

**MOLECULAR TOOLS FOR THE
CHARACTERIZATION OF
MYCOBACTERIUM AVIUM
SUBSPECIES
PARATUBERCULOSIS.**

A Thesis Submitted to the College of
Graduate Studies and Research
in Partial Fulfillment of the Requirements
For the Degree of Master of Science
In the Department of Veterinary Microbiology
Western College of Veterinary Medicine
University of Saskatchewan

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LIST OF ABBREVIATIONS

AFLP	Amplified fragment length polymorphism
AGID	Agar Gel Immunodiffusion
CF	Complement Fixation
DNA	Deoxyribonucleic acid
DTH	Delayed type hypersensitivity
ELISA	Enzyme-linked Immunosorbent Assay
HEYM	Herrold's Egg Yolk Media
IS	Insertion sequence
ITS	Internal transcribed spacer
MAC	<i>Mycobacterium avium-intracellulare</i> complex
PCR	Polymerase chain reaction
PCR-REA	PCR-Restriction endonuclease assay
PFGE	Pulse field gel electrophoresis
RAPD	Randomly amplified polymorphic DNA analysis
RFLP	Restriction fragment length polymorphism

ACKNOWLEDGEMENTS

I wish to acknowledge the important people that made this research possible. I wish to thank my supervisors, Dr. Murray Woodbury and Dr. Greg Appleyard, and the rest of my graduate committee, Dr. Manuel Chirino-Trejo, Dr. John Ellis and Dr. Lydden Polley. Financial support was provided by the Saskatchewan Agriculture Development Fund, the Saskatchewan Agri-Food Innovation Fund, the Government of the Northwest Territories and Parks Canada.

All of this work relied on the collection of *Mycobacterium avium* subspecies *paratuberculosis* isolates which the following people generously provided: Dr. Chirino-Trejo, Biljana Mihaljlovic, Dr. O'Brien, Dr. Elkin, Robin King, Dr. Stevenson, Dr. Manning, Letitia Curley, Gail Thomas and Dr. Stabel.

I am very grateful to Dr. Stevenson and the staff at the Moredun Research Institute in Scotland who kindly generated the PFGE data.

I am eternally grateful to my family and friends for their continued support, especially my mum, who readily admits to not understanding anything I do, but supports me just the same. I can't thank my office-mates, Kathy and Marla, enough for the endless "escape" lunches and Tony for his clarity as he constantly reminded me not to worry, "it's only graduate studies".

1. ABSTRACT

Several strain typing techniques are available to categorize *Mycobacterium avium* subspecies *paratuberculosis* (*M. paratuberculosis*) isolates into cattle, sheep, bison, and Intermediate groups. The majority of isolates studied were identified as members of the cattle associated group, regardless of sample host origin, suggesting that the cattle group of *M. paratuberculosis* isolates are very successful. This may be because host specificity is not critical for this group or the small differences required to demonstrate host specificity have not yet been found. A major limitation to the epidemiological study of *M. paratuberculosis* has been the difficulty associated with laboratory cultivation of this micro-organism. The new typing techniques described in this thesis do not require viable *M. paratuberculosis* bacteria and therefore open a door to novel typing practices.

The new molecular techniques, single stranded conformation polymorphism (SSCP) analysis and satellite typing, were applied to *M. paratuberculosis* isolates (n=75) from a broad range of ruminant hosts and geographic locations. SSCP analysis and satellite typing were compared to currently accepted techniques (PCR-REA, RFLP, PFGE) for their ability to rapidly and reliably differentiate among *M. paratuberculosis* isolates. PCR-REA segregated isolates (n=75) into cattle (n=72), sheep (n=1) or bison (n=2) associated strain types. Two isolates from cattle in Canada were typed as RFLP-BstEII C5 by RFLP analysis. PFGE grouped a subset (n=8) of *M. paratuberculosis* isolates into 4 different PFGE types. Satellite typing resulted in 4 different satellite types (A, B, C, D). SSCP analysis identified 2 regions (IS900-2 and HSP70) where sequence

polymorphisms could be targeted to display differences among *M. paratuberculosis* isolates.

2. LITERATURE REVIEW

2.1. *Mycobacterium avium-intracellulare* complex

Taxonomically, mycobacteria belong to a single genus *Mycobacterium*, within the family Mycobacteriaceae and the order Actinomycetales. The order Actinomycetales includes a large and diverse group of micro-organisms, but Mycobacteria are easily distinguished by their ability to synthesize mycolic acids found within their cell wall. Mycobacteria are bacterially defined as aerobic, acid-fast, rod-shaped, facultative intracellular organisms that are non-motile (115). Many *Mycobacterium* species, such as *Mycobacterium avium*, are pathogenic in both human and animal populations. *Mycobacterium avium* and a closely related species, *Mycobacterium intracellulare*, belong to a group of bacteria classified as the *Mycobacterium avium-intracellulare* complex (MAC). MAC is a large cluster of genotypically and phenotypically related organisms which can be divided into 28 serovars. Serovars 1-6, 8-11, 21 and 28 are considered to belong to the species *M. avium*, while serovars 7, 12-20 and 25 are assigned to *M. intracellulare* (122,153). Based on biochemical and DNA analysis, *M. avium* can be further subdivided into 3 subspecies corresponding to pathogenicity and host-range characteristics: *Mycobacterium avium* subspecies *avium*, *Mycobacterium avium* subspecies *silvaticum* and *Mycobacterium avium* subspecies *paratuberculosis* (147).

2.2. MAC Diseases

Mycobacterium avium subspecies *avium* (*M. avium*) is ubiquitous and the causative agent of avian tuberculosis. *M. avium* infections are found in all mammals, including humans, but disease is sporadic and rarely transmissible (146). In humans, *M. avium* can cause pulmonary disease in adults (112), submandibular adenopathies in children (48), and disseminated infections in the immuno-compromised (87). *Mycobacterium avium* subspecies *silvaticum* (*M. silvaticum*) isolates are obligate pathogens of animals causing tuberculosis in birds (40) and a paratuberculosis-like disease in mammals (94). The inability to grow on egg-based culture medium is characteristic of this subspecies (147). *Mycobacterium avium* subspecies *paratuberculosis* (*M. paratuberculosis*) causes granulomatous enteritis in ruminants, commonly referred to as Johne's disease. The requirement of mycobactin for growth is a characteristic of this subspecies (147).

2.3. *M. paratuberculosis* virulence, hosts and susceptibility

Mycobacteria are known to be extremely resistant to both physical and chemical damage due to the high impermeability of the mycobacterial cell wall (120). This permits *M. paratuberculosis* to survive in the environment for long periods of time, which is an important factor in Johne's disease transmission. *M. paratuberculosis* can survive for up to 1 year in feces and soil (89) and from 9 to 17 months in water, depending on temperature and pH (86,89). Heat tolerance is another virulence factor which may be important in the transmission of *M. paratuberculosis* to humans. Heat treatment at 63C for 30 minutes will kill 100% of *Mycobacterium bovis* isolates (29). Under the same conditions, 5 – 9% of *M. paratuberculosis* isolates will survive and as a consequence,

may be resistant to commercial pasteurisation conditions (29). *M. paratuberculosis* has a higher heat tolerance than other milk-borne pathogens, such as *Listeria monocytogenes* and *Coxiella* species (139). This knowledge has recently stimulated an increase in research into the survival of *M. paratuberculosis* in milk products. *M. paratuberculosis* bacteria can be isolated from raw milk (138) from both clinically (143) and subclinically infected cattle (138,142). *M. paratuberculosis* DNA can be found in retail pasteurized milk (97).

M. paratuberculosis infects ruminant livestock animals, such as cattle, sheep and goats. *M. paratuberculosis* has also been cultured from a number of wild ruminant (30,51,117) and non-ruminant species (13,14,49,50,67) such as foxes, wood mice, brown hares and crows. Viable *M. paratuberculosis* has been found in lesions associated with Crohn's disease in humans (145).

In experimental infection studies with cattle, animals less than 6 months of age are more susceptible to *M. paratuberculosis* infection than older animals (85). This age-related susceptibility is thought to be due to immature cellular immunity found in very young animals. Adult animals are susceptible to infection but require a higher infectious dose than younger animals (157).

Resistance to intracellular pathogens, such as mycobacteria, has been demonstrated in mice and suggested in humans, to be linked to the Natural Resistance Associated Macrophage Protein 1 (NRAMP1) (2,63). There is also evidence to support an

association between increased susceptibility to Crohn's disease and a defect in the NOD2 gene of humans (70).

2.4. Johne's disease

Johne's disease is characterized by a long incubation period and clinical signs vary depending on the stage of infection (31). There are 3 stages of Johne's disease, the first being the subclinical carrier stage where infected ruminants show no clinical signs of disease and do not shed *M. paratuberculosis* in their feces. The second stage is the subclinical shedder stage where there are still no clinical signs of infection, but infected animals are shedding *M. paratuberculosis* in their feces. The final stage of the disease is the clinical stage where infected animals are showing clinical signs of disease and are profusely shedding *M. paratuberculosis* in their feces. Under conditions of stress, more of the infected animals may develop clinical disease. Once clinical disease develops, affected animals eventually die.

2.5. *M. paratuberculosis* transmission, pathogenesis and pathology

The main route of *M. paratuberculosis* transmission is fecal-oral via contaminated soil, water, teats or fomite surfaces. The introduction of Johne's disease into a population generally occurs when an infected animal contaminates the grazing area with feces containing live *M. paratuberculosis* bacteria. Uninfected animals can also acquire infection by ingesting *M. paratuberculosis* contaminated feed or milk (142) or through in utero transmission (124). Wildlife species have been demonstrated to harbour *M. paratuberculosis* bacteria and could therefore be reservoirs of infection (50). Domestic ruminants may contact wildlife and/or their excreta when grazing in an area

contaminated with wildlife feces. Transmission from wildlife to domestic animals has not been documented, but can be postulated on the basis of genetic studies that demonstrate no differences between domestic and wildlife isolates (68). Transmission of strains between domestic species (108,111,118) and from domestic species to wildlife has been well documented (42) .

The pathogenesis of Johne's disease has been well described by Manning and Collins (91). The classical pattern of the host immune response is strongly biased towards a cell-mediated response during the early, subclinical stages of infection and later shifts to a humoral response during the late clinical stages of the disease. Once ingested, *M. paratuberculosis* bacteria cross from the lumen of the small intestine into the lymphoid system via M-cells of the Peyer's patches and are then taken up by epithelial macrophages, which become activated to drive a T cell response. The initial response is a TH1 or tuberculoid response which is characterised by the production of cell-mediated cytokines (IFN γ , IL-2, TNF α) and an infiltration of lymphocytes into the tissues. Typically, the number of *M. paratuberculosis* bacteria seen in the tissues at this stage is very low. The TH1 response or subclinical stage of Johne's disease can last for months to years. At some point in the disease, for unknown reasons, the TH1 response gives way to a TH2 or lepromatous response, which is characterised by IL-4, IL-5, IL-6 and IL-10 cytokine production and an influx of inflammatory cells to the site of infection. Inflammation of the intestine causes a thickening of the intestinal wall leading to poor nutrient absorption in affected animals (127,170). The TH2 response is responsible for activation and sustained antibody production. By this stage of the disease, clinical signs, such as weight loss and diarrhea, are evident.

The pathological changes occurring during an *M. paratuberculosis* infection have been well described (167). The earliest lesions begin as small granulomas in the Peyer's patches of the ileum and eventually extend to the adjacent lamina propria and villi. The fully-developed lesion is a chronic, granulomatous enteritis where macrophages, packed with bacteria, invade the lamina propria and submucosa of the intestine (128). *M. paratuberculosis* bacteria are then shed from the lamina propria into the lumen of the gut and can be found in the feces. Fecal shedding of *M. paratuberculosis* is sporadic but can occur quite early in the course of Johne's disease, before any detectable antibody response or observable clinical signs (96).

2.6. *M. paratuberculosis* prevalence, treatment and control

The spread of *M. paratuberculosis* from one geographic area to another is generally related to the trading of infected animals. The true prevalence of infection and the economic losses associated with Johne's disease are unknown because there are no practical diagnostic tests that reliably detect subclinical infections of *M. paratuberculosis*. National surveillance programs have been established in Australia (103), and proposed in Canada and the United States, to provide assurance that herds have a low probability of being infected. But because of the lack of a rapid, sensitive diagnostic assay, the reported prevalence of Johne's disease in each country is really a reflection of the quantity and strength with which testing is done. Effective surveillance programs will require that monitoring is ongoing, with repeated testing at specified intervals.

Johne's disease was first recorded in Australian cattle and sheep, 75 and 24 years ago, respectively (5). The number of cattle herds and sheep flocks officially recorded as being infected with *M. paratuberculosis* in 1998 was 1968 and 442, respectively (5,81). The reported seroprevalence of *M. paratuberculosis* infection in Belgian dairy cattle is 18% (19) and seropositive cattle of all ages were found in all districts of Austria, with a seroprevalence ranging from 0.5 to 7.1%, depending on the ELISA method used (61). Several studies across various regions of the United States of America have yielded seroprevalences of *M. paratuberculosis* infection in cattle populations ranging from 1.6 to 55% (20,74,84,156). Based on histological lesions, the prevalence of *M. paratuberculosis* infections in randomly selected Canadian slaughterhouse sheep was determined to be 3% in 2003 (9). A survey of commercial cow-calf operations in Saskatchewan, Canada demonstrated a herd prevalence of 3% (152). The seroprevalence of *M. paratuberculosis* infections in 90 dairy herds in the Maritimes provinces of Canada was 3.4% (27).

