Characterization and Development of an Assay for *Mycobacterium paratuberculosis* in Sheep

Honors Thesis

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PASS WITH DISTINCTION
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Spring 2002

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TO THE UNIVERSITY HONORS COLLEGE:

As faculty advisor for Marlene K. Bakko

I have read this paper and find it satisfactory.

[Signature]

Faculty Advisor

Date: 2/5/02
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Summary

*Mycobacterium avium* subspecies *paratuberculosis*, the causative agent of Johne's disease, is a bacterium of significant medical and economic importance to cattle and sheep producers. In contrast to cattle, diagnosis of sub-clinical and clinical ovine *M. paratuberculosis* infections is extremely difficult. In sheep, the clinical signs are vague, as a general loss of body condition often occurs unaccompanied by the characteristic diarrheal symptoms of cattle. Current serological tests implemented in bovine diagnosis are often inaccurate in detecting ovine strains. Moreover, ovine strains of *M. paratuberculosis* do not grow well, or at all, in culture.

To date, the reason for these differences is unknown. Nevertheless, the phenotypic differences are most likely linked to genotypic variation. The aim of this project was to evaluate a well characterized and distinct genetic marker of the mycobacterial genome, the 65kDa heat shock protein gene (*hsp65*), for sequence variations between bovine and ovine strains. Sequence variations have the potential to be used in the development of a polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) based assay that can detect and differentiate ovine strains of *M. paratuberculosis*. Ovine *M. paratuberculosis* isolates were obtained from the National Animal Disease Center (Ames, Iowa) and the RFLP profile of the ovine *hsp65* gene compared with those of bovine isolates. The ovine *hsp65* gene was sequenced and the sequence analyzed for novel restriction endonuclease cut sites. One enzyme candidate, *Faul*, was chosen for digestion of the ovine and bovine samples. Analysis of the RFLP revealed discernable differences between the ovine and bovine sequences that could be used as the basis for a combination PCR and RFLP based assay for ovine *M. paratuberculosis*. 
Introduction

*Mycobacterium avium* subspecies *paratuberculosis* (*M. paratuberculosis*), an acid-fast, Gram-positive bacillus (7), is the causative agent of Johne’s disease, a chronic infection of the mucosa of the distal small intestine, large intestine, ileum and lymph nodes of ruminants, including cattle, sheep, and goats. Progressive infection with the organism can lead to extensive tissue damage and thickening of the intestinal mucosa, ultimately resulting in protein malabsorption (7, 19). This damage is responsible for the clinical signs of progressive weight loss and chronic or intermittent diarrhea (7).

Johne’s disease is of great economic importance to the livestock industry worldwide. It is estimated that bovine *M. paratuberculosis* infections cause a loss of $1.5 billion annually in the USA (7). These large losses are due to the deaths of clinical cases, reduced milk yield in dairy animals, reduced weight gain and reduced fertility. In Australia, the disease has been of particular interest to the sheep industry as it is on the verge of becoming endemic in their large, economically important, flocks (23). The first report of ovine Johne’s disease in Australia was made in 1980 with the first field outbreak being reported the following year (6). The prevalence of Johne’s disease among Australian flocks has been rapidly increasing—578 flocks have been diagnosed as *M. paratuberculosis* positive, 442 of these flocks are still infected (1).

In order to curtail the advance of Johne’s disease towards endemic status the Australian Veterinary Committee has implemented a National Johne’s Disease Control Program (NJDP). As part of the NJDP, flocks are grouped into zones and categorized as either infected, residual, control, or protected based on factors such as Johne’s disease prevalence within that zone (2). Livestock owners face official restrictions that vary depending upon their zone’s classification. For example, sheep from infected
flocks may only be sold for slaughter, to a slaughter-only feedlot, or to another infected property (18). In addition, lawsuits and new trade practices have made Australian livestock owners increasingly aware of the legal liabilities of selling diseased livestock or contaminated land. This has resulted in market discrimination against contaminated land and breeding animals from known infected herds (16).

