Project No:
R80570

Project Leader
John Aitken; Inward Bound Ltd.

Report on
Final milestone of proposed research. The completion of an evaluation of the BD ProbeTec MTB in cattle and deer.

This report is divided into 5 sections:

1. Executive Summary
2. First Milestone Report
3. Second Milestone Report
4. Final report
5. Data Analysis (T.Ryan)

EXECUTIVE SUMMARY

The Animal Health Board (AHB) is committed to eradicating Mycobacterium bovis in New Zealand by 2015

1. Our task, supported by research funding from DeeResearch Ltd and the AHB, has been to source, modify and validate a PCR-based system to confirm TB infection of lymph nodes of target animals within an acceptable timeframe

2. We modified a commercially available testkit for the detection of TB in human patients to detect TB in the lymph nodes of reactor-positive deer and cattle.

3. Compared with culture as the gold standard, this test showed a sensitivity of 93% and a specificity of 100% in our hands.

4. This study has demonstrated satisfactorily the utility of the BD ProbeTec MTB test to reliably detect or exclude TB infection in lymph nodes within 24hours of sample receipt.

5. The study has also produced useful data on the comparative sensitivities and specificities of ZN stain and histological analysis for reliable TB diagnosis.

6. The cost of the test will be comparable to already available PCR TB tests. Price will be dependent on other factors, such as prospective volumes and/or the ability to pool samples without loss of sensitivity.

7. Rapid and accurate detection of TB is now a reality; this test will allow the AHB to speedily confirm TB infection and act with confidence on the result.

SECTION 2

5th April 2003

Project:
Rapid detection of tuberculosis infection in deer and cattle tissue.

Project No :
R80570

Project Leader :
John Aitken; Inward Bound Ltd.

Report on:
First milestone of proposed research. The modification and evaluation for technical purposes a range of commercial testkits and growth systems for the detection of Mycobacterium species, and the selection of a combination of methods that are optimal for the early detection of M.bovis in tissue samples from animals suspected of harbouring M.bovis.

Milestone 1 (March 2003)
We are confident that this milestone has been achieved

Results and recommendations

Introduction

Principle of DNA amplification
DNA amplification is the process of creating multiple copies of a specific target region of DNA. This technology has great diagnostic potential, as DNA based methods are able to detect very small amounts of pathogen DNA (or RNA) by amplifying specific genes characteristic of a given pathogen. As genes targeted may be specific to a given pathogen, molecular detection techniques tend to be highly discriminatory. Furthermore, nucleic acid amplification may generate many copies of a given target, from only a small quantity of initial nucleic acid. This lends these methods their high sensitivity.

Test Name	Amplification Technology	Target Region
Roche Amplicor®	PCR	16S rRNA gene
Gen-Probe® Amplified™ MTD	TMA	RRNA
BDProbeTec™	SDA	IS6110

Table 1 Comparison of amplification methods and target genes between three detection kits, SDA – Strand Displacement Amplification, PCR – Polymerase Chain Reaction, TMA – Transcription Mediated Amplification.

Principle of DNA detection

The Roche Amplicor® and Gen-Probe® Amplified™ MTD kits both detect the presence of an amplified DNA product at the termination of the amplification reaction. The BDProbeTec™ system differs from this in that it measures the change in accumulation of end product as the reaction progresses. The latter system is more in line with well-established systems of real-time PCR (e.g., Taqman®, LightCycler® etc), while the former systems are a little more antiquated. The advantages in measuring the progression of a reaction are two fold. Firstly, it saves time; amplification and detection are performed simultaneously, as opposed to being two distinct operations. Secondly there is no issue of contaminating samples with amplicons, post amplification; that is, negative sample cannot be contaminated with amplified products, which could subsequently be detected and interpreted as a positive result.

Sample preparation

An important consideration for the successful utilisation of any nucleic acid amplification technique is the quality of the template nucleic acid. To this end we established a total nucleic acid purification protocol based on established techniques. The utility of this protocol was verified initially by performing extractions on a culture of *Mycobacterium tuberculosis* suspended in 5 ml of saline to a turbidity approaching a #1 McFarland standard (cf. $1.5x10^8$ c.f.u. per ml). To optimise the extraction protocol, a number of pre-treatments were tested, including; (b) 15 minutes sonication, (c) 15 minutes dry heat (100°C), and (d) 15 minutes dry heat (100°C) followed by 15 minutes sonication. The two tubes which underwent sonication were supplemented with glass beads to aid the process. Specimens were kept refrigerated between steps. Following the various pre-treatments, the specimens were processed and total nucleic acids were extracted. The yield and purity of the resulting nucleic acid preparation was determined spectrophotometrically (Table 2) and visualised after agarose electrophoresis (Figure 1).

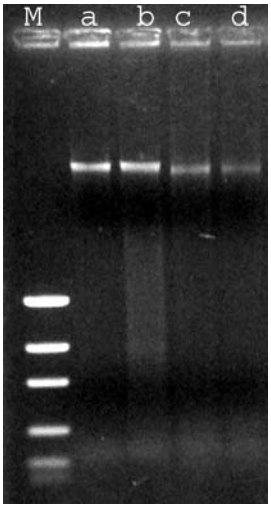

Figure 1 Nucleic acid purified from *M. tuberculosis* after different

Pre-treatment	Yield (μg/μl)	Purity (260/280 nm)
No pre-treatment	0.1	1.1
(b)	0.05	1.3
(c)	0.05	1.4
(d)	0.1	1.2

Table 2 Comparison of yield and purity of nucleic acids when various pre-treatments were used during extraction of nucleic acids.

The yields were satisfactory, appeared to be of sufficient purity and were similar between all three pre-treatments (and when no pre-treatment was used). The treatments which involved sonication appeared to have slightly lower yields of nucleic acid; this may have been due to binding of nucleic acids to the glass beads used to disrupt cells. From this it was concluded that our nucleic acid extraction technique was ideal for the extraction of nucleic acids from *M. tuberculosis* cell suspensions. This preparation of DNA was used to test the sensitivity of the three detection kits below. All three kits detected the presence of this purified DNA preparation, down to a dilution factor of 10^{-6}.

To estimate the effectiveness of this method in extracting mycobacterial nucleic acids from deer tissue, healthy deer tissue extract was spiked with a known quantity of mycobacterial cells and the total nucleic acids were extracted. In this instance it was not possible to quantify the mycobacterial nucleic acids in the specimen (either by agarose electrophoresis or spectrophotometrically) due to the presence of mammalian DNA. Instead the mycobacteria were detected using one of the commercial detection systems (Gen-Probe® Amplified™ MTD). Over a range of dilutions, the mycobacteria were adequately detected, suggesting that total nucleic acid extraction from infected deer tissue would provide a suitable template for each of direct detection systems. However, total nucleic acid precipitation in the presence of excess mammalian DNA may lead to lower yield of bacterial nucleic acid. Furthermore, proteins and other components in the tissue extract could conceivably provide the bacterial cells protection against proteolytic degradation during extraction.

Comparison of Three Commercial *Mycobacteria* Detection Systems

Work to date has focused on the evaluation of three commercial kits for the rapid detection of *Mycobacteria*. Each of these kits has been compared on a number of parameters, including sensitivity, ease of use and cost. Each of these is discussed and summarised in table 3 below. It should be noted that all the tests are designed for use on respiratory specimens. Application of such methodologies directly on tissue samples has inherent problems as tissue specimens often contain substances inhibitory to DNA amplification.

Sample Preparation
Each of the tests was validated using nucleic acids purified from infected tissue as a template for subsequent amplification, as described above. This was done for two reasons; firstly, as all three kits are designed for direct detection from respiratory specimens, no standard protocol exists for tissue extracts. Secondly, it was desirable for all three tests to utilise the same template as a baseline for accurate comparison. The recommended specimen preparation associated with each of the three kits is similar, typically consisting of heat inactivation and cell lysis by sonication or enzymatic degradation.

The BDProbeTec™ system is notable in this regard as all the addition equipment required for the completion of the test is supplied with the instrument.

Methodology

Theoretical considerations must be made in the evaluation of these three kits. The Gen-Probe® Amplified™ MTD method has been applied to non-respiratory specimens previously, with a degree of success (2, 5). This test is based on the rRNA itself, of which there are up to 2000 copies of in a given cell (as opposed to the rRNA gene itself, of which there might be three or four copies). Theoretically this would give this test a great advantage in sensitivity, although one must also consider the inherent instability of RNA. The time taken to perform this test is approximately two hours.

The Roche Amplicor® system is based on the proven technology of the polymerase chain reaction. The polymerase enzyme (Taq) used in this reaction has a known requirement for magnesium ions. This may be a limitation of this methodology, as nucleic acid extraction techniques often suggest resuspending purified nucleic acids in a buffer containing EDTA. This is known to chelate Mg^{2+} ions and render them inaccessible to the polymerase. Indeed it is suspected that may have been the cause of inhibition in some trial runs of the Amplicor® system. This kit detects the 16S rRNA gene. This is a sound target, yet will not be present in as high a copy number as the rRNA itself. The time taken to perform this test is approximately five hours.

The BDProbeTec™ system is based on the target IS6110 regions (4). These are horizontally mobile genetic elements; Insertion Sequences. These sequences tend to be randomly dispersed throughout out the genome, and have been found only in the *Mycobacterium tuberculosis* complex (*M. tuberculosis, M. bovis, M. africanum* and *M. microti*). Unlike the 16S rRNA gene, insertion sequences such as IS6110 are not necessary for the viability of the cell. The IS6110 is typically present in 8 to 20 copies in *M. tuberculosis* and present in two to six copies in *M. bovis* (9). A small number of isolates of *M. tuberculosis* have been found to not harbour any copies of the IS6110 element (3, 6), although it has not been found lacking in *M. bovis* isolates. Consequently, the BDProbeTec™ may be particularly well suited for the detection of bovine tuberculosis. The time taken to perform this test is approximately two hours.

