finalreport

Project code: **AHW.080**
B.AHW.0080
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Date published: **March 2006**
ISBN: **9781741912432**

PUBLISHED BY
Meat & Livestock Australia
Locked Bag 991
NORTH SYDNEY NSW 2059

Validation of pooled faecal culture for Caprine Johne's Disease

Meat & Livestock Australia acknowledges the matching funds provided by the Australian Government to support the research and development detailed in this publication.

NSW DEPARTMENT OF PRIMARY INDUSTRIES

Abstract

Goat populations infected with *M. avium* subsp. *paratuberculosis* (*Map*) contain individuals shedding a range of *Map* concentrations in their faeces, from clinical cases shedding high levels, to some subclinical or carrier animals 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 goats. Fourteen samples (stored for up to 4 years at -80^{o}C) of 21 selected on the basis of a prior positive culture result were found to yield *Map* on subsequent culture, including evaluation studies when samples were mixed with normal goat faeces at pooling rates from 1:5 to 1:50. A further two samples contained very low numbers of organisms ($< 2 \times 10^3$/g) and were only culture positive from undiluted faeces on re-culture. Three quarters of the 16 culture positive goats were considered to be shedding low to moderate levels of *Map*, estimated at less than 10^6/g of faeces. At pooling rates of up to 1:25, PFC was able to detect 13 of the 16 culture positive goats. An incubation period of at least 10 weeks at the 1:25 rate was needed to detect all 13 goats shedding $\geq 4 \times 10^4$ *Map* organisms/g of faeces, representing an estimated inoculum per vial of fewer than 6 organisms. This data supports a pooling rate of 1:25 for application of PFC as a diagnostic tool in caprine JD 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 goats. 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 at a 1:50 pooling dilution, but this technology has not been validated in goats. This study evaluated PFC at dilutions ranging from 1:5 to 1:50 in goats shedding a wide range of concentrations of *Map*. 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. Faeces from 17 goats naturally infected with the cattle (C) strain and four goats naturally infected with the sheep (S) strain of *Map* were evaluated.

Of 21 faeces from goats previously confirmed culture positive for *Map*, and stored at -80^{o}C for up to 4 years, 14 were found to yield *Map* on subsequent culture, including evaluation studies when samples were mixed with normal goat faeces at pooling rates of 1:5, 1:10, 1:20, 1:25. 1:30 and 1:50. An additional two samples yielded *Map* only on undiluted samples, and both were only intermittently positive in undiluted faeces. All samples were processed using procedures similar to those employed for OJD PFC, including a 12 week incubation period. Depending on the infecting strain, subcultures were made on Herrold's egg yolk medium (for C strain) or modified 7H10 media (for S strain), as the former is more suitable for growth of cattle strains of *Map,* and S strains only grow on modified 7H10 medium and not Herrold's.

Since the growth of S strains of *Map* from sheep faeces (Reddacliff *et al* 2003a) may differ from that of C or S strains from goat faeces, regression equations were developed to define the relationship between the number of *Map* cells in the caprine faecal culture inocula and the number of days to reach a cumulative growth index of 1000 (cgi1000). To quantify the shedding rate, endpoint titrations (Most Probable Number or MPN method) were undertaken from 10 samples from the original 21, selected at random. Their processed culture inocula were subjected to a 10-fold dilution series in Bactec broth (replicated 5-fold per dilution) to determine the relationship for goats. Of these 10, *Map* growth occurred in 9 animals, but only 5 (samples 2, 5, 8, 9, 21) yielded sufficient and repeatable growth at multiple dilutions to determine a reliable regression equation between the $\log_{10}$ inoculum (as determined by the MPN method) and the number of days to reach cgi1000 (dcgi1000). This data produced a final regression equation to describe the relationship between the rate of Bactec growth and the number of *Map* in the caprine faecal inoculum as follows:

$$\log_{10} \text{inoculum} = 7.3 - 0.121 \text{ dcgi1000}$$

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 16 culture positive goats generally contained between 10^2 and 7 x 10^6 viable *Map* cells per gram of faeces, and those that were culture positive when diluted with normal faeces were estimated to have contained > 1.5 x 10^3 *Map*/g.

At pooling rates up to 1:25, PFC was able to detect 13 of the 14 goat samples from goats shedding > 1.5 x 10^3 *Map*/g of faeces. In addition, an incubation period of 10 weeks was necessary to maximise detection of goats at a dilution rate of 1:25.

These results indicate that, for optimal results from pooling of caprine faeces, a dilution of 1:25 is recommended to detect goats shedding low to moderate levels of *Map.* At a 1:50 dilution, only animals shedding 10^5 *Map*/g or higher would be detected. Based on current laboratory fees, the laboratory costs for whole herd testing of goat herds for Johne's disease can be reduced by approximately 40% relative to serology and 75-90% relative to individual faecal culture.

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1 Background

1.1 Diagnostic testing for caprine JD in Australia

Following the first reports of caprine Johne's disease in Australia in Victoria in 1977 (Lenghaus *et al* 1977; Russell and Milner 1978), the disease was confirmed in NSW in 1978 and found to predominate among dairy rather than fibre breeds (Eamens 1989). In September 2004, 26 infected goat herds were known to be present in Australia; 10 in NSW, 8 in Victoria, 6 in Tasmania and one in South Australia (Animal Health Australia 2005). Goat producers are seeking more cost-effective herd based tests for Johne's disease to improve certification and control measures. In sheep, 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.

Recent work at this laboratory (Reddacliff *et al* 2003a) has also shown that the rate of shedding of *M. avium* subsp. *paratuberculosis* (*Map*) in sheep is proportional to the rate of growth of *Map* in Bactec cultures. This approach was adopted to determine the shedding rate of goats in the present study.

Goats with confirmed Johne's disease in Australia are predominantly infected with the cattle (C) strain of *Map*, but infection with the sheep (S) strain has been reported in goats reared or held on infected sheep properties at high stocking rates and with long term exposure to infected sheep flocks (Whittington and Taragel 2000). Recent work has suggested that goats are more severely affected by C strain than S strain infections, since *in vivo* and cultured cattle strains were much more pathogenic for Angora goats than sheep strains, and more likely to result in persistent faecal shedding, seroconversion and clinical disease (Stewart *et al* 2006). This report reflects earlier field findings where fibre goats showed a tendency to develop less severe disease than sheep on farms with ovine Johne's disease (OJD) in their sheep flocks (Whittington and Taragel 2000). These workers commented that goats infected with S strains may therefore have a tendency to milder disease and require laboratory testing to detect subclinical cases.

Since goats can be infected with C or S strains, and since these strains have different cultural requirements on solid media (Cousins *et al* 2003), it is important to choose appropriate solid media for subculture from Bactec broth. For C strains, Herrold's egg yolk medium supplemented with mycobactin J is considered an ideal solid subculture medium (Cousins *et al* 2003). However, for Australian S strains, a modified 7H10 medium with added antibiotics, egg yolk and mycobactin J is required to support growth on subculture (Whittington *et al* 1999; Cousins *et al* 2003). While the modified 7H10 medium supports C strain growth, isolated *Map* colonies are more difficult to see and growth is likely to be less luxuriant compared with Herrold's medium. In diagnostic laboratories in Australia, it is therefore important to provide a suitable solid subculture medium based on the history of the goat flock, and particularly any association with infected sheep flocks which may implicate S strain rather than C strain infection.

1.2 Overseas studies of caprine JD relative to PFC

Although several overseas reports describe PFC for bovine Johne's disease, none have addressed goats, and all 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 in cattle. Cattle simulation models from the Netherlands (Weber *et al* 2004) and the USA (van Schaik *et al* 2003) have reached similar conclusions.

No data is available on goats in relation to shedding rates and categories of shedders. 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.

While the distribution of low, medium and high shedders in infected goats is unknown, some reports indicate that a significant proportion of confirmed infected goats have multibacillary Johne's disease. Corpa *et al* (2000) reporting on histopathology of subclinical or clinical goats in Spain, indicated 50% of 68 diseased goats were multibacillary, and 15-27% of paratuberculous goats may be paucibacillary. This compares with an estimated prevalence of 31% for the paucibacillary form in paratuberculous sheep (Clarke and Little 1996).

1.3 Testing limitations

The length of incubation of Bactec media for routine *Map* cultures differs for sheep (12 weeks) compared with cattle or goat (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 or goat 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 can be a problem with faeces from ruminants 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 goats 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.

The search for cheaper tests for caprine paratuberculosis has included evaluation of a milk ELISA (Salgado *et al* 2005). However, the sensitivity was found to be 23% lower than that for serum, and may only be of merit for detection of heavy shedders (Salgado *et al* 2005).

1.4 Sample limitations

Access to an adequate volume of faeces from numbers of known infected Australian goats shedding *Map* at a given time-point is quite limited. This study used faecal samples obtained from routine culture submissions, and stored for variable periods, up to 4 years.

2 Project Objectives

2.1 Project Objectives

2.1.1 To obtain faeces from infected and uninfected goats to develop a cost-effective herd test for caprine Johne's disease

2.1.2 To utilise the above to identify which dilutions of faeces of single infected goats 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 shedder goats and testing by Pooled Faecal Culture (PFC) at a range of dilutions in known negative goat 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 goats naturally infected with C and S strains of *Map*, 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 goat herd certification.

3.2 Samples

Samples 1-18 were selected among diagnostic submissions to the Microbiology and Immunology Section at EMAI that showed growth of *Map* on initial radiometric faecal culture. An additional three samples (samples 19-21) were derived from interstate laboratories in Queensland and South Australia.

A total of 21 faeces from separate goats submitted in 2001-2005 from 10 herds met this criterion, and had been stored at -80^{o}C for up to 4 years after original collection for diagnostic testing. The source of these samples is outlined in Table 1. Four samples (Nos 2, 3, 4, 20) had been confirmed by IS*1311* PCR and REA as containing S strain of *Map*, while the remaining 17 were derived from goats infected with C strain. Table 2 outlines the laboratory findings in the source goats.

Normal (negative) faeces were collected from goats sourced from the Broken Hill area and slaughtered at Broken Hill abattoir. Several random faecal aliquots from a mixture of the faeces were cultured and found negative for *Map* by Bactec faecal culture, and then mixed and stored at -80^{o}C.

Table 1. Faecal samples collected from goats used in faecal dilution study

Sample	Lab submission reference	Date collected	Herd ID	Herd location (RLPB)	Accession sample serial number	Animal ID	Strain	Type	Vol (g)
1	MN014168	3/5/01	A	Moss Vale		Doe	C	Dairy	80
2	MN02B145	17/12/02	B	Goulburn		133	S	Meat/ Boer X	300
3	MN02B145	"	B	"		141	S	Meat/ Boer X	300
4	MN02B146	17/12/02	C	Goulburn		383	S	Meat/ Feral	300
5	MN032441	28/3/03	D	Moss Vale		376	C	Angora	80
6	MN034841	1/7/03	E	Moss Vale		Avon	C	Dairy	30
7	MN039637	2/12/03	D	Moss Vale		160	C	Angora	150
8	MN041367	13/2/04	F	Moss Vale	2		C	Saanen	300
9	MN045585	7/7/04	F	"	1	7904	C	Nubian x	70
10	MN045585	"	F	"	2	23838	C	Saanen	70
11	MN045895	15/7/04	F	"	1	157914	C	Saanen	140
12	MN045895	"	F	"	2	157941	C	Saanen x Anglo Nubian	140
13	MN045974	20/7/04	G	Moss Vale	7		C	Dairy	30
14	MN045974	"	G	"	17		C	Dairy	70
15	MN046121	23/7/04	F	Moss Vale	1	7908	C	Dairy	70
16	MN046121	"	F	"	2	7920	C	Dairy	70
17	MN046121	"	F	"	3	7984	C	Dairy	70
18	MN046152	"	F	"		7906	C	Dairy	70
19	MN047928	16/9/04	H	Iredale, Qld	11		C	Saanen	30
20	MN059418	1/4/05	I	Forbes	1	FH009	S	Meat	10
21	MN059418	1/4/05	J	Meningie, SA	2	0904	C	Dairy	14

Table 2. Laboratory findings in goats used as source of faecal samples in this study

Sample	Herd ID	Animal ID	Strain	H/P	H/P severity	Tissue culture	AGID	ELISA	Clinical status
1	A	Doe	C	Pos	Moderate to marked, multibacillary	Pos	Pos	Pos	Subclinical
2	B	133	S	Pos	Advanced, multibacillary	ND	Pos (ovine only)	Pos	Clinical
3	B	141	S	Pos	Focal, paucibacillary	ND	Pos (ovine only)	Pos	Subclinical
4	C	383	S	Pos	Mild, paucibacillary	Pos	Neg	Pos	Subclinical
5	D	376	C	Pos	Severe diffuse, paucibacillary	Pos	Pos	Pos	Subclinical
6	E	Avon	C	Pos	Advanced, multibacillary			Inconc	Clinical
7	D	160	C	NA	NA	NA	NA	NA	Vaccinated shedder
8	F		C	Pos	Severe, multibacillary	Pos	Pos	Pos	Subclinical
9	F	7904	C	Pos	Moderate; paucibacillary	Pos	Neg	Pos	Subclinical
10	F	23838	C	Pos	Early; no AFO's	Pos	Neg	Neg	Subclinical, early
11	F	157914	C	Pos	Moderate, paucibacillary	Pos	Neg	Pos	Subclinical
12	F	157941	C	Neg	Neg	Pos	Neg	Neg	Carrier
13	G		C	NA	NA	NA	ND	Neg	Subclinical
14	G		C	Pos	Marked, moderate AFO's	Pos	ND	Pos	Clinical
15	F	7908	C	NA	NA	ND	Neg	Inconc	Possible subclinical
16	F	7920	C	NA	NA	ND	Neg	Neg	Possible subclinical
17	F	7984	C	NA	NA	ND	Pos	Inconc	Subclinical
18	F	7906	C	NA	NA	ND	Neg	Neg	?Carrier
19	H		C						Subclinical
20	I	FH009	S	Pos	Mild, paucibacillary to moderate AFO's (aliquibacillary)	Pos	Pos	NT	Subclinical, in light condition
21	J	0904	C	Pos	Mild, paucibacillary to moderate AFO's (aliquibacillary)	Pos	Pos	NT	Subclinical, in light condition

NA: not available
NT: not tested

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 sample, 4-10 pellets from each of the 21 goats were homogenised with negative faeces at a dilution of 1:5 using a stainless steel Waring blender (Table 3). Similarly, 1-5 pellets were mixed with negative faecal pellets to produce dilutions of 1:10, 1:20, 1:25, 1:30 and 1:50, according to Table 3. Where faeces were not in pellet form, the average weight of a pellet, based on the mean of 10 normal (negative) pellets, was taken to represent a pellet and the dilutions performed according to weight. From all 21 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) For samples 1-19, 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. For samples 20-21, 1.5 g of faeces were cultured and similarly processed as described above.