Animals become infected by *M. paratuberculosis* through ingestion of bacteria from infected feces and milk, or by *in utero* transmission (15, 6). Young animals such as lambs and calves appear to be most susceptible to the disease and can contract it by suckling on fecally contaminated teats (15). The most common source of infection is food or water supplies (11) that have been contaminated by the feces of carrier animals (7). Cross-species contamination can also occur when sheep drink water that is contaminated by feces from *M. paratuberculosis*-infected cattle (15). In addition, there is evidence that subclinically infected sheep may be a factor in the epidemiology of *M. paratuberculosis* infections in cattle herds (20). While cross-species contamination is possible, the strains that are almost exclusively associated with sheep (S strains) are consistently difficult to culture while bovine-associated strains (C strains) are relatively easy to culture (27). Due to the fact that Johne’s disease has a long incubation period, carrier animals can shed the bacterium in their feces for eighteen months or more prior to the onset of clinical signs, allowing the infection to become well established in a flock or herd before it is identified (7).

In cattle, severe *M. paratuberculosis* infections are identifiable based on clinical signs of chronic diarrhea with accompanying weight loss. Although problematic, diagnosis of subclinical infection is possible. *M. paratuberculosis* infections are most
commonly detected by several culture and serology methods. Methods involving direct
detection of the organism include fecal culture, tissue culture, and radiometric fecal
culture tests. Alternatively, it is possible to detect the animal's immune response to *M.
paratuberculosis* by methods including the enzyme-linked immunosorbent assay, agar
gel immunodiffusion, and complement fixation, all of which assay for the animal's anti-
*M. paratuberculosis* antibodies. Unfortunately, all of these current tests have
disadvantages including long incubation time, low sensitivity, or the need for invasive
ante-mortem or post-mortem tissue collection. Moreover, ovine *M. paratuberculosis*
strains grow slower than bovine strains. Additionally, many of the serologic tests do not
detect ovine strains.

These disadvantages of the common culture and serology diagnostics have
hindered the eradication of Johne's disease in both sheep and cattle. In addition,
current tests do not differentiate between ovine and bovine strains, hindering elucidation
of an outbreak's epidemiology. Development of a diagnostic assay that is both rapid
and effective in detecting clinical and sub-clinical infections, that does not require post-
mortem or invasive tissue collection, and that differentiates between strains would
provide a valuable tool to livestock producers. Recently, the process of PCR has
attracted a great deal of attention because of its potential for providing rapid and
sensitive results. Several experimental PCR-based detection methods have been
developed for bovine *M. paratuberculosis*. The assays amplify either the 65kDa heat
shock protein gene (*hsp65*) (10) or the *IS900* (9,12) repetitive insertion sequence, both
of which have sequences unique to *M. paratuberculosis*. Although *IS900* is specific for
*M. paratuberculosis*, it may be lacking in some sheep-associated strains (5, 13). PCR
assays for *M. paratuberculosis* have most commonly been used to detect subclinical bovine infections while published studies of ovine diagnostics have largely relied on culture and serology. Because of the limitations associated with amplification of the IS900 sequence, this study focused on developing an assay that fulfills all of the above listed assay requirements using a combination of PCR amplification and restriction enzyme digestion (PCR-RFLP) of the *hsp65* genetic marker to detect and identify ovine strains of *M. paratuberculosis*.

**Materials and Methods**

*Sample Procurement:* Two samples of *M. paratuberculosis* of ovine origin were obtained from the National Animal Disease Center in Ames, Iowa. These samples consisted of isolate 1425 (sample 5001) and isolate 1434 (sample 5002). The isolates were originally collected from the Meat Animal Research Center, Clay Center, Nebraska. After receipt, the samples were divided and part of each culture suspended in Middlebrook 7H9 broth with OADC, Polysorbate 80, and Mycobactin J for further culture. The remainder was reserved for DNA extraction.

