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Environmental survivability of *Mycobacterium avium* subsp. *paratuberculosis* (Mptb) on northern grazing properties

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Executive summary

This study primarily examined the environmental survival of the Bison and Bovine strains of Mptb provided by Queensland Department of Agriculture and Fisheries from identified cases in Queensland cattle.

The study was conducted over a fifteen month period that allowed samples to be evaluated over a full summer and winter period to determine the survival attributes for the Mptb strain isolated in Queensland at four distinct sites using site specific soils.

The identification of two Queensland beef properties in 2012 and 2013 infected with Mptb not related to introduction to the properties of Australian cattle infected with known strains occurring in southern States, altered the epidemiological paradigm for Mptb in Queensland. It was necessary to establish environmental survival parameters of the new strain to provide guidance for control programs.

In addition, the potential for atypical mycobacteria, known to be present in the Queensland environment and previously isolated from cattle lymph nodes, to confound results of the recently approved HTJ PCR for diagnosis of presence of Mptb in faeces was investigated.

The final aspect of the research related to the development of techniques to demonstrate viability of bacteria in faecal and environmental samples positive to PCR testing for bacterial DNA.

Previous work (Whittington 2001) has indicated that Mptb ovine strain is capable of surviving in cool moist environments for periods of time exceeding 12 months. Temperature flux was thought to be a critical factor in survival times for these projects. This study has established that under certain conditions the Bovine and Bison strains can survive in northern Australia for periods exceeding 12 months. The effects of temperature flux were investigated but not clearly established. The previously proposed concept of dormancy for Mptb appears to be confirmed by this project.

The study confirmed that the PCR used in diagnostic tests was specific for Mptb isolates found in cattle in Australia. Environmental mycobacterial isolates were for the most part not a factor in confounding the results and any that were replicated were able to be easily distinguished by the product size.
The project also investigated the use of supplementary tests to PCR for determining viability of Mptb isolated from the environment and faecal samples. The use of propidium monoazide techniques to confirm viability was found to be unsatisfactory. Using commercial RNA extraction test kits proved to be more reliable than bacteriological culture to demonstrate the potential for recovery of viable organisms from faeces and environmental samples.

The study provides evidence that survival of viable Mptb organisms is possible in northern regions of Queensland for a period exceeding 12 months. Survival is enhanced in intact faecal pats protected from moisture.

While the study provides evidence of survivability of the organism in faeces and the environment it does not confirm the potential for infection of stock grazing land that has previously been contaminated by Mptb in faeces. The potential for infectivity of the environment over time was not tested in this study.

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1 Background

1.1 Detections in Queensland

The detection of a bison-like strain of Mptb in a bull from a stud breeding enterprise in Queensland in 2012 initiated a control and management plan for Queensland beef grazing enterprises. Further studies identified a further Mptb infected beef grazing property in 2013. Although still low in prevalence what was unusual about these cases was that other than a single identified case near Mackay in North Queensland, Mptb in beef cattle in Australia is generally confined to wet temperate environments of southern Australia. The strains of Mptb seen in the southern states are described as cattle (C) and sheep (S). Thus, the detection of the bison (B) strain in Queensland is the first known detection of this strain in Australia. Management plans for Mptb in southern environments and management systems are supported by the 52 week viability of ovine strain of Mptb in ideal conditions in temperate climates (Whittington et al., 2004). As yet, no work has been done to determine if the same would be true for the viability of bovine and bison strains of Mptb shed onto paddocks and aggregation points in tropical beef grazing environments. There has been no examination of the survivability of the Bison (B) strain of Mptb in the environment in Australia.

1.2 Significance for industry

At the time of project development Johne's disease was a regulated disease in Australia with detections invoking significant regulatory action including quarantine of properties to assist in the control of spread of the organism to other properties. Effective disease control is reliant on knowledge of the disease including parameters for environmental persistence. The contemporary thoughts were that Mptb would be unlikely to persist for lengthy periods in the harsh environments in northern Australia due to high temperatures and low moisture levels in the majority of the grazing lands of the region.

1.3 Overarching aim of the project

The low prevalence of Mptb in Queensland, particularly in beef herds and the strict level of regulatory control when the organism was detected, usually through entry of cattle or sheep from properties in endemic areas of Australia, reduced the need for extensive research on the parameters of the organism required for control or eradication of infection from Queensland stock and properties. The project was designed to test this assumption.

2 Project objectives

2.1 Objective 1 Environmental persistence

Identify the potential variables of persistence and viability to permit science based management and control strategies for beef Mptb in northern Queensland.

2.2 Objective 2 Effect of other environmental mycobacteria

Evaluate the effect of environmental mycobacteria isolated in Queensland on the sensitivity of the HTJ PCR test recently approved by SCAHLS for Mptb diagnostic testing.

2.3 Objective 3 Development of PCR based viability tests

Investigate a PCR based viability test which will reduce time and cost for testing of samples required to establish status of animals and properties.

3 Methodology

3.1 Objective 1: Environmental persistence

Identify the potential variables of persistence and viability to permit science based management and control strategies for beef Mptb in northern Queensland.

The primary objective of this project is to determine how long Mptb can survive in the North Queensland environment under different environmental conditions. In order to achieve this objective in a BJD Protected Zone, it was decided to make use of a controlled study where the presence of viable Mptb will be predominantly determined by viable bacterial counts by culture.

3.1.1 Setting up of field stations

3.1.1.1 Preparation of spiked samples

Bovine and Bison strains of Mptb were kindly donated by the Cooper's Plain, Department of Primary Industries (QDPI) laboratories in November 2013 and stored until early March 2014 where they were inoculated into prepared Middlebrook 7H9 broth (Difco Products) supplemented with Oleic Albumin Dextrose Catalase (OADC) and Glycerol (Oxoid Products) and then further supplemented with mycobactin J (Allied Health Monitor, USA) and 3.3% egg yolk emulsion and the antibiotic mixture PANTA (Polymyxin B, Amphotericin B, Nalidixic acid, Trimethoprim and Azlocillin) called M7H9S (Harris *et al.*, 2005 and Whittington *et al*., 2013). Their identity was confirmed by conventional PCR using 4 primer sets that amplified different parts of the *IS*900 gene (Table 1). Gel electrophoresis of products yielded bands of expected sizes for each primer set (Table 1 and Fig 1). DNA products were sent for sequencing (Macrogen, Korea). All sequences from DNA products showed 100 percent similarity to Mptb strain.

Table 1: Sets of *IS*900 primers used in the primer test

Primers	Primer sequences '3-'5	Product sizes	Reference
$P90_{Forward}$	GAAGGGTGTTCGGGGCCGTCGCTTAGG	413 bp	Cousins et al., 1999 and Kawaji et al., 2007
$P91_{Reverse}$	GGCGTTGAGGTCGATCGCCCACGTGAC		
$MP10_{Forward}$	ATGCGCCACGACTTGCAGCCT	183 bp	
$MP11_{Reverse}$	GGCACGGCTCTTGTTGTAGTCG		
$921_{Forward}$	CCGCGCTGCTGGAGTTGATT	724 bp	Cousins et al., 1999
$1051_{Reverse}$	CCGAATCCGGGCATGCTCAG		
IS900F	AAGGCCGACCATTACTGCAT	460 bp	In-house design
IS900R	CCAGCGCCGAAAGTATTCCAG		

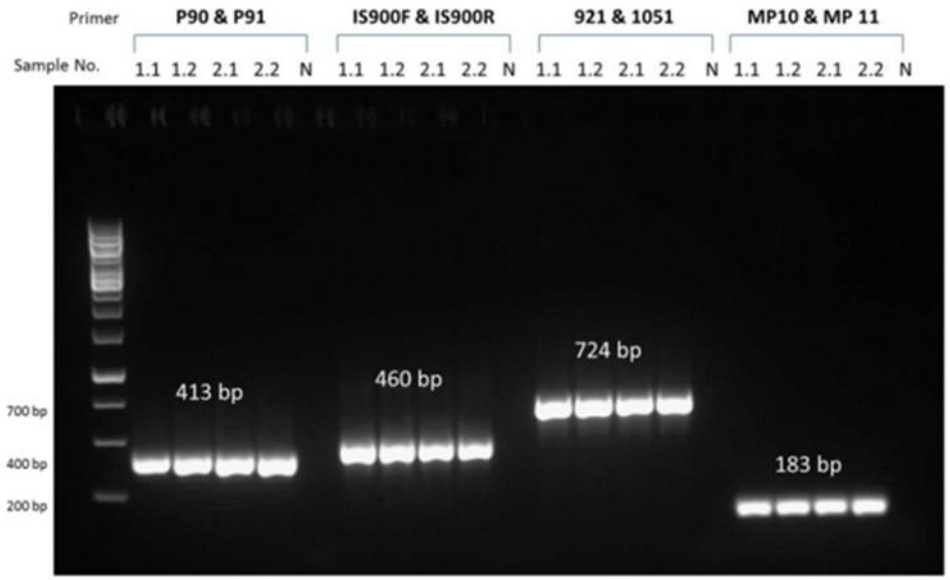


Fig 1: positive samples of *Mptb* were tested using P90 & P91 primers, IS900F and IS900R primers, 921 and 1051 primers and MP10 & MP11 primers.

Eight millilitres of bovine and bison Mptb was added respectively to 2 L of M7H9S and cultured until visible growth was obtained. Furthermore each strain was seeded into 250 mL of M7H9S to maintain cultures. Regular checking for contaminated cultures was carried out by sub-culturing an aliquot of 100 µL from each bottle onto blood agar base (Accumedia) supplemented with 5% citrated sheep blood and supplemented Middlebrook 7H11 agar (M711S) slopes incubated in air at 37°C. Any growths were microscopically examined for acid-fastness using Ziehl-Neelsen staining method and also the Gram's staining method for other bacteria.

Once visible growth had been obtained in the initial broth cultures, the species confirmation and quantification of the cultured bacteria was carried out by qPCR using a modified version of the quantitative real time Polymerase Chain Reaction (*q*PCR) of University of Sydney High Throughput - Johne's test (HT-J) PCR that detects the sequence peculiar to Mptb of the multi-copy *IS*900 gene (refer to 3.1.3).

As the bacteria only reached a maximum concentration of 10^5 bacteria/mL broth, the bacteria were concentrated by centrifugation at 3000 rpm for 30 minutes and the sediment was suspended to 250 mL volume in fresh liquid M7H9S to make a final concentration as described below. Mptb bovine and bison strains were mixed with cattle faeces which was sterilised twice by autoclaving at 121°C for 30 minutes. The mixed process was in a Class 2 Biosafety cabinet for the first and second sets of spiked samples. Using a sterile ice cream scoop, 20 mL of mixed faeces was added on the top of a 100 mL sterile soil sample (approximately 70g but varied due to the soil type) in hydroponic pots (50mm width x 50 mm length x 70 mm height) to make final concentrations of 2.4×10^5 bacteria/mL for bovine strain and 3.2×10^4 bacteria/mL for bison strain of Set 1 samples and 1.8×10^7 bacteria/mL and 7.9×10^6 bacteria/mL of set 2 samples.

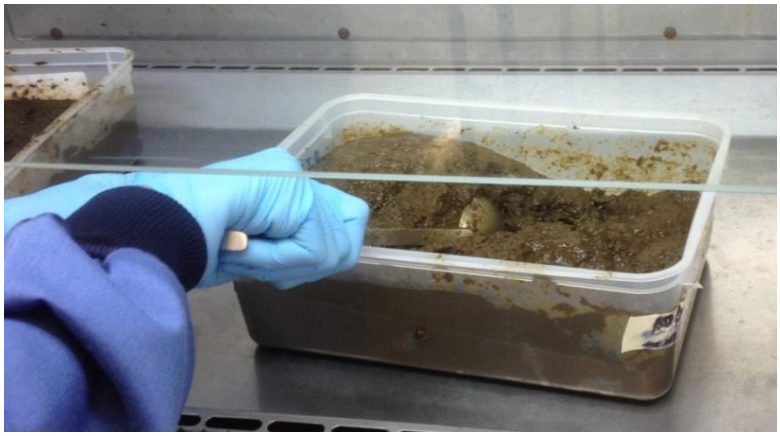


Fig 2: *Mycobacterium avium* subspecies *paratuberculosis* (Mptb) bovine and bison strains were mixed with sterile cattle faeces.

3.1.1.2 Selection of sites and soil analysis

Four sites were selected to represent different climatic conditions in northern Queensland. The James Cook University farm sites at Townsville and Malanda were chosen to represent coastal tropical and cool tropics (dairy area). The third site was the James Cook owned farm "Fletcherview" situated to the South West of Charters Towers representing the dry tropics (beef) grazing systems. The final site is in central Queensland at a private property at Dingo and represents the environment typical of the beef stud and fattening enterprises.

Soil samples were collected by spade scrapings from the surface of the uppermost parts of the top soil at four sites (Dingo, Malanda, Townsville and Charters Towers). The soil samples were submitted for chemical analysis to the Incitec Pivot Nutrient Advantage Laboratories, Werribee, Victoria. Soil pH, electrical conductivity, chloride content, exchangeable cation contents, particle size, organic carbon, plant-available phosphorus, potassium, nitrate nitrogen, sulphate, sulphur, plant micronutrients (copper, iron, manganese, zinc), and a phosphorus buffer index were tested. Detailed analysis is presented in Appendix 1

3.1.1.3 Site layout

One hundred and sixty faecal-soil samples as part of sample set 1 were placed at each site in July and August 2014. A repetition of "Sample Set 1", named "Sample Set 2" was placed at all the sites in January 2015. This was to check whether there was a seasonal effect on Mptb viability. They consisted of 80 of each Mptb strain which was further divided into 40 wet and 40 dry pots. The wet pots were maintained using a hydroponic system. Each of the two wet containers was supplied with water from an elevated 200 L supply water tank via tubing and the flow was controlled by a float valve. The float valve maintained the water depth at about 1-2 cm. Each tray was also covered with mosquito mesh. This was done to maintain sample integrity and avoid insect dissemination of Mptb. Although the stations were placed in livestock-free areas, there was still a possibility of interference from pets and wildlife. This was avoided by placing all the trays on shelving in a chicken coop (Fig 3). Automatic weather data loggers (EL-USB-2 Lascar Electronics Inc., UK) were installed at the sites (position indicated by the yellow arrow in Fig 3). The loggers were configured to record the local temperature and humidity, initially every six hours.

Each month a total of 8 samples namely, two samples of each variable (bovine and bison Mptb and wet and dry) were collected and submitted to James Cook University, Townsville

for analysis of the survivability of Mptb. At the same time data from the automatic weather data loggers (EL-USB-2 Lascar Electronics Inc., UK) were downloaded and compared to the meteorological data from that area. Due to the high fluctuations noted, it was decided in September 2014 to increase the reading frequency to hourly to more accurately collect temperature and humidity extremes and duration.

Fig 3: an example of the setup of the controlled field site situated at Malanda.

3.1.2 Monthly culturing of soil-faecal samples

Both *q*PCR and viable bacterial counts were performed on the mixed faeces. Since egg yolk emulsion had been added to M7H9S, the most probable number technique (MPN) that was developed as indicated in the technical manual provided in the Milestone 2 report had to be adjusted slightly. This was done by adding 50 µl of tetrazolium bromide [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT)] to the cultures 48 hours before reading the results. Contaminating bacteria reduce the tetrazolium from an uncoloured substance to red coloured formazan. Furthermore, it was also decided to run concurrently with the MPN a viable plate count. In brief, 100µL of each sample was spread onto 3 slants (2 7H11S and 1 Lowenstein-Jensen) and two plates with 7H11S. One set of plates was overlaid with plain bacteriological agar and one set of plates was overlaid with un-supplemented 7H11 agar. This was to provide moisture and protection to the colonies.

3.1.3 Development and utilisation of a manual PCR

Mptb positive samples (Bison and Bovine strains) as positive control for this study had been obtained in the initial broth cultures, the species confirmation of the cultured bacteria was carried out testing for the presence of DNA unique to Mptb with the four sets of primers in

Table 1 using conventional PCR (see above) and quantitative real time PCR (qPCR) was used to count the number of bacteria in the samples. For qPCR, a standard curve was made from Mptb genomic DNA reagent which is supplied in freeze-dried form the School of Veterinary Science, University of Sydney. TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0) was used to reconstitute the DNA standard samples and were prepared at concentrations of 2 µM, 200 nM, 20 nM, 2 nM and 200 pM for making a standard curve (10^4 to 10^0 bacteria). SensiFAST SYBR Lo-ROX kit (Bioline, Australia) was used for optimisation of qPCR parameters. DNA was amplified by real time PCR (RotorGene Q, Australia) using *IS*900 primers. The primers were described by Kawaji et al., 2007 (forward primer 5' ATG CGC CAC GAC TTG CAG CCT 3', reverse primer 5' GGC ACG GCT CTT GTT GTA GTC G 3' and product size 183 bp). qPCR Mastermix was used to make a total volume of 20 mL: 0.8 µL of 10 µM forward primer, 0.8 µL of 10 µM reverse primer, 10 µL of SensiFAST reaction mix, 4.4 µL of molecular grade water and 4 µL of template DNA. The reaction conditions were optimised using initial denaturation at 95ºC for 10 min for 1 cycle, 40 cycles of denaturation at 95ºC for 30 sec, annealing at 68ºC for 30 sec, extension at 72ºC for 30 sec, and melting curve at 65-95ºC.

3.1.4 Detection of Mptb in monthly samples

3.1.4.1 DNA detection

MagMAX Total Nucleic Acid Isolation Kit was used for DNA extraction and purification according to the manufacturer's instructions. 0.3 g of mixed samples (soil and faecal samples) and 1 mL of PBS buffer were added into 2 mL sterile screw cap tube and the tube was placed into mechanical cell disruptor (Mini-Beadbeater-96 bead beater) and then, ran at the maximum speed (36 oscillations/sec) for 90 sec. 235 µL of Lysis/Binding mixed solution was added to the tube. The tube was put into a mechanical cell disruptor and run at the maximum speed for 90 sec. The tube was centrifuged at 16000 x g for 1 min and supernatant was removed to a new sterile 1.5 mL tube, and then centrifuged at 16000 x g for 3 min. 230 µL of supernatant was transferred to a new sterile 1.5 mL tube and 130 µL of 100% isopropanol was added, and then mixed for 1 min. 20 µL of bead solution was added and mixed for 5 min. The tube was placed in the magnet and left for 5 min. The supernatant was discarded and the tube was removed from the magnet. 150 µL of wash solution 1 was added and mixed for 1 min. The tube was placed in the magnet and left for 1 min. The supernatant was again discarded and the tube was removed from the magnet. This wash solution 1 step was repeated twice. 150 µL of wash solution 2 was added and mixed for 1 min. The tube was placed in the magnet and left for 1 min. The supernatant was discarded and the tube was removed from the magnet. This wash solution 2 step was repeated twice. The tube was left to dry for 2 min and 50 µL of 65ºC Elution Buffer was added to the tube, and then mix well. The tube was put in the heat block at 65ºC for 1 min. The tube was removed from the heat block and mixed well (this step was repeated 3 times). The tube was placed in the magnet and left for 30 min. The supernatant (extracted DNA) was removed into the new 1.5 mL sterile tube. Then, SensiFAST SYBR Lo-ROX kit (Bioline, Australia) was used for manual qPCR (see 3.1.3).

3.1.4.2 RNA detection

0.05 g of faecal sample was weighted and transferred into 2 mL sterile screw cap tube. 350 µL of RNA Lysis buffer (RLA) and 0.3 g of 0.1 mm Zirconia/Silica Beads were added. The tube was placed into the mechanical cell disruptor (Mini-Beadbeater-96 bead beater) and ran at the maximum speed (36 oscillations/sec) for 90 sec. 350 µL of RNA Dilution Buffer (RDA) was added and mixed well, then centrifuged at 16000 x g for 10 min. Supernatant was transferred to a new 1.5 mL sterile tube. 200 µL of 95% Ethanol was added and mixed well. 350 µL of mixed sample was transferred to a spin basket, centrifuged at 16000 x g for 1

min and discarded the column. The spin basket was washed with 600 µL of RNA wash Solution (RWA), centrifuged at 16000 x g for 1 min and the liquid was discarded. 50 µL of DNase solution mix was added into the spin basket and incubated at room temperature for 15 min. 200 µL of Dnase Stop solution (DSA) was added, centrifuged at 16000 x g for 1 min and discarded the column. Then, the spin basket was washed with 600 µL of RWA, centrifuged at 16000 x g for 1 min and discarded the column. 250 µL of RWA was added, centrifuged at 16000 x g for 2 min and discarded the column. The spin basket was removed to a new 1.5 mL sterile tube and 100 µL of Nuclease Free Water was added to the spin basket. Then, the tube was centrifuged at 16000 x g for 1 min to extract RNA.

