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Possibilities for improving the D-PCR test for M. a. paratuberculosis from faeces

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Abstract

The rapid diagnosis of *M. a. paratuberculosis* infection in the early stages of disease, or where prevalence is low, is still critical for the management of ovine Johne's disease. Recently we reported the ability to detect *M. a*. *paratuberculosis* directly from sheep faecal samples by PCR (D-PCR) but the sensitivity of this test was less than the current gold standard test, pooled faecal culture (faecal culture). This project was undertaken to improve the sensitivity of the D-PCR test so that it was greater than or equal to faecal culture, without increasing the cost to the producer. Unfortunately, the new D-PCR described in this report failed to meet this objective. However, another D-PCR test developed in Japan that utilizes real-time PCR technology was also evaluated in the concluding stages of the project. The preliminary results with this test suggest that it has sensitivity equivalent to or greater than faecal culture. We highly recommended that this D-PCR test be further examined and developed for Australian conditions as a replacement or substitute test for faecal culture. As part of this project we successfully developed a new conventional multiplex PCR for *M. a*. *paratuberculosis* that includes an internal control. This new conventional PCR also warrants further investigation and should also be included in the future development of the D-PCR test to provide improved diagnosis and quality assurance for laboratories that are currently unable to use real-time-PCR technology for routine diagnostics.

Executive Summary

Seven years ago a major break through in the diagnosis of ovine Johne's disease (OJD) was reported with the introduction of pooled faecal culture (faecal culture). Faecal culture is based on radiometric culture and could successfully be used to culture the ovine strains of *Mycobacterium avium* subsp. *paratuberculosis* (*M. a*. *paratuberculosis*)[37]. These strains were almost impossible to routinely culture up until this time and while faecal culture has been a valuable tool in the diagnostic arsenal for OJD, it is still a time consuming procedure with long lag times between sample submission and the delivery of results. At around the same time faecal culture was reported the results from a study that identified a rapid means of detecting *M. a*. *paratuberculosis* by PCR directly from faeces (D-PCR)[20]. However, D-PCR lacked the sensitivity of faecal culture.

Since then other methods such as abattoir inspection have been introduced and are extremely efficient and economical means of regional surveillance for OJD but are not applicable to live animals, and can only ever detect animals which have progressed to have gross lesions. Sheep without gross lesions can shed *M. a. paratuberculosis* in their faeces and are a risk to other livestock. Faecal culture is still the "Gold Standard" test for OJD diagnosis in Australia. However, even with the changes in the management of ovine Johne's disease (OJD) in Australia there still exists the need for a rapid cost effective diagnostic test.

The aim of this project was to elevate the sensitivity of the original D-PCR to be equal or greater than that of faecal culture while maintaining the technical practicalities of the test and the low cost. To address this we undertook a series of experiments that focused on three separate components of the new test and then brought them together for evaluation on a test bank of faecal samples, these included:

- A new *M. a*. *paratuberculosis* isolation and DNA extraction procedure.
- The inclusion of an internal PCR control to more accurately interpret negative results.
- A new multiplex PCR to overcome the need for restriction endonuclease analysis for the confirmation of an *M. a*. *paratuberculosis* positive result.

Unfortunately, we were only successful in achieving a desirable outcome for two out of three of these components. A preliminary investigation of the new *M. a*. *paratuberculosis* isolation and DNA extraction procedure on spiked faecal samples indicated that it had sensitivity equal to or greater than faecal culture. However, when this procedure was examined, in conjunction with the newly developed multiplex PCR that included the internal control, using real faecal samples from sheep, the test failed to even achieve the sensitivity of the original D-PCR let alone come near faecal culture.

Because of the dramatic changes in the management of OJD in NSW since the time this project commenced, a severe downturn in the number and type of samples being submitted for OJD diagnosis has occurred. Consequently, we had to rely on stored samples and a few recent samples for the initial evaluation of the new test. Because these stored samples are now quite valuable for the future development of molecular based tests for *M. a*. *paratuberculosis* we decided to stop further evaluation of the new test and preserve the samples. Further evaluation would have exhausted many of these samples. Although we were able to establish that the poor performance of the test was directly attributable to the new *M. a*. *paratuberculosis* isolation and DNA extraction procedure, not the multiplex PCR or the internal control, time and financial constraints prevented us from developing this component of the test.
Fortunately, just before the conclusion of this project it became possible to evaluate a real time PCR methodology that was initially trialled in Japan at the Japanese National Institute of Animal Health combined with a Japanese faecal extraction technique (JohnePrep™, Shimadzu

Corporation). This test is currently not available in Australia but is being developed for use in sheep by the Farm Animal Health Unit of the Faculty of Veterinary Science at the University of Sydney by a student undertaking her Doctorate of Philosophy. The new D-PCR test (RT-J D-PCR) was evaluated using the same stored and recent faecal samples used to evaluate the new D-PCR procedure and successfully identified 86% of the faecal culture positive samples including all five recent faecal culture positive faecal samples. Furthermore, the DNA samples derived from the new procedure were used to more thoroughly examine the new multiplex PCR incorporating the long internal control. Once again the results were very encouraging with the multiplex PCR detecting 63% of the faecal culture positive samples. The discrepancies between the two PCR assays can be attributed to the increased sensitivity of real-time PCR over conventional agarose/ethidium bromide detection used for conventional PCR. However, the multiplex PCR and the internal control warrants further investigation and should also be included in the future development of faecal tests to allow for the laboratories that are currently unable to use or offer real-time-PCR technology for routine diagnostics.

Overall the preliminary results from the RT-J D-PCR are very promising and indicate that a more thorough examination of this test is required. Optimal results appear to be achieved when the new faecal extraction method is used in conjunction with real-time PCR. Future evaluation of this procedure should include both real-time and conventional PCR using the new multiplex PCR and long internal control developed as part of this project. Furthermore, an internal control could/should also be considered for the real-time PCR for the same reasons it was used in this project.

A new rapid test of the diagnosis of ovine Johne's disease is still an important requirement for the management of this disease in NSW, Australia and Internationally. It is highly recommended that the new faecal extraction method be further examined and developed for Australian conditions as a replacement/substitute test for faecal culture. Should this prove successful it could then be applied and optimised for the detection of *M. a*. *paratuberculosis* in bovine samples and once again be optimised for Australian conditions.

Contents

1 Background and Introduction

The diagnosis of *Mycobacterium avium* subsp. *paratuberculosis* (*M. a. paratuberculosis*) infection in the early stages of disease, or where prevalence is low, is critical for the management of ovine Johne's disease (OJD)[39]. The two most useful diagnostic tools currently available to the industry are abattoir surveillance and pooled faecal culture.

Abattoir inspection is an extremely efficient and economical method of regional surveillance for OJD. But of course, it is not applicable to live animals, and can only ever detect animals which have progressed to have gross lesions. Sheep without gross lesions can shed *M. a. paratuberculosis* in their faeces and are a risk to other livestock.

Pooled faecal culture (faecal culture) is the most sensitive practical current test to detect OJD infection in a living mob of sheep[30] [27;37]. The test relies on growing the *M. a. paratuberculosis* organisms present in faeces in a liquid culture medium, and detecting this growth radiometrically (metabolism of a radioactive substrate releases radioactive C_{14}). Once growth is detected polymerase chain reaction (PCR) and restriction endonuclease analysis (REA) are used to examine the DNA of the cultured organisms, to determine whether they are *M. a. paratuberculosis*. Thus faecal culture is expensive, and may take many months for definitive results.

The use of molecular techniques to directly detect the DNA of *M. a. paratuberculosis* in faeces, without having to first grow the organism in culture (Direct PCR or D-PCR) would theoretically allow the rapid (several days) and sensitive diagnosis of OJD in living sheep.

A D-PCR test was developed here at EMAI under an MLA-funded project led by Richard Whittington, with most of the laboratory work done by Ian Marsh. The Final Report was completed in 2000, and the work subsequently published in the peer-reviewed literature[20]. The test was further evaluated at EMAI in 2004 under routine diagnostic conditions in our laboratory and at Victorian Institute for the Advancement of Science (VIAS) and was also included in several on-going OJD research projects.

The sensitivity of the original D-PCR test is less than faecal culture. At the level of the pooled sample, the sensitivity for D-PCR compared to faecal culture varied from 64% in the early trials down to about 40% in the most recent investigations. At the farm level (more than one pool is normally examined), sensitivity was 79% in the early trials and 66% in the recent work. It is, however, always misleading to quote sensitivity estimates in isolation – they depend on the population being tested, and this has varied over the years. For example, the early trials probably included more flocks with a higher prevalence of infection, and D-PCR detected all the high prevalence flocks in the recent trials. When looked at from the view of number of *M. a. paratuberculosis* organisms in the sample examined, D-PCR appears to require about ten times as many to yield a positive result – about 10^4 for D-PCR compared to about 10^3 for culture. In practical terms, this all means that D-PCR will readily detect pools with large numbers of *M. a. paratuberculosis* organisms. This translates to sheep with severe multibacillary disease, and these are the animals of greatest immediate risk of transmitting infection.

The cost of D-PCR is similar to culture, but its great advantage is the short time for results. The current D-PCR test is now approved for use in NSW, and it is proving of use to some producers in the management of infected flocks, by providing a rapid assessment of whether sheep are shedding large numbers of *M. a. paratuberculosis*. However, because of the lower sensitivity compared to culture, negative results cannot be used for ABC points or to remove suspicion of infection.

There is opportunity to improve the current test, and some of the avenues for this were already suggested in the final report from the above project. Several papers on *M. a. paratuberculosis*-specific D-PCR from faeces or tissues (often from humans) have been published, and their techniques can also be investigated for adaptation to D-PCR from sheep faeces[3;4;6;11;12;14;15;18;21;23;26;31;36]. Many of the techniques as published, while apparently sensitive, are too time consuming, costly or at risk of cross-contamination, for use as routine diagnostic tests. The areas considered in the improvement process included:

- Increasing the starting sample size
- Removing competing DNA from the reaction
- Improving the lysis of the resistant mycobacteria
- Removal of DNA inhibitors
- Developing an internal control target
- Examination of DNA losses
- Use of a second *M. a. paratuberculosis*-specific PCR target
- The use of real-time PCR

Use of the existing D-PCR test in diagnostic investigations

The current diagnostic procedures within the NSW Department of Primary Industries, pooled faeces are subjected to DNA extraction based on a commercial resin and then tested in the PCR. The test can be completed within a week, and relies on two phases. The initial phase involves a “conventional” PCR assay on the DNA extract based on the IS*900* gene sequence of *M. a. paratuberculosis*, using a special combination of IS*900* primers, and two sample dilution rates to reduce PCR inhibition. The resultant PCR product is assessed on an agarose gel for molecular size to ensure it conforms to the specific molecular size expected for *M. a. paratuberculosis*. The second (confirmatory) phase then involves a restriction endonuclease assay (REA), but requires a sufficiently strong DNA gel band from the first phase before it can be run. If there is a product of the correct size but too weak to run in the REA, the result of the D-PCR testing is classified as a trace PCR reaction and is considered inconclusive. A positive D-PCR result requires both an IS*900* PCR product of the correct size and the correct REA profile to confirm that *M. a. paratuberculosis* is present.

To date, routine diagnostic application has been limited, with just 61 pools from 10 accessions in the past 2 years. Concurrent faecal culture testing was carried out on some accessions. Six accessions containing 31 pools were D-PCR negative. Two of these submissions (representing 15 pools) were also tested by faecal culture and found to be negative. From the remaining 4 accessions, reactions were detected in the D-PCR as shown in Table 1. In two flocks (Table 1) rapid confirmation of infection was possible by detection of a positive D-PCR.

Table 1: Diagnostic ovine pooled faecal accessions yielding a reaction in the *M. avium paratuberculosis* D-PCR

Flock	Reason	No pools submitted	D-PCR result (pools)	faecal culture result relevant to D-PCR results on specific pools
1	Show testing	5	1 Trace*	1 Neg
			4 Neg	4 Neg
2	Trace forward Introductions from high risk SA flock	9	1 Pos	1 Pos
			3 Trace	1 Pos, 2 Neg
			5 Neg	5 Neg
2	As above	9	1 Pos	1 Pos
			1 Trace	1 Neg
			7 Neg	7 Neg
3	Surveillance testing	7	3 Pos	3 Not done
			4 Neg	4 Not done
		30	5 Pos	2 Pos, 3 Not done
			5 Trace	1 Pos, 4 Neg
			20 neg	20 Neg

* indicates trace reaction in PCR, insufficient for confirmatory REA test; classified inconclusive

2 Project Objectives

- By July 2006: complete laboratory workup towards a practical, reliable and cost-effective PCR test that will have sensitivity as good as or better than radiometric culture. If this is not achievable, the project will be terminated at this point.
- By Jan 2007: Evaluate the test on a panel of real samples and on diagnostic submissions in parallel with culture.
- By April 2007: Evaluate the test in a second laboratory and submit to SCAHLS for consideration as an approved test

Proposed objectives to improve the D-PCR test
Specific areas for improvement of the existing D-PCR test included:

Increasing the starting sample size
Pooled faecal culture uses a 1.5 g sample and the original D-PCR uses only 0.2g. Increasing this to 2.0 g has the possibility of increasing sensitivity (theoretically up to 10 fold). A preliminary examination of this in the previous project did not show marked improvement, but it was not further investigated. Digestion techniques to concentrate *M. a. paratuberculosis* organisms from the larger faecal samples will be trialled. Similar techniques have already been applied to meat samples and material suitable for preliminary testing in the proposed project has been stored from a current MLA-funded project on *M. a. paratuberculosis* in meat – Project number: PRMS.044.)

Removing competing DNA from the reaction
Because of the very resistant cell wall of mycobacteria, a pre-treatment targeted at more labile micro-organisms may be effective. This seems not to have been used in any of the molecular work published to date.

Improving the lysis of the resistant mycobacteria
In the current protocol it is likely that many *M. a. paratuberculosis* cells remain intact, and thus their DNA is unavailable to the subsequent PCR. Alternate techniques to the simple boiling used in the current protocol will be trialled to improve DNA extraction. We have experience with several potentially useful procedures through the proteomics work done by Ian Marsh during his MLA-funded PhD studies.

Removal of DNA inhibitors
One of the difficulties with performing PCR on materials such as faeces is the loss of sensitivity due to inhibition of the PCR reaction by many normal faecal components. At present, the D-PCR test attempts to overcome this problem using a commercial product to separate the target organisms from such inhibitory components. However, four years in molecular biology is a long time, and there are now many newer commercial kits or materials for DNA extraction from faecal, soil and other contaminated samples which might be trialled. Such kits are not targeted at mycobacteria, and will not give optimum results with “off-the-shelf” use.

Developing an internal control target
This would be used in each test to identify PCR inhibition. Currently, when we get a negative result in D-PCR, we have no way of knowing whether the negative result is because no *M. a. paratuberculosis* DNA was present (i.e. a true negative) or whether inhibitors in that particular sample prevented the PCR from amplifying any specific DNA that was present. A control target that can be added to each reaction, and which gives a distinct band of different size to specific bands, allows assessment of possible inhibition. Such a modification would allow more

confidence in a negative D-PCR result for *M. a. paratuberculosis*. This confidence is important if D-PCR is to be acceptable for “negative” regulatory purposes.

Use of a second *M. a. paratuberculosis*-specific PCR target
Currently, even using PCR/REA as confirmation of growth in BACTEC medium, the identification of *M. a. paratuberculosis* DNA is not considered to be fully confirmatory of infection unless sub-culture to solid media/mycobactin dependence is also positive. We are at present investigating the use of other targets in multiplex PCR with IS*900,* that would satisfy regulators as definitive diagnostic criteria for *M. a. paratuberculosis*. These techniques would be applicable to D-PCR.