Amplification Inhibition Controls

Of the three kits, two have the option of detecting a co-amplified internal control. Amplification controls are a useful way of determining the success of an amplification reaction (7). The inclusion of an internal control also increases sensitivity of a test by avoiding false positives. Unless inhibitory specimens are identified, negative amplification reactions do not necessarily indicate an absence of an organism.

The BDProbeTec™ System incorporates an internal control into every test. This is co-amplified and detected simultaneously as the main reaction progresses. This is extremely convenient in that no extra preparation is required and the reaction can be monitored. The Roche Amplicor® system also allows an internal control to be utilised. In this instance the internal control is co-amplified (as is the case with the BDProbeTec™ System) however it has an individual detection protocol for the internal control – distinct from the main test. Effectively this doubles the number of samples for detection if the controls are to be used. The Gen-Probe® Amplified™ MTD system does not include an internal control, and many investigators have suggested this is to the kits detriment (8).

While the inclusion of an internal control is useful for dismissing false negatives and increasing sensitivity, it is also very useful when trouble shooting and establishing protocol. It has highlighted important information with regard to preparation of samples for amplification (following BDProbeTec™ method) and the DNA preparation when using Roche Amplicor®.

Ease of use

The Roche Amplicor® and Gen-Probe® Amplified™ MTD kits involve multiple pipette steps of multiple solutions during the amplification and detection stages. In the case of the Roche kit a multi-channel pipette reduces some of the pipette work, but this is still a laborious procedure. Both these kits require addition of multiple solutions with multiple incubation times. This increases the opportunity for contamination – either of the reactions or reagents. The BDProbeTec™ circumvents some of these problems as reagents are supplied lyophilised within their respective reaction vessels. Consequently, the BDProbeTec™ was the most "user-friendly" of the three methods trialed.

Cost

The development and implementation of any new diagnostic test will carry a cost. This is particularly the case with test utilising molecular technology. Obviously it is desirable to limit this cost and make the test as affordable as possible. A factor to be considered is the number of tests that can be performed with each kit; the Gen-Probe® Amplified™ MTD contains reagents for 50 tests while the Roche Amplicor® and BDProbeTec™ contain reagents for 96 tests. This effectively sets Gen-Probe® at twice the price.

Test Name	Methodology	Amplification Inhibition	Ease of use	Time	Cost	Overall
Amplicor®	*	***	*	*	***	**
Amplified™ MTD	**	*	**	***	*	**
BDProbeTec™	***	***	***	***	***	***

Table 3 Overall comparisons of the three detection kits. Each category it ranked from lowest (*) to highest (***).

Growth Detection

The BD MGIT broth detection system, in conjunction with Lowenstein Jensen media was selected as the culture system. The MGIT system allows for extraction of inoculated broth samples during the incubation phase, and this aspect may be useful for the subsequent probing of the broth to increase sensitivity of the detection system.

Conclusion

Overall, all three kits performed well. All three have been shown to be highly sensitive, and the results between tests correlate well. Ultimately the decision to recommend for further development, the BDProbeTec™ system, is based on factors as highlighted in Table 3. Either of the two other methods would be very satisfactory as a backup method. Of these two, the Amplicor® is better suited to high throughput scenarios, while the Gen-

Probe® Amplified™ MTD is better suited for the rapid verification of results as obtained by the initial method.

Further Development

Selection of the BDProbeTec™ system will require further development of the protocol to ensure the sensitivity of this system is maximised. Pre-treatment of specimens is necessary to ensure rupture of mycobacterial cells. To avoid inhibition, these cell lysates will be processed further to remove potentially inhibitory compounds. This can be performed using the standard nucleic acid purification system established. Provisional investigation into this combination of treatments has so far proved favourable. An example of this is shown in table 4. Tissue lysates that were initially inhibitory, may have the inhibitory compounds removed by nucleic acid purification (isolates 717 and 718). Furthermore, it can be confidently noted that the nucleic acid purification procedure itself has no deleterious effect on the target DNA itself, as is demonstrated in specimen 710.

Specimen #	BD treatement	Nucleic acid extraction only	BD treatement followed by nucleic acid extraction
710	Positive	Negative	Positive
717	Inhibited	Negative	Negative
718	Inhibited	Negative	Negative
2703-1	Negative	Negative	Negative

Table 4 Comparison of selected specimen preparation results when run on the BDProbeTec™ system. Note full BD treatment increases sensitivity (presumably by efficient lysis of cells), while the nucleic acid extraction procedure is useful for the removal of inhibitory compounds.

Furthermore, the amount of template nucleic acid added to the amplification reaction will have to be optimised. In the case of the Gen-Probe® Amplified™ MTD sensitivity was increased when using 500 µl of decontaminated sediment as a template, rather than the recommended 50 µl (1). This was performed on respiratory tract specimens (sputa, tracheobronchial secretions and bronchioloalveolar washings), affect attributed to the low bacterial load in many specimens. Sensitivity was increased from 71.4% to 83.3%, while specificity remained largely unaffected (99.4% to 99.0%). Such an increase in specificity may also be feasible with the BDProbeTec™ system.

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SECTION 3

Rapid detection of *Mycobacterium bovis* from bovine tissue specimens

June progress report, 2003.

Introduction

Bovine tuberculosis presents a major problem to New Zealand agriculture as it may severely limit the export-market access of many of the country's meat products. Consequently a great deal of research has been conducted into the epidemiology of bovine tuberculosis (Barlow 1994; Barlow et al. 1997; Barlow et al. 1998). The major proposed wildlife vector in New Zealand is thought to be the brush-tail possum (*Trichosurus vulpecula*), although many other species may also play important roles (Coleman et al. 2001). Many strategies have been proposed to control bovine Tb by intervening with this transmission route. To date the most effective method of intervention has involved eradication, although more novel solutions such as the vaccination of possums have also been suggested (Corner et al. 2002). While controlling the spread of bovine Tb is the ultimate goal of such projects, the aforementioned strategies of achieving this are very ambitious. Herein we describe the development of a rapid diagnostic test for bovine Tb. This test could be implemented in a very short period of time, and may help control inter- and intra- herd transmission of this disease.

Previously we trialled and compared three commercial systems for the rapid detection of mycobacteria from tissue specimens. The three systems evaluated were the Roche Amplicor® kit, the Gen-Probe® Amplified™ MTD kit and the BDProbeTec™ system. Although each of the three systems had their merits, the ultimate outcome of the previous report was that the BDProbeTec™ would be the platform of choice for further development. Consequently, the research objectives for the ensuing months have been;

(a) Development of appropriate pre-treatments suitable to be employed on tissue specimens.
(b) Optimisation of the template nucleic acid preparation procedure for the BDProbeTec™ system.
(c) Pilot study assessing the efficacy of using BDProbeTec™ on tissue specimens with suspected mycobacterial lesions.

The first two of these three objectives will not be fully considered in the current report, suffice to say many combinations of treatments have been evaluated before deciding on an appropriate protocol. All the results presented here in were obtained using the same protocol.

Methods

Specimen collection

Tissue specimens were collected from April 3 to May 28, 2003. A total of seventy-six tissue specimens were collected during this time. All specimens were excised lymph nodes collected at the time of slaughter. Tissue specimens were stored in sterile plastic containers and stored at 4°C until transported back to the laboratory (typically not longer than 48 hours).

Sample processing

Tissue specimens had lesions excised and macerated. The tissue homogenate was then decontaminated using the standard NALC-NaOH treatment. Decontaminated specimens were cultured and a smear was prepared for Ziehl-Neelsen (ZN) staining. Decontaminated specimens also underwent further treatment, and were subsequently used as template for the BDProbeTec™.

Results

Using the protocol established for the BDProbeTec™ a pilot study was undertaken to assess the efficacy of the system. The goal of this study was to assess the sensitivity of the BDProbeTec™ system compared to traditional identification methods such as culture and the acid-fast (ZN) stain. Tissue specimens were obtained from 76 animals suspected of carrying a mycobacterial infection, and tested using all three procedures. Of these specimens 59 (78%) were positive for detection of *Mycobacterium tuberculosis* complex by the BDProbeTec™ system.

Sixty-six specimens have confirmed culture results to date. A further ten specimens have been identified as being culture negative, but as these have not been incubated for six weeks they have not been included at this stage. Of the 66 specimens with culture results, 50 (76%) were proven positive by culture, while 55 (83%) were positive by BDProbeTec™. Sixty-eight of the total 76 specimens had ZN stains performed and in 44 (65%) of these AFB were observed.

BDProbeTec™ vs. Culture

Given that to date culture remains the gold standard for the diagnosis of mycobacterial infection, the molecular detection method would appear to correlate well. In our hands, the sensitivity and specificity of the BDProbeTec™ system is 100% (100-100) and 69% (58-80%) respectively (95% confidence interval shown in brackets). The positive predictive value (PPV) is 91% (84-98%) and the negative predictive value (NPV) is 100% (100-100%).

These results suggest the BDProbeTec™ system is an ideal system for the rapid detection of *Mycobacteria* from bovine tissues. The high sensitivity (i.e. all culture positives were detected by the BDProbeTec™) makes it an excellent screening procedure. It is worth considering these results in light of previously published studies using the BDProbeTec™ system. Below are the results of six similar trials, conducted on human respiratory specimens. It must be noted, however, that the data indicated below are the raw figures, and have not taken discrepancies into account.

