffers 1 and 4 were further evaluated over a range of final volumes for the hybridisation reaction as high volume is known to reduce the efficiency of hybridisation[39] and the optimal combination was hybridisation buffer 4 in 150 µL final volume and wash buffer A. This combination was then used to evaluate the optimal temperature and time for capture of the probe-target by the magnetic beads and the 4 hour incubation at 37 ^{o}C was chosen, which allowed the test to be completed in 2 days.

It became apparent during the course of this study that one of the most critical points in the procedure was the treatment of the bead-probe-target DNA complex during capture and washing procedures. Several methods of mixing the beads during the capture process, including no mixing, were examined. The more vigorous methods of mixing (eg. end over end rotation) were detrimental to the procedure. Presumably these methods resulted in forces that were able to shear the probe off the beads or break the IS*900* gene beyond the region of the capture probe, thereby excluding the appropriate 5 prime region of IS*900* from the detection PCR. These outcomes would prevent PCR amplification. The results suggest that gentle mixing is required, and that no mixing, while resulting in some amplification, had a limiting effect. Gentle mixing using a platform rocker where the tubes lie horizontally was chosen. Extreme care was exercised when handling the beads during the washing procedure. The wash buffer was dispensed down the side of the tube opposite to the side the beads had collected on. This was done to avoid shear forces produced by the fluid moving rapidly over the beads. After washing, the beads were resuspended in 5 µL of TE. Early attempts at using centrifugation to force the beads down into the TE were found to be detrimental and prevented amplification. Gently washing the beads off the wall of the tube with the 5 µL of TE appeared to have no adverse effect. It should also be noted that the beads should be washed without delay and resuspended in TE immediately after washing. Leaving the beads for 30 minutes in the class II biological safety cabinet before resuspending resulted in failure of amplification. This was attributed to either drying out of the beads or the effects of vibration caused by the motor in the cabinet. When these conditions were not adhered to a marked reduction or failure of PCR was observed.

During preliminary evaluations of faecal samples it became apparent that DNA purification using the glass bead method was prone to sample to sample cross contamination as the faecal extraction process control was positive in 3 out of the 5 replicate HC-PCR tests. Subsequent investigations indicated that the contamination was occurring during the glass bead purification step possibly due to aerosols of genomic DNA. During the purification process the tubes were repeatedly opened and closed to enable pipetting of samples or reagents thus allowing cross contamination of genomic DNA to occur. Although HC-PCR significantly reduces the impact of cross contamination with amplified product[32] it does not overcome cross contamination with genomic DNA. To address this problem, a closed system was developed using a resin based DNA purification kit. This technique was performed in a sterile 2.5 mL syringe and 1.5 mL screw cap centrifuge tube that did not require opening during the procedure. This eliminated the problems of cross contamination. The resin based method was also quicker and less laborious but care should be exercised to avoid needle stick injuries. The modified HC-PCR method was capable of detecting 5000 organisms per 200 mg of faeces based on agarose gel electrophoresis stained with ethidium bromide. Whilst this represents a reduction in analytical sensitivity compared to the results of Millar *et al*.[32], this is explained by the removal of Southern blotting from the procedure. However, amplified product could be observed by eye at a concentration of 500 organisms per 200 mg of faeces but at a concentration too low to be confirmed by restriction endonuclease analysis in agarose gels stained with ethidium bromide. This result indicates that the reduction in sensitivity after the omission of Southern blotting is compounded by limitations associated with routine agarose gel electrophoresis. Opportunities may exist to overcome these issues by incorporating more sensitive detection methods such as polyacrylamide gel electrophoresis combined with silver staining and is being investigated as part of the ongoing evaluation of HC-PCR.

Preliminary evaluation of the optimised HC-PCR protocol combined with the resin-based faecal DNA purification was conducted on 13 faecal samples. The test correctly identified all negative faeces on two separate occasions and all but 2 positive faeces in test 6 and all but 1 positive faeces in test 7. Sheep 13 which was weakly positive in test 6 but more strongly positive in test 7 was considered to be a paucibacillary case based on Ziehl-Neelsen staining and agar gel immunodiffusion precipitation test results. The variation in the amount of amplified product for sheep 13 observed in both tests and the results for sheep 6, which was negative in test 6 but positive in test 7, indicate that replicates may be required in PCR to normalise the results. Sheep 9 which was negative in both tests was considered to be infected based on histological examination only but was negative in the agar gel immunodiffusion precipitation test. Furthermore it was encouraging that the procedure identified a single infected animal amongst up to as many as 100 non-infected animals in a simulated pooled sample demonstrating that the technique may be suitable for screening flocks for Johne's disease. However, further work is required to confirm this. In particular to determine the sensitivity of HC-PCR in detecting single animals with low bacterial loads such as paucibacillary cases is required.