Infections due to *Mycobacterium* species are among the most difficult to treat because of the high lipid content and complexity of the bacterial cell wall (114). Also, mycobacteria are capable of surviving within host macrophages, which are largely responsible for eliminating pathogenic microbes (11). The chronic nature of *M. paratuberculosis* infections requires prolonged therapy, with multiple drug regimens of low toxicity. Suitable drugs are therefore expensive, making the treatment of *M. paratuberculosis* infections economically unfeasible.

As therapeutic measures have proved inefficient, identification of subclinically infected animals and their eradication form the basis of treatment and control. To prevent new infections, animals should be born in a clean, dry environment, free of fecal contamination. Newborn animals should be fed only colostrum from animals that routinely test negative for the presence of *M. paratuberculosis*. The control of Johne's disease could conceivably be affected by the ability of wildlife species to acquire and then pass infection back to domestic animals, but this relationship has never been characterised. The prevalence of *M. paratuberculosis* in wild ruminants is relatively low (107), whereas the prevalence in farmed animals has been demonstrated to be higher, probably due to the higher density of animals in farmed situations (107).

Other factors influencing the ecology of *M. paratuberculosis* infections are the increase in the number of susceptible hosts and environmental pollution leading to the acidification of soils and water (91). Eradication of *Mycobacterium tuberculosis* and *Mycobacterium bovis* from human and animal populations may have left animals with increased *Mycobacterium* susceptibility. Environmental pollution may have opened a niche for *M. paratuberculosis* since it has been demonstrated that *M. paratuberculosis* is far more resistant to inactivation by low pH than other pH-resistant pathogens, such as *Yersinia enterocolitica* and *Listeria monocytogenes* (140).

2.7. Diagnosis of *M. paratuberculosis* infection

Clinical signs of Johne's disease are manifested by weight loss and sometimes diarrhea (31). Mastitis, infertility and decreased milk production are additional signs of Johne's disease (31). The symptoms of Johne's disease were first described in 1895 by H. A.

Johne and L. Frothingham, who demonstrated a connection between cattle enteritis and the presence of acid-fast micro-organisms in sections of the intestinal mucosa (35).

Diagnosis of Johne's disease is usually based on clinical signs and the presence of *M. paratuberculosis* is confirmed by an established diagnostic test. Control of infection using this diagnostic protocol has proven to be difficult because of the different stages of Johne's disease. It is important to identify infected animals before they reach the subclinical shedder stage and become an unapparent source of infection to other animals. It is unfortunate that most diagnostic tools available for the detection of *M. paratuberculosis* are less than satisfactory (35). It is customary to perform whole herd culture tests to detect subclinical shedders and to determine the prevalence of the infection. As no test is 100% sensitive, control of the Johne's disease depends on repeated tests at 6 month or yearly intervals over a number of years to eliminate positive animals.

There are 2 main types of tests used to diagnose Johne's disease: Tests for the detection of *M. paratuberculosis* bacteria and tests that detect an immunological response to infection.

2.7.1. Detection of *M. paratuberculosis* bacteria

Cultivation of *M. paratuberculosis* bacteria from feces or tissues remains the most reliable method of detecting infected animals (132,141). The estimated sensitivity of fecal culture is roughly 33% and the specificity has been accepted as 100% (158). A disadvantage of using conventional culture methods is the long incubation time and the variation in sensitivity as some strains are more difficult to isolate than others. The

addition of certain amino acids or biochemical products, such as pyruvate, to culture media has been shown to both enhance (159) and inhibit (105) the growth of certain *M. paratuberculosis* isolates.

Traditional culture techniques are usually performed using culture slants of Herrold's Egg Yolk Media (HEYM) supplemented with mycobactin J, a chelating agent which aids the bacterium in acquiring iron needed for growth (144). This method requires up to 20 weeks of incubation, depending on the host origin of the isolate. An increasing number of *Mycobacterium* species have been identified possessing similar, if not identical, biochemical characteristics. This renders identification by classical culture methods ineffective. In the past, *M. paratuberculosis* was distinguished from *M. avium* and *M. silvaticum* by its dependence on mycobactin but recently, *M. paratuberculosis* isolates that are not mycobactin-dependent have been described (4).

Radiometric methods of culture, with automated monitoring of ¹⁴C-labelled bi-products from bacterial metabolism, are a less time-consuming form of bacterial growth because a positive culture can be detected before the visual formation of bacterial colonies.

Radiometric culture has a higher sensitivity and specificity when compared to conventional culture methods (131,163).

Broth-based cultivation of *M. paratuberculosis* bacteria from feces has also been described. A comparison of 7H10 Tween broth and HEYM yielded minimum *M. paratuberculosis* detection times of 27 and 49 days, respectively (47). However, evaluation procedures for the growth of *M. paratuberculosis* in broth media have not

been performed with actual clinical specimens, therefore the high background turbidity of fecal and tissue specimens subsequent to processing may change the detection times. Stich *et al* (137) evaluated a broth-based, non-isotopic automated system, called MB/BacT, and found it to be considerably more sensitive and rapid than using HEYM for the detection of *M. paratuberculosis* in bovine feces. Unfortunately, automated assays require a higher equipment investment.

Another method for the direct detection of *M. paratuberculosis* bacteria is acid fast staining of fecal smears. This is a rapid method for detecting the presence of mycobacteria, but it is not as specific or sensitive as culture (15). The direct detection of *M. paratuberculosis* bacteria in feces or tissues should always be confirmed with other detection methods to rule out the existence of other *Mycobacterium* species that can be present in these samples types.

2.7.2. Detection of host immunological response to infection

An immunological response to an *M. paratuberculosis* infection can be detected by measuring host antibody production. Antibody detection tests, such as Agar Gel Immunodiffusion (AGID), Complement Fixation (CF) and Enzyme-linked Immunosorbent Assay (ELISA), can be very sensitive when antibody production is at its highest, as in the late stages of disease. But, because the sensitivity of antibody detection tests increases with the progression of disease, actual clinical signs may be evident before a positive test result is achieved. The ELISA assay can be performed in as little as a few hours, but the overall sensitivity has been estimated at only 45% since antibodies may not be detectable until late in infection (38). Ferreira *et al.*(58) evaluated

the AGID assay for its possible adoption as a diagnostic test in field conditions. AGID was demonstrated to be unsatisfactory as a screening diagnostic test for subclinically infected animals, though it may be useful as a confirmatory test for suspect animals demonstrating the clinical signs of Johne's disease. Several studies have demonstrated the low sensitivity of CF when applied to subclinically infected animals (38,126,132). The consensus in the literature is that antibody detection assays are of little help in preventing the spread of infection because of the associated lag time between *M. paratuberculosis* shedding and a positive result.

Detection of the subclinical stage of the disease can be done by measuring the hosts' cellular response with such assays as delayed type hypersensitivity (DTH) or gamma interferon (IFN γ). DTH tests measures a cutaneous T-cell-mediated inflammatory response due to the increase in skin thickness produced by the intradermal inoculation of the Johnin antigen (31). A positive reaction is indicative of prior exposure to antigen or *M. paratuberculosis* infection. Since this test detects a cell-mediated immune response, it is useful for the detection of the early stages of an *M. paratuberculosis* infection.

However, DTH has a low specificity because of cross-reactions with other *Mycobacterium* species. In the IFN γ assay, the release of the cytokine, IFN γ , from host derived, sensitized lymphocytes after an overnight incubation with antigen is quantified by immunodetection. This assay may be useful for detection of the subclinical stage of an *M. paratuberculosis* infection, but is plagued with non-specific reactions (117).

A newly marketed commercial ELISA kit (Johne's Absorbed EIA, CSL Pharmaceuticals, Parkville, Vic), was evaluated for test sensitivity and specificity for the detection of *M. paratuberculosis* in subclinically infected cattle (39). The kit demonstrated a sensitivity of 47.3% and a specificity of 99.0%. The comparison of the sensitivity and specificity of this test with other tests for the detection of subclinically infected animals indicated that this test is, at present, the most efficient commercially available test for Johne's disease in cattle.

2.7.3. Molecular methods of detection

Several polymerase chain reaction (PCR) assays, targeting the IS900 region, have been developed for the detection of *M. paratuberculosis* DNA (25,43,52,55,92,151,164,166). IS900 is an insertion sequence or small mobile genetic element containing genes related to transposition. Unfortunately, PCR-based detection of *M. paratuberculosis* can be limited by poor recovery of DNA due to the highly specialized, lysis-resistant *Mycobacterium* cell wall (21) and by the presence of inhibitory substances in preferred clinical specimens, such as fecal samples (3,73,149,169). To address some of these limitations, different methods of cell wall lysis and removal of inhibitors have been developed. Immunomagnetic bead separation coupled with bead beating and real-time PCR was found to be an effective procedure for the detection of *M. paratuberculosis* DNA in fecal samples (82). The use of buoyant density centrifugation and sequence capture PCR has also proved to be an efficient means of purifying and concentrating *M. paratuberculosis* bacteria from fecal samples (69). Recently, a real-time PCR method was developed (57) with a sensitivity equal to that of fecal culture and the ability to detect amplified products without electrophoresis. Real-time PCR has the advantage of

being a more rapid assay and can be performed in a single tube to limit cross-sample contamination.

The discovery of the *M. paratuberculosis* specific genetic element, IS900, and the use of PCR based techniques have greatly improved the diagnosis of Johne's disease (99,151). But limited numbers of *M. paratuberculosis* bacteria in subclinically infected animals and the high concentration of inhibitors in clinical specimens, means that accurate detection of infected animals is still relatively poor making further improvement a necessity.

Currently favoured assays for *M. paratuberculosis* detection vary. Studies in Austria indicate that a combination of serological examination and detection of causative agent (PCR or culture) should be used for *M. paratuberculosis* diagnosis (61). Researchers in Argentina suggest using a combination of ELISA for screening and culture for confirmation of *M. paratuberculosis* infection, but emphasize the importance of continued IFN γ test development for the early detection of Johne's disease (105). The Australian state of Victoria has a voluntary bovine Johne's disease test and control program which relies on the use of an absorbed ELISA (Johne's Absorbed EIA Kit, CSL Pharmaceuticals, Parkville, Vic), but recent studies suggest that pooled fecal culture is more economical (161). The University of Minnesota (Minnesota, USA) also supports pooled fecal culture as a valid and cost-effective method for the detection of *M. paratuberculosis* infection in dairy cattle herds (154). Each country has preference towards certain brand-name assays, but the underlying technique remains the same.

Definitive diagnosis should follow the Office International des Epizooties suggestions that confirmation of *M. paratuberculosis* infection be based on the finding of gross or microscopic pathognomonic lesions and isolation of *M. paratuberculosis* bacteria in culture.

2.8. Genetics and strain typing

The entire *M. paratuberculosis* genome was recently sequenced by University of Minnesota researchers with collaborators at the United States Department of Agriculture's (USDA) National Animal Disease Center in Ames, Iowa. The *M. paratuberculosis* genome contains nearly 5 million base pairs in a circular chromosome with more than 4,500 predicted genes.

Widely studied genes in the *M. paratuberculosis* genome include the rDNA genes, the internal transcribed spacers and IS900. In mycobacteria, ribosomal genes are linked in a single operon starting with 16s rRNA, then 23s rRNA and finally 5s rRNA. This operon is present as a single copy in slowly growing mycobacteria, such as *M. paratuberculosis*, and as two copies in the more rapidly growing *Mycobacterium* species (17). Sequencing of the rDNA genes has been used to distinguish between different species of bacteria (46,54), but the rDNA genes are fairly conserved within species and are not useful for differentiating between isolates of the same species.

Between the 16s and 23s rRNA genes and between the 23s and 5s rRNA genes are 2 internal transcribed spacers ITS1 and ITS2, respectively. This arrangement is present in all bacteria (130). Ribosomal spacers have proven to be extremely useful tools for

typing and identifying closely related bacteria due to their high size and sequence variability (62).

A subspecies-specific region of the *M. paratuberculosis* genome has been identified and well characterized. Each *M. paratuberculosis* cell can contain 15 to 20 copies of a putative transposase known as IS900 (155). This insertion sequence is said to be the sole genetic element that distinguishes *M. paratuberculosis* from *M. avium* and therefore gene-based diagnostics have tried to capitalize on this. It was originally thought that IS900 was specific to *M. paratuberculosis* (36,98), but it has since been demonstrated that IS900-like sequences exist in other *Mycobacterium* species (56).

2.8.1. Restriction fragment length polymorphism analysis (RFLP)

Due to the above mentioned difficulties associated with the detection of *M. paratuberculosis*, additional molecular knowledge has been sought that might aid in the understanding of Johne's disease. A common way of differentiating among closely related species is to exploit differences found in the genome. Many molecular methods of genotyping, known as DNA fingerprinting or strain typing, have been described. RFLP analysis combined with southern blotting using various probes such as 5s rRNA (28), IS900 (148) and IS1311 (160) were the first of these to be described. This form of strain typing involves the digestion of the whole genome with restriction enzymes, fixation of the resultant DNA fragments onto a nylon membrane and then the detection of specific sequences with various, *M. paratuberculosis*-specific probes. The RFLP assay has been standardized by Pavlik and coworkers (109). An analysis of 1008 isolates of *M. paratuberculosis* resulted in 28 distinctive RFLP profiles or strain types,

using a section of IS900 as a probe. Although RFLP analysis is an accepted form of strain typing, drawbacks associated with this type of analysis include labour intensiveness and technical difficulties, such as high DNA concentration requirements. RFLP analysis is also limited in its ability to provide epidemiological information mainly because one RFLP pattern seems to predominate within a group of infected animals and a recent study demonstrated a poor relationship between RFLP type and species of ruminant host or clinical status (107).