3.1.4.3 Quantitative Reverse Transcriptase real time PCR (qRT-PCR)

SensiFAST SYBR Lo-ROX One Step kit (Bioline, Australia) was used for optimisation of qPCR parameters. RNA was amplified by real time PCR (RotorGene Q, Australia) using IS900 primers. The primers were described by Kawaji et al., 2007 (forward primer 5' ATG CGC CAC GAC TTG CAG CCT 3', reverse primer 5' GGC ACG GCT CTT GTT GTA GTC G 3' and product size 183 bp). qRT-PCR mastermix were included in a total volume of 20 ml: 0.8 µL of 10 µM forward primer, 0.8 µL of 10 µM reverse primer, 10µL of SensiFAST reaction mix, 0.2 µL of Reverse Transcriptase, 0.4 µL of RiboSafe RNase Inhibitor, 3.8 µL of molecular grade water and 4 µL of template RNA. The reaction conditions were optimised using Reverse Transcriptase step at 45 ºC for 10 min, initial denaturation at 95ºC for 10 min for 1 cycle, 40 cycles of denaturation at 95ºC for 30 sec, annealing at 68ºC for 30 sec, extension at 72ºC for 30 sec, and melting curve at 65-95ºC.

3.1.4.4 Propidium monoazide (PMA)

0.05 g of faecal sample was weighed and put into a 0.2 mL sterile screw cap tube. 500 µL of PBS was added to the tube. The tube was placed into a mechanical cell disruptor (Mini-Beadbeater-96 bead beater) and ran at the maximum speed (36 oscillations/sec) for 90 sec. 1.25 µL of PMA (20 mM stock solution) was added for a final concentration of 50 µM and mixed well. The tube was incubated in the dark for 5 min at room temperature. PMA was activated by blue LED light of 460 - 490 nm to cross-link PMA to DNA for 15 min. Then, DNA was extracted using MagMAX kit. Extracted DNA was used for qPCR using a qPCR protocol above.

The results from DNA, RNA and PMA detection methods were compared to determine effective detection method and survivability of Mptb in the environmental samples.

3.1.4.5 Quality control of PCR results

Two sets of blind samples were sent to School of Veterinary Science, University of Sydney and tested using HT-J PCR and compared the results with manual qPCR results. 8 samples of faeces mixed with Mptb, 8 Mptb DNA samples, 2 negative faecal samples and 2 DNA negative samples were sent in the first set. 2 sets of 5 samples of a serial dilution (105 to 00) of faeces mixed with Mptb were sent in the second set.

3.2 Objective 2: Effect of other mycobacteria

Evaluate the effect of environmental mycobacteria on the sensitivity of the HTJ PCR test

During the Brucellosis and Tuberculosis Eradication Campaign (BTEC) and until 2013, a large number of environmental *Mycobacterium* cultures were stored at the Queensland Department of Agriculture, Fisheries and Forestry (QDAFF), formally QDPI laboratory site at Townsville. Permission was granted to use these cultures in the project, with the proviso that we would send all recovered pure viable cultures to the DAFF Cooper's Plains Laboratory.

The primary aim was to check whether the environmental mycobacteria will cross-react or produce *IS*900-like sequences in the PCR test as there was some evidence in the literature that this was possible (Cousins et al., 1999). These bacteria are common in faeces and in the environment. Some of these environmental mycobacteria may also be unique to the environment of tropical biomes.

In late February 2014, 88 *Mycobacterium* species stored on beads at -80°C were collected. Some were identified as belonging to various *Mycobacterium* species, including, *M. avium, M. fortuitum*, *M. marinum* and *M. intracellulare.* Most, however, were not identified to species level. Bacterial coated beads of each stored isolate was cultured on purchased Lowenstein-Jensen (LJ) slopes with glycerol supplement (Oxoid) and incubated at 37°C (78 on 3/3/14 and the remaining 10 on 8/4/14). Cultures that did not grow on the LJ slopes were also placed on Middlebrook 7H11 agar. The cultured bacteria were examined microscopically by Ziehl-Neelsen staining method to determine acid-fast status. A picture of one of the cultures is shown in Fig 4. Furthermore, seven samples of soil were gathered from each station, then decomtaminated and cultured for *Mycobacterium* species using the Mptb soil culture protocol (Appendix 2).

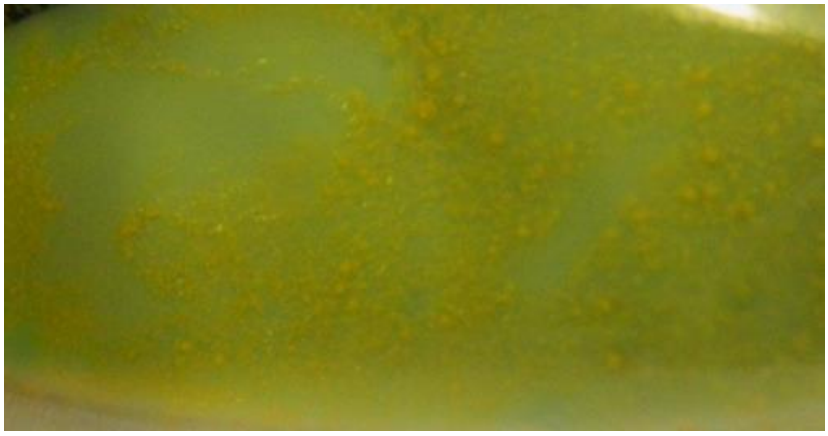


Fig 4: Small yellow colonies from Sample number 26 that yielded a PCR product with 921 and 1051 primers.

Due to the non-viability or severe contamination of some of the stored cultures, only 53 yielded acid-fast bacteria. They were tested with conventional PCR for *IS*900 sequences using 4 different primer sets, the sequences are shown in Table 1. All these primers amplified a different portion of the *IS*900 gene.

The cell walls of the bacteria were mechanically disrupted by suspending colonies in a 2 mL sterile screw cap tube containing 300 µL of lysis buffer and 0.3 g of zirconia/silica beads and beating them in a Mini-Beadbeater-96 bead beater at maximum speed (36 oscillations/sec) for 90 seconds. After centrifugation at 16 000xg for one minute the supernatant was transferred to a mini-column (Wizard SV Genomic DNA Purification System) and the manufacturer's instructions followed.

For conventional PCR, each 25 µL reaction consisted of template DNA, a primer set (reverse and forward primers) (Table 1) and PCR Master mix (GoTaq® Hot Start Polymerase, Promega, Australia) in the proportions recommended by the manufacturer. The thermocycle parameters consisted of an initial denaturing step at 95°C for 4 min, followed by 35 cycles at 94°C for 30s (DNA denaturing) and 60 or 70°C for 30s (primer annealing) depending sets of primers (see Table1) and 72°C for 1 min (nucleotide elongation) and finally 5 min at 72°C (final elongation). The PCR products were electrophoresed on 0.8%

agarose gels and the product size compared to a DNA ladder containing known sizes of DNA.

Any of the strains whose DNA was amplified i.e. produced *IS*900-like sequences were submitted to Macrogen, South Korea for sequencing. The oligonucleiotide sequences were checked for complementary sequences on the nucleotide BLAST program and database of the NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

3.3 Objective 3: Investigate a PCR based viability test

The aim of Objective 3 was to test molecular methods that could only detect nucleotides from viable bacteria. This would reduce time and cost associated with false positive results from non-viable bacteria. Two methods were selected, the detection of RNA with the Mptb-specific IS900 sequence and propidium monoazide treatment of samples prior to extraction of the DNA. In comparison to DNA, RNA has only a short lifespan, direct extraction of RNA would only be possible if viable bacteria were present prior to the extraction process. Propidium monoazide is only able to penetrate the outer membrane of dead bacteria, and when light activated, it will irreversibly bind DNA. Thus any DNA extracted from the sample after this treatment would have had to originate from viable bacteria.

Extraction of DNA for exposure to propidium monoazide and extraction of Mptb specific rRNA was performed as outlined in Objective 1. The method is briefly outlined below. These results were compared to viable counting methods using culture.

3.3.1 RNA detection

After the cells in 0.05 g of sample diluted in 350 µL of RNA Lysis buffer were mechanically disrupted by agitating them with 0.3 g of 0.1 mm Zirconia/Silica Beads. It was centrifuged and the supernatant placed in a spin column. The Promega RNA extraction kit was used and the manufacturer's instructions followed. The extracted RNA was then subjected to a qPCR using the IS900 primers as described by Kawaji et al., 2007 (forward primer 5' ATG CGC CAC GAC TTG CAG CCT 3', reverse primer 5' GGC ACG GCT CTT GTT GTA GTC G 3' and product size 183 bp). qRT-PCR mastermix were included in a total volume of 20 mL: 0.8 µL of 10 µM forward primer, 0.8 µL of 10 µM reverse primer, 10µL of SensiFAST reaction mix, 0.2 µL of Reverse Transcriptase, 0.4 µL of RiboSafe RNase Inhibitor, 3.8 µL of molecular grade water and 4 µL of template RNA. The reaction conditions were optimised using Reverse Transcriptase step at 45 ºC for 10 min, initial denaturation at 95ºC for 10 min for 1 cycle, 40 cycles of denaturation at 95ºC for 30 sec, annealing at 68ºC for 30 sec, extension at 72ºC for 30 sec, and melting curve at 65-95ºC.

3.3.2 Propidium monoazide (PMA) inactivation of naked DNA

PMA™ (propidium monoazide) is a high affinity photoreactive DNA binding dye invented by scientists at Biotium. The dye is weakly fluorescent by itself but becomes more fluorescent after binding to nucleic acids. It preferentially binds to dsDNA with high affinity. Upon photolysis, the photoreactive azido group on the dye is converted to a highly reactive nitrene radical, which readily reacts with any hydrocarbon moiety at the binding site to form a stable covalent nitrogen-carbon bond, thus resulting in permanent DNA modification. The dye is nearly completely cell membrane-impermeable, and thus can be selectively used to modify only exposed DNA from dead cells while leaving DNA from viable cells intact. This feature makes the dye highly useful in the selective detection of viable pathogenic cells by quantitative real-time PCR in the presence dead cells whose DNA has been PMA-modified and thus cannot be amplified. Since Biotium first developed PMA dye, there have been numerous publications on the use of the dye in pathogenic bacterial detection related to food

and water safety, medical diagnosis and biodefense; download the PMA Reference List. PMA dye is also available in H2O (40019), a convenient ready-to-use format.

For this method, 0.05 g of the faecal sample was weighed and put into 0.2 mL sterile screw cap tube. 500 µL of PBS was added to the tube. The tube was placed into mechanical cell disruptor (Mini-Beadbeater-96 bead beater) and ran at the maximum speed (36 oscillations/sec) for 90 sec. A final concentration of 50 µM PMA was added and mixed well. The tube was incubated in the dark for 5 min. at room temperature. PMA was activated by a ring of blue LED lights of 460 - 490 nm to cross-link PMA to DNA for 15 min. Remaining DNA was extracted using MagMAX kit and subjected to qPCR using the protocol as described in Objective 1

4 Results

4.1 Objective 1 Environmental persistence in northern Queensland

4.1.1 Soil analysis

Results from soil analysis showed Dingo soil was found to have a neutral pH with pH 6.8 while Townsville soil was acidic (pH 5.7), and the remaining two samples (Malanda and Charters Towers) with soil pH of less than 5.5 were strongly acidic. Chloride in all four soil samples was low (21 – 48 mg/kg) which demonstrated that salinity was not an issue for bacterial survival and growth. High nitrate nitrogen and high plant-available phosphorus contents were found in Malanda and Charters Towers soil samples that demonstrated high electrical conductivity. (Table 2) Townsville and Charters Towers soil samples had low soil water-holding capacities and were likely to produce relatively rapid drying conditions in the topsoil after rain. While, heavier textures and higher soil moisture retention characteristics were found in Dingo and Malanda soil samples that showed these sites could hold soil moisture longer into the dry season. The soil characteristics are typical of the soils of the respective regions.

Table 2. Soil properties of selected sites

	pH	Conductivity	Chloride	Org Carbon	Nitrate Nitrogen	Phosphorus
Malanda	5.4	0.30	21	3.4	140	42
Townsville	5.7	0.14	48	1.1	48	11
Ch Towers	5.5	0.41	26	2.4	190	24
Dingo	6.8	0.16	32	7.0	7.1	48
Desirable Range	6 - 7	<0.25	<300	>1.8	>10	10 - 60

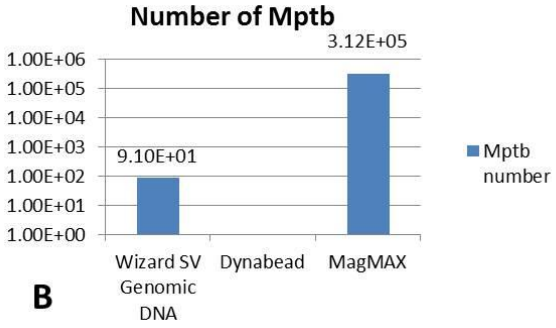
4.1.2 Methodology for detection of Mptb

When using stock spiked solutions, qPCR results showed that the number of bacteria from MagMAX magnetic bead kit was about 3 log 10 greater than Wizard SV Genomic DNA kit (Fig 5B) and significantly different from Wizard SV Genomic DNA kit and Dynabead kit ($P < 0.05$). Dynabead kit did not detect bacterial DNA from the samples assayed. For soil testing, qPCR results determined that different extracted DNA of Mptb from 4 different mixed soil samples can be detected using MagMAX kit and show high recovery bacterial number from the samples (Fig 5).

From this work it was decided to utilize Mag MAX extraction protocols for all work to determine the presence of DNA or RNA in trial samples.

Samples	Wizard	Dynabead	MagMAX
1	1.02E+02	0	6.13E+05
2	8.23E+01	0	1.05E+04
3	1.12E+02	0	6.17E+05
4	6.76E+01	0	7.83E+03
Average	9.10E+01	0	3.12E+05

A



B

Fig 5: (A) Number of Mptb from qPCR using SV, Dynabead and MagMAX test kit. (B) Graph shows qPCR results of Mptb number from extracted DNA from 3 different DNA extraction test kits

4.1.3 Initial content of trial samples

Assessment was made of a number of mixed samples prior to deployment to assess the validity of the process of mixing the Mptb infected stock solutions with the sterilized faeces and layering on top of the soil samples. It is noted as in Table 3, that there were variable numbers of bacteria in each sample pot. To counter this difference each monthly sample consisted of two random pots from the site being processed and the result reported as the highest product after processing.

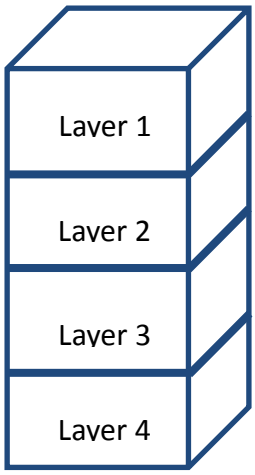
Table 3: Bacterial counts using quantitative real time Polymerase Chain Reaction (qPCR) on extracted DNA from 4 different soil samples mixed with Mptb.

Locations	Averages (Bacteria)	Ranges (Bacteria)
Dingo	3.22×10^5	1.79×10^4 - 9.12×10^5
Malanda	1.31×10^5	6.83×10^3 - 2.83×10^5
Townsville	7.09×10^5	4.83×10^5 - 1.02×10^6
Charter Towers	1.94×10^5	5.10×10^4 - 3.33×10^5

4.1.4 Movement of bacteria within the faeces/soil test samples

To determine the effect of holding some of the trial material in a high moisture condition for the duration of the trial, samples were 'layered and examined for presence of Mptb other than in the uppermost faecal layer. Small numbers of bacteria were able to be demonstrated to penetrate the soil column in both wet and dry conditions (Table 4).

Table 4: qPCR results of the bacterial number from different soil layers



Samples	Dingo	Malanda	Townsville	Charters Towers
Dry Layer 1	1.01E+04	2.92E+03	1.36E+04	9.81E+04
Dry Layer 1	9.18E+03	3.84E+03	1.36E+04	7.44E+04
Dry Layer 2	2.49E+01	1.32E+01	1.06E+02	2.67E+01
Dry Layer 2	1.98E+01	6.37E+00	1.14E+02	1.26E+01
Dry Layer 3	7.23E+00	1.46E+01	3.53E+01	7.96E+00
Dry Layer 3	6.22E+00	1.59E+01	3.78E+01	1.57E+01
Dry Layer 4	7.90E+00	1.69E+01	3.62E+01	7.39E+00
Dry Layer 4	1.04E+00	1.73E+01	2.21E+01	1.10E+01
Wet Layer 1	9.26E+02	2.81E+02	5.10E+04	1.13E+04
Wet Layer 1	9.19E+02	1.08E+03	3.48E+04	1.54E+04
Wet Layer 2	2.83E+00	2.19E+01	1.32E+01	4.56E+03
Wet Layer 2	9.71E+00	2.00E+01	1.35E+01	6.07E+03
Wet Layer 3	5.39E+00	1.94E+01	2.33E+01	1.56E+01
Wet Layer 3	3.00E+00	1.81E+01	9.58E+00	2.07E+01
Wet Layer 4	6.47E+00	2.38E+01	2.21E+01	2.40E+01
Wet Layer 4	6.84E+00	3.06E+01	4.95E+00	2.82E+01

These results demonstrated that Mptb can move down from soil surface to the bottom layer of soil in both dry and wet conditions.

Some water samples were also collected from the trays held in water baths. qPCR results determined that Mptb had moved from the top of the soil layer down into the water (Table 5). Culture confirmed this in one instance.

This result indicated that without force of water movement the Mptb had dissipated in the single case from the sample pots to the water contained within the tray. It is possible some contamination of the sides of the pots occurred during transport to the various sites as well so the findings must be viewed with caution.

Table 5: Water samples collected after 6 months from Dingo and Malanda and 12 months from Townsville and Charters Towers were tested using qPCR and culture

Location and strain	qPCR	Culture
Dingo Bison	2.97E+02	Negative
Dingo Bovine	2.23E+02	Negative
Malanda Bison	9.61E+02	Negative
Malanda Bovine	3.39E+03	Negative
Townsville Bison	4.85E+01	Negative
Townsville Bovine	2.28E+01	Negative
Cht Tws Bison	3.41E+03	**Positive**
Cht Tws Bovine	3.83E+04	Negative

4.2 Objective 1 and Objective 3: Detection of viable Mptb over time

4.2.1 Survival results for Mptb in all locations

The terminology set 1 and set 2 was developed to identify the first series of spiked samples deployed in mid year 2014 (set 1) and the second set (set 2) of spiked samples deployed in January 2015.

For ease of presentation only data for DNA is presented in the following figures. Full details of the culture and results of all samples can be found provided by contacting the authors.

Comparison of DNA results showed that Mptb in wet condition started to die after 3 months of the study (Fig 6) while Mptb in dry conditions were still be able to be detected after 16 months of this study (Fig 7).

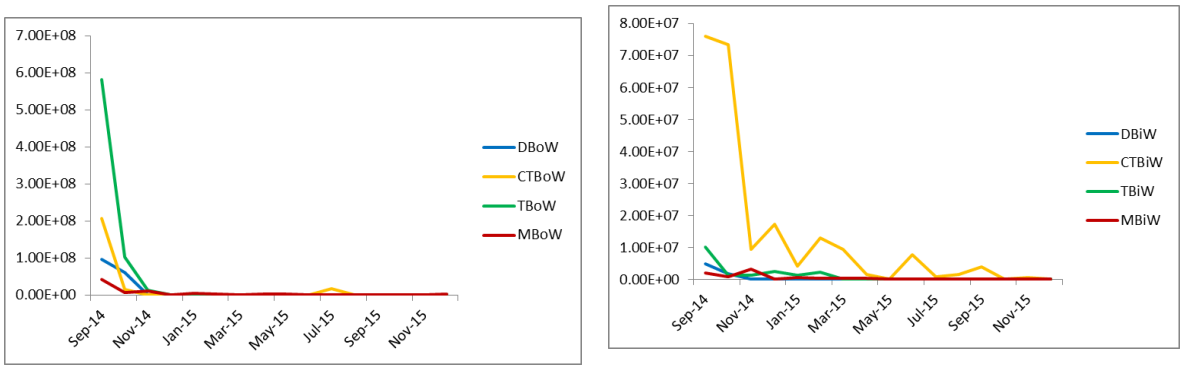


Fig 6: Mptb Bovine strain (left) and Bison strain (right) held under wet conditions (Set 1)

Similar results were obtained for the second set (set 2) of spiked samples under wet and dry conditions (Fig 8 and Fig 9). It is clear that the period of survival for wet samples was less than for the initial set deployed during cooler months.