The use of real-time PCR
This can further reduce turn-around time by a day, and would facilitate the quantification of a positive result. We are at present conducting preliminary investigations into the use of this technology for confirmation of growth from BACTEC cultures. If successful, we could also apply this to D-PCR.

3 Methodology and Results

Overview

The development of a new D-PCR for the detection of *M. a*. *paratuberculosis* in faecal samples was undertaken in a series of experiments addressing a number of issues. All developmental work and analysis was unless otherwise stated undertaken using the IS*900* specific 900VM PCR[20]. The first set of experiments included:

- Preparation of a spiked ovine faecal dilution series that was evaluated by faecal culture and the original D-PCR method.
- A comparison of faecal culture, original D-PCR, Qiagen QIAamp DNA Stool Mini Kit and MO BIO UltraClean Fecal DNA Kit using the spiked faecal dilution series.

Following the evaluation of the commercial kits we developed a new in-house D-PCR method and this included:

- Development of ethanol-based *M. a*. *paratuberculosis* isolation and DNA extraction procedure.
- DNA purification to address false negative samples.
- Development of an internal PCR control for use in conjunction with *M. a*. *paratuberculosis* specific PCR.
- Development of a multiplex PCR assay for the detection of *M. a*. paratuberculosis.

At the conclusion of these experiments a test bank of stored and recent faecal samples were examined using:

- The new D-PCR procedure, incorporating the multiplex PCR and internal control mimic.
- The RT-J direct detection test for faeces.

3.1 Preparation of an *M. a*. *paratuberculosis* spiked faecal dilution series and its evaluation by faecal culture and the original D-PCR method

An OJD faecal dilution series, ranging from undiluted OJD positive faeces to a 1 in 100,000 dilution OJD positive faeces in OJD negative faeces was prepared (Table 2). The status of the undiluted positive and negative faeces was confirmed by radiometric culture prior to the commencing this work. Large quantities of the dilution series was prepared such that all initial optimisation and validation experiments could be performed using aliquots of the same faecal preparations providing more meaningful inter-experimental comparisons of the results. Similar dilution series were prepared for later experiments using the same negative and positive faecal samples. However, subsequent preparations were evaluated by the original D-PCR method only as faecal culture was no longer performed at EMAI beyond October 2005 and the cost of examining these dilutions externally was prohibitive and not factored into the original budget.
The analytical sensitivity of both faecal culture and the original D-PCR[20] was determined using the faecal dilution series. The results indicated that with this dilution series the original D-PCR and faecal culture were capable of detecting *M. a. paratuberculosis* at dilutions of 1 in 500 and 1 in 5000, respectively. These results became the benchmarks for subsequent sensitivity experiments and demonstrated that the goal of this project was to develop an improved D-PCR technique with at least 10 times greater sensitivity than the original D-PCR.

Table 2: Faecal culture and original D-PCR on diluted OJD positive faecal samples.

Faecal dilution	Test	
	faecal culture	Original D-PCR
Undiluted	+	+
1 in 5	+	+
1 in 10	+	+
1 in 50	+	+
1 in 100	+	+
1 in 500	+	+
1 in 1000	+	-
1 in 5000	+	-
1 in 10,000	-	-
1 in 50,000	-	-
1 in 100,000	-	-
Negative faeces	-	-
Extraction control	-	-
Process control*	**not applicable**	-

* Process control has no DNA and is included to assess the integrity of the PCR reagents.

3.2 Comparison of faecal culture, original D-PCR, Qiagen QIAamp DNA Stool Mini Kit and MO BIO UltraClean Fecal DNA Kit using the spiked faecal dilution series

The key areas that needed to be addressed with the original D-PCR method in the DNA extraction component included:

- increasing the starting sample size
- removing competing DNA from the reaction
- improving the lysis of the resistant mycobacteria
- removal of DNA inhibitors
- examination of DNA losses

Prior to commencing work on the development of new in-house DNA extraction technique, a review of the literature on DNA extraction techniques from faeces identified two new commercial kit-based DNA extraction techniques that might be suitable for the DNA extraction component of the new D-PCR test. The Qiagen QIAamp DNA stool mini kit (Cat. No. 51504) and the MO BIO UltraClean Fecal DNA Kit (Cat. No. 12811-50) were both available in Australia and were purchased for evaluation as part of this project.

Both kits were thought to be capable of addressing many of the issues described above particularly the removal of DNA inhibitors from the reaction. Each kit was examined using the faecal dilution series prepared in Experiment 3.1. Both kits demonstrated that they were capable of extracting *M. a*. *paratuberculosis* DNA from the faecal samples when the resulting DNA samples were examined by the 900VM PCR routinely used with the original D-PCR method (Table 3). The Qiagen QIAamp DNA stool mini kit looked promising in terms of analytical sensitivity, capable of detecting *M. a*. *paratuberculosis* in the 1 in 5000 dilution. This was the target dilution required for the new test. However, this result was not reproducible and significant problems were incurred with cross contamination of the negative controls using this kit. While the MO BIO UltraClean Fecal DNA Kit had no cross contamination issues, it suffered from a lack of analytical sensitivity compared to the original D-PCR method.

Neither kit proved to be suitable for the task of improving the D-PCR test for a variety of practical and economic reasons. Both kits were in our opinion not suited to routine diagnostic use based on the amount sample manipulation required which increases the potential for cross contamination problems. Both kits required substantial amounts of labour and this would make

them expensive to use routinely. These kits were abandoned and the focus of the project shifted to developing a new in-house *M. a*. *paratuberculosis* isolation and DNA extraction technique.

Table 3: Summary of the results comparing faecal culture, original D-PCR and a modified D-PCR's using a Qiagen QIAamp DNA stool mini kit and MO BIO UltraClean faecal DNA kit on diluted OJD positive faecal samples.

Faecal dilution	Test			
	faecal culture	Original D-PCR	Qiagen-based D-PCR	MO BIO D-PCR
Undiluted	+	+	+	+
1 in 5	+	+	+	+
1 in 10	+	+	+	+
1 in 50	+	+	+	+
1 in 100	+	+	+	+
1 in 500	+	+	+	-
1 in 1000	+	-	+	-
1 in 5000	+	-	+	-
1 in 10,000	-	-	-	-
1 in 50,000	-	-	-	-
1 in 100,000	-	-	-	-
Negative faeces	-	-	-	-
Extraction control	-	-	-	
Process control*	not applicable	-	-	

* Process control has no DNA and is included to assess the integrity of the PCR reagents.

3.3 Development of a new EMAI D-PCR method

We assumed that the two main causes of problems in the DNA extraction process were excessive amounts of non-*M. a*. *paratuberculosis* DNA that may be inhibitory to the PCR and/or the presence of non-DNA PCR inhibitory substances. The type of non-DNA PCR inhibitory substances was difficult to determine within the scope of this project but we did investigate the effect of excessive amounts of non-*M. a*. *paratuberculosis* DNA.

Experiment 3.3.1: Determining the effects of non-*M. a*. *paratuberculosis* DNA on the sensitivity of the *M. a*. *paratuberculosis* PCR

A series of experiments where undertaken to identify the effect of excessive amounts of non-*M. a*. *paratuberculosis* DNA on the 900VM assays. DNA was extracted from multiple aliquots of OJD negative faeces using the original D-PCR method, producing a sample that contained sheep DNA and the DNA from other microbial and plant sources within the faeces. The non-*M. a*. *paratuberculosis* sample concentration was determined spectrophotometrically to be approximately 1.0 mg/mL. Ten fold DNA dilution series were prepared by diluting a concentrated *M. a*. *paratuberculosis* DNA sample from the 316V strain, used in our laboratory to routinely produce control DNA for PCR, in the non-*M. a*. *paratuberculosis* DNA and phosphate buffered saline (PBS). The latter was used as a control. Both dilution series were evaluated by the 900VM PCR and compared with a control 316V DNA dilution series. The results (Table 4) demonstrated that even in the presence of large amounts of non-*M. a*. *paratuberculosis* DNA, the 900VM PCR assay was capable of detecting *M. a. paratuberculosis* DNA at the same dilution (10^{-6}) in all three dilution series. Based on these results it would appear that non-*M. a*. *paratuberculosis* DNA is not a major cause of inhibition with the 900VM PCR assay.

Table 4: Comparison of the IS*900* PCR assay to detect *M. a. paratuberculosis* DNA diluted in non-specific DNA from sheep faeces and PBS (no-DNA control).

Dilution	316V control DNA	Dilution series in	
		non-*M. a. paratuberculosis* DNA	PBS
10^{-1}	+++	+++	+++
10^{-2}	++	++	++
10^{-3}	++	++	++
10^{-4}	+	+	+
10^{-5}	+	+	+
10^{-6}	Trace	Trace	Trace
TE (negative control)	-	-	-
Process control*	-	-	-

* Process control has no DNA and is included to assess the integrity of the PCR reagents.

Experiment 3.3.2: Development of a new DNA extraction method

Background

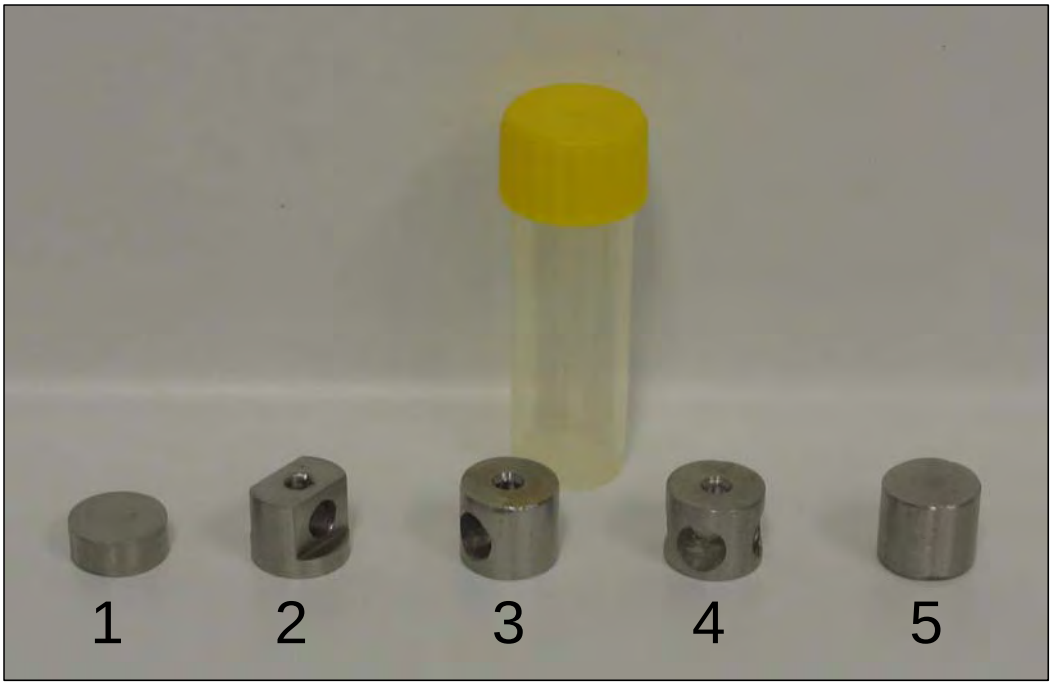
The results from Experiment 3.3.1 demonstrated that excessive amounts of non-*M. a. paratuberculosis* DNA was not the problem and that non-DNA PCR inhibitory substances appeared to be the major problem with the original D-PCR format. However, we also believed that sample size was also a limiting factor on the overall sensitivity of the test. To overcome these issues we evaluated a number of different approaches. The first of these involved using larger samples volumes with a mechanical type approach to separating the solid and liquid phases of the initial sample suspension. Hopefully the *M. a. paratuberculosis* cells would be confined to the liquid phase and be separated from the remaining faeces. Post-separation techniques could then be used to concentrate the *M. a. paratuberculosis* cells followed by DNA extraction. We also considered using a technique incorporating an acid-pepsin digestion of the faecal sample in a procedure similar to that describe recently for the isolation of *M. a. paratuberculosis* from meat samples. Another option became apparent upon examination of the faecal culture procedure. During the development of the faecal culture procedure it was observed that the egg yolk in the radiometric culture medium inhibited PCR[38]. To overcome this, a procedure was developed where absolute ethanol was used to separate the egg yolk and *M. a. paratuberculosis* cells prior to PCR without excessive detrimental effects on the *M. a. paratuberculosis* cells. We used this as the basis of a series of experiments to incorporate absolute ethanol into a procedure to isolate *M. a. paratuberculosis* cells from faeces.

Experiment 3.3.2.1: Mechanical separation of the solid and liquid phases

To increase the amount of faeces used in the D-PCR test we attempted using variety of metal plungers (Figure 1), designed and constructed at EMAI, to partition the liquid column containing the *M. a. paratuberculosis* cells from the remaining solid phase containing the bulk of the faecal material. The plungers were used on 2.0 g of faeces that was suspended in 3.0 mL of PBS in 5 mL tubes (Figure 1). Several of the plungers were designed to locate a pipette tip so that the pipette could be used to plunge the sample and then draw off the liquid phase without interfering with end of the pipette tip (plungers 2-4, Figure 1). The liquid phase was transferred to a fresh tube that was then centrifuged at high speed. The supernatant was discarded and the pellet was resuspended in 700 µL of PBS prior to completion of the DNA extraction as per the original D-PCR method. Unfortunately, this approach failed to reach the sensitivity required and also proved to be a potential source of cross contamination during the DNA extraction process and was not considered further.

Figure 1: Metal plungers used to partition the liquid column containing the *M. a. paratuberculosis* cells from the remaining faecal material (1) 1 cm solid (2) 2 cm shaved sides

(3) 2 cm single hole (4) 2 cm double hole (5) 2 cm solid. The tube at the rear is the 5 mL tube that the plungers were engineered to fit in.



Experiment 3.3.2.2: Acid–pepsin digestion of faecal samples to isolate *M. a*. *paratuberculosis*

A procedure similar to that described in the recently completed an MLA funded project titled "Potential pathogens in Australian Meat Products" (Project code: PRMS.044) was developed for acid-pepsin digestion of the pooled faecal sample. In the previous project acid pepsin was found to be an effective means of digesting meat samples without severe adverse effects on *M. a*. *paratuberculosis* cells. For this project we digested 0.2 g of faeces with acid–pepsin and PBS (controls) for 0.5, 1.0, 3.0 hr and overnight (12 -15 hr). The samples were allowed to rest for 0.5 hr before being centrifuged at 13,000 rpm (approximately 16,060 x*g*) for 10 mins at room temperature. The supernatant was removed and the remaining pellet was resuspended in 700 µL of PBS. DNA was then extracted as per the original D-PCR method.

M. a. *paratuberculosis* was only detected in the PBS controls indicating that acid –pepsin digest was severely detrimental when used on faecal samples and was not explored further.

Experiment 3.3.2.3: Ethanol-based extraction using 0.2 g of faeces

In the following method, used to isolate *M. a*. *paratuberculosis* from the faecal dilutions series, all steps were conducted using standard aseptic techniques. Absolute ethanol, 700 µL, was added to 0.2 g of faeces in a sterile screw top 2.0 mL tube. The contents were vortexed briefly. The samples were centrifuged at 300 rpm (approximately 9 x*g*) for 10 mins and then 200 µL of the supernatant was carefully transferred to a new sterile screw top 2.0 mL tube. The sample was centrifuged at 13,000 rpm (approximately 16,060 x*g*) for 5 min in a benchtop microfuge. The supernatant was discarded and 200 µL of sterile PBS was added to the tube and the sample was resuspended by vortexing. The sample was centrifuged at 13,000 rpm (approximately 16,060 x*g*) for 5 min and the supernatant was discarded after which 50 µL of sterile distilled water was added. The pellet was resuspended by vortexing the tube and the samples were then heated in a dry heating block at 105°C for 20 minutes. Samples were stored

at –20°C or evaluated immediately by the 900VM PCR. This procedure was evaluated using the faecal dilution series prepared in experiment 3.1 and was capable of detecting *M. a*. *paratuberculosis* at a dilution of 1 in 100 (Table 5). Based on this result the procedure was scaled up for use with 2.0 g of faeces.