2.8.2. Amplified fragment length polymorphism analysis (AFLP)

AFLP is a new tool for the detection and evaluation of genetic variation. AFLP's are a more rapid and less labour intensive alternative to the RFLP technique. The AFLP technique is used to selectively amplify a subset of DNA fragments obtained after the genomic digest of a micro-organism (Figure 2.1). Genetic differences between organisms are displayed through the different patterns of DNA fragments on a polyacrylamide gel.

The difference between RFLP analysis and the AFLP technique is that only a proportion of the DNA fragments generated by restriction digest will be amplified in the AFLP technique. This reduces the complexity of the initial DNA fragment mixture and therefore DNA fragment patterns are easier to distinguish on polyacrylamide gels. Genetic differences are detected as the absence or presence of DNA fragments due to mutations in restriction sites and insertions and/or deletions within a DNA fragment. AFLP analysis has been used to strain type different bacterial (75,104), plant (79,106) and parasite (18,34,66) species. Whether AFLP offers better discriminatory power over

PFGE depends on the micro-organism studied (45,88). AFLP analysis is technically preferred over RFLP analysis and has a higher discriminatory power than RFLP (26).

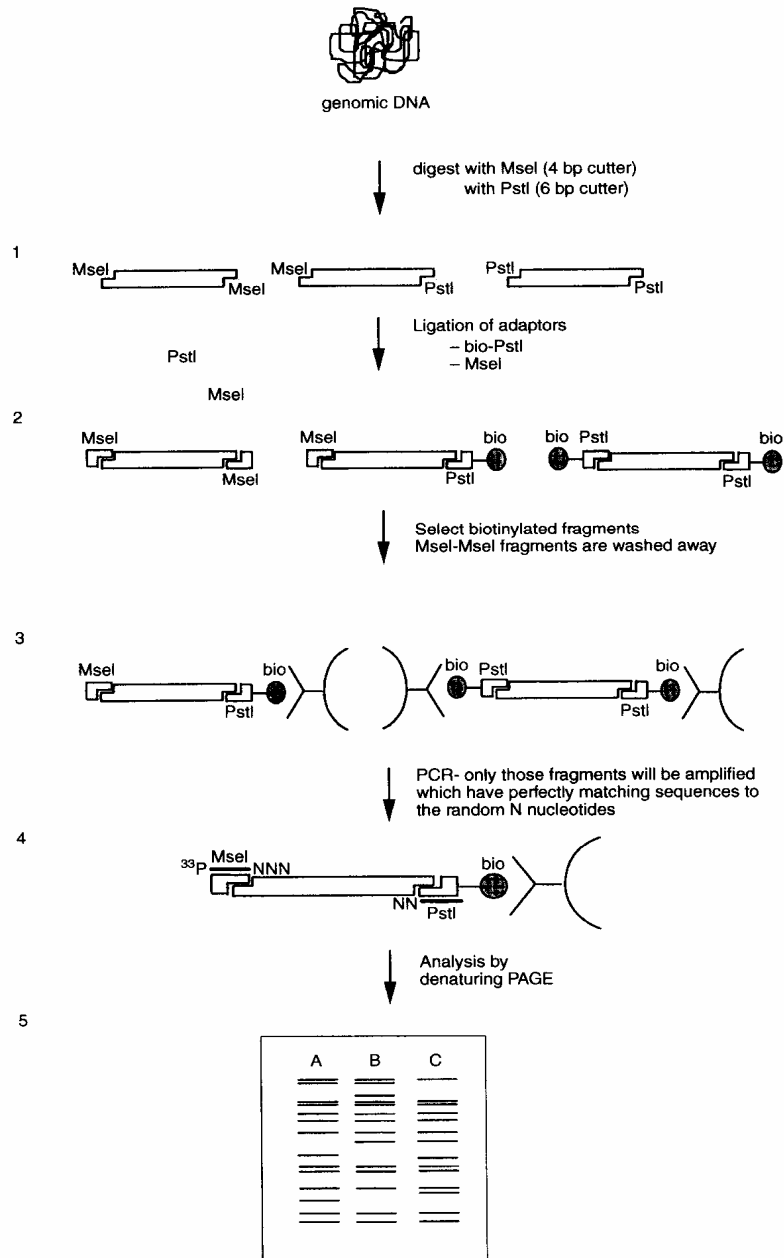


Figure 2.1 - Schematic representation of AFLP technique. Figure adapted from Matthes *et al.* (93).

2.8.3. Randomly amplified polymorphic DNA analysis (RAPD)

RAPD analysis was a popular typing technique because of its simple and straightforward protocol. This technique is similar to a standard PCR protocol, except RAPD analysis uses only a single, randomly chosen oligonucleotide which acts as both the forward and reverse primer. This single primer will hybridize to many different sites within the sample DNA, but a PCR fragment will only be generated if the primer anneals at 2 sites on opposite strands of the DNA, within 2 kb, the maximum length of a PCR product (53). Genetic variation is determined by changes in the pattern of amplification products displayed after agarose gel electrophoresis. RAPD analysis has been used to study the genetic diversity of different bacterial (8,78), viral (41,135), parasite (113,133) and plant (123,172) species. RAPD is technically less-demanding than RFLP analysis (129), but problems of interpretation due to inconsistent intensity of bands in different polymerase chain reaction runs may arise (135) and reproducibility between laboratories is questionable (76).

2.8.4. PCR-Restriction endonuclease assay (PCR-REA)

A more time efficient form of strain typing is PCR-REA. Through RFLP analysis, using the insertion sequence IS1311 as a probe, it was discovered that IS1311 exists in many *Mycobacterium* species (80), but specific sequence differences could be used to distinguish between and within species (160). An assay was developed which requires PCR amplification of a region of IS1311 and subsequent digestion of the PCR product with restriction enzymes. This form of strain typing permits distinction between cattle and sheep associated strains of *M. paratuberculosis* and has since been expanded to include a bison strain (165). PCR-REA has been demonstrated to be a rapid, reliable

method for strain typing of *M. paratuberculosis* isolates, but this technique also provides limited epidemiological information in that most *M. paratuberculosis* isolates are typed by this technique as cattle strains.

It is important to point out that “strain” refers to the genetically characterized digestion pattern associated with a particular group of *M. paratuberculosis* isolates and in this case, the “strain” has been named for the host in which it was originally isolated or most often found.

2.8.5. Pulse-field gel electrophoresis analysis (PFGE)

PFGE has been adopted and used as a variation of the RFLP assay in an attempt to resolve the different restriction digest profiles and to lessen the technical time required of a typical RFLP. To date, 16 PFGE profiles have been described from 93 isolates (136) and when compared to RFLP, PFGE could segregate the previously established RFLP profiles into smaller, more epidemiologically useful groups. The drawback to this method of strain typing is the requirement of large amounts of un-sheared DNA and time required (up to 6 months) for the growth of *M. paratuberculosis* bacteria in a liquid media.

Strain typing or genotyping of micro-organisms may provide insight into the epidemiology of Johne’s disease. The same molecular techniques used for strain typing bacterial genomes can be applied to host genomes and result in information that could explain why some hosts are more susceptible to disease than others. The recent identification of Johne’s disease-like lesions in the intestines of carnivores in Scotland

demonstrates that disease caused by *M. paratuberculosis* is not limited to ruminant animals (50). Strain typing of the carnivore isolates demonstrated that the same strain was found in both cattle and carnivores in Scotland. It can be hypothesized that the carnivores acquired this infection by eating rabbits, which have also been demonstrated to be carriers of this same strain. Furthermore, this unusual report of disease in carnivores could also be due to the pathogenic characteristics of this particular strain. It is in this way that strain typing identifies transmission patterns and pinpoints particular areas of *M. paratuberculosis* research that will aid in the overall understanding of Johne's disease.

The primary goal of research presented in this thesis was to find the appropriate molecular tools to identify additional polymorphic regions of the *M. paratuberculosis* genome. The specific objectives were:

- (1) Collect *M. paratuberculosis* isolates from a broad range of ruminant hosts and geographic locations.
- (2) Adopt published strain typing protocols to identify isolates falling into the established bison, cattle, sheep and Intermediate groups.
- (3) Identify polymorphic or variable genomic regions which could be used to further subdivide isolates into epidemiologically useful groups.

3. PCR-SURVEY OF NORTHERN CANADIAN BISON (*BISON BISON*) FOR THE PRESENCE OF *MYCOBACTERIUM AVIUM* SUBSPECIES *PARATUBERCULOSIS*

3.1. Introduction

bison (*Bison bison* subspecies *athabascae*) in Wood Buffalo National Park (WBNP) are one of the last genetic resources of wild wood buffalo in Canada. At one point, the population of wood bison that roamed North America was well over 100,000 but in the 1900s, bison numbers decreased to just 250 due to unregulated hunting (64). The last of the remaining wood bison were located in what is now known as WBNP. In an effort to increase this population of wood bison, the Canadian government shipped 6600 plains bison (*Bison bison* subspecies *bison*) from Alberta to WBNP. Unfortunately, the introduction of the plains bison was associated with the introduction of new diseases; bovine brucellosis (*Brucella abortus*) and tuberculosis (*Mycobacterium bovis*).

Creating brucella and tuberculosis-free bison herds is now a top priority for bison preservation in Canada. Monitoring the status of other possible pathogens, such as *M. paratuberculosis*, has been included in the preservation plan. Johne's disease has been previously diagnosed in farmed bison (23,24), but has not been diagnosed in the free-ranging bison from WBNP. Because of the chronic, intermittent nature of Johne's disease and the need to test live animals, a non-invasive, economical assay is needed.

Most non-invasive tests require the collection of blood or feces. Blood can be tested, using ELISA, AGID or CF, for the presence of an immune response to *M. paratuberculosis* infection, but these assays suffer from low sensitivity and specificity. An easier way to sample a wild population is to collect recently deposited fecal samples. In the recent past, fecal samples were often tested immediately by HEYM culture, despite the high costs and long incubation times. Now, with the advantages of PCR, it is more economical to look for the presence of *M. paratuberculosis* DNA in fecal samples and then culture only the positive samples. Recently, methods for the purification of DNA directly from fecal samples have been described (22,119). These procedures allow for the rapid purification of DNA directly from fecal matter, without the time and cost associated with culture. Once *M. paratuberculosis* has been isolated from feces further characterization or strain typing can be done.

Strain typing of *M. paratuberculosis* isolates from wild bison is important to understanding the epidemiology of Johne's disease in bison herds. Knowing what strain is infecting a herd and where that strain originated is important to bison management. Captive bison are susceptible to infection with cattle-related and bison-related *M. paratuberculosis* isolates (134), but the susceptibility of wild bison is unknown.

This section describes the nested PCR detection and strain typing of *M. paratuberculosis* DNA directly from bison fecal samples and was designed to partially achieve the objective of the collection and characterization of *M. paratuberculosis* isolates from a

broad range of ruminant hosts and geographic locations. PCR detection of DNA directly from fecal samples is a recently developed, highly beneficial technique. The direct detection technique and a method for strain typing *M. paratuberculosis* independent of the long incubation period required for cultivation is described.

3.2. Materials and Methods

3.2.1. Sample collection

A total of 835 fecal samples were opportunistically collected by biologists from the NWT Dept of Natural Resources from 6 bison herds across Northern British Columbia, WBNP, Nahanni Butte and Fort Liard (Figure 3.1). Samples were obtained from a pilot study performed to assess the existence of *M. paratuberculosis* in the Northern Canadian herds. Each herd was approached and fecal samples were collected immediately after deposition and kept frozen at -20C for up to 2 years. All fecal samples were screened for the presence of *M. paratuberculosis* DNA using a *M. paratuberculosis*-specific PCR assay. A representative of the *M. paratuberculosis* positive fecal samples were typed and sequenced as well as sent for confirmation by BACTEC culture.