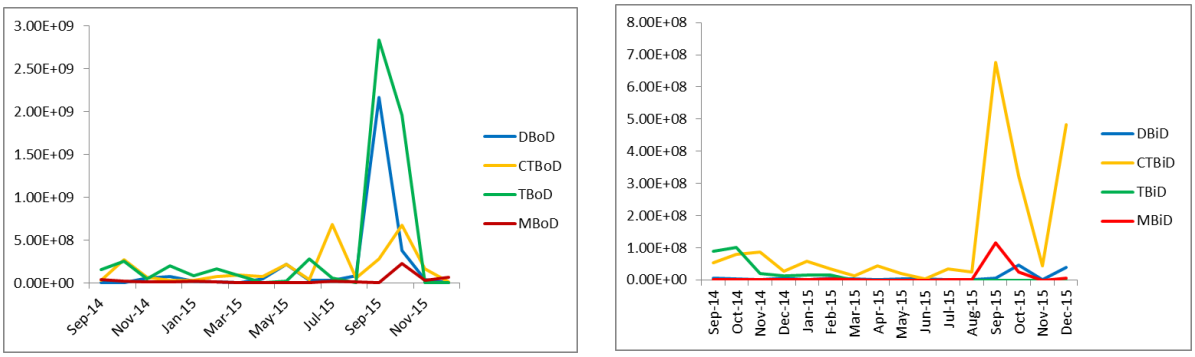


Fig 7: Mptb Bovine strain (left) and Bison strain (right) held under dry conditions (Set 1)

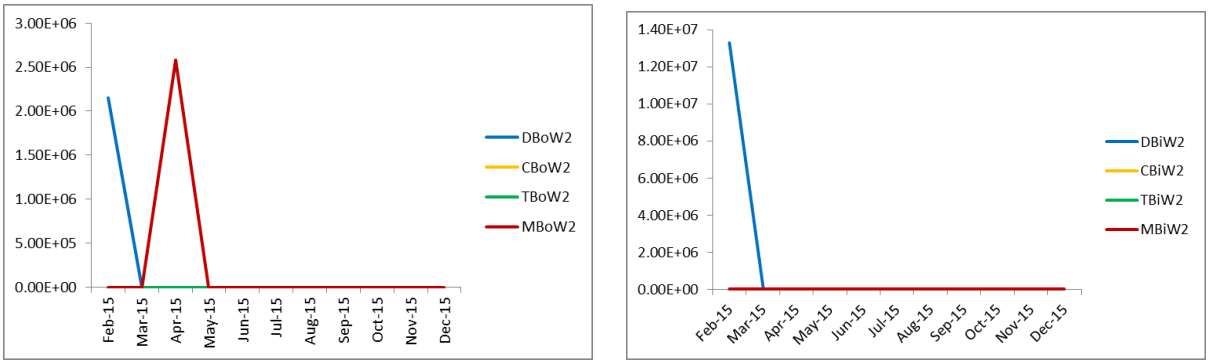


Fig 8: Mptb Bovine strain (left) and Bison strain (right) held under wet conditions (Set 2)

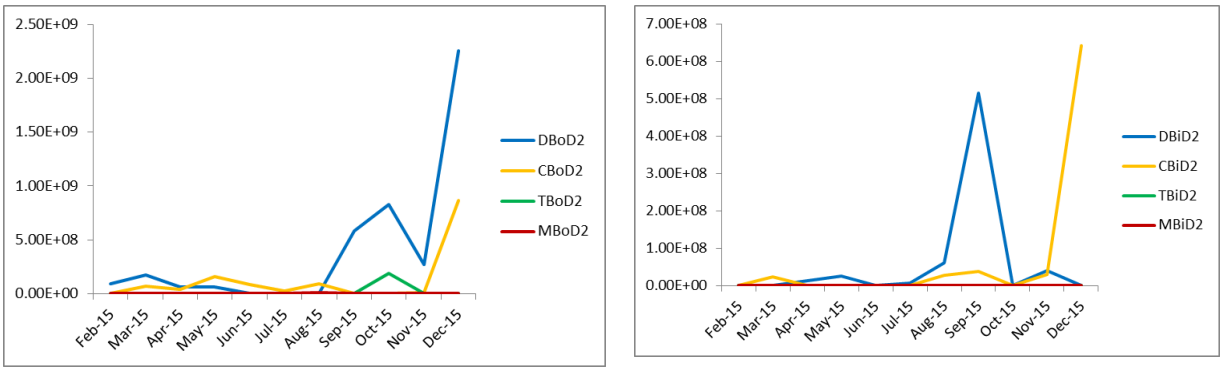


Fig 9: Mptb Bovine strain (left) and Bison strain (right) held under dry conditions (Set 2)

Similarly qPCR results from DNA, RNA and PMA detections showed that Bison and Bovine strains of Mptb had similar results in dry and wet conditions. (Table 6)

Results from PCR tests also showed numbers of bacteria from DNA, RNA and PMA tests were statistically different between all locations ($P<0.05$). In dry and wet conditions, numbers

of bacteria from DNA, RNA and PMA tests were statistically different between wet and dry samples (P<0.05).

A

Set 1 (16 months)

Locations	DNA	RNA	PMA	Total
Dingo	2/4 (50%)	0/4 (0%)	0/4 (0%)	2/12 (16.67%)
Malanda	3/4 (75%)	3/4 (75%)	2/4 (50%)	8/12 (66.67%)
Townsville	0/4 (0%)	0/4 (0%)	0/4 (0%)	0/12 (0%)
Charter Towers	3/4 (75%)	0/4 (0%)	3/4 (75%)	6/12 (50%)
Total	8/16 (50%)	3/16 (18.75%)	5/16 (31.25%)	16/48 (33.33%)

D

Set 1 (16 months)

Conditions	DNA	RNA	PMA	Total
Dry	6/8 (75%)	2/8 (25%)	3/8 (37.5%)	11/24 (45.83%)
Wet	2/8 (25%)	1/8 (12.5%)	2/8 (25%)	5/24 (20.83%)
Total	8/16 (50%)	3/16 (18.75%)	5/16 (31.25%)	16/48 (33.33%)

B

Set 2 (11 months)

Locations	DNA	RNA	PMA	Total
Dingo	2/4 (50%)	2/4 (50%)	0/4 (0%)	4/12 (33.33%)
Malanda	0/4 (0%)	2/4 (50%)	0/4 (0%)	2/12 (16.67%)
Townsville	0/4 (0%)	0/4 (0%)	0/4 (0%)	0/12 (0%)
Charter Towers	2/4 (50%)	2/4 (50%)	1/4 (25%)	5/12 (41.67%)
Total	4/16 (25%)	6/16 (37.5%)	1/16 (6.25%)	11/48 (22.92%)

E

Set 2 (11 months)

Conditions	DNA	RNA	PMA	Total
Dry	4/8 (50%)	4/8 (50%)	1/8 (12.5%)	9/24 (37.5%)
Wet	0/8 (0%)	2/8 (25%)	0/8 (0%)	2/24 (8.33%)
Total	4/16 (25%)	6/16 (37.5%)	1/16 (6.25%)	11/48 (22.92%)

C

Merge data

Locations	DNA	RNA	PMA	Total
Dingo	4/8 (50%)	2/8 (25%)	0/8 (0%)	6/24 (25%)
Malanda	3/8 (37.5%)	5/8 (62.5%)	2/8 (25%)	10/24 (41.67%)
Townsville	0/8 (0%)	0/8 (0%)	0/8 (0%)	0/24 (0%)
Charter Towers	5/8 (62.5%)	2/8 (25%)	4/8 (50%)	11/24 (45.83%)
Total	12/32 (37.5%)	9/32 (28.13%)	6/32 (18.75%)	27/96 (28.13%)

F

Merge data

Conditions	DNA	RNA	PMA	Total
Dry	10/16 (62.5%)	6/16 (37.5%)	4/16 (25%)	20/48 (41.67%)
Wet	2/16 (12.5%)	3/16 (18.75%)	2/16 (12.5%)	7/48 (14.58%)
Total	12/32 (37.5%)	9/32 (28.13%)	6/32 (18.75%)	27/96 (28.13%)

Table 6: A to F: Percentages of positive results of samples after experimental study (set 1: 16 months and set 2: 11 months) from DNA, RNA and PMA detections in different locations.

In the last sample collection, no bacterium (0%) was detected using DNA, RNA and PMA methods from Townsville samples compared to all PCR results from Malanda samples at 75%, 75% and 50%, respectively.

Table 7C shows combined data from Set 1 and 2 samples which showed percentages of positive and negative DNA results compared to positive and negative RNA and PMA results. Merged DNA positive results had 82.65% and 39.29% positive results from RNA and PMA tests, respectively. The PMA method confirmed that less than half of DNA positive results came from live bacteria. While DNA negative results had 23.66% RNA positive which showed that the RNA protocol maybe had higher sensitivity than the DNA protocol to detect Mptb in environmental samples.

The tables also presented the level of detection errors in this study.

A

Set 1 (16 months)

	DNA (+)	DNA (-)
	161	95
RNA (+)	135/161 (83.85%)	21/95 (22.11%)
RNA (-)	26/161 (16.15%)	74/95 (77.89%)
PMA (+)	75/161 (46.58%)	2/95 (2.11%)
PMA (-)	86/161 (53.42%)	93/95 (97.89%)

B

Set 2 (11 months)

	DNA (+)	DNA (-)
	35	222
RNA (+)	27/35 (77.14%)	54/222 (24.32%)
RNA (-)	8/35 (22.86%)	168/222 (75.68%)
PMA (+)	2/35 (5.71%)	4/222 (1.8%)
PMA (-)	33/35 (94.29%)	218/222 (98.2%)

C

Merge data

	DNA (+)	DNA (-)
	196	317
RNA (+)	162/196 (82.65%)	75/317 (23.66%)
RNA (-)	34/196 (17.35%)	242/317 (76.34%)
PMA (+)	77/196 (39.29%)	6/317 (1.89%)
PMA (-)	119/196 (60.74%)	311/317 (98.11%)

Table 7:A to C: Percentages of positive and negative results from DNA detection compared to positive and negative results from RNA and PMA detections

4.2.2 Comparison of Molecular and Culture methods for detection of Mptb

Fig 10 and Fig 11 represent results of the various detection methods to assess presence and viability of Mptb over 7 and 11 months respectively. Percentage of positive and negative results are plotted to reduce the variability of individual samples.

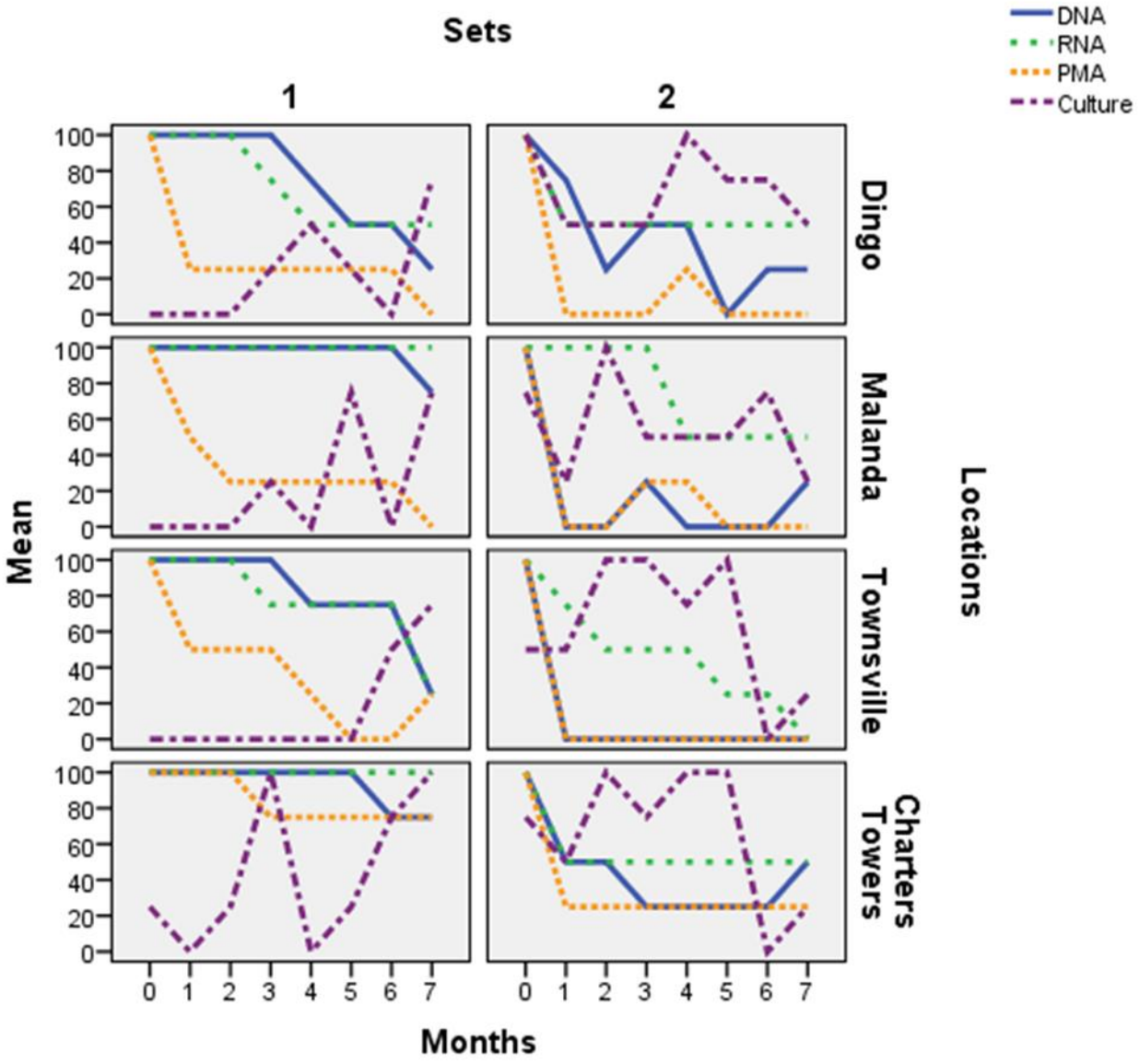


Fig 10: Graphs show seasonal effects of Mptb detection in the first 7 months of environmental study in samples set1 and 2 using DNA, RNA, PMA and culture detections.

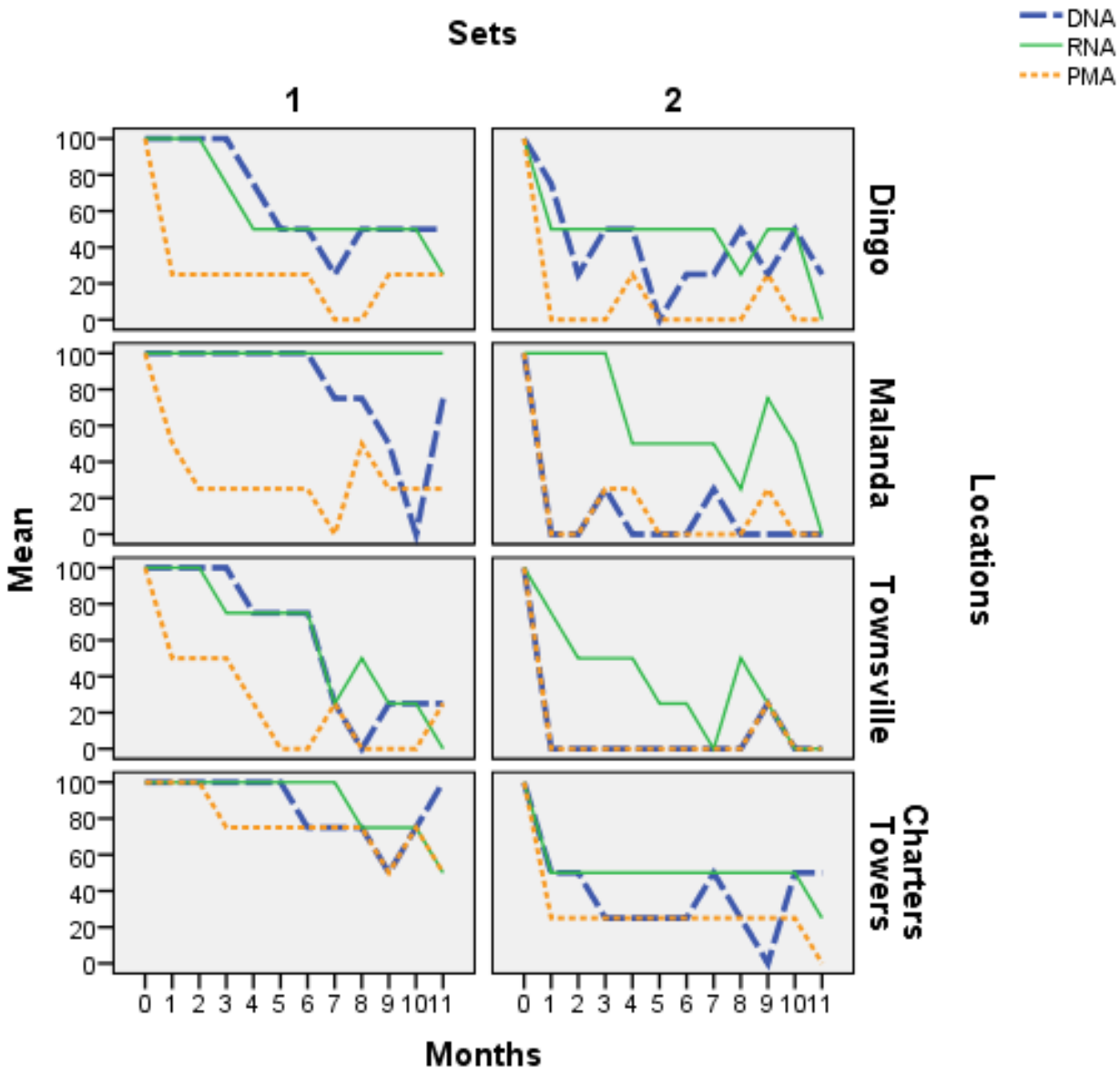


Fig 11: Graphs show seasonal effects of Mptb detection in the first 11 months of environmental study in samples set1 and 2 using DNA, RNA and PMA detections.

4.2.3 Quality control of PCR results

In 75% of the tests the results correlated well with the University of Sydney (Table 8). However in 5 DNA extraction tests there was a discrepancy of positive and negative results. As the DNA originated from the original sample, it should be concluded that, it is likely that JCU had false negatives in 3 samples and University of Sydney in 2 samples.

From these results, it is clear that false positive results at JCU are rare and that false negatives may be of greater concern.

The second set of samples prepared from faeces, showed 3 out of 10 samples did not correlate with University of Sydney results which may be false negative because of lower sensitivity of the JCU manual qPCR test. The qPCR results from JCU and University of

Sydney determined the tests have high specificity (100%) but low sensitivity (66.67%) (Table 6).

Set 1

Samples	JCU	Uni of Sydney
F1	4.04E+06	5.10E+04
F2	9.88E+05	4.28E+04
F3	3.52E+05	3.18E+01
F4	9.48E+06	2.67E+05
F5	4.74E+07	3.74E+06
F6	0.00E+00	0.00E+00
F7	0.00E+00	3.27E+05
F8	0.00E+00	0.00E+00
F9	0.00E+00	0.00E+00
F10	0.00E+00	0.00E+00
F11	5.40E+06	6.86E+04
F12	8.77E+07	4.83E+05
F13	9.42E+07	7.83E+05
F14	4.66E+07	4.00E+05
F15	1.07E+08	1.19E+07
F16	0.00E+00	2.51E+02
F17	0.00E+00	4.65E+04
F18	0.00E+00	1.87E+05
F19	0.00E+00	1.65E+05
F20	0.00E+00	0.00E+00
D1	4.04E+06	2.49E+05
D2	9.88E+05	4.62E+04
D3	3.52E+05	1.98E+04
D4	9.48E+06	3.96E+05
D5	1.00E+04	1.83E+04
D6	0.00E+00	9.21E+03
D7	0.00E+00	3.53E+05
D8	0.00E+00	0.00E+00
D9	0.00E+00	1.27E+03
D10	0.00E+00	0.00E+00
D11	5.40E+06	3.14E+05
D12	8.77E+07	5.12E+06
D13	9.42E+07	4.99E+06
D14	4.66E+07	2.65E+06
D15	1.00E+04	5.64E+03
D16	0.00E+00	0.00E+00
D17	0.00E+00	0.00E+00
D18	0.00E+00	1.03E+06
D19	0.00E+00	2.05E+04
D20	0.00E+00	0.00E+00

Set 2

Samples	JCU	Uni of Sydney
1	1.87E+06	3.50E+04
1	2.12E+06	3.88E+04
2	2.53E+02	5.80E+03
2	6.29E+02	5.20E+03
3	2.84E+02	5.60E+03
3	1.89E+02	6.60E+03
4	0.00E+00	4.00E+02
4	0.00E+00	1.00E+03
5	0.00E+00	0.00E+00
5	0.00E+00	0.00E+00
6	2.11E+07	2.08E+06
6	1.41E+07	2.38E+06
7	2.32E+03	5.13E+05
7	2.06E+03	5.10E+05
8	2.45E+03	7.71E+05
8	5.02E+02	7.33E+05
9	0.00E+00	1.11E+06
9	0.00E+00	1.11E+06
10	0.00E+00	2.46E+05
10	0.00E+00	2.25E+05

Different results

F = faecal samples
D = DNA samples

		Uni of Sydney	
		Positive	Negative
JCU	Positive	32	0
	Negative	16	12

Table 8: Table shows qPCR results of set 1 and set 2 samples from JCU compared to University of Sydney including summary of positive and negative results.