	Sensitivity (%)	**Specificity (%)**	**PPV (%)**	**NPV (%)**
Current study	*100*	*69*	*91*	*100*
Study 1 (Bergmann et al. 1998)	95.8	96.2	54.8	99.8
Study 2 (Pfyffer et al. 1999)	97.6	95	63.9	99.9
Study 3 (Bergmann et al. 2000)	87.5	99.0	70	99.7
Study 4 (Barrett et al. 2002)	92.7	96.0	97	90
Study 5 (Johansen et al. 2002)	82.7	98.5	--	--
Study 6 (Mazzarelli et al. 2003)	76.5	95.9	--	--

Table 1 Comparison of results obtained in the current study as compared with published results of other research groups using the BDProbeTec™ system. Note raw data shown, discrepancies have not been considered. Dashes indicate that data was not included.

Our pilot study suggests a sensitivity that was higher than, or similar to several other studies. The figures cited in the table above correspond to all samples which were culture positive were also positive by BDProbeTec™. However, in the current study, the specificity observed was considerably lower than that of previous reports. This arises because the high number of culture

negative specimens which were positive by BDProbeTec™. This lack of specificity is unlikely to be due to the introduction of contaminating nucleic acids during the preparation, as the negative controls consistently give the anticipated negative result. A more plausible explanation is decline in viability of the bacterial cells. This could be induced by several factors in the processing of the specimen, and the nature of the specimens themselves. This would hinder the ability to culture the organism, but should not affect the presence or absence of mycobacterial nucleic acid. This is supported by the observation that one of the five specimens was also positive by ZN stain. This further supports the notion that organisms were present initially, but could not be subsequently cultured.

ZN stain vs. Culture

Prior to the implementation of nucleic acid detection systems for bacterial pathogens, the most practical means of determining the presence of *Mycobacteria* was by direct examination of a specimen by microscopy. The ZN stain (or Acid-fast stain) is a differential stain that exploits the resistance of *Mycobacteria* to decolourisation by acid-alcohol. Consequently *Mycobacteria spp.* retain their initial stain, and are considered acid-fast bacilli (AFBs).

Sixty specimens had both culture and ZN stains performed. Direct examination of ZN stained smears detected AFBs in 41/60 (68%) of the specimens. The sensitivity and specificity of the ZN stain was 82% (72-92%) and 69% (57-80%) respectively. The positive predictive value was 88% (80-96%) and the negative predictive value was 58% (45-70%).

	Sensitivity (%)	**Specificity (%)**	**PPV (%)**	**NPV (%)**
Current study	82	69	88	58
Study 1 (Vuorinen et al. 1995)	80.8	99.1	--	--
Study 2 (Selvakumar et al. 2002)	84	--	--	--
Study 3 (Ulukanligil et al. 2000)	61	100	100	91
Study 4 (Farnia et al. 2002)	83	97	93	--
Review (Watterson et al. 2000)	60-70	--	--	--

Table 2 Comparison of results obtained in the current study as compared with published results of other research groups using the ZN stain. Dashes indicate that data was not included.

A comparison of the sensitivity and specificity observed in the current study with those of previous reports shows that the ZN stain performed on bovine tissue samples is at least as sensitive as on human respiratory specimens (Table 2). The specificity of the ZN stain was lower than expected, with 5 of 16 culture negative specimens (31%) yielding ZN positive stains. However, three of the five appeared as atypical AFBs in morphology and as such it was suspected they were not, in fact, part of the *M. tuberculosis* complex but possibly *M. avium*. Should atypical AFBs be considered as negative, (and histology reports indicated that this was the case), the amended specificity PPV and NPV would be 88%, 95% and 74% respectively. Furthermore, the choice of tissue samples used in this pilot study (i.e. cases of suspected TB) unavoidably favoured the recovery of positive specimens. This in turn results in the recovery of a low number of negative specimens, which may have affected the specificity result.

	Sensitivity (%)	**Specificity (%)**	**PPV (%)**	**NPV (%)**
BDProbeTec™	100	69	91	100
ZN Stain	82	69	88	58

Table 3 Comparison of results obtained using the BDProbeTec™ system and ZN staining in the current study.

Although the sensitivity of the ZN stain is considerably lower than that of the BDProbeTec™, it does have some utility in the rapid identification of the acid-fast bacilli other than those of the *M. tuberculosis* complex. It also has much utility as a rapid and inexpensive method of screening specimens, adding further sensitivity. As such it may complement the BDProbeTec™. Sixty-eight specimens had both ZN performed and have been processed by BDProbeTec™. Of the 44 positive ZN smears 38 (86%) of were also positive by BDProbeTec™ and of the 24 negative ZN smears, 13 (54%) were positive by BDProbeTec™.

Discussion

Comparison of the sensitivity and specificity results obtained using the BDProbeTec™ in the current study, correlate well with those published previously (Table 1). Furthermore it must be considered that these kits have been marketed to be used exclusively on human respiratory specimens. Nevertheless, reports have been made of its utility in non-respiratory specimens including urine, tissue and CSF (Johansen et al. 2002). The Johansen study showed the BDProbeTec™ to have an overall sensitivity and specificity of 60.7% and 96.7%, respectively, on non-respiratory specimens. In our hands, the BDProbeTec™ has proven to be considerably more sensitive and an extremely robust method for detection of *M. tuberculosis* complex from bovine tissue. The protocol we have established fully warrants further field-trials to establish the value in a commercial environment.

In three culture-positive specimens AFBs of an unusual morphology were observed in some of the ZN stains. It was suspected that these may in fact be *M. avium*. *Mycobacterium avium* subspecies *paratubeculosis* is the etiological agent of paratuberculosis (Johnes disease) in ruminants (Valentin-Weigand et al. 1999). The BDProbeTec™ is specific for the *M. tuberculosis* complex only, and as such was negative for these three specimens. It may be possible to increase reliability of the test by using histological reports to augment an algorithm aimed at early prediction of disease status.

Inhibition

Initially we reported some inhibition problems when using the BDProbeTec™. This problem was addressed by performing DNA purification. Of all the 76 specimens tested using the BDProbeTec™ in the current study, not one instance of inhibition was noted.

Increasing sensitivity

Reports have been published documenting a variety of methods used to increase the sensitivity of existing protocols for the molecular detection of pathogens. Most such protocols are based on more efficient lysis of the bacterial cells and consequently increasing yields of target DNA. We have performed a number of treatments on a standard suspension of mycobacterial cells in saline, and culture positive tissue specimens (data not shown). None of the procedures trialled gave a significant increase in sensitivity above that of the current protocol.

Non-invasive sampling

The current report shows the great utility for the early diagnosis of mycobacterial infections in cattle. Currently this takes many weeks to get a positive identification, and with the molecular diagnosis we have implemented, the entire procedure can be easily implemented within 24 hours, with very good sensitivity.

Both culture and nucleic acid amplification based techniques are currently performed on excised lymph nodes post slaughter. There may be some potential application in the collection of specimens from living animals suspected of having Tb without the destruction of the animal

itself. This may allow elimination or isolation of affected animals, before spreading the illness through an entire herd.

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Appendix I – Isolate information (culture complete, *n*=66)

This shows the raw data for the 66 samples which have had culture performed, and incubated for an appropriate period of time. ND = not determined.

Spec. number	Spec. Collected	ZN stain	Culture	BDProbeTec™
001	03/04/2003	+++	Negative	Negative
002	05/04/2003	+	Positive	Positive
003	05/04/2003	+++	Positive	Positive
004	05/04/2003	ND	Positive	Positive
005	09/04/2003	NEG	Negative	Negative
006	09/04/2003	NEG	Positive	Positive
007	09/04/2003	NEG	Positive	Positive
008	09/04/2003	++	Positive	Positive
009	09/04/2003	NEG	Positive	Positive
010	09/04/2003	+	Positive	Positive
011	09/04/2003	+	Positive	Positive
012	09/04/2003	+	Positive	Positive
013	09/04/2003	++	Positive	Positive
014	09/04/2003	++	Positive	Positive
015	09/04/2003	++	Positive	Positive
016	09/04/2003	+++	Positive	Positive
017	09/04/2003	+	Positive	Positive
018	09/04/2003	NEG	Negative	Positive
019	09/04/2003	NEG	Positive	Positive
020	09/04/2003	+	Positive	Positive
021	09/04/2003	++	Positive	Positive
022	10/04/2003	NEG	Negative	Negative
023	10/04/2003	++	Positive	Positive
024	10/04/2003	+	Positive	Positive
025	10/04/2003	+	Positive	Positive
026	10/04/2003	NEG	Positive	Positive
027	10/04/2003	ND	Positive	Positive
028	10/04/2003	++	Positive	Positive
029	10/04/2003	ND	Positive	Positive
030	10/04/2003	NEG	Negative	Negative
031	10/04/2003	NEG	Positive	Positive
032	10/04/2003	++	Positive	Positive
033	10/04/2003	NEG	Positive	Positive
034	12/04/2003	ND	Positive	Positive
035	12/04/2003	ND	Positive	Positive
036	16/04/2003	+++	Negative	Negative
037	16/04/2003	ND	Positive	Positive
038	16/04/2003	+++	Positive	Positive
039	24/04/2003	NEG	Negative	Negative
040	30/04/2003	++	Positive	Positive
041	01/05/2003	+++	Negative	Negative
042	01/05/2003	NEG	Negative	Negative
043	02/05/2003	NEG	Negative	Negative

Spec. number	Spec. Collected	ZN stain	Culture	BDProbeTec™
044	07/05/2003	++	Positive	Positive
045	07/05/2003	+	Positive	Positive
046	07/05/2003	NEG	Negative	Positive
047	07/05/2003	++	Negative	Positive
048	07/05/2003	++	Positive	Positive
049	07/05/2003	NEG	Negative	Positive
050	07/05/2003	NEG	Negative	Positive
051	07/05/2003	NEG	Positive	Positive
052	08/05/2003	++	Positive	Positive
053	08/05/2003	++	Positive	Positive
054	08/05/2003	+++	Positive	Positive
055	08/05/2003	++	Positive	Positive
056	08/05/2003	+++	Positive	Positive
057	08/05/2003	+	Positive	Positive
058	08/05/2003	+	Positive	Positive
059	08/05/2003	NEG	Negative	Negative
060	08/05/2003	+	Positive	Positive
061	08/05/2003	+	Positive	Positive
062	08/05/2003	++	Positive	Positive
063	08/05/2003	+++	Positive	Positive
064	08/05/2003	+++	Negative	Negative
065	09/05/2003	+	Positive	Positive
066	10/05/2003	+	Positive	Positive

Appendix II – Isolate information (culture incomplete, *n*=10)

This shows the raw data for the 66 samples which have had culture performed, and incubated for an appropriate period of time. ND = not determined. Culture results shown as negative may be due to in sufficient incubation time. * Specimen obtained 03-20/06/03 (to be confirmed).