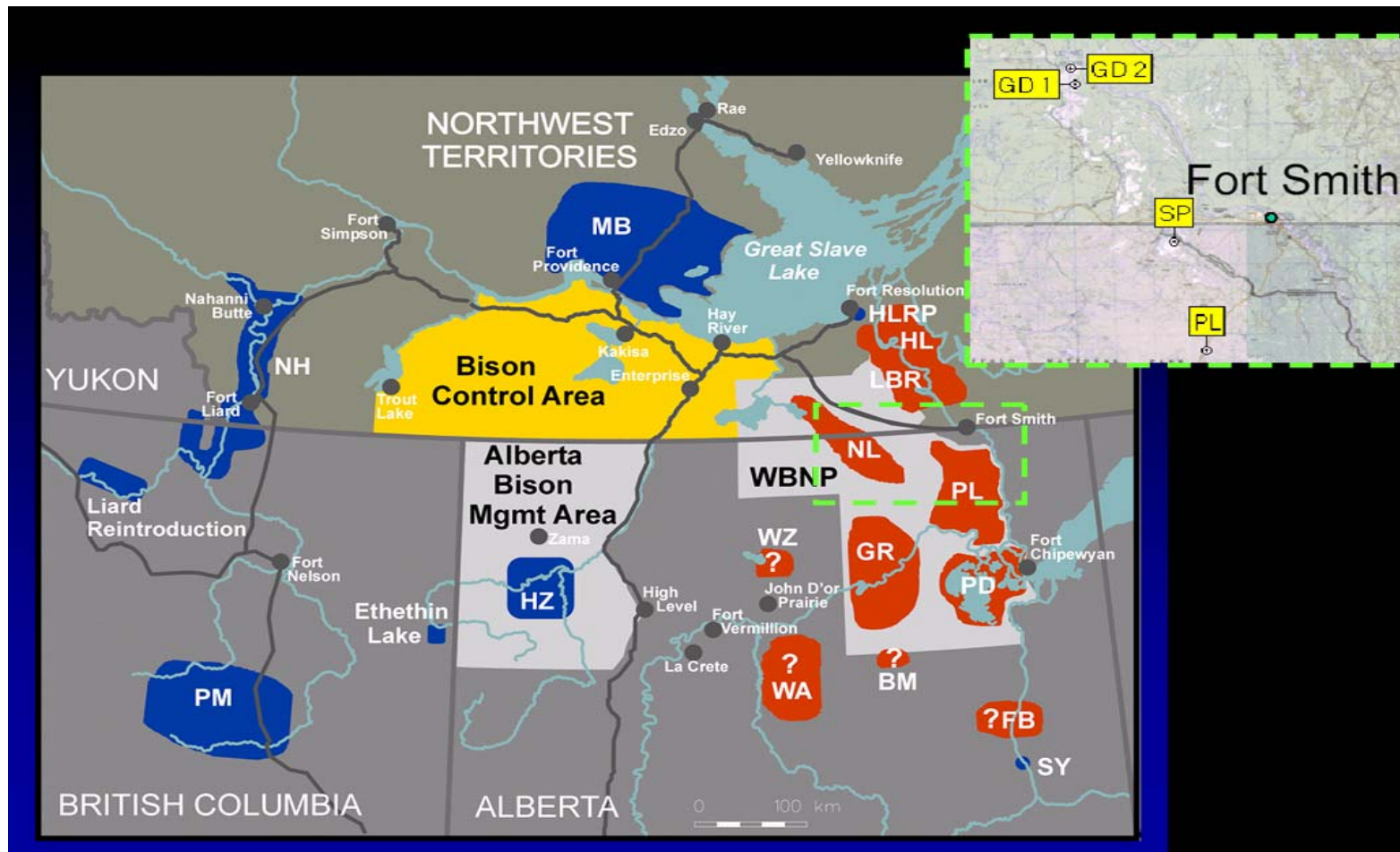


Figure 3.1 - Bison (*Bison bison*) Distribution and Fecal Sampling Areas: Hook Lake (HL), Pine Lake (PL), Grand Detour (GD), Sweet Grass (SG), Salt Plains (SP), Nahanni (NH). Figure supplied by Dr. Elkin (Fort Smith, NWT, Canada).

3.2.2. Fecal preparation

The isolation of bacteria directly from bison fecal samples was modified from the technique described by Chui *et al.* (32). Approximately 4 grams of each fecal sample was suspended in 50mL Falcon tubes (BD Biosciences, Ontario, Canada) containing 25mL of 0.6% SDS and 3 (3mm) glass beads (VWR, Canada). Tubes were sealed with paraffin and vigorously shaken, horizontally, for 30 min to break up large fecal chunks. After sitting vertically at room temperature for 30 min, the supernatant was transferred to a clean 50mL Falcon tube. Any bacteria in the supernatant were washed with sterile, purified water 3× by centrifugation (800 x g, 30 min) then pellet and slurry were re-suspended in 20mL of sterile, purified water. After the last centrifugation, the pellet and slurry was not re-suspended in water. The sample was mixed and 250µl was extracted using the MagaZorb® DNA Mini-Prep Kit (Cortex Biochem, California, USA), as outlined below. A positive extraction control was performed by spiking 10µl of a McFarland standard 3 suspension of *M. paratuberculosis* (ATCC #19698) into a 4 gram sample of *M. paratuberculosis* negative feces. A negative extraction control was not performed because feces free of any bacterial DNA was not obtainable.

3.2.3. DNA extraction

DNA extraction from fecal samples was performed using a protocol modified from the manufacturer's instructions and using the reagents provided with the MagaZorb DNA Mini-Prep Kit unless otherwise stated. A volume (250µl) of fecal wash was added to a sterile ribolyzer tube containing 300µl of 12mM Tris (pH 8.0) and 100µl of 100-120 mesh glass beads (Mandell Scientific, Ontario, Canada). Samples were ribolyzed at

speed 6.0 for 45 sec. After centrifugation (100 x g, 5 min) 200µl of supernatant was mixed with 20µl of proteinase K and 200µl lysis buffer, vortexed (30 sec) and incubated at 56°C for 10 min. Five hundred microliters of binding buffer and 20µl of well-mixed MagaZorb beads were added, samples were hand mixed and then placed on a rotator to mix for 10 min. MagaZorb beads and DNA were separated from supernatant by placing the tube on a magnet for 3 min. Supernatant was discarded and beads were washed 2× with 1mL of wash buffer. DNA was eluted from the beads by adding 500µl elution buffer and placed on a rotator for 10 min. After sedimentation of beads using a magnet (3 min), the supernatant was retained and kept at -20C until the PCR assay was performed. A positive extraction control was performed by processing a known spiked fecal sample and a negative extraction control was performed with water.

3.2.4. Polymerase chain reaction (PCR)

Nested PCR was performed using gene 254 of the *M. paratuberculosis*-specific locus 251 (10), as the target sequence. The primers used in the primary reaction (254-F, 5'-*TGG GCA GCC CGG TGT CCC G-3'* and 254-R, 5'-*CAC GCG CTC CTT TCA GCC TT-3'*) were previously established (10), while the secondary primers (254-F2, 5'-*TCG GGG CTG GAT TCG TAT TC-3'* and 254-R2, 5'-*GCC AAC TTT CCG GTG CTC AA-3'*) were specifically designed for this nested protocol.

The size of the amplified products were; 293bp for the primary reaction and 134bp for the secondary reaction. For the primary reaction 2µl of MagaZorb extracted DNA was added to a PCR cocktail containing 1× *Taq* Buffer with (NH₄)₂SO₄, 3mM MgCl₂,

0.25mM dNTP, 1.6 pmol each primer, 0.025 U/ μ l recombinant Taq polymerase and sterile, filtered water up to 48 μ l. All reagents were supplied by Fermentas Life Sciences (Ontario, Canada). PCR (MJ Research) cycling parameters for the primary reaction were as follows: 94°C for 5 min; [94°C for 45 s, 55°C for 1 min and 72°C for 2 min] \times 40 cycles, and a final extension at 72°C for 10 min. Two microliters of the primary reaction was added to a PCR cocktail containing 1 \times *Taq* Buffer with (NH₄)₂SO₄, 2mM MgCl₂, 0.25mM dNTP, 1.6 pmol each primer, 0.025 U/ μ l recombinant Taq polymerase and sterile, filtered water up to 48 μ l. PCR cycling parameters for the secondary reaction were the same as the primary reaction with the exception of 30 instead of 40 cycles. PCR amplicons were analysed by electrophoresis through a 2.5% agarose gel, staining with ethidium bromide and visualizing with a UV transilluminator. MagaZorb extracted DNA samples were processed at 2 different concentrations, neat and 1/100 dilution. A positive control consisted of DNA extracted from an ATCC (#19698) isolate of *M. paratuberculosis* and a negative control consisted of water instead of DNA. The specificity of the nested PCR assay was determined to be 100% by testing all available DNA from *Mycobacterium* species and other closely related organisms (Table 3.1).

Table 3.1- *Mycobacterium* species and other related micro-organisms used to determine specificity of PCR assays.

Organism	# Isolates	Locus 251 Result	IS1311 Result
<i>Brucella suis</i>	1	Negative	Negative
<i>Corynebacterium</i> species	3	Negative	Negative
<i>Moraxella bovis</i>	1	Negative	Negative
<i>Mycobacterium avium</i> subspecies <i>avium</i>	12	Negative	Positive
<i>Mycobacterium avium</i> subspecies <i>silvaticum</i>	1	Negative	Negative
<i>Mycobacterium bovis</i>	2	Negative	Negative
<i>Mycobacterium fortuitum</i>	1	Negative	Negative
<i>Mycobacterium gordonae</i>	1	Negative	Negative
<i>Mycobacterium intracellulare</i>	1	Negative	Negative
<i>Mycobacterium kansasii</i>	2	Negative	Negative
<i>Mycobacterium microti</i>	2	Negative	Negative
<i>Mycobacterium nonchromogenicum</i>	1	Negative	Negative
<i>Mycobacterium tuberculosis</i>	2	Negative	Negative
<i>Mycobacterium ulcerans</i>	1	Negative	Negative
<i>Mycoplasma bovis</i>	2	Negative	Negative
<i>Rhodococcus</i> species	2	Negative	Negative

3.2.5. Culture

PCR positive fecal samples (n=8) from each of the different fecal sampling areas (Figure 3.1) were sent to the Animal Health Monitoring Lab (Abbotsford, BC, Canada) for confirmation with this laboratory's PCR assay and BACTEC culture.

3.2.6. Strain typing (PCR-REA)

Strain typing was modified from a previously described protocol (165). Nested PCR was performed using MagaZorb extracted DNA as a template for the amplification of a region of IS1311. The primers used in the primary reaction (M56, 5'-GCG TGA GGC TCT GTG GTG AA-3' and M119, 5'-ATG ACG ACC GCT TGG GAG AC-3') were previously established to amplify a region of the IS1311 insertion sequence (165), while

the secondary primers (M42, 5'-TGG ACC AGT CTG CCT TGC TG-3' and M545, 5'-TGC AGT AAG TGG CGT CGA GG-3') were specifically designed for this nested protocol. The primary and secondary amplified products were 608bp and 503bp, respectively.

For the primary reaction 2µl of MagaZorb extracted DNA was added to a PCR cocktail containing 1× *Taq* Buffer with (NH₄)₂SO₄, 2mM MgCl₂, 0.25mM dNTP, 0.8µmol each primer, 0.025 U/µl recombinant *Taq* polymerase and sterile, filtered water up to 48µl.

All reagents were supplied by Fermentas Life Sciences (Ontario, Canada). PCR cycling parameters for the primary reaction were as follows: 94°C for 3 min; [94°C for 30 s, 62°C for 15 s and 72°C for 1 min] × 40 cycles, and a final extension at 72°C for 5 min.

Two microliters of the primary reaction was added to the secondary PCR cocktail containing the same reagents and cycling conditions as the primary reaction. PCR amplicons were analysed by electrophoresis through a 2.5% agarose gel, staining with ethidium bromide and visualizing with a UV transilluminator. Negative and positive controls as well as assay specificity (Table 3.1) were as described for the locus 251 PCR assay.

Restriction digest was carried out at 37°C for 2 hours in a 16µl volume containing 1× restriction buffer, 1× Bovine Serum Albumin (BSA), 1U of each *Hinf*I and *Mse*I (New England Biolabs, Ontario, Canada), 10µl secondary PCR product and sterile, filtered water up to 16µl. The restriction fragment sizes are shown in Table 3.2. Digested DNA fragments were electrophoresed through 2.5% agarose gels at 100V for 1 hour.

Table 3.2 - Restriction patterns generated from nested PCR amplification of IS1311 and digestion with enzymes HinfI and MseI.

Isolate type	Secondary product size (bp)	Restriction pattern sizes (bp)
<i>M. paratuberculosis</i> (sheep)	503	226, 277
<i>M. paratuberculosis</i> (cattle)	503	67, 159, 226, 277
<i>M. paratuberculosis</i> (bison)	503	67, 159, 277
<i>Mycobacterium avium</i> subspecies <i>avium</i>	503	134, 146, 224

3.2.7. Sequencing

A region of the IS1311 element from a bison, cattle, sheep strain of *M. paratuberculosis* was amplified and sequenced (Plant Biotechnology Institute, Saskatchewan, Canada) using primers M42 and M545. Amplified products from 2 different bison fecal samples were also sequenced in this manner. Sequences were aligned manually with previously established *M. paratuberculosis* IS1311 sequences (GenBank AJ223975 and AJ223974) using MegAlign software (DNASar, Madison WI.).

3.3. Results

Out of 835 fecal samples, a total of 26 were positive for the presence of the *M. paratuberculosis*-specific gene 254 from Locus 251. No positive results were obtained when other *Mycobacterium* species or other bacterial species were tested (Table 3.1). The positive fecal samples were collected from the Sweet Grass (n=2), Pine Lake (n=2),

Hook Lake (n=1) and Grand Detour (n=21) herds (Table 3.3). Of the 8 samples sent to the Animal Health Monitoring Lab, 3 were positive by this laboratory’s diagnostic PCR assay. No *M. paratuberculosis* colonies were isolated with BACTEC culture. Therefore further molecular analysis was performed to characterize *M. paratuberculosis* directly from bison fecal samples.

Table 3.3 – Geographic location and number of bison fecal samples positive for the *M. paratuberculosis*-specific gene 254.

Bison Herd Location	Number Tested	Number Positive
Grand Detour	373	21
Hook Lake	315	1
Nahanni	27	0
Pine Lake	17	2
Salt Plains	61	0
Sweet Grass	42	2
	n=835	n=26

Strain typing was achieved using a nested protocol targeting a region of IS1311. Of the 26 fecal samples positive for the presence of *M. paratuberculosis* DNA, 2 yielded sufficient *M. paratuberculosis* DNA for strain typing. Strain typing revealed the presence of a sheep-related strain in the Sweet Grass herd and a cattle-related strain in the Grand Detour herd. The amplified IS1311 region from the Sweet Grass isolate and Grand Detour isolate was sequenced and revealed a unique 3bp region not found in other previously sequenced isolates in Genbank (Figure 3.2).