(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^{o}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. Depending on the source strain of *Map*, the B samples were routinely subcultured to Herrold's egg yolk medium with mycobactin J (C strains) or 7H10 medium with egg yolk, mycobactin J and antibiotics (S strains), and incubated for up to 10 weeks for confirmation of typical, mycobactin-dependent colonies (Cousins *et al* 2003).

Table 3. Dilution of samples (by pellet number or weight) for PFC study

Samples	1-5, 8-9, 11-12, 15-16, 18		10, 17		14*		6, 13		20-21	
Final dilution	Pos faeces	Neg faeces	Pos faeces	Neg faeces	Pos faeces	Neg faeces	Pos faeces	Neg faeces	Pos faeces	Neg faeces
1 in 5	10	40	5	20	5 g	20 g	4	16	1 g	4 g
1 in 10	5	45	5	45	5 g	45 g	2	18	1 g	9 g
1 in 20	2	38	2	38	2 g	38 g	1	19	1 g	19 g
1 in 25	2	48	2	48	2 g	48 g	1	24	1 g	24 g
1 in 30	2	58	2	58	2 g	58 g	1	29	1 g	29 g
1 in 50	1	49	1	49	1 g	49 g	1	49	1 g	49 g
Total neat faeces	22		17		17 g		10		6 g	

* unpelleted faeces

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

Ten of the 21 neat faecal samples (Samples 2, 3, 4, 5, 7, 8, 9, 18, 20 and 21) 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 or modified 7H10 medium containing mycobactin J, depending on whether the source strain of *Map* was of C or S type. BACTEC vials were incubated for up to 12 weeks at 37°C. Herrold's and 7H10 slopes were incubated for up to 20 weeks 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 caprine faeces.

4 Results and Discussion

4.1 PFC dilution results

From the 21 selected faeces cultured neat and also subjected to the dilution study in normal faeces, only 14 (samples 1-6, 8-11, 14, 16-17 and 21) were found to yield *Map* on subsequent culture.

One sample (No. 3) was culture positive at the neat and 1:10 dilution only, whereas all other samples (n = 13) were culture positive at a dilution of 1:25 or greater. The results for each dilution are shown in Table 4. In sample 4 at the 1:30 dilution, 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). In this sample and 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 two instances, IS*900* PCR/REA was positive but *Map* was not grown on subculture, due to contamination (Table 4; see also Appendix 1).

Two additional samples (samples 7 and 20) were found to be culture negative in the PFC dilution series, even at the neat dilution, but were culture positive in some replicates of the neat dilution in the ten-fold dilution series. Sample 7 was in fact tested in duplicate in both series (referred to as 7A and 7B), and was positive in one of the two neat samples cultured in the ten-fold dilution series, in one of five replicates at that dilution. Sample 20 yielded 4/5 replicates positive at the nil dilution (neat) in the ten-fold dilution study, but not when cultured undiluted for the PFC series.

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 caprine samples suspected of containing C strains of *Map*. Table 5 shows the time taken to reach a suitable growth index to confirm the presence of *Map* in the 14 samples that were culture positive in the PFC dilution series. In comparing cumulative detection of culture positive samples (presented in Table 6), an incubation of at least 10 weeks was required at a dilution of 1:25 for optimal results (detection of 13 of the 14 samples at dilutions above 1:10).

Table 4. **Success of PFC for detection of *M. avium* subsp *paratuberculosis* in goats 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
1	+	+	+	+	+	+	-
2	+*	+	+	+	+	+	+
3	+	-	+	-	-	-	-
4	+	+	+	+	+*	+**	-
5	+	+	+	+	+	+	+
6	ND	+	+	+	+	+	+
8	+	+	+	+	+	+	+
9	+	+	+	+	+	+	+
10	+	+	+	-	+	+	-
11	+	+	+	+	+	+	+
14	ND	+	+	+	+	+	+
16	+	+	+	+	+	+	-
17	+	+	+	+	+	+	+
21	+	+	+	+	+	-	+
12, 13, 15	ND	-	-	-	-	-	-
18, 19	-	-	-	-	-	-	-
7, 20	+#	-	-	-	-	-	-

* positive by PCR/REA only; contaminant overgrowth on solid media
** positive by subculture (mycobactin dependency) only
# grew in ten-fold dilution series only, not in PFC dilution series

Table 5. Incubation time in Bactec required to reach high growth index (GI 999) to enable confirmation of positive cultures from 14 goats shedding *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**
1	10	7	8	9	8	8	na
2	6	7	8	8	7	8	8
3	7	na	11	na	na	na	na
4	6	8	8	9	8	12*	na
5	6	7	7	8	8	7	9
6	na	4	5	5	5	5	5
8	4	5	6	6	5	6	6
9	5	5	5	5	5	5	5
10	8	8	11	na	10	9	na
11	6	8	8	8	8	9	9
14	na	8	7	8	7	9	8
16	6	8	8	8	8	8	na
17	7	6	8	8	8	7	7
21	6	9	8	8	7	na	8#

* only detected by subculture

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

Table 6. Cumulative positive results for 14 *Map* shedder goat samples at different dilutions according to weeks incubated

	Cumulative culture positive samples after Various weeks of incubation								
Dilution	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
1:5	1	3	4	7	12	13	13	13	13
1:10	0	2	3	5	12	12	12	14	14
1:20	0	2	3	3	10	12	12	12	12
1:25	0	3	3	6	12	12	13	13	13
1:30	0	2	3	5	8	11	11	11	12*
1:50	0	2	3	4	7	9	9	9	9

* includes one sample only detected by subculture

4.2 Results of ten-fold dilution series

From the 10 faeces where additional studies were performed on 10-fold dilutions of VAN-treated inoculum injected into five-fold replicates of BACTEC media, nine samples produced growth of *Map* at the neat (nil) dilution, seven at the 10^{-1} dilution, six at the 10^{-2} dilution, five at the 10^{-3} dilution and two at the 10^{-4} dilution (Table 7). The resultant MPN estimates of inocula for the nine culture positive samples from those studied in the ten-fold dilution series is also shown in Table 7. In terms of establishing a regression line requiring similar rates of growth at multiple points in the dilution series, three samples (nos. 5, 8, 9) were useful in providing at least three points to determine a regression equation, while samples 2 and 21 provided two such points for 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 and 1:10 dilutions for the samples showing repeatability among all five replicates positive (samples 2, 5, 8, 9 and 21). This was also calculated at the 1:100 and 1:1000 dilutions for samples that showed similar rates of growth in all five tubes at those dilutions (viz samples 5, 8, 9 at the 1:100 dilution and samples 8 and 9 at the 1:1000 dilution). From this data the number of days to reach a cgi of 1000 (dcgi1000) was calculated (Figures 1a-5a). 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 each sample produced a series of regression equations (as shown in Figures 1b-5b) of:

Sample 2 $\log_{10}$ inoculum = 6.2 – 0.099 dcgi1000
Sample 5 $\log_{10}$ inoculum = 8.8 – 0.152 dcgi1000
Sample 8 $\log_{10}$ inoculum = 7.9 – 0.134 dcgi1000
Sample 9 $\log_{10}$ inoculum = 7.5 – 0.115 dcgi1000
Sample 21 $\log_{10}$ inoculum = 6.0 – 0.109 dcgi1000

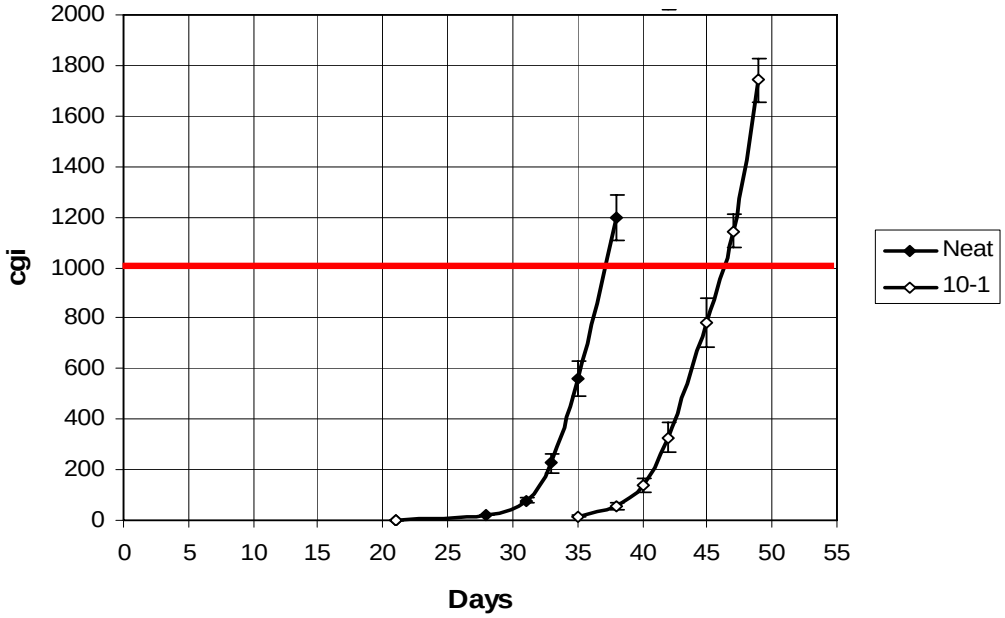
Table 7. Growth of *Map* in 5-fold replicate BACTEC broths in a 10 fold dilution series

	No. positive/no. tested at varying dilutions					MPN result	MPN/g*	Range
	Neat	**1:10**	**1:100**	**1:1000**	**1:10000**			
2	5/5	5/5	5/5	1/5	0/5	5-5-5-1-0	3300	1,000-10,000
3	4/5	2/5	0/5	0/5	0/5	4-2-0-0-0	22	6.8-50
4	5/5	5/5	2/5	2/5	0/5	5-5-2-2-0	940	340-2,300
5	5/5	5/5	5/5	2/5	0/5	5-5-5-2-0	4900	1,500-15,000
7	1/5	0/5	0/5	0/5	0/5	1-0-0-0-0	2	0.1-10
8	5/5	5/5	5/5	5/5	3/5	5-5-5-5-3	92000	22,000-260,000
9	5/5	5/5	5/5	5/5	2/5	5-5-5-5-2	54000	15,000-170,000
20	4/5	0/5	0/5	0/5	0/5	4-0-0-0-0	13	4.1-35
21	5/5	5/5	2/5	0/5	0/5	5-5-2-0-0	540	150-1,700
18	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 2

a. Plot of growth as a cumulative growth index (cgi). The lines represent results derived from the mean (± SEM) cgi for the neat and 1:10 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 2

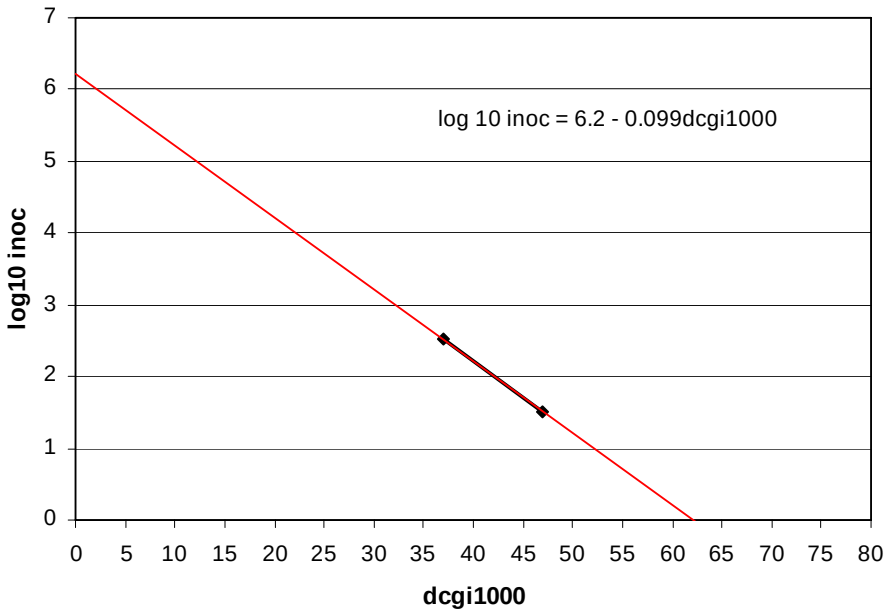
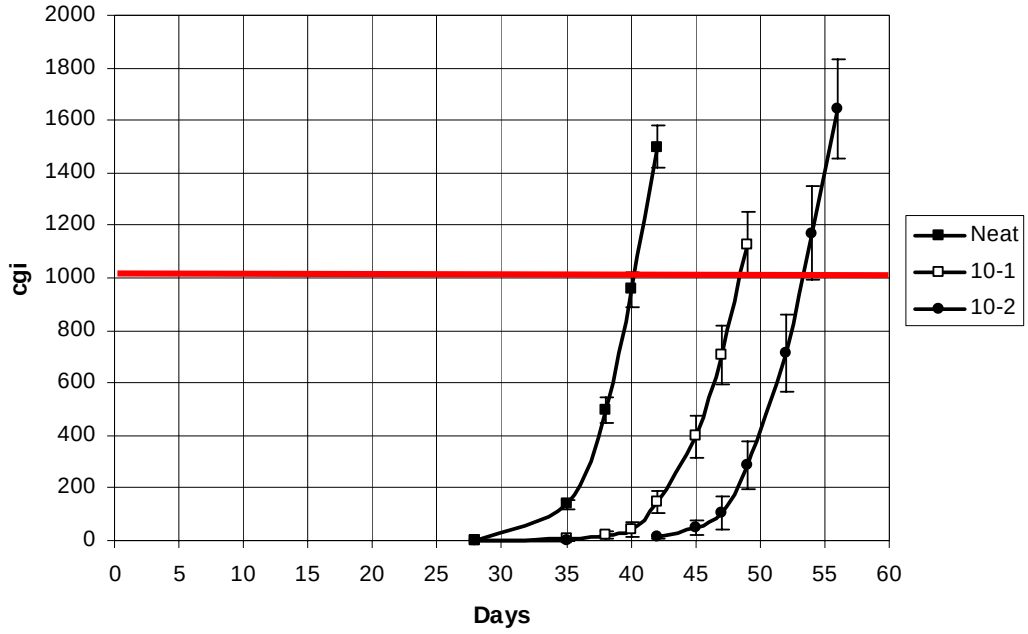