4.2.4 Temperature and humidity recordings

Temperature and humidity recordings were initially recorded six hourly and average values associated with the day of the recording. After an initial period of data it was realized that averages from six recordings would not adequately provide a quantitative value that would be useful in detecting differences between sites.

Hourly recordings were used for the duration of the project and reduced to day and hour data sets for the hours a particular day achieved a reading of 30^{o}C and 40^{o}C. These points were assessed as ones which would provide significantly different values for each site and may be related to the differences in survival times seen with the Mptb organism. (Table 9).

For relative humidity the points were determined to be hours for which readings were less than 50% RH and less than 40% RH. (Table 10)

Dingo

Months	Hrs>30	Hrs>40	Days/mth
Oct-14	235	15	30
Nov-14	319	41	30
Dec-14	341	19	31
Jan-15	268	26	28
Feb-15	260	20	28
Mar-15	336	36	29
Apr-15	149	3	22
May-15	51	0	10
Jun-15	1	0	1
Jul-15	9	0	3
Aug-15	55	0	13
Sep-15	106	0	23
Oct-15	217	7	30
Nov-15	296	29	29
Dec-15	161	6	16

Malanda

Months	Hrs>30	Hrs>40	Days/mth
Oct-14	104	15	28
Nov-14	198	23	30
Dec-14	220	41	31
Jan-15	115	0	17
Feb-15	102	3	16
Mar-15	157	37	25
Apr-15	78	0	23
May-15	56	0	18
Jun-15	2	0	2
Jul-15	10	0	8
Aug-15	38	0	16
Sep-15	63	0	21
Oct-15	20	0	7
Nov-15	60	0	18
Dec-15	24	0	16

Townsville

Months	Hrs>30	Hrs>40	Days/mth
Oct-14	202	0	31
Nov-14	163	0	30
Dec-14	299	4	31
Jan-15	255	16	29
Feb-15	260	27	28
Mar-15	266	22	31
Apr-15	162	6	25
May-15	134	0	27
Jun-15	33	0	12
Jul-15	32	0	10
Aug-15	75	0	26
Sep-15	130	0	29
Oct-15	160	0	29
Nov-15	225	0	30
Dec-15	148	0	16

Charters Towers

Months	Hrs>30	Hrs>40	Days/mth
Oct-14	222	21	31
Nov-14	284	76	30
Dec-14	311	99	31
Jan-15	271	60	30
Feb-15	252	28	28
Mar-15	174	20	18
Apr-15	177	0	25
May-15	61	0	15
Jun-15	2	0	2
Jul-15	15	0	6
Aug-15	58	0	12
Sep-15	154	0	30
Oct-15	226	18	30
Nov-15	326	82	30
Dec-15	183	34	16

Table 9: Number of hours temperatures reached or exceeded 30 and 40 degree Celsius and number of days of temperature over 30 degree Celsius in Dingo, Malanda, Townsville and Charters Towers

Dingo

Months	Hrs <50%	Hrs <40%	Days/Mth
Oct-14	400	292	31
Nov-14	347	259	30
Dec-14	281	158	28
Jan-15	165	60	24
Feb-15	177	57	23
Mar-15	298	159	29
Apr-15	294	172	27
May-15	265	137	26
Jun-15	114	51	15
Jul-15	302	159	24
Aug-15	400	255	31
Sep-15	416	313	30
Oct-15	359	239	31
Nov-15	280	195	28
Dec-15	162	115	15

Malanda

Months	Hrs <50%	Hrs <40%	Days/Mth
Oct-14	139	97	29
Nov-14	199	127	28
Dec-14	192	115	31
Jan-15	45	9	11
Feb-15	50	10	11
Mar-15	91	46	25
Apr-15	43	10	11
May-15	52	11	15
Jun-15	6	0	4
Jul-15	35	15	10
Aug-15	71	21	20
Sep-15	98	22	23
Oct-15	16	4	5
Nov-15	0	0	0
Dec-15	0	0	0

Townsville

Months	Hrs <50%	Hrs <40%	Days/Mth
Oct-14	209	70	29
Nov-14	162	17	25
Dec-14	137	15	25
Jan-15	57	1	14
Feb-15	76	15	14
Mar-15	118	18	23
Apr-15	176	69	24
May-15	176	92	23
Jun-15	67	20	13
Jul-15	187	117	24
Aug-15	189	89	30
Sep-15	217	73	28
Oct-15	167	25	30
Nov-15	115	11	26
Dec-15	78	8	15

Charters Towers

Months	Hrs <50%	Hrs <40%	Days/Mth
Oct-14	381	303	31
Nov-14	313	215	30
Dec-14	308	209	31
Jan-15	209	122	27
Feb-15	237	141	28
Mar-15	157	98	19
Apr-15	341	212	29
May-15	338	202	28
Jun-15	122	36	16
Jul-15	348	162	31
Aug-15	356	253	31
Sep-15	349	264	30
Oct-15	318	232	31
Nov-15	298	213	30
Dec-15	166	113	16

Table 10: Humidity values under 50% and 40% and number of days of humidity under 50% in Dingo, Malanda, Townsville and Charters Towers

4.3 Objective 2 Cross-reactions in PCR by saprophytic mycobacteria

Of the seven original soil samples that had been cultured prior to use in the study, no mycobacteria were cultured, however, 4 yielded *Rhodococcus* species. These were also tested for the presence of *IS*900-like sequences but were negative.

Twenty nine (29) cultures of the 53 QDAFF cultures gave positive bands on a 1% agarose gel using one or more of the primers that amplified the IS900-like sequence. However, the size of the PCR product was larger than what would be expected for the Mptb *IS*900 sequence. The results are shown in Table 11.

Table 11: A summary of the *IS*900-like sequences detected with the different primer sets of 29 cultures.

Primers	**P90 & P91**	**IS900F & IS900R**	**921 & 1051**	**MP10 & MP11**	**Total positive samples**
Positive	10	5	10	12	29
Number of positive cultures where more than one primer set detected IS900-like sequences	4 also amplified with the 921&1051 primers	3 also amplified with the 921&1051 primers MP10&MP11 primers	1 also amplified with the MP10&MP11 primers		8

Most of the amplicons obtained when using the P90 and P91 primers showed larger sizes than the 413 bp that was expected as they were up to 2000 bp. These are shown in a picture of the gel in Fig 12.

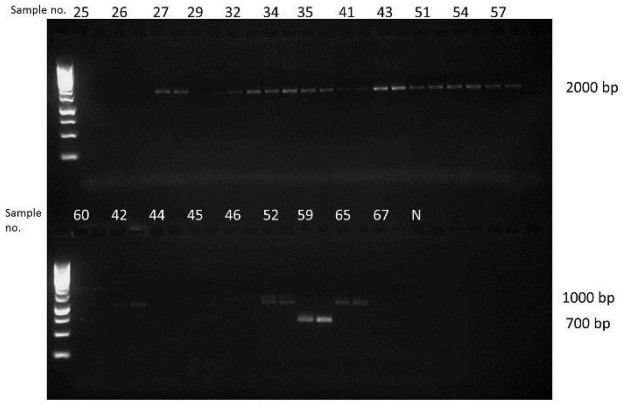


Fig 12: A picture of the gel showing different sizes of amplified DNA with the P90 and P91 primers showed for 21 cultures

All the samples that had produced bands, a total of 35 as it also included some of the products where multiple bands had been obtained, were submitted to Macrogen, South Korea for sequencing. 14 PCR products out of 35 produced clean sequences that could be cross-checked for complementary sequences on the nucleotide BLAST program and database of the NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Seven of these sequences did not match to other sequences on the NCBI data base. Four of the sequences had low similarity to *Mycobacterium* sp. JDM601 (6%) and 3 PCR products had high similarity to *Rhodococcus* sp. (94%) which can be found in the environment and it may cause cross reaction and false positive result in PCR.

Twenty on (21) of the cultures showed that they were mixed. Once mycobacteria were purified in these cultures, no *IS*900-like sequences could be detected.

5 Discussion

5.1 Ability of Mptb to survive in northern Queensland and remain viable

The primary objective of the project was to determine survival periods and develop methodologies for identification of viable organisms in environmental samples.

Objective 1: Identify the potential variables of persistence and viability to permit science based management and control strategies for beef Mptb in northern Queensland.

Objective 3: Investigate a PCR based viability test which will reduce time and cost for testing of samples required to establish status of animals and properties.

5.1.1 Effect of temperature

Temperature data were recorded within the enclosures, initially on a six hourly cycle and after two months data was recorded hourly to provide more accurate records.

Analysis of data included average and median temperatures on a daily and monthly basis. This yielded large data sets that did not conclusively identify differences between sites.

A likely parameter of the effect of temperature on survival of Mptb was the number of hours per month where temperatures exceeded 30^{o}C and 40^{o} C.

It is difficult to directly relate significance of temperature differences between sites to the detection of DNA within the samples. This could be due to the effects of shading within each site or to the penetration of bacteria to deeper levels of the sample pot.

Fig 13 plots the temperature values of each site. Townsville clearly has the greatest number of days exceeding 30^{o}C during the trial period and also consistently exceeded this threshold in every month of the trial providing relatively less variation between seasons or months. Dingo has the greatest monthly variations followed by Charters Towers and Malanda.

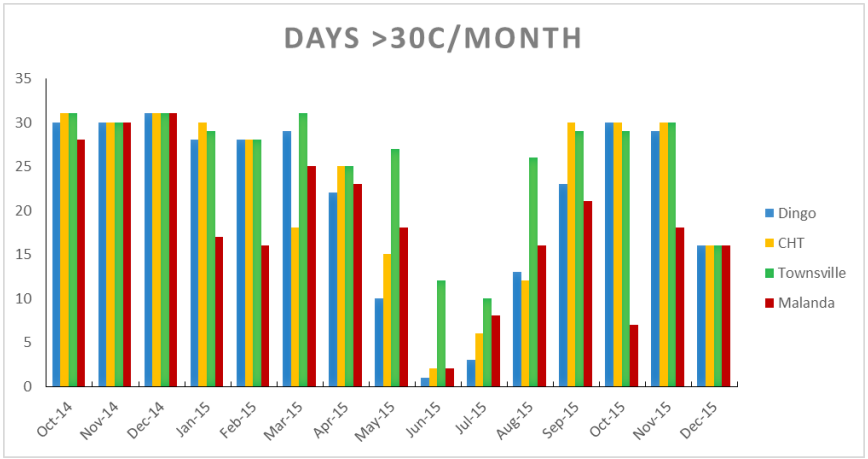


Fig 13: Comparison of days exceeding 30^{o}C at each site.

In considering the effect of temperature on viability it was considered that the highest temperature achieved may be more significant than repeated lower level recordings for the survival of Mptb. Fig 14 provides a visual representation of the hours per month where temperature records exceeded 40^{o}C. This is interesting as the peak temperatures achieved during the summer of 2014 should have had an effect on the survival of the first set of samples. This is not supported by recovery of DNA and RNA.

The potential for temperature alone to be a predictor of survivability is not consistent with the findings of this study or others (Manning 2001). This study supports the findings of others that the lipid rich bacterial membrane of Mptb protects the organism from temperatures and pH fluctuations.

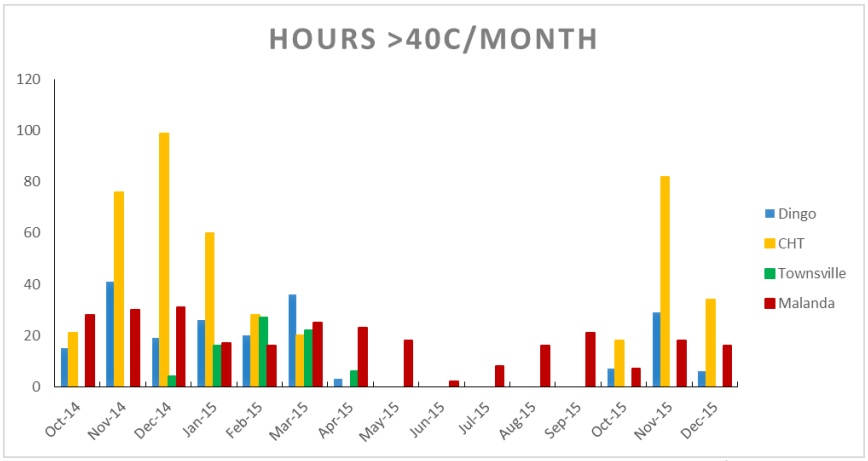


Fig 14: Number of hours each site recorded temperatures above 40^{o}C each month

However this study notes a decline in recovery rates for samples deployed in the hotter summer months for set 2 samples. It is not possible to identify temperature as the sole determinant in these cases as samples from sites with lower relative humidity did not show a similar decline.

It has been recorded that Mptb is able to survive the standard milk pasteurisation process (Manning 2001). This process utilises a minimum temperature of 62.8^{o}C for 30 minutes. Assuming this temperature is critical then the environmental temperatures are unlikely to exceed this value and be responsible for denaturation of the Mptb bacterium.

5.1.2 Effect of humidity

As with temperature, the variability of data makes any simple determination of significance of relative humidity over the trial period difficult. The project was undertaken during a particularly dry period with below average rainfall for the region during the study period. In general with the exception of Malanda, humidity was consistently below 50% for the majority of days at the other sites (Fig 15).

When the number of hours per month where humidity was recorded to be below 40% are assessed (Fig 16) it becomes obvious that the inland sites of Dingo and Charters Towers. In this case Dingo and Charters Towers as inland sites recorded more hours where humidity was at this low level than the coastal site at Townsville and the wet tropics site at Malanda.

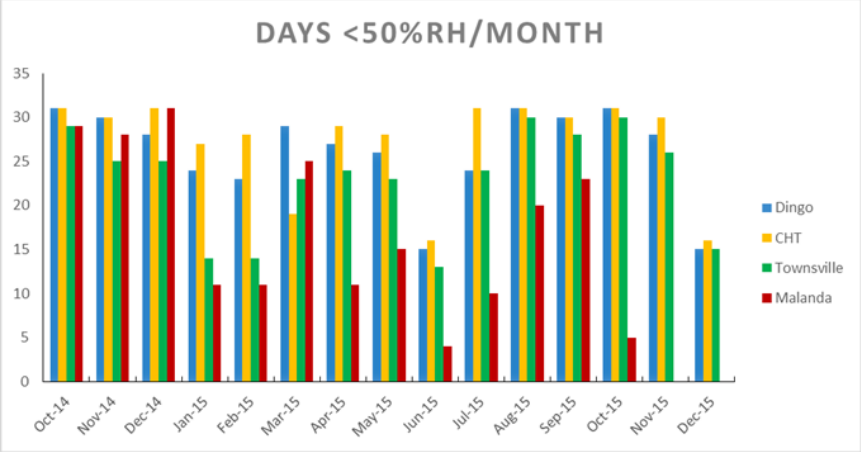


Fig 15: Number of days where RH was less than 50% each month of the trial

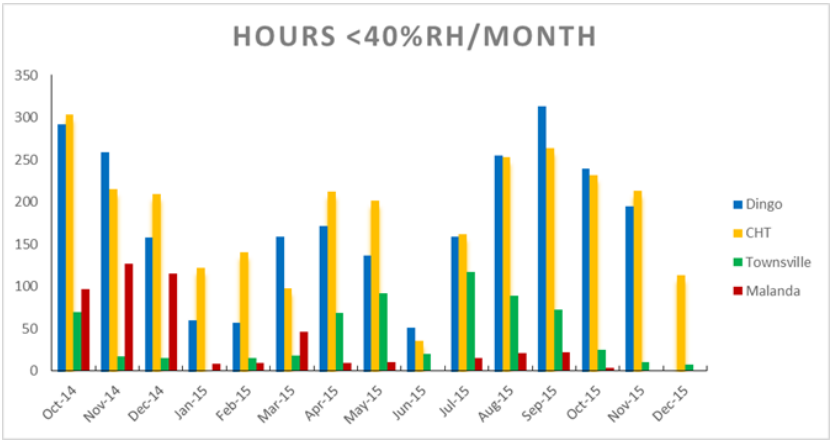


Fig 16: Number of hours that RH was less than 40% each month

The relationship between soil moisture, as demonstrated by the difference in survival of organisms in the permanently wet pots and atmospheric humidity may be significant.

Samples held under high soil moisture (wet) had generally lower persistence of the organism over time (see discussion below). There is a similar association with the sites at Malanda and Townsville where the higher relative humidifies encountered may have impacted on the higher decline in the persistence of organisms. This relationship cannot be statistically substantiated. It is possible to speculate that higher soil moisture permitted growth of saprophytic bacteria that may have been detrimental to the survival of the Mptb organisms.

5.1.3 Effect of pH

The pH differences were not great between the study sites. Dingo was neutral (6.8) followed by slightly acid Charters Towers (5.7) more acidic Townsville (5.5) and strongly acid Malanda (5.4). Previous studies (Schroen et al, 2000) have suggested a relationship between alkaline soils and greater decay rates for detection of Mptb. Greatest decay rates

over all samples in this trail were achieved at Townsville followed by Malanda, Dingo and finally Charters Towers. These results do not relate survival or degradation to soil pH values.

5.1.4 Effect of soil moisture

This parameter had the most clearly defined effect on recovery of Mptb from samples. Where samples were maintained in water the recovery rapidly declined and remained at zero for the duration of the project. The exception was for the first deployment of samples at Charters Towers where irregular detection of DNA was possible for periods up to 15 months. (Fig 6, Fig 7, Fig 8, Fig 9.).

The recoveries in dry samples demonstrated a pattern similar to previous studies (Whittington, 2001) where after a long period of failure to detect DNA or culture the organism, the later samples recorded detection of Mptb in significant numbers. This is presented as evidence for the apparent dormancy potential of the Mptb organism.

In our study the lack of a dormancy state being recorded in samples held at water saturation compared to samples held in completely dry conditions is most likely explained by the failure of the organism to enter dormancy in the presence of moisture, or other soil microbial activity resulted in an adverse impact on the survival of Mptb organisms.

The soil used in our study had been autoclaved to remove potential for environmental mycobacteria to confuse the results but the samples would have been contaminated by windborne microorganisms as it was noted at stages during the project that algae and primitive ferns and moss were detected in the trays containing water at all locations.

The particularly dry climatic conditions experienced during the study would have contributed to the effect of drying on the samples held without water in the trays to replenish soil moisture.

The study design did not permit analysis of the factors involved in the observation that apparent dormancy lasted approximately 12 months in the first set of samples and only seven (7) months in the second set of samples. Speculation that a combination of temperature and humidity may have been responsible is unable to be confirmed by the recording systems used for this study.

5.1.5 Assessment of viability of Mptb detections in soil and faeces

After six months of the study the results from tests for DMA, RNA and PMA at this time point were tabulated. A lower percentage (18.75%) of qPCR negative results from RNA extraction compared to the higher percentage (68.75%) of qPCR negative results for PMA process compared to the 25 percent obtained by DNA extraction test (Table 12). The inference is that PMA at this point was overestimating the percentage of non-viable bacterial DNA and RNA was likely to be correctly identifying the viability of organisms in the test samples or slightly underestimating the viability of the organisms.

Locations	DNA test	RNA test	PMA test
1. Dingo	2/4 (50%)	2/4 (50%)	3/4 (75%)
2. Malanda	0/4 (0%)	0/4 (0%)	3/4 (75%)
3. Townsville (JCU)	1/4 (25%)	1/4 (25%)	4/4 (100%)
4. Charters Towers	1/4 (25%)	0/4 (0%)	1/4 (25%)
Total	4/16 (25%)	3/16 (18.75%)	**11/16 (68.75%)**

Table 12: Comparison of negative qPCR results (6 mth) from DNA, RNA and PMA protocols

For both the RNA and PMA testing the faecal pad was found to be a more reliable sample when compared to a soil/faeces mixture.

Specifically for the RNA test, the loss of sensitivity could be due to soil humic acid RNA degradation, soil inhibiting RNA adherence to the column membrane or soil colour interfering with the qPCR. Different protocols would have to be tested to find the most optimal one.

The likely interference to the PMA protocol for binding to naked DNA is that particulate matter, especially clay particles, within samples can decrease the light path of the blue light and result in lack of activation and binding of bacterial DNA leading to false negative viability results.

Initially the PMA test appeared to perform better than the RNA qPCR, however conclusions cannot be made without reliable culture results. Culture results were negatively impacted by the presence of soil contamination from the samples.

After 16 months of data collection, qPCR results of extraction of DNA, RNA and PMA, were significantly different in all locations ($P>0.05$) using Kruskal-Wallis test and also showed a significantly different between dry and wet conditions ($P>0.05$) using Mann-Whitney U test. qPCR results also determined that no significantly difference of RNA results between set 1 (winter) and set 2 (summer) ($P>0.05$) while DNA and PMA results showed a significantly difference between set 1 and set 2 ($P<0.05$) using Mann-Whitney U test.