GD Isolate	TGGACCC C AT G CTGCCTTGCTGGAGGTGCTGGACGCATTACGCAATG
SG Isolate	TGGACCC C AT G CTGCCTTGCTGGAGGTGCTGGACGCATTACGCAATG
USA Bison	TGGACCCAGT-CTGCCTTGCTGGAGGTGCTGGACGCATTACGCAATG
CAN Cattle	TGGACC-AGT-CTGCCTTGCTGGAGGTGCTGGACGCATTACGCAATG
NZ Sheep	TGGAC C A- A T A CTGCCTTGCTGGAGGTGCTGGACGCATTACGCAATG
AJ223974	TGGACC-AGT-CTGCCTTGCTGGAGGTGCTGGACGCATTACGCAATG
AJ223975	TGGACC-AGT-CTGCCTTGCTGGAGGTGCTGGACGCATTACGCAATG

Figure 3.2 - Partial IS1311 nucleotide sequence of *M. paratuberculosis* isolates from Grand Detour (GD), Sweet Grass (SG), United States (USA), Canada (CAN), New Zealand (NZ) and Genbank (AJ223974, AJ223975).

3.4. Discussion

This report demonstrates the existence of *M. paratuberculosis* DNA in wild Canadian bison. The presence of *M. paratuberculosis* DNA was demonstrated with the use of a nested *M. paratuberculosis*-specific PCR assay and a nested *M. paratuberculosis* typing protocol. It is significant to find *M. paratuberculosis* DNA in the Northern Canadian bison herds because the bison in and around WBNP do not demonstrate clinical signs of Johne's disease and repeated attempts to culture *M. paratuberculosis* from these bison have been un-successful. It is possible but unlikely that wild bison can become infected with *M. paratuberculosis* and eventually clear the infection. If this was true, it would not be unrealistic to hypothesize that wild animals are better equipped than domestic species to deal with paratuberculosis infections.

It may be that Northern Canadian bison are quietly dying of Johne's disease, but dead or dying bison are not being discovered due to the vast territory inhabited by these herds. Perhaps *M. paratuberculosis* infections and Johne's disease are highly prevalent in all

bison herds in Northern Canada, but this hasn't been demonstrated due to a lack of efficient diagnostic techniques and insufficient sampling.

Another possibility for the apparent lack of disease in the Northern Canadian bison is that the isolates obtained from Northern Canada may not be as virulent as isolates from clinically affected animals. This study suggests that the strains infecting the Northern Canadian bison are distinct from those previously described. Although the sequences from the Northern Canadian bison (Figure 3.2) only differ by 3 nucleotides, this is significant considering the current strain typing technique, PCR-REA, relies on a single nucleotide polymorphism. Whether or not this unique sequence is limited to bison or more specifically to bison in Northern Canada remains unknown. Unfortunately, supported conclusions can not be established with this study due to the low number of fecal samples positive for the presence of *M. paratuberculosis* DNA.

The unsuccessful cultivation of *M. paratuberculosis* from Northern bison fecal samples could be due to the possibility that unsuitable laboratory growth conditions were used for these isolates. Previous evidence of toxicity associated with growth elements (sodium pyruvate) in *M. paratuberculosis* culture media have been described (77). PCR may be more sensitive than culture for the detection of mycobacteria (121), therefore low levels *M. paratuberculosis* shed in the feces may not be detected by culture. Reduced viability of organisms at the time of culture, due to repeated freeze-thawing of fecal samples is also possible.

The existence of *M. paratuberculosis* infection in Northern Canadian bison has not yet been demonstrated, partly because of logistical difficulties in collecting samples from free-ranging animals in the vastness of the Canadian north. Samples were obtained from a pilot study performed to assess the existence of *M. paratuberculosis* in the Northern Canadian herds. A random collection of fecal samples from each herd was not performed, therefore these results should not be used to extrapolate *M. paratuberculosis* prevalence to other Northern bison herds. Now that a more efficient diagnostic assay is available, directed routine sampling of the Northern Canadian bison herds is possible. Eventual cultivation of an *M. paratuberculosis* isolate from the Northern Canadian bison herds would also provide essential support to the suggestion that Northern bison are infected with a unique bison associated strain of *M. paratuberculosis*.

Since the Northern Canadian bison represent the last genetic resource of wild wood bison in Canada (171), considerable effort has been made towards their preservation. Previously identified *M. paratuberculosis* isolates from bison (n=2), from the United States examined in this study did not demonstrate the unique nucleotide sequence found in isolates from the Northern Canadian herds. The isolates from Northern Canadian bison appear to be unique in their IS1311 sequence and should be characterized further to shed some light on epidemiological questions related to host susceptibility, virulence and geography. Further studies with larger sample numbers will determine if the Northern Canadian bison IS1311 nucleotide sequence is unique. Epidemiological information will impact management, treatment and control of *M. paratuberculosis* infections in Northern Canadian bison. The Northern Canadian bison-associated strain

may have always existed in Canadian bison and should therefore remain unmanaged to preserve the natural ecology of these herds.

The fecal strain typing technique used in this study is a non-invasive assay that could be used to screen large numbers of fecal samples for the presence of cattle, sheep, bison or Northern Canadian bison-associated *M. paratuberculosis* strains. A comparison of the fecal strain typing technique with serological tests could strengthen the significance of detecting *M. paratuberculosis* DNA in the Northern Canadian bison herds. The fecal strain typing technique is a convenient, cost effective, specific assay which can be exploited for the generation of isolates for large scale epidemiological studies.

4. INTRA-SPECIES HETEROGENEITY FOUND WITHIN ISOLATES OF *MYCOBACTERIUM AVIUM* SUBSPECIES *PARATUBERCULOSIS*

4.1. Introduction

Differentiation or genetic typing of bacterial, fungal or viral isolates is important for understanding the epidemiology of disease. An understanding of the dynamics of infection transmission within populations or host species provides information about host susceptibility and lays the groundwork for infection control. An analysis of the molecular diversity within a microbial species can facilitate our understanding of the evolution of the species and its corresponding disease. The association of a specific strain with certain disease characteristics is important to understanding the population genetics of a particular microbe.

Targeting different loci of a microbial genome, for the purposes of identification, will result in different levels of discrimination, revealing genetic variations between different species or within a single species. The goal of strain typing is to find the right tool that will target the right region of the genome to give you the variability that is desired.

RFLP, AFLP, RAPD, PCR-REA and PFGE are some of the molecular based typing techniques that have been applied to the investigation of genetic variation within the bacterial pathogen, *M. paratuberculosis*. IS900 RFLP profiles have been used extensively in epidemiological studies to compare isolates from different host species and geographic locations. IS900 RFLP analysis has demonstrated that cattle and sheep are preferentially infected with cattle-associated and sheep-associated strains, respectively, while other ruminant (12,37,51,162,168) and non-ruminant (67,108) host species appear to be equally susceptible to either type. A variation on the RFLP assay, PFGE can subdivide RFLP profiles and distinguish between pigmented and non-pigmented *M. paratuberculosis* isolates (136).

Using PCR-REA, Whittington *et al.*, demonstrated a bison-associated strain that, to date, has only naturally been found in bison (165). AFLP is a reliable and effective tool for *M. paratuberculosis* strain typing, yielding 15 AFLP patterns from a group of 24 *M. paratuberculosis* isolates (6). Unfortunately, a comparison of discriminatory powers of AFLP versus RFLP or PFGE, for *M. paratuberculosis* typing, has not been published. A study of 208 *M. paratuberculosis* isolates, resulted in 6 RAPD patterns (110), which, when compared to AFLP analysis, demonstrates the moderate abilities of RAPD to differentiate among *M. paratuberculosis* isolates.

M. paratuberculosis has been described as extremely homogenous (101) and therefore finding genetic polymorphisms that can distinguish between epidemiologically significant isolates or facilitate a new diagnostic test, makes genotyping a hot topic in Johne's research. PCR-REA, RFLP and RAPD techniques are generally unable to

resolve *M. paratuberculosis* isolates into meaningful epidemiological groups. PFGE can deliver higher resolution, but the procedure requires the growth of isolates in a liquid medium and not all *M. paratuberculosis* isolates can survive the transfer from a solid to a liquid medium.

Satellite typing and single stranded conformation polymorphism analysis (SSCP) are 2 new DNA fingerprinting techniques that could be used to explore the *M. paratuberculosis* genome for polymorphic regions. Microsatellites (short tandem repeats, short sequence repeats, simple sequence repeats or variable number tandem repeats) are tandemly repeated units of DNA, with each unit being between 1 and 10 bp in length (33). Minisatellites are also tandemly repeated DNA units, but are between 15 to 70 bp in length (83). Both micro and minisatellites are widely dispersed throughout eukaryotic and prokaryotic genomes and are often highly polymorphic due to variation in sequence and the number of repeat units. Due to their high variability, micro and minisatellites are becoming increasingly important in gene mapping and population studies. Micro and minisatellites are used for human forensic and paternity testing (102) and have been identified in bacteria (7,59,60,65) and parasites (90,150).

A very recent study using multilocus short sequence repeats (MLSSR) enabled the differentiation of *M. paratuberculosis* isolates that were indistinguishable by AFLP (6). A comparison of the resolving power of MLSSR with RFLP or PFGE analysis has not been established.

SSCP analysis is a rapid method used for the screening of DNA fragments for nucleotide sequence polymorphisms. SSCP analysis relies on mobility differences of DNA secondary structures formed by single stranded DNA fragments of different nucleotide sequence. SSCP has been applied to DNA fragments from bacteria (72,125), viruses (1,95) and parasites (44,173).

This section was designed to collect *M. paratuberculosis* isolates from a broad range of ruminant hosts and geographic locations, to identify isolates that fall into the established bison, cattle, sheep and Intermediate groups and to identify new polymorphic regions useful in the classification of *M. paratuberculosis* isolates.

4.2. Materials and Methods

4.2.1. Collection and cultivation of *M. paratuberculosis* isolates

A total of 75 *M. paratuberculosis* isolates were solicited from research organizations in Canada, United States, New Zealand and Scotland (Table 4.1). All isolates were subsequently grown on Herrold's egg yolk media (HEYM) at 37°C for up to 2 years. Colonies were confirmed to be *M. paratuberculosis* by acid fast stain, IS900 PCR (99) and locus 251 PCR (10).

Table 4.1 - Geographic and host distribution of *M. paratuberculosis* isolates (n=75).

Geographic Origin	Host Origin						Total
	Cattle	Sheep	Goat	Bison	Elk	Deer	
Canada	11	1	6	3	3	10	34
USA	1	0	0	5	7	3	16
Scotland	0	0	0	0	0	15	15
New Zealand	0	1	0	0	0	9	10
Total	12	2	6	8	10	37	75

4.2.2. Polymerase chain reaction-restriction endonuclease assay (PCR-REA)

M. paratuberculosis DNA was isolated from HEYM culture slants using a standard phenol extraction technique (71). Three to five colonies were homogenized in 600µl of lysis buffer (100mM NaCl, 500mM Tris (pH 8.0), 10% sodium dodecylsulfate, 0.2mg/mL proteinase K), vortexed for 5 s and then incubated at 65°C for 2 hours. An equal volume of Tris-buffered phenol-chloroform (1:1 v/v, pH 8.0) was added and the mixture, vortexed for 30s and then centrifuged for 5 min at 15,000 x g. The aqueous phase was removed to a clean tube and the phenol-chloroform extraction was repeated. The aqueous phase was again removed to a clean tube containing 2.5 volumes of ice cold 95% ethanol and 0.3M sodium acetate. Samples were mixed by gentle inversions and incubated overnight at -20°C. DNA was recovered by centrifugation for 15 min at 4°C and 15,000 x g. The DNA pellet was washed twice with 80% ethanol, dried under vacuum for 5 to 10 min and then dissolved in 30µl of sterile, purified water.

PCR-REA of a region of IS1311 was performed using the primers M56 and M119 (M56, 5'-GCG TGA GGC TCT GTG GTG AA-3' and M119, 5'-ATG ACG ACC GCT TGG GAG AC-3'), as previously described (165). Phenol-extracted DNA (2µl) was added to a PCR cocktail containing 1× *Taq* Buffer with (NH₄)₂SO₄, 2mM MgCl₂, 0.25mM dNTP, 0.8µmol each primer, 0.025 U/µl recombinant *Taq* polymerase and sterile, filtered water up to 48µl. All reagents were supplied by Fermentas Life Sciences (Ontario, Canada). PCR cycling parameters were as follows: 94°C for 3 min; [94°C for 30 s, 62°C for 15 s and 72°C for 1 min] × 40 cycles, and a final extension at 72°C for 5 min. PCR amplicons (608bp) were analysed by electrophoresis through a 2.5% agarose gel, staining with ethidium bromide and visualizing with a UV transilluminator. A positive control consisted of DNA extracted from an ATCC (#19698) isolate of *M. paratuberculosis* and a negative control consisted of water instead of DNA. Assay specificity is demonstrated in Table 3.1.

Restriction digest was carried out at 37°C for 2 hours in a 16µl volume containing 1× restriction buffer, 1× BSA, 1U of each *Bst*EII and *Mse*I, 10µl PCR product and sterile, filtered water up to 16µl. Digested DNA fragments were electrophoresed through 2.5% agarose gels at 100V for 1 hour. Products were detected by staining with ethidium bromide and visualizing with a UV transilluminator.