Figure 2. Determination of regression equation data for sample 5

a. Plot of growth as a cumulative growth index (cgi). The lines represent results derived from the mean (± SEM) cgi for the neat and 1:10 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 5

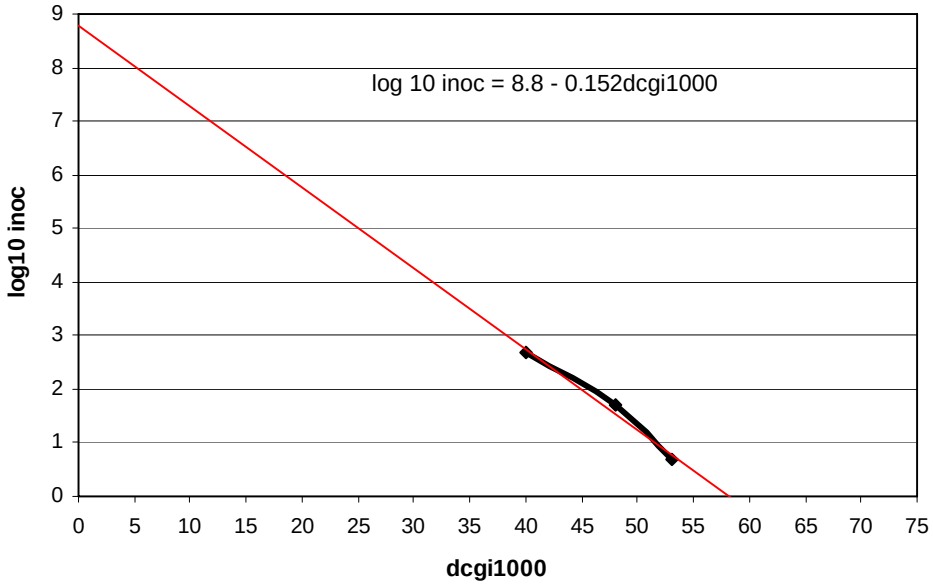
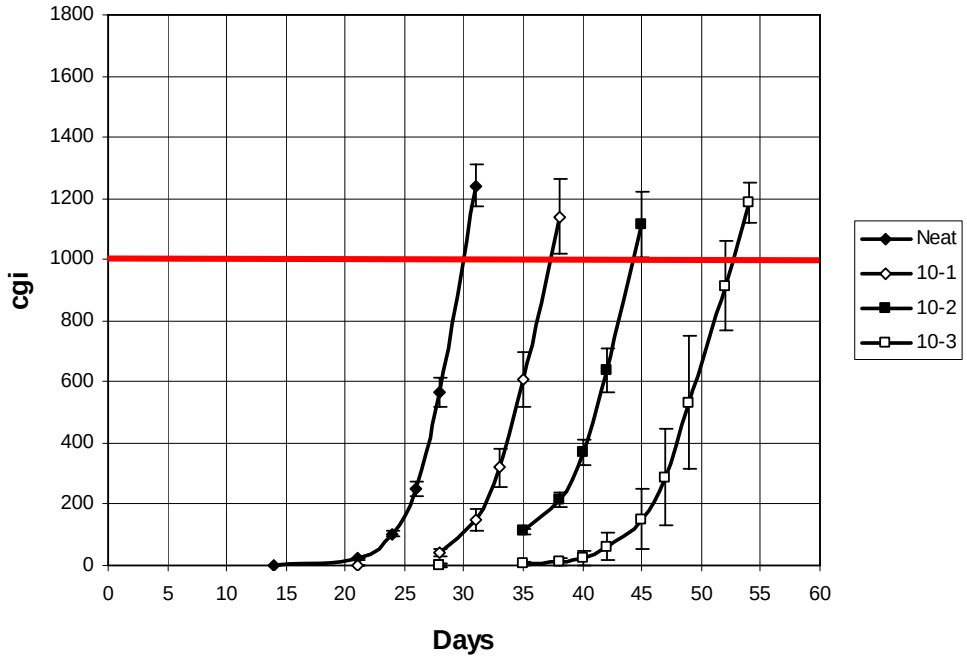


Figure 3. Determination of regression equation data for sample 8

a. Plot of growth as a cumulative growth index (cgi). The lines represent results derived from the mean (± SEM) cgi for the neat and 1:10 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 8

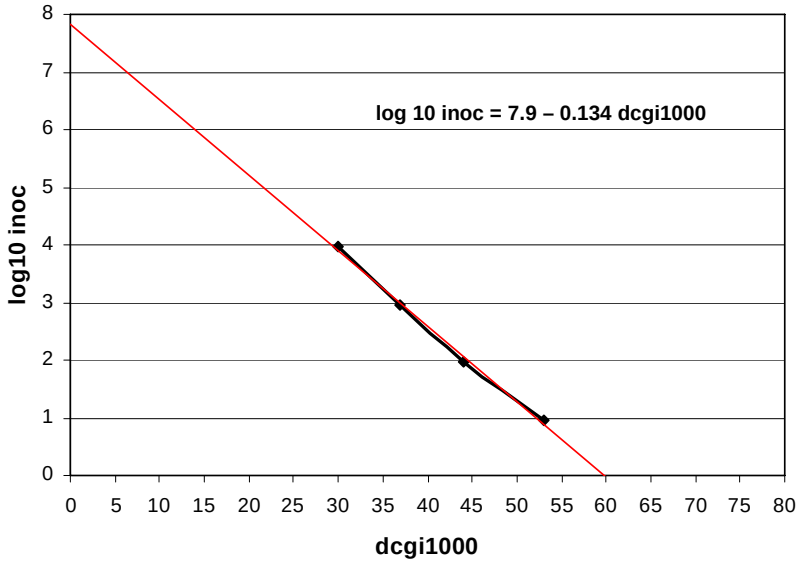
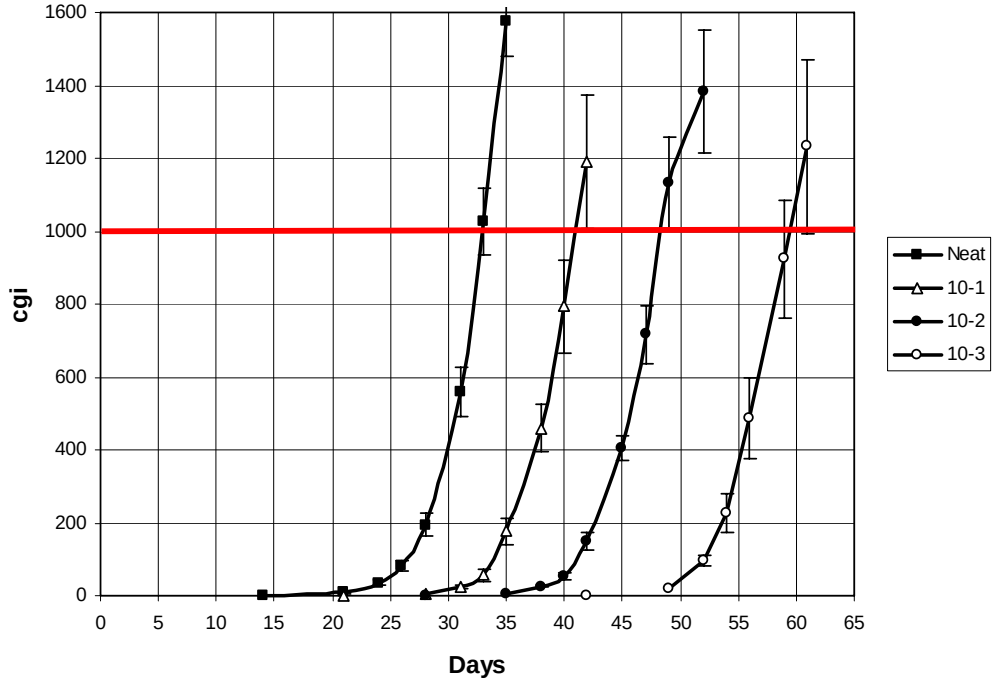


Figure 4. Determination of regression equation data for sample 9

a. Plot of growth as a cumulative growth index (cgi). The lines represent results derived from the mean (± SEM) cgi for the neat and 1:10 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 9

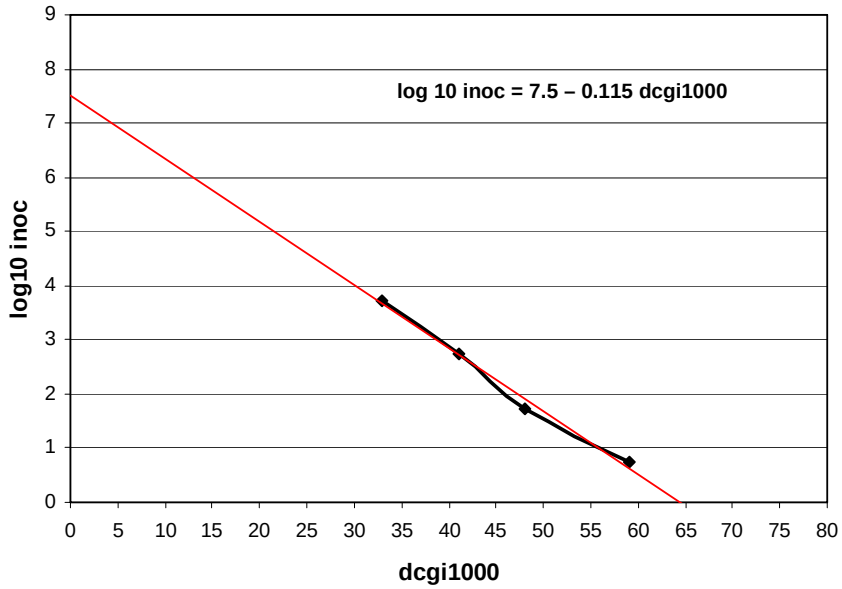
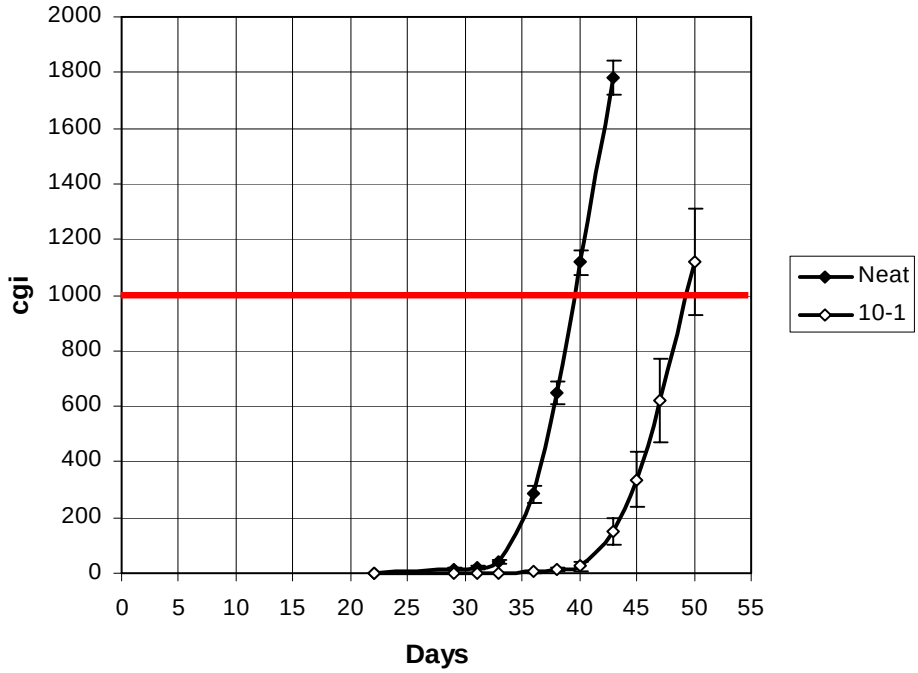
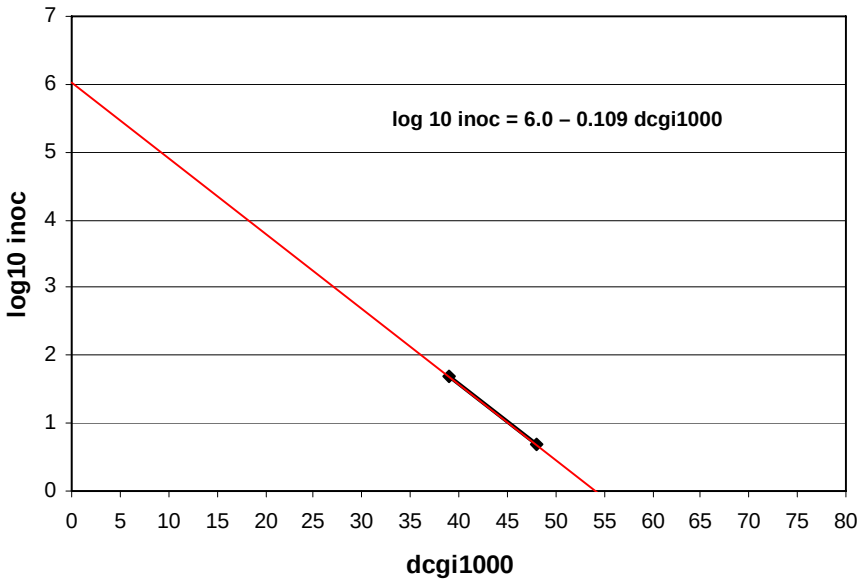


Figure 5. Determination of regression equation data for sample 21

a. Plot of growth as a cumulative growth index (cgi). The lines represent results derived from the mean (± SEM) cgi for the neat and 1:10 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 21



4.3 Calculation of regression equation and shedding rates

From a plot of the full dataset on one graph (Figure 6), the final linear regression equation for caprine faeces was determined as:

$$\log_{10} \text{inoculum} = 7.3 - 0.121 \text{ dcgi1000}$$

Based on this regression equation, the inoculum was calculated for all samples using dcgi1000 data derived from the pooled faecal samples and from the 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. Since *Map* S strain data has indicated a loss of 50 fold (1.7 $\log_{10}$) due to processing (Reddacliff *et al* 2003b), this was taken into account in estimating the final shedding rate estimated for all culture positive goats. This data is presented in Table 8. In this table, estimates based on MPN in the dilution series for nine samples are compared with estimates for all goats based on the regression equation, derived from dcgi1000 determined from the pooled faecal culture and/or ten-fold dilution series.