*DNA Isolation for PCR:* Bacteria were suspended in TE buffer with 200 μg/ml Proteinase K and 10 mg/ml Lysozyme. Following overnight incubation the samples were centrifuged, the supernatant discarded and the cell pellet resuspended in Chaotrope Solution (4M guanidine thiocynate, 25mM sodium citrate, 0.5% sarkosyl). Bacterial lysis was accomplished by vortexing with zirconium beads. Beads were pre-washed in hot water and bicarbonate solution and rinsed several times with distilled water. Beads were then added to the bacterial solution (1:4) and the tubes were vortexed for ten minutes. The beads were allowed to settle before the supernatant was
removed and subjected to two phenol-chloroform (24:1) extractions followed by ethanol precipitation. Precipitated product was resuspended in 10mM Tris, pH 9.0.

**Polymerase Chain Reaction:** Sample identity was confirmed using the Mptb I (5'-GCC GCT GCT GAT CAT CGC-3') and Mptb II (5'-CCT CGA TGC GGT GCT TGC -3') primers, which amplify a 458 base pair region of the *M. paratuberculosis* hsp65 gene. To increase sensitivity, a nested PCR was completed using the primers Int I (5'-ACC CTG GTC GTC AAC AAG-3') and Int II (5'-AAC CAC TGC TGC TGG AAC-3'), amplifying a 199 bp region within the Mptb I and II amplicon. In both cases, each 50μl reaction contained 10-100 ng of DNA, 200 μM of each dNTP, 1 μM each primer, 1.25 U DNA polymerase (Expand Taq, Roche Applied Science, Indianapolis, IN), 10 mM Tris HCl (pH 8.3), 50 mM KCl, and 1.5 mM MgCl₂. Samples were amplified in a Perkin-Elmer 9600 thermocycler (Foster City, CA) for 30 cycles of denaturing (95°C) for 45 seconds, annealing (60°C) for 45 seconds, and extension (72°C) for 90 seconds. The amplification products were visualized on a 1% agarose gel containing GelStar nucleic acid stain (FMC Bioproducts, Rockland, ME) using an Alpha Imager 2000 (Alpha Innotech Corp., San Leandro, CA).

**Restriction Enzyme Digestion of Amplified DNA:** RFLP analysis was used to verify species identity as *M. paratuberculosis* (10). *PstI* digestion of the nested PCR product of *M. avium* produces an 88 bp and a 111 bp fragment. *M. paratuberculosis* is not cleaved within this region. Each 20 μl reaction contained 10 U *PstI* (GibcoBRL, Carlsbad, CA), 10-100 ng of DNA, 50mM Tris-HCl (pH 8.0), 10 mM MgCl₂, 50 mM NaCl, and 16 μl water. Samples were incubated at 37°C for two hours. Control reactions
consisted of known *M. avium* or *M. paratuberculosis* DNA with no enzyme added. Digest products were visualized as previously described.

**Hsp65 Cloning:** To obtain good quality sequence data, PCR amplicons from both sheep samples of *M. paratuberculosis* were cloned into a plasmid vector. PCR products were generated using Pfu Turbo DNA polymerase (Stratagene, La Jolla, CA) with the primers MKMTB13A (5'-AGG CGA TGG ACA AGG T-3') and Mptb II following the same PCR protocols as previously described. The product was cloned into the pCR 4Blunt-TOPO cloning vector (Invitrogen, Carlsbad, CA) following the Zero Blunt TOPO PCR Cloning kit instructions and transfected into *Escherichia coli*. Ligation of the *hsp65* insert into the cloning vector disrupts a lactose metabolism gene promoter. Consequently, successful transformants with kanamycin resistance were readily identified using blue/white screening with X-gal. Five successful transformant colonies for each sample were selected for plasmid isolation using a Wizard Plus Miniprep DNA Purification kit (Promega, Madison, WI). The isolated plasmids were analyzed for the presence of the *hsp65* insert by restriction endonuclease analysis using EcoRI (GibcoBRL, Carlsbad, CA). Further confirmation of insert presence was obtained by PCR amplification of MKMTB13A/Mptb II amplicon from the isolated plasmids. The plasmid DNA was quantified using a Beckman DU-64 spectrophotometer (Beckman-Coulter, Fullerton, CA), and representatives with the highest concentration were chosen for sequencing.