Sequence capture-PCR has been used to extract DNA and RNA from viruses and bacteria from a variety of environmental and clinical samples with varying degrees of success. Muir *et al.* [33] described a sequence capture-PCR protocol for identifying enterovirses from clinical samples including cerebrospinal fluid, stool, saliva, blood, pericardial fluid, urine and tissues from humans. While this method was simple the sensitivity was no greater than

simpler extraction techniques. When a similar procedure was applied to stool and shellfish samples to detect hepatitis A, Arnal *et al*.[29] reported disappointing results with sequence capture-PCR, favouring other extraction techniques to eliminate PCR inhibitors and non-specific RNA and DNA. These results suggest that sequence capture-PCR success maybe dependent on the particular application. In both of these studies sequence capture was performed using probes that were pre-bound to magnetic beads. Other studies using sequence capture-PCR suggest that this reduces the efficiency of capture. Hybridisation in solution followed by capture on magnetic beads (two-step capture) maybe more efficient[31]. Successful two-step capture has been reported for the detection of a number of mycobacterial species including; *M. tuberculosis*[31], *M. paratuberculosis*[32] and *M. bovis*[34]. However, Roring *et al*.[34] suggested that a pre-enrichment procedure such as radiometric culture prior to sequence capture-PCR maybe required to detect paucibacillary cases while Muir *et al*.[33] suggested using nested-PCR to improve sensitivity.

We plan to undertake a larger scale evaluation of the optimised HC-PCR procedure for flock diagnosis of ovine Johne's disease based on pooled samples. The results of this study indicate that extensive optimisation experiments may be required to achieve adequate performance on an application by application basis, and that published protocols can only be considered as a guide for new applications.

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Captions to figures

Figure 1: PCR results for the various methods of mixing the magnetic beads during capture. Lane 1 is a molecular size marker. Lanes 2 to 9 are combinations of end over end rotation at different angles and speeds: steep/fast (2 and 3), steep/slow (4 and 5), shallow/fast (6 and 7) and shallow/slow (8 and 9). For the remaining lanes agitation was achieved with a platform rocker (10 and 11), microtitre plate shaker (12 and 13) and no agitation for lanes 14 and 15.

Figure 2: HC-PCR was performed on a dilution series (10^{-1} to 10^{-4}, lanes 3 to 6) of *M. paratuberculosis* DNA. After capture and washing, the beads were either re-suspended in TE immediately (panel A) or allowed to sit for 30 minutes before re-suspension in TE (panel B). Lane 1 is a molecular size marker, lane 2 is a blank and lane 7 is a negative control.

Figure 3: Comparison of DNA purification techniques, glass bead (lanes 2-9) and resin (lanes 12-19) on faeces spiked with a serial dilution of *M. paratuberculosis* whole cells (5×10^6 to 0.5 per 200 mg of faeces). Lane 1 is the molecular size marker, lane 11 is blank and lanes 10 and 20 are the negative control faeces (the same faeces used for spiking).

Figure 4: The results from HC-PCR tests 6 (top row) and 7 (bottom row) (Table 4). HC-PCR, incorporating the resin DNA purification method, was performed on the group of 13 faecal samples. For each row lanes 1 -13 are the 13 faecal samples, lane 14 is a molecular size marker. Lanes 15, 16 and 17 are the HC-PCR positive, negative and reagent control, respectively. Lanes 18 and 19 are the hybridisation and capture controls, respectively.

Figure 5: Results from HC-PCR performed on faeces diluted to represent pooled faecal samples. Lanes 1 and 6 are the undiluted faeces from two sheep known to have Johne's disease. Lanes 2 and 7, 3 and 8, 4 and 9 and 5 and 10 are dilutions of the infected faeces with faeces from a non-infected sheep equal to 1 in 10, 1 in 25, 1 in 50 and 1 in 100,

respectively. Lane 11 is a molecular size marker. Lanes 12, 13 and 14 are the HC-PCR positive, negative and reagent control, respectively. Lanes 15 and 16 are the hybridisation and capture controls, respectively.

Table 1 : Hybridisation buffers.

Buffer	Ingredients	Reference
1	6x SSPE (900 mM NaCl, 60 mM NaH_2PO_4-$2H_2O$, 6 mM EDTA, pH 7.4), 0.1% SDS	based on [32]
2	Buffer 1 plus 5% w/v polyethylene glycol 8000	
3	6x SSC (0.9 M NaCl, 0.09 M Na3 citrate), 1x Denhardts reagent, 0.1% SDS	based on [39]
4	HEPES (30 mM HEPES, 3 mM EDTA, pH7.5), 1 M NaCl, sterile water	based on [40]

Table 2 : Wash buffers.

Buffer	Ingredients
A	1x PBS, 0.1% w/v BSA
B	6x SSPE (900 mM NaCl, 60 mM NaH_2PO_4-$2H_2O$, 6 mM EDTA, pH 7.4), 0.1% SDS, 0.1% w/v BSA
C	6x SSPE (900 mM NaCl, 60 mM NaH_2PO_4-$2H_2O$, 6 mM EDTA, pH 7.4), 0.1% SDS
D	TE (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.8), 0.1% w/v BSA
E	TE (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.8)

Table 3: The ✎ symbol indicates the levels of the procedure where the control was included and processed.

Procedure	JD positive faeces	JD negative faeces	Faecal extraction process (PBS)	Hybridisation (sterile water without target DNA)	Capture (beads in water)	PCR positive (target DNA)	PCR negative (water)	PCR reagent (PCR cocktail without target DNA or water)
Extraction	✎	✎	✎					
Purification	✎	✎	✎					
Hybridisation	✎	✎	✎	✎				
Capture	✎	✎	✎	✎	✎			
Washing	✎	✎	✎	✎	✎			
PCR	✎	✎	✎	✎	✎	✎	✎	✎
Electrophoresis	✎	✎	✎	✎	✎	✎	✎	✎
Expected result	positive	negative	negative	negative	negative	positive	negative	negative

Table 4: Results for HC-PCR on the test group of 13 faecal samples. Tests 1-5 were performed after purification of the DNA with the glass bead method and tests 6 and 7 were performed after purification of the DNA with the resin method.