Table 13: Percentages of positive results of samples after experimental study (set 1: 16 months and set 2: 11 months) from DNA, RNA and PMA detections including 7 months of culture result in different locations.

Locations	DNA	RNA	PMA	Culture	Total
Dingo	4/8 (50%)	2/8 (25%)	0/8 (0%)	3/8 (37.5%)	9/32 (28.13%)
Malanda	3/8 (37.5%)	5/8 (62.5%)	2/8 (25%)	3/8 (37.5%)	13/32 (40.63%)
Townsville	0/8 (0%)	0/8 (0%)	0/8 (0%)	1/8 (12.5%)	1/32 (3.13%)
Charters Towers	5/8 (62.5%)	2/8 (25%)	4/8 (50%)	2/8 (25%)	13/32 (40.63%)
Total	12/32 (37.5%)	9/32 (28.13%)	6/32 (18.75%)	9/32 (28.13%)	36/128 (28.13%)

5.1.6 Implications from the study for objectives 1 and 3

Objective 1: Identify the potential variables of persistence and viability to permit science based management and control strategies for beef Mptb in northern Queensland.

Objective 3: Investigate a PCR based viability test which will reduce time and cost for testing of samples required to establish status of animals and properties.

There is now a body of published evidence that Mptb survives in northern and southern Australian environments for periods in excess of 12 months.

Whether infective doses remain after the initial three months is still unclear but if there was an intention to declare biological freedom following detection of infection there would be a requirement to implement a testing of sentinel animals to determine absence of the organism.

The study has added evidence to the observation that Mptb is capable of a period of dormancy and reactivation particularly where the environment is supportive of a spore like transformation and reactivation.

This study clearly demonstrated that soil water saturation was detrimental to the development and subsequent reactivation of Mptb. The implications for arid grazing lands is not quantified but should be regarded as significant and worthy of a precautionary biosecurity approach and possible further investigation.

There is a need to further develop the RNA and PMA protocols to validate the qPCR results for viable bacteria.

The HT-J PCR test is able to be refined to allow low cost reliable detection of Mptb in faecal samples. This will enable diagnostic testing of low throughput samples. Encouragement should be provided to private animal health laboratories to offer Mptb testing to enhance surveillance.

5.2 Ability of environmental mycobacteria to confound the HT–J PCR test

The eradication of bovine tuberculosis in northern Australia was complicated by the presence of mycobacteria that cross reacted to the tuberculin and gamma interferon tests.

Objective 2: the effect of environmental mycobacteria isolated in Queensland on the sensitivity of the HTJ PCR test recently approved by SCAHLS for Mptb diagnostic testing.

5.2.1 Effect of environmental mycobacteria on HT-J PCR

Eighty eight stored cultures were re-cultured and purified for extraction of DNA. Cross reactions for the IS900 sequence used in the HT-J PCR test were eliminated once contaminating Rhodococcus bacteria were removed.

With adequate training in the purification and preparation of the samples the risk of cross reaction is considered small. Products from non Mptb have significantly different size to Mptb.

5.2.2 Implication from the study for objective 3

With adequate training and establishment of positive controls there is little risk of cross reactions from environmental mycobacteria either contaminating environmental samples or infecting live animals.

6 Conclusions/recommendations

6.1 Future research questions

6.1.1 Dormancy of Mptb

The obvious finding from this project that was supported by previous research is the apparent ability of Mptb to be regarded as dormant and to regenerate at a later date poses a risk for infected properties needing to confirm eradication of the organism for livestock market access.

Controlled research to determine the potential for dormancy and the conditions under which it is induced and return to vegetative state is required to ensure management decisions for biosecurity can be made with confidence.

6.2 Practical applications of project results

The project finding that it is possible for Mptb to persist in northern soils for extended periods has bearing on the management programs for known infected properties. Management and biosecurity recommendation should include this knowledge into property management plans. Animal health authorities and veterinarians need to recognise the potential risk of reinfection from dormant Mptb and include long term surveillance and monitoring programs to ensure compliance with biosecurity obligations.

The development of a manual method for identification of PCR products suitable for small scale laboratory testing of diagnostic samples requires refinement but will enable other laboratories to undertake health testing to achieve market access goals.

Refinement of the propidium monoazide (PMA) and RNA assay to determine viability of organisms identified by DNA will allow a more scientific approach to management programs.

Culture results will always be gold standard for identification of Mptb but in the absence of reliable molecular diagnostic tests are prone to high false negative rates when applied to environmental samples due to contamination and the potential of decontamination protocols to affect viable organisms.

6.3 Development and adoption activities

6.3.1 Survival of Mptb in northern environments

Management of previously infected herds must consider the potential for survival of organisms for periods in excess of 12 months and in areas of low soil moisture the potential for dormancy and reactivation after periods of 15 months or longer.

6.3.2 Application of available tests for diagnostic purposes

The project demonstrates the limitations to any single environmental or faecal PCR positive sample. While there is little risk of cross reaction from non Mptb environmental bacterial there is a risk of detection of non-viable bacteria and potentially detection of bacteria that would not cause infection in the host animals.

Examination of animals for the potential of infection with Mptb should employ serological as well as molecular tests on a number of animals or groups of animals to increase sensitivity of testing.

Examination of environmental samples should include tests for viability as well as direct molecular detection of DNA to increase sensitivity of the detection system. The use of culture should always be included with attempts to decontaminate the sample without the use of antibiotics prior to culture.

6.3.3 Adoption of a standard for notification of Johne's disease

Culture positive results from animals are unquestionably a reason to notify the presence of Johne's disease for the animals. However faecal PCR positive animals with a negative culture result require clarification and assessment of risk prior to notification and application of export restrictions. Intervals for repeat testing, interpretation when more than a single individual animal is positive to molecular tests need to be developed and agreed to assist the management of the disease in Australia.

Similarly the negative culture and molecular testing of environments cannot be assumed to be evidence of absence unless the testing is repeated over a 24 month period of time.

7 Key messages

7.1 Environmental survival of Mptb in northern soils

Mptb will survive for periods of approximately 3 months in northern environments. Survival in some circumstances may be prolonged by dry conditions and the organism's ability to enter a dormant state for periods exceeding 12 months.

Management strategies to minimise infection risks from the environment need to be applied on previously confirmed infected properties.

High value animals intended to remain in the breeding herd or for export should be subject to molecular and culture examination to minimise the risk of infection being present.

Environmental mycobacteria are a low risk of cross reaction in current molecular tests.

Control of risk of infection is possible using available technologies.

Enhancement of the understanding of the process of dormancy may reduce the risk of reoccurrence of the disease in previously infected properties and animals.

8 Acknowledgements

The funding of this project by Meat and Livestock is acknowledged particularly the interest and support provided by Dr Johann Schröder.

Technical assistance in developing the project concept was provided by Dr Douglas Begg of the University of Sydney and also Dr Karen Plain who provided invaluable advice on the molecular and microbiology challenges of the project.

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The commitment of the people who allowed this project to be undertaken on their property is recognised and valued. The researchers are always indebted to the co-operators who accept the concept as worthwhile and are prepared to offer unqualified support and facilities. We thank you Dr Bill Tranter, Dr Carlie and Lauchie Ward and Mr Martin Holzwart.

Finally to the staff within the Research and Finance sections at James Cook University who ensure the financial and contractual details are effectively completed.

With our thanks and deep appreciation, Robert, Jackie, Orachun and Liz.

9 Bibliography

Cousins DV, Whittington R, Marsh I, Masters A, Evans RJ, Kluver P (1999) Mycobacteria distinct from *Mycobacterium avium* subsp. *paratuberculosis* isolated from the faeces of ruminants possess *IS*900-like sequences detectable by IS900 polymerase chain reaction: implications for diagnosis*. Molecular and Cellular Probes* **14**, 431–442

Harris NB, Robbe-Austerman S, Payeur JB (2005). Effect of egg yolk on the detection of *Mycobacterium avium* subsp. *paratuberculosis* using the ESP II liquid culture system. *Journal of Veterinary Diagnostic Investiga*tion, **17**, 554–560.

Kawaji S, Taylor DL, Mori Y, Whittington RJ (2007) Detection of *Mycobacterium avium* subsp. *paratuberculosis* in ovine faeces by direct quantitative PCR has similar or greater sensitivity compared to radiometric culture. *Veterinary Microbiology* **125**, 36-48

Manning EJB, (2001) *Mycobacterium avium* subspecies *paratuberculosis*: A review of current knowledge. Journal of Zoo and Wildlife Medicine 32(3): 293-304.

Schroen C, Kluver P, McDonald W, Butler K, Condron R, Hope A. (2000) Survival of Mycobacterium paratuberculosis. Project TR.055. Meat and Livestock Australia Limited Sydney. ISBN 174036 4082.

Whittington RJ, (2001) Survival of Johne's disease in the environment. Project OJD.003. Meat and Livestock Australia. Sydney. ISBN 0 7347 11293 6.

Whittington RJ, Marshall DJ, Nicholls PJ, Marsh IB, Reddacliff LA, (2004) Survivability and dormancy of Mycobacterium a*vium* subsp. *paratuberculosis* in the environment. *Applied Environmental Microbiology,* **70**, 2989-3004.

Whittington RJ, Whittington A-M, Waldron A, Begg DJ, de Silva, K, Purdie A, Plain K, (2013). Development and Validation of a Liquid Medium (M7H9C) for Routine Culture of *Mycobacterium avium* subsp. *paratuberculosis* To Replace Modified Bactec 12B Medium. *Journal of Clinical Microbiology*, 51, 3993-4000.

10 Appendices

10.1 Appendix 1 - Detailed soil analysis

Soil Horizons Pty Ltd, Townsville

Technical Report 2014-20

Client: School of Veterinary and Biomedical Sciences, James Cook University, Townsville

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2 November 2013

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Coventry, R.J. (2013). Bovine Johne's Disease Project, James Cook University: Properties of topsoils at four test sites in Central and North Queensland. Technical Report 2014–20, Soil Horizons Pty Ltd, Townsville (unpublished).

Table of contents

Executive summary

This report sets out the results of a blind trial where the properties of a set of soils have been interpreted in terms of their likely impact on the growth of plants and also on the possible vigour and persistence of populations of Mycobacterium paratuberculosis, the bacterium causing Bovine Johne's Disease.

Four topsoil samples (one or more having been collected from a paddock infected with M. paratuberculosis) were collected by members of the James Cook University's Bovine Johne's Disease Project and were delivered to Soil Horizons for analysis. In order to assess the major constraints to plant growth presented by the soil materials a set of their chemical and particle-size analyses were carried out by the NATA-registered and ASPAC-affiliated laboratories of the Incitec Pivot Laboratories, Werribee, Victoria. No soil biological assessments were made.

The soils were found to be strongly acidic to neutral in pH, non-saline, non-sodic, and had a range of properties affecting their fertility. Interpretations of the soil test results, allied with the field morphological properties of the soils, suggest the following likelihood of soil infection with the M. paratuberculosis bacteria:

- Most likely infected: sample Mptb 1 with neutral pH (6.8), very high organic matter content, high clay content and associated water-holding capacity;
- Probably – possibly infected: sample Mptb 3 with the next highest soil pH (5.7), but low organic carbon and clay contents;
- Unlikely to be infected: samples Mptb 2 and Mptb 4, both of which would provide adequate organic matter for survival of the soil microbes, but their strongly acidic soil conditions (pH 5.4 and 5.5) are likely to have favoured strongly the growth of fungi rather than bacteria.

If this blind assessment matches the knowledge of the Bovine Johne's Disease Project personnel about which of the four analysed soil samples were from infected paddocks, then there are opportunities to manage the populations of the bacterial pathogens by way of managing the acidity of the soils supporting the pastures in infected paddocks.

A small research project has been outlined to define the target soil pH to which the pH of the soils supporting infected pastures should be lowered by soil acidification processes. How long the pasture soils need to remain in an acidic condition to eliminate active populations of paratuberculosis bacteria could be derived from long- term monitoring of acidified, formerly infected sites.

If the blind assessment is incorrect, then further work is required to revise the present report and to identify any obvious link between the occurrence of the pathogens and the properties of the soils of infected paddocks.

1. Introduction

1.1 Background

Animal Health Australia (2014) has indicated that Bovine Johne's Disease is an incurable infection of cattle, which causes wasting and chronic diarrhoea. It results in lost production and can end in the animal's death. The disease is caused by a bacterium (Mycobacterium paratuberculosis) that lives mainly in the intestines of infected animals. It causes the intestinal wall to thicken and reduces the normal absorption of nutrients from grazing, so the animal can eventually starve to death. Infected cattle excrete the bacteria in their manure, which contaminates pastures, soils, and watercourses, spreading infection to other cattle sharing the same paddocks or yards.

It is also known that the micro-organisms causing Bovine Johne's Disease, Mycobacterium paratuberculosis, are resilient and can live for a long time in the environment. Recent research in southern Australia has shown that heat and sunlight destroy the pathogenic bacteria and that, under normal summer conditions in paddocks and waterways, around 90% of the bacteria die within six weeks. However, in the right conditions – moist, shaded areas – the M. paratuberculosis bacteria can survive for longer than 12 months.

1.2 Aims of this report

This report relates to the nature and properties of the uppermost topsoil layers occurring at four locations in Central and Northern Queensland of interest to a project aimed at controlling Bovine Johne's Disease and led by Associate Professor Robert Hedlefs of the School of Veterinary and Biomedical Science of James Cook University.

The report sets out the results of analyses of the chemical and particle-size properties of the soil samples and interprets the results in terms of the desirable conditions for the growth of agricultural and horticultural plants and their possible impacts on soil bacterial populations.

The overall aim of this part of the research project was to determine if there might be some relationship between soil properties and the survival of the bacterial pathogens. Therefore, one (or more) of the soil samples was collected from a site that was thought to host the M. paratuberculosis bacterium, but those sample numbers were not disclosed to the soil analyst. No soil biological assays were carried out.

2. The soil samples

2.1 Soil sampling methods

Soil samples were collected by spade scrapings from the surface of the uppermost parts of the topsoil at four sites in Central and Northern Queensland by the Bovine Johne's Disease Project personnel. Sample numbers Mptb 1 – Mptb 4 were assigned in the field. The labelled samples were delivered to Soil Horizons, Townsville, for the description of the soil morphological properties and to make arrangements for the chemical and particle-size analysis of the samples.

No details of the sampling sites were provided with the samples. Nor was any indication given of which samples may have come from infected paddocks.

2.2 Field morphological characteristics of the soils

The following morphological properties of each of the soil samples were recorded by Soil Horizons (Table 1):

- soil colour, using Munsell soil colour charts;
- field texture, using the method of Northcote (1979) by manipulating moist soil samples between the fingers;
- soil structure and consistence of the samples using the method of the National Committee for Soil and Terrain (2009).

Table 1. Morphological properties of soil samples from Bovine Johne's Disease study sites in Central and North Queensland. Source of data: Soil Horizons Pty Ltd, Townsville.

Soil sample no.	Soil colour Munsell Colour System	Soil field texture	Soil structure and consistence	Comments
Mptb 1	Very dark greyish brown (10YR 1/2 moist, 10YR 4/2 dry)	Gravelly, histic loam	Modertately developed, fine polyhedral structure (8 - 12 mm); dry and loose to weakly friable	Gravel (5 - 10%) consisted of small (2 - 10 mm), angular, quartz and rock fragments. Sample contained many dry grass fragments; strongly water repellent.
Mptb 2	Dark reddish brown (2.5YR 3/4 moist)	Light clay	Very strongly developed, fine polyhedral structure (2 - 12 mm); moist and plastic	No sand or gravel fraction evident.
Mptb 3	Brown (10YR 4/3 moist, 10YR 6/3 dry)	Gritty sandy loam (fine sandy)	Massive (?); dry and loose to weakly friable	Grit (< 5 %) consisted of small (2 - 4 mm), angular, quartz and rock fragments.
Mptb 4	Dark reddish brown (5YR 3/3 moist)	Slightly gritty sandy loam (fine sandy)	Massive (?); moist and friable	Grit (< 1%) consisted of a few, small (2 - 4 mm), angular, quartz and rock fragments. Sample contained abundant fragments of eucalypt leaves.

3. Scope of the soil analyses

The soil samples were submitted for chemical analysis to the Incitec Pivot Nutrient Advantage Laboratories, Werribee, Victoria. The laboratories are registered with the National Association of Testing Authorities (NATA) and are certified with the Australasian Soil and Plant Analysis Council (ASPAC), which ensures quality control over the soil testing procedures.

Analyses of selected soil chemical and particle-size properties were carried out on all of the soil samples in order to assess their major constraints to plant growth:

- soil pH, to determine soil acidity or alkalinity levels,
- electrical conductivity and chloride content, to determine potential soil salinity levels,

- exchangeable cation contents, to determine basic soil fertility levels and the extent of cation imbalances that may affect soil physical properties such as soil dispersion, water infiltration and transmission properties, and erosional stability;
- particle-size (% coarse sand, % fine sand, % silt, % clay) to assess the soil texture and help interpret cation exchange information and the uniformity of soil parent materials.

An additional suite of soil analyses was undertaken to explore the chemical fertility of the topsoil materials. These soil tests included:

- organic carbon, plant-available phosphorus and potassium, nitrate nitrogen, sulphate sulphur, plant micronutrients (copper, iron, manganese, zinc), and a phosphorus buffer index that provides an indicator of the potential of the soil to lock up any phosphorus applied in phosphatic fertilisers.

No soil biological assays were undertaken.

4. Soil test results and their significance

4.1. 'Desirable values' for the soil test results

The results of the soil analyses are listed in Tables 2 and 3.

The main aim of the soil testing program was to determine how well the properties of the soils sampled from the field matched a set of 'desirable values' for different analytes for soils used in agriculture and horticulture. The desirable values shown in Tables 2 and 3 have been derived from an evaluation of the data of Landon (1984), Reuter and Robinson (1997), Peverill et al. (1999), the Australian Standard AS 4419 (2003) 'Soils for Landscaping and Garden Use', Hazelton and Murphy (2007), the Queensland Department of Main Roads and Transport (2012), and the author's experience of over 40 years of working with tropical soils and plants in North and Central Queensland.

Just as the soils showed strong differences from one another in their field morphological properties (Table 1), the data of Tables 2 and 3 indicate that the analysed soil samples presented some large differences from one another in soil pH, and in their contents of organic matter and macronutrients; their properties also differed from the 'desirable values' for agricultural and horticultural soils.

These soil properties are discussed in the following sections of this report.

Table 2. Values for soil pH, salinity, macronutrient, and micronutrient contents of soil samples from four sites in Central and Northern Queensland.

Soil analytical results from the NATA-registered and ASPAC-affiliated Nutrient Advantage Laboratory of Incitec Pivot Ltd, Werribee, Victoria.

Colour coding of the cells: blue cells indicate analytes with values higher than desirable values for agricultural and horticultural soils, tan cells indicate analytes with lower than desirable values.

Sample details			Acidity, alkalinity, salinity			Macronutrients							Micronutrients			
Sample number	Upper depth limit	Lower depth limit	pH (1:5 soil:water)	Electrical Conductivity	Chloride	Organic Carbon	Nitrate Nitrogen	Available Phosphorus (Colwell)	Available Phosphorus (BSES)	Phosphorus Buffer Index	Available Potassium (Colwell)	Sulphate Sulphur	Copper	Iron	Manganese	Zinc
	cm			dS/m	mg/kg	%	mg/kg	mg/kg	mg/kg		mg/kg	mg/kg	mg/kg			
	Desirable values:		*6 - 7*	*< 0.25*	*< 300*	*> 1.8*	*> 10*	*10 - 60*	*> 30*	*< 100*	*80 - 200*	*10 - 80*	*0.3 - 20*	*2 - 400*	*2 - 45*	*3 - 10*
Mptb 1	0	2	6.8	0.16	32	7.0	7.1	48	150	43	420	5	1.0	26	70	6.2
Mptb 2	0	2	5.4	0.30	21	3.4	140	42	37	410	370	45	3.1	91	35	5.3
Mptb 3	0	2	5.7	0.14	48	1.1	48	11	16	50	190	8	1.1	110	25	4.2
Mptb 4	0	2	5.5	0.41	26	2.4	190	24	38	19	340	10	0.3	74	12	3.9

Table 3. Values for exchangeable cation and particle-size properties of soil samples from four sites in Central and Northern Queensland.

Soil analytical results from the NATA-registered and ASPAC-affiliated Nutrient Advantage Laboratory of Incitec Pivot Ltd, Werribee, Victoria.

Colour coding of the cells: blue cells indicate analytes with values higher than desirable values for agricultural and horticultural soils, tan cells indicate analytes with lower than desirable values.