**DNA Sequencing:** The ovine *hsp65* was sequenced from the recombinant pCR 4Blunt-TOPO vector via the terminal dideoxy fluorescence method using the primers T3 (5'-ATT AAC CCT CAC TAA AGG GA-3') and T7 (5'-TAA TAC GAC TCA CTA TA-3').
Each 10 μl reaction contained 0.5 to 1.0 μg DNA, 3.2 pm each primer, and 4.0 μl sequencing dye mix (BigDye Terminator Cycle Sequencing V 2.0 Ready Reaction Sequencing Kit, Applied Biosystems, Foster City, CA) containing fluorescent dideoxynucleoside triphosphates. Samples were labeled during 30 cycles of denaturing (96°C) for 30 seconds, annealing (50°C) for 15 seconds, and 4 minutes of extension (60°C) in a Perkin-Elmer 9600 thermocycler. The sequencing PCR reaction products were purified using gel spin columns (Edge Biosystems, Gaithersburg, MD). Both strands were sequenced using an ABI Prism 377 DNA Sequencer (Applied Biosystems, Foster City, CA).

**Sequence Analysis:** PHRAP software (11) was used to assemble a consensus sequence from the five sequencing runs of each ovine isolate. A consensus sequence for each isolate was created and those two sequences were combined to produce an overall ovine *M. paratuberculosis* consensus sequence. The resulting sequence was aligned to the bovine *M. paratuberculosis* sequence using FASTA software (21) and sequence differences between the ovine consensus and known bovine sequence were identified. Ovine consensus sequence corresponding to *hsp65* was identified and analyzed along with the known bovine *M. paratuberculosis hsp65* sequence using Webcutter V 2.0 (14) in order to identify restriction endonucleases that cut at the polymorphic sites. The enzymes identified during this analysis were used in a simulated digest of the Mptb amplicon using NEBcutter V 1.0 (25). Candidate enzymes were screened on the basis of the number of fragments they produced from the Mptb I and II amplicon and the ease of interpretation of digestion results on a standard 1% agarose gel.
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Faul Digestion: Amplicons from previous Mptb I and II PCR were digested with Faul to evaluate the predicted bovine and ovine RFLP differences. Each 20 µl cutting reaction contained 1 U Faul (New England Biolabs, Beverly, MA), 10 mM Tris-HCl, 10 mM MgCl₂, 1 mM DTT (pH 7.6), 10-100 ng DNA, and 16 µl water. Control reactions consisted of known M. paratuberculosis DNA or sample DNA with no enzyme added. Reactions were incubated at 55°C for two hours and visualized as previously described.

Results

PCR and RFLP Analysis: The Mptb I and Mptb II and the Int I and Int II primer combinations amplified both sample 5001 and 5002, supporting the NADC characterizations of the samples as M. paratuberculosis. Initial PstI digestion of Int I and II amplicons produced fragments within the 100bp range for sample 5001 while no reaction product was visible for sample 5002 (Fig 1). Results such as those seen for sample 5001 are characteristic of M. avium, not M. paratuberculosis. These results were confirmed by repeat digestion of a nested PCR product with PstI. Subsequent PstI digestion of the Mptb I and Mptb II amplicon resulted in RFLP similarities between sample 5001 and both M. avium and M. paratuberculosis while 5002 was consistent with M. paratuberculosis (Fig 2).

Sequence Analysis: Alignment and comparison of the ovine sequence consensus created from 5001 and 5002 and comparison to the known bovine M. paratuberculosis hsp65 sequence revealed three differences (Fig. 3). There was a G→A transition at position 654, a C→T transition at position 1192, and a G→C transversion at position 1263. Only the polymorphisms at positions 1192 and 1283 were within the targeted Mptb I and Mptb II amplification region. Comparison of the ovine hsp65 consensus and bovine hsp65 sequences with the analogous M. avium gene showed the identified
polymorphisms do not correspond to positions of sequence dissimilarity between *M. avium* and *M. paratuberculosis*.

