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Validation of Pooled Faecal Culture for Bovine Johne's Disease with Low Level Shedder Cattle

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Abstract

Cattle populations infected with *M. avium* subsp. *paratuberculosis* (*Map*) contain individuals shedding a range of *Map* concentrations in their faeces, from clinical cases shedding in excess of 10^8 organisms per gram, to some subclinical cases shedding fewer than 10^3/g. Pooled faecal culture, which is designed to reduce testing costs for infected herds, is based on thresholds of infection that can be detected using current cultural procedures. A sensitive procedure for PFC, based on radiometric culture and IS*900* PCR/REA confirmation, was used to examine pooled faecal culture of faeces from low shedder cattle, defined by slow growth of *Map* on initial radiometric culture (first growth index at 5 weeks or later). Eight samples (stored for up to 17 months at -80^{o}C) of 14 selected were found to yield *Map* on subsequent culture, including evaluation studies when samples were mixed with normal cattle faeces at pooling rates from 1:5 to 1:50. All were considered to be shedding relatively low levels of *Map*, estimated at less than 10^5/g of faeces in seven of the eight cases and less than 5 x 10^5/g in the remaining animal. At pooling rates of more than 1:5, PFC sensitivity was found to be low, and an incubation period of at least 10 weeks at the 1:5 rate was needed to detect cattle shedding $\leq 10^4$ *Map* organisms/g of faeces, representing an estimated inoculum per vial of fewer than 20 organisms. These results will assist the cattle industries to clarify acceptable pooling rates for PFC as a diagnostic tool in BJD control.

Executive Summary

Whole herd faecal culture, based on individual culture of samples, is recognised as a sensitive, but expensive diagnostic tool to evaluate herd infection rates of *M. avium* subsp. *paratuberculosis* (*Map*) in cattle. Pooled faecal culture (PFC), based on radiometric (Bactec) culture procedures with confirmation by IS*900* PCR and REA, has been proven to offer cost savings in detecting and evaluating infection rates in sheep flocks, and prior studies at EMAI indicated this technique was of merit in cattle. In the earlier studies, samples from cattle that were shedding moderate to high levels of *Map* were investigated. To augment that work, this study used similar radiometric culture procedures and confirmatory testing steps to investigate pooling rates suitable to low shedder cattle. In addition, since prior work with sheep samples by Reddacliff *et al* (2003a) showed *Map* concentrations in inocula for Bactec culture correlate with their growth rate in the culture media, this approach was adopted to quantify the *Map* shedding rate of the animals under test.

The case definition in the selection of "low shedder" cattle was based on slow growth of *Map* on initial radiometric faecal culture. Such samples were selected on the results of their initial diagnostic culture, if initial growth (as a growth index measured weekly after inoculation) was only evident at 5 or more weeks of an 8 week incubation period. From 14 faeces which met this criterion, and had been stored at -80^{o}C for up to 17 months, eight were found to yield *Map* on subsequent culture, including evaluation studies when samples were mixed with normal cattle faeces at pooling rates of 1:5, 1:10, 1:20, 1:25. 1:30 and 1:50. All samples were processed using procedures similar to those employed for OJD PFC. This included a 12 week incubation period, but all subcultures were made on Herrold's egg yolk medium instead of modified 7H10 media, since the latter is more suitable for growth of cattle strains of *Map*.

Since the growth of sheep (S) strains of *Map* from ovine faeces (Reddacliff *et al* 2003a) may differ from cattle (C) strains from bovine faeces, regression equations were developed to define the relationship between the number of *Map* cells in the bovine faecal culture inoculum and the number of days to reach a cumulative growth index of 1000 (cgi1000). Our prior study of moderate to heavy shedders, and based on two representative animals (1085 and 38) in that study had defined such a regression equation. To augment that information, six samples from the original 14 were selected at random and their processed culture inocula subjected to a 10-fold dilution series in Bactec broth (replicated 5-fold per dilution) to determine the relationship for low shedder cattle. Of these six, *Map* growth occurred in four animals, but only one (sample 15) yielded sufficient growth at multiple dilutions to determine a reliable regression equation between the $\log_{10}$ inoculum (as determined by the Most Probable Number or MPN method) and the number of days to cgi1000 (dcgi1000). This data was added to that already established from prior results for animals 1085 and 38 to produce a final regression equation to describe the relationship between the rate of Bactec growth and the number of *Map* in the inoculum as follows:

$$\log_{10} \text{inoculum} = 6.55 - 0.121 \text{ dcgi1000}$$

This equation was then used to estimate, from the rate of growth in both the 10 fold dilution series and the growth in the PFC dilution series, the number of organisms inoculated from the original faeces prior to processing. Since Reddacliff *et al* (2003b) estimated a 1.7 log (50 fold) loss in viable cell concentration of S strains of *Map* due to routine decontamination procedures as used in this study, and allowing for dilution steps to reach the final inoculum to Bactec, the original numbers of viable cells of *Map* per gram of faeces prior to culture of each positive animal were estimated. These methods indicated that the samples from the eight low shedder cattle generally contained between 10^2 and 10^5 viable *Map* cells per gram of faeces.

At pooling rates greater than 1:5, PFC sensitivity was found to be low in the low shedder cattle, especially those shedding $\leq 10^4$ *Map* organisms/g of faeces. In addition, an incubation period of 10 weeks was necessary to maximise detection of low shedder cattle at a dilution rate of 1:5.

These results indicate that, for optimal results from pooling of bovine faeces, a dilution of 1:5 is recommended to detect cattle shedding low levels of *Map.* At higher dilutions, only animals shedding 10^4 *Map*/g or higher would be detected. Based on current laboratory fees, the laboratory costs for whole herd testing of infected herds where clinical signs of Johne's disease are not apparent can be reduced by approximately 35%.

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1 Background

1.1 Diagnostic herd testing developments of PFC for BJD in Australia

Australian cattle producers are seeking more cost-effective herd based tests for Johne's disease to improve certification and control measures. In other species, development of pooled faecal culture (PFC) (Whittington *et al* 1999, 2000) and direct PCR (D-PCR) tests have recently undergone validation for use in control of Johne's disease.

In prior work at the Elizabeth Macarthur Agricultural Institute (EMAI), studies of PFC in cattle shedding moderate to high levels of *M. paratuberculosis* were completed. These studies, funded by NSW DPI, examined the dilution rates for detection of naturally infected cattle faeces. In those studies, dilution rates of 1:30 were quite successful when combined with a Bactec culture system and confirmation of growth by IS*900* PCR and REA.

Recent work at this laboratory (Reddacliff *et al* 2003a) has also shown that the rate of shedding of *M. paratuberculosis* in sheep is proportional to the rate of growth of *M. paratuberculosis* in Bactec cultures. This approach was adopted to determine the shedding rate of all cattle used in the earlier BJD PFC study, and determined that shedder rates would be rated as moderate to high. However, to avoid a falsely high claim for sensitivity of BJD PFC, this dataset needed to be complemented by faeces from naturally infected cattle that are confirmed to be shedding *M. paratuberculosis* at the lower end of the scale.

1.2 Overseas studies of BJD PFC

Overseas studies of PFC for bovine Johne's disease (BJD) have been generally restricted to solid media based systems. In the Netherlands (Kalis *et al* 2000, 2004) and USA (Wells *et al* 2002a, 2002b, Wells *et al* 2003; Tavornpanich *et al* 2004), PFC using small pool sizes has been shown to be of value. Simulation models from the Netherlands (Weber *et al* 2004) and the USA (van Schaik *et al* 2003) have reached similar conclusions.

In cattle, quantitative assessments of shedding rates have been based on colony forming units on solid media, with arbitrary estimates of low, medium and high shedders based on tube counts of 1-30, 30-300 and >300 cfu/0.1 g (van Schaik *et a*l 2003). In simulation models, these authors assumed that the proportion of low, medium and high shedders among infected cattle that excrete cultivable organisms is 70%, 10% and 20% respectively. Allowing for losses in processing of 1.7 log for Bactec media (and likely more for solid media), and dilution of samples in testing, these categories would equate to at least $< 1.5 \times 10^4$, up to 1.5×10^5 and $>1.5 \times 10^5$ organisms per g of faeces.

1.3 Testing limitations

The length of incubation of Bactec media for routine *Map* cultures differs for sheep (12 weeks) and cattle (8 weeks) samples. These incubation times adopted generally reflect the fact that lower numbers of *M. paratuberculosis* may be present in pooled OJD faecal samples compared with individual cattle faecal samples, and thus may require a longer incubation phase for maximal detection. A comparison of 8 vs 12 week culture is therefore important in the present study.

Bactec culture reduces the time taken for diagnostic testing compared to conventional (solid medium) culture, although one limitation of a liquid-base culture system such as Bactec is the issue of contaminant overgrowth. This is a particular problem with faeces from cattle on silage, where bacterial and fungal spores may not be readily destroyed in decontamination procedures. Direct testing of Bactec growth by PCR/REA can overcome these problems to some extent, and pre-treatment of faecal samples by freezing can also reduce the impact of contaminant overgrowth.

The cost of testing cattle for Johne's disease on a herd basis is expensive because only individual tests, based on blood (ELISA) or faecal culture-based assays, are currently validated. In sheep, cost savings are possible because of the availability of a validated test based on pooled faecal culture (PFC). Here groups of 50 sheep can be pooled into one sample for testing, at an approximate laboratory test cost of $2.30 per animal plus veterinary sampling charges. PFC has proven to be more sensitive than serology on sheep, and has very high specificity.

A report prepared by Sykes *et al* (2000) suggested that “if the pool size cannot be increased to 20 or more animals then economics do not favour the use of PFC on a broad scale in cattle”. This information was based on relative comparison of the cost and sensitivities of alternate methods, particularly absorbed ELISA. However, it overestimated sensitivities of ELISA and underestimated of the cost of ELISA testing.

1.4 Sample limitations

Access to an adequate volume of faeces from numbers of known infected cattle shedding low numbers of *M. paratuberculosis* at a given time-point is quite limited. A diagnostic laboratory can only assess a low shedder rate after the samples have been tested, and are therefore dependent on sufficient residual material to be available for PFC development studies. Re-sampling the same animal after this time is problematic because several weeks may have elapsed and the shedding rate may have increased. This study used a slow growth rate (growth evident after 5 weeks of incubation) as the determinant for low shedder rate in obtaining and storing faecal samples from routine culture submissions.

2 Project Objectives

2.1 Project Objectives

2.1.1 To obtain faeces from infected, low shedder cattle and uninfected cattle to develop a new test for bovine Johne's disease

2.1.2 To utilise the above to identify which dilutions of faeces of single infected cattle afford a sensitive and cost-effective herd test, based on pooled samples, for *M. avium* subsp *paratuberculosis.* The technology to be investigated is pooled faecal culture based on Bactec culture with confirmation by PCR/REA.

3 Methodology

3.1 General methodology

This was a laboratory-based diagnostic project using faeces from low level shedder cattle and testing by Pooled Faecal Culture (PFC) at a range of dilutions in known negative cattle faeces. The individual dilutions were cultured and, where applicable, growth confirmed as *M. avium* subsp. *paratuberculosis (Map)*. The number of *Map* in each sample of faeces was also calculated using established techniques that relate rate of growth in radiometric culture to actual concentration in faeces from prior endpoint titration (Most Probable Number, MPN) studies.

Based on samples from approximately 10 cattle, appropriate dilution rates were assessed to provide an assay of sufficient sensitivity that it will be able to be used in diagnostic laboratories in Australia for the purpose of cattle herd certification.