Experiment 3.3.2.4: Ethanol-based extraction using 2.0 g of faeces

Similar to experiment 3.3.2.3 except 5 mL of ethanol was used to extract *M. a*. *paratuberculosis* from 2.0 g of faeces in 10 mL centrifuge tubes. The samples were centrifuged at 200 rpm (approximately 10 x*g*) for 10 mins at room temperature. Four millilitres of the supernatant was transferred to a sterile 10.0 mL tube before centrifuging at 3600 rpm (approximately 3000 x*g*) for 20 min at room temperature and the supernatant was discarded. Sterile PBS, 1.0 mL, was added to the tube. The samples were resuspended by aspiration with the pipette and transferred to sterile screw top 2.0 mL tubes and centrifuged at 13,000 rpm (approximately 16,060 x*g*) for 5 mins at room temperature. The supernatant was discarded and 50 µL of sterile distilled water was used to resuspend the pellet by vortexing. The samples were heated in a dry heating block at 105°C for 20 minutes. After boiling the samples were centrifuged at 13,000 rpm (approximately 16,060 x*g*) for 5 mins at 4^{o}C. The supernatant was transferred to a new labelled 2.0 mL screw top tube and then stored at –20°C or evaluated immediately by 900VM PCR. This procedure was evaluated using the faecal dilution series prepared in experiment 3.1 and was capable of detecting *M. a*. *paratuberculosis* at a dilution of 1 in 1000 (Table 5). Based on this result the procedure was scaled up for use with 10.0 g of faeces.

Experiment 3.3.2.5: Ethanol-based extraction using 10.0 g of faeces

The method described in experiment 3.3.2.4 was scaled up using 40.0 mL absolute ethanol and 10.0 g of faeces in a sterile 50 mL tube. The samples were centrifuged at 200 rpm (approximately 10 x*g*) for 10 mins at room temperature. The supernatant (20-25 mL) was transferred to a sterile 50.0 mL tube before centrifuging at 3600 rpm (approximately 3000 x*g*) for 20 min at room temperature and the supernatant was discarded. Sterile PBS, 1.0 mL, was added to the tube. The samples were resuspended by aspiration with the pipette and transferred to sterile screw top 1.5 mL tubes and centrifuged at 13,000 rpm (approximately 16,060 x*g*) for 5 mins at room temperature. The supernatant was discarded and 50 µL of sterile distilled water was used to resuspend the pellet by vortexing. The samples were heated in a dry heating block at 105°C for 20 minutes. After boiling the samples were pulsed (approximately 2000 x*g*) for 5 secs at 4^{o}C. The supernatant was transferred to a new labelled 1.5 mL screw top tube and then stored at –20°C or evaluated immediately by 900VM PCR. This procedure was evaluated using the faecal dilution series prepared in experiment 3.1 and was capable of detecting *M. a*. *paratuberculosis* at a dilution of 1 in 10,000 (Table 5) which gave this technique analytical sensitivity beyond what was required for this project. The new ethanol-based DNA extraction technique appeared to be highly suitable for the detection of *M. a*. *paratuberculosis* in large volume faecal samples, was easy to perform and was ready for evaluation on stored and current faecal samples.

Table 5: PCR results from the scaled up of the ethanol extraction procedure evaluated using the faecal dilution series.

Faecal dilution	Amount of faeces (g)		
	0.2	2.0	10.0
Undiluted	**4+**	**3+**	**4+**
1 in 5	**3+**	**4+**	**4+**
1 in 10	**2+**	**4+**	**4+**
1 in 50	**1+**	**2+**	**2+**
1 in 100	**Tr**	**2+**	**3+**
1 in 500	**-**	**1+**	**2+**
1 in 1000	**-**	**Tr**	**2+**
1 in 5000	**-**	**-**	**1+**
1 in 10,000	**-**	**-**	**Tr**
1 in 50,000	**-**	**-**	**-**
1 in 100,000	**-**	**-**	**-**
Negative faeces	**-**	**-**	**-**
Extraction control	**-**	**-**	**-**
Process control*	**-**	**-**	**-**

* Process control has no DNA and is included to assess the integrity of the PCR reagents.

3.4 DNA purification to address false negative samples

To address the issue of false negative samples that were the result of PCR inhibitory substances in the faeces, we needed to obtain a large quantity of negative faeces containing PCR inhibitory substances. Sheep faeces was collected from the EMAI pastures and pooled. Five replicate samples were prepared by diluting the OJD positive faeces at a rate of 1 in 50, as per the faecal culture requirements. These samples were then examined using the new D-PCR procedure (experiment 3.3.2.5) and the 900VM PCR. All DNA samples were evaluated in PCR undiluted and at a 1 in 10 dilution. The 1 in 10 dilutions were used with the hope that this would dilute out the PCR inhibitors allowing for amplification of the 900VM product. As can be seen in Figure 2 this was the case for three of the five replicates. The third replicate (Figure 2, lane 3) was then used for further examination of the inhibitory faeces.
Inhibitory sample number 3 was used to produce a faecal dilution series (1 in 50, 1 in 500 and 1 in 5000) spiked with the OJD positive control faeces. These were then used to examine a number of post extraction DNA purification options. For these experiments the DNA was isolated from the faeces using the new D-PCR procedure and then examined by 900VM PCR as both pre and post purification samples. DNA purification was achieved using: Eppendorf Phase Lock Gels (Cat. No. 0032 007.961 and 0032 007.953), Millipore Microcon filters (Cat. No. 42412) and Montage filters (Cat. No. UFC7 PCR 50), Eppendorf PerfectPrep DNA purification kit (Cat. No. 0032 007.759) and by simply diluting the DNA samples. The first three procedures were performed according to the manufacturer's recommendations and although each of these was successful in purifying the DNA sample and removing the inhibitors, procedural complications and cost prevented their suitability.

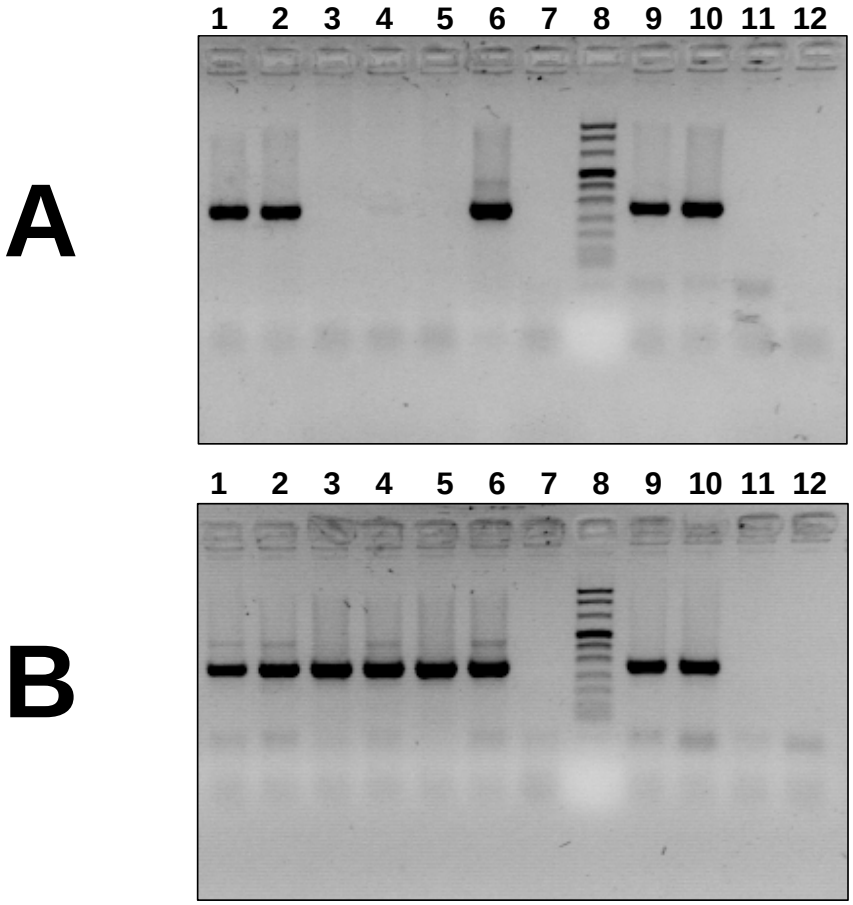
Better results were achieved by simply diluting the DNA sample. Following extraction of the total DNA sample from the faeces using the new-DPCR technique, the DNA samples and controls were further diluted at the following rates: 1 in 2, 1 in 5 and 1 in 10. The results (Table 6) clearly demonstrate that the undiluted samples were inhibited in PCR but as the DNA sample became more dilute, inhibition ceased and the 900VM amplification product was achieved. However, a new concern became apparent. While limiting the effect of the PCR inhibitors, the dilution effect also compromised overall sensitivity as a result of the diluting the *M. a*. *paratuberculosis* DNA also. For future experiments we selected a 1 in 5 dilution rate, however, optimal results for the removal PCR inhibitors were achieved with the Eppendorf PerfectPrep DNA purification kit. Unfortunately like the other kit-based techniques the additional cost of the kit and the labour was considered to be an issue with this technique but this method could be used to further evaluate

samples that were deemed to be falsely negative when used in conjunction with an internal control for the PCR.

Table 6: 900VM PCR results achieved when evaluating the post-extraction removal of PCR inhibitors by dilution of the DNA sample and Eppendorf PerfectPrep DNA purification kit.

Sample	Non-purified DNA	Purification technique					
		Diluted DNA only				PerfectPrep	PerfectPrep
		Neat	1 in 2	1 in 5	1 in 10	Neat	1 in 5
B3 1 in 50	-	-	-	2+	3+	3+	2+
B3 1 in 500	-	-	1+	1+	1+	2+	1+
B3 1 in 5000	-	-	Tr	Tr	Tr	Tr	-
Positive control	1+	1+	1+	Tr	Tr	1+	-
Negative control	-	-	-	-	-	-	-
Process control	-	-	-	-	-	-	-

Figure 2: PCR results from the evaluation of EMAI faecal samples collected from pasture spiked with OJD positive faeces to produce a source of false negative faeces. Lanes 1 to 5 are the spiked faecal samples. Lane 6 and 7 are the positive and negative control faeces, respectively. Lane 8 is the molecular size marker and lanes 9 to 12 are the PCR controls. Undiluted samples are shown in (A) and the 1 in 10 samples are shown in (B).



3.5 Development of an internal PCR control for use in conjunction with *M. a. paratuberculosis* specific PCR

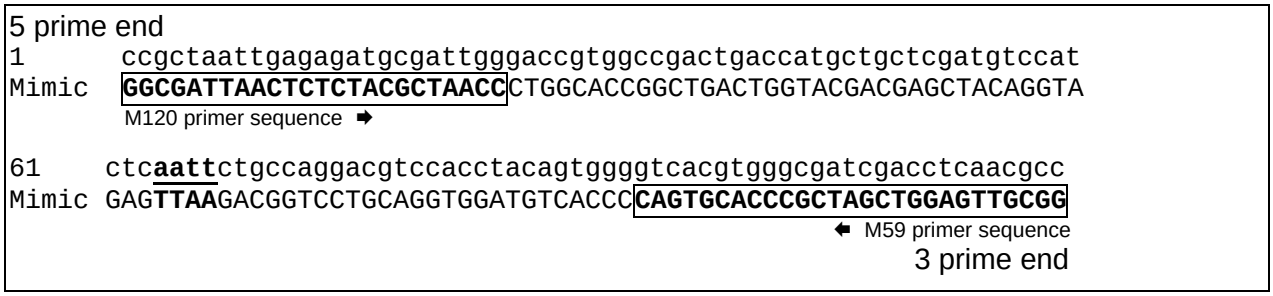
Background

A limitation of diagnostic PCR assays is false negative results, that is, samples that are positive but do not amplify due to inhibition of the assay. PCR assays that do not include an internal control for each individual reaction can at best produce an interpretable result from non-inhibited positive samples and this makes it difficult to interpret negative results. Under these conditions diagnosticians cannot determine if a negative result is truly negative or if the reaction failed as a result of inhibitory substances present in the samples that have prevented amplification from occurring. Even though the appropriate positive and negative controls may have been included within a PCR run, generally run as individual reactions, these are only useful for establishing the integrity of the PCR reagents and act as references for which the samples can be compared against. To overcome this situation PCR-based diagnosticians have begun to introduce internal controls such that each amplification reaction can be monitored. A number of these internal controls have been reported[8;9;2] but have yet to be adopted widely by the *M. a*. *paratuberculosis* research community. Generally, these are produced using DNA cloning techniques and this may be a limitation on their widespread use, as many laboratories may not have time, finances or the laboratory expertise to prepare these internal controls. To overcome this we have designed a PCR-based method for producing an internal control, based on a method described and used in the detection of *Bordetella pertussis* by PCR[24]. However, a limitation of multiplex PCRs, a PCR where multiple targets are amplified in a single reaction, is the preferential amplification effect. That is, one amplification product can be amplified preferentially over the other(s). Therefore, great care must be taken when designing these assays. One of the factors that can determine this preferential amplification effect is the size of the product to be amplified, smaller amplification products can dominate the assay. For this reason we designed one short internal control and one long internal control. To minimise the complexity of the reactions we decide to use the same PCR primers used in the 900VM reaction.

Experiment 3.5.1: Short internal control mimic

To produce the short internal control mimic we designed a 120 base pair single stranded DNA sequence (Figure 3) that included the M120 and M59 primers from the 900VM reaction. The complementary sequence for the M120 primer (24 base pairs) was located at the five prime end and the M59 primer sequence (27 base pairs) at the three prime end. The remaining 69 bp of sequence, between the two primers, was derived from the *Rhodococcus erythropolis* linear plasmid pBD2, complete sequence (GenBank accession AY223810). This was identified as the closest non-*M. a*. *paratuberculosis* sequence matching the 900VM amplification product sequence when this was used in a BLASTn search [(1)] of the nr database from the National Centre of Biotechnology Information (NCBI) website (www.ncbi.nlm.nih.gov). The *Rhodococcus erythropolis* sequence was used as this was sufficiently different from the IS*900* sequence but possessed similar melting temperature characteristics. The short internal control sequence (Figure 3) was modified to include an *Mse* I restriction endonuclease site so the amplified product could be digested and not interfere with interpretation of the REA of the specific 900VM product, should this be required.

Figure 3: The single strand mimic sequence for the short internal control (lower sequence in UPPERCASE) including the complementary sequence for the M120 primer **boxed and bold** at the 5 prime end and the M59 primer at the 3 prime end **boxed and bold**. The lower case sequence is the sequence that would be synthesised during PCR after initiation with the M120 primer. The *Mse* I restriction endonuclease site(TTAA) is shown at base position 64-67 in bold.



The mimic sequence shown in Figure 3 was purchased from a commercial primer supplier and used as the template in a PCR to produce double stranded amplified product with the primers M120 and M59. Briefly, a reaction volume of 50 µl containing 5 µL of the mimic template at the dilutions shown in Table 10, 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, 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 in 96-place thermal cycler (Corbett Research, Sydney, Australia). PCR cycling conditions were: one cycle of denaturation at 94°C for 3 min followed by 37 cycles of denaturation at 94°C for 30 sec, annealing at a range of temperatures (see Table 7) 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. PCR results were assessed by electrophoresis in 2% agarose gels stained with ethidium bromide using the molecular size marker number VIII (Roche). 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 prepared in a clean location, without DNA sample). The process control tube was not opened until the completion of the PCR and was designed to assess the integrity of the PCR reagents. The aim of the PCR was to use the M120 primer in the early rounds of amplification to produce the double stranded copies of the mimic that could be used as template in later rounds with the M59 primer also to ultimately produce large amounts of double stranded mimic.