From the estimated faecal shedding rates of *Map* in 16 culture positive goats (Table 8), two goats (nos 7, 20) shedding up to 1.5×10^3 organisms per gram of faeces, were undetected at dilutions of 1:5 or above. One animal (no. 3) estimated to be shedding 1.6×10^3 (by MPN) was found to be test positive at 1:10. Based on an assumed loss of 50 fold due to processing, this would equate to a Bactec inoculum of $1500/50 \times 14.3 = 2$ organisms from neat faeces and < 1 organism in the current procedure at the 1:10 dilution. On the estimated numbers of cells available for culture from goats, relatively small inocula of *Map* into Bactec cultures thus appear to yield detectable growth after prolonged incubation. The remaining 13/16 animals were detected at dilutions of 1:30 or 1:50, and 9 at 1:50, and these results correlated with the estimated shedding rates. One anomaly was sample 21 that was positive at 1:25 and 1:50 but negative at 1:30. Thus animals shedding 10^4 to 10^5 /g were generally found positive at 1:30 and, except for sample 16 from a goat estimated to be shedding 7.4×10^5 *Map*/g and which was positive at 1:30, those shedding > 10^5 *Map*/g were all positive at a 1:50 dilution.

The results were also 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 4 years 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 12, 13, 15, 18 and 19. However, this may also indicate that the results represent a conservative estimate of the benefit of PFC in goats, and that a dilution of 1:25 or 1:30 may represent a conservative dilution to detect infection on a herd basis.

Figure 6. Regression equation for caprine faeces based on MPN and cgi1000 data derived from 5 goats (2, 5, 8, 9, 21) in current study

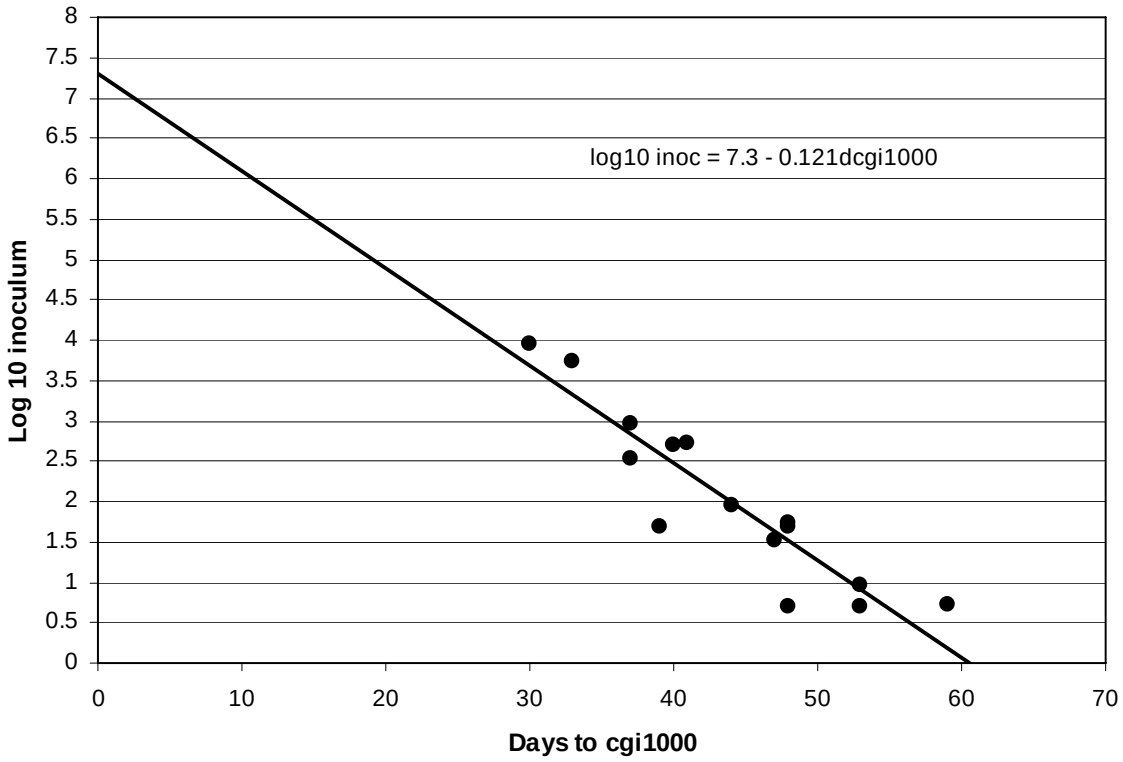


Table 8. Estimation of faecal shedding rate of *Map* from goats, based on an assumed 50-fold loss during processing, and ordered from lowest to highest rate of shedding

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)
1	9.7×10^4	30		
2	4.8×10^5	50	2.4×10^5	$7.2 \times 10^4 – 7.2 \times 10^5$
3	9.7×10^3	10	1.6×10^3	$4.9 \times 10^2 – 3.6 \times 10^3$
4	1.8×10^5	30	6.7×10^4	$2.4 \times 10^4 – 1.6 \times 10^5$
5	2.1×10^5	50	3.5×10^5	$1.1 \times 10^5 – 1.1 \times 10^6$
6	4.3×10^6	50		
7	(2.9×10^3)#	1	1.4×10^2	$7.2 \times 10^0 – 7.2 \times 10^2$
8	3.3×10^6	50	6.6×10^6	$1.6 \times 10^6 – 1.9 \times 10^7$
9	1.5×10^6	50	3.9×10^6	$1.1 \times 10^6 – 1.2 \times 10^7$
10	4.4×10^4	30		
11	2.1×10^5	50		
14	2.2×10^5	50		
16	7.4×10^5	30		
17	5.1×10^5	50		
20	8.4×10^4	1	1.2×10^3	$3.9 \times 10^2 – 3.3 \times 10^3$
21	3.6×10^5	50	5.1×10^4	$1.4 \times 10^4 – 1.6 \times 10^5$
12, 13, 15, 18, 19	-	-	-	-

* based on days to cumulative growth index of 1000

\# based on growth rate of a single replicate (1/5) from neat faeces; mean would be 1/5 this value i.e. 5.8×10^2

4.4 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 are compared against the MPN estimates from Bactec growth in Tables 9 and 10. These are split for convenience of presentation between goats infected with the S strain (Table 9) and those infected with C strain (Table 10). From the columns in these tables, it is clear that Bactec culture was more sensitive than solid media culture, since Bactec cultures were positive at the 10^{-3} dilution on three occasions when only the 10^{-2} dilution was positive on solid media (e.g. samples 2, 4 and 5). Further, the counts on solid media were difficult to assess because a 10 fold dilution often resulted in a plate count changing from > 50 cfu to 0 (e.g. samples 2, 5, 8, 21), and would therefore be considered less reliable than those based on MPN from growth in Bactec media.

These results suggest that solid media may underestimate counts by a factor of approximately 1 $\log_{10}$. Allowing for an arbitrary count of 100 on samples that gave a result of >50 cfu on solid media, there was reasonable agreement between these two methods of determining the *Map* inocula for the nine samples (nos 2-5, 7-9, 20-21). However, in general, arbitrary estimates of cfu in samples 2, 5, 8 and 9 (due to an inability to accurately count cfu on the solid media at > 50 cfu) tended to result in an apparent overestimate (by 0.6 $\log_{10}$) of the inocula compared to the MPN data.

Table 9. Comparison of counts to determine inocula from ten-fold dilutions on modified 7H10 and in Bactec media used for MPN determinations (S strain goats)

Sample ID		Neat	10^{-1}	10^{-2}	10^{-3}	10^{-4}	Estimate of inoculum
2	**MPN**	5	5	5	1	0	330 (100 - 1000)
	Solid medium colony counts	ND*	>50	>50	0	0	
		ND	5	1	0	0	
		ND	>50	>50	0	0	
		ND	>50	25	0	0	
		Mean inoc/ 0.1 mL	763#	3150	0	0	1957
3	**MPN**	4	2	0	0	0	2.2 (0.7 – 5)
	Solid medium colony counts	ND*	C	C	0	C	
		ND	C	C	C	C	
		ND	0	C	0	C	
		ND	C	C	C	C	
		Mean inoc/ 0.1 mL	na	na	na	na	Not assessed
4	**MPN**	5	5	2	2	0	94 (34 – 230)
	Solid medium colony counts	ND*	C	0	0	0	
		ND	C	0	0	0	
		ND	C	0	0	0	
		ND	0	0	0	0	
		Mean inoc/ 0.1 mL	na	0	0	0	< 100
20	**MPN**	4	0	0	0	0	1.3 (4.1 – 35)
	Solid medium colony counts	ND*	0	0	0	0	
		ND	0	0	0	0	
		ND	0	0	0	0	
		ND	0	0	0	0	
		Mean inoc/ 0.1 mL	0	0	0	0	< 10

* ND: not done
na not able to be assessed
C contaminants prevented meaningful count
count > 50; arbitrarily set at 100 to calculate mean

Table 10. Comparison of counts to determine inocula from ten-fold dilutions on Herrold's and in Bactec media used for MPN determinations (C strain goats)

Sample ID		Neat	10^{-1}	10^{-2}	10^{-3}	10^{-4}	Estimate of inoculum
5	**MPN**	5	5	5	2	0	490 (150 – 1500)
	Solid medium colony counts	ND*	27	>50	0	0	
		ND	23	0	0	0	
		ND	22	0	0	0	
		ND	>50	13	0	0	
		Mean inoc/ 0.1 mL	430	2,830	0	0	1,630
7	**MPN**	1	0	0	0	0	0.2 (0.01 -1)
	Solid medium colony counts	ND*	0	0	0	0	
		ND	0	0	0	0	
		ND	0	0	0	0	
		ND	0	0	0	0	
		Mean inoc/ 0.1 mL	0	0	0	0	<10
8	**MPN**	5	5	5	5	3	9,200 (2,200 – 26,000)
	Solid medium colony counts	ND*	>50	>50	55	0	
		ND	>50	>50	>50	0	
		ND	>50	>50	6	1	
		ND	>50	>50	>50	>50	
		Mean inoc/ 0.1 mL	1,000	10,000	65,250	Na	37,625
9	**MPN**	5	5	5	5	2	5,400 (1500 – 17000)
	Solid medium colony counts	ND*	>50	>50	>50	ND	
		ND	>50	>50	5	ND	
		ND	>50	>50	>50	ND	
		ND	>50	>50	7	ND	
		Mean inoc/ 0.1 mL	1,000	10,000	28,000	Na	19,000
21	**MPN**	5	5	2	0	0	54 (15 - 170)
	Solid medium colony counts	ND*	9	10	0	0	
		ND	3	3	0	0	
		ND	7	>50	0	0	
		ND	6	48	0	0	
		Mean inoc/ 0.1 mL	62.5	4,030#	0	0	2,046

* ND: not done
na not able to be assessed
C contaminants prevented meaningful count
\# count > 50; arbitrarily set at 100 to calculate mean

4.5 Relevance of findings to shedding rates among goats in infected herds

The estimates of shedding rates for the 16 goats shedding *Map* in this study 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 bovine shedders of *Map* as either low, moderate or high based on colony counts on solid media, the estimated shedding rates among the 16 caprine shedders were therefore compared to such figures described in the literature.

From the report of van Shaik *et al* (2003) (based on cattle), 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 $<1.5 \times 10^4$, $1.5 \times 10^4 – 1.5 \times$

10^5, and > 1.5 x 10^5 per gram of faeces. These "old criteria", for which van Shaik *et al* (2003) suggested 70% of infected animals (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 suggests a factor of 1 $\log_{10}$ increased sensitivity due to culture on Bactec media, it is probable 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 16 goats 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 11. Based on either "old" or "new" criteria, it is clear that the shedding level of the animals investigated in this study was not biased toward a "high shedder" category.

Although there are no earlier studies to confirm shedding rates in infected goat populations, one report on histopathology of subclinical or clinical goats in Spain, indicated 50% of 68 diseased goats were multibacillary (Corpa *et al* 2000). These workers also found 10/68 (14.7%) had a diffuse lymphocytic form of Johne's disease with 7/10 showing low numbers of mycobacteria in the intestinal sections, while the remaining 24/68 (35.3%) were divided among goats with focal lesions and goats with diffuse mixed lesions. Low to moderate numbers of *Map*-like organisms were observed in 6/16 goats with focal mucosal lesions, and all 8 goats with diffuse mixed lesions had scarce but consistently observable acid-fast organisms on intestinal sections. While the overall proportion of goats with mycobacteria on sections was relatively high (55/68; 81%), the cultural success rate from ileal tissue of 62 of the goats was relatively low (58%), including only 71.4% of multibacillary cases. This was attributed to limited sample selection for culture (from cases where ileum had no prominent lesion), but may have been further exacerbated by the use of only solid LJ medium, and severe decontamination processes not used in Australian laboratories.