3.2 Samples

Samples were selected among diagnostic submissions to the Microbiology and Immunology Section at EMAI. The case definition in selection of “low shedder” cattle for this study was based on slow growth of *Map* on initial radiometric faecal culture. Such samples were selected based on the results of their initial diagnostic culture, if initial growth (as a growth index measured weekly after inoculation) was only evident at 5 or more weeks of an 8 week incubation period.

A total of 15 faeces submitted in 2004 from 3 beef and 8 dairy herds met this criterion, and had been stored at -80^{o}C for up to 17 months after original collection for diagnostic testing. The source of these samples is outlined in Table 1; two samples (numbers 8 and 13) were derived from the same animal at different sampling times, four months apart.

Table 1. Faecal samples collected from low shedder cattle used in faecal dilution study

Sample	Lab submission reference	Date collected	Herd ID	Herd location (RLPB)	Accession sample serial number	Animal ID	Breed	Vol (g)
1	MN041164	5/2/04	A	South Coast	3	132	Beef	20
2	MN042086	9/3/04	B	Kempsey	NA	NA	Dairy (Friesian)	70
3	MN041165	5/2/04	C	South Coast	6	933	Dairy	12
4	MN041166	5/2/04	D	South Coast	8	5049	Dairy (Friesian)	10
5	MN042581	25/3/04	E	Riverina	2	1571	Dairy (Friesian)	25
6	MN044386	31/5/04	F	Tweed-Lismore	2	NA	Beef (Greyman/ Murray Grey)	70
7	MN044828	15/6/04	G	Maitland	5	696	Dairy (Friesian/ Jersey)	10
8	MN043339	21/4/04	H	South Coast	1	1759	Dairy	17
9	MN043339	"	"	"	11	1854	"	18
10	MN043339	"	"	"	12	1902	"	12
11	MN048073	22/9/04	I	Casino	13	NA	Beef (Angus)	45
12	MN048073	"	"	"	94	NA	"	48
13	MN046981	19/8/04	H	South Coast	2	1759	Dairy (Friesian)	99
14	MN049457	8/11/04	J	Riverina	7	5353	Dairy (Friesian)	15
15	MN049805	18/11/04	K	Riverina	NA	NA	Dairy (Friesian)	57

Normal (negative) faeces were collected from six dairy cows in a commercial dairy at EMAI with a National BJD Market Assurance Plan status of MN3. Faecal aliquots from each cow were individually cultured and found negative for *Map* by individual Bactec faecal culture, and then mixed and stored at -80^{o}C.

3.3 Pooled Faecal Culture procedures

All samples were pooled with normal (negative) faeces at final dilutions of 1:5, 1:10, 1:20, 1:25 and 1:50. Depending on the volume of faeces available from each low shedder sample, 1.75-7.5 g faeces from each of the 15 cattle were homogenised with negative faeces at a dilution of 1:5 and, in some cases 1:10 and 1:25, using a stainless steel Waring blender (Table 2). From these pooled samples, further dilutions of 1:10, 1:20, 1;25 and 1:50 in normal faeces were then made and similarly homogenised (Table 2). From all 15 neat faeces and from each of the six dilutions of faecal homogenates for those faeces, 2 g aliquots were decontaminated according to the following method:

(a) 2 g faeces were added to 10 mL saline in screw-topped polypropylene tubes and mixed thoroughly with a swab stick, then after removal of the swab thoroughly mixed by shaking the tube vigorously.
(b) After the faeces had settled in the tube for 30 min, 3.5 mL of the top portion of the supernatant was transferred using a sterile plastic transfer pipette to a 30 mL polypropylene tube containing 25 mL 0.9% (w/v) HPC/BHI, avoiding any floating debris and ensuring that the pipette tip did not touch the inside of the tube.
(c) Following incubation at 37°C for 20-26 hr, the material was centrifuged at 2,300 rpm (900 *g*) for 30 min in a benchtop centrifuge (Beckman).
(d) After discarding the supernatant, 1 mL of antibiotic mixture VAN (0.1 mg/mL vancomycin; 0.05 mg/mL amphotericin B; 0.1 mg/mL nalidixic acid) was added to each tube and the pellet completely resuspended.

(e) Following incubation at 37°C for 70-74 hours, 0.1 mL of the resuspended pellet was inoculated using a 1 mL syringe into a vial of BACTEC 12B medium (Becton Dickinson) previously supplemented with a mixture of (per vial) egg yolk (1 mL), mycobactin J (100 μL of 50 μg/mL), PANTA PLUS antibiotic supplement (Becton Dickinson)(200 μL) and sterile water (0.7 mL).

Each Bactec vial was incubated at 37°C for up to 12 weeks, and examined weekly for growth in a Bactec 460 machine (Becton Dickinson). Growth was recorded weekly as a growth index (GI) between 0 and 999, generated from the ion chamber within the machine.

Samples from Bactec vials were collected when a growth index of 999 was reached ("A" sample), and one week thereafter ("B" sample) in accord with routine diagnostic laboratory practice at EMAI. For samples that showed growth above GI 200 but did not reach a GI of 999, the A and B samples were collected in the two weeks following the maximal GI. The B samples were examined following ethanol extraction (Whittington *et al* 1998) by routine IS*900* PCR (Moss *et al* 1992), with primers according to Millar *et al* (1995) and confirmed using *Mse*1 REA procedures according to Whittington *et al* (2000). If PCR negative on the B sample, these procedures were repeated on the A sample. The B samples were routinely subcultured to Herrold's egg yolk medium with mycobactin J and incubated for up to 10 weeks for confirmation of typical, mycobactin-dependent colonies (Cousins *et al* 2003).

Table 2. Dilution of samples (by weight) for PFC study

Samples	**1, 2, 6, 8, 11-15**		**3**		**4,10**		**5**		**7**		**9**	
Final dilution	Pos faeces	Neg faeces	Pos faeces	Neg faeces	Pos faeces	Neg faeces	Pos faeces	Neg faeces	Pos faeces	Neg faeces	Pos faeces	Neg faeces
1 in 5	7.5 g neat	30 g	3 g neat	12 g	4 g neat	16 g	7.5 g neat	30 g	1.75 g neat	7 g	5 g neat	20 g
1 in 10	3.75 g neat	33.75 g	4 g of 1 in 5	4 g	10 g of 1 in 5	10 g	10 g of 1 in 5	10 g	3 g of 1 in 5	3 g	3.75 g neat	33.75 g
1 in 20	10 g of 1 in 10	10 g	3 g of 1 in 10	3 g	8 g of 1 in 10	8 g	8 g of 1 in 10	8 g	2 g of 1 in 10	2 g	10 g of 1 in 10	10 g
1 in 25	5 g of 1 in 5	20 g	3 g of 1 in 5	12 g	4 g of 1 in 5	16 g	5 g of 1 in 5	20 g	1 g of 1 in 5	4 g	5 g of 1 in 5	20 g
1 in 30	10 g of 1 in 10	20 g	2 g of 1 in 10	4 g	4 g of 1 in 10	8 g	5 g of 1 in 10	10 g	2 g of 1 in 10	4 g	10 g of 1 in 10	20 g
1 in 50	3.75 g of 1 in 5	33.75 g	7 g of 1 in 25	7 g	8 g of 1 in 25	8 g	3.75 g of 1 in 5	33.75 g	2 g of 1 in 5	2 g	3.75 g of 1 in 5	33.75 g
Total neat faeces	11.25 g		3 g		4 g		7.5 g		1.75 g		8.75 g	

3.4 Determination of *Map* inocula and excretion rates by dilution series

Six of the 15 neat faecal samples (Samples 2, 6, 11, 12, 13 and 15) with adequate residual material after the PFC dilution series were selected at random and processed as described above until inoculation into Bactec media. With the 1 mL of pellet suspended in VAN by vigorous vortexing for 30 sec, 0.1 mL aliquots were inoculated into each of 5 BACTEC vials containing PANTA PLUS, egg yolk and mycobactin J supplements as described above. From the remaining VAN material, this was

again vortexed vigorously and 400 μL was transferred into 3600 μL of PBS containing 0.1% v/v Tween 80 (PBSTw) [PBSTw: 200 mL autoclave-sterilised PBS; 800 μL filter sterilised 25% v/v Tween 80 solution]. This represented a 10^{-1} dilution of the VAN inoculum. From this dilution, and following vortexing as described above, further 10-fold dilutions were made in PBSTw by transfer of 400 μL into 3600 μL. From dilutions at 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} of the VAN-treated inoculum, each was vortexed and 0.1 mL then immediately inoculated in each of five BACTEC vials containing supplements as previously described. From each of these dilutions, 0.1 mL was also inoculated onto each of four slopes of Herrold's egg yolk medium containing mycobactin J. BACTEC vials and Herrold's slopes were incubated for up to 12 weeks and 20 weeks respectively at 37°C. When growth was apparent in BACTEC vials, these were additionally examined every 2-3 days until a cumulative growth index (cgi) exceeded 1000.

Growth in BACTEC vials exhibiting growth was examined for the presence of *Map* by IS*900* PCR, REA and subculture as described previously.

Using the end point titration (Most Probable Number; MPN) method based for a 5 tube dilution series (USDHHS 2001), the numbers of *Map* in the inocula were calculated. These inocula were compared with the cumulative growth index and at each dilution exhibiting growth, the results were graphed to determine the number of days to reach a cgi of 1000 (dcgi1000).

The log of the inocula (calculated from MPN figures) were graphed against the dcgi1000 to generate a linear trendline (in Microsoft Excel®) and thence determine a regression equation to describe the relationship between growth and inoculum size for bovine faeces. The resultant data was added to previous calculations derived from earlier studies (based on two moderate to heavy shedders of *Map*, animals 1085 and 38), to determine a regression equation to describe the relationship between inoculum and growth rate for bovine faeces from all levels of shedder cattle.

4 Results and Discussion

4.1 PFC dilution results

From the 15 selected faeces cultured neat and also subjected to the dilution study in normal faeces, only eight (Samples 2, 3, 5, 6, 7, 9, 11 and 15) were found to yield *Map* on subsequent culture. Two of these samples (Nos. 7, 11) were culture positive only at the 1:5 or 1:50 dilutions respectively, and at no other concentration (including neat). Thus at the range of dilutions studied, 6/8 of the culture positive samples were detected as positive at both the neat and the 1:5 dilution rate, 3/8 at the 1:10 rate, 2/8 at the 1:20 and 1:30 dilution rates, 4/8 at the 1:25 dilution rate and 4/8 at the 1:50 dilution rate. The results for each dilution are shown in Table 3. For sample 3 at the 1:10 and 1:25 dilution, and for sample 15 at the 1:20 dilution, IS*900* PCR testing for confirmation of Bactec growth was negative but *Map* was able to be confirmed by subculture to Herrold's media. In these three instances, growth of morphologically typical, mycobactin-dependent colonies was observed. For sample 3 at both the 1:10 and 1:25 dilutions, the growth in Bactec media was delayed and did not reach a growth index of 999 within the 12 week incubation period (refer Appendix 1).

Table 3. Success of PFC for detection of *M. avium* subsp *paratuberculosis* in low shedder cattle at different dilutions, when inoculated to Bactec culture media. All positive were results confirmed by IS*900* PCR and REA unless otherwise specified.

Sample	Culture result at varying dilution rates						
	Neat	1:5	1:10	1:20	1:25	1:30	1:50
2	+	+	+	+	+	+	+
3	+	+	+*	-	+*	-	+
5	+	+	-	-	-	-	-
6	+	-	-	-	-	-	-
7	-	-	-	-	-	-	+
9	+	+	-	-	+	-	-
11	-	+	-	-	-	-	-
15	+	+	+	+*	+	+	+
1,4, 8, 10, 12, 13, 14	-	-	-	-	-	-	-

* positive by subculture (mycobactin dependency) only

The incubation time taken for detection by PCR/REA or subculture after reaching maximal growth index was typically longer than the 8 week incubation period typically used for individual bovine samples. Table 4 shows the time taken to reach a suitable growth index to confirm the presence of *Map* in the eight culture samples. In comparing cumulative detection of culture positive samples, Table 5 demonstrates that an incubation of at least 10 weeks was required for optimal results.