Table 7: First round PCR conditions and results to produce the double stranded short internal mimic control

Sample	Dilution	Annealing temperature (^oC)		
		54	**58**	**62**
Short mimic	Undiluted	Smear	Smear	Smear
Short mimic	10^{-1}	1+	Tr	Tr
Short mimic	10^{-2}	Tr	-	-
316V positive control	Not applicable	2+	2+	2+
TE negative control	Not applicable	-	-	-
Process control*	Not applicable	-	-	-

* Process control has no DNA and is included to assess the integrity of the PCR reagents.

The amplified product derived from the 54^{o}C annealing temperature and using the mimic template at 1 in 10 and 1 in 100 (Table 7) was purified to remove remaining primers and other PCR reagents. The purified double stranded product was then re-amplified in the 900VM PCR with the M120 and M59 primers (Table 8).

Table 8: Second round PCR results to evaluate short internal mimic control

Sample	Dilution	1st round dilution	
		1 in 10	1 in 100
Purified short mimic	Undiluted	1+	1+ (diffuse)
Purified short mimic	10^{-1}	1+	1+ (diffuse)
Purified short mimic	10^{-2}	1+	1+ (diffuse)
316V positive control	Not applicable	2+	2+
TE negative control	Not applicable	-	-
Process control*	Not applicable	-	-

* Process control has no DNA and is included to assess the integrity of the PCR reagents.

The short internal control mimic was then validated in the 900VM PCR using serial DNA dilutions derived from cattle (C) and sheep (S) strains of *M. a*. *paratuberculosis*. The DNA dilutions were run in PCR with and without the short internal control mimic to determine any adverse effects the mimic may have on the amplification of the *M. a*. *paratuberculosis* specific product. The results (Table 9) clearly demonstrated that with both *M. a*. *paratuberculosis* strains the PCR favoured the amplification of the mimic over the specific *M. a*. *paratuberculosis* product. As stated previously this is not unusual as multiplex PCR can often favour the amplification of smaller targets. The short internal control mimic was found to be of no value and work ceased on its development.

Table 9: 900VM PCR results to validate the use of the short internal control mimic in conjunction with control serial DNA dilutions of DNA derived from cattle (C) and sheep (S) strains of *M. a*. *paratuberculosis*.

Dilution	Cattle strain		Sheep strain	
	C strain only	C strain / mimic	S strain only	S strain/mimic
10^{-2}	3+	Tr /1+	2+	Tr /1+
10^{-3}	2+	- / 1+	2+	- / 1+
10^{-4}	2+	- / 1+	2+	- / 1+
10^{-5}	2+	- / 1+	2+	- / 1+
10^{-6}	1+	- / 1+	1+	- / 1+
10^{-7}	Tr	- / 1+	Tr	- / 1+
10^{-8}	-	- / 1+	-	- / 1+
TE negative control	-	- / 1+	-	- / 1+
Process control*	-	- / 1+	-	- / 1+

* Process control has no DNA and is included to assess the integrity of the PCR reagents

Experiment 3.5.2: Long internal control mimic

A long internal control mimic was produced using a variation on the method described by Müller *et al.* (1998)[24]. In that study Müller *et al.* (1998) used a PCR MIMIC™ construction kit where the researchers effectively ligated their specific primers to a MIMIC DNA fragment. While this may be satisfactory for many PCR assays, no sequence specific criterion is used to select the internal mimic region. Therefore, we chose to use a similar process (Figure 4) that amplified a region of the *Mycobacterium avium* subsp. *avium* (*M. a. avium*) genome that is not present in the *M. a*. *paratuberculosis* genome and does not include an *Mse* I restriction endonuclease site. The absence of this site would allow for REA of the specific product without interference from the mimic. The rationale for using the *M. a. avium* sequences was that the mimic sequence would have characteristics similar to those of the IS*900* sequence given that it was from a closely related mycobacterial species. Furthermore, we already had the *M. a*. *paratuberculosis* and *M. a. avium* isolates and the *M. a*. *paratuberculosis* primers. All we needed to purchase was the *M. a*. *paratuberculosis*/*M. a. avium* composite primers (Table 10). The composite primers were designed to incorporate regions unique to both *M. a*. *paratuberculosis* and *M. a. avium*. The *M. a. avium* region would be used to amplify the region from *M. a. avium* in a primary PCR and then the *M. a*. *paratuberculosis* primers would be used to produce large quantities of the mimic in a secondary PCR using the 900VM PCR.

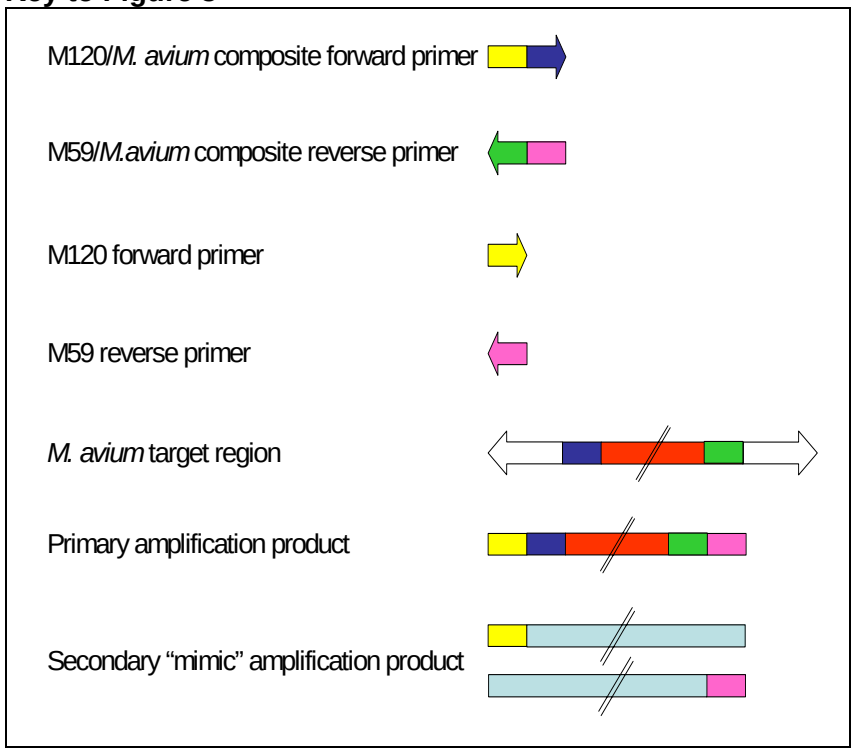
Table 10: Primers used to produce the long internal control mimic. The primary PCR primers (M706/M707) are the *M. a*. *paratuberculosis*/*M. a. avium* composite primers with the *M. a*. *paratuberculosis* region in normal text and the *M. a. avium* region in ***bold italics***. The secondary PCR primers (M120/M59) are the standard IS*900* reaction 900VM primers.

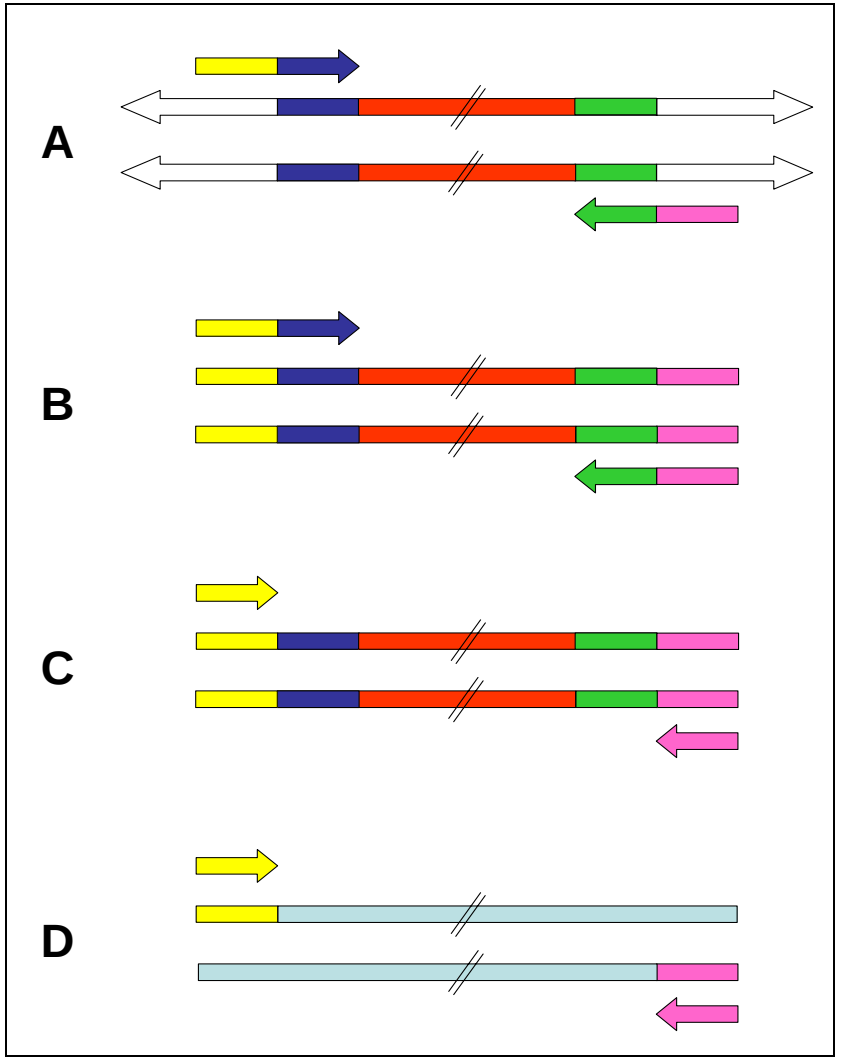
M	Direction	Sequence	Amplified product (bp)
706	Forward	ccgcTAATTgAgAgATgcgATTgg***ccATggTggAgAcgAgcAATTcgcc***	400
707	Reverse	ggcgTTgAggTcgATcgcccAcgTgAc***cAcTTggcTTTTcgAcTcATcAcgg***	
120	Forward	ccgcTAATTgAgAgATgcgATTgg	400
59	Reverse	ggcgTTgAggTcgATcgcccAcgTgAc	

The primary PCR was undertaken on DNA from an *M. a. avium* isolate formerly used at EMAI for the production of *M. a*. *paratuberculosis* ELISA reagents. Briefly, a reaction volume of 50 µl containing 5 µL of the *M. a. avium* template, 250 ng of the M706 and M707 primers, 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, 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 in 96-place thermal cycler (Corbett Research, Sydney, Australia). PCR cycling conditions were: one cycle of denaturation at 94°C for 3 min followed by 37 cycles of denaturation at 94°C for 30 sec, annealing at a range of temperatures (see Table 10) 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. PCR results were assessed by electrophoresis in 2% agarose gels stained with ethidium bromide using the molecular size marker number VIII (Roche). The following controls were included: negative control (sterile water) and a process control (PCR cocktail prepared in a clean location, without DNA sample). The process control tube was not opened until the completion of the PCR and was designed to assess the integrity of the PCR reagents. The PCR amplified a single product of approximately 400 bp long. The amplified product was purified using an Eppendorf PerfectPrep DNA purification kit. The purified product was then diluted in a 10 fold dilution series (10^{-1} to 10^{-21}) and the 10^{-6} to 10^{-21} dilutions were used as the template for the secondary PCR with the M120 and M59 primers using the same reaction conditions described above. The following controls were included: positive control DNA (100 fg/µL *M. paratuberculosis* DNA), negative control (sterile water) and a process control (PCR cocktail prepared in a clean location, without DNA sample). Twenty microlitres of the amplified product from each dilution was run on a 2% agarose gel (Figure 5).

Figure 4: Process followed to amplify the long internal control mimic. Steps A-B occurs during the primary amplification round. In the initial cycles of amplification the composite primers amplify the *M. a. avium* specific product (A), the double stranded mimic product is amplified continually filling in the M120/M59 primer sequences (B). Steps C-D is undertaken in secondary amplification round using the M120/M59 *M. a*. *paratuberculosis* specific primers only.

Key to Figure 3





Based on the results shown in Figure 5 we selected to further evaluate the 10^{-8} to 10^{-11} dilutions (Figure 5, lanes 3-6) of the mimic in a chequerboard titration against an *M. a*. *paratuberculosis* DNA dilution series. The results (Figure 6) clearly indicated that the 10^{-10} dilution (Figure 6, lanes 18-25) was the most appropriate.

Figure 5: Secondary PCR for the preparation of the long internal control mimic using the primers M120 and M59 and the 10^{-6} to 10^{-21} dilutions of the purified amplification product from the primary PCR (M706 and M707) as the target. Lanes 1 to 16 are the results for the 10^{-6} to 10^{-21} dilutions; lane 17 is the 316V positive control and lane 18 is the molecular size markers. Negative results (not on image) were all negative.

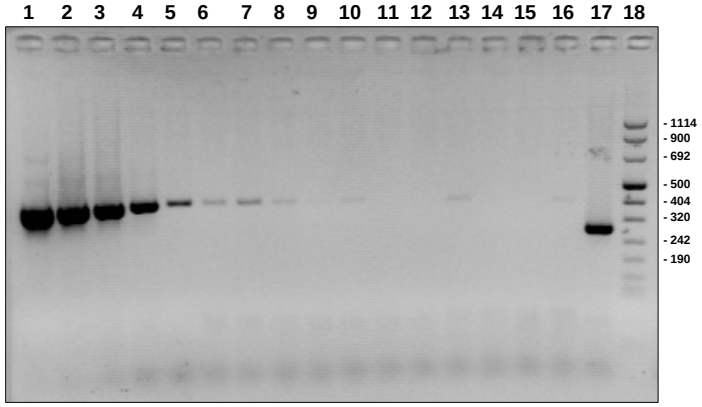
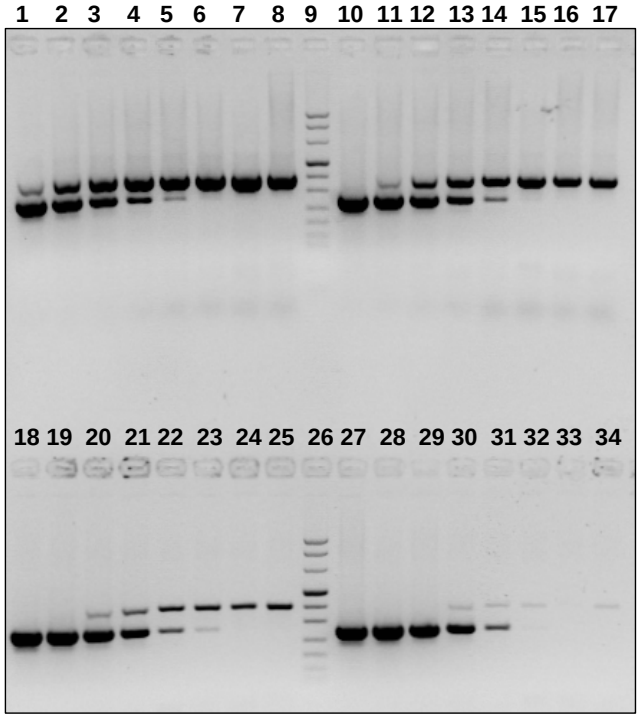


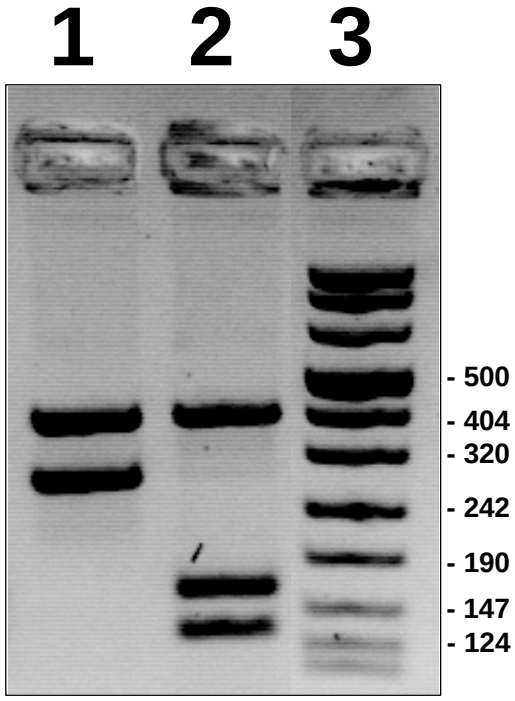
Figure 6: Chequerboard titration of *M. a*. *paratuberculosis* control DNA (10^{-3} to 10^{-8}) against four dilutions of the long internal control mimic; lanes 1 to 6 – 10^{-8}, lanes 10 to 15 – 10^{-9}, lanes 18 to 23 – 10^{-10} and lanes 27 to 32 – 10^{-11}. Lanes 9 and 26 are the molecular size markers. The remaining lanes are negative controls.



