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Mycobacterium avium subspecies *paratuberculosis* in Bison (*Bison bison*) from Northern Canada

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ABSTRACT: Nested polymerase chain reaction (PCR) using the *Mycobacterium avium* subspecies *paratuberculosis* (*Map*)–specific region, locus 251, was used as a screening tool for the detection of *Map* DNA in fecal samples from northern Canadian bison herds. Further characterization of positive samples (26/835) was performed because *Map* DNA was found without signs of disease. Strain typing, using PCR-Restriction endonucleas assay (REA), was limited to two samples but revealed that the samples corresponded to a cattle-related strain and a sheep-related strain. Sequencing of part of the IS1311 region from the two samples revealed a unique three base-pair region, which is only found within the northern Canadian bison isolates.

Key words: Bison, *Mycobacterium paratuberculosis*, PCR, PCR-REA.

Bison (*Bison bison 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 in North America was well over 100,000, but in the 1900s, due to unregulated hunting, wood bison numbers decreased to just 250 animals (Government of the Northwest Territories [NWT], 2004). From 1923 to 1928, the Canadian government moved 6,600 plains bison (*Bison bison bison*) from Alberta to WBNP to increase the general bison population. Unfortunately, the translocation of plains bison was associated with the introduction of bovine brucellosis (*Brucella abortus*) and tuberculosis (*Mycobacterium bovis*) to the area. Creating brucella- and tuberculosis-free bison herds is now a high priority for bison conservation in Canada. Monitoring the status of other possible pathogenic infections, such as those from *Mycobacterium avium* subsp. *paratuberculosis* (*Map*), has been included in the preservation plan. Infection with this organism has been previously demonstrated in farmed bison (Buergelt and Ginn, 2000), but never in the free-ranging bison from the WBNP area.

There is an extended preclinical phase of *Map* infection and intermittent fecal shedding of *Map* organisms from infected animals. A noninvasive, economical assay is needed to test asymptomatic animals for evidence of infection. Blood tests are available, but aside from the difficulty in obtaining samples from free-ranging bison, these assays suffer from low sensitivity and specificity. An easier way to sample a wild population is to collect freshly deposited fecal samples. In the recent past, fecal samples have often been tested immediately after collection by culture, despite the high costs and long incubation times. However, using polymerase chain reaction (PCR) technology, it is more economical to look for the presence of *Map* DNA in fecal samples and subsequently culture only the positive samples. Recently, methods for the purification of DNA directly from fecal samples have been described (Rudi et al., 2004). These procedures allow for the rapid purification of DNA directly from fecal matter, which permits further characterization or strain typing without the time and cost associated with culture. This communication describes the use of nested PCR for detection and strain typing of *Map* DNA directly from bison fecal samples to determine if *Map* is present in

northern Canadian bison herds and reports the finding of *Map* DNA in wild, northern Canadian bison.

Biologists from the Northwest Territory (NWT) Department of Natural Resources opportunistically collected 835 fecal samples from six bison herds at various locations across northern British Columbia, and the WBNP (59°21′N, 112°17′W), Nahanni Butte (61°05′N, 123°23′W), and Fort Liard (60°13′N, 123°22′W) areas in the NWT. There is no domestic cattle or sheep production in these areas.

The method for direct isolation of bacteria from bison feces was described by Chui et al. (2004). A positive extraction control was performed by spiking 10 µl of a McFarland standard 3 suspension of *Map* (ATCC #19698) (Becton, Dickenson and Co., Sparks, Maryland, USA) into a 4 g sample of known *Map*-negative feces. This positive control was carried through all assays described in this communication.

A volume (250 µl) of fecal wash was extracted using the MagaZorb® DNA Mini-Prep Kit (Cortex Biochem, San Leandro, California, USA). Nested PCR for the detection of *Map* DNA was performed using gene 254 of the *Map*-specific locus 251 (Bannantine et al., 2002), as the target sequence. The primers used in the primary reaction have previously been established (Bannantine et al., 2002), 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 secondary primers were designed by analyzing *Map* DNA sequences from Genbank using Lasergene software (DNASTAR Inc., Madison, Wisconsin, USA). The secondary primers were checked for specificity in silico through Genbank and in vitro by testing other bacterial species similar to *Map* (Table 1). The PCR conditions are shown in Table 2. A subset ($n=8$) of the PCR positive fecal samples from different geographic areas was sent to the Animal Health Monitoring

TABLE 1. Results of polymerase chain reaction (PCR) testing of *Mycobacterium* species and other related microorganisms showing the specificity of PCR (locus 251 and IS1311) and strain-typing assay (restriction endonuclease [REA]) used to detect the presence and strain of *Map* in bison fecal samples.