Sample	Species	JD Status[(a)]	Test 1	2	3	4	5	6	7
1	alpaca	-	-	+	-	-	-	-	-
2	alpaca	-	-	-	-	-	n.t.	-	-
3	alpaca	-	-	-	+	-	-	-	-
4	alpaca	-	-	+	+	-	-	-	-
5	sheep	-	-	+	-	-	-	-	-
6	sheep	+	+	+	+	+	+	-	+
7	sheep	-	-	+	-	-	-	-	-
8	sheep	-	-	+	+	-	-	-	-
9	sheep	+	+	-	-	-	-	-	-
10	sheep	+	+	+	+	+	+	+	+
11	sheep	+	+	+	+	+	+	+	+
12	sheep	+	+	+	+	+	+	+	+
13	sheep	+	+	+	-	-	-	+	+
Controls									
Positive faeces			+	+	+	+	+	+	+
Negative faeces			-	-	-	-	-	-	-
Faecal extraction process control			-	+	+	+	-	-	-
Hybridisation control			n.t.	n.t.	-	-	-	-	-
Capture control			n.t.	n.t.	-	-	-	-	-
PCR positive			+	+	+	+	+	+	+
PCR negative			-	-	-	-	-	-	-
PCR reagent			-	-	-	-	-	-	-

(a) - JD status of the animal based on the results from: BACTEC culture, Ziehl-Neelsen staining of tissue samples, agar gel immunodiffusion precipitation test, gross histological examination. n.t. not tested

Figure 1

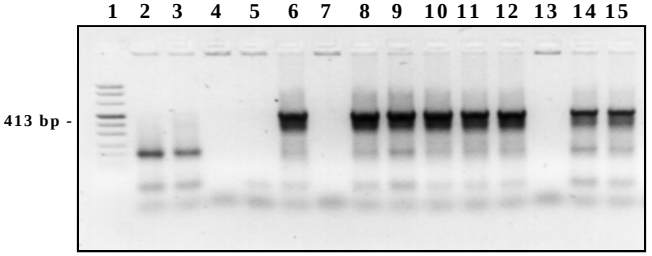


Figure 2

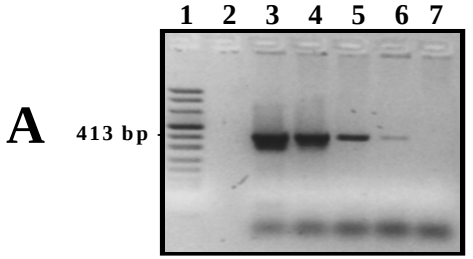


Figure 3

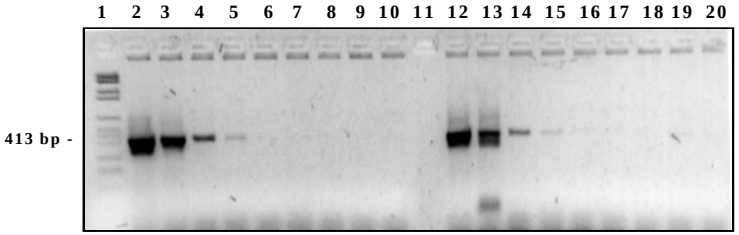


Figure 4

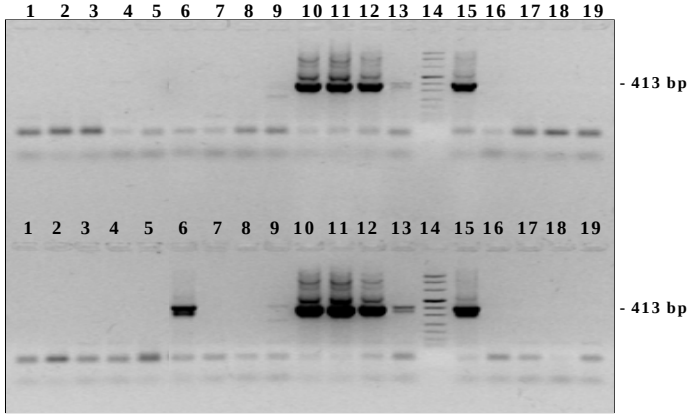
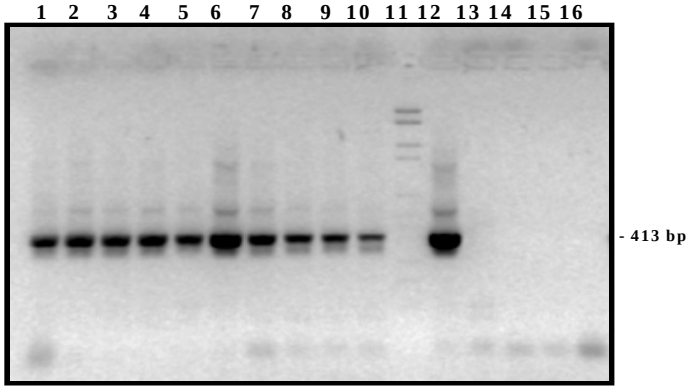