In our experience, cultural success rate is very high from tissues with observable acid fast rods in lesions, and suggests the methods used in the current PFC study would be more sensitive than some employed overseas to confirm individual infections. Nevertheless, the study of Corpa *et al* (2000) highlights the probability that if the diffuse form of Johne's disease in goats is paucibacillary, then 15-27% of paratuberculous goats may be paucibacillary (Corpa *et al* 2000; Catton 2002). Such figures are consistent with the findings in the current study, where 13/16 goats (81%) were estimated to be shedding > 1.5 x 10^4 *Map*/g and detectable at a dilution of 1:25. Of some interest is that three of the 16 goats (nos 2, 6 and 14, Table 2) were clinical cases, and all were estimated to be moderate to high shedders and detectable at 1:50 dilutions (Tables 8, 11). Based on the new criteria suggested for Bactec cultures, 75% fell into the low to moderate shedder category, and were not biased toward heavy shedders. From this data it is likely that the selection of a 1:25 or 1:30 dilution represents a conservative recommendation for application of PFC in herds for disease risk assessment.

Table 11. Categorisation of animals by *Map* excretion rate, based on two separate criteria, and divided among 16 shedders from this study. Results based on MPN where available; otherwise based on common regression equation

	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 x 10^4	**1.5 x 10^4 to 1.5 to 10^5**	**> 1.5 x 10^5**	**< 1.5 x 10^5**	**1.5 x 10^5 to 1.5 to 10^6**	**> 1.5 x 10^6**
Sample IDs	3, 7, 20	1, 4, 10, 21	2, 5, 6, 8, 9, 11, 14, 16, 17	1, 3, 4, 7, 10, 20, 21	2, 5, 11, 14, 16, 17	6, 8, 9
Highest dilution positive (range)	1-10	30-50	30-50	1-50	30-50	50
Total no. samples	3	4 (3*)	9 (10*)	7 (6*)	6	3 (4*)

* result relevant to sample 21 if based on common regression equation

5 Success in Achieving Objectives

5.1 Determine if any obstacles prevent SCAHLS approval and/or uptake of technology as a routine diagnostic assay for herd certification purposes

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 faecal samples and test dilutions by radiometric (Bactec) culture

5.2.1 Obtain adequate volumes of faeces from infected goats (n =19) and sufficient large volume of negative faeces to enable dilutions from 1:5 to 1:50.

At the start of the project, faeces were available to test from a total of 16 C strain infected goats and 3 S strain infected goats. The budget allowed for the sourcing of an additional 7 S strain goats if these could be sourced during the project. As described in Table 1, 18 of the original 19 faeces were sourced from NSW goats herds, and one from a Qld herd. In late 2005, two additional faeces were obtained from SA DPI, one an S-strain-infected goat from SA and the second a C-strain-infected goat killed in SA but derived from a NSW flock. The latter sample was useful when it became apparent that some of the original samples of C strain infected goats were showing poor *Map* viability on repeat (neat and diluted) culture. However, the volume available from one of these (S strain goat sample 20) was quite low and this in turn may have affected the Map viability within that sample.

While work was based on samples from 21 goats, 5 were not viable on re-culture and two were not culture positive when assessed at the neat dilution in the PFC dilution series, although both were positive in the neat dilution in the 10-fold dilution series. These latter two samples were still of value in delineating the sensitivity of the assays relevant to faecal shedding rates of *Map*.

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

As described in the Materials and Methods, all 21 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 and ovine strains, depending on the strain involved.

5.3 Evaluate cultures using growth in Bactec for *Map* by routine laboratory procedures

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.

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 of 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 or modified 7H10 medium with mycobactin J and incubated for up to 10 weeks for confirmation of typical, mycobactin-dependent colonies.

In addition to detection of culture positive samples among the dilution series in negative faeces, the concentration of *Map* in each original sample was examined using an additional 10-fold dilution series of Bactec inoculum in PBSTw. Using ten 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 3.3 as the positive/negative outcome for each. From nine 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 five samples was also sufficient to determine a regression equation relating the rate of growth at multiple dilutions to the MPN of *Map* in inoculum, according to the dcgi1000 method previously described by Reddacliff *et al* (2003a).

The regression data from these samples were combined 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 culture positive 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 in goats at dilutions of 1:25 will have a high impact on the goat industry, since this represents a saving of 75-90% in the cost of whole herd testing by faecal culture, or 40% in the cost of whole herd testing by ELISA.

Current herd status is defined by ELISA testing at a cost for a 100 goat herd of $8.30 (incl GST) per head = $830 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 individual faecal culture at a cost of approx. $38-100, totalling approx. $ 868-930. The cost of PFC (on a 1:25 pooling regime) would equate to 4 pools @ $130 = $520, 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 1:10, 1:20, 1:25, 1:30 and 1:50 based on current charges in NSW is shown in Figures 7 and 8. From this, it is clear that dilutions $\geq$ 1:20 become much more cost-effective than lower dilutions.

Figure 7. **Cost of whole herd testing for JD in goat herds, assuming follow up culture on 1% of goats tested by ELISA**

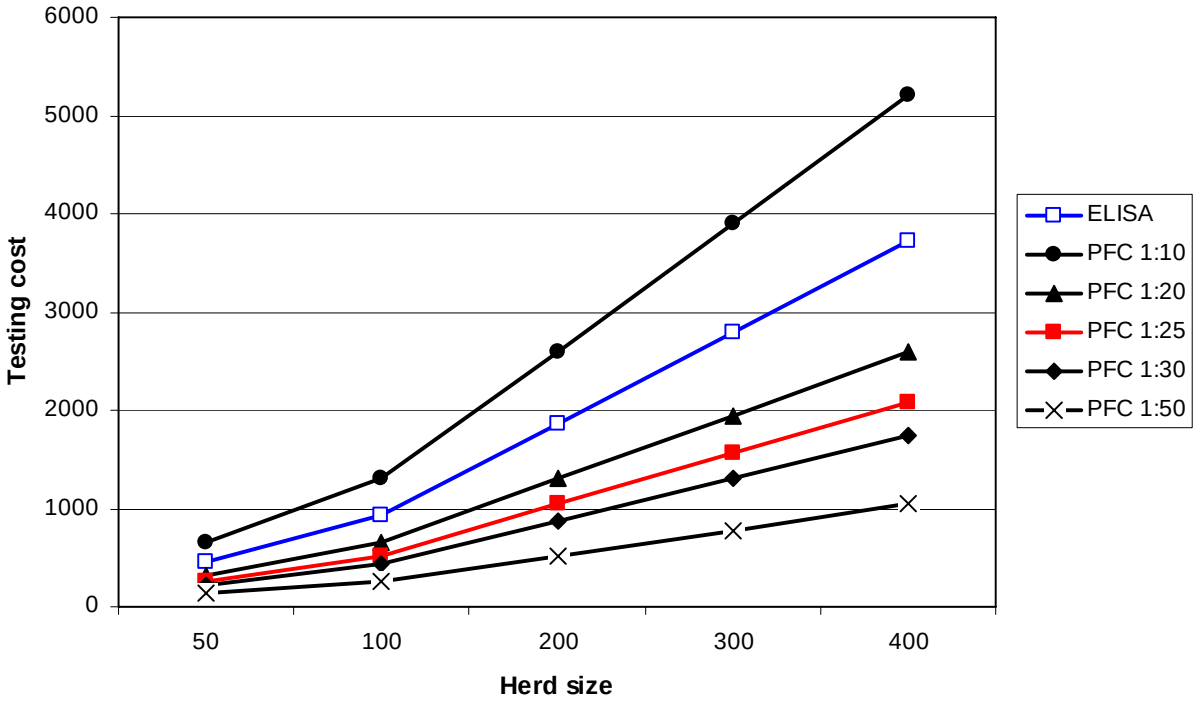
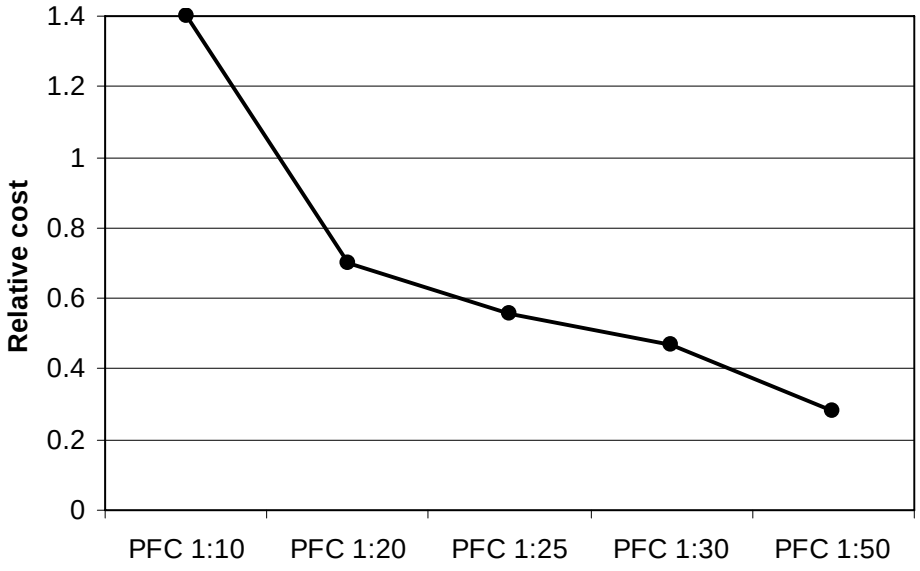


Figure 8. **Relative laboratory testing costs for goat herds, assuming follow up culture on 1% of goats tested by ELISA**



6.2 Impact on Meat and Livestock Industry in five years time

The potential improved sensitivity of a culture-based assay in goat herds is likely to gain wide adoption in the goat industry when testing costs can be significantly reduced. A cost-effective whole herd assay that provides detection of goats at the 1:25 dilution could be further improved in sensitivity 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 increase detection of lower shedder goats by increasing the base inoculum by a factor of 2.86, and therefore enable even a 50-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 detects low shedder animals. In addition, more information on the prevalence of the different ranges of *Map* shedding among infected goats 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 infected shedder goats among dilutions of 1:25 or higher.

7 Conclusions and Recommendations

7.1 Dilution and incubation time for PFC for goats

As expected, the dilution rate for a positive culture in PFC procedures reflected the number of *Map* organisms that infected goats are shedding, together with the losses attributable to processing. Thus the limiting factor affecting the success of Bactec culture is in the number of viable cells surviving to the Bactec inoculum. In this study, faeces from 16 goats with prior evidence of shedding were estimated to contain (from stored samples) up to 9200 *Map* in their Bactec inocula (sample 8, Table 10), with those giving a reliable positive culture result having a minimum number in their inoculum of 1.3 *Map* (sample 20, Table 10). The Bactec liquid culture medium thus appears quite sensitive in culturing very low levels of viable *Map* cells. These inocula translated to goat shedding rates of *Map* in the range $1.2 \times 10^3 – 6.6 \times 10^6$ per gram of faeces, based on data relevant to processing losses of S strain *Map* prior to Bactec inoculation (Reddacliff *et al* 2003b).

While prior data is not available for goats regarding the prevalence of different shedding rates, and particularly shedding rates relevant to determinations from Bactec culture with PCR/REA confirmation, findings from overseas work and from this study have been used to draw some conclusions. Data from the USA based on solid media counts, has suggested that 70% of infected cattle are categoried as shedding $< 1.5 \times 10^4$ *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).

The current study estimated that, based on growth in Bactec media, goats shedding $< 10^4$ *Map* cells/g of faeces are not reliably detected at dilutions in faeces $\geq$ 1:10 with the current procedure as routinely applied to sheep. However, since 13/16 goats were considered to be shedding $> 10^4$ *Map*/g and all were detected at a 1:25 dilution, this dilution combined with an incubation period of at least 10 weeks is recommended, when coupled with confirmation of Bactec growth by IS*900* PCR and

REA. Twelve of these 13 animals were also detected at a 1:30 dilution, with the exception being one goat that was also detected at a 1:50 dilution.

The cost-effectiveness of testing at a 1:25 dilution should enable the goat industries (fibre and dairy) to replace testing based on ELISA serology with PFC. The detection limit of > 1.5 x 10^4 organisms/g of faeces at this dilution is consistent with information from cattle populations that this is likely to represent detection of both low and medium shedders, not just high shedder animals. When compared to excretion rates reported for cattle and adjusted for improved Bactec culture sensitivity, 9-10 goats from the 13 detected at a dilution of 1:25 (69-77%, depending on excretion rates estimated from MPN or growth rate data) would be classified low to medium shedders, and 3-4 as high shedders.

One overseas study of paratuberculous goats subjected to histopathological examination has suggested that a high proportion of such animals show either multibacillary disease (50%) or consistently observable mycobacteria on intestinal sections in a further 31% (Corpa *et al* 2000). This data can be interpreted to suggest that 15-27% of paratuberculous goats are paucibacillary (Catton 2002), and therefore is consistent with the hypothesis that PFC at a 1:25 dilution should be able to detect a high proportion of goats with Johne's disease.

Based on the likelihood that the number of viable cells had deteriorated in some samples due to storage for up to 4 years, this represents a conservative recommendation. However, if additional information can be ascertained that a significant proportion of infected goats are shedding at levels lower than 10^4/g, then procedures would have to be modified to overcome the limitation of the base number of cells that survive to the Bactec inoculum, to enable the cost-effective dilutions (≥ 1:20) to be maintained for herd certification.

7.2 Recommendations for future studies in this area

The following issues would merit attention 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 50 fold due to processing and a further 10-14.3 fold by selection of sub-aliquots 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 sensitivity, and possibly 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

Data is known concerning the distribution of low, medium and high shedders in cattle herds based on arbitrary cut-points relevant to solid medium (conventional) culture on Herrold's egg yolk medium. No such information is available in goat herds. 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 goats 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 goats are excreting using Bactec methodology, then we could determine the likely success rate of all dilutions of PFC. Considering the low number of known infected goat herds in Australia, this would be a challenging assignment.