**Restriction Enzyme Selection:** Enzyme selection was based on the following requirements: 1) that the digestion products differ between the ovine and bovine Mptb I/Mptb II amplicon sequences, and 2) that the number of restriction fragments produced be few enough and of large enough size to be easily differentiated on a standard agarose electrophoresis gel. Four enzymes that cut within the polymorphic sites were identified: *AciI*, *BstUI*, *FauI*, and *TaqI* (Table 1). Of the four enzymes identified, only *FauI* was chosen for further evaluation based on the prediction that it would fulfill the above stated requirements.

**FauI Digestions:** Gel visualization of the *FauI* digest showed RFLP do exist between the bovine and ovine sequences (Fig 4). Although weak, restriction fragments for samples 5001 and 5002 are present between 100 and 200 bp while the fragment for the known bovine sample, U6, was slightly larger than 200 bp. The locations of the bovine restriction fragments are consistent with the predicted RFLP patterns but the ovine results are rather weak and not completely conclusive. The U6 PCR product was present in greater quantities than the 5001 and 5002 products, which appears to have resulted in incomplete digestions of the sample with *FauI*. However, RFLP differences do appear to exist between the bovine and ovine *M. paratuberculosis* sequences.

**Discussion**

Sub-clinical *M. paratuberculosis* infections are currently detected by the methods of culture or serology, all of which have disadvantages. For example, *M. paratuberculosis* is slow to grow in culture, requiring up to twelve to sixteen weeks for visible bacterial growth (28). Ovine strains may require up to nine months for visible growth in culture
(26). Although culture time is shortened to three to seven weeks by radiometric fecal culture (28), this is still a relatively long time for test results. Additionally, sensitivity of fecal culture is not high, particularly when testing subclinically infected animals that may be shedding low numbers of organisms. Tissue cultures have increased sensitivity, but require the same extended incubation period. The long incubation period combined with invasive tissue sample retrieval methods (post-mortem or surgical ante-mortem sampling) make tissue culture an undesirable method of detection.

Many serological tests are available to detect the immune response to the bacterial infection. The most commonly used serological test is the enzyme linked immunosorbent assay (ELISA), which detects the presence of antibodies to \textit{M. paratuberculosis} in serum. This test has the advantage of being both inexpensive and rapid. Nevertheless, it has relatively poor sensitivity (43.4\% to 58.8\% for sub-clinical cases) (8) and specificity. The low sensitivity may be due, in some cases, to clinically infected animals becoming anergic so that they do not produce the targeted anti-\textit{M. paratuberculosis} antibodies, resulting in false negatives. Specificity can also be problematic over the course of clinical disease as the test may be cross reactive with other species of mycobacteria, resulting in false positives (24).

Similar sensitivity problems are seen with the agar gel immunodiffusion (AGID), another serum antibody test (17). AGID has a sensitivity level of only 26.6\% for sub-clinical cases, but is estimated to have a 100\% specificity level (8). The widely used complement fixation (CF) test assays for the activation of the complement fixation immune response (4) and is the test required by many nations when importing animals. Nevertheless, the test has a sensitivity level of only 38.4\% for sub-clinical cases (8).
Despite the fact that detection by radiometric fecal culture is relatively lengthy and serology tests such as ELISA and AGID have sensitivity and specificity problems, they are the approved screening methods for the National Johne’s Disease Program (3). In addition, since *M. paratuberculosis* is slow to grow and animals may not mount a detectable immune response until two years post infection, sheep are not eligible for Johne’s disease screening until they are older than twenty-four months (3). In recent years, PCR based assays for *M. paratuberculosis* have been developed by several research groups in attempts to improve detection methods (9, 12). Many of these assays, however, still require tissue samples or are developed for detection from milk samples. Moreover, they do not differentiate between ovine and bovine strains and are therefore not useful in tracking the epidemiology of an infection.