Figure 5



Appendix 2 : HC-PCR evaluation of faeces from 126 individual sheep - Results

Sample	Farm Status	Culture	W. to 999	900M	HC-PCR
1	Infected	+	4	+	
2	Infected	+	4	+	
3	Infected	+	5	+	
4	Infected	+	5	+	
5	Infected	+	5	+	
6	Infected	+	6	-	+
7	Infected	-		-	+
8	Infected	+	5	+	
9	Infected	+	6	+	
10	Infected	+	8	-	-
11	Infected	+	6	+	
12	Infected	+	7	+	
13	Infected	+	6	+	
14	Infected	+	5	+	
15	Infected	+	3	+	
16	Infected	+	5	+	
17	Infected	+	5	+	
18	Infected	-		-	-
19	Infected	+	5	+	
20	Infected	-		-	-
21	Infected	-		-	-
22	Infected	+	7	+	
23	Infected	-		-	-
24	Infected	+	5	+	
25	Infected	+	3	+	
26	Infected	+	6	-	-
27	Infected	+	7	-	-
28	Infected	+	3	+	
29	Infected	+	3	+	
30	Infected	+	4	+	
31	Infected	+	5	-	+
32	Infected	+	6	+	
35	Infected	+	4	+	
36	Infected	+	5	+	
39	Suspect	-		+	
40	Infected	+	7	-	-
41	Infected	-		+	
42	Infected	-		-	-
43	Infected	-		-	+
44	Infected	-		-	-
45	Infected	-		-	+
46	Infected	+	4	+	
47	Infected	+	6	-	-
48	Infected	+	5	-	+
49	Infected	+	5	-	-
50	Infected	-		-	+
51	Infected	-		-	-
52	Infected	-		+	
53	Infected	-		+	
54	Infected	+	6	-	
55	Infected	+	3	+	
56	Infected	-		-	-
57	Infected	-		-	+

Sample	Farm Status	Culture	W. to 999	900M	HC-PCR
58	Infected	+	3	+	
59	Suspect	-		-	-
60	Suspect	-		-	-
61	Suspect	-		-	-
62	Suspect	-		-	-
63	Suspect	-		-	-
64	Suspect	-		-	-
65	Suspect	-		-	-
66	Suspect	-		-	-
67	Suspect	-		-	-
68	Suspect	-		+	
69	Suspect	-		-	-
70	Suspect	-		-	-
71	Infected	+	3	+	
72	Infected	+	3	+	
73	Infected	+	3	+	
74	Infected	+	4	+	
75	Infected	+	3	+	
76	Infected	+	4	+	
77	Infected	+	4	+	
78	Infected	-		-	-
79	Infected	+	4	-	-
80	Infected	-		-	-
81	Infected	-		-	-
82	Infected	-		-	+
83	Infected	-		-	+
84	Infected	-		-	-
85	Infected	+	5	-	-
86	Infected	-		-	-
87	Infected	-		-	-
88	Infected	-		-	-
89	Infected	-		-	-
90	Infected	-		-	-
91	Infected	-		-	-
92	Infected	-		-	-
93	Infected	-		-	-
94	Infected	-		-	-
95	Infected	-		-	-
96	Infected	+	5	-	-
97	Infected	-		-	-
98	Suspect	-		-	-
99	Suspect	-		-	-
100	Suspect	-		-	-
101	Suspect	-		-	-
102	Suspect	-		-	-
103	Suspect	-		-	-
104	Suspect	-		-	-
105	Suspect	-		-	-
106	Suspect	-		-	-
107	Suspect	-		-	-
108	Suspect	-		-	-
109	Suspect	-		-	-
110	Suspect	-		-	-
111	Suspect	-		-	-

Sample	Farm Status	Culture	W. to 999	900M	HC-PCR
112	Suspect	-		-	-
113	Suspect	-		-	-
114	Infected	+	4	+	
115	Infected	-		-	-
116	Infected	-		-	-
117	Infected	+	4	+	
118	Infected	-		-	-
119	Infected	+	4	+	
120	Infected	+	4	+	
121	Suspect	-		-	-
122	Suspect	-		-	-
123	Suspect	-		-	-
124	Suspect	-		-	-
125	Suspect	-		-	-
126	Suspect	-		+	-
127	Suspect	-		-	-
128	Suspect	-		-	-
129	Unknown	+	4	+	-
130	Unknown	+	3	-	-

Appendix 3 :HC-PCR evaluation of 155 pooled faecal samples - Results

Sample	Farm Status	Culture	W. to 999	900M	HC-PCR
8	Un-infected	-		+	-
12	Un-infected	-		-	-
13	Un-infected	-		-	-
14	Infected	+	4	-	-
22	Infected	+	3	-	-
23	Unknown	-		-	-
24	Unknown	-		-	-
31	Infected	+	7	-	-
32	Infected	-		-	-
35	Infected	+	6	-	-
37	Infected	-		-	-
44	Unknown	-		-	-
51	Un-infected	-		-	-
52	Infected	+	6	-	-
54	Infected	-		-	-
60	Un-infected	+	5	-	-
65	Un-infected	-		-	-
66	Unknown	+	5	-	-
67	Unknown	-		-	-
73	Unknown	+	5	+	-
74	Un-infected	+	6	-	-
75	Infected	-		-	-
80	Infected	+	5	-	-
82	Infected	+	3	+	+
88	Infected	+	5	-	-
92	Infected	-		-	-
96	Infected	+	5	-	-
114	Infected	+	5	+	+
137	Infected	+	7	-	-
138	Infected	-		-	-
139	Infected	-		-	-
140	Infected	-		-	-
141	Infected	-		-	-
142	Infected	+	6	+	+
143	Infected	+	5	+	+
145	Infected	+	8	-	-
146	Infected	+	8	-	-
147	Infected	+	6	+	+
148	Infected	+	6	-	-
149	Infected	-		+	+
161	Infected	+	8	-	-
206	Un-infected	-		-	-
207	Infected	+	6	-	-
209	Un-infected	-		-	-
210	Infected	+	4	-	+
211	Infected	+	4	-	+
212	Infected	+	4	-	-
213	Infected	+	4	-	+
214	Un-infected	-		-	-
215	Unknown	-	4	-	-
216	Infected	+	4	+	+