One of the most encouraging aspects of the long internal control mimics results, is that when the mimic is used at a dilution of 10^{-10} (Figure 6, lanes 18 to 25) and with high concentrations of *M. a*. *paratuberculosis* DNA the 900VM reaction favours the amplification of the smaller *M. a*. *paratuberculosis* specific IS*900* specific product. However, as the amount *M. a*. *paratuberculosis* DNA becomes limiting the reaction shifts to the amplification of the larger mimic product. This is ideal for use in a diagnostic context.

Should REA be required to confirm an *M. a*. *paratuberculosis* result, we needed to be certain that the long internal control mimic would not interfere with the interpretation of the result. Therefore, we performed a PCR with 900VM reaction including the long internal control mimic and the 316V control DNA as the template. The resulting amplified product (Figure 7, Lane 1) was digested with *Mse* I (Figure 7, Lane 2) and the two samples compared on a 2% agarose gel. Clearly, the long internal mimic (top band) has not cut and does not interfere with the interpretation of the digested lower IS*900* specific band.

Figure 7: Comparison the *Mse* I undigested (lane 1) and digested (lane 2) amplification products from the 900VM reaction, including the long internal control mimic, when using 316V DNA as the target. Lane 3 is the molecular size marker.



To conclude the development of the long internal control mimic we needed to sequence the entire length of the 400 bp long internal control mimic to be certain that we had the correct *M. a*. *paratuberculosis* primer sequences attached to the desired *M. a. avium* region. Full length sequence was obtained (Figure 8) using the M120 and M59 *M. a*. *paratuberculosis* primers and this clearly demonstrated that mimic contained the correct *M. a. avium* region. This sequence was then used in a BLASTn search of the *M. a. avium* genome to confirm the absence of the *M. a*. *paratuberculosis* specific primer sequences.

Figure 8: The consensus sequence derived from the amplified product from the two mimic PCR's (upper sequence) aligned with the predicted sequence (lower sequence) designed to include the *M. a. avium* region (lower case) including the M706 and M707 primers (**bold**

underlined lower case) plus the *M. a*. *paratuberculosis* specific primers M120 and M59 (**BOLD UPPER CASE**).

```
  1 ccgctaattgagagatgcgattggggtaccacctctgctcgttaagcggc 50
    ||||||||||||||||||||||||||||||||||||||||||||||||||
  1 CCGCTAATTGAGAGATGCGATTGGggtaccacctctgctcgttaagcggc 50
            .         .         .         .         .
 51 cacagccacccctacatcatgtgctagctgtaccggcccggcagcaacgc 100
    ||||||||||||||||||||||||||||||||||||||||||||||||||
 51 cacagccacccctacatcatgtgctagctgtaccggcccggcagcaacgc 100
            .         .         .         .         .
101 tgccgccatcggcttgtgcgtccacgccacccacgcgtgcttgagcgcgg 150
    ||||||||||||||||||||||||||||||||||||||||||||||||||
101 tgccgccatcggcttgtgcgtccacgccacccacgcgtgcttgagcgcgg 150
            .         .         .         .         .
151 cgagcctcccgtcgtacagcgccagccagccccgccacttgccgagccgg 200
    ||||||||||||||||||||||||||||||||||||||||||||||||||
151 cgagcctcccgtcgtacagcgccagccagccccgccacttgccgagccgg 200
            .         .         .         .         .
201 cggttgccgtcctcgtagtgcttgacaggagcgacagatccccaagcgac 250
    ||||||||||||||||||||||||||||||||||||||||||||||||||
201 cggttgccgtcctcgtagtgcttgacaggagcgacagatccccaagcgac 250
            .         .         .         .         .
251 gcccgttacaaacgccgacgcgctcactgatatatcagcaccggtgcctc 300
    ||||||||||||||||||||||||||||||||||||||||||||||||||
251 gcccgttacaaacgccgacgcgctcactgatatatcagcaccggtgcctc 300
            .         .         .         .         .
301 tcctttgggtccggcactggctttggcaaccgggtcgcgcgcgcttcggg 350
    ||||||||||||||||||||||||||||||||||||||||||||||||||
301 tcctttgggtccggcactggctttggcaaccgggtcgcgcgcgcttcgGG 350
            .         .         .         .         .
351 cactactcagcttttcggttcacgtcacgtgggcgatcgacctcaacgcc 400
    ||||||||||||||||||||||||||||||||||||||||||||||||||
351 cactactcagcttttcggttcacGTCACGTGGGCGATCGACCTCAACGCC 400
```

An initial evaluation of the long internal control mimic was performed using the false negative control faecal dilution series (1 in 50, 1 in 500 and 1 in 5000) prepared in experiment 3.4. DNA extraction was undertaken on the false negative faeces as well as the appropriate controls using the new-D-PCR technique described in experiment 3.3.2.5. DNA samples were then examined using the 900VM reaction including the long internal control mimic. The DNA samples were examined either undiluted or diluted 1 in 5 and both before and after purification with the Eppendorf PerfectPrep DNA purification kit as described in experiment 3.4. The results (Figure 9) confirm earlier findings showing that the undiluted/un-purified samples were inhibited producing false negative results as indicated by the absence of the long internal control mimic. However, the 1 in 5 dilution promoted the amplification of the IS*900* product at the lower faecal dilutions but still without amplification of the mimic. The PerfectPrep purified DNA amplified both the IS*900* and mimic products at all dilutions tested with both the undiluted and 1 in 5 dilutions. However, as we suspected in earlier experiments the 1 in 5 dilution did appear to limit the sensitivity of the IS*900* component of the PCR. To confirm this we undertook a final experiment comparing this procedure on 1 in 50, 1 in 500 and 1 in 5000 dilutions of non-inhibitory samples prepared in experiment 3.1. Once again the PCR was performed on DNA samples that were undiluted and diluted 1 in 5. The results (Figure 10) clearly demonstrate the adverse effects of the 1 in 5 dilution. Therefore, in the event of a negative result as indicated by the absence of the long internal control mimic we would recommend purification of the DNA sample using the

Eppendorf PerfectPrep DNA purification kit and then running these DNA samples in PCR undiluted.

Figure 9: Evaluation of the long internal control mimic using false negative faeces spiked with OJD positive faeces at the dilution rates of 1 in 50, 1 in 500 and 1 in 5000 plus negative and positive control faeces and a process control with the 900VM PCR. Treatments include: PCR on undiluted DNA without purification (lanes 1 to 6), PCR on 1 in 5 diluted DNA without purification (lanes 7-12), PCR on undiluted DNA with purification (lanes 18 to 23) and PCR on 1 in 5 diluted DNA with purification (lanes 24 to 29). Lanes 13 and 30 are the molecular size markers and lanes 14 to 17 and 31 to 34 are replicates of the PCR controls.

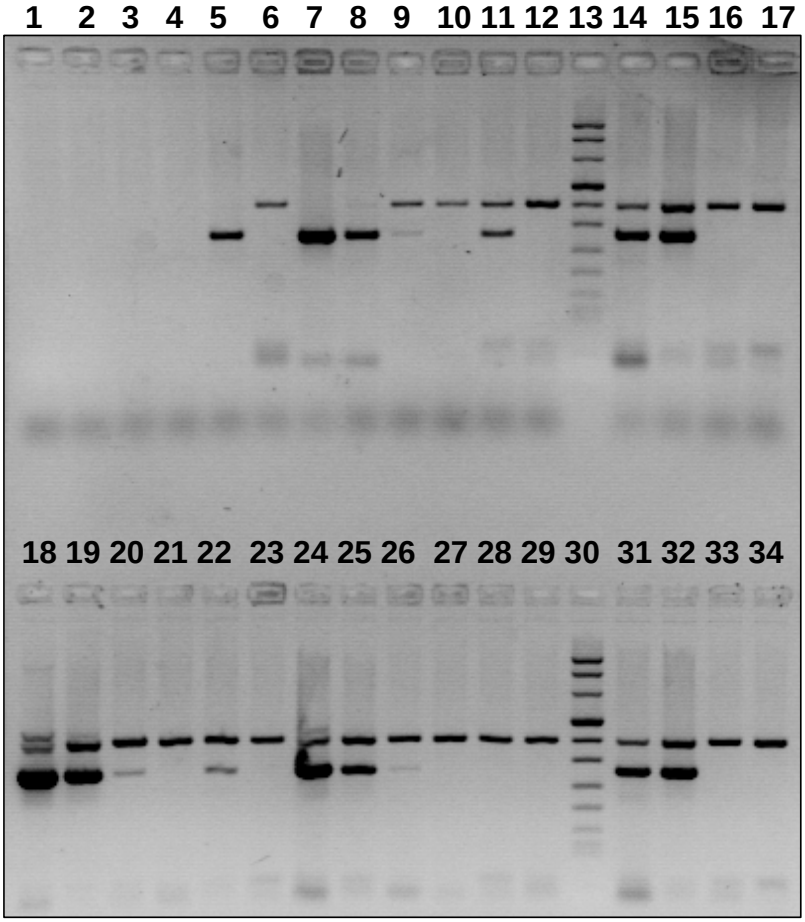
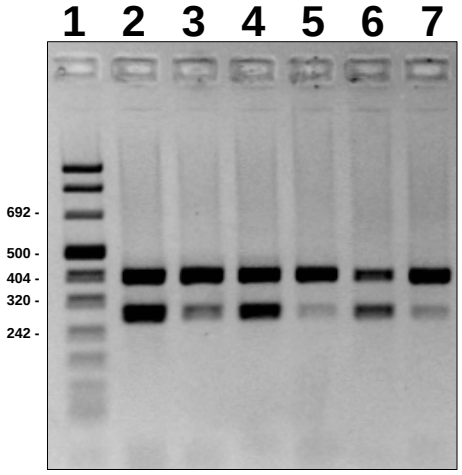


Figure 10: Evaluation of the long internal control mimic using 1 in 50, 1 in 500 and 1 in 5000 of *M. a*. *paratuberculosis* spiked faeces with the 900VM PCR. Lane 1 is the molecular size marker. Lanes 2 and 3 are the undiluted and 1 in 5 diluted DNA samples, respectively from the 1 in 50 faeces dilution. Lanes 4 and 5 are the undiluted and 1 in 5 diluted DNA samples, respectively from the 1 in 500 faeces dilution. Lanes 6 and 7 are the undiluted and 1 in 5 diluted DNA samples, respectively from the 1 in 5000 faeces dilution.



3.6 Development of a multiplex PCR assay for the detection of *M. a. paratuberculosis*

Background

At present the identification of *M. a*. *paratuberculosis* by conventional PCR relies on using IS*900* as the target sequence to confirm the presence of *M. a*. *paratuberculosis*[13;5]. IS*900* is considered to be unique to *M. a*. *paratuberculosis* and is a multicopy gene with up to 18 copies per cell, making it an ideal target for PCR. However, studies have shown that similar genomic sequences exist in non-*M. a*. *paratuberculosis* environmental mycobacterial species that may cross react in an IS*900*-based PCR producing false positive results[7]. To overcome this, REA of the amplified IS*900* product was introduced to eliminate false positive results[7]. Unfortunately, a recent study identified a non-*M. a*. *paratuberculosis* species that managed to circumvent this approach[10]. Therefore, to re-new the specificity of conventional PCR based diagnosis of *M. a*. *paratuberculosis*; IS*900* can be used in conjunction with other recently identified *M. a*. *paratuberculosis* specific targets in multiplex reactions. In recent years two new unique gene sequences have been identified that may be suitable for this task. Like IS*900*, IS*Mav2*[34] and IS*Map02*[33] are multicopy genes and to date have been shown to be unique to *M. a*. *paratuberculosis*. Unfortunately, there are fewer copies of each sequence in the *M. a*. *paratuberculosis* genome compared to IS*900* and therefore PCR assays based on these sequences may suffer from reduced sensitivity. However, if used in conjunction with IS*900*, samples that amplify both sequences may be considered positive for the presence of *M. a*. *paratuberculosis* without the need for any further examination by REA.

The aim of this set of experiments was to develop a multiplex assay for the detection of *M. a*. *paratuberculosis* by conventional PCR with visualisation by agarose gel electrophoresis. The new PCR was to incorporate IS*900* as the primary target and either IS*Mav2* or IS*Map02* as the secondary unique target using the assay conditions described in Marsh *et al.* 2001[20]. A number of PCR assays were evaluated using the primers described in Table 11 in the combinations described in Table 12. A number of alternate PCR platforms including: the Eppendorf MasterTaq (Cat. No. 0032 002.404), Eppendorf Hotmaster Taq (Cat. No. 0032 002.676) and Applied Biosystems AmpliTaq Gold (Cat. No. N808-0241) were compared with the routine PCR platform [20] (based on Millar *et al.* 1996 [22]) used at EMAI. None of these provided any improvement over the routine PCR platform and were not examined further. The requirement for β-mercaptoethanol in the routine PCR platform was also evaluated throughout this set of experiments and it was concluded that all *M. a*. *paratuberculosis* PCR can be successfully undertaken without β-mercaptoethanol. β-mercaptoethanol was omitted from the EMAI PCR protocol.

Table 11: Primer and amplification product details for the PCR assays evaluated in development of a multiplex PCR for the detection of *M. a*. *paratuberculosis*.

Target	Name	M	Direction	Sequence	Amplified product (bp)	Ref.
IS*900*	900M	58 59	Forward Reverse	gAAgggTgTTcggggccgTcgcTTAgg ggcgTTgAggTcgATcgcccAcgTgAc	413	(22)
IS*900*	900VM	120 59	Forward Reverse	ccgcTAATTgAgAgATgcgATTgg ggcgTTgAggTcgATcgcccAcgTgAc	289	(20)
IS*Mav2*	MAV2	694 695	Forward Reverse	gTgAgTTgTccgcATcAgAT gcATcAAAgAgcAccTcgAc	494	(32)
IS*Map02*	MAP02-1	696 697	Forward Reverse	gcAcggTTTTTcggATAAcgAg TcAAcTgcgTcAcggTgTccT	278	(33)
IS*Map02*	MAP02-2	688 689	Forward Reverse	ggATAAcgAgAccgTggATgc AAccgAcgccgccAATAcg	117	(33)
IS*Map02*	MAP02-3	674 675	Forward Reverse	gcATcTTcTgAAggccgAcA gcATccAcggTcTcgTTATc	188	This study

Table 12: Combinations of individual PCR assays and their predicted amplification products used to evaluate the possibility of an *M. a*. *paratuberculosis* multiplex PCR

Reaction 1	Reaction 2	Expected Amplification products
900VM	MAV2	289, 494
900VM	MAP02-2	289, 117
900M	MAV2	413, 494
900M	MAP02-1	413, 278
900M	MAP02-2	413, 117
900M	MAP02-3	413, 188
900VM	MAP02-3	289, 188

All PCR assays were evaluated for analytical sensitivity both individually (Table 13) and when used in the combinations described in Table 14. The results from the individual assays were used to determine if any adverse effects were produced when the assays were used in the multiplex combinations. From these results the optimal combination was shown to include the 900VM and MAP02-3 assays using the primers M120 and M59 plus M674 and 675, respectively. The analytical sensitivity of the individual reactions was maintained in the multiplex reaction as can be seen in Figure 10.