7.2.3 Determine the proportion of infected goats detectable by PFC at 1:25 dilution, with and without test modification

Since dilutions higher than 1:20 are likely to be cost effective, criteria to define 70% of the infected population and the equivalent dilution rate to detect this would be of great benefit in determining the ultimate sensitivity of the test method as recommended. If the majority of infected goats are shedding levels of > 1.5×10^4/g then these would be a likely to be detected at a 1:25-1:30 dilution. Based on such information, we would then have greater assurance of the likely impact on disease control of these dilutions (1:25, 1:30) 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

This project was financially supported by NSWDPI and MLA.

The capable technical assistance of the technical staff within the Microbiology and Immunology Section at EMAI is gratefully acknowledged. Deb Walker and Naomi Porter provided excellent assistance in the set-up and culture of samples in both the PFC and dilution-enumeration series, and initial confirmation of *Map* cultures. Shayne Fell and Sue Austin provided excellent technical support in completion of these efforts and Shayne undertook completion of the technical confirmation on the cultures, as well as completion of the data entry for the project.

I am indebted to Mr Greg Curran, NSWDPI Senior Regional Animal Health Manager - Western Division, who collected the bulk volume of negative goat faeces (ex Broken Hill abattoir) that was used in the dilution series for the PFC testing. Mr Keith Hart, District Veterinarian Moss Vale RLPB, and goat owners within the Moss Vale RLPB kindly provided valuable samples from a number of infected flocks in their district to assist in this study. Mr Jack van Wijk and Mr Jeremy Rogers, South Australian Dept of Primary Industries kindly provided the samples from two herds at the end of the

study, to augment the number of infected goats evaluated. The sample from the Qld doe was kindly forwarded by Mr A Mackenzie, Ipswich, Qld.

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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 ten samples in 10-fold dilutions in PBSTw (for MPN estimations and regressions) respectively.

Abbreviations:

P: positive on subculture from Bactec broth (colonies of typical *Map* growth rate, morphology and mycobactin dependent)m
N: negative on subculture
C: contaminated on subculture
ETOH: ethanol extraction
TR/1+/2+/3+/4+:
Indicates strength of PCR gel band from Trace to 4+
MP: Consistent with *Map* on PCR/REA profile

10.1 Appendix 1

Growth of samples (expressed as growth index) 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 +/-	SUB
		10.6.05	17.6.05	24.6.05	1.7.05	8.7.05	15.7.05	22.7.05	29.7.05	5.8.05	12.8.05	19.8.05	26.8.05	DATE	REA	DATE	REA	CULTURE
1	1	0	0	0	0	26	365	655	852	948	937	893	818	19.8.05	4+ / MP	19.8.05	2+ / MP	P
	1 in 5	0	0	0	0	8	323	999	999	999	999	999	876	25.7.05	3+ / MP			P
MN01/4168	1 in 10	0	0	0	0	0	32	555	999	999	999	999	999	4.8.05	1+ / MP			P
CM01/0541	1 in 20	3	0	0	0	0	5	86	654	999	999	999	999	19.8.05	4+ / MP			P
	1 in 25	3	2	0	0	0	27	619	999	999	999	999	999	4.8.05	3+ / MP			P
Doe	1 in 30	3	0	0	0	1	2	226	999	999	999	999	999	4.8.05	2+ / MP			P
	1 in 50	0	0	0	0	0	0	5	0	0	0	0	0					
2	1	0	1	0	159	994	999	999	999	999	999	831	707	4.8.05	4+ / MP			C
	1 in 5	0	0	0	0	23	143	999	999	999	999	999	837	4.8.05	4+ / MP			P
MN02/B145	1 in 10	4	0	0	0	0	20	290	999	999	999	999	999	4.8.05	4+ / MP			P
CM02/1262	1 in 20	3	0	0	0	3	32	456	999	999	999	999	999	4.8.05	3+ / MP			P
	1 in 25	3	0	0	0	19	209	999	999	999	999	999	853	4.8.05	3+ / MP			P
Animal # 133	1 in 30	2	0	0	0	4	40	444	999	999	999	999	999	4.8.05	2+ / MP			P
	1 in 50	4	0	0	0	1	17	223	999	999	999	999	999	4.8.05	4+ / MP			P
3	1	0	0	0	0	14	655	999	999	999	999	999	992	4.8.05	4+ / MP			P
	1 in 5	2	0	0	0	0	0	0	0	0	0	0	0					
MN02/B145	1 in 10	2	0	0	0	0	3	3	21	403	853	999	999	29.8.05	2+ / MP			P
CM02/1262	1 in 20	0	0	0	0	0	1	0	0	0	0	0	0					
	1 in 25	5	0	0	0	0	0	0	0	0	0	0	0					
Animal # 141	1 in 30	3	0	0	0	0	0	0	0	0	0	0	0					
	1 in 50	3	0	0	0	0	1	0	0	0	0	0	0					
4	1	0	0	0	27	919	999	999	999	999	999	812	597	4.8.05	3+ / MP			P
	1 in 5	2	0	0	0	3	69	645	999	999	999	999	999	4.8.05	1+ / MP			P
MN02/B146	1 in 10	3	0	0	0	5	91	714	999	999	999	999	999	4.8.05	3+ / MP			P
CM02/1263	1 in 20	2	0	0	0	0	4	15	257	999	999	999	999	19.8.05	2+ / MP			P
	1 in 25	2	22	65	28	50	516	836	999	999	999	999	999	4.8.05	2+ / MP			C
Animal # 383	1 in 30	3	1	0	0	0	0	0	0	17	126	579	916	29.8.05	N	29.8.05	N	P
	1 in 50	3	2	1	0	0	0	0	0	0	0	0	0					
5	1	0	3	0	22	377	999	999	999	999	999	854	745	4.8.05	2+ / MP			P
	1 in 5	2	0	0	0	52	579	999	999	999	999	999	888	4.8.05	3+ /MP			P
MN03/2441	1 in 10	3	1	0	0	18	379	999	999	999	999	999	937	4.8.05	3+ / MP			P
CM03/0247	1 in 20	0	0	0	0	2	88	792	999	999	999	999	999	4.8.05	4+ / MP			P
	1 in 25	2	0	0	0	6	82	936	999	999	999	999	999	4.8.05	4+ / MP			P
Animal # 376	1 in 30	2	3	0	0	13	327	999	999	999	999	999	960	4.8.05	4+ / MP			P
	1 in 50	3	2	0	0	2	20	428	843	999	999	719	523	19.8.05	3+ / MP			P

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 +/-	SUB
		10.6.05	17.6.05	24.6.05	1.7.05	8.7.05	15.7.05	22.7.05	29.7.05	5.8.05	12.8.05	19.8.05	26.8.05	DATE	REA	DATE	REA	CULTURE
6	1	Insufficient faeces to culture Neat																
	1 in 5	0	6	11	999	999	999	999	999	999	764	555	283	28.7.05	1+ / MP			P
MN03/4841	1 in 10	3	4	24	332	999	999	999	999	999	982	999	667	28.7.05	2+ / MP			P
CM03/0484	1 in 20	3	0	14	228	999	999	999	999	999	957	999	644	28.7.05	4+ / MP			P
	1 in 25	1	0	37	485	999	999	999	999	999	870	852	464	28.7.05	3+ / MP			P
Avon	1 in 30	4	0	27	373	999	999	999	999	999	948	981	622	SAMPLE	MISSING	28.7.05	4+ / MP	P
	1 in 50	2	0	19	346	999	999	999	999	999	960	862	542	28.7.05	2+ / MP			P
7A.	1	0	0	0	0	0	0	9	8	8	0	0	0					
MN03/9637	1 in 5	2	0	2	0	2	0	0	0	0	2	0	0					
CM03/0913	1 in 10	0	0	0	0	0	0	0	0	0	0	0	0					
MN03/9681	1 in 20	3	0	0	0	0	1	0	0	0	0	0	0					
CM03/0927	1 in 25	2	0	0	0	0	0	0	0	0	0	0	0					
	1 in 30	1	0	0	0	0	1	0	0	0	0	0	0					
Animal # 160	1 in 50	0	0	0	0	0	0	0	0	0	0	0	0					
7B.	1	0	1	0	0	0	0	1	4	3	0	0	0					
MN03/9744	1 in 5	1	0	0	0	0	0	0	0	0	0	0	0					
CM03/0928	1 in 10	3	0	0	0	0	0	0	0	0	0	0	0					
MN03/9847	1 in 20	1	0	0	0	0	0	0	0	0	0	0	0					
CM03/0934	1 in 25	1	0	0	0	0	0	0	0	0	0	0	0					
	1 in 30	3	0	0	0	0	0	0	0	0	0	0	0					
Animal # 160	1 in 50	2	0	0	27	12	4	1	4	0	1	0	0					
8	1	0	34	794	999	999	999	999	999	934	705	457	340	28.7.05	2+ / MP			P
	1 in 5	2	0	22	358	999	999	999	999	999	938	789	612	28.7.05	4+ / MP			P
MN04/1367	1 in 10	2	0	5	140	820	999	999	999	999	999	912	734	28.7.05	1+ / MP			P
CM04/0125	1 in 20	3	0	2	79	834	999	999	999	999	776	676	369	28.7.05	2+ / MP			P
	1 in 25	3	0	10	12	999	999	999	999	999	773	533	236	28.7.05	4+ / MP			P
Animal # 2	1 in 30	3	0	0	42	522	999	999	999	999	999	999	672	28.7.05	2+ / MP			P
	1 in 50	2	0	0	59	608	999	999	999	999	999	999	716	28.7.05	2+ / MP			P
9	1	0	3	130	877	999	999	999	999	999	911	667	562	28.7.05	2+ /MP			P
	1 in 5	2	3	33	463	999	999	999	999	999	857	885	580	28.7.05	3+ / MP			P
MN04/5585	1 in 10	2	1	14	238	999	999	999	999	999	818	933	458	28.7.05	3+ / MP			P
CM04/0535	1 in 20	3	0	16	308	999	999	999	999	999	864	952	507	28.7.05	2+ / MP			P
	1 in 25	5	1	15	247	999	999	999	999	999	849	915	471	28.7.05	2+ / MP			P
Animal # 7940	1 in 30	3	0	10	214	999	999	999	999	999	909	791	351	28.7.05	4+ / MP			P
	1 in 50	8	0	8	157	999	999	999	999	999	979	730	360	28.7.05	2+ / MP			P
10	1	0	0	1	0	0	18	518	999	999	999	999	999	19.8.05	1+ / MP			P
	1 in 5	8	0	0	0	0	39	809	999	999	999	999	890	4.8.05	4+ / MP			P
MN04/5585	1 in 10	3	0	0	0	0	0	4	19	342	998	999	999	1.3.06	3+/MP			#
CM04/0535	1 in 20	0	0	0	0	0	0	0	0	2	3	1	2					
	1 in 25	7	0	0	0	0	0	2	113	724	999	999	999	1.12.05	1+ / MP			P
# 23838/2	1 in 30	3	0	0	0	0	24	395	903	999	999	999	981	19.8.05	2+ / MP			P
	1 in 50	6	0	0	0	0	0	0	0	0	0	2	0					