Table 4. Incubation time in Bactec required to reach high growth index (GI 999) to enable confirmation of positive cultures from 8 cows shedding low numbers of *Map*

	Incubation time (weeks) required for detection of *Map* at varying dilution rates						
Sample	**Neat**	**1:5**	**1:10**	**1:20**	**1:25**	**1:30**	**1:50**
2	7	7	8	7	7	8	12
3	10	10	12*#	-	12*#	-	11
5	7	8*	-	-	-	-	-
6	12	-	-	-	-	-	-
7	-	-	-	-	-	-	8
9	11	10*	-	-	10	-	-
11	-	8	-	-	-	-	-
15	7	8	6	6*	7	6	7

* only detected by subculture

\# 1 week after maximal GI failed to reach GI 999

Table 5. Cumulative positive results for 8 low *Map* shedder cattle samples at different dilutions according to weeks incubated

	Cumulative culture positive samples after various weeks of incubation						
Dilution	**6**	**7**	**8**	**9**	**10**	**11**	**12**
1:5	0	1	4	4	6	6	6
1:10	1	1	2	2	2	2	3*
1:20	1*	2	2	2	2	2	2
1:25	0	2	2	2	3	3	4*
1:30	1	1	2	2	2	2	2
1:50	0	1	2	2	2	3	4

* includes one sample only detected by subculture

4.2 Results of ten-fold dilution series

From the six faeces where additional studies were performed on 10-fold dilutions of VAN-treated inoculum injected into five-fold replicates of BACTEC media, four samples (Nos. 2, 6, 11, 15) produced growth of *Map* at the neat (nil) dilution, two (Nos. 11 and 15) at the 10^{-1} dilution and one (Sample 15) at dilutions of 1:100 or higher (Table 6). Thus in terms of establishing a regression line requiring growth at three points in the dilution series, only sample 15 was useful in determining a regression equation.

The mean cumulative growth index (cgi) for the replicates at each dilution was calculated for confirmed *Map* positive dilutions at the neat, 1:10 and 1:100 dilutions for Sample 15. From this data the number of days to reach a cgi of 1000 (dcgi1000) was calculated (Figure 1a). The dcgi1000 was also determined for other individual positive cultures of other samples at each dilution which failed to produce positive results across all five replicates.

The data from sample 15 produced a regression equation (as shown in Figure 1b) of:

$\log_{10}$ inoculum = 6.4 – 0.107 dcgi1000

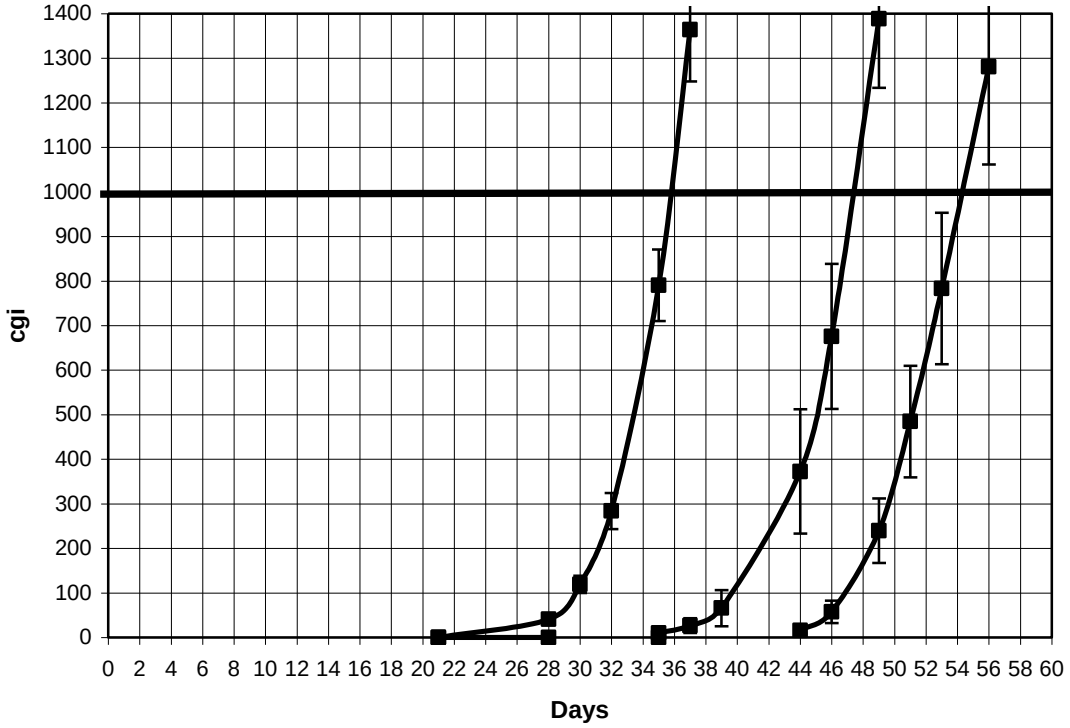
Table 6. Growth of *Map* in 5-fold replicate BACTEC broths in a 10 fold dilution series

	No. positive/no. tested at varying dilutions					MPN result	MPN/g*	Range
	Neat	**1:10**	**1:100**	**1:1000**	**1:10000**			
2	5/5	0/5	0/5	0/5	0/5	5-0-0-0-0	23	6.8-70
6	1/5	0/5	0/5	0/5	0/5	1-0-0-0-0	2	0.1-10
11	2/5	1/5	0/5	0/5	0/5	2-1-0-0-0	6.8	1.8-17
15	5/5	5/5	5/5	1/5	0/5	5-5-5-1-0	3300	1,000-10,000
12, 13	0/5	0/5	0/5	0/5	0/5	0	0	

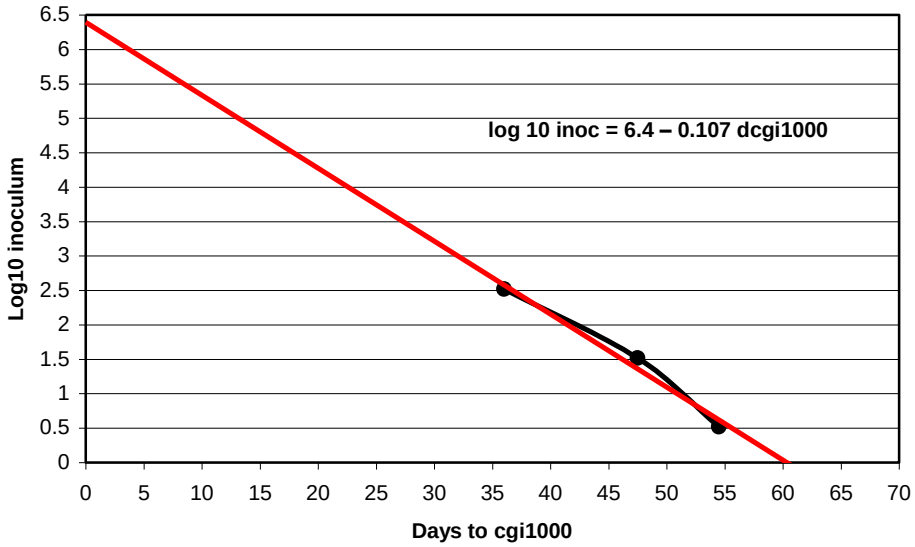
* expressed as Most Probable Number (MPN) per gram of inoculum, considering the neat inoculum represents 0.1 mL (0.1 g) of the VAN-treated inoculum. Actual inoculum in *Map* cells is 1/10 of these figures.

Figure 1. Determination of regression equation data for Sample 15

a. Plot of growth as a cumulative growth index (cgi). The lines represent results derived from the mean (± SEM) cgi for the neat, 1:10 and 1:100 dilution series and allow the days to cgi1000 to be calculated for each dilution



b. Determination of regression equation between log_{10} inoculum (determined from MPN calculations) and days to cgi1000 for sample 15

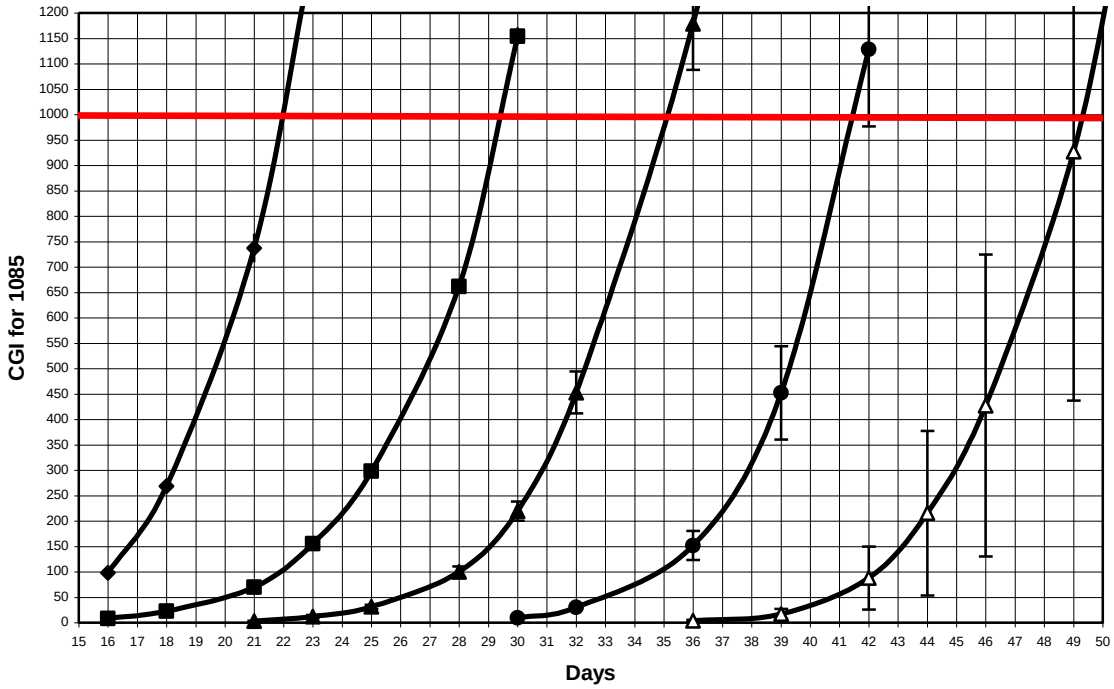


4.3 Comparison with data from earlier BJD PFC studies at EMAI

Data derived from the earlier studies to determine the relationship between inoculum size and growth in Bactec media for two cattle (1085 and 38) is shown in Figures 2 and 3.