Table 13: Analytical sensitivity of the individual PCR assays

Reaction	*M. a. paratuberculosis* DNA dilution						
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	Neg
900M	4+	4+	4+	3+	2+	2+	-
900VM	4+	4+	4+	3+	2+	2+	-
MAV2	3+	2+	2+	2+	1+	Tr	-
MAP02-1	3+	3+	2+	2+	1+	Tr	-
MAP02-2	2+	2+	2+	1+	Tr	Tr	-
MAP02-3	3+	3+	2+	2+	2+	1+	-

Table 14: Analytical sensitivity of the multiplex PCR assays

Reactions	*M. a. paratuberculosis* DNA dilution						
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	Neg
900VM/MAV2	3+/1+	3+/1+	3+/Tr	3+/Tr	3+/Tr	3+/-	-/-
900VM/ MAP02-2	3+/1+	3+/1+	3+1+	3+/Tr	3+/Tr	3+/Tr	-/-
900M/MAV2	2+/4+	2+/4+	2+/4+	1+/3+	Tr/2+	Tr/1+	-/-
900M/ MAP02-1	4+/2+	3+/2+	2+/2+	1+/1+	Tr/Tr	-/-	-/-
900M/ MAP02-2	3+/2+	3+/2+	3+/2+	2+/1+	2+/1+	1+/Tr	-/-
900M/ MAP02-3	4+/3+	3+/3+	3+/3+	2+/2+	1+/1+	Tr/Tr	-/-
900VM/ MAP02-3	4+/4+	4+/4+	4+/4+	2+/2+	2+/2+	1+/Tr	-/-

The final experiment undertaken in the development of the multiplex reaction was to evaluate the analytical sensitivity of the multiplex reaction in the presence of the long internal control mimic using the 316V control DNA dilution series as the target. As seen previously, the multiplex reaction favoured the amplification of the IS*900* and IS*Map02* products at high concentrations of *M. a*. *paratuberculosis* DNA and the long internal control mimic was amplified only when *M. a*. *paratuberculosis* DNA became limiting (Figure 11). While the analytical sensitivity of the 900VM product was not affected the analytical sensitivity for the IS*Map02* product may have been reduced slightly. In the case of the IS*Map02* product not amplifying the IS*900* product would need to be confirmed by *Mse* I REA.

To complete this component of the project, the multiplex PCR assay including the long internal control mimic was evaluated on 70 DNA samples that had been kept from the original D-PCR project and stored at -20^{o}C. All 70 samples had been previously examined by faecal culture and the original D-PCR method. The complete set of results for these samples can be found in Appendix 1 and are summarised in Table 15. The multiplex reaction compared favourably with the original D-PCR results for these samples. Fifty six (87.5%) of the 64 faecal culture positive samples (one more than the original D-PCR result) were identified by the multiplex reaction. Of these 44 produced both the IS*900* (289 bp) and IS*Map02* (188 bp) bands and a further 12 produced the IS*900* band only. Of the eight faecal culture positive samples that failed to be detected by the multiplex reaction four amplified the long internal control mimic and four did not. This suggests that the original D-PCR did in fact suffer from the presence of PCR inhibitors and that false negative results may have been issue. All six faecal culture negative samples did amplify the long internal control mimic and therefore were considered truly negative. This completed the development of the new D-PCR test that now required evaluation using real faecal samples.

Figure 10: Analytical sensitivity results for the 900VM/MAP02-3 multiplex reaction. Lane 1 is the molecular size marker. Lanes 2 to 8 represent the 10^{-2} to 10^{-8} dilutions of the 316V DNA dilution series and lanes 9 and 10 are the PCR and process negative controls, respectively.

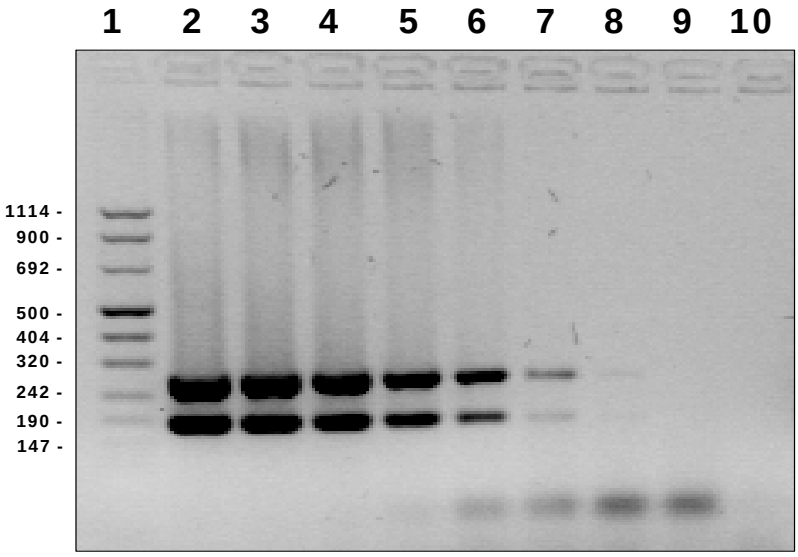


Figure 11: Analytical sensitivity results for the 900VM/MAP02-3 multiplex reaction including the long internal control mimic. Lanes 1 to 6 represent the 10^{-3} to 10^{-8} dilutions of the 316V DNA dilution series. Lanes 7 and 8 are the PCR and process negative controls, respectively and lane 9 is the molecular size marker.

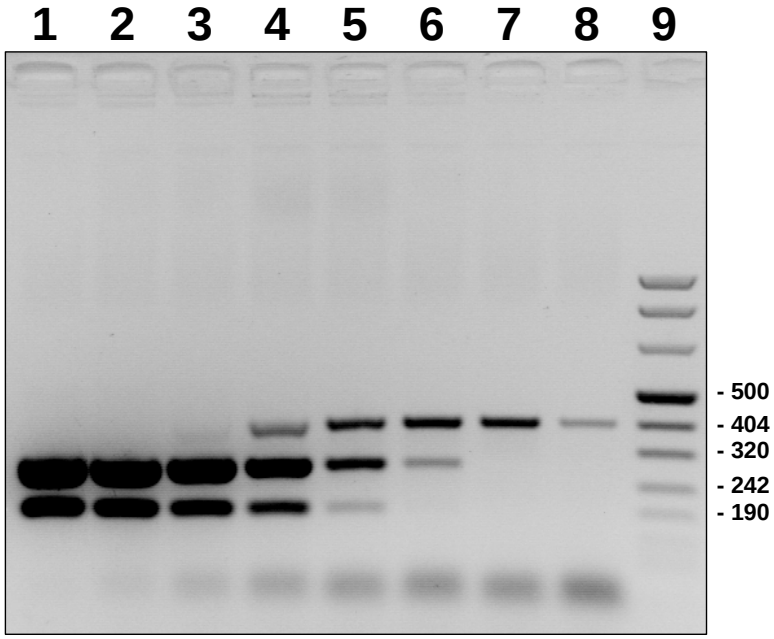


Table 15: Multiplex PCR results for the 70 DNA samples previously examined by faecal culture and the original D-PCR.

		Original D-PCR result with 900M PCR		New D-PCR result with Multiplex PCR	
		D-PCR positive (n = 55)	D-PCR negative (n = 15)	D-PCR positive (n = 56)	D-PCR negative (n = 14)
Faecal culture results (n = 70)	Positive (n = 64)	55	9	56 (44[b], 12[c])	8 (4[a], 4[d])
	Negative (n = 6)	0	6	0	6[a]

(a) – all samples amplified the long internal control mimic
(b) – all samples amplified the correct bands from IS*900* and IS*Map02*
(c) – all samples amplified the IS*900* band only
(d) – all samples failed to amplify the long internal control mimic

3.7 Initial validation of the new EMAI D-PCR test incorporating the IS*900*/IS*Map02* multiplex PCR and the long internal control mimic on both stored and recent faecal samples

An initial evaluation of the new D-PCR method was undertaken using 15 faecal samples (stored) that had been kept from the original D-PCR project and stored at -20^{o}C. All 15 samples had been previously examined by faecal culture and the original D-PCR method (for the complete set of results for these samples see Appendix 2). These samples were selected on that basis that there were large volumes, > 20g per sample, of each faecal sample and this would allow for repeated testing if required.

All 15 samples plus the appropriate controls were processed using the new D-PCR protocol described in experiment 3.3.2.5 and evaluated with the 900VM/MAP02-3 multiplex assay incorporating the long internal control mimic. Unfortunately, the results (Table 16) were quite disappointing with the new D-PCR showing a marked reduction in sensitivity compared to the original D-PCR test. Only three (37.5%) of the eight samples originally identified were found to be positive with the new D-PCR test. However all three amplified the correct IS*900* (289 bp) and IS*Map02* (188 bp) bands in the multiplex PCR. Interestingly, all 15 samples amplified the long internal control mimic indicating that the test suffers from reduced sensitivity even in the absence of PCR inhibitors.

Concern was raised over the integrity of these samples given that they had been in storage for greater than five years at -20^{o}C. Therefore the new D-PCR protocol was further evaluated using faecal culture positive diagnostic samples that have recently been submitted. Due to the recent downturn in samples being submitted for OJD diagnosis in NSW as result of the new OJD management guidelines, only five positive samples could be sourced of which there was only sufficient faeces from four. The four positive samples were tested along with five replicates of the negative control faeces. The new D-PCR failed to detect any of the positive faecal culture samples (Table 17). All five culture negative samples and two of the culture positive samples amplified the long internal control but the remaining two culture positive samples did not, indicating the presence of PCR inhibitors.

Based on these results no further evaluation of the new D-PCR protocol was undertaken using stored of recent pooled faecal culture samples in order to preserve these samples for future work given the current lack of OJD submissions for diagnosis.

Table 16: Results from the initial evaluation of the new D-PCR method using 10 g of faeces and the multiplex PCR with the long internal control mimic on 15 faecal samples from the original D-PCR project that have been stored at -80°C.

		Original D-PCR result with 900VM PCR		New D-PCR result with Multiplex PCR	
		D-PCR positive (n = 9)	D-PCR negative (n = 6)	D-PCR positive (n = 3)	D-PCR negative (n = 12)
Faecal culture results (n = 15)	Positive (n = 11)	8	3	3 [(a), (b)]	8 [(a)]
	Negative (n = 4)	1	3	0	4 [(a)]

(a) – all samples amplified the long internal control mimic
(b) – all samples amplified the correct bands from IS*900* and IS*Map02*

Table 17: Results from the initial evaluation of the new D-PCR method using 10 g of faeces and the multiplex PCR with the long internal control mimic on five recent faecal samples that had been recently examined by faecal culture and stored at -80°C.

		New D-PCR result with 900VM PCR	
		D-PCR positive (n = 0)	D-PCR negative (n = 9)
Faecal culture results (n = 9)	Positive (n = 4)	0	4 (2 [(a)], 2 [(b)])
	Negative (n = 5)	0	5 [(a)]

(a) – all samples amplified the long internal control mimic
(b) – all samples failed to amplify the long internal control mimic

3.8 Initial evaluation of the RT-J D-PCR on stored and recent faecal samples

At the 7th international Colloquium on Paratuberculosis in Bilbao Spain, representatives of the Shimadzu Corporation and the Japanese National Institute of Animal Health reported the results from a study that included a novel method of DNA preparation and amplification for the direct detection of *M. a*. *paratuberculosis* from cattle faeces[19]. This method includes two components know as the JohnePrep™ DNA preparation reagents for *Mycobacterium avium* subsp. *paratuberculosis* in bovine faeces and the JohneAmp™ Gene amplification reagent for *Mycobacterium avium* subsp. *paratuberculosis* DNA and are both manufactured by the Shimadzu Corporation. These are currently only commercially available in Japan and would be too expensive to use here. However, they have been used to develop a new D-PCR test (RT-J D-PCR) for use with sheep by the Farm Animal Health Unit of the Faculty of Veterinary Science at the University of Sydney by a student undertaking her Doctorate of Philosophy.

To determine the integrity of the 15 stored samples used in the initial evaluation of the new D-PCR test (experiment 3.7) using a second test, we sent the 15 stored samples and the 10 recent samples to the University of Sydney for examination using the RT-J D-PCR. The results (see Appendix 2 for the complete set of results) were extremely encouraging. Overall, 86% of the faecal culture positive samples were identified by the RT-J D-PCR including all five recent pooled faecal culture positive faecal samples (Table 18). Interestingly, the RT-J D-PCR also

identified *M. a*. *paratuberculosis* in one sample that had been previously found negative by faecal culture. Of the stored samples nine out of the 11 faecal culture samples were found to be positive by the RT-J D-PCR demonstrating that the integrity of these samples had been maintained.

Two conclusions could made from these results (1) the RT-J D-PCR is a very sensitive method of detecting *M. a*. *paratuberculosis* in ovine faecal samples and (2) the real- time PCR appears to superior to the conventional PCR. To establish this we ran the DNA samples derived from the RT-J DNA extraction method in the new multiplex PCR incorporating the long internal control. Once again the results were very encouraging with the multiplex PCR detecting 10 (63%) of the faecal culture positive samples. Of these nine amplified both the IS*900* and IS*Map02* products and only one amplified the IS*900* product on its own. All five recent faecal culture positive samples were correctly identified. The long internal control mimic was successfully amplified in all nine faecal culture negative samples but failed to amplify in one of the faecal culture positive-multiplex PCR negative samples. Overall this demonstrates that the RT-J D-PCR appears to have addressed the issue of PCR inhibitors.

Table 18: Results from the evaluation of the RT-J D-PCR and the EMAI multiplex PCR with the long internal control mimic on the 25 stored and recent faecal samples.

		RT-J D-PCR		Multiplex PCR with long internal control mimic using the DNA prepared with the RT-J method	
		D-PCR positive (n = 15)	D-PCR negative (n = 10)	D-PCR positive (n = 10)	D-PCR negative (n = 15)
Faecal culture results (n = 25)	Positive (n = 16)	14	2	10 (9 [b],1 [c])	6 (5 [a], 1 [d])
	Negative (n = 9)	1	8	0	9 [a]

(a) – all samples amplified the long internal control mimic
(b) – all samples amplified the correct bands from IS*900* and IS*Map02*
(c) – all samples amplified the IS*900* band only
(d) – all samples failed to amplify the long internal control mimic

4 Discussion

Even with the introduction of faecal culture for the diagnosis of OJD the need for a rapid (<48 hr) diagnostic test still exists for both sheep and cattle strains of *M. a*. *paratuberculosis*. We reported the development of a PCR for the detection of *M. a*. *paratuberculosis* directly from faeces[20]. However, this test was lacking in sensitivity compared to faecal culture. By combining our knowledge of the original D-PCR test[20] with recent advances in molecular based diagnostics we undertook this project to improve the original D-PCR test with the hope that the new test would have sensitivity greater than or equal to the routine pooled faecal culture test currently used for the diagnosis of ovine Johne's disease[38]. However, this was not going to be an easy task as biological samples such as faeces are well known to be complicated samples for obtaining DNA from for diagnostic PCR purposes[28]. In developing the new test we need to take into consideration three main factors (1) dealing with the many PCR inhibitory substances commonly associated with faeces[28] (2) incorporating a suitable internal PCR control to monitor their presence in each reaction tube and (3) designing a new multiplex PCR for the specific detection of *M. a*. *paratuberculosis* that would negate the need for REA as previously required[7].