Spec. number	Spec. Collected	ZN stain	Culture	BDProbeTec™
067	02/05/2003	+++	Negative	Negative
068	17/05/2003	NEG	Negative	Positive
069	17/05/2003	NEG	Negative	Negative
070	17/05/2003	ND	Negative	Negative
071	28/05/2003	ND	Negative	Positive
072	*	NEG	Negative	Negative
073	*	NEG	Negative	Negative
074	*	+	Negative	Positive
075	*	NEG	Negative	Negative
076	*	+	Negative	Positive

SECTION 4

28th June 2005

Project No:
R80570

Project Leader
John Aitken; Inward Bound Ltd.

Report on
Final milestone of proposed research. The completion of an evaluation of the BD ProbeTec MTB in cattle and deer.

This is the third and final report on the above project and should be read in conjunction with the two previous reports. (Sections 1 & 2)

Summary
After the removal of animals that displayed typical lesions, but were culture negative ("indeterminate") from the data, a total of 322 animals of both species were tested. The distribution of species and disease states are detailed in table 1

TABLE 1

				Count	Column N %
Species	Cattle	TB Status	Not TB	73	45.6%
			TB	87	54.4%
			Total	160	100.0%
	Deer	TB Status	Not TB	77	47.5%
			TB	85	52.5%
			Total	162	100.0%
	Total	TB Status	Not TB	150	46.6%
			TB	172	53.4%
			Total	322	100.0%

In cattle the BD Probetec MTB test showed a sensitivity of 92.0% and a specificity of 100% (Table 2)
In deer the BD Probetec MTB test initially showed a sensitivity of 82.4% and a specificity of 100% (Table 2)
In both species, the test showed a sensitivity of 87.2% and a specificity of 100%

TABLE 2

				TB Status			
				TB		Not TB	
				Count	Column Total N %	Count	Column Total N %
Species	Cattle	PCR	Positive	80	92.0%	0	.0%
			Negative	7	8.0%	73	100.0%
			Total	87	100.0%	73	100.0%
	Deer	PCR	Positive	70	82.4%	0	.0%
			Negative	15	17.6%	77	100.0%
			Total	85	100.0%	77	100.0%
	Total	PCR	Positive	150	87.2%	0	.0%
			Negative	22	12.8%	150	100.0%
			Total	172	100.0%	150	100.0%

Subsequently 14 deer discrepant samples (ie TB-positive but PCR-negative) primarily from the "Taylor Herd" were reanalyzed. Using these results a sensitivity for deer of 94.1% and a specificity of 100% were obtained (Table 3)
This correction resulted in a revised result for both species of a sensitivity of 93.0% and a specificity of 100%

TABLE 3

				TB Status			
				TB		Not TB	
				Count	Column Total N %	Count	Column Total N %
Species	Cattle	PCR 2	Positive	80	92.0%	0	.0%
			Negative	7	8.0%	73	100.0%
			Total	87	100.0%	73	100.0%
	Deer	PCR 2	Positive	80	94.1%	0	.0%
			Negative	5	5.9%	77	100.0%
			Total	85	100.0%	77	100.0%
	Total	PCR 2	Positive	160	93.0%	0	.0%
			Negative	12	7.0%	150	100.0%
			Total	172	100.0%	150	100.0%

Overall the test performed in line with our expectations, and in our opinion the technology is very suitable for the timely detection of TB in lymph nodes of cattle and deer. There is also the strong possibility, given the sensitivity of the test, that it could also be useful for early detection of TB in animals from other biological sites.

ROC analyses suggest that the current BD ProbeTec MTB cutoff point of 4000 could be adjusted back to around 1000, enhancing sensitivity without reducing specificity. using the 1000-4000 range as a suspect category could be considered.

Methods.

Samples were submitted to our testing laboratory via courier systems from various meatworks throughout NZ.
Representative samples from the same nodes were also submitted to a Reference Laboratory (Wallaceville) for mycobacterial culture and to another independent testing laboratory (Gribbles) for histological examination.
On receipt in the laboratory samples were either kept at 4^{o}c (if prompt processing was likely) or stored at -20^{o}c until processing could be undertaken.
After processing, all samples were retained at -70^{o}c for subsequent reanalysis if this was required.

Primary processing

Initial processing was undertaken using a biohazard hood in a containment area suitable for TB processing.

The biopsy sample was placed on a sterile surface and a representative section of tissue, preferably with a visible lesion, was excised using a sterile scalpel blade. The selected piece of tissue was then placed in a sterile tissue processing tube and homogenised in approximately 2 ml of distilled water.
The homogenised suspension was then decanted into a 50 ml sterile test tube for subsequent processing.
All materials used were disposable to avoid the possibility of contamination by bacteria adherent to inadequately cleaned instruments.

Decontamination

Decontamination of the homogenised sample was carried out according to the method outlined below.

N-Acetyl L Cysteine (NALC) Digestion Procedure

Reagents

1 M/15 Phosphate Buffer
- a) stock solution
  - 9.47 gm/L of Na2HPO4 =M/15
  - 9.07 gm/L of KH2PO4 =M/15
- b) pH 6.8 solution
  - 50 ml M/15 Na2HPO4
  - 50 ml M/15 KH2PO4

2 NALC-NaOH Digestant-Decontamination Solution

a) 1N NaOH (4%) 25ml
b) 0.1M sodium Citrate 25ml
c) 20% N-acetyl –L-Cysteine 1.25 ml
or
N-acetyl-L- Cysteine (powder) 0.25 gm

Sterilise soln a. and b. separately by autoclaving. Mix and store at 4 -10^{o}c (Mixture is stable for several weeks)
Add soln c. within 24 hours of use.

3. 0.2% Bovine Albumin Fraction V in 0.85% saline
Adjust to 7.0 pH with 10% NaOH.. Sterilise by filtration.

Digestion and Culture

1. Transfer the homogenized sample to a 50 ml aerosol-free screw cap centrifuge tube.
2. Add an equal volume of NALC-NaOH solution.
3. Mix by hand until digested (in a safety cabinet)
4. Let stand for 15 minutes at room temperature.
5. Fill tube to within ¾ inch of the top with sterile M/15 Phosphate buffer
6. Centrifuge 15 minutes at approximately 3000rpm
7. Decant supernatant into a splash-proof container. Retain sediment for PCR and culture.
8. From the sediment prepare a smear for ZN stain.
9. To remaining sediment add 1ml of 0.2% bovine albumin solution. (optional)
10. Make a 1:10 dilution of albumin-digest by adding 5ml of sterile water or 0.85% saline.

This method is suitable for both PCR and culture. If culture is omitted then it is possible to modify the procedure for PCR only.

Specimen preparation for BD ProbeTec MTB test.
Any method that involves detection of TB DNA is dependent on an efficient method for rupture of the bacterial cell wall and exposure of the contents to the amplification process.
A number of methods are available; all rely on a combination of physical disruption augmented by chemical and enzymatic enhancement. Freezing of the suspension may also be beneficial as the mycobacterial cell wall is susceptible to freeze fractionation.
The specimen preparation method included with the BD ProbeTec MTB uses centrifugation, heating to 105^{o}c followed by enzymatic degradation in the presence of heat (65^{o}c) and a period of sonication. (see below)
This method had been optimised for the processing of human sputum samples.

Tissue processing inevitably results in microscopic clumps of connective tissue with TB bacilli enmeshed within the cells. This situation is less likely to occur in respiratory secretions where there is not a strong tissue matrix present.
After processing the first batch of reactor-positive deer for that stage of the study it was realized that the samples could benefit from an initial sonication step and this was integrated into the BD methodology for all samples subsequently collected.

BD Probetec MTB Assay

Post-decontamination, specimens were sonicated for 20 minutes in a warm water bath. 0.5 ml of sample was withdrawn from the tube and added to 1ml of DTB wash buffer I, followed by centrifugation at 12000 rpm for 3 minutes. The resulting pellet was placed into a drying oven at 05^{o}c for 30 minutes. 0.1 of lysis buffer was then added to the pellet and the tube was then suspended in a waterbath/sonicator at 65^{o}c. for 45 minutes. The lysed material was then neutralized by the addition of 0.6 ml of the neutralization buffer. The detection assay was then performed according to manufacturer's instructions contained in the package insert. Results were generally available on the day of analysis.

Internal Amplification Control

.
PCR, particularly when run as an in-house* or "home brew" method,; is carried out as is prone to false negative results These occur when the target DNA sequence is present in the sample (ie the sample is TB positive) but the PCR reaction is inhibited by other substances present in the same sample. This is known as inhibition, and the result is falsely negative. The SDA method used in the BD ProbeTec MTB assay contains an internal amplification control. This internal control ensures that inhibition is detected and possible false negative reactions are avoided. Additionally, SDA is generally less prone to inhibition when compared to PCR.
*In-house ("home brew")PCR is a term used to describe the preparation of all reagents and primers by the laboratory performing the test. This is distinct from our use of commercial testkits, which are prepared external to the laboratory by a diagnostic company.