Figure 2. Growth curve and regression equation for cow 1085 from prior study

a. Determination of days to cgi1000 from growth curves representing 10 fold dilutions of Bactec inoculum, from neat to 10^{-4}



b. Resultant regression equation for cow 1085, based on MPN of Bactec inoculum

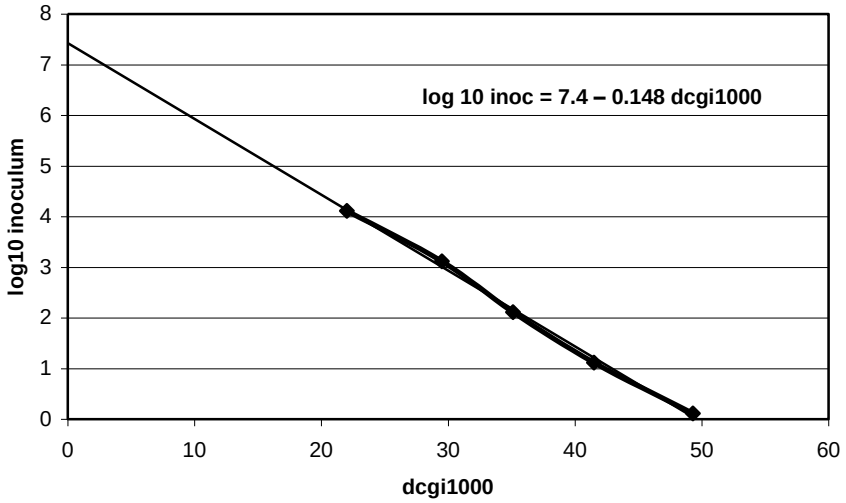
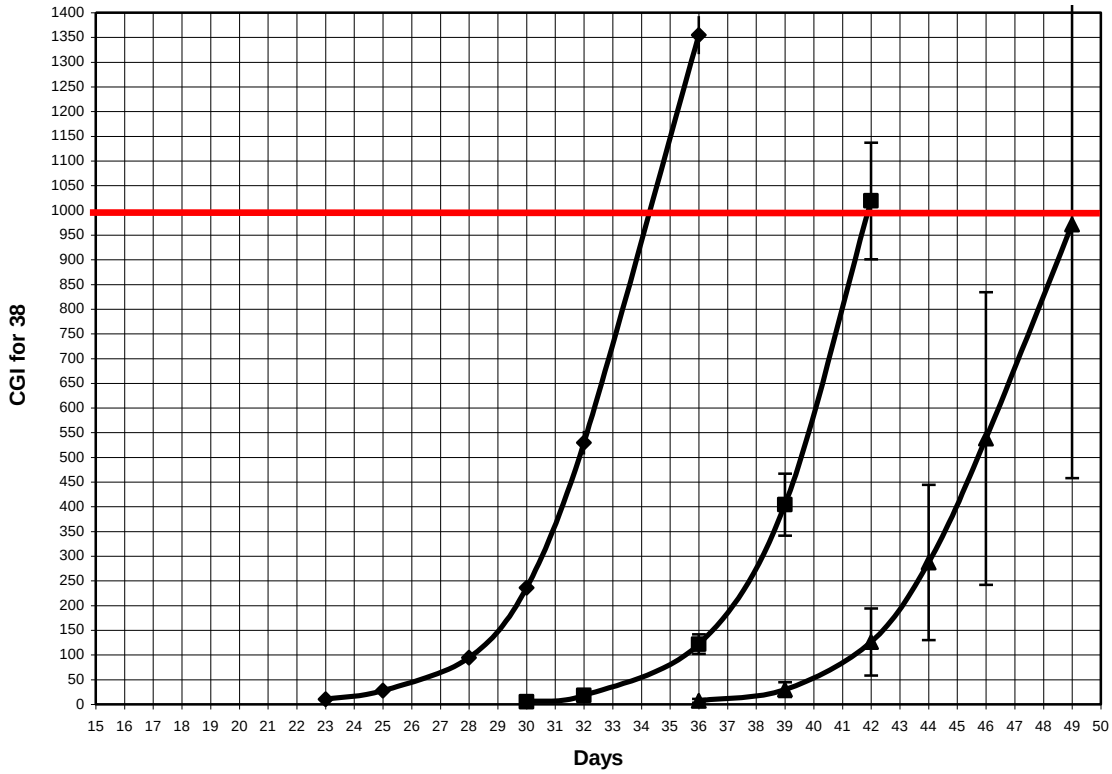
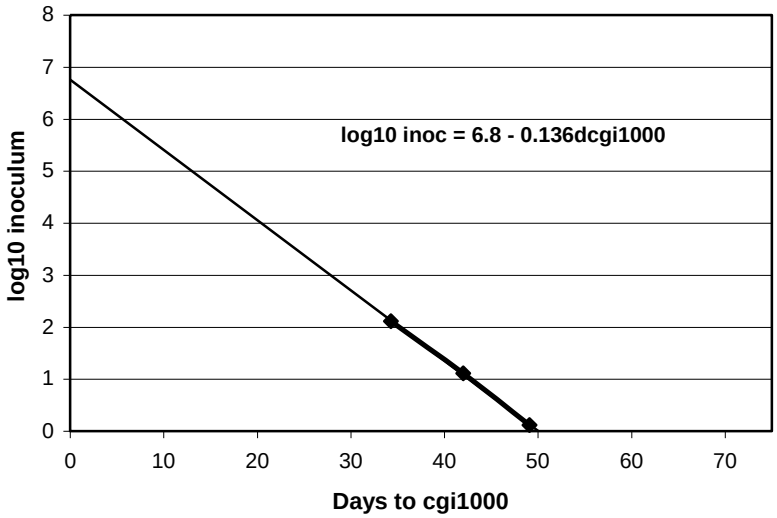


Figure 3. Growth curve and regression equation for cow 38 from prior study

a. Determination of days to cgi1000 from growth curves representing 10 fold dilutions of Bactec inoculum, from neat to 10^{-4}



b. Resultant regression equation for cow 38, based on MPN of Bactec inoculum



4.4 Calculation of regression equation and shedding rates

Figure 4 shows a plot of the full dataset from sample 15 together with similar datasets for two other samples (1085 and 38), whose individual regression equations had been calculated from the prior

study (1085: $\log_{10}$ inoculum = 7.4 - 0.148dcgi1000; 38: $\log_{10}$ inoculum = 6.8 - 0.136dcgi1000). This provided the final linear regression equation for bovine faeces shown in Figure 4, as:

$$\log_{10} \text{inoculum} = 6.55 - 0.121 \text{ dcgi1000}$$

Based on this regression equation, the inoculum size calculated for all samples using dcgi1000 data derived from the pooled faecal samples and from the ten fold dilution series is shown in Table 7. Based on *Map* S strain data that indicated a loss of 50 fold (1.7 $\log_{10}$) due to processing (Reddacliff *et al* 2003b), the final shedding rate estimated for the eight "low shedder" cattle is also presented in Table 7. In this table, estimates based on MPN in the dilution series for four samples are compared with estimates for all cattle based on the regression equation, derived from dcgi1000 determined from the pooled faecal culture and/or ten-fold dilution series. In cultures that showed a slow growth of *Map* and that subsequently yielded a $\log_{10}$ inoculum result from the regression equation below 0, these were adjusted to $\log_{10}$ inoculum = 0, as it was assumed that such (positive) cultures contained an inoculum of at least one organism.

From the estimated faecal shedding rates of *Map* (Table 7), cattle shedding up to 1×10^4 organisms per gram of faeces, were generally undetected at dilutions above 1:5. Based on an assumed loss of 50 fold due to processing, this would equate to a BACTEC inoculum of $10{,}000/50 \times 14.3 = 14$ organisms from neat faeces and 2.8 organisms in the current procedure at a 1:5 dilution. On the estimated numbers of cells available for culture from low shedder cattle, relatively small inocula of *Map* into Bactec cultures appear to yield detectable growth after prolonged incubation.

The results were consistent with the assumption that C strain and S strain *Map* are similarly affected by a 50-fold loss during processing. However, it is also known that C strain *Map* cells may clump readily, producing underestimations of *Map* cells in dilution series, despite PBS-Tween 80 treatment that is known to break-up S strain organisms (Reddacliff *et al* 2003b). This could mean that the *Map* concentrations estimated from the regression equations or MPN series may represent an underestimate of the true numbers of viable organisms in the faeces.

The long-term storage of the samples for up to 17 months may have affected the viability of the *Map* cells in all samples, which may explain the failure to re-culture from even neat (nil) dilutions of samples 1, 4, 8, 10, 12, 13 and 14. However, this may also indicate that the results represent a conservative estimate of the benefit of PFC in low shedder cattle, and that a dilution of 1:5 may represent a conservative dilution to detect infection on a herd basis.

Figure 4. Regression equation for bovine faeces based on cgi1000 data derived from three cattle (1085, 38, and Sample 15 from current study)

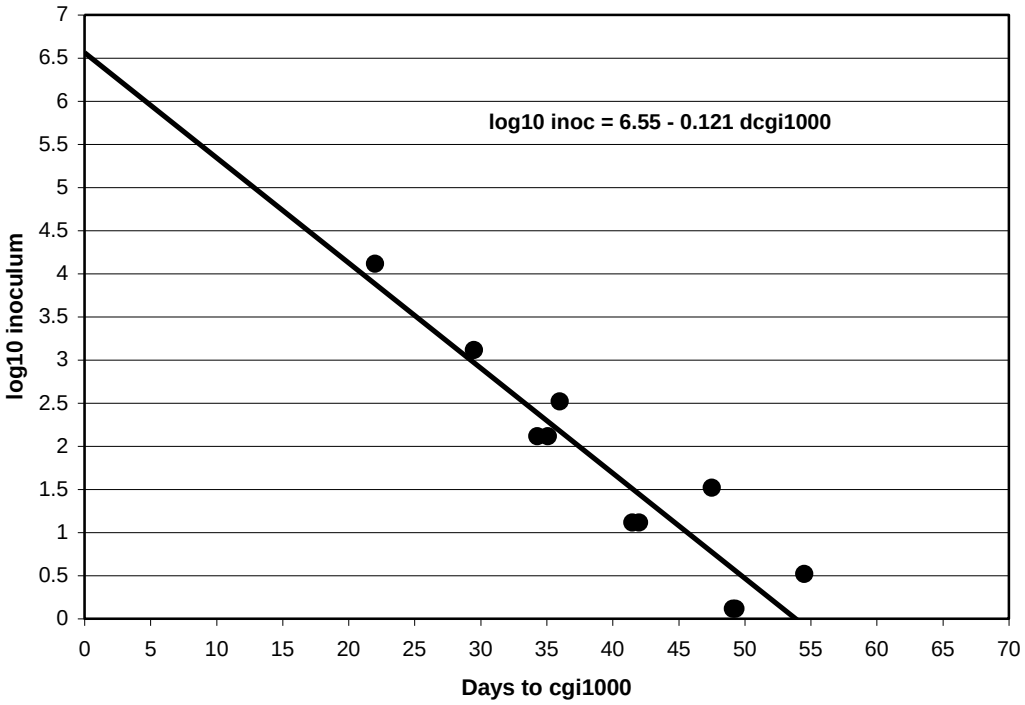


Table 7. Estimation of faecal shedding rate of *Map* from low shedder cattle, based on an assumed 50-fold loss during processing, and ordered from lowest to highest rate of shedding

Sample	*Mapl* g faeces by regression equation from PFC and ten-fold dilution series*	Highest dilution positive	*Mapl* g faeces by MPN from ten-fold dilution series	95% CI (from MPN)
6	7.1×10^{2}	1	1.4×10^{2}	$7 \times 10^{0} – 7.2 \times 10^{2}$
11	3.3×10^{3}	5	4.9×10^{2}	$1.3 \times 10^{2} – 1.2 \times 10^{3}$
5	1.0×10^{4}	5		
3	2.1×10^{3}	50		
9	7.4×10^{3}	30		
2	1.8×10^{4}	50	1.6×10^{3}	$4.9 \times 10^{2} – 5.0 \times 10^{3}$
7	4.9×10^{4}	50		
15	7.6×10^{4}	50	2.4×10^{5}	$7.2 \times 10^{4} – 5.1 \times 10^{5}$
1, 4, 8, 10, 12, 13, 14	-	-	-	-

* based on days to cumulative growth index of 1000

4.5 Comparison of *Map* in inocula determined from Bactec dilutions and from colony counts on solid media

The results of colony counts of the inoculum prepared for the Bactec MPN dilution series is compared against the MPN estimates from Bactec growth in Table 8. While there appeared minor differences for Sample 15 in these estimates, there were lower counts on solid media for the two samples examined in the earlier study (Samples 1085 and 38), by a factor of approximately 1 $\log_{10}$.