4.2.3. Restriction fragment length polymorphism analysis (RFLP)

The preparation of DNA for RFLP analysis was adapted from a procedure designed by Stevenson *et al* (136). All *M. paratuberculosis* isolates listed in Table 3 were propagated in Middlebrook 7H9 broth media supplemented with 0.4%(wt/vol) Tween 80, 10% (vol/vol) OADC enrichment and 2µg/mL mycobactin J. A single colony from each isolate was inoculated into 9mL of 7H9 media, incubated at 37°C with constant agitation to prevent clumping of cells. All isolates were incubated for a 6 month period.

When the culture cell density was comparable to a McFarland standard 2, 1mL of culture was harvested by centrifugation (15,000 x g, 5 min). The cell pellet was washed once with spheroplasting buffer (20mM Citrate phosphate buffer pH 5.6 [0.2M citric acid, 0.5M sodium hydrogen phosphate], 50mM EDTA, 0.1% (wt/vol) Tween 80) and resuspended in 125µl. This suspension was warmed to 55°C and mixed with 125µl of 1.5% (wt/vol) low melting point agarose (Sigma-Aldrich Canada Ltd) and poured into plug molds (Bio-Rad Laboratories, Canada Ltd). Solidified plugs were incubated in 1mL lysis buffer (10mM Tris-HCL (pH 8), 1mM EDTA, 1mg/mL lysozyme) at 37°C for 24 hours. Lysis buffer was removed and replaced with 1mL ESP solution (0.5M EDTA (pH 8), 1% (wt/vol) lauryl sarcosine, 1mg/mL proteinase K) for incubation at 55°C for 7 days.

Plugs were washed 10 times in 1mL TE buffer (10mM Tris-HCL, 1mM EDTA (pH 8)). Three to five millimetre slices were cut from plugs and incubated in 50µl commercial (New England Biolabs, Ontario, Canada) restriction buffer mix (1× BstEII buffer, 0.1× BSA, 2U/µl BstEII) at 60°C for 16 hours. The remainder of plug were stored in TE

buffer at 4°C. After the 16 hour digestion, a further 2U of BstEII was added and incubation continued for 2 hours. The majority of restriction buffer mix was removed, without disturbing the agarose plug, and plugs were melted at 65°C for 10 minutes. Melted plugs were loaded into a 1% (wt/vol) agarose gel and electrophoresed in 1× TAE (242g Tris-HCL, 57.1g Glacial Acetic Acid, 100mL 0.5M EDTA) at 100 volts for 3 hours. Digestion patterns were detected by staining with ethidium bromide and visualizing with a UV transilluminator. A digoxigenin-labeled DNA molecular weight marker (Roche Diagnostics, Quebec, Canada, Cat# 1218603) and a positive control (IS900 PCR product) were also included in each gel.

The transfer of *M. paratuberculosis* DNA from agarose gels to a positively-charged nylon membrane was adapted from the protocol provided with the DIG DNA Labelling and Detection Kit (Roche Diagnostics, Quebec, Canada, Cat# 1093657). Agarose gels were prepared for transfer by washing 4× (15 minutes/wash) with each denaturing (1.5M sodium chloride, 0.5M sodium hydroxide) and neutralizing (1.5M sodium chloride, 1M Tris-HCL (pH 7.5)) solutions. DNA was transferred for 24 hours under standard capillary conditions (71) using 1.5M ammonium acetate. Following transfer, the nylon membrane was immediately baked at 80°C for 2 hours in a standard oven. After baking, membranes were hybridized at 55°C for 24 hours in 35mL of hybridization-solution (5×SSC [(0.6M sodium chloride, 0.06M sodium citrate) pH 7], 0.5% blocking agent (Roche, Cat# 1096176), 0.1% (wt/vol) sarcosyl, 0.02% (wt/vol) SDS) containing 200µl of DIG-labelled IS900 probe. DIG-labelled probe was generated by following the protocol provided with the DIG DNA Labelling and Detection Kit (Roche Diagnostics, Quebec, Canada, Cat# 1093657).

Membranes were washed 2×5 minutes with wash buffer 1 (2×SSC, 0.1% (wt/vol) SDS) and 2×15 minutes at 55°C with wash buffer 2 (0.1×SSC, 0.1% (wt/vol) SDS). A final wash of 5 minutes with maleic acid washing buffer (0.1M maleic acid, 0.15M sodium chloride, 0.3% Tween 20, pH 7.5) was performed. The membrane was placed in 35mL blocking solution (0.1M maleic acid, 0.15M sodium chloride, 1% blocking agent (Roche Cat#1096176) for 30 minutes. The blocking solution was decanted and replaced with 10mL of fresh blocking solution containing 5µl of anti-Digoxigenin-AP solution (Roche, Cat#1093274) and incubation continued for a further 30 minutes. The membrane was then washed 2×15 minutes with maleic acid washing buffer and then incubated in 10mL of detection buffer (0.1M tris, 0.1M sodium chloride, pH 9.5) containing 200µl of colour substrate solution (Roche, Cat#1681451). The membrane was incubated in colour substrate solution for up to 16 hours.

4.2.4. Pulse-field gel electrophoresis (PFGE)

M. paratuberculosis isolates (Table 4.1) were propagated and immobilized in low melting point agarose plugs as was described for RFLP analysis. Plugs were washed and prepared for restriction digest in a 50µl volume containing commercial (New England Biolabs, Ontario, Canada) restriction buffer mix (1× restriction buffer, 0.1× BSA, 2U/µl SnaBI) at 37°C for 16 hours. After the 16 hour digestion, a further 2U of SnaBI was added and incubation continued for 3 hours. The majority of restriction buffer mix was removed, without disturbing the agarose plug, and plug was melted at 65°C for 10 minutes. Melted plugs were loaded into a 1% (wt/vol) pulse-field certified agarose gel and electrophoresed in 0.5× TBE (44.5mM tris, 44.5mM boric acid, 1mM

EDTA). Electrophoresis was performed using a CHEF Mapper system (Bio-Rad, Hercules, CA). Electrophoresis parameters are shown in Table 4.2. PFGE patterns were detected by staining with ethidium bromide and visualizing with a UV transilluminator. A lambda midrange marker (New England Biolabs, Cat#N3552S) was also loaded with each gel.

Table 4.2 - Parameters for PFGE performed with *M. paratuberculosis* isolates digested with the restriction enzyme SnaBI.

Restriction enzyme	SnaBI
Initial switch time (s)	6.75
Final switch time (s)	26.29
Gradient (V/cm)	6
Angle	120
Temperature (celsius)	14
Time (hours)	40

4.2.5. Satellite typing

Micro and minisatellites were identified by running the *M. paratuberculosis* genome sequence (NC002944, GenBank) through the software Tandem Repeats Finder (16). A total of 6 microsatellites and 4 minisatellites were randomly chosen, out of a total of 36, for analysis (Table 4.3). Primers were designed (Primer Designer, Scientific & Educational Software, NC, USA) to amplify the chosen micro or minisatellite and as little of the flanking DNA as possible.

DNA from all 75 *M. paratuberculosis* isolates (Table 4.1) was prepared using a standard phenol extraction technique (71). Two microlitres of *M. paratuberculosis* DNA was added to a PCR master mix containing 1× *Taq* Buffer with $(\text{NH}_4)_2\text{SO}_4$, 2mM MgCl_2 , 0.25mM dNTP, 0.8µmol of each primer belonging to 1 primer set from Table 4.3, 0.025 U/µl recombinant *Taq* polymerase and sterile, filtered water up to 48µl. All reagents were supplied by Fermentas Life Sciences (Ontario, Canada). PCR cycling parameters were as follows: 94°C for 5 min; [94°C for 1 min, 55°C for 1 min and 72°C for 1 min] × 40 cycles, and a final extension at 72°C for 10 min. PCR amplicons were analysed by electrophoresis through a 15% acrylamide gel, staining with ethidium bromide and visualizing with a UV transilluminator. A positive control consisted of DNA extracted from an ATCC (#19698) isolate of *M. paratuberculosis* and a negative control consisted of water instead of DNA.

Table 4.3 - Micro and Minisatellites regions of the *M. paratuberculosis* genome examined in this study.

Satellite Sequence	Target Region or Gene	Satellite Size (bp)	Copy Number
CCG GCC CAA	85C Complex gene, GenBank #AF280068	9	5
CCC CGG CCC GCA ATT TTT TCA	gidB, rnpA, rpmH, dnaA, dnaN, recF, gyrB genes, GenBank #AF222789	21	2
TAC GGC CAA TAC GGG CAA	34KDa antigen gene, GenBank #AF411607	18	2
CAC	Multiple regions throughout <i>M. paratuberculosis</i> , genome	3	3 to 5
GCA CCT	Multiple regions throughout <i>M. paratuberculosis</i> , genome	6	2
CGG TCA GCC GGG	Multiple regions throughout <i>M. paratuberculosis</i> , genome	12	3 to 4
TCG GTT GAT GCG TGA CGT TGT T	ISMav2 Transposase gene, GenBank #AF286339	22	3
AT	Multiple regions throughout <i>M. paratuberculosis</i> , genome	2	2 to 14
TTA TTA ATA A	Multiple regions throughout <i>M. paratuberculosis</i> , genome	10	2 to 4
GTA	Multiple regions throughout <i>M. paratuberculosis</i> , genome	3	2 to 9

4.2.6. Single stranded conformation polymorphism analysis (SSCP)

Suspect polymorphic regions were identified by analysing other closely related *Mycobacterium* species (GenBank) and finding the corresponding region within the *M. paratuberculosis* genome. In total, 8 different genes or regions of the *M. paratuberculosis* genome were analysed with SSCP (Table 4.4). IS900-1 corresponds to the region between the TetR gene (putative transcription regulator) and the beginning of the insertion sequence, IS900, on locus 6. IS900-2 corresponds to the region between the Pks gene (putative polyketide synthase) and the end of IS900 on locus 6 (GenBank AJ250023). HSP70 refers to the 70KDa heat shock protein gene and HSP65 refers to the 65KDa heat shock protein gene. ITS1 and ITS2 correspond to the internal transcribed spacers between the 16S - 23s ribosomal RNA genes and the 23s – 5s ribosomal RNA genes, respectively. Ogt and RpsL are genes found to be variable in *Mycobacterium tuberculosis* isolates and correspond to DNA repair and Streptomycin resistance, respectively.

DNA was extracted from all *M. paratuberculosis* isolates (n=75) listed in Table 4.1, using a standard phenol extraction technique (71). Two microlitres of *M. paratuberculosis* DNA was added to a PCR master mix containing 1× *Taq* Buffer with $(\text{NH}_4)_2\text{SO}_4$, 2mM MgCl_2 , 0.25mM dNTP, 0.8µmol of each primer belonging to 1 primer set from Table 4.4, 0.025 U/µl recombinant *Taq* polymerase and sterile, filtered water up to 48µl. All reagents were supplied by Fermentas Life Sciences (Ontario, Canada). PCR cycling parameters were as follows: 94°C for 5 min; [94°C for 1 min, 55°C for 1

min and 72°C for 1 min] × 30 cycles, and a final extension at 72°C for 10 min. PCR amplicons were analysed by electrophoresis through a 2.5% agarose gel, staining with ethidium bromide and visualizing with a UV transilluminator. A positive control consisted of DNA extracted from an ATCC (#19698) isolate of *M. paratuberculosis* and a negative control consisted of water instead of DNA.

Table 4.4 - *M. paratuberculosis* regions/genes and corresponding primer sets examined with SSCP analysis.

Target Gene/Region	Primers		Product Size (bp)
IS900-1	IS900-1	CCG AAC ACC CTT CAA GAA AG	326
	IS900-3	CTT GGA GGG GAA GTG GTT AT	
IS900-2	IS900-2	GGC TTG ACA ACG TCA TTG AG	484
	IS900-4	GTG TTG AAC GCC TTG ATC AC	
HSP70	HSP70-F	CCA GGA GGA ATC ACT ATG GC	383
	HSP70-383-R	TTG AAG TAC GCC GGT ACG GT	
HSP65	HSP65-F	GGC GCC GAG CTG GTC AAG GAA GTC	352
	HSP65-R	CGA GCT GCA GGC CGA AGG TGT TGG	
ITS1	ITS1	GAT TGG GAC GAA GTC GTA AC	400
	ITS2	AGC CTC CCA CGT CCT TCA TC	
ITS2	ITS2-F	GCA CTA ACC GGC CGA AAA CT	204
	ITS2-R	AGG CTT AGC TTC CGG GTT CG	
Ogt	Ogt-F	CGC GAT CCG GTT CTG ACG AA	252
	Ogt-R	CGA TCT GCT CGG CGA TTT CG	
RpsL	RpsL-F	CAA GGG TCG TCG GGA CAA GA	339
	RpsL-R	CTC CTT CTT GGC GCC GTA AC	

A diagrammatic explanation of SSCP is demonstrated in Figure 4.1. Ten microliters of PCR product was mixed with 10µl loading dye, boiled for 10 minutes and immediately cooled on ice. Five microliters of cooled sample was loaded into 1× MDE gel and run for 1 hour at 120 volts. DNA fragments (Table 4.4) were analysed by staining with ethidium bromide and visualizing with a UV transilluminator.