Unfinished at time of writing; negative at 5 weeks of a 10 week culture period

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 +/-	SUB
		10.6.05	17.6.05	24.6.05	1.7.05	8.7.05	15.7.05	22.7.05	29.7.05	5.8.05	12.8.05	19.8.05	26.8.05	DATE	REA	DATE	REA	CULTURE
11	1	0	0	0	10	321	999	999	999	999	999	913	770	4.8.05	2+ / MP			P
	1 in 5	5	0	0	0	6	172	997	999	999	999	999	999	4.8.05	3+ / MP			P
MN04/5895	1 in 10	3	0	0	0	0	52	657	999	999	999	999	999	4.8.05	4+ / MP			P
CM04/0556	1 in 20	6	0	0	0	3	110	974	999	999	999	999	875	4.8.05	4+ / MP			P
	1 in 25	0	0	0	0	2	126	857	999	999	977	964	752	4.8.05	2+ / MP			P
Animal # 1	1 in 30	5	0	0	0	0	9	255	884	999	999	999	999	19.8.05	2+ / MP			P
	1 in 50	1	0	0	0	0	1	97	889	999	999	999	999	19.8.05	3+ / MP			P
12	1	Insufficient faeces left for Neat to be cultured																
	1 in 5	4	0	0	0	0	0	2	0	2	2	1	2					
MN04/5895	1 in 10	4	0	0	0	0	0	1	0	64	690	999	999	29.8.05	N	20.9.05	N	C
CM04/0556	1 in 20	3	0	0	0	0	0	1	0	0	0	0	2					
	1 in 25	2	0	0	0	0	0	3	0	0	0	0	0					
Animal # 2	1 in 30	6	0	0	0	0	0	2	0	1	3	1	0					
	1 in 50	2	0	0	0	0	0	2	0	1	0	1	0					
13	1	Insufficient faeces left for Neat to be cultured																
	1 in 5	1	0	0	0	0	0	2	0	0	0	0	0					
MN04/5974	1 in 10	7	0	0	0	0	0	2	0	1	2	1	0					
CM04/0560	1 in 20	8	0	0	0	0	0	1	0	1	1	0	0					
	1 in 25	0	0	0	0	0	0	1	0	0	3	0	0					
Animal # 7	1 in 30	8	2	0	0	0	0	1	0	0	2	1	0					
	1 in 50	1	0	0	0	0	0	1	0	2	2	0	0					
14	1	Insufficient faeces left for Neat to be cultured																
	1 in 5	1	0	0	0	0	5	167	999	999	999	999	999	4.8.05	4+ / MP			P
MN04/5974	1 in 10	2	0	0	2	0	80	999	999	999	999	999	999	4.8.05	4+ / MP			P
CM04/0560	1 in 20	7	0	0	0	0	7	156	999	999	999	999	999	4.8.05	4+ / MP			P
	1 in 25	6	0	0	0	3	78	999	999	999	999	999	538	4.8.05	4+ / MP			P
Animal # 17	1 in 30	3	0	0	0	0	16	362	915	999	999	999	994	19.8.05	2+ / MP			P
	1 in 50	3	0	0	0	0	17	542	999	999	999	999	705	4.8.05	4+ / MP			P
15	1	Insufficient faeces left for Neat to be cultured																
	1 in 5	4	0	0	0	0	0	4	10	10	9	3	1					
MN04/6121	1 in 10	0	0	0	1	0	0	1	3	0	3	1	0					
CM04/0568	1 in 20	0	0	0	0	0	0	1	2	3	2	3	0					
	1 in 25	0	1	0	0	0	0	3	1	4	2	3	0					
Animal # 7908	1 in 30	2	0	0	0	0	0	3	3	3	2	1	0					
	1 in 50	3	0	0	0	0	0	2	1	1	3	2	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	ETOH 1	PCR +/-	ETOH 2	PCR +/-	SUB
		10.6.05	17.6.05	24.6.05	1.7.05	8.7.05	15.7.05	22.7.05	29.7.05	5.8.05	12.8.05	19.8.05	26.8.05	DATE	REA	DATE	REA	CULTURE
16	1	0	0	0	47	715	999	999	999	999	999	897	749	4.8.05	3+ / MP			P
	1 in 5	2	2	0	0	0	37	495	999	999	999	999	444	4.8.05	4+ / MP			P
MN04/6121	1 in 10	3	0	0	0	1	42	626	999	999	999	999	497	4.8.05	Tr / MP			P
CM04/0568	1 in 20	2	1	1	0	0	35	624	999	999	999	999	679	4.8.05	4+ / MP			P
	1 in 25	0	2	0	1	0	39	605	999	999	999	999	999	4.8.05	3+ / MP			P
Animal # 7920	1 in 30	2	0	0	0	51	999	999	999	999	999	999	718	28.7.05	2+ / MP			P
	1 in 50	2	2	0	0	0	0	8	4	4	5	3	1					
17	1	0	0	0	0	43	655	999	999	999	999	999	923	4.8.05	2+ / MP			P
	1 in 5	3	1	2	2	61	999	999	999	999	999	999	763	28.7.05	1+ / MP			P
MN04/6121	1 in 10	1	0	0	0	1	40	721	999	999	999	999	999	4.8.05	4+ / MP			P
CM04/0568	1 in 20	2	0	0	0	5	96	744	999	999	999	999	921	4.8.05	4+ / MP			P
	1 in 25	1	0	0	0	2	42	904	999	999	999	999	632	19.8.05	3+ / MP			P
Animal # 7984	1 in 30	3	0	1	2	10	304	999	999	999	999	999	675	4.8.05	1+ / MP			P
	1 in 50	1	2	0	0	7	215	999	999	999	999	999	971	4.8.05	3+ / MP			P
18	1	0	0	0	0	0	0	4	10	7	0	0	0					
	1 in 5	2	2	2	1	0	1	4	7	4	8	6	3					
MN04/6152	1 in 10	3	0	0	0	0	2	3	3	4	4	2	1					
CM04/0573	1 in 20	1	0	0	0	0	0	1	3	2	2	1	3					
	1 in 25	0	0	0	28	12	2	8	5	5	2	4	3					
Animal # 7906	1 in 30	1	2	0	0	2	0	0	2	2	2	2	1					
	1 in 50	2	0	0	2	0	1	2	2	1	2	2	3					
19	1	0	1	1	1	14	724	999	999	999	999	648	415	4.8.05	N	1.9.05	N	C
	1 in 5	2	1	0	1	0	0	2	3	2	2	3	2					
MN04/7928	1 in 10	2	0	0	0	0	0	1	0	1	3	2	2					
CM04/0704	1 in 20	1	0	0	0	1	1	2	2	4	2	1	1					
	1 in 25	3	0	0	0	0	0	1	3	3	0	1	2					
	1 in 30	2	0	0	0	0	0	2	2	3	0	2	1					
	1 in 50	3	1	0	2	0	0	2	2	1	3	2	3					

		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 +/-	SUB
		not read	3.1.06	9.1.06	16.1.06	23.1.06	30.1.06	6.2.06	13.2.06	20.2.06	27.2.06	6.3.06	13.3.06	DATE	REA	DATE	REA	CULTURE
20	1		0	0	0	0	0	0	0	0	0	0	0					
	1 in 5		0	0	0	0	0	0	0	0	0	0	0					
MN05/9418	1 in 10		0	0	0	0	0	0	0	0	0	0	0					
FH009	1 in 20		0	0	0	0	0	0	0	0	0	0	0					
	1 in 25		0	0	0	0	0	0	0	0	0	0	0					
	1 in 30		0	0	0	0	0	0	0	0	0	0	0					
	1 in 50		0	0	0	0	0	0	0	0	0	0	0					
21	1		0	0	0	509	999	999						23.2.06	N	1.3.06	4+/MP	P
	1 in 5		0	0	0	0	80	447	903	999	999			27.2.06	3+ /MP			P
MN05/9418	1 in 10		0	0	0	46	654	870	999	999				22.2.06	N	1.3.06	1+/MP	#
ID0904	1 in 20		0	0	0	13	444	597	999	914				22.2.06	N	1.3.06	3+/MP	#
	1 in 25		0	0	0	119	999	999	999					22.2.06	N	1.3.06	4+/MP	P
	1 in 30		0	0	0	0	0	0	0	0	0	0	0					
	1 in 50		0	0	0	0	160	596	264	946				22.2.06	N	1.3.06	4+/MP	P

Unfinished at time of writing; negative at 5 weeks of a 10 week culture period

10.2 Appendix 2

Growth of samples (as growth index) 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	ETOH 1	PCR +/-	ETOH 2	PCR +/-	SUB	CONV
		1.7.05	8.7.05	15.7.05	18.7.05	20.7.05	22.7.05	25.7.05	27.7.05	29.7.05	1.8.05	3.8.05	5.8.05	8.8.05	10.8.05	12.8.05	15.8.05	17.8.05	19.8.05	22.8.05	24.8.05	26.8.05	29.8.05	31.8.05	2.9.05	5.9.05	7.9.05	9.9.05	12.9.05	14.9.05	16.9.05	DATE	REA	DATE	REA	CULTURE	CULTURE
2	N	0	0	0			13	31	81	248	615	565	569			999			999			999			999			999			839	29.8.05	2+ / MP			N	
	N	0	0	0			26	90	229	448	723		991			999			999			999			999			999			770	29.8.05	1+ / MP			N	
MN02/B145	N	0	0	0			21	56	150	344	628		853			999			999			999			999			999			805	29.8.05	N	20.9.05	1+ / MP	N	
CM02/1262	N	0	0	0			21	50	123	298	583		787			999			999			999			999			971			791	29.8.05	N	20.9.05	1+ / MP	N	
	N	0	0	0			21	58	156	352	608		894			999			999			999			999			999			834	29.8.05	2+ / MP			N	
Animal # 133	10-1	0	0	0			0			21	58	143	299	571		860			999			999			999			999			960	1.9.05	4+ / MP			N	>50
	10-1	0	0	0			0			16	36	86	213	502	509	557			999			999			999			999			999	1.9.05	4+ / MP			N	5
	10-1	0	0	0			0			11	28	60	151	417	447	520			999			999			999			999			999	20.9.05	3+ / MP			N	>50
	10-1	0	0	0			0			10	17	38	110	356	427	533			999			999			999			999			915	20.9.05	3+ / MP			N	>50
	10-1	0	0	0			0			28	53	89	175	418	433	513			999			999			999			999			999	20.9.05	3+ / MP			N	
	10-2	0	0	0			0			0			22	50	115	259	524	495	514			999			999			999			999	20.9.05	1+ / MP			N	>50
	10-2	0	0	0			0			0			0			10	10	4	13	27	36	83	229	277	316	513		743			999	23.9.05	4+ / MP			N	1
	10-2	0	0	0			0			0			0			0			12	12	16	42	158	229	293	448		667			999	23.9.05	3+ / MP			N	>50
	10-2	0	0	0			0			0			11	3	7	37	210	286	344	541		817			999			999			999	20.9.05	2+ / MP			N	25
	10-2	0	0	0			0			0			0			20	16	49	117	345	392	429			999			999			999	20.9.05	1+ / MP			N	
	10-3	0	0	0			0			0			0			0			11	11	17	44	196	302	356	570		771			999	23.9.05	4+ / MP			N	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						
3	N	0	0	0			0			0			0			19	59	178	308	519		727			999			999			999	20.9.05	2+ / MP			N	
	N	0	0	0			0			0			0			16	80	203	381	603		805			999			999			999	20.9.05	2+ / MP			N	
MN02/B145	N	0	0	0			0			0			20	50	113	322	693		964			999			999			999			999	20.9.05	2+ / MP			N	
CM02/1262	N	0	0	0			0			0			0			0			0			0			0			0			0						
	N	0	0	0			0			0			0			126	394	480	539			999			999			999			999	20.9.05	2+ / MP			N	
Animal # 141	10-1	0	0	0			0			0			0			0			0			0			0			0			0						C
	10-1	0	0	0			0			0			0			0			0			0			0			0			0						C
	10-1	0	0	0			0			0			0			0			308	548	522	506			999			999			999	20.9.05	2+ / MP			N	N
	10-1	0	0	0			0			0			0			0			0			0			0			0			0						C
	10-1	0	0	0			0			0			0			76	257	316	360	489		691			999			999			999	20.9.05	2+ / MP			N	
	10-2	0	0	0			0			0			0			0			0			0			0			0									C
	10-2	0	0	0			0			0			0			0			0			0			0			0									C
	10-2	0	0	0			0			0			0			0			0			0			0			0									C
	10-2	0	0	0			0			0			0			0			0			0			0			0									C
	10-2	0	0	0			0			0			0			0			0			0			0			0									
	10-3	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									C
	10-3	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									C
	10-3	0	0	0			0			0			0			0			0			0			0			0									
	10-4	0	0	0			0			0			0			0			0			0			0			0									C
	10-4	0	0	0			0			0			0			0			0			0			0			0									C
	10-4	0	0	0			0			0			0			0			0			0			0			0									C
	10-4	0	0	0			0			0			0			0			0			0			0			0									C
	10-4	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	ETOH 1	PCR +/-	ETOH 2	PCR +/-	SUB	CONV
		1.7.05	8.7.05	15.7.05	18.7.05	20.7.05	22.7.05	25.7.05	27.7.05	29.7.05	1.8.05	3.8.05	5.8.05	8.8.05	10.8.05	12.8.05	15.8.05	17.8.05	19.8.05	22.8.05	24.8.05	26.8.05	29.8.05	31.8.05	2.9.05	5.9.05	7.9.05	9.9.05	12.9.05	14.9.05	16.9.05	DATE	REA	DATE	REA	CULTURE	CULTURE
4	N	0	0	0			0			293	668	649	594			999			999			999			999			999			676	1.9.05	2+ / MP			N	
	N	0	0	0			0			81	221	364	481			999			999			999			999			999			800	1.9.05	2+ / MP			N	
MN02/B146	N	0	0	0			0			0			133	346	415	496			999			999			999			999			999	20.9.05	2+ / MP			N	
CM02/1263	N	0	0	0			0			257	548	544	548			999			999			999			999			999			764	1.9.05	2+ / MP			N	
	N	0	0	0			0			127	428	552	646			999			999			999			999			999			684	1.9.05	3+ / MP			N	
Animal # 383	10-1	0	0	0			0			0			0			0			54	87	140	228	457	486	427			999			999	23.9.05	4+ / MP			N	C
	10-1	0	0	0			0			0			18	58	135	277	497	479	482			999			999			999			928	20.9.05	TR / MP			N	C
	10-1	0	0	0			0			0			347	609	582	611			999			999			999			999			863	20.9.05	3+ / MP			N	C
	10-1	0	0	0			0			0			0			12	76	146	217	386	398	374			999			999			999	20.9.05	1+ / MP			N	N
	10-1	0	0	0			0			0			179	432	443	510			999			999			999			999			878	20.9.05	3+ / MP			N	
	10-2	0	0	0			0			95	349	473	596			999			999			999			999			999			667	1.9.05	2+ / MP			N	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			27	89	123	170	280	295	303			790			999	27.9.05	4+ / MP			N	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			15	59	146	278	510	467	443			999			999			999			999	20.9.05	3+ / MP			N	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			190	332	357	370			981			999			999	23.9.05	3+ / MP			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						N
	10-4	0	0	0			0			0			0			0			0			0			0			0			0						
5	N	0	0	0			0			160	378	426	486			999			999			999			999			999			822	29.8.05	3+ / MP			P	
	N	0	0	0			0			147	368	444	529			999			999			999			999			999			844	29.8.05	2+ / MP			P	
MN03/2441	N	0	0	0			0			109	323	513	629			999			999			999			999			999			816	29.8.05	2+ / MP			P	
CM03/0247	N	0	0	0			0			92	270	381	514			999			999			999			999			999			859	29.8.05	N	20.9.05	1+ / MP	P	
	N	0	0	0			0			177	473	521	560			999			999			999			999			999			816	29.8.05	3+ / MP			P	
Animal # 376	10-1	0	0	0			0			11	43	64	144	338	357	429			999			999			999			999			917	1.9.05	2+ / MP			P	27
	10-1	0	0	0			0			0			106	245	329	426			999			999			999			999			948	1.9.05	3+ / MP			P	23
	10-1	0	0	0			0			0			66	180	284	421	804		999			999			999			999			999	1.9.05	4+ / MP			P	22
	10-1	0	0	0			0			0			58	140	216	362	717		917			999			999			999			999	20.9.05	3+ / MP			P	>50
	10-1	0	0	0			0			13	36	53	130	349	373	466			999			999			999			999			838	1.9.05	4+ / MP			P	
	10-2	0	0	0			0			0			21	59	103	214	544	542	539			999			999			999			999	20.9.05	4+ / MP			P	>50
	10-2	0	0	0			0			0			0			163	374	450	472			999			999			999			999	20.9.05	4+ / MP			P	N
	10-2	0	0	0			0			0			27	80	131	237	512	460	442			999			999			999			999	20.9.05	4+ / MP			P	N
	10-2	0	0	0			0			0			0			15	11	2	2	12	16	41	186	259	298	449		574			999	23.9.05	2+ / MP			P	13
	10-2	0	0	0			0			0			0			106	281	380	445			999			999			999			999	20.9.05	4+ / 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			11	14	4	10	109	219	298	465		667			999	23.9.05	3+ / MP			P	N
	10-3	0	0	0			0			0			0			0			0			0			0			0			0						N
	10-3	0	0	0			0			0			0			109	236	321	372	568		743			999			999			999	20.9.05	1+ / 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						