MOTA Scores

The Metric Other Than Acceleration (MOTA) score is a number derived as part of the analysis of the amplification process. The number is specific to an individual sample, and the manufacturer's specifications advise against using this number as a primary cutoff point to indicate a positive or negative sample. Other investigators have however, demonstrated a relationship between the MOTA score and numbers of bacilli, and suggested that the score may be useful in monitoring human response to treatment. (de la Calle et al 2003) Analysis of our data is in agreement with this observation, and we agree with his finding that the MOTA scores can be segmented into three categories : <1000; 1000-4000; and >4000. These categories can be equated with negative, suspicious, and positive respectively.

Those samples in the second category (1000-4000, or "suspicious") demonstrate some amplification activity and can either be rerun, or considered presumptively positive.

"The Taylor Group"

This group consisted of 14 deer, (mainly Taylor Holdings) where the preliminary SDA result was reported as negative. These results skewed the data on deer, and were analysed on commencement of the trial. A decision was made to rerun those samples at the conclusion of the trial to eliminate the possibility that a TB strain may not have had the target sequence.

Two factors may have contributed to the original negative results.

- *Sampling of the nodes* at the meatworks/laboratory was not optimal. Our laboratory had also carried out culture on the samples and those results showed low numbers of bacilli, consistent with the NVL status of the received node.
- *A major modification* was made to the method subsequent to the initial analysis of the majority of animals in this group. This modification, we believe, improved the sensitivity of the test.

The repeated samples on these were less than optimal, as very small amounts of lymphatic tissue were available in some instances.

Culture positive and SDA PCR negative samples, including the 14 repeated samples, can be seen in table 4.

TABLE 4

						Culture	Score	PCR	Score 2	PCR 2
PCR Ref	Run 01.05.04	Cattle	R Johnson/E Monk	Tag	03/17518	Positive	84	Negative	84	Negative
					03/17514	Positive	90	Negative	90	Negative
	Run 10.02.05	Deer	SL Harris	Tag	320	Positive	966	Negative	966	Negative
	Run 10.05.04	Deer	MR & LL Thomas	Tag	03/7989	Positive	2,794	Negative	28,054	Positive
	Run 12.10.04	Deer	Taylor Holdings	Tag	01/21513	Positive	173	Negative	1,384	Negative
					03/12718	Positive	181	Negative	34,211	Positive
					03/12727	Positive	316	Negative	19,399	Positive
					03/12716	Positive	367	Negative	28,417	Positive
					03/12728	Positive	596	Negative	34,827	Positive
					03/12736	Positive	669	Negative	33,266	Positive
			GW, AI & RJ Smith	Tag	03/14032	Positive	1,295	Negative	6,268	Positive
	Run 13.05.04	Cattle	CA & CA Nimmo	Tag	04/5503	Positive	333	Negative	333	Negative
	Run 15.04.04	Cattle	J & S Lee	Tag	02/99596	Positive	0	Negative	0	Negative
	Run 18.03.04	Deer	Taylor Holdings	Tag	03/12651	Positive	0	Negative	28,459	Positive
					03/12669	Positive	0	Negative	109	Negative
					03/12636	Positive	59	Negative	42,668	Positive
					03/12665	Positive	146	Negative	66	Negative
					03/12688	Positive	304	Negative	439	Negative
					03/12633	Positive	1,837	Negative	16,174	Positive
	Run 20.07.04	Cattle	Te Namu Farms	Tag	01/21938	Positive	166	Negative	166	Negative
	Run 25.10.04	Cattle	RJ & B Frame	Tag	94/936	Positive	75	Negative	75	Negative
	Run 31.08.04	Cattle	DA Hutton/Kinzett	Tag	03/15168	Positive	132	Negative	132	Negative

Indeterminate Results

Table 5 below, shows the analysis of those samples which exhibited gross lesions, but were culture negative. Of the 17 culture negative samples, 3 (18%) were PCR positive. All 17 indeterminate samples were ZN stain negative, and 10 (75%) of the 17 samples showed either positive or suspect histology. This observation may indicate low numbers of TB present in the lesions, or may indicate presence of non-viable TB bacilli. PCR will detect non-viable bacilli

TABLE 5

ZN				PCR		Total
				Positive	Negative	
Negative	Histology	Typical	Count	3	7	10
			% within Histology	30.0%	70.0%	100.0%
		Suspicious	Count	0	3	3
			% within Histology	.0%	100.0%	100.0%
		Negative	Count	0	4	4
			% within Histology	.0%	100.0%	100.0%
	Total		Count	3	14	17
			% within Histology	17.6%	82.4%	100.0%

Pricing

The most significant expense associated with this test is the testkit. As with other medical testkits, this cost is dependent on the volume of tests processed (including other PCR tests used from the same manufacturer), other contractual arrangements with the manufacturer, and the relative strength of the NZ dollar.
Reducing the price of each test is also possible by pooling individual samples, and this needs to be explored further. Labour is also a significant expense, and this contribution to test price can be reduced depending on volume of samples, batching, and the number of times the assay is run each week. An optimal sized run is 24 samples, but it is possible for one operator to complete >100 amplifications in the course of a day. (and this is commonly achieved when chlamydia analysis is performed in medical laboratories using the BD Probetec Chlamydia assay.) The larger the run size, the more economies of scale can be achieved.

Discussion.

The advent of molecular tests has revolutionized the detection of TB infections in humans. The BD ProbeTec MTB complex direct detection assay is an example of the portfolio of tests currently available to provide early detection of TB infection. We have adapted this test for use in animals.

The performance of the MTB test in this study demonstrates the usefulness of the assay in both cattle and deer lymph nodes and is a valuable addition to the current battery of tests available for TB detection.
Although lymph node tissue has potential for inhibition of the SDA reaction, the inclusion of an internal amplification control contained in the BD ProbeTec MTB allows the user to detect interference.
No inhibition was detected in the course of the study.
Advances in the medical technology field towards the rapid and accurate diagnosis of tuberculosis (of which the BD ProbeTec MTB evaluated here is one) are undergoing rapid development, refining and upgrading. The primary patents for the PCR process will lapse in 2005 and this will also mean a quantum leap in available technology and innovative testkit methodologies for the detection of TB infection. In addition, the global pressures exerted by international events (bioterrorism) have spun off robust portable instruments capable of PCR-based early detection of biological agents ***in the field***.

The above developments have signaled significant advances in the area of TB detection. Our study has validated and verified some of the possibilities for TB control through the merging of medical and infection control advances currently available, and soon to be considerably expanded.
It is our strong belief that our approach to the modification of medical technology for animal TB diagnosis has been successful and continued developmental research on this path will produce further benefits for TB diagnosis and control.

Acknowledgements

The collection of suitable samples in different categories across New Zealand was a time-consuming and arduous process. We wish to acknowledge the great work done by Kevin Crews to enable us to have sufficient samples to ensure a successful trial.
The input of Terry Ryan was key to the analysis of the large amount of data generated in the course of the study, and in the derivation of a useful and succinct set of conclusions. His contribution is tremendously appreciated by the mathematically challenged amongst us.
The solid support in mycobacterial culture work provided by Dr Geoff DeLisle and his team at Wallaceville enabled us to complete this study with total confidence in the impartial and technically excellent support provided by his Unit. Thanks Guys!
Lastly, we wish to express our gratitude to DeeResearch Ltd and the AHB for jointly funding this study, and for providing logistical support so willingly to ensure the successful completion of our project.

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SECTION 5

Summary Data from PCR test files

Classification of animals

TB : Culture Positive

Not TB : Animals from TB-free herds

Indeterminate : Animals with typical lesions but culture-negative

				Count	Column N %
Species	Cattle	TB Status	TB	87	50.0%
			Not TB	73	42.0%
			Indeterminate	14	8.0%
			Total	174	100.0%
	Deer	TB Status	TB	85	50.6%
			Not TB	77	45.8%
			Indeterminate	6	3.6%
			Total	168	100.0%
	Total	TB Status	TB	172	50.3%
			Not TB	150	43.9%
			Indeterminate	20	5.8%
			Total	342	100.0%

Listing of animals that will be used for the primary evaluation of PCR

				Count	Column N %
Species	Cattle	TB Status	Not TB	73	45.6%
			TB	87	54.4%
			Total	160	100.0%
	Deer	TB Status	Not TB	77	47.5%
			TB	85	52.5%
			Total	162	100.0%
	Total	TB Status	Not TB	150	46.6%
			TB	172	53.4%
			Total	322	100.0%

PCR Sensitivity & Specificity

Initial Data (PCR 1)

				TB Status			
				TB		Not TB	
				Count	Column Total N %	Count	Column Total N %
Species	Cattle	PCR	Positive	80	92.0%	0	.0%
			Negative	7	8.0%	73	100.0%
			Total	87	100.0%	73	100.0%
	Deer	PCR	Positive	70	82.4%	0	.0%
			Negative	15	17.6%	77	100.0%
			Total	85	100.0%	77	100.0%
	Total	PCR	Positive	150	87.2%	0	.0%
			Negative	22	12.8%	150	100.0%
			Total	172	100.0%	150	100.0%

With repeated data (PCR 2)

				TB Status			
				TB		Not TB	
				Count	Column Total N %	Count	Column Total N %
Species	Cattle	PCR 2	Positive	80	92.0%	0	.0%
			Negative	7	8.0%	73	100.0%
			Total	87	100.0%	73	100.0%
	Deer	PCR 2	Positive	80	94.1%	0	.0%
			Negative	5	5.9%	77	100.0%
			Total	85	100.0%	77	100.0%
	Total	PCR 2	Positive	160	93.0%	0	.0%
			Negative	12	7.0%	150	100.0%
			Total	172	100.0%	150	100.0%