Table 8. Comparison of counts to determine inocula from ten-fold dilutions on Herrold's medium and in Bactec media used for MPN determinations

Sample ID		Neat	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	Estimate of inoculum
1085	**MPN from Bactec dilutions**	5	5	5	5	4	0	13000 (3600 – 40000)
	Solid medium colony counts	ND*	TNTC**	3	1	1	ND	
		ND	TNTC	11	4	0	ND	
		ND	TNTC	11	1	0	ND	
		ND	TNTC	14	0	0	ND	
		Mean inoculum (per 0.1 mL)		975	1500	2500#		1240
38	**MPN from Bactec dilutions**	5	5	4	0	0	0	130 (36 – 400)
	Solid medium colony counts	ND*	4	1	0	0	ND	
		ND	4	0	0	0	ND	
		ND	4	0	0	0	ND	
		ND	4	0	0	0	ND	
		Mean inoculum (per 0.1 mL)	40	25#				40
15	**MPN from Bactec dilutions**	5	5	5	1	0	ND	330 (100 – 1000)
	Solid medium colony counts	ND*	28	0	0	0	ND	
		ND	56	0	0	0	ND	
		ND	32	4	0	0	ND	
		ND	> 50 ##	0	0	0	ND	
		Mean inoculum (per 0.1 mL)	540	100				320

* ND: not done
** TNTC: too numerous to count
\# excluded from consensus mean calculation due to high variance and single colony at this dilution
count > 50; arbitrarily set at 100 to calculate mean

4.6 Relevance of findings to shedding rates among cattle in infected herds

The estimates of shedding rates for the 8 "low shedder" cows could be determined from either the regression equation (as applied to growth in the PFC or 10-fold dilution series) or the MPN data generated from the 10-fold dilution series for four of the animals. Since others have categorised

shedders of *Map* as either low, moderate or high based on colony counts on solid media, the estimated shedding rates among the 8 "low shedders" were therefore compared to such figures described in the literature. From the report of van Shaik *et al* (2003), and taking into account an estimated loss of 50 fold due to processing, based on studies on S strain by Reddacliff *et al* (2003b) the suggested ranges for low, medium and high shedders would equate to $<1.5 \times 10^4$, 1.5×10^4 – 1.5×10^5, and $> 1.5 \times 10^5$ per gram of faeces. These "old criteria", for which van Shaik *et al* (2003) suggested 70% of infected cattle fell into the "low" category, do not take into account the likely difference in sensitivity between solid media and Bactec liquid media. Since the current study indicated a factor 1 $\log_{10}$ increased sensitivity due to culture on Bactec media, it is suggested that "new criteria" for classifying shedding rates based on Bactec growth should be 10 fold higher than those as applied to solid (Herrold's) media

The estimates of shedding rates for the eight cattle studied, based on the either the regression equation or the MPN data and categorised by the "old criteria" and "suggested new criteria" is given in Table 9. Using similar criteria, the results based on the same regression equation and applied to 35 samples tested in prior studies on 14 cattle, were compared with those of the eight cattle in the present study (Table 9). Based on either "old" or "new" criteria, it is clear that the shedding level of the eight animals investigated in this study were quite distinct from those of the prior study. However, it remains to be proven what proportion of infected cattle would reside in the "low shedder" category (based on either the old or new criteria), to be sure that a particular PFC dilution is applicable to the majority of infected shedder cattle for disease detection and control purposes.

From this data it is likely that the selection of a 1:5 dilution represents a very conservative recommendation for application of PFC in herds for disease risk assessment.

Table 9. Categorisation of cattle by *Map* excretion rate, based on two separate criteria, and divided among 8 low shedders from this study and 14 higher level shedders from prior study

	Old criteria based on solid media (per g of faeces)			**Suggested new criteria for Bactec cultures (per g of faeces)**		
	Low	**Medium**	**High**	**Low**	**Medium**	**High**
	$< 1.5 \times 10^4$	**1.5×10^4 to 1.5 to 10^5**	**$> 1.5 \times 10^5$**	**$< 1.5 \times 10^5$**	**1.5×10^5 to 1.5 to 10^6**	**$> 1.5 \times 10^6$**
8 "low shedders" based on regression	5 (6*)	3 (1*)	0 (1*)	8 (7*)	0 (1*)	0
14 "moderate and high shedders"	0	0	14	0	2	12

* result if based on MPN data; all other results refer to common regression equation

5 Success in Achieving Objectives

5.1 Discuss suitability of potential results with representative from SCAHLS

The suitability of potential results was discussed with representatives advising SCAHLS to determine any obstacles preventing SCAHLS approval and /or uptake of technology as a routine diagnostic assay for herd certification purposes. MLA was advised of these outcomes in a milestone report in 2005, prior to proceeding to the next stage.

There were no obstacles identified to prevent uptake of the technology as a routine diagnostic test. The chairman of SCAHLS (Dr Andrew Gregory) was contacted with regard to this project and project AHW.080, as these share common methodologies and approaches.

Research protocols regarding the two MLA projects relating to development of PFC for cattle and goats were forwarded by Dr Gregory to the JD writing group (excepting G. Eamens, as he was the author), the Johne's Disease Reference Laboratory and the New Test Development (NTD) Working Group. Comments were received from 3 of the 5 recipients.

[The NTD working group is chaired by Dr Deb Cousins, who is also a member of the JD writing group. (Western Australia currently has responsibility for the NTD WG as a SCAHLS activity, inherited from Barry Richards when Deb Cousins took over as Manager of the WADPI Animal Health laboratory, South Perth).]

The NTD working group response was as follows:

1. The purpose of the test must be clearly stated and the research should aim to determine the Se and Sp of the test for this purpose as is required for nucleic acid detection (NAD) and serology tests.

2. SCAHLS encourages researchers to maximise the numbers of positive and negative samples that are used for validation of a new test;

3. SCAHLS recognises that in certain situations, ideal numbers cannot be obtained, especially when a disease is rare or specimens are difficult to collect, and will take this into account when evaluating new tests;

4. SCAHLS cannot categorically state that certain numbers will be accepted as it is not privy to the full research design. SCAHLS is reluctant to recommend absolute numbers required for validation of any test as the numbers required will depend on the particular population of animals being tested, the extent of disease in that population, the Se of the test and the level of confidence required in a test;

5. SCAHLS relies on individual researchers applying good scientific principles to the research design and validation of new tests; the study should be of a quality that can be submitted for publication in a peer reviewed journal;

6. As SCAHLS does not have access to the research strategy it cannot determine whether the design will provide sufficient data on the value of various numbers of pools that can be reliably used for diagnosis (detection of M. paratuberculosis) of Johne's disease in the herd situation.

7. SCAHLS recognises that in the case of PFC for cattle and goats the culture method itself is not being evaluated, as the culture method that will be used (once samples are pooled) is a nationally agreed standard that has already been subjected to rigorous technology transfer and evaluation;

8. As long as the researcher can provide convincing evidence that the sample mixing can be reproduced using the methods stated, the test (culture) should not have to be validated in another laboratory.

9. SCAHLS understands the difficulty in finding goats infected with paratuberculosis.

10. SCAHLS considers collaboration with the national reference laboratory should provide additional samples to assist the research effort in cattle.

Comments were also solicited and received by the Principal Investigator from two members (Dr R. Whittington, Dr D. Cousins) of the JD Writing Group, whose individual comments were as follows:

Prof R. Whittington

Analytical sensitivity (sensitivity to pooling). I believe that what you propose with goats and cattle will suffice. For cattle, the additional existing data on consistency of shedding and distribution in the dung pat will be useful.

Analytical specificity. Unlike the situation in 1998 with sheep, there should be no questions about the analytical specificity of the method provided that an SOP that mitigates against sample-to-sample cross contamination is followed.

Diagnostic sensitivity. Can be inferred by modelling the proportion of low and high shedders in a herd.

Overall I think this should satisfy requirements.

Dr D. Cousins

My personal comments are consistent with that which was supplied from the New Test Development Working Group.

In addition, in terms of strategy, in my opinion the selection of dilutions you have quoted seem fair and reasonable, and you should aim to test as many samples as possible (It is very difficult to prescribe an actual number, you need to feel comfortable (and be able to argue the point) with the numbers). I agree with your comments on specificity, and pooling method for goat faeces. I believe you will have to describe and validate the methods you choose for pooling the bovine faeces.

I would still hold to the view that collaboration with the Ref Lab should be pursued as much as possible to increase the numbers of samples being assessed.

The Working Group has developed two new templates that will be used in validation assessment in line with the OIE approval for new tests. The SCAHLS templates (believe it or not) are more simple than that being used by OIE (and evaluation is currently free). We had in mind to develop a template for culture (esp for JD) but it has not been done yet. I attach the two templates we have developed

so far; The Nucleic Acid Detection one is final, the serology one still in draft) so you can see the sort of information you will need to provide for assessment.

The information that needs to be completed for assessment by the NTDWG as referred to by Dr Cousins are described in summary form below: (in this instance taken from requirements for a new serological test, as the requirements for a culture based test are not finalised):

- Intended purpose of assay
- Description and references
- Assay protocol
- Assay development information
- Analytical sensitivity and specificity
- Interpretation
- Precautions to avoid false negatives and positives
- Selection and sampling of reference population
- Diagnostic sensitivity and specificity
- Comparison with gold standard (i.e. Individual faecal culture)
- Technology transfer and reproducibility
- Monitoring assay performance, including validation criteria and additional testing in a target population
- Diagnostic implementation, incl reagents required and QA

5.2 Obtain adequate faeces from 10 low shedder cattle and set up for culture

5.2.1 Obtain adequate volumes of faeces from 10 confirmed low shedder cattle and sufficient large volume of negative faeces to enable dilutions from 1:5 to 1:50.

The budget allowed for the sourcing of an additional 5 low shedder samples (total 15 samples) if these were available. A total of 15 low shedder cattle were sourced from 9 NSW herds, and additional samples were requested from the JD reference laboratory at Attwood, Vic. However, the amount of faeces the reference laboratory was able to supply (from a total of 3 cases suspected of being low shedders) was insufficient for the proposed dilution studies.

While work was based on samples from 15 low shedders, only eight were found suitable. However, these are in addition to work already completed on 37 samples from high and medium shedders, and will enable a strong case to be put forward to meet future SCAHLS requirements.

5.2.2 Homogenise a range of dilutions and test by radiometric (Bactec) culture

As described in the Materials and Methods, all 15 samples were homogenised in a dilution range including neat (nil) dilutions, and dilutions in normal (culture negative) faeces of 1:5, 1:10, 1:20, 1:25, 1:30 and 1:50 as originally planned. All dilutions were cultured by radiometric culture using routine procedures as used in OJD PFC with the selection of a solid subculture medium suited to bovine strains.

5.3 Evaluate cultures up to 12 weeks of incubation

All cultures were incubated up to 12 weeks and tested weekly before discarding as negative. This was undertaken because it was anticipated the routine 8 week incubation period for individual faeces may be insensitive when concentrations of *Map* in inocula are reduced as a result of dilution with negative faeces.

5.4 Confirm growth and concentrations of *Map* by routine laboratory procedures

5.4.1 Confirm growth in Bactec media by routine procedures

Samples from Bactec growth were collected when a growth index of 999 was reached ("A" sample), and one week thereafter ("B" sample) in accord with routine diagnostic laboratory practice at EMAI. For samples that showed growth above GI 200 but did not reach a GI if 999, the A and B samples were collected in the two weeks following the maximal GI. The B samples were examined by routine IS*900* PCR and REA procedures, and repeated on the A sample if negative. The B samples were routinely subcultured to Herrold's egg yolk medium with mycobactin J and incubated for up to 10 weeks for confirmation of typical, mycobactin-dependent colonies.