	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 +/-	SUB	CONV
		1.7.05	8.7.05	15.7.05	18.7.05	20.7.05	22.7.05	25.7.05	27.7.05	29.7.05	1.8.05	3.8.05	5.8.05	8.8.05	10.8.05	12.8.05	15.8.05	17.8.05	19.8.05	22.8.05	24.8.05	26.8.05	29.8.05	31.8.05	2.9.05	5.9.05	7.9.05	9.9.05	12.9.05	14.9.05	16.9.05	DATE	REA	DATE	REA	CULTURE	CULTURE
7A	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						
MN03/9637	N	0	0	0			0			0			0			0			0			0			0			0			0						
CM03/0913	N	0	0	0			0			0			0			0			0			0			0			0			0						
MN03/9681	N	0	0	0			0			0			0			0			0			0			0			0			0						
CM03/0927	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
Animal # 160	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						
	10-4	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						
	10-4	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						
7B	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						
MN03/9744	N	0	0	0			0			0			0			97	265	397	476			999			999			999			999	20.9.05	2+ / MP				P
CM03/0928	N	0	0	0			0			0			0			0			0			0			0			0			0						
MN03/9847	N	0	0	0			0			0			0			0			0			0			0			0			0						
CM03/0934	10-1	0	0	0			0			0			0			0			0			0			0			0			0						
	10-1	0	0	0			0			0			0			0			0			0			0			0			0						
Animal # 160	10-1	0	0	0			0			0			0			0			0			0			0			0			0						
	10-1	0	0	0			0			0			0			0			0			0			0			0			0						
	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						
	10-2	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						
	10-2	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						
	10-3	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						
	10-3	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						
	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						
	10-4	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						
	10-4	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						
8	N	0	0	15	57	106	239	576	581	599			999			999			999			999			935			760			602	19.8.05	2+ / MP			P	
	N	0	0	23	85	149	328	708		999			999			999			999			999			887			689			505	19.8.05	4+ / MP			P	
MN04/1367	N	0	0	21	76	140	303	653		999			999			999			999			999			941			764			585	19.8.05	4+ / MP			P	
CM04/0125	N	0	0	23	78	142	317	780		999			999			999			999			999			784			498			314	19.8.05	1+ / MP			P	
	N	0	0	34	109	195	392	663		999			999			999			999			999			906			740			582	19.8.05	3+ / MP			P	
Animal # 2	10-1	0	0	0			76	195	270	388	672		898			999			999			999			999			851			600	29.8.05	4+ / MP			P	>50
	10-1	0	0	0			45	117	172	278	490		744			999			999			999			999			933			755	29.8.05	4+ / MP			P	>50
	10-1	0	0	0			30	83	149	283	523		825			999			999			999			999			917			708	29.8.05	4+ / MP			P	>50
	10-1	0	0	0			22	58	105	210	483	504	547			999			999			999			999			928			712	29.8.05	1+ / MP			P	>50
	10-1	0	0	0			34	84	155	279	497		759			999			999			999			999			875			681	29.8.05	1+ / MP			P	
	10-2	0	0	0			0			58	111	150	259	488		747			999			999			999			999			923	1.9.05	4+ / MP			P	>50
	10-2	0	0	0			0			41	79	118	199	405	411	449			964			999			999			999			999	20.9.05	N	23.9.05	3+ / MP	P	>50
	10-2	0	0	0			0			43	80	111	194	398	410	467			999			999			999			999			967	1.9.05	2+ / MP			P	>50
	10-2	0	0	0			0			62	123	192	346	603		964			999			999			999			999			903	1.9.05	4+ / MP			P	>50
	10-2	0	0	0			0			72	138	205	323	504		753			999			999			999			999			946	1.9.05	4+ / MP			P	
	10-3	0	0	0			0			16	34	53	112	316	365	456			999			999			999			999			933	1.9.05	4+ / MP			P	55
	10-3	0	0	0			0			0			18	13	40	110	396	437	507			999			999			999			999	20.9.05	4+ / MP			P	>50
	10-3	0	0	0			0			0			22	28	51	152	414	436	425			999			999			999			999	20.9.05	1+ / MP			P	6
	10-3	0	0	0			0			0			13	32	86	217	494	484	507			999			999			999			999	20.9.05	4+ / MP			P	>50
	10-3	0	0	0			0			0			26	58	140	291	604		874			999			999			999			999	20.9.05	N	23.9.05	3+ / MP	P	
	10-4	0	0	0			0			0			0			0			0			12	19	45	124	317	334	339			999	23.9.05	3+ / MP			P	N
	10-4	0	0	0			0			0			0			21	78	183	305	554		782			999			999			999	20.9.05	2+ / MP			P	N
	10-4	0	0	0			0			0			0			0			0			0			0			0			0						1
	10-4	0	0	0			0			0			0			0			0			0			0			0			0						>50
	10-4	0	0	0			0			0			0			11	17	52	150	430	452	459			999			999			999	20.9.05	2+ / MP			P	

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 +/-	SUB	CONV
		1.7.05	8.7.05	15.7.05	18.7.05	20.7.05	22.7.05	25.7.05	27.7.05	29.7.05	1.8.05	3.8.05	5.8.05	8.8.05	10.8.05	12.8.05	15.8.05	17.8.05	19.8.05	22.8.05	24.8.05	26.8.05	29.8.05	31.8.05	2.9.05	5.9.05	7.9.05	9.9.05	12.9.05	14.9.05	16.9.05	DATE	REA	DATE	REA	CULTURE	CULTURE
9	N	0	0	11	23	40	99	355	502	578			999			999			999			999			999			777			559	19.8.05	2+ / MP			P	
	N	0	0	0	21	33	85	292	392	504			999			999			999			999			999			820			671	19.8.05	3+ / MP			P	
MN04/5585	N	0	0	0	23	33	79	283	408	557			999			999			999			999			999			842			651	19.8.05	4+ / MP			P	
CM04/0535	N	0	0	13	30	53	127	435	535	606			999			999			999			999			941			619			412	19.8.05	4+ / MP			P	
	N	0	0	15	43	73	167	470	499	507			999			999			999			999			974			776			601	19.8.05	4+ / MP			P	
Animal # 7940	10-1	0	0	0			19	63	98	216	486	484	467			999			999			999			999			989			721	29.8.05	2+ / MP			P	>50
	10-1	0	0	0			10	28	65	144	370	429	449			999			999			999			999			999			747	29.8.05	2+ / MP			P	>50
	10-1	0	0	0			0			113	261	338	456			999			999			999			999			999			777	29.8.05	N	20.9.05	1+ / MP	P	>50
	10-1	0	0	0			0			54	132	175	260	410	373	358			969			999			999			999			904	20.9.05	3+ / MP			P	>50
	10-1	0	0	0			0			74	166	239	358	557		677			999			999			999			999			907	1.9.05	3+ / MP			P	
	10-2	0	0	0			0			13	37	60	131	356	392	481			999			999			999			999			899	1.9.05	2+ / MP			P	>50
	10-2	0	0	0			0			11	30	45	108	316	379	492			999			999			999			999			895	1.9.05	2+ / MP			P	>50
	10-2	0	0	0			0			0			49	93	141	271	608		866			999			999			999			999	20.9.05	4+ / MP			P	>50
	10-2	0	0	0			0			10	22	37	85	302	369	463			999			999			999			999			921	1.9.05	3+ / MP			P	>50
	10-2	0	0	0			0			0			105	221	275	360	651		828			999			999			999			999	1.9.05	3+ / MP			P	
	10-3	0	0	0			0			0			0			0			59	184	423	361	557		678			999			999	20.9.05	2+ / MP			P	>50
	10-3	0	0	0			0			0			0			69	214	316	422	689		940			999			999			999	20.9.05	2+ / MP			P	5
	10-3	0	0	0			0			0			0			10	68	150	251	437	420	391			996			999			999	20.9.05	2+ / MP			P	>50
	10-3	0	0	0			0			0			0			0			290	450	274	406			990			999			999	23.9.05	2+ / MP			P	7
	10-3	0	0	0			0			0			0			28	97	184	267	433	424	387			975			999			999	23.9.05	3+ / MP			P	
	10-4	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						
	10-4	0	0	0			0			0			0			0			15	91	191	300	498		637			999			999	23.9.05	3+ / MP			P	
	10-4	0	0	0			0			0			0			0			0			0			0			0			0						
	10-4	0	0	0			0			0			0			0			0			39	178	290	338	508		635			999	23.9.05	2+ / MP			P	
18	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						
MN04/6152	N	0	0	0			0			0			0			0			0			0			0			0			0						
CM04/0573	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 # 7906	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						
	10-4	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						
	10-4	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						

		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 +/-	SUB	CONV
		not read	3.01.06	9.01.06			16.01.06			23.01.06	25.01.06	27.01.06	30.01.06	1.02.06	3.02.06	6.02.06	8.02.06	10.02.06	13.02.06	15.02.06		20.02.06			27.2.06			6.3.06			13.3.06	DATE	REA	DATE	REA	CULTURE	CULTURE
20	N		0	0			0			45	108	248	452	447		806			999			999										23.2.06	N	1.3.06	4+/MP	P	#
	N		0	0			0			0			162	253	356	505			999			999										23.2.06	N	1.3.06	4+/MP	P	#
MN05/9418	N		0	0			0			21	35	96	349	404	441	546			999			999										23.2.06	N	1.3.06	4+/MP	P	#
FH009	N		0	0			0			67	117	272	517	495		907			999			999										23.2.06	N	1.3.06	4+/MP	P	#
	N		0	0			0			0			0			0			0			0			0			0			0						
	10-1		0	0			0			0			0			0			0			0			0			0			0						
	10-1		0	0			0			0			0			0			0			0			0			0			0						
	10-1		0	0			0			0			0			0			0			0			0			0			0						
	10-1		0	0			0			0			0			0			0			0			0			0			0						
	10-1		0	0			0			0			0			0			0			0			0			0			0						
	10-2		0	0			0			0			0			0			0			0			0			0			0						
	10-2		0	0			0			0			0			0			0			0			0			0			0						
	10-2		0	0			0			0			0			0			0			0			0			0			0						
	10-2		0	0			0			0			0			0			0			0			0			0			0						
	10-2		0	0			0			0			0			0			0			0			0			0			0						
	10-3		0	0			0			0			0			0			0			0			0			0			0						
	10-3		0	0			0			0			0			0			0			0			0			0			0						
	10-3		0	0			0			0			0			0			0			0			0			0			0						
	10-3		0	0			0			0			0			0			0			0			0			0			0						
	10-3		0	0			0			0			0			0			0			0			0			0			0						
	10-4		0	0			0			0			0			0			0			0			0			0			0						
	10-4		0	0			0			0			0			0			0			0			0			0			0						
	10-4		0	0			0			0			0			0			0			0			0			0			0						
	10-4		0	0			0			0			0			0			0			0			0			0			0						
	10-4		0	0			0			0			0			0			0			0			0			0			0						
21	N		0	0			15	2	18	254	395	514	723			999			999													23.2.06	N	1.3.06	4+/MP	P	9
	N		0	0			12	2	10	200	342	465	673			999			999													23.2.06	N	1.3.06	4+/MP	P	3
MN05/9418	N		0	0			21	9	30	281	409	492	667			999			999													23.2.06	N	1.3.06	3+/MP	P	7
ID0904	N		0	0			10	2	12	182	327	500	734			999			999													23.2.06	N	1.3.06	1+/MP	P	6
	N		0	0			19	9	28	308	350	370	513			933			999			999										23.2.06	N	1.3.06	4+/MP	P	
	10-1		0	0			0			0			69	104	216	431	457		877			999			999							27.2.06	3+/MP			##	10
	10-1		0	0			0			10	17	44	236	341	459	658			999			999										23.2.06	N	1.3.06	4+/MP	P	3
	10-1		0	0			0			0			109	175	275	468			999			999										23.2.06	N	1.3.06	4+/MP	P	>50 v small
	10-1		0	0			0			0			40	69	151	423	393		767			999			999							27.2.06	4+/MP			##	48 v small
	10-1		0	0			0			11	15	32	166	247	330	488			999			999										23.2.06	1+/no rea	1.3.06	4+/MP	P	
	10-2		0	0			0			0			0			0			0			0			0			0			0						1
	10-2		0	0			0			0			0			0			0			0			0			0			0						#
	10-2		0	0			0			0			50	87	205	423	447		808			999			999							27.2.06	4+/MP			##	#
	10-2		0	0			0			0			0			0			0			0			0			0			0						54 v small
	10-2		0	0			0			0			0			17	32	118	334	337	423	775			999			999				27.2.06	4+/MP			##	
	10-3		0	0			0			0			0			0			0			0			0			0			0						
	10-3		0	0			0			0			0			0			0			0			0			0			0						
	10-3		0	0			0			0			0			0			0			0			0			0			0						
	10-3		0	0			0			0			0			0			0			0			0			0			0						
	10-3		0	0			0			0			0			0			0			0			0			0			0						
	10-4		0	0			0			0			0			0			0			0			0			0			0						
	10-4		0	0			0			0			0			0			0			0			0			0			0						
	10-4		0	0			0			0			0			0			0			0			0			0			0						
	10-4		0	0			0			0			0			0			0			0			0			0			0						
	10-4		0	0			0			0			0			0			0			0			0			0			0						

Negative at 16 weeks of 20 week incubation
Negative at 5 weeks of 10 week incubation
Note: Dark cells in quadruplicate under far right column are negative at 16 weeks of a 20 week incubation but not expected to be positive.