Sample details			Exchangeable cations: Amounts						Exchangeable cations: Proportions						Particle size				
Sample number	Upper depth limit	Lower depth limit	Cation Exchange Capacity	Exch. Calcium	Exch. Magnesium	Exch. Potassium	Exch. Sodium	Exch. Aluminium	Exch. Calcium	Exch. Magnesium	Exch. Potassium	Exch. Sodium	Exch. Aluminium	Ca : Mg ratio	Coarse sand *2.0 - 0.2 mm*	Fine sand *0.2 - 0.02 mm*	Silt *0.02 - 0.002 mm*	Clay *< 0.002 mm*	Silt + Clay *< 0.020 mm*
	cm		meq / 100 g of soil						percent					ratio	percent				
	Desirable values:		*> 5*	*> 3*	*> 0.15*	*> 0.3*			*60 - 80*	*15 - 25*	*2 - 4*	*< 6*	*< 10*	*3 - 7*				10 - 35	15 - 50
Mptb 1	0	2	22.9	16.0	6.0	0.8	0.1		70	26	4	1		2.7	22	44	14	20	34
Mptb 2	0	2	7.7	3.8	3.2	0.6	0.1	0.1	49	41	8	1	<1.3	1.2	8	45	30	18	48
Mptb 3	0	2	5.6	3.7	1.4	0.3	0.1	0.1	66	25	5	2	<1.8	2.6	11	64	19	6	25
Mptb 4	0	2	9.0	6.0	2.1	0.7	0.0	0.2	67	23	8	0	2	2.9	34	49	10	8	18

Only one of the analysed soil samples was found to have a neutral pH (Mptb 1 with pH 6.8; Table 2). One sample (Mptb 3 with pH 5.7) was acidic, and the remaining two samples (Mptb 2 and Mptb 4), with soil pH of less than 5.5 were strongly acidic.

Because soil pH exerts a major control over the availability of plant nutrients, it is possible that the strong acidity of sites Mptb 2 and Mptb 4 may have impacted on the composition and quality of the pasture at those sites and also, perhaps, on the soil microbiological environment.

All soil microorganisms have a soil pH in which they survive and grow best. It is well known that bacterial species thrive under high calcium, near-neutral soil conditions which, under favourable moisture conditions, usually produce large and diverse bacterial populations (e.g. Brady and Weil 2002; Handreck and Black 2002). On the other hand, acidic soil environments tend to allow fungi to become dominant, but they will tolerate neutral or alkaline soils (Table 4).

Table 4. Ranges of soil pH preferred by various soil microorganisms.

Source: Handreck and Black (2002, Table 11.6).

Microorganism	Preferred soil pH range	Comments
Rhizobium bacterium	Above 5	H^+ is toxic to this bacterium. Effective legume root inoculation requires Ca.
Ectomycorrhizal fungi	4 – 6; some to 7	Higher pH inhibits their seedling infection and their spread to seedlings.
Endomycorrhizal fungi	4.5 – 8	Some species still effective at higher soil pH.
Decomposers of organic matter	5 – 9	Bacteria and actinomycetes become less numerous as soil pH declines; fungi dominate at low soil pH.
Bacteria that convert ammonium to nitrate	above 6	Ammonium from fertilisers is only slowly converted to nitrates for plant uptake in soils with pH less than 6.
Bacteria that attack fungi	6.5 – 7.5	Some are active at higher or lower soil pH but their effectiveness is greatest near neutral soil pH.

4.2 Soil salinity

The salinity status of the soil is indicated by the values of two analytes: electrical conductivity and chloride content. The former is simply a measure of the content of soluble salts in the soil and is determined by how readily an electrical current may be passed through a thoroughly mixed suspension of 1 part of soil to 5 parts of water. Some soluble salts such as gypsum (calcium sulphate) or lime (calcium carbonate) may have little impact on plant growth, but may accumulate in the soil under dry climatic conditions, may be dissolved by the water use in electrical conductivity testing, and may produce a relatively high value for the conductivity determination. Hence, high electrical conductivity values, when considered in isolation, do not necessarily indicate a salinity problem in the soil.

In Australian agriculture, most of the soil salinity issues that are detrimental to plant growth are related to elevated contents of sodium chloride in the soil. Hence the chloride content of the soil is always included in a rigorous analysis of soil salinity issues. If the soil electrical conductivity and chloride content exceed values of 0.25 dS/m or 300 mg/kg, respectively, then it is possible that the salinity of the soil may impact on the growth of salt-sensitive vegetation.

All of the chloride determinations made on the four analysed soils were very low (21 – 48 mg/kg) indicating that there is no salinity issue in any of the soils. Therefore, the relative sizes and persistence of soil bacterial populations are unlikely to be influenced by soil salinity effects.

The electrical conductivity of two of the soil samples (Mptb 2 and Mptb 4) were found to lie at a higher than desirable level. While this is indicating a significant content of soluble salts in the two soils, it is also associated with high nitrate nitrogen and high plant-available phosphorus contents (Table 2). The data suggest that the pastures at sites Mptb 2 and Mptb 4 had been fertilised with a fertiliser mix that probably included potassium nitrate; the fertiliser application occurred prior to sampling the soils and there was little rain between fertilising and sampling to leach the soluble nutrients from the topsoils.

4.3 Soil fertility

4.3.1 Organic carbon

The organic carbon content of a soil indicates the status of the reserves of organic matter that may be broken down by microbially-mediated decomposition processes in the soil to produce plant nutrients to be taken up by the next generation of plants to grow at the site. Generally, the soil microbial population will be higher where there are more plant residues (organic carbon sources) in the soil to provide nourishment for the soil microbes.

The soil of site Mptb 1 had the highest organic carbon content and, therefore, the highest potential population of soil microbes; the near-neutral pH of the soil suggests that the microbial population is likely to be dominated by bacteria (as discussed in Section 4.2, above). Hence, it might be expected

that the effects of the favourable soil pH and soil organic matter content of this site may have been combined to also provide an effective niche for the vigorous growth of M. paratuberculosis bacteria.

Sites Mptb 2 and Mptb 4 also presented desirable contents of organic carbon but had strongly acidic soil pH (Table 2). While providing good reserves of organic matter to sustain soil microbial populations, the strongly acidic conditions are likely to be detrimental to soil bacteria and may be inhospitable environments for the survival of M. paratuberculosis bacteria.

Of the four analysed soils, only the soil of site Mptb 3 had a lower than desirable content of organic carbon, hence its soil microbial population is likely to be lower than that at the other three sites. The acidic soil pH (5.7; Table 2) may allow populations of M. paratuberculosis bacteria to persist.

4.3.2 Plant nutrient contents

Given their moderate to high soil organic matter reserves, three of the four analysed soils appeared to be moderately to strongly fertile and capable of supporting good pasture growth. The chemical fertility of the soil of site Mptb 3 appears to be somewhat rundown with lower than desirable contents of organic carbon, plant-available phosphorus and potassium, and sulphur.

The high nitrate nitrogen and plant-available potassium contents of the soils of site Mptb 2 and Mptb 4 (Table 2) appear to be related to recent fertilising practices at the sites (as discussed in Section 4.3, above). The high potassium contents of these soils is also evident in their higher than desirable proportions of exchangeable potassium (8%; Table 3). The soil of site Mptb 1 also presented a high plant-available potassium content, but it is not readily evident why this aspect of the soil chemistry was not also reflected in a high percentage of exchangeable potassium in the soil (4%, Table 3).

Soil testing results have been reported for two determinations of plant-available phosphorus that were designed to attempt to mimic processes in the root-zones of plants: the BSES phosphorus content was extracted from the soil by shaking with a weakly acidic solution and is best used as a fertility indicator for acidic soils (as in the present project), and the Colwell phosphorus content, extracted by a weakly alkaline solution, is better suited for assessing the phosphorus levels of alkaline – neutral soils (Reef Wise Farming 2010). The two methods may return up to 10-fold differences in values for the amount of phosphorus available in the soil for plant uptake.

At the time of submitting the soil samples to the analytical laboratory, the pH of each soil was unknown; therefore, both determinations of the plant-available phosphorus contents in the soils were requested and are reported in Table 2. The BSES phosphorus contents, relevant to the analysed soil samples, all indicate that there were adequate contents of phosphorus to support pasture growth in all of the analysed soil except for the rundown soil at site Mptb 3.

The contents of the soil micronutrients (the ‘trace elements’) all lay within the desirable ranges for agricultural soils (Table 2).

4.4 Exchangeable cations

Soils consist of gravel, sand, silt, and clay particles, often bound together in aggregates, with intervening pore spaces that hold the air and water which are essential for plant growth. Many of a plant’s nutritional needs are met from cations held in the water in the soil (the ‘soil

solution'). The most commonly occurring cations in soils are calcium [Ca2+], magnesium [Mg2+], potassium [K+], and sodium [Na+], the 'basic cations', and the first three are essential for growth in all plants. Hydrogen [H+] and aluminium [Al3+], the 'acidic cations', build up in more strongly weathered soils from which the basic cations have been lost by weathering and leaching. The ability of a soil to retain and supply nutrients as cations to plants (i.e. the 'cation exchange capacity of the soil') is controlled primarily by the composition and mineralogy of the soil constituents, particularly that of the clay fraction.

Many soil processes are driven by the electrochemical charge characteristics of the soil components. The strongly reactive components of the soil are the 'colloids', which are very tiny fragments of mineral (clay) or organic (humic) materials in the soil. They are all finer than two thousandths of a millimetre (< 0.002 mm) and present large surface areas in the soil upon which many chemical reactions take place. The surfaces of the colloidal particles usually carry a net negative electrical charge. To maintain a neutral electrical state in the soil, positively charged cations are attracted from the soil solution and are held loosely on the negatively charged sites on the surfaces of the colloids by electrostatic forces that operate similarly to the way iron filings may be held by a magnet. Different soil colloids have different abilities to attract and hold cations in the soil solution. The cations may swap, or 'exchange' positions between the surfaces of the colloids and the soil solution depending on the mineralogical and chemical properties of the soil. The overall effect determines the 'cation exchange capacity' of the soil which is, therefore, a measure of the soil's fertility.

Except for the soil at site Mptb 1 with a moderate cation exchange capacity (23 meq / 100 g of soil; Table 3), all the other analysed soils had low cation contents (6 – 8 meq / 100 g of soil; Table 3) that are likely to be sufficient to sustain pasture growth, but their low abundances may impact on soil reactions to fertiliser inputs and other land management strategies.

To ensure adequate plant growth, however, it is not sufficient just to have a good supply of exchangeable cations available in the soil; the relative contents of exchangeable cations in each soil horizon should be in a desirable balance for plant growth and aggregate stability in the soil. The ways in which imbalances in the proportions of exchangeable cations in the soil may control the physical stability of the soil are well known, and in stable productive soils, the exchangeable cations should be present in the following proportions (Hazelton and Murphy 2007):

- exchangeable calcium: 60 – 80 % of the cation exchange capacity,
- exchangeable magnesium: 15 – 25% of the cation exchange capacity,
- exchangeable potassium: 2 – 4 % of the cation exchange capacity,
- exchangeable sodium: less than 6% of the cation exchange capacity,
- exchangeable aluminium less than 10 % of the cation exchange capacity (likely to be a problem only in soils with pH less than 5.5).

The only cation imbalance evident in any of the analysed soils was a calcium : magnesium imbalance in the most acidic of the soils occurring at site Mptb 2 (Table 3) and the productivity of the soil enhanced by the addition of calcium to the soil.

Because of its strong acidity, the red clay soil at site Mptb 2 is unlikely to host the M. paratuberculosis bacteria. If lime were applied to the soil to ameliorate its Ca : Mg imbalance, the associated rise in soil pH could turn the soil into a more attractive host for pathogenic bacteria.

Hence, any increase in calcium content of this soil to promote better pasture growth should be brought about by gypsum (calcium sulphate) applications that will not affect the soil pH.

4.5 Soil morphological properties

The light field textures (Table 1) and low clay contents (Table 3) of samples Mptb 3 and Mptb 4 suggest relatively low soil water-holding capacities for the soil samples and are likely to produce relatively rapid drying conditions in the topsoil after rain. On the other hand, however, the heavier textures and higher soil moisture retention characteristics of the topsoil samples at site Mptb 1 and Mptb 2 suggest that, all else being equal, these sites could hold soil moisture longer into the dry season than the lighter textured soils.

These data imply that should the soils be infected with a bacterial pathogen, the bacterial populations may persist longer into the dry season at the sites with heavier topsoil textures, that is at sites Mptb 1 and 2).

5. Discussion

5.1 Soils and M. paratuberculosis bacterial populations

It is now possible to use the soils data presented and discussed above to rank the four sites from which the analysed were collected in terms of their possible susceptibility to the development and persistence of populations of M. paratuberculosis.

The following ranking is based entirely upon the interpretations made from the field morphological, chemical, and particle-size properties of the analysed soil samples. Moist emphasis has been placed on the soil pH conditions whose impact on soil bacterial and fungal populations is well known in the soils literature. Some importance has been placed on the organic matter contents of the soil samples and less on their field textures and associated water-holding capacities.

1. The soil sample most likely to be infected by M. paratuberculosis is Mptb 1 with neutral pH (6.8), very high organic matter content, high clay content and associated water-holding capacity. The pH of the soil will favour the development of bacteria over fungi at the site; the very high soil organic carbon contents will provide a good food sources for the bacteria, whose populations will also be sustained by the favourable soil moisture-holding capacity of the soil.

2. If any other soil sample is infected by M. paratuberculosis, it will be sample Mptb 3 with the next highest soil pH (5.7), but low organic carbon and clay contents. It is possible that this sample has a sufficiently low soil pH, however, to prevent the build up of strong bacterial populations. This topsoil is also likely to dry out quickly after rain providing a less hospitable environments for the persistence of any bacterial population that may initially establish at the site.

3. The soil samples least likely to be infected by M. paratuberculosis populations are sites Mptb 2 and Mptb 4. . Both soil samples would provide adequate organic matter for survival of the soil microbes, but their strongly acidic soil conditions (pH 5.4 and 5.5; Table 3) and likely to favour strongly the growth of fungi rather than bacteria. Although site Mptb 2 presented the highest (silt + clay) content of all of the analysed soils, and is likely to have the highest water-holding capacity of all of the soils, that soil moisture would favour the growth and persistence of fungi rather than by M. paratuberculosis populations.

The foregoing soil assessments and site rankings should now be tested against the distributions of M. paratuberculosis populations across the four analysed topsoils in Central and Northern Queensland that are known to the Bovine Johne's Disease Project personnel at James Cook University. Poor agreement means that other, less obvious soil properties are likely to be controlling the bacterial populations in the soil and further soil analysis may be required.

5.2 Soil management practices to reduce pathogenic bacteria populations

If there is good agreement between the soil properties, especially soil pH, and the vigour and survival of M. paratuberculosis populations, as suggested in this report, then a small research project is proposed. The outcomes from the research may be used to set targets for land management practices that have high potential for controlling the bacterial pathogens. But before discussing the proposed research project, it is important to outline the ease with which soils may be acidified.

5.2.1 Soil acidification processes

A number of agrichemicals and agricultural practices are capable of acidifying soils. Some of the more common ones are discussed below.

5.2.1.1 Sulphur

Possibly the most common practice used to acidify the soil, is the application of elemental sulphur. Most soil pH kits for home garden use offer the following rule of thumb: the pH of the uppermost 10 cm of a medium-textured soil (i.e. loamy soil that is not excessively sandy or excessively clayey) may lowered by 1 pH unit by the application of 0.5 – 0.7 t of sulphur / ha. The amount of sulphur needed to change the pH of a sandy soil is about half of that of the rule of thumb, and for a clay-rich soil is about twice the rule of thumb. Modifying the pH of a greater depth of soil, or by a larger number of pH units, can be determined by simple multiplication.

There are several disadvantages in using elemental or ‘agricultural sulphur’:

- the dust from the sulphur is irritating to the eyes and nose of the operator and is nasty to use;
- the sulphur is relatively slowly soluble and has to go through a microbial decomposition process in the soil before it is effective as a soil amendment, so it takes a reasonable period of time to start working effectively after its application.

The first problem may be overcome by the use of 'prilled sulphur' which is available as small, waxy beads or 'prills' that are much easier to work with, but slower to break down in the soil. But the second problem remains.

5.2.1.2 Nitrogenous fertilisers

Nitrogen is the element most needed by pasture plants and usually the element that is most deficient in the soil. Nitrogenous fertilisers (e.g. urea, ammonium-, or nitrate-based fertiliser) are designed to lift the nitrogen content of the soil and thereby sustain pasture growth. The nitrogen is converted by microbial processes to ammonium salts and then nitrates before uptake by growing plants. Any nitrogen applied to the soil in excess of the plant requirements will be leached from the soil, predominantly as Ca(NO3)2, produces a relative enrichment of acidic cations (H+, Al3+), thereby acidifying the soil:

The form of nitrogenous fertiliser strongly influences soil acidification rates, with the most effective being ammonium sulphate and monoammonium phosphate (Table 4). Alkaline N-fertilisers (e.g. calcium nitrate) do not acidify the soil.

Table 4. Soil acidification rates induced by different nitrogenous fertilisers

Fertiliser and soil acidification	No nitrate	100% nitrate
Most acidifying = ammonium fertilisers:	**3.**	**7.**
Medium acidification:	**1.**	**5.**
Low acidification:	**0**	**3.**
Urea, ammonium nitrate, aqua ammonia, anhydrous		

The values in the cells indicate the weight (kg) of lime needed / kg of N added to neutralise the acidity induced by fertiliser leaching. Source: Pratley and Robinson (1998).

The major benefits from using a nitrogenous fertiliser to acidify the soil are two-fold:

- the pasture grass is provided with nitrogen – a major nutrient that is almost inevitably deficient in Central and North Queensland pastures;
- the leaching of any excess fertiliser below the root-zone of the grasses will gradually acidify the soil in more pasture-friendly manner than that from a heavy sulphur application.

5.2.1.3 Grow pasture legumes

Infections of bacteria of Rhizobium spp in legume roots will secrete atmospheric nitrogen and with the passage of time that nitrogen is released into the soil. Like excessive

nitrogenous fertiliser the legume-fixed nitrogen can also be leached from the soil and will gradually create acidic conditions.

Legumes crops are sown into sugarcane paddocks at the end of each cropping cycle in many districts for two main reasons:

- to break cycles of pests and diseases caused by soil-borne microbial pathogens hosted by sugarcane residues that persist in the soil after plough-out of poor crops;
- to provide a biological source of nitrogen that will offset the need for heavy nitrogen fertiliser applications at the time of planting of the next crop.

Legumes can provide significant inputs of nitrogen into the soil. The data of Table 5 show the amounts of nitrogen that will accumulate in the soil if high- and low-yielding crops (8 and 2 t/ha, respectively) of three different legumes are ploughed into the soil, and also the reduced amounts of nitrogen returned to the soil if the grain of the legume crop is harvested before the stubble is incorporated into the soil.

Table 5. Nitrogen contributed to the soil from three good and poor legume crops used in sugarcane cultivation in tropical Queensland. Data from 'BSES Six Easy Steps', Schroeder et al. 2009).

Legume	Crop yield (t/ha)	Total N contribution (kg/ha)	N contribution if grain harvested (kg/ha)
Soybean	8	360	120
(3.5% N)	2	90	30
Cowpea	8	290	100
(2.8% N)	2	70	25
Lablab	8	240	80
(2.3% N)	2	60	20

Leaching of nitrogen from decomposing leguminous residues has increased the acidity of the soils of some pastures of the Southern Highlands of New South Wales by 1 – 2 pH unit over 50 years (Williams 1980); comparable soil acidification rates were determined by Noble et al. (1997) under stylo-dominated pastures in tropical Queensland.

The legume-induced soil acidity in some NSW Southern Highlands pasture soils has risen sufficiently to depress the growth of the native grass pastures that were not well adapted to acidic soil conditions and have impacted negatively on sheep production (Russell 1986). Estimates of the amount of lime that would be required to neutralise the current rate of soil acidification lie in the range of 40 – 250 kg of calcium carbonate / ha each year (Helyar and Porter 1989; Ridley et al.1990a, 1990b).

Thus, soils under legume-dominated pastures may be acidified by nitrate leaching over somewhat longer time periods than may occur under the use of nitrogenous fertilisers.

5.2.1.4 Use of other inputs

A variety of other chemical methods such as the application of ferrous sulphate, phosphoric acid solution, or peat may be used to acidify small areas of horticultural soils. Their use at paddock scales for the amendment of pastoral soils is either impractical or prohibitively expensive.

5.2.2 Proposed research project

If soil pH is found to be the primary control over M. paratuberculosis populations in tropical and subtropical pastures, then there are several effective methods that may be used to acidify agricultural soils. But before implementing a soil acidification program over infected pastures, it is necessary to identify the soil pH threshold below which the pathogenic bacteria cannot survive. This may be attempted through field trials, but a simple laboratory study may be effective in identifying the target pH to which the pH of the pastoral should be amended.