Summary of records where status = TB but PCR = negative

						Culture	Score	PCR	Score 2	PCR 2
PCR Ref	Run 01.05.04	Cattle	R Johnson/E Monk	Tag	03/17518	Positive	84	Negative	84	Negative
					03/17514	Positive	90	Negative	90	Negative
	Run 10.02.05	Deer	SL Harris	Tag	320	Positive	966	Negative	966	Negative
	Run 10.05.04	Deer	MR & LL Thomas	Tag	03/7989	Positive	2,794	Negative	28,054	Positive
	Run 12.10.04	Deer	Taylor Holdings	Tag	01/21513	Positive	173	Negative	1,384	Negative
					03/12718	Positive	181	Negative	34,211	Positive
					03/12727	Positive	316	Negative	19,399	Positive
					03/12716	Positive	367	Negative	28,417	Positive
					03/12728	Positive	596	Negative	34,827	Positive
					03/12736	Positive	669	Negative	33,266	Positive
			GW, AI & RJ Smith	Tag	03/14032	Positive	1,295	Negative	6,268	Positive
	Run 13.05.04	Cattle	CA & CA Nimmo	Tag	04/5503	Positive	333	Negative	333	Negative
	Run 15.04.04	Cattle	J & S Lee	Tag	02/99596	Positive	0	Negative	0	Negative
	Run 18.03.04	Deer	Taylor Holdings	Tag	03/12651	Positive	0	Negative	28,459	Positive
					03/12669	Positive	0	Negative	109	Negative
					03/12636	Positive	59	Negative	42,668	Positive
					03/12665	Positive	146	Negative	66	Negative
					03/12688	Positive	304	Negative	439	Negative
					03/12633	Positive	1,837	Negative	16,174	Positive
	Run 20.07.04	Cattle	Te Namu Farms	Tag	01/21938	Positive	166	Negative	166	Negative
	Run 25.10.04	Cattle	RJ & B Frame	Tag	94/936	Positive	75	Negative	75	Negative
	Run 31.08.04	Cattle	DA Hutton/Kinzett	Tag	03/15168	Positive	132	Negative	132	Negative

Animal Status Indeterminate

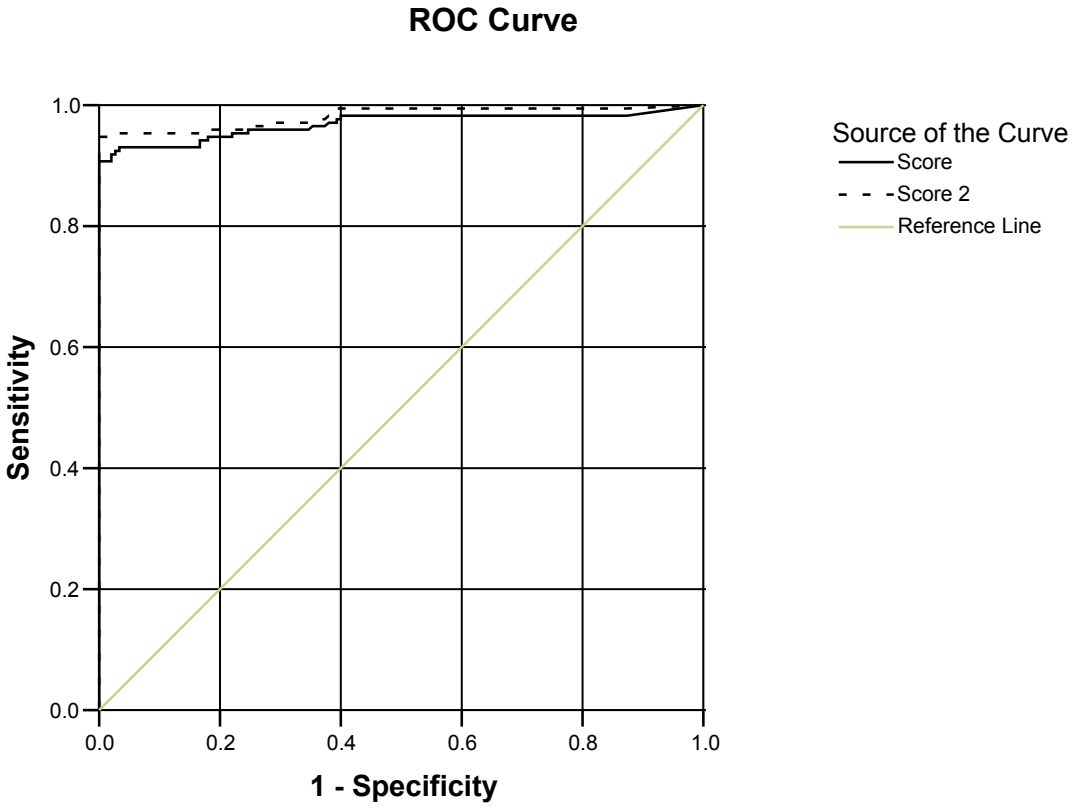
Typical gross lesions but negative culture

All ZN negative, 10 histology typical, 3 histology suspicious, 3 (18%) PCR positive (all histology typical)

				PCR		
ZN				Positive	Negative	Total
Negative	Histology	Typical	Count	3	7	10
			% within Histology	30.0%	70.0%	100.0%
		Suspicious	Count	0	3	3
			% within Histology	.0%	100.0%	100.0%
		Negative	Count	0	4	4
			% within Histology	.0%	100.0%	100.0%
	Total		Count	3	14	17
			% within Histology	17.6%	82.4%	100.0%

ROC Analysis

Cattle and Deer



Diagonal segments are produced by ties.

Data (see table 1 & 2) suggests adoption of a cutpoint of around 1,000 rather than 4,000 (higher sensitivity without compromising specificity)

Table 1. PCR 1, relationship between cutpoint, sensitivity and specificity

Cutpoint	Sensitivity	1 - Specificity	Specificity
-1.00	1.000	1.000	0.000
0.50	0.983	0.873	0.127
1.50	0.983	0.800	0.200
2.50	0.983	0.753	0.247
3.50	0.983	0.733	0.267
4.50	0.983	0.713	0.287
6.00	0.983	0.693	0.307
7.50	0.983	0.687	0.313
8.50	0.983	0.680	0.320
10.00	0.983	0.653	0.347
12.00	0.983	0.647	0.353
13.50	0.983	0.627	0.373
14.50	0.983	0.620	0.380
16.00	0.983	0.600	0.400
17.50	0.983	0.587	0.413
18.50	0.983	0.580	0.420
19.50	0.983	0.573	0.427
20.50	0.983	0.567	0.433
24.50	0.983	0.553	0.447
28.50	0.983	0.547	0.453
29.50	0.983	0.533	0.467
30.50	0.983	0.520	0.480
31.50	0.983	0.513	0.487
32.50	0.983	0.507	0.493
33.50	0.983	0.500	0.500
35.50	0.983	0.493	0.507
38.00	0.983	0.487	0.513
39.50	0.983	0.473	0.527
40.50	0.983	0.460	0.540
41.50	0.983	0.453	0.547
43.00	0.983	0.447	0.553
45.00	0.983	0.440	0.560
46.50	0.983	0.433	0.567
48.00	0.983	0.427	0.573
50.00	0.983	0.420	0.580
52.50	0.983	0.413	0.587
54.50	0.983	0.407	0.593
57.00	0.983	0.400	0.600
61.00	0.977	0.400	0.600
69.00	0.977	0.393	0.607
75.50	0.971	0.393	0.607
78.00	0.971	0.387	0.613
82.00	0.971	0.380	0.620
84.50	0.965	0.373	0.627
87.00	0.965	0.360	0.640
89.50	0.965	0.353	0.647
90.50	0.959	0.347	0.653

92.00	0.959	0.340	0.660
94.00	0.959	0.333	0.667
95.50	0.959	0.327	0.673
98.00	0.959	0.320	0.680
100.50	0.959	0.313	0.687
102.00	0.959	0.307	0.693
111.50	0.959	0.280	0.720
122.50	0.959	0.273	0.727
126.50	0.959	0.260	0.740
128.50	0.959	0.253	0.747
130.50	0.959	0.247	0.753
137.00	0.953	0.247	0.753
143.00	0.953	0.240	0.760
144.50	0.953	0.227	0.773
145.50	0.953	0.220	0.780
147.00	0.948	0.220	0.780
148.50	0.948	0.213	0.787
150.00	0.948	0.200	0.800
153.00	0.948	0.193	0.807
157.50	0.948	0.187	0.813
163.00	0.948	0.180	0.820
168.50	0.942	0.180	0.820
171.50	0.942	0.173	0.827
172.50	0.942	0.167	0.833
177.00	0.936	0.167	0.833
183.00	0.930	0.167	0.833
186.50	0.930	0.160	0.840
188.50	0.930	0.147	0.853
190.00	0.930	0.140	0.860
192.50	0.930	0.133	0.867
201.50	0.930	0.127	0.873
213.00	0.930	0.120	0.880
218.00	0.930	0.113	0.887
219.50	0.930	0.107	0.893
222.50	0.930	0.100	0.900
228.00	0.930	0.093	0.907
234.50	0.930	0.087	0.913
240.00	0.930	0.080	0.920
253.50	0.930	0.073	0.927
273.00	0.930	0.067	0.933
283.00	0.930	0.060	0.940
289.00	0.930	0.047	0.953
297.50	0.930	0.040	0.960
303.00	0.930	0.033	0.967
307.50	0.924	0.033	0.967
313.50	0.924	0.027	0.973
319.00	0.919	0.027	0.973
327.50	0.919	0.020	0.980
350.00	0.913	0.020	0.980
378.00	0.907	0.020	0.980