The results from this study present the possibility of using a combination of PCR-RFLP analysis using DNA isolated from feces as an assay that would be sensitive and specific for ovine *M. paratuberculosis* and which could be used at an earlier age. Sequencing of the ovine strains of *M. paratuberculosis* and analysis of that sequence led to the identification of a restriction enzyme (*Faul*) that theoretically fulfills the study’s criteria for selection (digestion products that differ between the ovine and bovine Mptb I/Mptb II amplicon sequences and restriction fragments that can be easily differentiated on a standard agarose electrophoresis gel). *Faul* is predicted to cleave the ovine *hsp65* at one of the sites of sequence variation, producing a RFLP pattern that would be discernibly different from that of the bovine strain.

Based on the initial RFLP analysis, sample 5001 was shown to have characteristics of both *M. avium* and *M. paratuberculosis*, as agarose gel visualization of the PstI
digestion products revealed fragment sizes corresponding to both *M. avium* and *M. paratuberculosis* (Fig 2). Restriction enzyme digestion of sample 5002 consistently produced fragment sizes characteristic of *M. paratuberculosis* (Figs 1 and 2). However, when the ovine consensus sequence was analyzed for *PstI* restriction sites the results were identical to those of *M. paratuberculosis*. The reason for these differences seems to be in sequence variation between samples 5001 and 5002 that was not represented in the final consensus sequence. It is possible that sample 5001 is a heterogeneous culture that contains representatives of more than one *M. paratuberculosis* strain and could possibly contain a low number of *M. avium* organisms. A heterogeneous culture could result in the appearance of sequence variations and variations in restriction fragment patterns similar to those noted in this study.

Close analysis of the individual 5001 sequencing results revealed that 5001 did indeed have sequence similarities to *M. avium*. Position 986 of the *M. paratuberculosis hsp65* gene and the similar region of sample 5002 contains a T while the same position of the *M. avium hsp65* gene and sample 5001 contains a G. It is the G that is contained within the 5'-CTGCAG-3' recognition sequence of *PstI*. When the computer software assembled the ovine consensus sequence these similarities between 5001 and *M. avium* were for an unknown reason discarded by the computer algorithm and instead the 5002 sequence from this region was used.

While the sequence variations that were discovered do not explain any phenotypic or pathogenic differences between the ovine and bovine strains, they do aid in development of an assay to differentiate between the strains. For a restriction enzyme to be diagnostically useful it must cut the internal PCR product and produce fragments
that are prominently different between the two strains that could be easily seen on a standard (1%) agarose gel. The identified restriction enzyme, *Faul*, is predicted to fulfill these criteria. Although weak, the fragments for 5001 and 5002 are present at a level different that that of the fragments for the *M. paratuberculosis* control (Fig. 4). However, those fragments do appear very weak and slightly below the predicted level. The poor gel quality could be attributed to the fact that the gel was allowed to run too long, resulting in fragments that are either present at slightly lower than predicted levels or not present at all. More likely, these results are due to two factors: 1) the age of the sample from which the DNA was obtained and 2) the age of the PCR product used in the digest. Since ovine *M. paratuberculosis* is slow to grow in culture, difficult to identify subclinically, and is not of as great of a concern in the United States as it is in Australia, obtaining the two samples used in this project was problematic. The samples that were obtained were labeled as being collected “years ago” and therefore may have had some viability and stability limitations that complicated this study’s analysis. In addition, the PCR product used in the digestion had been stored at -20°C for over twelve months and therefore the DNA integrity may have been compromised.