It is proposed that a laboratory and field study be undertaken in four easy stages to test the hypothesis that the vigour and persistence of M. paratuberculosis can be controlled by reducing the pH of infected soils to a level that makes the soil inhospitable for the pathogenic bacteria. Those stages are set out below:

Stage 1. In the laboratory, grow M. paratuberculosis populations (or populations of whatever bacteria occur in Bovine Johne's Disease-infected soils) on an agar substrate and then assess their susceptibility to increasing acidity of the growing medium in order to define the most acidic pH that the pathogenic bacterium can tolerate.

Stage 2. Repeat the experiment using an inoculum of soil infected with M. paratuberculosis in the growing medium to demonstrate that the threshold acidity for the survival of the bacteria under sterile laboratory conditions (Stage 1) also apply to real-world conditions.

Stage 3. Using the results from Stages 1 and 2, define the soil pH that will be toxic to M. paratuberculosis populations (the ‘target soil pH’). Collect topsoil samples systematically from field site(s) which are known to be the infected by M. paratuberculosis and determine their soil pH values by laboratory testing. Determine the level of acidification required to reduce the pH of each soil to the ‘target soil pH’. Return to the infected sites and use one of the methods discussed in Section 5.2.1 (above) to reduce the pH of the paddock topsoil to the ‘target soil pH’.

Stage 4. After there has been sufficient rainfall, or irrigation water applications, to mobilise the soil acidification reagents through the topsoil, return to the field site and collect soil samples for the determination of both soil pH (to check that the ‘target soil pH’ had been achieved) and the size and vigour of M. paratuberculosis populations, and carry out the necessary laboratory testing to quantify the results, and perhaps modify the methods for future use.

Acidification of the soil may be required for only a short period to control the bacterial population. It may turn out to be that once a soil has been temporarily acidified to eliminate a vigorous and persistent population of bacterial pathogens, then the pH of the formerly infected soil may possibly be raised to a more productive pH by liming. On-going paddock

monitoring will be required to determine the time frames for bacterial control and the elimination of the pathogens.

6. Conclusions

Four soil samples (one or more having been collected from a paddock infected with M. paratuberculosis) have been subjected to laboratory analysis as part of the Bovine Johne's Disease Project at James Cook University. Interpretations of the soil test results, allied with the field morphological properties of the soils, suggest the following likelihood of soil infection with the M. paratuberculosis bacteria:

- Most likely infected: sample Mptb 1 with neutral pH (6.8), very high organic matter content, high clay content and associated water-holding capacity;
- Probably – possibly infected: sample Mptb 3 with the next highest soil pH (5.7), but low organic carbon and clay contents;
- Unlikely to be infected: samples Mptb 2 and Mptb 4, both of which would provide adequate organic matter for survival of the soil microbes, but their strongly acidic soil conditions (pH 5.4 and 5.5) are likely to favour strongly the growth of fungi rather than bacteria.

If this blind assessment matches the knowledge of the Bovine Johne's Disease Project personnel about which of the four analysed soil samples were from infected paddocks, then there are opportunities to manage the populations of the bacterial pathogens by way of managing the acidity of the soils supporting the pastures in infected paddocks.

A small research project has been outlined to define the target soil pH to which the pH of the soils supporting infected pastures should be lowered by soil acidification processes. How long the pasture soils need to remain in an acidic condition to eliminate active populations of paratuberculosis bacteria could be derived from long-term monitoring of acidified, formerly infected sites.

If the blind assessment is incorrect, then further work will be required to identify any obvious link between the occurrence of the pathogens and the properties of the soils of infected paddocks.

7. References

Animal Health Australia (2014). National Johne's Disease Program. http://www.animalhealthaustralia.com.au/programs/johnes-disease/bjd-home-page/about- bjd/ Viewed 30 October 2014.

Brady, N.C., and Weil, R.R. (2002). Nature and properties of soils. 13th Edition, Prentice-Hall Inc., Upper Saddle River, New Jersey.

Handreck, K.H., and Black, N.D. (2002). Growing media for ornamentals and turf. 3rd Edition, University of New South Wales Press, Randwick, NSW.

Hazelton, P.A., and Murphy, B.W. (2007). Interpreting soil test results: What do the numbers mean?CSIRO Publishing, Collingwood.

Helyar. K.R., and Porter, W.M. (1989). Soil acidification: its measurement, and the processes involved. In: A.D. Robson (ed), Soil acidity and plant growth, Academic Press, Sydney, pp. 61 – 102.

Landon, J.R. (1984). Booker tropical soil manual. A handbook for soil survey and agricultural land evaluation in the tropics and subtropics. Booker Agriculture International Ltd., London.

National Committee on Soil and Terrain (2009). Australian soil and land survey field handbook. Third edition, CSIRO Publishing, Melbourne.

Noble, A.D., Cannon, M.G., and Muller, D. (1997). Evidence of accelerated soil acidification under Stylosanthes dominated pastures. Australian Journal of Soil Research, 35, 1309-1322.

Northcote, K.H. (1979).A factual key for the recognition of Australian soils. Rellim Technical Publications, Glenside, South Australia.

Peverill, K.I., Sparrow, L.J., and Reuter, D.J. (1999). Soil analysis: an interpretation manual. CSIRO Publishing, Collingwood, Victoria.

Pratley, J., and Robertson, A. (1998). Agriculture and the environmental imperative. CSIRO Publishing, Collingwood, Victoria.

Queensland Department of Transport and Main Roads (2012). Main Roads Technical Standard MRTS16B. Vegetation Ground Works – Appendix. http://www.tmr.qld.gov.au/Business-industry/Technical-standards-publications/Standard- specifications-roads.aspx Viewed 14 February 2014.

Reef Wise Farming (2010). Calculating phosphorus application rates on alkaline soils. Technical Advisory Note No. 1, Queensland Department of Primary Industries, Brisbane.

Reuter, D.J., and Robinson, J.B. (1997). Plant analysis: an interpretation manual. CSIRO Publishing, Collingwood, Victoria.

Ridley, A.M., Slattery, W.J., Helyar, K.R., and Cowling, A. (1990a). The importance f the carbon cycle to acidification of a grazed annual pasture. . Australian Journal of Experimental Agriculture, 30, 529 - 537.

Ridley, A.M., Slattery, W.J., Helyar, K.R., and Cowling, A. (1990b). Acidification under grazed annual and perennial grass pastures. Australian Journal of Experimental Agriculture, 30, 539-544.

Russell, J.S. (1986).). Improved pastures. In: Russell, J.S., and Isbell, R.F. (eds), “Australian soils: the human impact”, University of Queensland Press, Brisbane, 374 – 396.

Schroeder, B.L., Wood, A.W., Moody, P., Park, G., Panitz, J.H., Stewart, R.L. (2009). Delivering nutrient management guidelines to growers in the Central Region of the Australian sugar industry. Proceedings of the Australian Society of Sugar Cane Technologists, 31, 177-185.

Williams, C.H. (1980). Soil acidification under clover pasture. Aust. J. Exp. Agric. Anim. Husb., 20, 561-567.

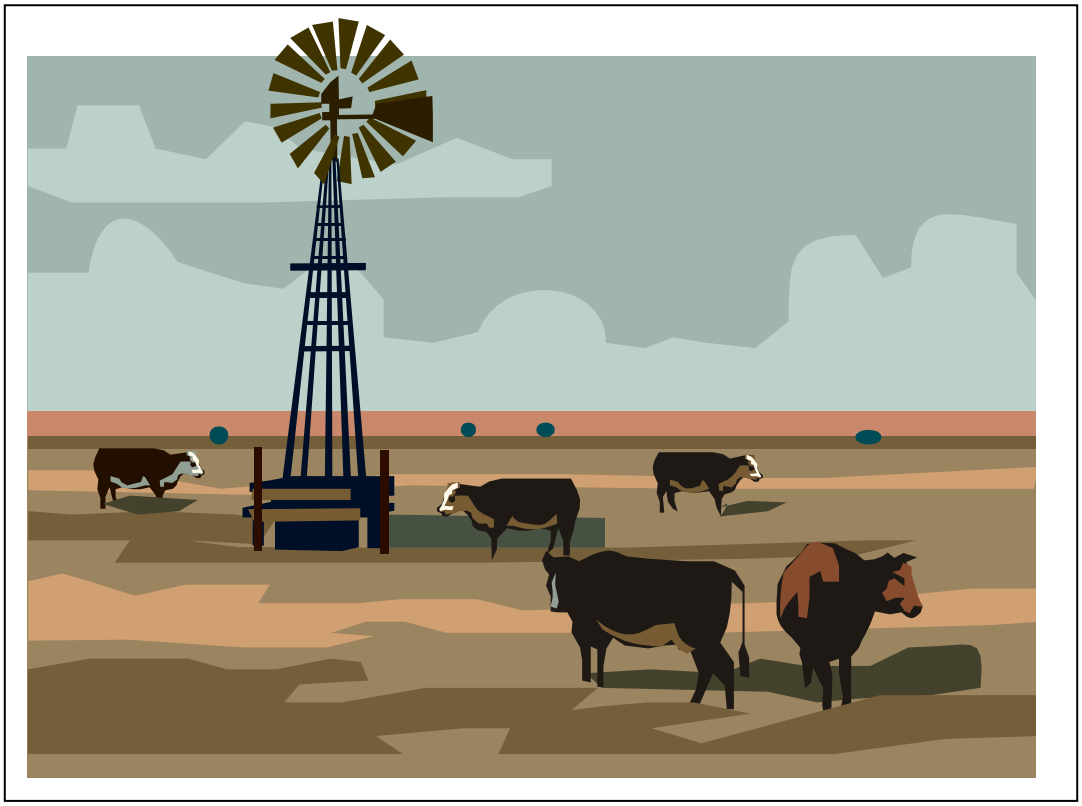
10.2 Appendix 2 - Environmental sample culture protocol

BOVINE JOHNES DISEASE –

Mycobacterium *avium*

subsp. *paratuberculosis*

CULTURE PROCEDURE



List of contents

1 Scope and application

1.1 This procedure describes the culture of M.*avium* subspecies *paratuberculosis* {M. *paratuberculosis*}from faeces/soil samples that have been exposed to environmental conditions in the field. This project is to determine the longevity of the organism in the environment.

2 Principle

2.1 The organism M.*avium* subsp. *Paratuberculosis* is the causative agent for Bovine Johnes Disease {BJD or Johnes}. It is chronic enteritis of ruminants transmitted by the faecal-oral route, previously confined to southern Australia, where climate and soil conditions are thought to account for the persistence of the organism in faecally contaminated soil.

A recent outbreak of BJD on properties in Central and North Queensland has initiated this project.

2.2 Isolation of M. *paratuberculosis* is the definitive test for BJD. Organisms shed in the faeces of BJD affected cattle remain for an undetermined time on and in soil. The purpose of this project is provide an answer to determining viability of M. *paratuberculosis* in tropical Australia.

2.3 Organism spiked soil/faeces samples will be collected at specific time periods from four field sites, then processed through two decontamination steps. One half of each collected sample will be processed, with the second half frozen at -80^{0}C, to be available if further testing is required.

There will be two techniques used for this project: the first is the culture of organisms from the samples using the medium BBL 7H11 agar with the addition of Mycobactin J (an iron chelator), Pyruvate (a growth promotant), and Oleic/Albumin/Dextrose/Catalase (OADC) supplement.

The second technique to be used is a micro-method for the determination of the “Most Probable Number {MPN}” of Mycobacterium *paratuberculosis* present in each sample. Growth of M. *paratuberculosis* is usually evident at 6 – 8 weeks, but may take up to 16 weeks.

3 Reagents

3.1 Middlebrook 7h11 agar slopes with OADC Supplement, Mycobactin J, PANTA, egg yolk, & Malachite green.

3.2 Middlebrook 7H9 Broth with OADC Supplement , Mycobactin J, PANTA, egg yolk.

3.3 Middlebrook 7H9 Broth without supplements

3.4 HPC/50%BHI Solution (Hexadecylpyridinium Chloride/Brain Heart Infusion)

3.5 VAN/50%BHI Solution (**V**ancomycin,**A**mphotericinB,**N**aladixic Acid/50% Brain Heart Infusion)

3.6 Sterile deionised Water plus Tween 80 (100μl)

3.7 Zeihl-Neelson (ZN) Stain Reagents

3.8 Gram Stain (Gm) Reagents

3.9 Positive Controls:– 7H9 Broth w/- all supplements plus *Mptb bovis & bison*.

4 Equipment

4.1 Centrifuge

4.2 Centrifuge tubes, 50ml, disposable

4.3 Biohazard hood, Class 2

4.4 Glass mixing beads, 6mm, sterilised in vials

4.5 Incubator @ 37^0C

4.6 10ml disposable pipettes

4.7 Pipette-boy for graduated pipettes

4.8 Pipettor adjustable 2 -200μl

4.9 Pipettor adjustable 20 - 1000μl.

4.10 Tips, sterile, disposable, to fit automatic pipettes

4.11 Transfer pipettes, 3ml, disposable, sterile

4.12 Microscope slides

4.13 Microscope

4.14 Vials, sterile, 50ml, polypropylene

4.15 Vials, sterile, 20ml polyproylene

4.16 Vials, sterile, 5ml.

4.17 Sterile tissue culture plates, 24 well, Suspension cell, flat bottom.

4.18 Sterile tissue culture plates, 24 well, flat bottom.

5 Samples and sampling

5.1 Samples will be a mix of faeces and soil, preweighed, and spiked with a pre-determined number of M. *paratuberculosis* organisms. The faeces will be collected from Johnes Disease free cattle based at James Cook University. The soil used will be collected from each test site.

5.2 Samples will be processed within 48hours of receipt. If this is not possible, the samples will be stored at -70^{0}C to -80^{0}C till processing can be done.

5.3 Collected samples, upon receipt, will be given an identifying number [BJD *n*] (see Appendix 9.1) and labelled in the following format "BJD*n.*1.*"*(where'1' is the first group of samples in the week, '2' is the second and so on) The individual site sample replicates will always be numbered as follows:- 1/1, 1/2 (bison DRY); 2/1, 2/2 (bison WET); 3/1, 3/2 (bovis DRY); 4/1, 4/2(bovis WET). This will correspond with the field data will be entered onto laboratory data sheets (see Appendix 9.2); and sample data entered onto laboratory culture sheets(see Appendix 9.3).

5.4 All jars and tubes used to process each sample will be labelled to match sample number.

5.5 Each sample will also be tested by "Most Probable Number"{**MPN**}technique to determine bacteria load in low numbers. To facilitate handling, this will be done by using a microtitre methodology.

6 Procedure

6.1 Sample preparation

6.1.1 The top layer of sample is divided in half and remaining sample halved again. The full half is added to a labelled 20ml sterile vial to be cultured fot M*ptb.* Half of the remaining sample is placed in a labelled 5ml sterile vial and stored at -80^{o}C for PCR section. The final portion of sample is handed on for PCR screening.

6.1.2 10ml of Sterile deionised water + Tween 80 is added to the DRY Samples to rehydrate for processing.

6.2 Sample decontamination

6.2.1 Samples are pulverised by hand using disposable plastic spoon handle, then approx. 3grams is added to labelled 50ml vial, and remaining sample is split between x2 5ml vials for storage at -80^{o}C. , [1] to -80^{o}C{long term storage}, and [2] to -80^{o}C {short term storage – for reprocessing if required}

6.2.2 Add 35ml 0.5%HPC in 50% BHI broth (3.4) to vial and mix vigorously. Incubate overnight [24 hours]at 37^{o}C.

Control: Add 5ml Mptb control culture to 35ml 0.5%HPC/BHI(3.4). Process as for test samples.

6.2.3 After incubation, 30mL of the supernatant is transferred (avoiding fibrous sediment) to a new 50mL tube. Centrifuge [3,500RPM] for 30 minutes (keep temperature at >10^0C to avoid precipitation).

6.2.4 Discard supernatant and suspend pellet in 1mL VAN/BHI(3.5). Incubate is at 37^0C overnight.

6.3 Slope inoculation

6.3.1 Add 200µl to each sample to x2 7H11 slopes. Spread inoculum evenly over slopes with a sterile 10µl inoculation loop. Add 200ul to 6ml of 7H9(with supplements) liquid media{9.4.3}.

POSITIVE CONTROLS: Inoculate a 7H11 slope with processed Mptb cultures.

6.3.2 Place slopes in labelled rack for incubation at 37^0C.

6.4 Plate count

6.4.1 Inoculate a 7H11 agar slope {70ml Vial} with 200µl of sample and streak for Plate Count analysis. Check plates weekly for growth.

6.4.2 Once easily discernable colonies are present, count all colonies present.

6.4.3 Calculate total colonies per soil/faeces sample based on 2000µl inoculum.

6.5 Broth inoculation for MPN

6.5.1 Dilution of sample: Set up 24-well tissue culture plates as shown {1 plate = x4 samples}:

(Plate notation is written on lid only)

Eg: Culture BJD 13-1, samples 1/1 to 2/2.

BJD 13-1	1	2	3	4	5	6
	10^{-1} 1/1	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
	1/2					
	2/1					
	2/2					

6.5.2 To dilute each sample: mark a 24 well tissue culture plate with sample number per row. Add 1800µl 7H9Broth (3.2) in wells 1 to 5. Add 200µl of sample to Well 1[10^{-1}], then add 200 µl 10^{-1}sample to Well2(10^{-2}); mix carefully, and continue to Well 5(10^{-5}). There are now x5 10-fold dilutions for each sample. **Change Oct2015- volume now 2/3 of stated volume ie 1200µl + 134µl. To minimise slippage from wells.**

6.5.3 MPN Set-Up: Add 1800µl Supplemented 7H9 broth (No EGG) to required cells of 24 well Tissue culture SUSPENSION CELL Plates.

Blank/ Negative Control
Test Sample
Positive Control – Mptb *bison*
Positive Control - Mptb *bovis*
No Test

1	2	3	4	5	6
10^{-0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
X1 well only					
BLANK	Positive control *Mptb bison*	**Sample details: BJD#,**		Positive control *Mptb Bovis*	BLANK

6.5.4 To each column [x3 wells] add 200µl of each dilution of sample.

6.5.5 Add 200µl sup. 7H9 broth to BLANK wells to maintain volume.

POSITIVE" CONTROLS:- Add known Myc.*paratubulosis (bovis & bison)* to marked wells in Row 4.

6.5.6 Seal plates with Parafilm. Leave plate on bench surface while laying paprafilm around – parafilm can snap and this jars the plate causing spillage.

6.5.7 Incubate plates in strong clipseal bags at 37^{o}C in a sealed plastic box.

6.5.8 Reading MPN Plates:- From 4 weeks incubation, check plates for growth. Add 400µl Tetrazolium dye (9.6.10) to each well. Check for colour development, then read cutoff point (takes about 48-72 hours)

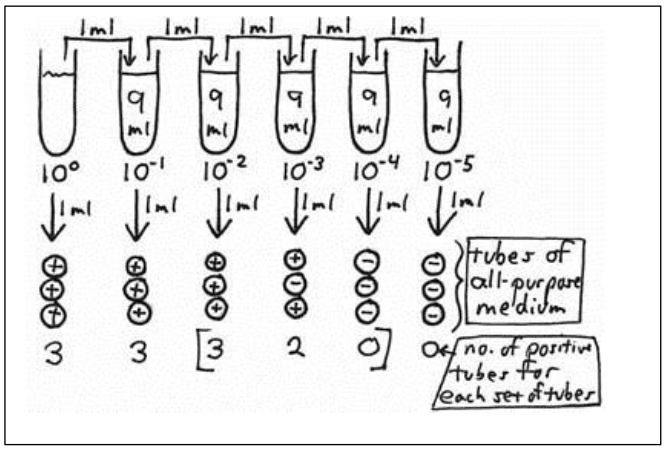
Use Table 1 to determine MPN score for each sample.

6.6 Incubation

6.6.1 Incubate all culture slopes and MPN plates at 37^{0}C till growth is present, approximately 6 – 16 weeks. Record observations on sheet "BJD PROJECT CULTURE RECORD", "MPN Data Results", and "BJD PROJECT PLATE COUNT RESULTS" (Sect. *9.3)

6.6.2 Any growth on slopes is to be checked by making a smear of growth, and staining with Ziehl-Neelson staining. Cultures positive for acid fast bacilli (AFB's), are to be passed on for PCR confirmation.

6.7 MPN determination (using text, diagram and Table below, referenced from web page: John Lindquist, Department of Bacteriology, University of Wisconsin – Madison)



6.7.1 After incubation, the number of tubes showing growth is recorded. As succeeding dilutions were made, the organisms were diluted to such an extent that none were in the inocula of the seven tubes [marked negative]. In order to estimate the number of organisms per ml of the sample which would cause this kind of growth response, we locate the three sets of tubes which show the dilution of the organisms "to extinction" – ie, those tubes which were inoculated from 10^{-2}, 10^{-3}, and 10^{-4} dilutions. The result is a score of 3-2-0.