A sound and logical approach was taken to identify the potential shortcomings of the original D-PCR procedure and make the appropriate modifications to overcome these to produce a new D-PCR method that would provide sensitivity as good as or possibly better than radiometric culture.

Prior to commencing work on the development of new in-house D-PCR, two commercial kits; the Qiagen QIAamp DNA Stool Mini Kit and MO BIO UltraClean Fecal DNA Kit, both designed specifically for the extraction of DNA samples from faeces, were evaluated and compared with faecal culture and the original D-PCR method. Although both kits were capable of extracting *M. a*. *paratuberculosis* DNA from spiked faecal samples, problems were identified with each kit that prevented their inclusion in the new D-PCR test. The Qiagen kit appeared to be capable of detecting *M. a*. *paratuberculosis* at very high dilutions of faeces but this was not reproducible and this extraction procedure was also prone to cross contamination, whereas the MO BIO kit suffered from poor analytical sensitivity. However, the major concern with both kits was cost. While the reagent and consumable cost per sample was not that bad, both kits were very labour intensive and this would make the cost of the test prohibitive. The focus of the work was re-directed to the development of new in-house procedure.

The two main sources of PCR inhibition were thought to be non-specific DNA and PCR inhibitory substances. After experimentally demonstrating that excessive amounts of non-specific DNA was probably not the problem, we focused on developing a technique that would hopefully remove inhibitory substances and concentrate *M. a*. *paratuberculosis* from larger faecal samples than have been used before. The aim of using larger faecal samples was to increase the sensitivity of the test to be able to detect low shedding or sub-clinical cases of OJD.

To achieve this we modified and applied the ethanol-based extraction technique used on radiometric cultures to separate *M. a*. *paratuberculosis* cells from the egg white used in the culture medium, prior to PCR. Proteins in the egg white are also inhibitory in PCR. When this approach was used with 0.2 g of faeces, the volume used in the original D-PCR, the new test demonstrated reduced analytical sensitivity compared to the original D-PCR. However, as the test was scaled up to incorporate 2.0 g and 10.0 g of faeces the analytical sensitivity increased and was equal to or possibly greater than faecal culture with the 10.0 g samples. Unfortunately, when the new D-PCR test was evaluated on real world samples the sensitivity plummeted and was even less than the original D-PCR method. The failure of the new *M. a*. *paratuberculosis* isolation and DNA extraction procedure became the rate limiting step in achieving the desired

objectives of the project. This was confirmed when the internal control demonstrated that the new test was still prone to extracting sufficient concentrations of PCR inhibitory substances to prevent the PCR from working. Furthermore, several culture positive samples failed to amplify the *M. a*. *paratuberculosis* specific bands even when the long internal control mimic did amplify indicating that the new test was inherently less sensitive even in the absence of PCR inhibitors.

Although the main objective of the project was not fulfilled the project did manage to develop some new and important contributions for the molecular based diagnosis of *M. a*. *paratuberculosis*.

With the ever increasing use of PCR for the diagnosis of microbial pathogens from a variety of biological samples, concern has been raised over the interpretation of negative results or more importantly false negative results[40]. While much attention has been directed to dealing with false positive results that arise from cross contamination of samples, very little attention has been direct at understanding the sources and functions of PCR inhibitory substances[40]. Consequently, some researchers have called for greater regulation in the introduction of PCR assays to be used for diagnostic purposes and have request the mandatory introduction of internal amplification controls[16]. This has led to collaboration between the European Standardization Committee and the International Standard Organisation (ISO) resulting in the proposed general guidelines for the use of PCR for the detection of food-borne pathogens[17]. Several approaches for the development of internal PCR controls have been described and reviewed[17], however, many of these are quite complex and may be beyond the scope of many laboratories for reasons such as time, cost or technical expertise. This may account for why internal PCR controls have not been widely accepted.

To date, only a few studies have been published on the introduction of internal PCR controls for *M. a*. *paratuberculosis* PCR assays[2;8;9;29;35] but these have yet to be widely adopted by the *M. a*. *paratuberculosis* research community, possibly for the reason stated above. As part of this project we developed a simple PCR-based technique, based on the method used to produce an internal control for *Bordetella pertussis*[24], for the production of an internal control mimic that can be used in conjunction with the M120 and M59 PCR primers used in the 900VM PCR for *M. a*. *paratuberculosis*[20]. When we examined this control for the effects it may have on the analytical sensitivity of the IS*900* specific target in the same reaction we found that it had no adverse effects and more importantly that the IS*900* target was preferentially amplified over the mimic. Therefore, in the presence of large amounts of the *M. a*. *paratuberculosis* target the PCR amplified the IS*900* target only and not the mimic. Under these circumstances the mimic is not needed but when the IS*900* target became limiting or absent the mimic did amplify confirming a weak *M. a*. *paratuberculosis* result or a true negative result. However, if neither product were to be amplified the sample would be deemed to be inhibited and the samples would need to be re-examined with fresh starting material or further purification of the DNA sample.

The next issue we addressed was the modification of the 900VM PCR assay that currently only amplifies a single product from the IS*900* gene. Over the last ten years studies have been published demonstrating the potential of non-*M. a*. *paratuberculosis* environmental mycobacteria to produce false positive results in IS*900*-based PCR as a result of IS*900*-like elements present in their genomes[7;10]. To overcome this, *M. a*. *paratuberculosis* specific restriction endonuclease sites in IS*900* were identified and are now routinely used in REA of the amplified product to confirm an *M. a*. *paratuberculosis* diagnosis. However, this can be costly and time consuming. Several new *M. a*. *paratuberculosis* specific DNA sequences: F57[25], IS*Mav2*[34] and IS*Map02*[33] have been identified and these can now be used to in PCR to circumvent the need for REA[32;33;35] . These new elements are present as either single copies, F57, or like IS*900* as multi-copy sequences but with far fewer copies than IS*900* making them less desirable targets than IS*900*.

We examined the potential of using the two multicopy sequences, IS*Mav2* and IS*Map02* in a multiplex PCR with the IS*900* target using the 900M[22] and 900VM assays[20]. The best results were achieved using the 900VM assay with IS*900* and IS*Map02* sequences as the targets. This reaction was evaluated including the long internal control mimic. Once again the analytical sensitivity of the IS*900* target was maintained in the presence of the long internal control mimic but the IS*Map02* target may have suffered a little in the multiplex reaction. The multiplex PCR was used to examine 70 DNA samples derived samples previously tested by faecal culture and the original D-PCR using the 900M assay. The multiplex reaction successfully identified 87.5% of the faecal culture positive samples which was one more than the when the 900M reaction was used in the initial stages of the original D-PCR. Of the 56 samples that were positive 44(78.5%) amplified both the IS*900* and IS*Map02* targets and the remaining 12 (21.5%) amplified the IS*900* target only and would require confirmation by REA. Eight faecal culture samples were PCR negative of which 4 did amplify the long internal control and 4 did not. This result demonstrated that PCR inhibitors were probably a source of false negative results with the original D-PCR and may have contributed to the reduced overall sensitivity of the test.

To complete the project we commenced evaluating the new D-PCR test now including the multiplex PCR and the long internal control mimic on faecal samples that had been evaluated by faecal culture. Dramatic changes in the management of OJD in NSW since the time this project commenced resulted in a severe downturn in the number and type of samples being submitted for OJD diagnosis. Therefore, we had to rely on stored samples and a few recent samples for the initial evaluation of the new test. The results were very disappointing with the sensitivity of the new test being lower than the original D-PCR. This was clearly an issue with the DNA extraction and the multiplex PCR and long internal control performed very well. Due to time and financial constraints we could not progress with the development of the *M. a*. *paratuberculosis* isolation and DNA extraction component of the procedure and decided to cease any further evaluation in order to preserve the stored faecal samples.

Just before the conclusion of this project it became possible to evaluate a new method developed by the Shimadzu Corporation and the Japanese National Institute of Animal Health. This test, first presented at the 7th international Colloquium on Paratuberculosis in Bilbao Spain[19], includes two components know as the JohnePrep™ DNA preparation reagents for *Mycobacterium avium* subsp. *paratuberculosis* in bovine faeces and the JohneAmp™ Gene amplification reagent for *Mycobacterium avium* subsp. *paratuberculosis* DNA These are currently not available in Australia but have been used to develop the RT-J D-PCR for use with sheep by the Farm Animal Health Unit of the Faculty of Veterinary Science at the University of Sydney by a student undertaking her Doctorate of Philosophy.

The RT-J D-PCR was evaluated using the same stored and recent faecal samples used to evaluate the new D-PCR procedure developed at EMAI. The RT-J D-PCR identified 86% of the faecal culture positive samples including all five recent faecal culture positive samples. Of the stored samples nine out of the 11 faecal culture samples were found to be positive by the RT-J D-PCR demonstrating that the integrity of these samples had been maintained.

One of the interesting aspects of the results from the RT-J D-PCR was that they were achieved using real-time PCR. Not all laboratories have access to real-time PCR and cannot currently offer it. Therefore, the same DNA samples derived from the RT-J method were used to evaluate the new multiplex PCR incorporating the long internal control. Once again the results were very encouraging with the multiplex PCR detecting 10 (63%) of the faecal culture positive samples. Of these nine amplified both the IS*900* and IS*Map02* products and only one amplified the IS*900* product on its own. Once again all five recent faecal culture positive samples were correctly identified. The long internal control mimic was successfully amplified in all nine faecal culture

negative samples but failed to amplify in one of the faecal culture positive-multiplex PCR negative samples.

Overall the results from the RT-J D-PCR were very promising and indicate that a more thorough examination of this test is required. Optimal results appear to be achieved using the RT-J method in conjunction with real-time PCR. However, future evaluation of this procedure should include both real-time and conventional PCR using the new multiplex PCR and long internal control developed as part of this project. Furthermore, the long internal control could also be redesigned to work in conjunction with the real-time PCR for the same reasons it was used in this project.

A new rapid test of the diagnosis of ovine Johne's disease is still an important requirement for the management of this disease in NSW, Australia and Internationally. Unfortunately, this project failed to develop a satisfactory replacement for the original D-PCR but the preliminary results from the RT-J D-PCR developed by the Shimadzu Corporation and the Japanese National Institute of Animal Health were extremely encouraging and indicate that this test should be evaluated further. However, further evaluation should also include the conventional multiplex PCR with long internal control as an alternative to real-time PCR to accommodate those laboratories that cannot offer real-time PCR in a diagnostic capacity at this point in time.

5 Success in Achieving Objectives

Although there was strong evidence during the early and middles stages of this project that a new D-PCR method was achievable based on establishing the analytical sensitivity of the new test on spiked faecal samples, disappointing results were achieved when the test was finally evaluated on real world samples. This meant the subsequent objectives in this project were not satisfactorily meet.

- A practical and cost-effective PCR test was developed with analytical sensitivity as good as or possibly better than radiometric culture when evaluated on spiked faecal samples. However this test failed to maintain its sensitivity when applied to real world samples and proved to be less sensitive that the original D-PCR.
- As the issue of poor real world sensitivity was only identified in the latter stages of the project it was not possible in to meet the subsequent objectives of testing a complete panel of real world samples and diagnostic submissions in parallel with culture.
- For the reasons stated above, establishing the test in a second laboratory and submitting the new test to SCAHLS for consideration was not possible. However, a new D-PCR test initially trialled in Japan at the Japanese National Institute of Animal Health combined with a Japanese faecal extraction technique (JohnePrep™, Shimadzu Corporation). This test is currently not available in Australia but is being developed for use in sheep by the Farm Animal Health Unit of the Faculty of Veterinary Science at the University of Sydney by a student undertaking her Doctorate of Philosophy. The early results strongly indicate that this test is an ideal replacement for the original D-PCR test and faecal culture.

6 Impact on Meat and Livestock Industry

A new rapid method, the RT-J D-PCR, for identification of *M. paratuberculosis* in pooled faecal samples from sheep has been recommended. However, this test requires further validation under Australian conditions but if approved, 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 an improvement by comparison with faecal culture where results are not available for months, causing significant inconvenience to producers. There are constraints to approval of this test and it is not possible to forecast when it may be used routinely.

The technology in the test 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.

7 Conclusions and Recommendations

A new rapid test of the diagnosis of ovine Johne's disease is still an important requirement for the management of this disease in NSW, Australia and Internationally.

Unfortunately, the new D-PCR described in this report failed to meet the objectives of this project and this requirement for industry. However, the preliminary results for the detection of *M. a*. *paratuberculosis* in faecal samples from sheep with the RT-J D-PCR are extremely promising. It is highly recommended that the RT-J D-PCR be further examined and developed for Australian conditions as a replacement test for faecal culture. Should this prove successful the RT-J D-PCR could then be applied and optimised for the detection of *M. a*. *paratuberculosis* in bovine samples and once again optimised for Australian conditions.

The successful development of the multiplex PCR and the long internal control as part of the project warrants further investigation and should also be included in the future development of the RT-J D-PCR to allow for the laboratories that are currently unable to use or offer real-time-PCR technology for routine diagnostics.

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9 Bibliography

1. Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389-402.

2. Brey, B. J., R. P. Radcliff, D. L. Clark Jr, and J. L. Ellingson. 2006. Design and development of an internal control plasmid for the detection of *Mycobacterium avium* subsp. *paratuberculosis* using real-time PCR. Mol Cell Probes 20:51-9.

3. Bull, T. J., E. J. McMinn, K. Sidi-Boumedine, A. Skull, D. Durkin, P. Neild, G. Rhodes, R. Pickup, and J. Hermon-Taylor. 2003. Detection and verification of *Mycobacterium avium* subsp. *paratuberculosis* in fresh ileocolonic mucosal biopsy specimens from individuals with and without Crohn's disease. J Clin Microbiol 41:2915-23.

4. Christopher-Hennings, J., M. A. Dammen, S. R. Weeks, W. B. Epperson, S. N. Singh, G. L. Steinlicht, Y. Fang, J. L. Skaare, J. L. Larsen, J. B. Payeur, and E. A. Nelson. 2003. Comparison of two DNA extractions and nested PCR, real-time PCR, a new commercial PCR assay, and bacterial culture for detection of *Mycobacterium avium* subsp. *paratuberculosis* in bovine faeces. J Vet Diagn Invest 15:87-93.

5. Collins, D. M., D. M. Gabric, and G. W. De Lisle. 1989. Identification of a repetitive DNA sequence specific to *Mycobacterium paratuberculosis*. FEMS Microbiol Lett 51:175-8.

6. Collins, D. M., D. M. Stephens, and G. W. de Lisle. 1993. Comparison of polymerase chain reaction tests and faecal culture for detecting *Mycobacterium paratuberculosis* in bovine faeces. Vet Microbiol 36:289-99.

7. Cousins, D. V., R. Whittington, I. Marsh, A. Masters, R. J. Evans, and P. Kluver. 1999. Mycobacteria distinct from *Mycobacterium avium* subsp. *paratuberculosis* isolated from the faeces of ruminants possess IS900-like sequences detectable IS900 polymerase chain reaction: implications for diagnosis. Mol Cell Probes 13:431-42.

8. Englund, S., A. Ballagi-Pordany, G. Bolske, and K. E. Johansson. 1999. Single PCR and nested PCR with a mimic molecule for detection of *Mycobacterium avium* subsp. *paratuberculosis*. Diagn Microbiol Infect Dis 33:163-71.

9. Englund, S., G. Bolske, A. Ballagi-Pordany, and K. E. Johansson. 2001. Detection of *Mycobacterium avium* subsp. *paratuberculosis* in tissue samples by single, fluorescent and nested PCR based on the IS900 gene. Vet Microbiol 81:257-71.