Before any statements of complete project success or of diagnostic usefulness of the *Mptb-Faul* PCR-RFLP method can be made, a larger number of ovine *M. paratuberculosis* samples need to be tested. In addition, due to our inability to obtain fecal samples from infected sheep, the fecal sample aspect of the assay remains to be tested. As part of the validation process for this assay, uninfected fecal samples should be spiked with known concentrations of ovine *M. paratuberculosis* organisms and assayed using the proposed system in order to determine the minimum number of
organisms detectable. Nevertheless, the sequence differences discovered between the ovine consensus sequence and the bovine sequence were also present between the ovine sequence and the *M. avium hsp65*. Therefore, if the predictions are supported by further sample testing, an assay developed based on these findings would be specific in differentiating the ovine strain of *M. paratuberculosis* from both the bovine strain and *M. avium*. However, *PstI* digestions would still need to be needed in order to differentiate between bovine *M. paratuberculosis* and *M. avium*. The ability to differentiate between *M. paratuberculosis* strains and *M. avium* would be useful in elucidating the epidemiology of a flock or herd's infection in order to prevent further disease propagation as well as providing a tool to increase our understanding of disease transmission.

Combination of the current experimental PCR/*PstI* RFLP analysis diagnostic assay with *Faul* RFLP analysis could be used to rapidly differentiate between *M. avium* and bovine and ovine strains of *M. paratuberculosis*. A properly equipped diagnostic lab using such an assay would be able to produce results within several days rather than months. As long as *M. paratuberculosis* is being shed in feces bacterial DNA could be obtained from fecal samples for testing. Additionally, because an organism’s genetic sequence is unique and PCR can produce results from small amounts of DNA, sensitivity and specificity should be relatively high.

The advantages of such an assay are numerous. Infected individual animals in a flock or herd could be quickly identified so that proper actions could be taken in a timely manner. Moreover, the results can be obtained from live animals that are still subclinical but shedding the bacteria, providing a tool than can be used in disease
eradication from a herd or flock. Early detection would especially be of aid to the Australian sheep industry. The eligible testing age could be decreased, enabling flock managers to identify infected animals earlier and thus implement National Johne's Disease Program guidelines sooner. Information provided by the proposed assay would also be useful in elucidating the epidemiology of an infection so that future exposure to the bacteria source can be eliminated. For example, if cattle were grazing with sheep and the Johne's assay results were positive for the ovine strain (or vice versa) a producer would have the knowledge of which species seeded *M. paratuberculosis* into the operation. Overall, a livestock producer would be able to use the results of the assay proposed by this study to reduce the economic losses that persistent *M. paratuberculosis* infection is known to cause.

**Acknowledgements**

The author would like to thank Ms. Carlene Emerson for her help with experiment design and completion, Mr. Derek Pouchnik of the WSU LBB2 Analytical Laboratory for his assistance in the sequencing portion of this project, Mr. Paul Sheppard of ZymoGenetics, Inc. (Seattle, WA) for his expertise in sequence analysis, Drs. Timothy Baszler and Douglas Call for their editorial comments, and Dr. Inge Eriks for her mentoring and assistance during every step of the project. This work was supported in part by the Johnson Scholarship from the Washington State University Honors College.
References


Fig 1. *PstI* digestion of Int I and Int II amplicons. Sample 5001 (arrow) appears to be cutting similar to *M. avium* (sample not shown) while sample 5002 and the *M. paratuberculosis* control, U6, are not cut.
Fig 2. *PstI* digestion of *Mptb* I and *Mptb* II amplicons. As with the *Int I* and *Int II* amplicon digestions, sample 5001 appears to be cleaved similar to *M. avium* (arrows) while also retaining the approximately 350 bp fragment consistent with *M. paratuberculosis* (circled). Sample 5002 shows fragment patterns consistent with *M. paratuberculosis*. 
Fig 3. Sequence alignment of bovine \textit{M. paratuberculosis} \textit{hsp65} and the corresponding region of the ovine consensus sequence highlighting the positions of sequence variation.

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Table 1. Predicted restriction fragment lengths (base pairs).

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Figure 4. 1% Agarose Gel Visualization of *Faul* Digestion of *M. paratuberculosis* *hsp65*. Samples 5001 and 5002 show restriction fragments between 100 bp and 200 bp (arrows). The known bovine sample, U6, shows restriction fragments at greater than 200 bp (circled).