5.4.2 Determine the concentration of *Map* in each original sample.

Using six of the samples, 10-fold dilution series of Bactec inoculum in a 5 tube endpoint titration (Most Probable Number, MPN) format were undertaken using Bactec media and confirmation of growth as described in 4.4.1 as the positive/negative outcome for each. From 4 samples yielding growth, an MPN for *Map* was estimated per gram of Bactec inoculum, and one tenth of this was equivalent to the number of *Map* cells in the Bactec inoculum. Data from one sample was also sufficient to determine a regression equation relating the rate of growth at three dilutions to the MPN of *Map* in inoculum, according to the dcgi1000 method previously described by Reddacliff *et al* (2003a).

The regression data from this sample was combined with data from two other samples from earlier studies to identify a regression equation for growth of bovine strain in Bactec broth (in days to reach cgi1000) relative to the inoculum.

This equation was then used to estimate, from growth in the pooled faecal culture series and the ten-fold dilution series, the number of organisms in each inoculum. Based on studies that showed a 50 fold reduction in *Map* (S strain) concentration due to decontamination procedures before Bactec inoculation, estimates of *Map* excretion rates (per g of faeces) were calculated for each low shedder animal.

6 Impact on Meat and Livestock Industry – now & in five years time

6.1 Impact on Meat and Livestock Industry now

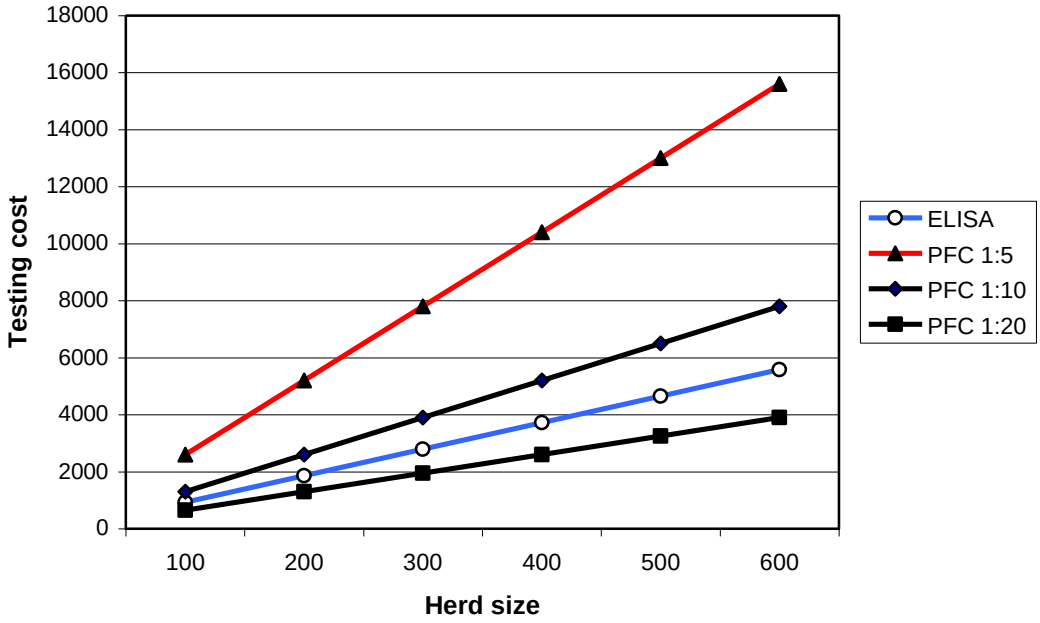
A recommendation to allow PFC to be used for herd testing at dilutions of 1:5 will have a low impact on the cattle industry, since this represents a saving of only 35% in the cost of whole herd testing. Current herd status is defined by ELISA testing at a cost for a 300 cow herd of $8.30 (incl GST) per head = $2490 with an estimated sensitivity of 25% and a specificity of approx 99%. This total would be increased, as follow up of seroreactors would be required for overall test specificity, and to satisfy

regulators of disease control. At a rate of 1% reactors, this would equate to an additional three individual faecal cultures at a cost of approx. $300, totalling approx. $ 2800. The cost of PFC (on a 1:5 pooling regime) would equate to 60 pools @ $130 = $7800, with an estimated sensitivity of 45%, a specificity of 100% but a delay in results of 11-12 weeks.

The improved sensitivity of whole herd Bactec culture by PFC compared to serology would be of benefit in infected herds. In addition, costs could be reduced by targeted surveillance of at risk stock in herds of uncertain status.

The comparative cost of whole herd ELISA testing compared with PFC testing at 1:5, 1:10 and 1:20 based on current charges in NSW is shown in Figure 5. From this, it is clear that dilutions of 1:10 and 1:20 become much more cost-effective than 1:5.

Figure 5. Relative laboratory testing costs for cattle herds, assuming follow up culture on 1% of cattle tested by ELISA



6.2 Impact on Meat and Livestock Industry in five years time

The potential improved sensitivity of a culture-based assay in cattle herds is unlikely to gain wide adoption in the cattle industry while testing costs are high. A cost-effective whole herd assay that provides detection of low shedder cattle at the 1:10 dilution could be developed using increased inocula. The current method utilised only 3.5 mL of base inocula from a 10 mL decontamination liquid to concentrate *Map* cells in 1 mL, of which 0.1 mL was inoculated to the liquid culture medium. It is feasible that a 5 mL base inoculum, coupled with a larger Bactec inoculum (0.2 mL) may overcome the limitations of low shedding rate of “low shedder cattle” by increasing the base inoculum by a factor of 2.86, and therefore enable the 10 fold dilution to detect *Map* in the same animals.

Other methods that are less damaging to the viability of *Map* cells may also be able to further increase the base inoculum. Over the next five years, it is recommended that an effort be made to determine how PFC at higher dilutions can be achieved for reliable detection of infection in low shedder animals. In addition, more information on the prevalence of the different ranges of *Map* shedding among infected cattle would assist greater adoption of PFC.

In 5 years, it is anticipated that the current culture platform of Bactec culture will become more obsolete, and alternative procedures will need to be validated. It is critical that such procedures be examined for their ability to confirm low shedder cattle among dilutions of 1:10 or higher.

7 Conclusions and Recommendations

7.1 Dilution and incubation time for PFC for low shedder cattle

This study has demonstrated that the limiting factor affecting the success of Bactec culture is in the number of viable cells surviving to the Bactec inoculum. While the Bactec liquid culture medium appears quite sensitive in culturing very low levels of viable *Map* cells, the limiting factors appear to be low starting number of organisms in faeces, and the losses attributed to processing.

Data from the USA based on solid media counts, has suggested that 70% of infected cattle are categoried as shedding < 1.5×10^4 *Map* cells/g of faeces (van Shaik *et al* 2003). However, there is a high likelihood that solid media may underestimate the number of viable cells of *Map*, and that such animals if tested by Bactec culture may yield a 10 fold higher count (viz 1.5×10^5/g). This study has estimated that, based on growth in Bactec media, cattle shedding < 10^4 Map cells/g of faeces are not reliably detected at dilutions in faeces > 1:5 with the current procedure as routinely applied to sheep. For optimal detection, a dilution of 1:5 and an incubation period of at least 10 weeks is recommended, when coupled with confirmation of Bactec growth by IS*900* PCR and REA.

Based on the likelihood that the number of viable cells had deteriorated in some samples due to storage for up to 18 months, this represents a conservative recommendation.

The cost-effectiveness of testing at this dilution is unlikely to enable cattle industries to replace testing based on ELISA serology with PFC, where a detection limit of 10^4 organism/g of faeces is required. However, if additional information can be ascertained that a significant proportion of infected cattle are shedding at higher levels, and/or if procedures can be modified to overcome the limitation of the base number of cells that survive to the Bactec inoculum, then more cost-effective dilutions (e.g. 1:10) could be demonstrated to be appropriate.

7.2 Recommendations for future studies in this area

The following issues need to be addressed in future studies:

7.2.1 Modifications that can be undertaken with existing methodology to increase the *Map* inoculum from faeces, without adverse effects on detection rates

It is believed that the currently applied method is likely to reduce the inoculum from each gram of bovine faeces by a factor of 50X due to processing and a further 10-14.3 fold by selection of subaliquots from HPC-decontaminated faecal material for further processing into Bactec media. In the current study, it is estimated a shedding rate of 10^4/g could result in an inoculum of fewer than 15 viable *Map* cells. There is potential to examine the loss of viability during processing using materials other than HPC and VAN, of concentration steps in the procedures, and the eventual effect on contamination rates in herd testing. For example, a modification that doubles the *Map* inoculum to Bactec without increased adverse effects such as contamination would have a considerable impact on cost-efficiency.

7.2.2 Define the cut-off point for Bactec detection (in number of *Map* per gram of faeces) of the majority of low shedders, which make up a high proportion of infected animals

As described in 4.6, data is known concerning the distribution of low, medium and high shedders in a herd based on arbitrary cut-points relevant to solid medium (conventional) culture on Herrold's egg yolk medium. Information should be sought to confirm criteria that define low, medium and high level shedders, and in particular the proportion of different shedding rates of infected cattle in known infected herds, based on quantification by Bactec procedures similar to those used in this study.

In doing so, if we can define the level of *Map* that (for example) 70% of infected cattle are excreting using Bactec methodology, then we could determine the likely success rate of higher dilutions than the 1:5 currently proposed to detect infected shedder cattle in an infected herd.

7.2.3 Determine the proportion of infected cattle detectable by PFC at 1:10 and 1:20 dilutions, with and without test modification

Since only dilutions higher than 1:5 are likely to be cost effective, if it is confirmed that the criteria to define 70% of the infected population as low shedders is equivalent to the criteria as suggested in this study, then it is clear that improved methods to detect lower level shedders are needed. If however the majority of infected cattle are shedding levels of > 10^4/g then a higher proportion would be detectable at a 1:10 dilution. Based on such information, we would then have greater assurance of the likely impact on disease control of these higher dilutions (1:10 and 1:20) for PFC.

7.2.4 Determine similar levels for other detection systems, based on culture or other methods, including direct PCR

Since Bactec culture systems are likely to become more difficult to maintain with ageing equipment and paucity of replacement parts, the success of alternate culture based platforms when applied to known concentrations of *Map* in pooled faeces need to be considered. Ongoing work to improve direct PCR may reduce the cost of testing but only if this is competitive in cost and sensitivity with culture-based systems.

8 Acknowledgements

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10 Appendices

Appendices 1 and 2 show data for all samples in Bactec media at varying dilutions in faeces (PFC dilutions) and for six samples in 10-fold dilutions in PBSTw (for MPN estimations and regressions) respectively.