10. Englund, S., G. Bolske, and K. E. Johansson. 2002. An IS900-like sequence found in a *Mycobacterium* sp. other than *Mycobacterium avium* subsp. *paratuberculosis*. FEMS Microbiol Lett 209:267-71.

11. Erume, J., J. Spergser, and R. Rosengarten. 2001. Rapid detection of *Mycobacterium avium* subsp. *paratuberculosis* from cattle and zoo animals by nested PCR. Afr Health Sci 1:83-9.

12. Fang, Y. , W. H. Wu, J. L. Pepper, J. L. Larsen, S. A. Marras, E. A. Nelson, W. B. Epperson, and J. Christopher-Hennings. 2002. Comparison of real-time, quantitative PCR with molecular beacons to nested PCR and culture methods for detection of *Mycobacterium avium* subsp. *paratuberculosis* in bovine fecal samples . J Clin Microbiol

40:287-91.

13. Green, E. P., M. L. Tizard, M. T. Moss, J. Thompson, D. J. Winterbourne, J. J. McFadden, and J. Hermon-Taylor. 1989. Sequence and characteristics of IS900, an insertion element identified in a human Crohn's disease isolate of *Mycobacterium paratuberculosis*. Nucleic Acids Res 17:9063-73.

14. Halldorsdottir, S., S. Englund, S. F. Nilsen, and I. Olsaker. 2002. Detection of *Mycobacterium avium* subsp. *paratuberculosis* by buoyant density centrifugation, sequence capture PCR and dot blot hybridisation. Vet Microbiol 87:327-40.

15. Hampson, S. J., F. Portaels, J. Thompson, E. P. Green, M. T. Moss, J. Hermon-Taylor, and J. J. McFadden. 1989. DNA probes demonstrate a single highly conserved strain of *Mycobacterium avium* infecting AIDS patients. Lancet 1:65-8.

16. Hoorfar, J., N. Cook, B. Malorny, M. Wagner, D. De Medici, A. Abdulmawjood, and P. Fach. 2004. Diagnostic PCR: making internal amplification control mandatory. J Appl Microbiol 96:221-2.

17. Hoorfar, J., B. Malorny, A. Abdulmawjood, N. Cook, M. Wagner, and P. Fach. 2004. Practical considerations in design of internal amplification controls for diagnostic PCR assays. J Clin Microbiol 42:1863-8.

18. Khare, S., T. A. Ficht, R. L. Santos, J. Romano, A. R. Ficht, S. Zhang, I. R. Grant, M. Libal, D. Hunter, and L. G. Adams. 2004. Rapid and sensitive detection of *Mycobacterium avium* subsp. *paratuberculosis* in bovine milk and feces by a combination of immunomagnetic bead separation-conventional PCR and real-time PCR. J Clin Microbiol 42:1075-81.

19. Kojima, K., N. Nishimura, Y. Mori, and Y. Yokomizo. 2002. Efficiency of polymerase chain reaction using a novel method of DNA preparation and amplification for detection of *Mycobacterium avium* subsp. *paratuberculosis* in bovine fecal samples. 7th International Colloquium on Paratuberculosis,June 11-14, 2002, Bilbao, Spain .

20. Marsh, I. B. and R. J. Whittington. 2001. Progress towards a rapid polymerase chain reaction diagnostic test for the identification of *Mycobacterium avium* subsp. *paratuberculosis* in faeces. Mol Cell Probes 15:105-18.

21. McNab, W. B., A. H. Meek, S. W. Martin, and J. R. Duncan. 1991. Associations between dairy production indices and lipoarabinomannan enzyme-immunoassay results for paratuberculosis. Can J Vet Res 55:356-61.

22. Millar, D. S., S. J. Withey, M. L. Tizard, J. G. Ford, and J. Hermon-Taylor. 1995. Solid-phase hybridization capture of low-abundance target DNA sequences: application to the polymerase chain reaction detection of *Mycobacterium paratuberculosis* and *Mycobacterium avium* subsp. silvaticum. Anal Biochem 226:325-30.

23. Momotani, E., M. Kubo, Y. Ishikawa, Y. Matsubara, Y. Nakajima, and T. Yoshino. 1993. Immunohistochemical distribution of S-100 alpha-positive cells in bovine mycobacterial and non-mycobacterial granulomas. J Comp Pathol 108:291-301.

24. Muller, F. M., N. Schnitzler, O. Cloot, P. Kockelkorn, G. Haase, and Z. Li. 1998. The rationale and method for constructing internal control DNA used in pertussis polymerase chain reaction. Diagn Microbiol Infect Dis 31:517-23.

25. Poupart, P., M. Coene, H. Van Heuverswyn, and C. Cocito. 1993. Preparation of a specific RNA probe for detection of *Mycobacterium paratuberculosis* and diagnosis of Johne's disease. J Clin Microbiol 31:1601-5.

26. Prantera, C., G. Bothamley, S. Levenstein, R. Mangiarotti, and R. Argentieri. 1989. Crohn's disease and mycobacteria: two cases of Crohn's disease with high anti-mycobacterial antibody levels cured by dapsone therapy. Biomed Pharmacother 43:295-9.

27. Prantera, C. and M. L. Scribano. 1999. Crohn's disease: the case for bacteria. Ital J Gastroenterol Hepatol 31:244-6.

28. Radstrom, P., R. Knutsson, P. Wolffs, M. Lovenklev, and C. Lofstrom. 2004. Pre-PCR processing: strategies to generate PCR-compatible samples. Mol Biotechnol 26:133-46.

29. Rodriguez-Lazaro, D., M. D'Agostino, M. Pla, and N. Cook. 2004. Construction strategy for an internal amplification control for real-time diagnostic assays using nucleic Acid sequence-based amplification: development and clinical application. J Clin Microbiol 42:5832-6.

30. Sergeant, E. S., R. J. Whittington, and S. J. More. 2002. Sensitivity and specificity of pooled faecal culture and serology as flock-screening tests for detection of ovine paratuberculosis in Australia. Prev Vet Med 52:199-211.

31. Sherman, D. M., J. M. Gay, D. S. Bouley, and G. H. Nelson. 1990. Comparison of the complement-fixation and agar gel immunodiffusion tests for diagnosis of subclinical bovine paratuberculosis. Am J Vet Res 51:461-5.

32. Shin, S. J., Y. F. Chang, C. Huang, J. Zhu, L. Huang, H. S. Yoo, K. S. Shin, S. Stehman, S. J. Shin, and A. Torres. 2004. Development of a polymerase chain reaction test to confirm *Mycobacterium avium* subsp. *paratuberculosis* in culture. J Vet Diagn Invest 16:116-20.

33. Stabel, J. R. and J. P. Bannantine. 2005. Development of a nested PCR method targeting a unique multicopy element, ISMap02, for detection of *Mycobacterium avium* subsp. *paratuberculosis* in fecal samples. J Clin Microbiol 43:4744-50.

34. Strommenger, B., K. Stevenson, and G. F. Gerlach. 2001. Isolation and diagnostic potential of ISMav2, a novel insertion sequence-like element from *Mycobacterium avium* subspecies *paratuberculosis*. FEMS Microbiol Lett 196:31-7.

35. Tasara, T., L. E. Hoelzle, and R. Stephan. 2005. Development and evaluation of a *Mycobacterium avium* subspecies *paratuberculosis* (MAP) specific multiplex PCR assay. Int J Food Microbiol 104:279-87.

36. Vannuffel, P., C. Dieterich, B. Naerhuyzen, P. Gilot, M. Coene, R. Fiasse, and C. Cocito. 1994. Occurrence, in Crohn's disease, of antibodies directed against a species-specific recombinant polypeptide of *Mycobacterium paratuberculosis*. Clin Diagn Lab Immunol 1:241-3.

37. Whittington, R. J., S. Fell, D. Walker, S. McAllister, I. Marsh, E. Sergeant, C. A. Taragel, D. J. Marshall, and I. J. Links. 2000. Use of pooled fecal culture for sensitive and economic detection of *Mycobacterium avium* subsp. *paratuberculosis* infection in flocks of sheep. J Clin Microbiol 38:2550-6.

38. Whittington, R. J., I. Marsh, S. McAllister, M. J. Turner, D. J. Marshall, and C. A. Fraser.

1999. Evaluation of modified BACTEC 12B radiometric medium and solid media for culture of *Mycobacterium avium* subsp. *paratuberculosis* from sheep. J Clin Microbiol 37:1077-83.

39. Whittington, R. J. and E. S. Sergeant. 2001. Progress towards understanding the spread, detection and control of *Mycobacterium avium* subsp *paratuberculosis* in animal populations. Aust Vet J 79:267-78.

40. Wilson, I. G. 1997. Inhibition and facilitation of nucleic acid amplification. Appl Environ Microbiol 63:3741-51.

10 Appendices

10.1 Appendix 1: The complete set of results for the evaluation of the *M. a. paratuberculosis* multiplex PCR.

Sample No.	P No.	Status	Pool	GI(+) PCR(+)	Week to 999	DPCR 900VM	REA	DPCR 900M	Mimic	900VM	ISMap02
1	97	Y	2	+	5	+	+	+	+	+	+
2	98	Y	3	+	4	+	+	+	+	+	+
3	99	Y	4	+	5	+	+	+	+	+	+
4	101	Y	6	+	5	+	+	+	+	+	+
5	102	Y	7	+	5	+	+	+	+	+	+
6	103	Y	8	+	5	+	+	+	+	+	+
7	104	Y	9						+		
8	105	Y	10	+	5	+	+	+	+	+	+
9	106	Y	11	+	6				+	+	
10	116	Y	3	+	6	+	+	+			
11	117	Y	4	+	5	+		+	+	+	+
12	118	Y	5	+	5			+	+	+	+
13	119	Y	6	+	6			+	+	+	+
14	120	Y	7	+	5				+	+	+
15	121	Y	8	+	4	+	+	+	+	+	+
16	122	Y	9	+	6			+		+	+
17	123	Y	10	+	5	+	+	+	+	+	+
18	124	Y	11	+	5	+	+			+	+
19	125	Y	12	+	5	+	+	+		+	+
20	126	Y	13	+	4	+	+	+	+	+	+
21	127	Y	14	+	6	+	+	+	+	+	+
22	128	Y	15	+	5			+	+	+	+
23	129	Y	16	+	6	+	+	+		+	+
24	130	Y	17	+	6	+		+	+	+	
25	131	Y	18	+	5			+	+	+	

Sample No.	P No.	Status	Pool	GI(+) PCR(+)	Week to 999	DPCR 900VM	REA	DPCR 900M	Mimic	900VM	ISMap02
26	132	Y	19	+	5			+	+	+	
27	133	Y	20	+	5			+	+	+	
28	134	Y	21	+	6	+	+		+	+	
29	135	Y	22	+	7	+	+	+	+	+	
30	136	Y	23	+	5	+	+	+		+	+
31	150	Y	B9	+	6	+	+		+	+	+
32	151	Y	C1	+	8				+	+	+
33	152	Y	C2	+	6	+	+	+	+	+	+
34	153	Y	C3	+	7	+		+	+	+	+
35	154	Y	D1	+	7			+			
36	155	Y	D2	+	6	+	+	+			
37	156	Y	D3	+	6			+	+	+	+
38	157	Y	D4	+	5	+	+	+	+	+	+
39	338	Y	2	+	5	Not done		+	+	+	+
40	339	Y	3	+	6	Not done		+	+		
41	340	Y	4	+	3	Not done		+		+	+
42	341	Y	5	+	5	Not done		+	+		
43	342	Y	6	+	6	Not done		+	+		
44	343	Y	7	+	4	Not done		+	+	+	
45	344	Y	8	+	4	Not done		+	+	+	+
46	345	Y	9	+	4	Not done		+	+	+	+
47	346	Y	10	+	4	Not done		+	+	+	+
48	347	Y	11	+	6	Not done		+	+	+	+
49	348	Y	12	+	6	Not done					
50	349	Y	13	+	5	Not done		+	+	+	+
51	350	Y	14	+	3	Not done			+	+	+
52	351	Y	15	+	5	Not done		+	+	+	+
53	352	Y	16	+	4	Not done		+	+	+	

Sample No.	P No.	Status	Pool	GI(+) PCR(+)	Week to 999	DPCR 900VM	REA	DPCR 900M	Mimic	900VM	ISMap02
54	353	Y	17	+	5	Not done		+	+	+	
55	354	Y	18	+	4	Not done		+	+	+	
56	355	Y	19	+	4	Not done		+	+	+	+
57	356	Y	20	+	6	Not done		+	+	+	
58	374	Y	8	+	5	Not done		+		+	+
59	375	Y	9	+	5	Not done		+	+	+	+
60	376	Y	10			Not done			+		
61	377	Y	11			Not done			+		
62	378	Y	12			Not done			+		
63	379	Y	13			Not done			+		
64	380	Y	14			Not done			+		
65	381	Y	15	+	5	Not done		+		+	+
66	382	Y	16	+	5	Not done		+	+	+	+
67	383	Y	17	+	5	Not done		+	+	+	+
68	384	Y	18	+	5	Not done		+	+	+	+
69	385	Y	19	+	5	Not done			+		
70	386	Y	20	+	5	Not done		+	+	+	+

10.2 Appendix 2: The complete set of results for the evaluation of the new EMAI and RT-J D-PCR methods.

Sample Number	Sample type	Status	PFC	Week to 999	Original D-PCR method PCR results with 900M reaction only (0.2 g)	Original D-PCR project PCR results with 900VM (0.2 g)		New EtOH-based D-PCR method (10 g samples)			RT-J D-PCR results (0.25 g)	RT-J DNA samples used with the 900VM PCR	RT-J DNA samples used with the EMAI multiplex PCR		
						PCR	REA	Mimic	900VM	ISMap02			Mimic	900VM	ISMap02
1	Stored	Y	+	4	+	+	+	+	+	+	+	+	+	+	+
2	Stored	Y	+	6	-	+	+	+	-	-	+	-	+		
3	Stored	Y	+	8	-	-		+	-	-	-	-	+		
4	Stored	Y	+	5	+	+	+	+	-	-	+	+	+		
5	Stored	Y	-		-	-		+	-	-	-	-	+		
6	Stored	Y	-		-	-		+	-	-	+	-	+		
7	Stored	Y	+	4	+	+		+	-	-	+	-	+		
8	Stored	Y	+	5	-	-		+	-	-	+	-			
9	Stored	Y	+	4	+	+	+	+	+	+	+	+	+	+	
10	Stored	Y	+	4	-	+	+	+	+	+	+	+		+	+
11	Stored	Y	+	3	+	+		+	-	-	+	+	+	+	+
12	Stored	Y	+	4	+	+	+	+	-	-	+	+	+	+	+
13	Stored	Y	+	8	-	-		+	-	-	-	-	+		
14	Stored	Y	-		-	+		+	-	-	-	-	+		
15	Stored	Y	-		-	-		+	-	-	-	-	+		
16	Recent	N	-		Not tested	Not tested	Not tested	+	-	-	-	-	+		
17	Recent	N	-		Not tested	Not tested	Not tested	+	-	-	-	-	+		
18	Recent	N	-		Not tested	Not tested	Not tested	+	-	-	-	-	+		
19	Recent	N	-		Not tested	Not tested	Not tested	+	-	-	-	-	+		
20	Recent	N	-		Not tested	Not tested	Not tested	+	-	-	-	-	+		
21	Recent	?	+	5	Not tested	Not tested	Not tested	+	-	-	+	+	+	+	+
22	Recent	?	+	7	Not tested	Not tested	Not tested	Insufficient sample			+	+	+	+	+
23	Recent	?	+	5	Not tested	Not tested	Not tested	-	-	-	+	+		+	+
24	Recent	?	+	6	Not tested	Not tested	Not tested	-	-	-	+	+	+	+	+
25	Recent	?	+	4	Not tested	Not tested	Not tested	+	-	-	+	+	+	+	+