412.00	0.907	0.013	0.987
435.50	0.907	0.007	0.993
516.00	0.907	0.000	1.000
632.50	0.901	0.000	1.000
817.50	0.895	0.000	1.000
1,130.50	0.890	0.000	1.000
1,566.00	0.884	0.000	1.000
2,315.50	0.878	0.000	1.000
3,732.00	0.872	0.000	1.000
4,861.00	0.866	0.000	1.000
5,443.50	0.860	0.000	1.000
6,160.00	0.855	0.000	1.000
6,498.50	0.849	0.000	1.000
6,533.50	0.843	0.000	1.000
6,612.50	0.837	0.000	1.000
7,252.50	0.831	0.000	1.000
8,424.50	0.826	0.000	1.000
9,760.50	0.820	0.000	1.000
10,687.50	0.814	0.000	1.000
11,037.50	0.808	0.000	1.000
11,765.50	0.802	0.000	1.000
12,578.50	0.797	0.000	1.000
13,089.50	0.791	0.000	1.000
13,436.50	0.785	0.000	1.000
13,680.00	0.779	0.000	1.000
13,970.00	0.773	0.000	1.000
15,704.50	0.767	0.000	1.000
17,734.50	0.762	0.000	1.000
19,471.00	0.756	0.000	1.000
21,267.50	0.750	0.000	1.000
21,943.00	0.744	0.000	1.000
22,198.50	0.738	0.000	1.000
22,308.00	0.733	0.000	1.000
22,517.00	0.727	0.000	1.000
22,695.00	0.721	0.000	1.000
24,516.50	0.715	0.000	1.000
26,625.00	0.709	0.000	1.000
27,099.50	0.703	0.000	1.000
27,574.50	0.698	0.000	1.000
27,975.50	0.692	0.000	1.000
28,188.00	0.686	0.000	1.000
28,567.00	0.680	0.000	1.000
28,921.00	0.674	0.000	1.000
30,388.00	0.669	0.000	1.000
31,784.50	0.663	0.000	1.000
32,906.50	0.657	0.000	1.000
34,588.00	0.651	0.000	1.000
35,381.50	0.645	0.000	1.000
35,727.00	0.640	0.000	1.000
36,302.00	0.634	0.000	1.000

36,977.50	0.628	0.000	1.000
37,332.50	0.622	0.000	1.000
37,518.50	0.616	0.000	1.000
37,659.50	0.610	0.000	1.000
38,412.00	0.605	0.000	1.000
39,199.00	0.599	0.000	1.000
39,345.50	0.593	0.000	1.000
39,397.50	0.587	0.000	1.000
39,476.50	0.581	0.000	1.000
39,747.00	0.576	0.000	1.000
40,081.50	0.570	0.000	1.000
40,303.00	0.564	0.000	1.000
40,746.00	0.558	0.000	1.000
41,248.00	0.552	0.000	1.000
41,455.50	0.547	0.000	1.000
41,749.00	0.541	0.000	1.000
42,103.00	0.535	0.000	1.000
42,280.00	0.529	0.000	1.000
42,403.50	0.523	0.000	1.000
42,499.50	0.517	0.000	1.000
42,843.50	0.512	0.000	1.000
43,165.50	0.506	0.000	1.000
43,274.50	0.500	0.000	1.000
43,704.00	0.488	0.000	1.000
44,403.50	0.483	0.000	1.000
44,793.00	0.477	0.000	1.000
45,105.00	0.471	0.000	1.000
45,691.00	0.465	0.000	1.000
46,007.00	0.459	0.000	1.000
46,297.00	0.453	0.000	1.000
46,587.00	0.448	0.000	1.000
46,636.00	0.442	0.000	1.000
46,696.50	0.436	0.000	1.000
46,737.00	0.430	0.000	1.000
46,763.50	0.424	0.000	1.000
46,913.00	0.419	0.000	1.000
47,126.50	0.413	0.000	1.000
47,212.00	0.407	0.000	1.000
47,256.50	0.401	0.000	1.000
47,309.50	0.395	0.000	1.000
47,514.00	0.390	0.000	1.000
47,794.50	0.384	0.000	1.000
48,168.50	0.378	0.000	1.000
48,447.50	0.372	0.000	1.000
48,474.50	0.366	0.000	1.000
48,923.50	0.360	0.000	1.000
49,658.50	0.355	0.000	1.000
49,988.50	0.349	0.000	1.000
50,086.00	0.343	0.000	1.000
50,228.00	0.337	0.000	1.000

50,316.00	0.331	0.000	1.000
50,429.50	0.326	0.000	1.000
50,645.00	0.320	0.000	1.000
50,839.50	0.314	0.000	1.000
51,119.50	0.308	0.000	1.000
51,479.50	0.302	0.000	1.000
51,798.50	0.297	0.000	1.000
52,034.50	0.291	0.000	1.000
52,127.50	0.285	0.000	1.000
52,173.50	0.279	0.000	1.000
52,255.50	0.273	0.000	1.000
52,378.00	0.267	0.000	1.000
52,512.50	0.262	0.000	1.000
52,605.50	0.256	0.000	1.000
52,780.00	0.250	0.000	1.000
53,067.50	0.244	0.000	1.000
53,216.50	0.238	0.000	1.000
53,377.00	0.233	0.000	1.000
53,787.00	0.227	0.000	1.000
54,102.00	0.221	0.000	1.000
54,268.00	0.215	0.000	1.000
54,458.00	0.209	0.000	1.000
54,540.50	0.203	0.000	1.000
54,592.00	0.198	0.000	1.000
54,696.50	0.192	0.000	1.000
54,811.50	0.186	0.000	1.000
54,884.00	0.180	0.000	1.000
56,077.50	0.174	0.000	1.000
57,701.00	0.169	0.000	1.000
58,481.00	0.163	0.000	1.000
58,971.00	0.157	0.000	1.000
59,483.50	0.151	0.000	1.000
60,116.00	0.145	0.000	1.000
60,452.50	0.140	0.000	1.000
60,706.50	0.134	0.000	1.000
61,372.50	0.128	0.000	1.000
62,202.50	0.122	0.000	1.000
62,635.00	0.116	0.000	1.000
62,967.50	0.110	0.000	1.000
63,242.50	0.105	0.000	1.000
63,422.00	0.099	0.000	1.000
63,835.50	0.093	0.000	1.000
64,127.00	0.087	0.000	1.000
64,495.50	0.081	0.000	1.000
64,909.00	0.076	0.000	1.000
65,014.00	0.070	0.000	1.000
65,526.00	0.064	0.000	1.000
66,036.50	0.058	0.000	1.000
66,151.00	0.052	0.000	1.000
66,506.50	0.047	0.000	1.000

67,576.50	0.041	0.000	1.000
68,753.00	0.035	0.000	1.000
69,217.00	0.029	0.000	1.000
69,913.50	0.023	0.000	1.000
71,103.00	0.017	0.000	1.000
77,765.00	0.012	0.000	1.000
88,798.00	0.006	0.000	1.000
93,762.00	0.000	0.000	1.000

Table 2. PCR 2, relationship between cutpoint, sensitivity and specificity

Cutpoint	Sensitivity	1 - Specificity	Specificity
-1.00	1.000	1.000	0.000
0.50	0.994	0.873	0.127
1.50	0.994	0.800	0.200
2.50	0.994	0.753	0.247
3.50	0.994	0.733	0.267
4.50	0.994	0.713	0.287
6.00	0.994	0.693	0.307
7.50	0.994	0.687	0.313
8.50	0.994	0.680	0.320
10.00	0.994	0.653	0.347
12.00	0.994	0.647	0.353
13.50	0.994	0.627	0.373
14.50	0.994	0.620	0.380
16.00	0.994	0.600	0.400
17.50	0.994	0.587	0.413
18.50	0.994	0.580	0.420
19.50	0.994	0.573	0.427
20.50	0.994	0.567	0.433
24.50	0.994	0.553	0.447
28.50	0.994	0.547	0.453
29.50	0.994	0.533	0.467
30.50	0.994	0.520	0.480
31.50	0.994	0.513	0.487
32.50	0.994	0.507	0.493
33.50	0.994	0.500	0.500
35.50	0.994	0.493	0.507
38.00	0.994	0.487	0.513
39.50	0.994	0.473	0.527
40.50	0.994	0.460	0.540
41.50	0.994	0.453	0.547
43.00	0.994	0.447	0.553
45.00	0.994	0.440	0.560
46.50	0.994	0.433	0.567
48.00	0.994	0.427	0.573
50.00	0.994	0.420	0.580
52.50	0.994	0.413	0.587
54.50	0.994	0.407	0.593
59.00	0.994	0.400	0.600

64.50	0.994	0.393	0.607
70.50	0.988	0.393	0.607
75.50	0.983	0.393	0.607
78.00	0.983	0.387	0.613
82.00	0.983	0.380	0.620
84.50	0.977	0.373	0.627
87.00	0.977	0.360	0.640
89.50	0.977	0.353	0.647
90.50	0.971	0.347	0.653
92.00	0.971	0.340	0.660
94.00	0.971	0.333	0.667
95.50	0.971	0.327	0.673
98.00	0.971	0.320	0.680
100.50	0.971	0.313	0.687
102.00	0.971	0.307	0.693
106.00	0.971	0.280	0.720
114.50	0.965	0.280	0.720
122.50	0.965	0.273	0.727
126.50	0.965	0.260	0.740
128.50	0.965	0.253	0.747
130.50	0.965	0.247	0.753
137.00	0.959	0.247	0.753
143.00	0.959	0.240	0.760
144.50	0.959	0.227	0.773
146.50	0.959	0.220	0.780
148.50	0.959	0.213	0.787
150.00	0.959	0.200	0.800
153.00	0.959	0.193	0.807
157.50	0.959	0.187	0.813
163.00	0.959	0.180	0.820
168.50	0.953	0.180	0.820
171.50	0.953	0.173	0.827
178.50	0.953	0.167	0.833
186.50	0.953	0.160	0.840
188.50	0.953	0.147	0.853
190.00	0.953	0.140	0.860
192.50	0.953	0.133	0.867
201.50	0.953	0.127	0.873
213.00	0.953	0.120	0.880
218.00	0.953	0.113	0.887
219.50	0.953	0.107	0.893
222.50	0.953	0.100	0.900
228.00	0.953	0.093	0.907
234.50	0.953	0.087	0.913
240.00	0.953	0.080	0.920
253.50	0.953	0.073	0.927
273.00	0.953	0.067	0.933
283.00	0.953	0.060	0.940
289.00	0.953	0.047	0.953
297.50	0.953	0.040	0.960