6.7.2 Using the 3-digit score for each sample, from TABLE 1, determine the result. Eg 3-2-0 gives a score result, 0.93. This is then multiplied by the dilution value of the middle set of tubes, 10^{-3}. Therefore, the Most Probable Number of organisms per one ml of the original undiluted sample would be: 0.93×10^3 or 9.3×10^2

Table 1 MPN result scoring

No. of Tubes Positive in:-			MPN in the inoculum of the Middle set of tubes
1st set	2nd set	3rd set	
0	0	0	<0.03
0	0	1	0.03
0	0	2	0.06
0	0	3	0.09
0	1	0	0.03
0	1	1	0.061
0	1	2	0.092
0	1	3	0.12
0	2	0	0.062
0	2	1	0.093
0	2	2	0.12
0	2	3	0.16
0	3	0	0.094
0	3	1	0.13
0	3	2	0.16
0	3	3	0.19
1	0	0	0.036
1	0	1	0.072
1	0	2	0.11
1	0	3	0.15
1	1	0	0.073
1	1	1	0.11
1	1	2	0.15
1	1	3	0.19
1	2	0	0.11
1	2	1	0.15
1	2	2	0.20
1	2	3	0.24
1	3	0	0.16
1	3	1	0.20
1	3	2	0.24
1	3	3	0.29
2	0	0	0.091
2	0	1	0.14
2	0	2	0.20
2	0	3	0.26
2	1	0	0.15
2	1	1	0.20
2	1	2	0.27
2	1	3	0.34
2	2	0	0.21
2	2	1	0.28
2	2	2	0.35
2	2	3	0.42
2	3	0	0.29
2	3	1	0.36
2	3	2	0.44
2	3	3	0.53
3	0	0	0.23
3	0	1	0.39
3	0	2	0.64
3	0	3	0.95
3	1	0	0.43
3	1	1	0.75
3	1	2	1.2
3	1	3	1.6
3	2	0	0.93
3	2	1	1.5
3	2	2	2.1
3	2	3	2.9
3	3	0	2.4
3	3	1	4.6
3	3	2	11
3	3	3	>24

7 Results

7.1 Culture results are to be recorded on the Laboratory Data sheets (Appendix 2).

7.2 All laboratory data is to be entered onto duplicate documents stored on Shared Drive 'SOVBS(\\fmhms-share.jcu.\fmhms)(V:) SVBS_BJD

8 References

8.1 'Australia and New Zealand Standard Diagnostic Procedures, April 2002' JD

8.2 OIE Terrestrial Manual 2008, Chapter 2.1.11, "Paratuberculosis (Johne's Disease) Journal of Clinical Microbiology, May 2011, p 1822 -1830. Vol. 49, No.5 "Culture Phenotypes of Genomically and Geographically Diverse *Mycobacterium avium* subsp *paratuberculosis* Isolates from Different Hosts. Richard J.Whittington, *etal.*

8.3 Journal of Clinical Microbiology, May 2011, pg 1822-1830. "Culture Phenotypes of Genomically & Geographically Diverse Mycobacterium *avium* subsp. *Paratuberculosis* Isolates from different Hosts." Richard J. Whittington *et al*.

8.4 Journal of Validation Technology [Summer 2010] Vol 16, No 3. 'The Most Probable Number Method and it's uses in Enumeration, Qualification and Validation' Scott Sutton.

8.5 Web Page:- "The Most Probable Number Method" {from Dilution Theory Pg 3}, by John Linguist, Dept of Bacteriology, University of Wisconsin. USA.

8.6 J. of Milk Food Technology, Vol 38. No:9, Pgs 540-545 (Sept 1975). "Understanding and Teaching the Most Probable Number Technique" J.L.Oblinger and J.A.Koburger, Food Science Dept. University of Florida, Gainesville. USA.

8.7 Applied Microbiology, June 1973, p956-960. American Society for Microbiology. "Preservation of Mycbacteria: 100% Viability of Suspensions Stored at -70^{0}C. Thomas H. Kim and George P. Kubica. Trudeau Institute, Inc., Saranac Lake. New York. 12983

8.8 US Food and Drug Administration. Food Science & research laboratory Methods. Bacteriological Analytical Manual (BAM) "BAM Appendix 2 Most Probable Number from Serial Dilutions" Robert Blogett (retired). October 2010.

8.9 US Dept of Agriculture Food Safety and Inspection Service, Office of Public Health Science. "Most Probable Number and Procedure and Tables". 28/1/2008.

8.10 Journal of Clinical Microbiology, May 2002 pg 1873-1874. "Tetrazolyium Microplate Ass as a Rapid and Inexpensive Colormetric Method for Determination of Antibiotic Susceptibility of *Mycobacterium tuberculosis."* Luz Caviedes, Jose Delago, Robert H. Gilman.

8.11 Jour. Of Vet Diagnostic Investigation17:554-560(2005), “Effect of egg yolk on the detection of *Mycobacterium avium paratuberculosis* using ESP II liquid culture system. N. Beth Harris; Suelee Robbe-Austermannn; Janet B. Payeur

8.12 Jour. Of Clinical Microbiology. Dec 2013; 51(12): 3993 – 4000. “Development and Validation of a liquid Medium (M7H9C) for routine culture of M*ycobacterium avium* subsp *paratuberculosis* to Replace Modified Bactec 12B Medium. Richard J. Whittington, Ann-Michele Whittington, Anna Waldron, Douglas J. Begg, Kumi De Silva, Auriol C. Purdie, and Karren Plain.

9 Appendices

9.1 BJD project sampling number schedule

BJD project sampling number schedule 2014-2015

Sample No:	Week Dates	Sample No:	Week Dates	Sample No:	Week Dates
BJD1	2014 March 3 - 7	BJD26	August 25 – 29	BJD51	Feb 16 – 20
BJD2	March 10 – 14	BJD27	Sept 1 – 5	BJD52	Feb 23 - 27
BJD3	March 17 - 21	BJD28	Sept 8 – 12	BJD53	March 2 - 6
BJD4	March 24 – 28	BJD29	Sept 15 –19	BJD54	March 9 - 13
BJD5	March 31 – Apr 4	BJD30	Sept 22 - 26	BJD55	March 16 - 20
BJD6	April 7 – 11	BJD31	Sept 29 – Oct 3	BJD56	March 23 - 27
BJD7	April 14 - 18	BJD32	October 6 – 10	BJD57	Mar 30 –Apr 3
BJD8	April 21 - 25	BJD33	October 13 – 17	BJD58	Apr 6 - 10
BJD9	April 28 – May 2	BJD34	October 20 - 24	BJD59	Apr 13 - 17
BJD10	May 5 - 9	BJD35	October 27 - 31	BJD60	Apr 20 - 24
BJD11	May 12 – 16	BJD36	November 3 - 7	BJD61	Apr 27 – May 1
BJ12D	May 19 - 23	BJD37	November 10 – 14	BJD62	May 4 - 8
BJD13	May 26 – 30	BJD38	November 17 – 21	BJD63	May 11 - 15
BJD14	June 2 – 6	BJD39	November 24 - 28	BJD64	May 18 - 22
BJD15	June 9 – 13	BJD40	December 1 - 5	BJD65	May 25 - 29
BJD16	June 16 – 20	BJD41	December 8 – 12	BJD66	June 1 - 5
BJD17	June 23 – 27	BJD42	December 15 - 19	BJD67	June 8 - 12
BJD18	June 30 – July 4	BJD43	December 22 – 26	BJD68	June 15 - 19
BJD19	July 7 – 11	BJD44	Dec 29 – Jan 2 {2015}	BJD69	June 22 - 26
BJD20	July 14 - 18	BJD45	**2015** Jan 5 –Jan 9	BJD70	Jun 29 – Jul 3
BJD21	July 21 – 25	BJD46	Jan 12 – Jan 16	BJD71	July 6 - 10
BJD22	July 28 – Aug 1	BJD47	Jan 19 – 23	BJD72	July 13 – 17
BJD23	August 4 – 8	BJD48	Jan 26 – 30	BJD73	July 20 - 24
BJD24	August 11 – 15	BJD49	Feb 2 – 6	BJD74	July 27 - 31
BJD25	August 18 – 22	BJD50	Feb 9 - 13	BJD75	August 3 - 7

9.2 BJD project sample data record

BJD PROJECT SAMPLE DATA RECORD

Sample Number:

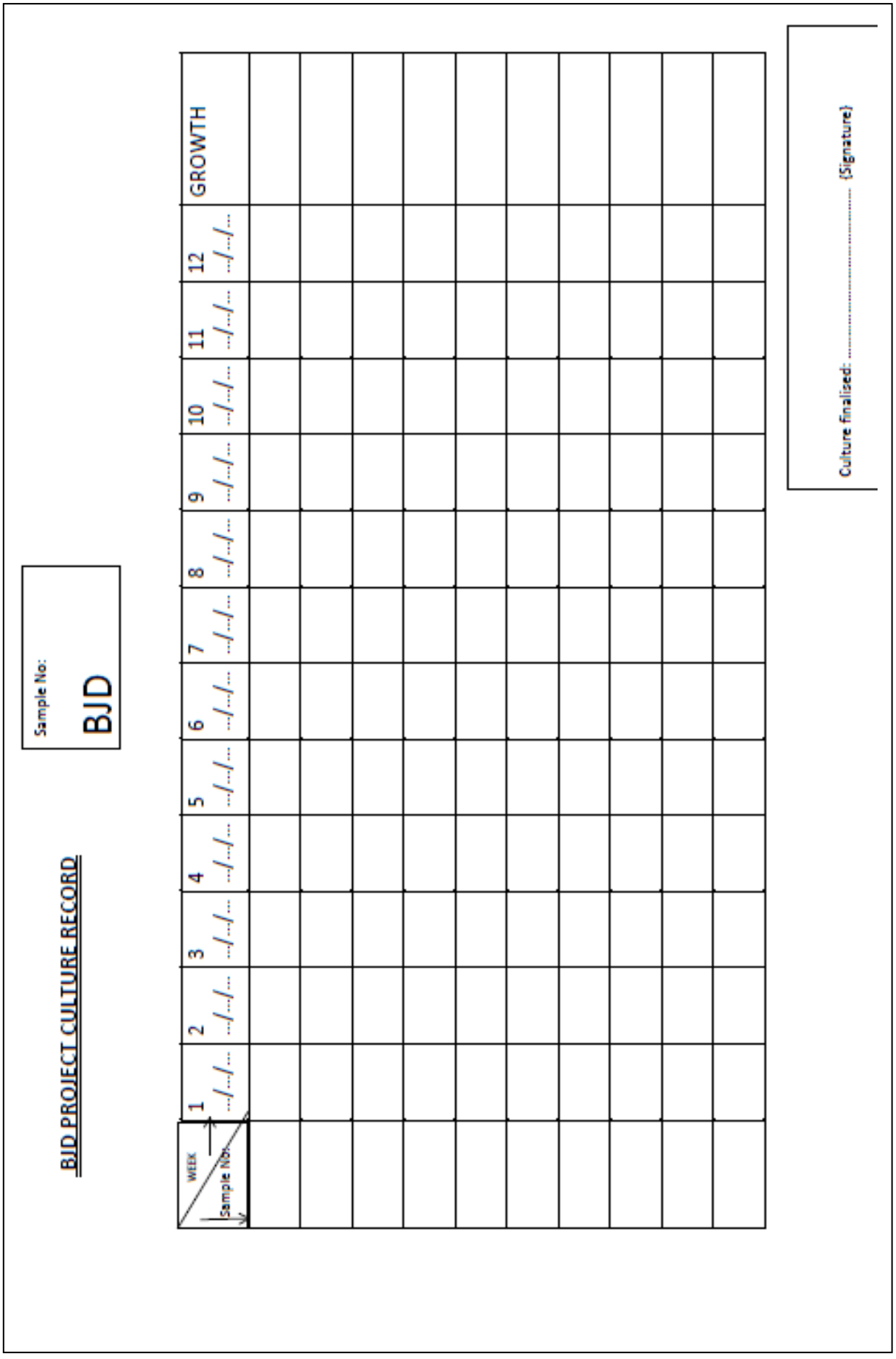
BJD

Date Received: ..... Date Collected: ..... Date Cultured: .....

Experimental Site: {A} JCU; {B} Fletcherview; {C} Dingo; {D} Malanda

Sample Data → / ↓Culture number	Season		BJD Strain		Replicate		Weight of soil/faeces			Culture Result		
	Wet	Dry	Bovine	Bison	1	2	Sample Weight	Vol. H_2O To 80	Total Wt for Processing.	Growth on Slope	AFB	PCR Result

9.3 BJD project culture record



9.4

MPN result record

MPN Data Results

Date Prepared: **Date read:**

Sample No: BJD

Sample No:	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	Score	RESULT
1/1								
1/2								
2/1								
2/2								
3/1								
3/2								
4/1								
4/2								
Positive Control *Mptb bison*								
Positive Control *Mptb bovis*								

Read by:

9.5 BJD project plate count results

Sample No: BJD

BJD PROJECT PLATE COUNT RESULTS Date Cultured: / /

WEEK / Sample No:	1 …/…/…	2 …/…/…	3 …/…/…	4 …/…/…	5 …/…/…	6 …/…/…	7 …/…/…	8 …/…/…	9 …/…/…	10 …/…/…	11 …/…/…	12 …/…/…	GROWTH
1/1													
1 /2													
2/1													
2/2													
3/1													
3/2													
4/1													
4/2													
POSITIVE CONTROL M.ptb													

Culture finalised: ..... {Signature/Date}

9.6 Media and reagent recipes

9.6.1 MIDDLEBROOK 7H11 AGAR SLOPES with supplements

Middlebrook 7H11 Agar Base (BD) 14.3gm

Deionised Water 900mL

Glycerol 3.76mL

Middlebrook OADC supplement (BD) 100mL

Antibiotic Mix (BBL PANTA MIX) 5mL

Mycobactin J 200mgm

Sterile Egg Yolk 33.0mL

Malachite green 2% 5.0mL

Mix 7H11 agar base, water and glycerol to obtain a smooth suspension, heat to boiling, then autoclave at 121^{0}C for 10mins.

Cool in 50^{0}C waterbath.

When cooled, add aseptically, 100ml Middlebrook OADC Enrichment, 50mL PANTA antibiotic mixture, Mycobactin J, egg yolk and Malachite green. Mix well.

Dispense as 12mL into sterile, 30mL screwcap vials. Allow to set at a slant to produce slope. Label with Media type, Date produced, Expiry Date {6months} and store at 4-6^{0}C.

Test for sterility by incubation at 37^{0}C for 48 hours, and, test for performance by inoculating with appropriate typical culture.

9.6.2 MIDDLEBROOK 7H11 AGAR PLATES

Make up agar as above, but pour into sterile (clean) petri dishes. Label as above and store in a sealed plastic bag in refrigerator. Leave out the egg yolk and malachite green, if required.

Alternative:

Add 26-30ml agar to sterile 70ml jars and allow to set on slant.

9.6.3 MIDDLEBRROK 7H9 Broth {with and without egg yolk}

Middlebrook 7H9 Broth Base (BD) 4.7gms

Deionised water 900mL

Glycerol 2mL

Middlebrook ADC supplement (BD) 100mL

Antibiotic Mix (In-House [PANTA] Mix) 50mL

Mycobactin J 200mgm

Sterile Egg Yolk 33.0mL

7H9 With supplements:- {for use in culturing Mptb *bison & bovis* strains for field trials, and determining MPN }

Mix 7H9 broth base, water and glycerol. Autoclave at 121^{0}C for 10 minutes. Cool in 500C waterbath. When cool, aseptically add 100mL Middlebrook ADC Supplement, Antibiotic Mix, egg yolk and Mycobactin J.

Label with Media type, Date produced, Expiry Date {6months} and store at 4-6^{0}C.

Test for sterility by incubation at 37^{0}C for 48 hours, and, test for performance by inoculating with appropriate typical culture.

9.6.4 HPC/50%BHI Solution (Hexadecylpyridinium Chloride/50% Brain Heart Infusion)

Hexadecylpyridinium Chloride (HPC) 5gm

Brain Heart infusion Broth (Acumedia) 18.5gms

Deionised Water .....1,000 mL

Mix BHI and water, dissolve, (heating to 580C may be required), then autoclave at 1210C for 20 mins. Test for sterility by incubation at 37^{0}C for 48 hours. . Label with Media type, Date produced, Expiry Date {6months} and store at 4-6^{0}C.

When required, aseptically add 5gm HPC powder and mix gently. Solution **WILL** froth.

This solution is STABLE at room temperature for ONE WEEK ONLY. Do not refrigerate.

9.6.5 VAN/50%BHI Solution (VAN/50% Brain Heart Infusion)

Reagents

50% Brain Heart Infusion Broth (Sterile) 200mL

Vancomycin 20mgm

*****Amphotericin B Solution 1mL

Naladixic Acid 20mgm

Mix Ingredients, then dispense 1mL volumes using a syringe filter and store at -20^{0}C

***Amphotericin B Stock Solution (10mgm/mL)**

Dissolve Amphotericin B in DMSO to strength 10mgm/mL, then dilute to volume 5mL with deionised water. Dispense into aliquots and store at -20^{0}C.

9.6.6 PANTA mix

Reagents

Polymixin B ..... 37,600 U

Amphotericin B ..... 3.8 mg

Nalidixic acid ..... 15 mg

Trimethoprim ..... 3.8 mg

Azlocillin ..... 3.8 mg

Samples and sampling

	%purity	re/litre	mg/mL	10x in 50 mL (mg)	solvent
Polymixin B	93.9	37600-Units	6.7	3335.7	water
Amphotericin B	75	3.8mg	5.1	2533.3	DMSO
Nalidixic acid	100	15mg	15	7500	1 M NaOH in water
Trimethoprim	100	3.8mg	3.8	1900	1 M NaOH in water or DMSO
Azlocillin	95.25	3.8mg	4.0	1994.8	water

Make up 10X concentration stock solutions of each antibiotic in 50mL solvent using the corresponding masses and solvents shown in the Table. Freeze the stock solution until required. Just before use, defrost rapidly, dilute 1 in 10 with sterile water and add 1 mL of the working antibiotic solution to 995 mL of media.

9.6.7 Malachite Green 2%

Malachite green powder 2gms

Water, deionised 100mls

Dissolve malachite green in water. Mix well, crystals may take a while to dissolve completely. Sterilise by filtaration.

9.6.8 ZEIHL-NEELSON Stain

Reagents:

Carbol Fuchsin Stain

Commercial product used, source Australian Biostain.

Acid Alcohol

C2H5OH (95%) 97mL

Conc. HCl 3mL

Mix reagents in hood. Add acid to alcohol to prevent flashback.

CounterStain – Methylene Blue

Commercial product used, source Australian Biostain.

Procedure:

- Prepare smears in Biohazard II Hood and air dry.
- Heat-fix smears over Bunsen flame. Cool.
- Flood each slide with conc. Carbol Fuchsin, heat gently till small amount of steam rises. Do not boil. ……5 - 10 minutes.
- Rinse in running tap water
- Rinse with acid-alcohol over slide till red colour no longer runs.
- Thoroughly rinse in running tap water.
- Flood with counterstain, methylene blue ……. 1 - 2 minutes.
- Rinse with running tap water, then air dry.

Read using x100 magnification and oil.

Results:

Acid Fast Organisms --- pink to red

Non-Acid Fast Organisms --- blue.

9.6.9 Gram Stain

Reagents:

Crystal Violet Stain

Gram's iodine Solution

Safranin O Stain

Alcohol 70% - Acetone 30% Decolourising solution.

All Stains are commercially sourced from Australian Biostain.

Procedure:

- Prepare smears in Biohazard II hood and air-dry.
- Heat-fix smears over Bunsen flame. Cool
- Flood slide(s) with Crystal Violet ……2minutes.
- Tip off excess, wash briefly with running tap water.
- Flood with Gram's Iodine Solution……..2 minutes.
- Rinse briefly with running tap water.
- Holding slide up [with forceps], dropwise rinse slide with alcohol/acetone mix till no further purple/blue colour runs
- Rinse briefly with running tap water.
- Flood slide[s] with Safranin O Stain…….2 minutes.
- Rinse briefly with running tap water.
- Air dry and examine under oil.

Results:

Gram positive bacteria --- dark to mid Blue

Gram Negative bacteria --- Pink.

9.6.10 TETRAZOLIUM Dye for MPN Plates (Ref #8.10)

Reagents:

Tetrazolium Bromide, (thiazolyl blue)

Final solution is mixed in the ratio of: 1mgTetBrom/1mL in absolute Ethanol is mixed with 1.5mL 10%Tween 80.

Make up mix to volume required.

Added to MPN plates at 50μl/well.

10.3 Appendix 3 - Culture results

Have been filed separately and are available on request.

10.4 Appendix 4 – Molecular testing data

Data for the molecular testing of samples and the comparison of PMA and RNA testing for viability have been filed separately and are available on request.

10.5 Appendix 5 – Temperature and humidity data

Data for each site have been filed separately and are available on request.