10.1 Appendix 1

Growth of samples in Bactec media at varying dilutions in faeces (PFC dilutions)

ID	DILN	WK 1	WK 2	WK 3	WK 4	WK 5	WK 6	WK 7	WK 8	WK 9	WK 10	WK 11	WK 12	ETOH 1	PCR +/-	ETOH 2	PCR +/-	REPEAT	SUB
		25.7.2005	1.8.2005	8.8.2005	15.8.2005	22.8.2005	29.8.2005	5.9.2005	14.9.2005	19.9.2005	26.9.2005	3.10.2005	10.10.2005	DATE	REA	DATE	REA	PCR/REA	CULTURE
1	1	9	5	3	3	2	1	0	0	3	0	0	0						
	1 in 5	8	5	4	2	3	2	0	0	3	999	999	999	25/10/2005	N	11/11/2005	N		C
MN04/1164	1 in 10	6	4	3	3	3	4	52	363	258	217	204	218	25/10/2005	N	11/11/2005	N		C
CM04/0101	1 in 20	7	5	2	1	2	3	0	69	393	895	894	870	25/10/2005	N	11/11/2005	N		C
	1 in 25	6	4	2	3	3	2	0	5	46	157	121	163						
Animal # 3	1 in 30	6	4	4	2	308	999	999	999	999	999	999	880	25/10/2005	N	11/11/2005	N		C
	1 in 50	9	4	2	3	3	5	0	0	16	145	632	532	25/10/2005	N	11/11/2005	N		C
2	1	3	4	0	2	10	179	999	999	999	999	999	937	25/10/2005	4+ / MP				P
	1 in 5	7	4	2	5	71	699	999	999	999	999	999	814	25/10/2005	4+ / MP				P
MN04/2086	1 in 10	5	5	3	4	9	111	726	999	999	999	999	999	25/10/2005	1+ / MP				P
CM04/0190	1 in 20	9	4	0	4	15	303	999	999	999	870		312	25/10/2005	2+ / MP				P
	1 in 25	9	5	0	3	18	317	999	999	999	999	999	999	25/10/2005	N	11/11/2005	1+ / MP		P
	1 in 30	5	4	0	2	7	73	777	999	999	999	999	854	25/10/2005	1+ / MP	25/10/2005	3+ / MP		P
	1 in 50	7	5	0	3	5	99	600	460	563	888		847	25/10/2005	1+ / MP				P
3	1	7	0	0	5	5	22	164	967	945	999	999	999	25/10/2005	3+ / MP				P
	1 in 5	8	0	0	3	1	0	18	464	581	999	999	999	25/10/2005	N	11/11/2005	2+ / MP		C
MN04/1165	1 in 10	6	0	0	2	3	0	0	28	87	542	617	627	25/10/2005	N	11/11/2005	N		P
CM04/0102	1 in 20	7	0	0	2	0	0	0	42	235	591	720	999	25/10/2005	N	11/11/2005	N		C
	1 in 25	1	0	0	2	2	0	0	427	429	529	791	287	25/10/2005	N	11/11/2005	N		P
Animal # 6	1 in 30	1	0	0	3	3	0	0	0	0	66	999	999	25/10/2005	N	11/11/2005	N		C
	1 in 50	1	0	0	1	2	0	0	35	132	282	999	191	25/10/2005	1+ / MP				C
4	1	1	0	0	1	2	0	0	0	0	0	0	0						
	1 in 5	1	0	0	3	2	0	4	568	999	853		385	25/10/2005	N	11/11/2005	N		C
MN04/1166	1 in 10	2	0	0	3	1	0	28	423	880	999	999	999	25/10/2005	N	11/11/2005	N		C
CM04/0103	1 in 20	0	0	0	2	2	0	0	0	1	74		999	25/10/2005	N	11/11/2005	N		C
	1 in 25	2	0	0	1	2	0	0	56	162	342		287	25/10/2005	N	11/11/2005	N		C
Animal # 8	1 in 30	0	0	0	0	1	0	0	57	219	639		999	25/10/2005	N	11/11/2005	N		C
	1 in 50	2	0	0	0	1	0	0	0	202	390		806	25/10/2005	N	11/11/2005	N		C
5	1	1	0	0	0	51	673	999	999	999	999	999	975	25/10/2005	4+ / MP				P
	1 in 5	2	0	0	0	8	181	684	665	420	257	147	146	24/11/2005	TR / MP	24/11/2005	1+ / MP		P
MN04/2581	1 in 10	1	0	0	0	2	0	0	466	723	999	999	999	25/10/2005	N	11/11/2005	N		C
CM04/0245	1 in 20	1	0	0	0	3	0	0	1	185	732	999	879	25/10/2005	N	11/11/2005	N		C
	1 in 25	1	0	0	0	1	0	0	493	589	725	975	999	25/10/2005	N	11/11/2005	N		C
Animal # 2	1 in 30	0	0	0	0	2	0	0	0	32	755	999	999	25/10/2005	N	11/11/2005	N		C
	1 in 50	1	0	0	0	0	0	0	195	418	742	999	999	25/10/2005	N	11/11/2005	N		C

ID	DILN	WK 1	WK 2	WK 3	WK 4	WK 5	WK 6	WK 7	WK 8	WK 9	WK 10	WK 11	WK 12	ETOH 1	PCR +/-	ETOH 2	PCR +/-	REPEAT	SUB
		25.7.2005	1.8.2005	8.8.2005	15.8.2005	22.8.2005	29.8.2005	5.9.2005	14.9.2005	19.9.2005	26.9.2005	3.10.2005	10.10.2005	DATE	REA	DATE	REA	PCR/REA	CULTURE
6	1	1	0	0	0	0	0	14	527	683	906	921	867	25/10/2005	1+ / MP				P
	1 in 5	1	1	0	0	0	0	0	0	0	0	98	516	25/10/2005	N	11/11/2005	N		C
MN04/4386	1 in 10	1	0	0	0	0	0	0	311	579	999	999	999	25/10/2005	N	11/11/2005	N		C
CM04/0433	1 in 20	1	0	0	0	0	0	0	41	165	999	999	999	25/10/2005	N	11/11/2005	N		C
	1 in 25	0	0	0	0	0	0	0	0	0	656	999	999	25/10/2005	N	11/11/2005	N		C
Animal # 2	1 in 30	1	0	0	0	0	0	0	0	0	0	169	622	25/10/2005	N	11/11/2005	N		C
	1 in 50	1	0	0	0	0	0	0	0	24	205	302	260	25/10/2005	N	11/11/2005	N		C
7	1	1	0	0	0	0	0	0	0	0	0	0	0						
	1 in 5	1	0	0	0	0	0	0	0	0	0	0	0						
MN04/4828	1 in 10	0	0	0	0	0	0	0	0	0	0	0	0						
CM04/0471	1 in 20	1	0	0	0	0	0	0	0	0	0	0	28						
	1 in 25	1	0	0	0	0	0	0	0	4	115	537	459	25/10/2005	N	11/11/2005	N		C
Animal # 5	1 in 30	0	0	0	0	0	0	0	0	0	29	297	596	31/10/2005	N	11/11/2005	N		C
	1 in 50	1	0	0	0	0	7	339	999	999	752	421	268	25/10/2005	TR	11/11/2005	TR / MP		P
8	1	1	0	0	0	0	0	0	0	0	0	0	0						
	1 in 5	2	0	0	0	0	0	0	0	0	0	0	0						
MN04/3339	1 in 10	0	0	0	0	0	0	0	56	158	999	999	999	25/10/2005	N	11/11/2005	N		C
CM04/0317	1 in 20	0	0	0	0	0	0	0	0	41	285	892	999	11/11/2005	N	11/11/2005	N		C
	1 in 25	1	0	0	0	0	0	0	0	0	209	999	999	25/10/2005	N	11/11/2005	N		N
Animal # 1	1 in 30	1	0	0	0	0	0	0	4	273	610	999	999	31/10/2005	N	11/11/2005	N		C
	1 in 50	2	0	0	0	0	0	0	0	0	0		154						
9	1	2	0	0	0	0	0	10	516	744	997	999	999	31/10/2005	4+ / MP				P
	1 in 5	1	0	0	0	0	0	4	732	859	629		960	25/10/2005	TR	11/11/2005	2+ / MP		P
MN04/3339	1 in 10	1	0	0	0	0	0	0	415	944	999	999	999	25/10/2005	N	11/11/2005	N		N
CM04/0317	1 in 20	1	0	0	0	0	2	52	183	176	241	250	237	31/10/2005	N	11/11/2005	N		C
	1 in 25	0	0	0	0	0	0	35	916	999	999	999	999	25/10/2005	N	11/11/2005	1+ / MP		C
Animal # 11	1 in 30	0	0	0	0	0	0	1	45	258	537	849	779	31/10/2005	N	11/11/2005	N		C
	1 in 50	0	0	0	0	0	0	0	0	0	9	94	174						
10	1	2	0	0	0	0	0	0	1	0	3	0	0						
	1 in 5	1	0	0	0	0	0	0	2	0	0	0	142						
MN04/3339	1 in 10	1	0	0	0	0	0	0	2	48	675	999	999	31/10/2005	N	11/11/2005	N		C
CM04/0317	1 in 20	0	0	0	0	0	1	0	120	324	525	803	842	31/10/2005	N	11/11/2005	N		C
	1 in 25	1	0	0	0	0	0	0	99	627	999	998	955	25/10/2005	N	11/11/2005	N		C
Animal # 12	1 in 30	1	0	0	0	0	0	0	52	152	454	912	999	31/10/2005	N	11/11/2005	N		C
	1 in 50	2	0	1	0	0	0	0	122	322	616	773	743	31/10/2005	N	11/11/2005	N		C

ID	DILN	WK 1	WK 2	WK 3	WK 4	WK 5	WK 6	WK 7	WK 8	WK 9	WK 10	WK 11	WK 12	ETOH 1	PCR +/-	ETOH 2	PCR +/-	REPEAT	SUB
		25.7.2005	1.8.2005	8.8.2005	15.8.2005	22.8.2005	29.8.2005	5.9.2005	14.9.2005	19.9.2005	26.9.2005	3.10.2005	10.10.2005	DATE	REA	DATE	REA	PCR/REA	CULTURE
11	1	1	2	0	0	0	0	0	2	0	0	0	0						
	1 in 5	2	1	0	0	0	3	59	999	999	999	906	808	25/10/2005	TR	11/11/2005	N	TR / MP	P
MN04/8073	1 in 10	0	0	0	0	0	0	0	5	0	3	0	0						
CM04/0714	1 in 20	2	1	0	0	0	0	0	2	0	2	0	0						
	1 in 25	7	2	0	0	0	0	0	0	7	89	478	936	31/10/2005	N	11/11/2005	N		C
Animal # 13	1 in 30	1	1	0	0	0	0	0	2	0	3	0	43						
	1 in 50	3	0	1	0	0	0	0	2	10	194	304	556	31/10/2005	N	11/11/2005	N		C
12	1	6	0	0	0	0	0	0	1	0	2	0	0						
	1 in 5	1	1	0	0	0	0	0	279	295	565	999	875	31/10/2005	N	11/11/2005	N		C
MN04/8073	1 in 10	2	0	0	0	0	0	0	21	55	363	656	661	31/10/2005	N	11/11/2005	N		C
CM04/0714	1 in 20	2	0	0	0	0	0	0	2	0	0	0	46						
	1 in 25	1	0	0	0	0	0	0	1	0	2	212	324	31/10/2005	N	11/11/2005	N		C
Animal # 94	1 in 30	2	0	0	0	0	0	0	2	0	3	350	828	31/10/2005	N	11/11/2005	N		C
	1 in 50	2	0	0	0	0	0	0	1	0	0	0	0						
13	1	2	0	0	0	0	0	0	0	0	2	0	0						
	1 in 5	2	0	0	0	0	0	0	0	0	1	0	0						
MN04/6981	1 in 10	2	0	0	1	0	0	0	0	54	206	670	681	31/10/2005	N	11/11/2005	N		C
CM04/0631	1 in 20	5	0	0	0	0	0	0	0	1	31	263	251	31/10/2005	N	11/11/2005	N		C
	1 in 25	6	0	0	0	0	0	0	0	15	570	778	655	31/10/2005	N	11/11/2005	N		C
Animal # 2	1 in 30	4	0	0	0	0	0	0	0	0	4	63	639	31/10/2005	N	11/11/2005	N		C
	1 in 50	1	0	1	0	0	0	0	0	0	1	18	379	31/10/2005	N	11/11/2005	N		C
14	1	2	0	0	0	0	0	0	0	0	0	0	0						
	1 in 5	0	0	0	1	0	0	0	0	36	197	999	999	31/10/2005	N	11/11/2005	N		C
MN04/9457	1 in 10	2	0	0	0	0	0	0	38	121	379	844	884	31/10/2005	N	11/11/2005	N		C
CM04/0857	1 in 20	5	3	1	0	0	0	0	0	15	69	507	645	31/10/2005	N	11/11/2005	N		C
	1 in 25	2	0	0	0	0	0	6	51	92	217	170	185	31/10/2005	N	11/11/2005	N		C
Animal # 7	1 in 30	5	2	1	0	0	0	0	32	58	373	698	999	31/10/2005	N	11/11/2005	N		C
	1 in 50	1	1	0	0	0	2	52	367	312	272	228	199	31/10/2005	N	11/11/2005	N		C
15	1	1	0	1	4	39	347	999	999	999	999	999	888	25/10/2005	1+ / MP				P
	1 in 5	4	3	0	2	12	122	847	999	999	999	999	979	25/10/2005	3+ / MP				P
MN04/9805	1 in 10	5	0	0	23	427	999	999	999	766	508	284	173	25/10/2005	4+ / MP				C
CM04/0883	1 in 20	3	2	0	62	766	999	999	999	999	999	974	609	25/10/2005	N	11/11/2005	N		P
	1 in 25	0	0	0	31	241	363	999	999	999	999	999	861	25/10/2005	3+ / MP				C
	1 in 30	0	0	2	33	449	999	999	999	999	785	346	158	25/10/2005	4+ / MP				P
	1 in 50	1	2	0	5	171	960	999	999	883	422	273	150	25/10/2005	4+ / MP				P