Sample	Farm Status	Culture	W. to 999	900M	HC-PCR
234	Infected	-		-	-
235	Infected	+	10	-	-
236	Infected	+	4	-	-
237	Infected	+	4	-	+
238	Infected	+	5	-	-
239	Infected	-		-	-
241	Infected	+	5	-	-
242	Infected	+	4	+	+
243	Infected	-		-	-
244	Infected	+	4	+	+
245	Infected	+	4	-	-
246	Infected	+	4	-	+
247	Infected	+	5	-	+
248	Infected	+	5	-	-
249	Infected	-		-	-
250	Infected	+	4	+	+
251	Infected	+	4	+	+
252	Infected	+	4	-	-
253	Infected	+	5	+	+
254	Infected	+	4	+	+
255	Infected	+	6	-	+
256	Infected	+	8	-	-
257	Infected	+	5	+	-
258	Infected	-		-	-
259	Infected	-		-	-
260	Infected	+	4	+	-
261	Infected	+	5	-	-
262	Infected	+	4	+	-
263	Infected	+	4	-	-
264	Infected	+	3	+	-
265	Infected	+	4	+	-
266	Infected	+	8	-	-
267	Infected	-		-	-
268	Infected	-		-	-
269	Infected	+	4	+	-
270	Infected	+	5	+	-
271	Infected	+	4	-	-
272	Infected	+	7	+	-
273	Infected	+	4	+	-
274	Infected	-		-	-
275	Infected	-		-	-
276	Infected	-		+	-
277	Infected	+	5	+	+
278	Infected	-		-	-
279	Infected	-		-	-
280	Infected	+	6	-	-
281	Infected	-		-	-
282	Infected	-		-	-
283	Infected	+	5	+	-
284	Infected	-		-	-
285	Infected	-		-	-
286	Infected	-		-	-

Sample	Farm Status	Culture	W. to 999	900M	HC-PCR
287	Infected	+	5	+	+
288	Infected	-		-	-
289	Infected	-		-	-
290	Infected	-		-	-
291	Infected	+	5	-	-
292	Infected	-		-	-
293	Infected	-		-	-
294	Infected	-		-	-
295	Infected	+	4	+	+
296	Infected	+	4	-	-
297	Infected	-		-	-
298	Infected	-		-	-
299	Infected	+	4	-	+
300	Infected	+	7	-	-
301	Infected	-		-	+
302	Infected	+	4	-	-
303	Infected	+	7	-	-
304	Infected	+	5	-	-
305	Infected	+	5	+	+
306	Infected	-		-	-
307	Infected	+	3	+	+
308	Infected	-		+	-
311	Infected	+	5	-	-
312	Infected	+	5	-	-
314	Infected	+	5	+	+
315	Infected	+	5	+	+
316	Infected	+	5	+	+
317	Infected	+	5	+	+
318	Infected	-		-	-
319	Infected	-		-	-
320	Infected	+	7	+	+
321	Infected	+	6	+	+
322	Infected	-		-	-
323	Infected	+	7	+	+
324	Infected	+	7	+	+
325	Infected	-		-	+
326	Infected	-		-	+
327	Infected	+	5	+	+
328	Infected	+	7	+	+
330	Infected	-		-	+
331	Infected	-		-	+
332	Infected	-		-	+
333	Infected	-		-	+
334	Infected	+	5	+	+
337	Infected	+	4	+	+
367	Infected	+	5	+	-
368	Infected	+	5	+	+
369	Infected	+	5	+	+
370	Infected	+	5	+	+
371	Infected	-		-	-
372	Infected	+	5	+	+
373	Infected	+	4	+	+

Appendix 4 : Problems identified with the HC-PCR method and options for correction and/or improvement as per Milestone report 1 TR.060 (2/3/99) and the results to date.