4.3. Results

4.3.1. Polymerase chain reaction-restriction endonuclease assay (PCR-REA)

PCR-REA is capable of typing *M. paratuberculosis* isolates into cattle, sheep or bison-associated strains. Most (72/75) *M. paratuberculosis* isolates tested in this study were typed as cattle-associated strains. The exceptions were 1 isolate from a sheep in New Zealand and 2 isolates from 2 different bison in the United States (Table 4.5). The bison-associated strains (n=2) were both isolated from bison and this is consistent with previous findings (165). Likewise, the 1 isolate from a sheep was typed as a sheep-associated strain. In contrast, isolates which typed as cattle-associated were recovered from all 6 animal hosts, including sheep and bison. All *M. avium* isolates (n=5) could be distinguished from *M. paratuberculosis* isolates (n=75). The restriction fragment sizes are shown in Figure 4.2.

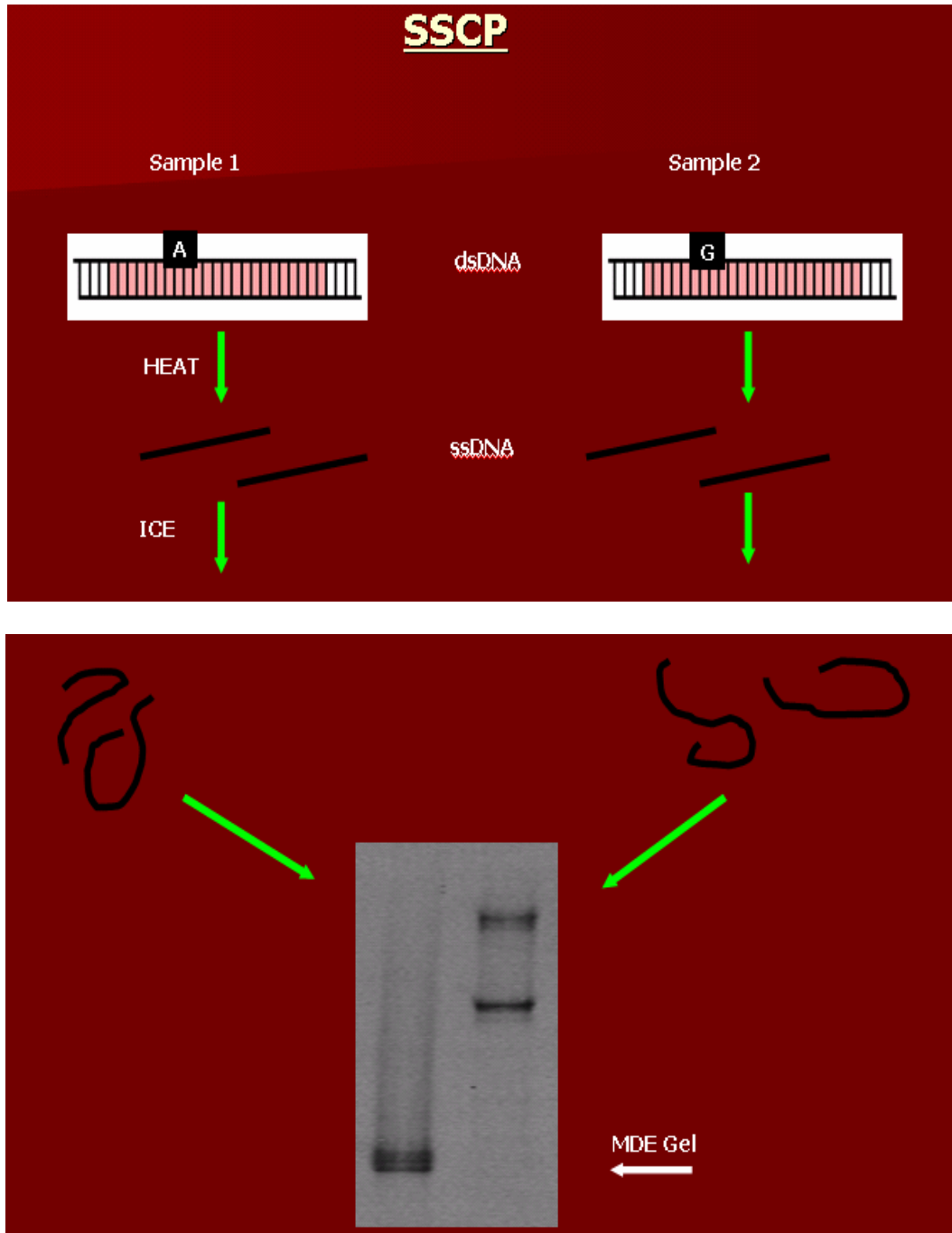


Figure 4.1 - Schematic representation of SSCP technique. Samples represent double stranded PCR fragments. PCR products were heated (95°C, 10 min), which results in single stranded PCR fragments, and rapidly cooled on ice, which causes the single strands to fold into stable conformations based on nucleotide sequence. Differences in nucleotide sequence were then detected as different banding patterns on acrylamide gels.

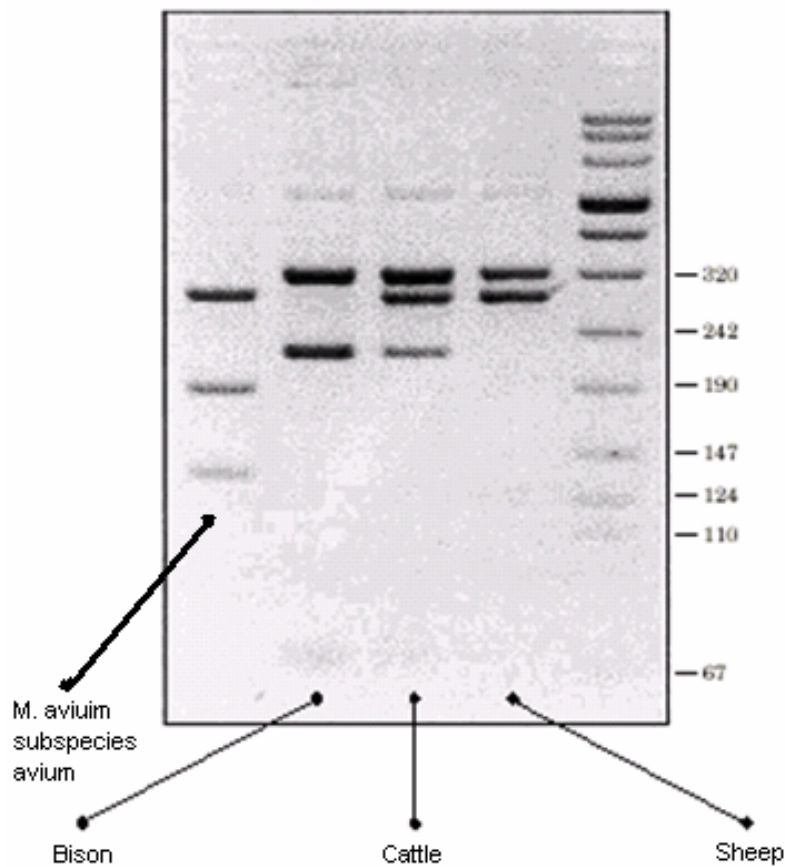


Figure 4.2 - PCR-REA of *M. paratuberculosis* isolated from bison, cattle and sheep. The different banding patterns are the result of a single nucleotide polymorphism in the IS1311 gene. Figure adapted from Whittington *et al* (165).

Table 4.5 - PCR-REA strain type and host distribution of *M. paratuberculosis* isolates (n=75).

PCR-REA Stain Type	Host Origin						Total
	Cattle	Sheep	Goat	Bison	Elk	Deer	
Bison-associated	0	0	0	2	0	0	2
Cattle-associated	12	1	6	6	10	37	72
Sheep-associated	0	1	0	0	0	0	1
Total	12	2	6	8	10	37	75

4.3.2. Restriction fragment length polymorphism analysis (RFLP)

Only 2 *M. paratuberculosis* isolates could be cultivated in Middlebrook 7H9 broth media. Therefore, only 2 isolates could be typed by RFLP analysis. Both isolates were typed as RFLP-BstEII C5 (Figure 4.3) and were isolated from cattle in Canada (Alberta and Ontario).

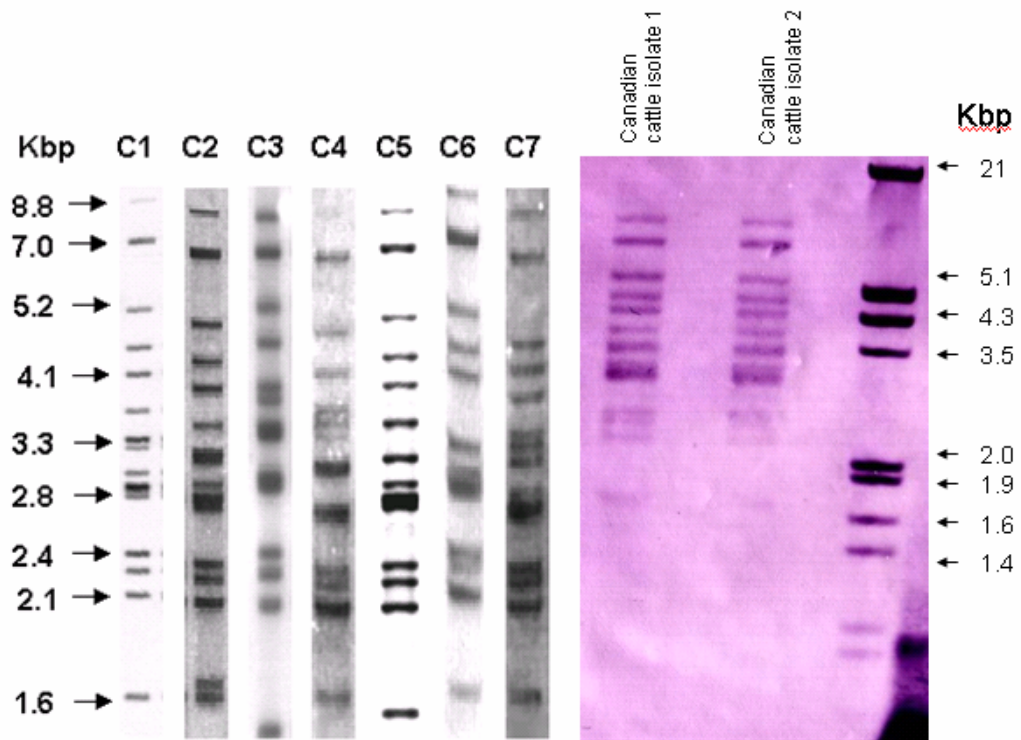


Figure 4.3 - BstEII RFLP digestion of *M. paratuberculosis* isolates (n=2) and subsequent southern blotting using IS900 as a probe. Diagram compares BstEII RFLP profiles C1 - C7 generated by Pavlik *et al* (109).

4.3.3. Pulse-field gel electrophoresis (PFGE)

PFGE analysis, performed on 8 isolates at the Moredun Research Institute (Penicuik, Midlothian, UK), resulted in 4 different PFGE types (1, 2, 3, 30). Types 1, 2 and 3 have been previously described (136), but type 30 is a new profile from 2 isolates from the United States. PFGE type 1 was found in cattle and goat isolates from Canada and Type 2 was found in a bison isolate and a deer isolate both from the United States. Type 3 was from a deer isolate in Scotland and Type 30 was found in a deer isolate and an elk isolate both originating from the United States.

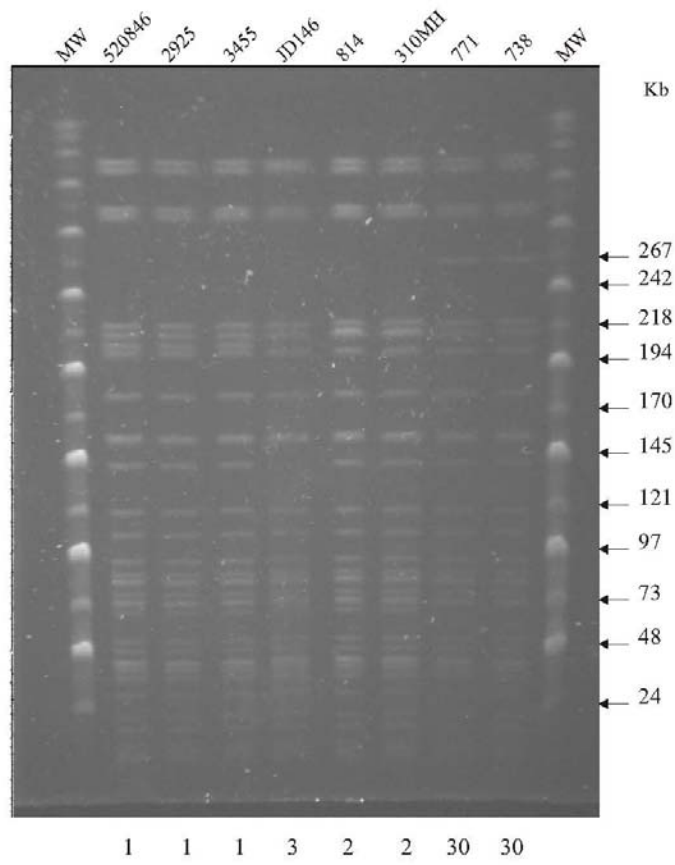


Figure 4.4 - Pulse field gel electrophoresis of *Sna*BII digested *M. paratuberculosis* isolates from Canada, Scotland, New Zealand and United States. Numbers on the bottom of the figure correspond to the different profiles.