10.2 Appendix 2

Growth of samples in Bactec media in 10-fold dilutions in PBSTw (for MPN estimations and regressions)

ID	DILN	WK 1	WK 2	WK 3	WK 4			WK 5			WK 6		WK 7			WK 8			WK 9			WK 10			WK 11			WK 12		DAY @ 999	ETOH 1	PCR +/-	ETOH 2	PCR +/-	REPEAT	SUB	CONV
		8.8.2005	15.8.2005	22.8.2005	29.8.2005	31.8.05	2.9.05	5.9.2005	7.9.05	9.9.05	14.9.2005	16.9.05	19.9.2005	21.9.2005	23.9.2005	26.9.2005	28.9.2005	30.9.05	4.10.2005	5.10.05	7.10.05	10.10.2005	12.10.05	14.10.05	17.10.2005	19.10.05	21.10.05	24.10.2005	26.10.05		DATE	REA	DATE	REA	PCR / REA	CULTURE	CULTURE
2	N	0	0	0	0			0			296	368	572	528		899			999			999			999			905			31/10/2005	2+ / MP				P	
	N	0	0	0	0			0			27	78	264	302	310	464			999			999			999			923			31/10/2005	3+ / MP				P	
MN04/2086	N	0	0	0	0			0			18	45	237	298	328	486			999			999			999			962			31/10/2005	4+ / MP				P	
CM04/0190	N	0	0	0	0			0			14	41	248	354	422	622			999			999			999			999			31/10/2005	TR	11/11/2005	N	3+ / MP	P	
	N	0	0	0	0			0			291	398	616			999			999			999			999			859			25/10/2005	TR	11/11/2005	N	2+ / MP	P	
	10-1	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-1	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-1	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-1	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-1	0	0	0	0			0			0		0			0			0			0			0			0									
	10-2	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-2	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-2	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-2	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-2	0	0	0	0			0			0		0			0			0			0			0			0									
	10-3	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-3	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-3	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-3	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-3	0	0	0	0			0			0		0			0			0			0			0			0									
	10-4	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-4	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-4	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-4	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-4	0	0	0	0			0			0		0			0			0			0			0			0									
6	N	0	0	0	0			0			0		73	136	226	374	327		628			629			788			704			31/10/2005	2+ / MP				P	
	N	0	0	0	0			0			0		0			0			0			0			0			0									
MN04/4386	N	0	0	0	0			0			0		0			0			0			0			0			0									
CM04/0433	N	0	0	0	0			0			0		0			0			0			0			0			0									
	N	0	0	0	0			0			0		0			0			0			0			0			0									
Animal # 2	10-1	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-1	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-1	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-1	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-1	0	0	0	0			0			0		0			0			0			0			0			0									
	10-2	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-2	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-2	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-2	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-2	0	0	0	0			0			0		0			0			0			0			0			0									
	10-3	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-3	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-3	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-3	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-3	0	0	0	0			0			0		0			0			0			0			0			0									
	10-4	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-4	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-4	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-4	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-4	0	0	0	0			0			0		0			0			0			0			0			0									

P.PSH.0184 - Validation of PFC for BJD with low level shedder cattle

ID	DILN	WK 1	WK 2	WK 3	WK 4			WK 5			WK 6		WK 7			WK 8			WK 9			WK 10			WK 11			WK 12		DAY @ 999	ETOH 1	PCR +/-	ETOH 2	PCR +/-	REPEAT	SUB	CONV
		8.8.2005	15.8.2005	22.8.2005	29.8.2005	31.8.05	2.9.05	5.9.2005	7.9.05	9.9.05	14.9.2005	16.9.05	19.9.2005	21.9.2005	23.9.2005	26.9.2005	28.9.2005	30.9.05	4.10.2005	5.10.05	7.10.05	10.10.2005	12.10.05	14.10.05	17.10.2005	19.10.05	21.10.05	24.10.2005	26.10.05		DATE	REA	DATE	REA	PCR / REA	CULTURE	CULTURE
11	N	0	0	0	0			0			0		10	28	104	327	323	330	577			799			940			832			31/10/2005	1+ / MP				P	
	N	0	0	0	0			0			0		0			0			0			0			0			0									
MN04/8073	N	0	0	0	0			0			0		19	52	167	412	385		765			933			999			907			31/10/2005	TR	11/11/2005	4+ / MP		P	
CM04/0714	N	0	0	0	0			0			0		0			0			0			0			0			0									
	N	0	0	0	0			0			0		0			0			0			0			0			0									
Animal # 13	10-1	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-1	0	0	0	0			0			0		0			0			0			21	77	70	127	124	197	295	250		31/10/2005	N	11/11/2005	N		N	N
	10-1	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-1	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-1	0	0	0	0			0			0		10	20	85	355	356	383	625			999			999			999			31/10/2005	TR	11/11/2005	N	1+ / MP	P	
	10-2	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-2	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-2	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-2	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-2	0	0	0	0			0			0		0			0			0			0			0			0									
	10-3	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-3	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-3	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-3	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-3	0	0	0	0			0			0		0			0			0			0			0			0									
	10-4	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-4	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-4	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-4	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-4	0	0	0	0			0			0		0			0			0			0			0			0									
12	N	0	0	0	0			0			0		0			0			0			0			0			0									
	N	0	0	0	0			0			0		0			0			0			0			0			0									
MN04/8073	N	0	0	0	0			0			0		0			0			0			0			0			0									
CM04/0714	N	0	0	0	0			0			0		0			0			0			0			0			0									
	N	0	0	999	999			936			549		234			227			271			243			241			202			25/10/2005	N	11/11/2005	N		C	
Animal # 94	10-1	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-1	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-1	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-1	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-1	0	0	0	0			0			0		0			0			0			0			0			0									
	10-2	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-2	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-2	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-2	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-2	0	0	0	0			0			0		0			0			0			0			0			0									
	10-3	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-3	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-3	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-3	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-3	0	0	0	0			0			0		0			0			0			0			0			0									
	10-4	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-4	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-4	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-4	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-4	0	0	0	0			0			0		0			0			0			0			0			0									

ID	DILN	WK 1	WK 2	WK 3	WK 4			WK 5			WK 6		WK 7			WK 8			WK 9			WK 10			WK 11			WK 12		DAY @ 999	ETOH 1	PCR +/-	ETOH 2	PCR +/-	REPEAT	SUB	CONV
		8.8.2005	15.8.2005	22.8.2005	29.8.2005	31.8.05	2.9.05	5.9.2005	7.9.05	9.9.05	14.9.2005	16.9.05	19.9.2005	21.9.2005	23.9.2005	26.9.2005	28.9.2005	30.9.05	4.10.2005	5.10.05	7.10.05	10.10.2005	12.10.05	14.10.05	17.10.2005	19.10.05	21.10.05	24.10.2005	26.10.05		DATE	REA	DATE	REA	PCR / REA	CULTURE	CULTURE
13	N	0	0	0	0			0			0		0			0			0			0			0			0									
	N	0	0	0	0			0			0		0			0			0			0			0			0									
MN04/6981	N	0	0	0	0			0			0		0			0			0			0			0			0									
CM04/0631	N	0	0	0	0			0			0		0			0			0			0			0			0									
	N	0	0	0	0			0			0		0			0			0			0			0			0									
Animal # 2	10-1	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-1	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-1	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-1	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-1	0	0	0	0			0			0		0			0			0			0			0			0									
	10-2	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-2	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-2	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-2	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-2	0	0	0	0			0			0		0			0			0			0			0			0									
	10-3	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-3	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-3	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-3	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-3	0	0	0	0			0			0		0			0			0			0			0			0									
	10-4	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-4	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-4	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-4	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-4	0	0	0	0			0			0		0			0			0			0			0			0									
15	N	0	0	0	31	64	144	509	609		999		999			999			999			993			816			675			25/10/2005	4+ / MP				P	
	N	0	0	0	26	51	116	378	440	453	785		876			999			999			996			829			719			25/10/2005	TR	11/11/2005	1+ / MP		P	
MN04/9805	N	0	0	0	32	60	133	460	546		999		999			999			999			977			863			703			25/10/2005	4+ / MP				P	
CM04/0883	N	0	0	0	55	107	216	575	614		999		999			999			999			915			813			665			25/10/2005	4+ / MP				P	
	N	0	0	0	59	110	216	609	659		999		999			999			999			948			808			637			25/10/2005	4+ / MP				P	
	10-1	0	0	0	0			26	38	89	524	23??	749			999			999			999			999			911			25/10/2005	1+ / MP				P	28
	10-1	0	0	0	0			0			140	311	713			999			999			999			999			547			25/10/2005	2+ / MP				P	56
	10-1	0	0	0	0			0			163	333	748			999			999			999			999			895			25/10/2005	3+ / MP				P	32
	10-1	0	0	0	0			0			134	299	687	764		999			999			999			999			950			25/10/2005	TR	11/11/2005	1+ / MP		P	>50
	10-1	0	0	0	0			24	45	107	572	551	665			999			999			999			999			922			25/10/2005	1+ / MP				P	
	10-2	0	0	0	0			0			0		40	82	165	362	339	349	561			862			999			999			31/10/2005	3+ / MP				P	N
	10-2	0	0	0	0			0			0		104	168	229	391	358		729			891			999			999			31/10/2005	1+ / MP				P	N
	10-2	0	0	0	0			0			19	55	229	296	332	556			999			999			999			999			31/10/2005	N	11/11/2005	1+ / MP		P	4
	10-2	0	0	0	0			0			22	63	235	305	353	569			999			999			999			999			31/10/2005	3+ / MP				P	N
	10-2	0	0	0	0			0			37	90	304	374	414	612			999			999			999			999			31/10/2005	N	11/11/2005	1+ / MP		P	
	10-3	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-3	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-3	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-3	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-3	0	0	0	0			0			0		10	18	46	211	250	309	568			847			999			999			31/10/2005	3+ / MP				P	
	10-4	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-4	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-4	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-4	0	0	0	0			0			0		0			0			0			0			0			0									N
	10-4	0	0	0	0			0			0		0			0			0			0			0			0									