Problem Area	Effect	Options	Improvement	Result
Location of capture probe on IS*900* gene.	Loss of sensitivity due to fragmentation of DNA.	Design and evaluate new capture probes in regions of IS*900* closer to the detection PCR region.	Improved sensitivity.	Several of the new capture probes out performed the Millar capture probe in HC-PCR but only 1 of the new capture probes produced a result similar to DPCR . DPCR once again out performed HC-PCR as observed in Milestone report 1.
Detection of the amplified product from HC-PCR.	Loss of sensitivity (10 fold) due to removal of Southern blotting from protocol.	Silver staining of agarose gels. Polyacrylamide gel electrophoresis with silver staining.	Improved sensitivity and may allow use of restriction endonuclease analysis for confirmation of *M. avium* subsp *paratuberculosis.*	Silver staining of agarose gels was unsuccessful. Polyacrylamide gel electrophoresis with silver staining resulted in a 10 to 100 fold increase in the analytical sensitivity over agarose gel electrophoresis with ethidium bromide staining for both PCR and REA.
Amount of faeces. DNA extraction method limited to 0.2 grams.	Impaired sensitivity due to insufficient *M. avium* subsp *paratuberculosis* DNA or excess non-specific DNA	Modify method to increase amount of faeces to 2 grams. Modify method to eliminate some of the non-specific DNA extracted from the faeces.	Improved sensitivity (10 fold).	Not Attempted
Deterioration of capture probe.	Loss of sensitivity or complete failure of reaction.	Replace current capture probe and re-evaluate samples. Regular QC of capture probe using control samples.	Improved sensitivity. Determine the shelf life of capture probe.	The results from the current experiment matched those of the experiment performed in 1997, indicating that the capture probe had not deteriorated.
IS*900* region *per se*.	Possible loss of sensitivity due to steric factors in magnetic capture.	Try capture and detection of IS*1311* with REA to identify and distinguish between sheep and cattle strains of *M. avium* subsp *paratuberculosis* and *M. avium* subsp *avium.*		Not Attempted
Evaluate new protocol				Not Attempted

Appendix 5: Silver staining procedure for PAGE.

Step	Time	Solution	Weight/Volume
Fix	1 hour or more (usually overnight)	Methanol Acetic Acid 37% Formaldehyde MQW	50ml 12ml 50µl 38ml
Wash	3 x 20 minutes	Ethanol MQW	50ml 50ml
Pre-treatment	1 minute	Sodium thiosulphate (10mg/ml) MQW	2ml 98ml
Rinse	3 x 20 seconds	MQW	100ml
Impregnate	20 minutes	Silver nitrate 37% Formaldehyde MQW	0.2g 75µl 100ml
Rinse	3 x 20 seconds	MQW	100ml
Developer	10 minutes	Sodium thiosulphate Sodium carbonate 37% Formaldehyde MQW	2ml 6g 75:l 98ml
Rinse	2 x 2 minutes	MQW	100ml
Destain	10 minutes	Methanol Acetic Acid MQW	50ml 12ml 38ml
Store		MQW	100ml

Appendix 6: Recommendations for the continued development of a rapid cost effective test for ovine Johne's disease based on testing of pooled faeces.

Order	Task	Action	Desired Outcome	Time
1	Continue research into direct PCR	• Agarose gel electrophoresis/ethidium bromide staining followed by polyacrylamide gel electrophoresis/silver staining on negatives. • Compare the sensitivity and specificity of direct PCR using the Millar primers with those using the Vary primers. • Evaluate alternate primers throughout the 5 prime region of the IS*900* gene that may result in a more sensitive reaction. • Develop and evaluate methods to increase the effects of faecal sample size 10 fold	Determine the optimal conditions for the detection of *M. paratuberculosis* from pooled faecal samples, using pooled faecal culture as the bench mark.	December 1999 - February 2000
2	Evaluate direct PCR with IS*1311*	As for task number 1 only replacing IS*900* as the target for PCR with IS*1311*.		Only if required
3	Retest a large collection of pooled faecal samples.	Evaluate the direct PCR procedure when we are confident that the method has been optimised.	To determine the sensitivity of the new test	February 2000 - April 2000

Appendix 7 : Preliminary evaluation of DPCR on pooled faecal samples - Results.

Sample	Status	Culture	W.to 999	DPCR 900M	DPCR 900VM	REA 900VM	PAGE 900VM	PAGE REA 900VM	DPCR 1311L	REA 1311L	DPCR 1311S	REA 1311S
92	Infected	-		-	-				-		-	
93	Infected	-		-	-				-		-	
94	Infected	-		-	-				-		-	
95	Infected	-		-	-				-		-	
96	Infected	+	5	-	+	+			-		+	+
97	Infected	+	5	+	+	+			+	+	+	+
98	Infected	+	4	+	+	+			+	+	+	+
99	Infected	+	5	-	+	+			+	+	+	+
101	Infected	+	5	-	+	+			-		+	+
102	Infected	+	5	-	+	+			-		+	+
103	Infected	+	5	-	+	+			+		+	+
104	Infected	-		-	-				-		-	
105	Infected	+	5	-	+	+			-		+	+
106	Infected	+	6	-	-		-		-		-	
114	Infected	+	5	+	+	+			+	+	+	+
115	Infected	+	6	+	+	+			-		+	
116	Infected	+	6	+	+	+			+	+	+	+
117	Infected	+	5	+	+		+	+	-		+	+
118	Infected	+	5	-	-		+	+	-		-	
119	Infected	+	6	-	-		+	+	+	+	+	+
120	Infected	+	5	-	-		+	+	-		+	+
121	Infected	+	4	+	+	+			+	+	-	
122	Infected	+	6	-	-		-		-		-	
123	Infected	+	5	-	+	+			-		-	
124	Infected	+	5	+	+	+			+	+	+	+
125	Infected	+	5	-	+	+			+	+	+	+
126	Infected	+	4	+	+	+			+	+	+	+
127	Infected	+	6	-	+	+			+	-	+	+
128	Infected	+	5	-	-		-		-		-	
129	Infected	+	6	+	+	+			+	+	+	+
130	Infected	+	6	-	+		+	+	-		+	
131	Infected	+	5	-	-		+	+	+		+	+
132	Infected	+	5	-	-		+	+	+		+	+
133	Infected	+	5	-	-		+	+	-		-	
134	Infected	+	6	-	+	+			-		+	
135	Infected	+	7	-	+	+			+	-	+	+
136	Infected	+	5	+	+	+			+	+	+	+
137	Infected	+	7	-	-		+	+	-		+	
138	Infected	-		-	-				-		-	
139	Infected	-		-	-				-		-	
140	Infected	-		-	-				-		-	
141	Infected	-		-	-				-		-	
142	Infected	+	6	+	+	+			+	+	+	+