4.3.4. Satellite Typing

After analyzing 10 satellite regions, 1 region (TCG GTT GAT GCG TGA CGT TGT T), a 22bp repeat, was variable among the *M. paratuberculosis* isolates tested. Isolates could be typed as A (29%), B (40%), C (16%) or D (15%) (Figure 4.4, Table 4.6). Type A was associated with a variety of animal hosts, as was type B. The majority of type C isolates (69%) were recovered from deer. Similarly, 67% of type D isolates originated from deer and elk. The lack of isolates in this study from sheep (n=2) limits any conclusions concerning the heterogeneity of isolates. Unfortunately, the discriminatory level of this region was very low therefore typing isolates using this region could not differentiate *M. paratuberculosis* isolates from *M. avium* isolates.

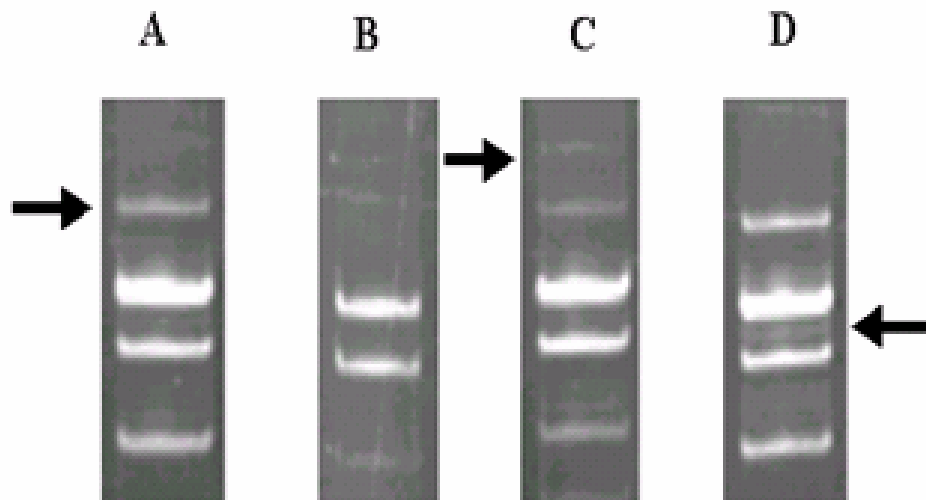


Figure 4.5 - Different satellite types arising from amplification of the TCG GTT GAT GCG TGA CGT TGT T microsatellite region and polyacrylamide gel electrophoresis. Arrows demonstrate the different banding patterns which distinguish each satellite type.

Table 4.6 - Satellite Type and host distribution of *M. paratuberculosis* isolates (n=75) and *M. avium* isolates (n=5).

Microsatellite Type	Host Origin							Total
	Cattle	Sheep	Goat	Bison	Elk	Deer	<i>M. avium</i>	
Type A	5	0	3	1	2	12	0	23
Type B	3	2	1	7	4	12	3	32
Type C	2	0	2	0	0	9	0	13
Type D	2	0	0	0	4	4	2	12
Total	12	2	6	8	10	37	5	80

4.3.5. Single stranded conformation polymorphism analysis (SSCP)

SSCP analysis was performed on 8 different regions or genes of the *M. paratuberculosis* genome (Table 4.7). SSCP analysis identified 2 regions (IS900-2 and HSP70) where sequence polymorphisms could be targeted to display differences among *M. paratuberculosis* isolates. SSCP analysis of the IS900-2 region demonstrated a polymorphism that existed in only 1 of the 75 isolates analysed in this study. This isolate was acquired from a sheep in New Zealand. SSCP analysis of the HSP70 gene revealed a unique polymorphism that was found in 4 *M. paratuberculosis* isolates from deer in Scotland. PCR amplification was not supported when *M. avium* isolates were tested.

Table 4.7 - Genes or regions examined by SSCP analysis and the resulting *M. paratuberculosis* isolate typing.

SSCP Region	Target Description	SSCP Type
IS900-1	Non-coding region between TetR gene and IS900	Type I (n=75)
IS900-2	Non-coding region between Pks gene and IS900	Type I (n=1) Type II (n=74)
HSP70	70KDa Heat Shock Protein gene	Type I (n=71) Type II (n=4)
HSP65	65KDa Heat Shock Protein gene	Type I (n=75)
ITS1	Internal Transcribed Spacer between 16s rRNA and 23s rRNA	Type I (n=75)
ITS2	Internal Transcribed Spacer between 23s rRNA and 5s rRNA	Type I (n=75)
Ogt	Gene associated with DNA repair in <i>M. tuberculosis</i>	Type I (n=75)
RpsL	Gene associated with Streptomycin resistance in <i>M. tuberculosis</i>	Type I (n=75)

4.4. Discussion

M. paratuberculosis has been described as extremely genetically homogenous (101), therefore finding genetic polymorphisms that can distinguish between epidemiologically significant isolates or facilitate a new diagnostic test are of great interest. Once a functional polymorphic region has been identified, an assay can be designed to exploit that polymorphism. The discriminatory power of this assay is of particular importance, especially when targeting organisms with limited genetic diversity, such as *M. paratuberculosis*.

Our current knowledge of strain typing has demonstrated the existence of cattle, sheep and bison-related strains of *M. paratuberculosis*. Among the available *M. paratuberculosis* typing assays, RFLP, PFGE and AFLP seem to be the most reproducible with the highest discriminatory value. Unfortunately, a comparison of discriminatory powers of AFLP versus PFGE versus RFLP, for *M. paratuberculosis* typing, is not available. In this study, RFLP was attempted over AFLP because it is the most widely used strain typing technique for *M. paratuberculosis* typing and was therefore needed as a comparison to any newly developed techniques.

Several studies have made reference to the difficulties associated with cultivating sheep isolates (116,163,164) and how this impacts genotyping studies which require the growth of *M. paratuberculosis* isolates. The majority of the isolates analysed in this study could not be cultivated in the liquid media required for both RFLP and PFGE, which limits the usefulness of these assays. On top of the growth requirements, both RFLP and PFGE assays require extensive preparation time and therefore results can take

many months, again limiting the usefulness of these assays. This study can not support or disprove the discriminatory value of the RFLP or PFGE assays because too few isolates were typed by these methods. What this study can advocate is new assays, such as satellite typing and SSCP, which do not rely on the growth of *M. paratuberculosis* for typing purposes.

An analysis of different mini and microsatellite regions resulted in 1 region where genetic differences could be detected among the *M. paratuberculosis* isolates analyzed in this study. The satellite typing assay described in this thesis closely parallels that of an RAPD assay. In silico analysis of the *M. paratuberculosis* genome provided evidence for a single locus, 22 base pair repeat region. But, it is obvious from Figure 4.5 that more than one region of the *M. paratuberculosis* genome was amplified by PCR. There are 2 explanations for these results. Only 1 genomic nucleotide sequence of *M. paratuberculosis* is available therefore in silico analysis is only accurate for this single sequenced isolate. Alternatively, the primers designed to amplify the 22 base pair repeat region could be homologous to other regions of the *M. paratuberculosis* genome. Regardless of the explanation, these results support the theory that there is more variation among *M. paratuberculosis* isolates than what is currently being detected.

SSCP analysis was demonstrated to be a rapid and reliable technique for the screening of *M. paratuberculosis* isolates for sequence polymorphisms which could be further targeted for diagnostic or typing purposes. The benefits of SSCP analysis are the lack of requirement of live organisms or large amounts of high quality genomic DNA. SSCP analysis could rapidly type all *M. paratuberculosis* isolates, regardless of host or sample

origin. Unfortunately, the regions analyzed by SSCP in this study do not provide the suggested high discriminatory power associated with RFLP, AFLP or PFGE.

Cattle-associated isolates are found in all ruminant hosts (Table 4.8), which either means that *M. paratuberculosis* isolates are not host specific or supports the theory that this subspecies is extremely homogenous. But, the molecular techniques applied in this study did find new genetic differences thought not to exist. The first important implication of this study is the suggestion that heterogeneity exists among *M. paratuberculosis* isolates. The second implication is that certain assays will aid in the selection of heterogeneity and some assays will not.

Table 4.8 demonstrates that satellite typing is the best assay for sub-dividing *M. paratuberculosis* isolates into smaller, manageable, but not meaningful groups as *M. paratuberculosis* can not be distinguished from *M. avium*. The next best assay appears to be PFGE, but as this study demonstrates, this is not a practical assay. Because PCR-REA is an accepted assay that can be applied independently of *M. paratuberculosis* cultivation, it is a useful assay to compare to different SSCP regions. The optimum way to find nucleotide polymorphisms is via genome sequencing. Due to the large time and cost associated with this type of research, SSCP is a practical alternative to sequencing. SSCP analysis is a more sensitive assay than PCR-REA and therefore the appropriate choice for further *M. paratuberculosis* typing studies.

Table 4.8 – Summary of *M. paratuberculosis* isolates (n=75) and typing techniques.

HOST	CATTLE	SHEEP	BISON	GOAT	ELK	DEER	<i>M. AVIUM</i>
	(n=12)	(n=2)	(n=8)	(n=6)	(n=10)	(n=37)	(n=5)
ACID FAST	Positive	Positive	Positive	Positive	Positive	Positive	Positive
IS900-PCR	Positive	Positive	Positive	Positive	Positive	Positive	No amplification
LOCUS 251 PCR	Positive	Positive	Positive	Positive	Positive	Positive	No amplification
PCR-REA	C	C or S (n=1)	C or B	C	C	C	Mbav pattern
	(n=12)	(n=1)	(n=6) (n=2)	(n=6)	(n=10)	(n=37)	(n=5)
RFLP	RFLP-BstEII C5 (n=2)	No growth	No growth	No growth	No growth	No growth	No hybridization
Satellite Type	Type A (n=5)	Type A (n=0)	Type A (n=1)	Type A (n=3)	Type A (n=2)	Type A (n=12)	Type A (n=0)
	Type B (n=3)	Type B (n=2)	Type B (n=7)	Type B (n=1)	Type B (n=4)	Type B (n=12)	Type B (n=3)
	Type C (n=2)	Type C (n=0)	Type C (n=0)	Type C (n=2)	Type C (n=0)	Type C (n=9)	Type C (n=0)
	Type D (n=2)	Type D (n=0)	Type D (n=0)	Type D (n=0)	Type D (n=4)	Type D (n=4)	Type D (n=2)
SSCP (IS900-2)	Type II (n=12)	Type I or Type II II (n=1) (n=1)	Type II (n=8)	Type II (n=6)	Type II (n=10)	Type II (n=37)	No amplification (n=5)
SSCP (HSP70)	Type I (n=12)	Type I (n=2)	Type I (n=8)	Type I (n=6)	Type I (n=10)	Type I or Type II (n=33) (n=4)	No amplification (n=5)
PFGE	Type 1 (n=2)	Not done	Type 2 (n=1)	Type 1 (n=1)	Type 30 (n=1)	Type 2/Type 3/Type 30 (n=1) (n=1) (n=1)	Not done

C = Cattle, B = Bison, S = Sheep

5. CONCLUSIONS AND FUTURE DIRECTIONS

There is evidence that different genotyping techniques, used on the same sample set, will yield patterns that are independent of one another, indicating the need to use more than one genotyping technique to distinguish between isolates (100). One purpose of this study was to compare and contrast different molecular typing assays on a wide range of *M. paratuberculosis* isolates from different geographic locations and host species and to find the appropriate molecular tool to rapidly and reproducibly distinguish between *M. paratuberculosis* isolates.

M. paratuberculosis bacteria grow very slowly and can be particularly challenging to cultivate in vitro. The lack of reliable cultivation techniques hinders investigations aimed at explaining why this group of organisms seems to be so homogenous and why current typing techniques can not distinguish between strains. Some of the variability that could exist within this subspecies could be related to growth requirements. This study supports the concept of development of genotyping techniques not requiring growth of the micro-organism or large quantities of genomic DNA. A combination of rapid, reliable assays will greatly assist a large scale, epidemiological study involving *M. paratuberculosis* isolates from a wide range of hosts and geographic locations.

This study has also demonstrated the benefits associated with screening fecal samples for the presence of *M. paratuberculosis* DNA before committing to the time and expense involved with microbial cultivation. The use of this sensitive screening technique combined with the PCR-REA (160) will confirm the presence of *M. paratuberculosis* DNA in any particular sample or host species. SSCP analysis can then be applied to look for genetic differences among *M. paratuberculosis* isolates.

This study clearly demonstrates the existence of *M. paratuberculosis* DNA in the fecal samples obtained from the bison in the Northwest Territories. The finding of *M. paratuberculosis* DNA that differs from what has already been described is exciting, but until an isolate can be grown in culture and further characterized, the implications of this discovery are still unknown.

The objectives of this study were to collect *M. paratuberculosis* isolates from a broad range of ruminant hosts in an attempt to understand the molecular diversity occurring among different isolates and to identify *M. paratuberculosis*-specific molecular markers which could distinguish isolates and therefore aid in disease detection, prevention and control. Overall, this study has demonstrated that there are techniques available for the identification of *M. paratuberculosis* DNA in a variety of sample types. What is still needed is a discriminative typing assay that can use the low levels of DNA isolated from different sample types. When this study was initiated, the complete *M. paratuberculosis* genome sequence was unavailable. Now that the sequence of one isolate is known, scanning for variable regions of the genome will occur at an exponential rate. Finding a polymorphic region or regions of the microbial genome which can be exploited for use

independently of microbial growth is the goal of any researcher studying molecular epidemiology. This study has demonstrated how SSCP analysis can be used as a powerful tool for genome screening to find that perfect polymorphic region.

6. References

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