306.50	0.953	0.033	0.967
316.50	0.953	0.027	0.973
327.50	0.953	0.020	0.980
361.00	0.948	0.020	0.980
412.00	0.948	0.013	0.987
435.50	0.948	0.007	0.993
437.50	0.948	0.000	1.000
702.50	0.942	0.000	1.000
1,175.00	0.936	0.000	1.000
3,027.00	0.930	0.000	1.000
4,861.00	0.924	0.000	1.000
5,443.50	0.919	0.000	1.000
6,051.50	0.913	0.000	1.000
6,376.50	0.907	0.000	1.000
6,498.50	0.901	0.000	1.000
6,533.50	0.895	0.000	1.000
6,612.50	0.890	0.000	1.000
7,252.50	0.884	0.000	1.000
8,424.50	0.878	0.000	1.000
9,760.50	0.872	0.000	1.000
10,687.50	0.866	0.000	1.000
11,037.50	0.860	0.000	1.000
11,765.50	0.855	0.000	1.000
12,578.50	0.849	0.000	1.000
13,089.50	0.843	0.000	1.000
13,436.50	0.837	0.000	1.000
13,680.00	0.831	0.000	1.000
13,970.00	0.826	0.000	1.000
15,140.50	0.820	0.000	1.000
16,738.00	0.814	0.000	1.000
17,734.50	0.808	0.000	1.000
18,783.00	0.802	0.000	1.000
20,087.00	0.797	0.000	1.000
21,267.50	0.791	0.000	1.000
21,943.00	0.785	0.000	1.000
22,198.50	0.779	0.000	1.000
22,308.00	0.773	0.000	1.000
22,517.00	0.767	0.000	1.000
22,695.00	0.762	0.000	1.000
24,516.50	0.756	0.000	1.000
26,625.00	0.750	0.000	1.000
27,099.50	0.744	0.000	1.000
27,574.50	0.738	0.000	1.000
27,961.00	0.733	0.000	1.000
28,068.50	0.727	0.000	1.000
28,188.00	0.721	0.000	1.000
28,355.00	0.715	0.000	1.000
28,438.00	0.709	0.000	1.000
28,650.00	0.703	0.000	1.000
28,921.00	0.698	0.000	1.000

30,388.00	0.692	0.000	1.000
31,784.50	0.686	0.000	1.000
32,530.00	0.680	0.000	1.000
33,642.50	0.674	0.000	1.000
34,115.00	0.669	0.000	1.000
34,519.00	0.663	0.000	1.000
34,992.00	0.657	0.000	1.000
35,381.50	0.651	0.000	1.000
35,727.00	0.645	0.000	1.000
36,302.00	0.640	0.000	1.000
36,977.50	0.634	0.000	1.000
37,332.50	0.628	0.000	1.000
37,518.50	0.622	0.000	1.000
37,659.50	0.616	0.000	1.000
38,412.00	0.610	0.000	1.000
39,199.00	0.605	0.000	1.000
39,345.50	0.599	0.000	1.000
39,397.50	0.593	0.000	1.000
39,476.50	0.587	0.000	1.000
39,747.00	0.581	0.000	1.000
40,081.50	0.576	0.000	1.000
40,303.00	0.570	0.000	1.000
40,746.00	0.564	0.000	1.000
41,248.00	0.558	0.000	1.000
41,455.50	0.552	0.000	1.000
41,749.00	0.547	0.000	1.000
42,103.00	0.541	0.000	1.000
42,280.00	0.535	0.000	1.000
42,403.50	0.529	0.000	1.000
42,499.50	0.523	0.000	1.000
42,607.50	0.517	0.000	1.000
42,904.00	0.512	0.000	1.000
43,165.50	0.506	0.000	1.000
43,274.50	0.500	0.000	1.000
43,704.00	0.488	0.000	1.000
44,403.50	0.483	0.000	1.000
44,793.00	0.477	0.000	1.000
45,105.00	0.471	0.000	1.000
45,691.00	0.465	0.000	1.000
46,007.00	0.459	0.000	1.000
46,297.00	0.453	0.000	1.000
46,587.00	0.448	0.000	1.000
46,636.00	0.442	0.000	1.000
46,696.50	0.436	0.000	1.000
46,737.00	0.430	0.000	1.000
46,763.50	0.424	0.000	1.000
46,913.00	0.419	0.000	1.000
47,126.50	0.413	0.000	1.000
47,212.00	0.407	0.000	1.000
47,256.50	0.401	0.000	1.000

47,309.50	0.395	0.000	1.000
47,514.00	0.390	0.000	1.000
47,794.50	0.384	0.000	1.000
48,168.50	0.378	0.000	1.000
48,447.50	0.372	0.000	1.000
48,474.50	0.366	0.000	1.000
48,923.50	0.360	0.000	1.000
49,658.50	0.355	0.000	1.000
49,988.50	0.349	0.000	1.000
50,086.00	0.343	0.000	1.000
50,228.00	0.337	0.000	1.000
50,316.00	0.331	0.000	1.000
50,429.50	0.326	0.000	1.000
50,645.00	0.320	0.000	1.000
50,839.50	0.314	0.000	1.000
51,119.50	0.308	0.000	1.000
51,479.50	0.302	0.000	1.000
51,798.50	0.297	0.000	1.000
52,034.50	0.291	0.000	1.000
52,127.50	0.285	0.000	1.000
52,173.50	0.279	0.000	1.000
52,255.50	0.273	0.000	1.000
52,378.00	0.267	0.000	1.000
52,512.50	0.262	0.000	1.000
52,605.50	0.256	0.000	1.000
52,780.00	0.250	0.000	1.000
53,067.50	0.244	0.000	1.000
53,216.50	0.238	0.000	1.000
53,377.00	0.233	0.000	1.000
53,787.00	0.227	0.000	1.000
54,102.00	0.221	0.000	1.000
54,268.00	0.215	0.000	1.000
54,458.00	0.209	0.000	1.000
54,540.50	0.203	0.000	1.000
54,592.00	0.198	0.000	1.000
54,696.50	0.192	0.000	1.000
54,811.50	0.186	0.000	1.000
54,884.00	0.180	0.000	1.000
56,077.50	0.174	0.000	1.000
57,701.00	0.169	0.000	1.000
58,481.00	0.163	0.000	1.000
58,971.00	0.157	0.000	1.000
59,483.50	0.151	0.000	1.000
60,116.00	0.145	0.000	1.000
60,452.50	0.140	0.000	1.000
60,706.50	0.134	0.000	1.000
61,372.50	0.128	0.000	1.000
62,202.50	0.122	0.000	1.000
62,635.00	0.116	0.000	1.000
62,967.50	0.110	0.000	1.000

63,242.50	0.105	0.000	1.000
63,422.00	0.099	0.000	1.000
63,835.50	0.093	0.000	1.000
64,127.00	0.087	0.000	1.000
64,495.50	0.081	0.000	1.000
64,909.00	0.076	0.000	1.000
65,014.00	0.070	0.000	1.000
65,526.00	0.064	0.000	1.000
66,036.50	0.058	0.000	1.000
66,151.00	0.052	0.000	1.000
66,506.50	0.047	0.000	1.000
67,576.50	0.041	0.000	1.000
68,753.00	0.035	0.000	1.000
69,217.00	0.029	0.000	1.000
69,913.50	0.023	0.000	1.000
71,103.00	0.017	0.000	1.000
77,765.00	0.012	0.000	1.000
88,798.00	0.006	0.000	1.000
93,762.00	0.000	0.000	1.000

Other Summary Data

H&E Histology by Culture

				Culture			
				Positive		Negative	
				Count	Column Valid N %	Count	Column Valid N %
Species	Cattle	Histology	Typical	84	96.6%	9	64.3%
			Suspicious	1	1.1%	2	14.3%
			Negative	2	2.3%	3	21.4%
			Total	87	100.0%	14	100.0%
	Deer	Histology	Typical	35	71.4%	1	33.3%
			Suspicious	13	26.5%	1	33.3%
			Negative	1	2.0%	1	33.3%
			Total	49	100.0%	3	100.0%
	Total	Histology	Typical	119	87.5%	10	58.8%
			Suspicious	14	10.3%	3	17.6%
			Negative	3	2.2%	4	23.5%
			Total	136	100.0%	17	100.0%

ZN Histology by Culture

				Culture			
				Positive		Negative	
				Count	Column Valid N %	Count	Column Valid N %
Species	Cattle	ZN	Typical	23	26.4%	0	.0%
			Suspicious	0	.0%	0	.0%
			Negative	64	73.6%	14	100.0%
			Total	87	100.0%	14	100.0%
	Deer	ZN	Typical	12	24.5%	0	.0%
			Suspicious	3	6.1%	0	.0%
			Negative	34	69.4%	3	100.0%
			Total	49	100.0%	3	100.0%
	Total	ZN	Typical	35	25.7%	0	.0%
			Suspicious	3	2.2%	0	.0%
			Negative	98	72.1%	17	100.0%
			Total	136	100.0%	17	100.0%