Sample	Status	Culture	W.to 999	DPCR 900M	DPCR 900VM	REA 900VM	PAGE 900VM	PAGE REA 900VM	DPCR 1311L	REA 1311L	DPCR 1311S	REA 1311S
143	Infected	+	5	-	+	+			+	-	+	+
145	Infected	+	8	-	+	+			-		-	
146	Infected	+	8	-	-		-		-		-	
147	Infected	+	6	-	+	+			+	-	+	+
148	Infected	+	6	-	-		+	+	-		-	
149	Infected	-		-	+	+			+	-	+	+
150	Infected	+	6	-	+	+			-		+	
151	Infected	+	8	-	-		-		-		-	
152	Infected	+	6	-	+	+			+	-	+	+
153	Infected	+	7	-	+		+	+	+		+	
154	Infected	+	7	-	-		-		-		-	
155	Infected	+	6	-	+	+			+	-	+	+
156	Infected	+	6	-	-		-		-		-	
157	Infected	+	5	+	+	+			-		+	+
158	Infected	+	6	-	+	+			-		+	
159	Infected	-		-	-				-		+	
160	Infected	-		-	-				-		-	
161	Infected	+	8	-	+	-	-		-		-	
206	Un-infetced	-		-	-				-		-	
207	Infected	+	6	+	+	+			+		+	+
208	Un-infected	-		-	-				-		-	
209	Un-infected	-		-	-				-		-	
210	Infected	+	4	+	+	+			+	+	+	+
211	Infected	+	4	+	+	+			+	+	+	+
212	Infected	+	4	-	+	+			+		+	+
213	Infected	+	4	+	+	+			+	+	+	+
214	Un-infected	-		-	-				-		-	
215	Unknown	-	4	-	-				-		-	
216	Infected	+	4	-	+	+			+	+	+	+
234	Infected	-		-	-				-		-	
235	Infected	+	10	-	-		+	-	-		-	
236	Infected	+	4	-	+	+			-		+	+
237	Infected	+	4	+	+	+			+	+	+	+
238	Infected	+	5	+	+	+			-		+	+
239	Infected	-		-	-				-		-	
240	Infected	-		-	-				-		-	
241	Infected	+	5	-	+	+			-		+	+
242	Infected	+	4	+	+	+			+	+	+	+
243	Infected	-		+	+	-			-		-	
244	Infected	+	4	+	+	+			+	+	+	+
245	Infected	+	4	+	+	+			+		+	+
246	Infected	+	4	+	+	+			+		+	+
247	Infected	+	5	+	+	+			-		+	+

Sample	Status	Culture	W.to 999	DPCR 900M	DPCR 900VM	REA 900VM	PAGE 900VM	PAGE REA 900VM	DPCR 1311L	REA 1311L	DPCR 1311S	REA 1311S
248	Infected	+	5	-	+		+	+	-		-	
249	Infected	-		-	+				-		-	
250	Infected	+	4	+	+	+			+	-	+	+
251	Infected	+	4	+	+	+			+		+	+
252	Infected	+	4	-	+		+	+	-		+	
253	Infected	+	5	+	+	+			+	-	+	+
254	Infected	+	4	+	+	+			+	+	+	+
255	Infected	+	6	-	+	+			-		+	
256	Infected	+	8	-	-		-		-		-	
257	Infected	+	5	+	+	+			-		+	
258	Infected	-		-	-				+		-	
259	Infected	-		-	-				-		-	
260	Infected	+	4	-	+		+	+	-		-	
261	Infected	+	5	-	-				-		+	+
262	Infected	+	4	+	+	+			-		+	+
263	Infected	+	4	-	+	+			-		+	+
264	Infected	+	3	-	+				+		-	
265	Infected	+	4	+	+	+			+	+	+	+
266	Infected	+	8	-	-		-		-		+	
267	Infected	-		-	+				-		+	
268	Infected	-		-	-				+		-	

Appendix 8: Blind evaluation of DPCR on pooled faecal samples - Results.

Sample	DPCR (900VM)	REA	Culture	W. to 999
1	-		-	
2	-		-	
3	-		-	
4	-		-	
5	-		-	
6	-		-	
7	-		-	
8	-		-	
9	-		-	
10	-		-	
11	-		-	
12	-		-	
13	-		-	
14	-		-	
15	-		-	
16	-		-	
17	-		-	
18	-		-	
19	-		-	
20	-		-	
21	-		-	
22	-		-	
23	-		-	
24	-		-	
25	-		-	
26	-		-	
27	-		-	
28	-		-	
29	+	+	+	4
30	-		+	6
31	-		-	
32	-		-	
33	-		-	
34	-		-	
35	-		-	
36	-		-	
37	-		-	
38	-		-	
39	-		-	
40	-		-	
41	-		-	
42	-		-	
43	-		-	
44	-		-	
45	-		-	
46	-		-	
47	-		-	
48	-		-	

Sample	DPCR (900VM)	REA	Culture	W. to 999
49	-		-	
50	-		-	
51	-		-	
52	-		-	
53	-		-	
54	-		-	
55	-		-	
56	-		-	
57	-		-	
58	-		-	
59	-		-	
60	-		-	
61	-		-	
62	-		-	
63	-		-	
64	-		-	
65	-		-	
66	+	+	+	4
67	-		-	
68	-		-	
69	-		-	
70	-		-	
71	-		-	
72	+	+	+	5
73	-		-	
74	-		-	
75	-		-	
76	-		-	
77	-		-	
78	+	+	-	
79	-		-	
80	-		-	
81	-		-	
82	-		-	
83	-		-	
84	-		-	
85	-		-	
86	-		-	
87	-		-	
88	-		-	
89	-		-	
90	-		-	
91	-		-	
92	-		-	
93	-		-	
94	-		-	
95	-		-	
96	-		-	

Sample	DPCR (900VM)	REA	Culture	W. to 999
97	-		-	
98	-		-	
99	-		-	
100	-		-	
101	-		-	
102	-		-	
103	-		-	
104	-		-	
105	-		-	
106	-		-	
107	-		-	
108	-		-	
109	-		-	
110	-		-	
111	-		-	
112	-		-	
113	-		-	
114	-		-	
115	+	+	+	4
116	+	-	-	
117	-		-	
118	-		-	
119	-		-	
120	+	+	+	4
121	+	+	+	4
122	-		+	5
123	-		-	
124	+	+	+	4
125	+	+	+	4
126	+	+	+	5
127	+	+	+	5
128	+	+	+	5
129	-		-	
130	-		-	
131	-		-	
132	-		-	
133	-		-	
134	-		+	6
135	-		-	
136	-		-	
137	-		-	
138	-		-	
139	-		-	
140	-		-	
141	-		-	
142	-		-	
143	-		-	
144	-		-	

Sample	DPCR (900VM)	REA	Culture	W. to 999
145	-		-	
146	-		-	
147	-		-	
148	-		-	
149	-		-	
150	-		-	
151	-		-	
152	-		-	
153	-		-	
154	-		-	
155	-		-	
156	-		-	
157	-		-	
158	-		-	
159	-		-	
160	-		-	
161	-		-	
162	-		-	
163	-		-	
164	-		-	
165	-		-	
166	-		-	
167	-		-	
168	-		-	
169	-		-	
170	-		-	
171	-		-	
172	-		-	
173	-		-	
174	-		-	
175	-		-	
176	-		-	
177	-		-	
178	-		+	8
179	-		-	
180	-		-	
181	-		-	
182	-		-	
183	-		-	
184	-		-	
185	-		-	
186	-		-	
187	-		-	
188	-		-	
189	-		-	
190	-		-	
191	-		-	
192	-		-	

Sample	DPCR (900VM)	REA	Culture	W. to 999
193	-		-	
194	-		-	
195	-		-	
196	-		-	
197	-		-	
198	-		-	
199	-		-	
200	-		-	
201	-		-	
202	-		-	
203	-		-	
204	-		-	
205	-		-	
206	-		-	
207	-		-	
208	-		-	
209	-		-	
210	-		-	
211	-		+	6
212	+	+	-	
213	-		+	6
214	-		-	
215	+	+	+	5
216	-		-	
217	-		-	
218	-		-	
219	-		-	
220	-		-	
221	-		-	
222	-		-	
223	-		-	
224	-		-	
225	-		-	
226	-		-	
227	-		-	
228	-		-	
229	-		-	
230	-		-	
231	-		-	
232	-		-	
233	-		-	
234	-		-	
235	-		-	
236	-		-	
237	-		-	
238	-		-	
239	-		-	
240	-		-	

Sample	DPCR (900VM)	REA	Culture	W. to 999
241	-		-	
242	-		-	
243	-		-	
244	-		-	
245	-		-	
246	-		-	
247	-		-	
248	-		-	
249	-		-	
250	-		-	
251	-		-	
252	-		-	
253	-		-	
254	-		-	
255	-		-	
256	-		-	
257	-		-	
258	-		-	
259	-		-	
260	-		-	
261	-		-	
262	-		-	
263	-		-	
264	-		-	
265	-		-	
266	-		-	
267	-		-	
268	-		-	
269	-		-	
270	+	+	+	4
271	-		+	6
272	-		+	12
273	+	+	+	5
274	+	+	+	6
275	+	+	+	5
276	-		-	
277	-		-	
278	-		-	
279	-		-	
280	-		-	
281	-		-	
282	-		-	
283	-		-	
284	-		-	
285	-		-	
286	-		-	
287	-		-	
288	-		-	

Sample	DPCR (900VM)	REA	Culture	W. to 999
289	-		-	
290	+	+	+	6
291	+	+	+	6
292	+	+	+	5
293	-		+	6
294	-		+	5
295	-		-	
296	+	+	+	6
297	-		-	
298	+	+	-	
299	-		-	
300	-		-	
301	-		-	
302	-		-	
303	-		-	
304	-		-	
305	-		-	
306	-		-	
307	-		-	
308	-		-	
309	-		-	
310	+	+	-	
311	-		-	
312	-		-	
313	-		-	
314	-		-	
315	-		-	
316	+	+	-	
317	-		-	
318	-		-	
319	-		-	
320	-		-	
321	-		-	
322	-		-	
323	+	+	-	
324	-		-	
325	-		-	
326	-		-	