Organism	Locus 251 PCR (base pair)	IS1311 PCR (base pair)	Strain-typing PCR-REA (base pair)
Brucella suis	Negative	Negative	Negative
Corynebacterium sp.	Negative	Negative	Negative
Moraxella bovis	Negative	Negative	Negative
Mycobacterium bovis	Negative	Negative	Negative
Mycobacterium fortuitum	Negative	Negative	Negative
Mycobacterium gordonae	Negative	Negative	Negative
Mycobacterium intracellulare	Negative	Negative	Negative
Mycobacterium kansasii	Negative	Negative	Negative
Mycobacterium microti	Negative	Negative	Negative
Mycobacterium nonchromogenicum	Negative	Negative	Negative
Mycobacterium tuberculosis	Negative	Negative	Negative
Mycobacterium ulcerans	Negative	Negative	Negative
Mycoplasma bovis	Negative	Negative	Negative
Rhodococcus sp.	Negative	Negative	Negative
Mycobacterium avium subsp. *avium*	Negative	503	134, 146, 224
Mycobacterium avium subsp. *silvaticum*	Negative	Negative	Negative
M. avium subsp. *paratuberculosis* (sheep)	134	503	226, 277
M. avium subsp. *paratuberculosis* (cattle)	134	503	67, 159, 226, 277
M. avium subsp. *paratuberculosis* (bison)	134	503	67, 159, 277

TABLE 2. Polymerase chain reaction (PCR) conditions for *Mycobacterium avium* subsp. *paratuberculosis* detection from fecal samples and strain typing of IS1311.

	Map detection: locus 251, Gene 254		Strain typing: PCR-REA of IS1311	
	Primary reaction	Secondary reaction	Primary reaction	Secondary reaction
Size (base pairs)	293	134	608	503
DNA (μl)	2	2	2	2
10× *Taq* Buffer with $(NH_4)_2SO_4$	1×	1×	1×	1×
$MgCl_2$ (mM)	3	2	2	2
dNTP (mM)	0.25	0.25	0.25	0.25
Primers (pmol)	1.6	1.6	0.8	0.8
Recombinant *Taq* polymerase (U/μl)	0.025	0.025	0.025	0.025
Step 1, 94 C	5 min	5 min	3 min	3 min
Step 2, 94 C	45 sec	45 sec	30 sec	30 sec
Step 3	55 C for 1 min	55 C for 1 min	62 C for 15 sec	62 C for 15 sec
Step 4, 72 C	2 min	2 min	1 min	1 min
Step 5, 72 C	10 min	10 min	5 min	5 min
Cycles (steps 2–4)	40	30	40	40

Lab (Abbotsford, British Columbia, Canada) for confirmation with this laboratory's PCR assay and BACTEC culture.

Samples positive for gene 254 were strain typed based on a modified, previously described protocol (Whittington et al., 2001). The primers used in the primary reaction have previously been established to amplify a region of the IS1311 insertion sequence (Marsh et al., 1999), 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 PCR conditions are shown in Table 2. PCR-restriction endonuclease (PCR-REA) digest of IS1311 was carried out as previously described (Whittington et al., 2001), with the exception that the digest was performed on the secondary PCR product, and therefore the digest sizes are different than previously described. The expected, modified restriction fragment sizes are shown in Table 1.

The amplified region of the IS1311 element was also sequenced (Plant Biotechnology Institute, Saskatoon, Saskatchewan, Canada) using primers M42 and M545. *Mycobacterium paratuberculosis* DNA from five amplified products from bison, cattle, and sheep strains, and two different northern bison fecal samples were sequenced in this manner. Sequences were aligned manually with previously established *M. paratuberculosis* IS1311 sequences (Genbank AJ 223975 and AJ223974) using MegAlign software (DNASTAR, Inc).

Twenty-six of 835 fecal samples were positive for the presence of the *Map*-specific gene 254 from locus 251, but no positive results were obtained when other *Mycobacterium* or other bacterial species were similarly tested (Table 1). *Map* DNA positive fecal samples were collected from the Sweet Grass (58°83′ N, 111°96′W) ($n=2$), Pine Lake (59°67′N, 112°19′W) ($n=2$), Hook Lake (60°72′N, 112°76′W) ($n=1$), and Grand Detour (60°36′N, 112°80′W) ($n=21$) herds in the NWT. Samples from Nahanni (61°05′N, 123° 23′W) and Salt Plains (59°96′N, 112° 34′W) herds were negative. Of the eight samples sent to the Animal Health Monitoring Laboratory, three were positive for *Map* DNA by this laboratory's diagnostic PCR assay, but no *Map* colonies were isolated using BACTEC culture. Therefore, further molecular analysis was performed at the Western College of Veterinary Medicine (Saskatoon, Saskatchewan,

Canada) to characterize *Map* directly from bison fecal samples.

Of the 26 fecal samples positive for the presence of *Map* DNA, two yielded sufficient *Map* 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 (Table 1). The amplified IS1311 region from the Sweet Grass isolate and Grand Detour isolate was sequenced and revealed a unique 3 base-pair (bp) region (CA-T, located at position 32 of IS1311) not found in other previously sequenced isolates in Genbank. Furthermore, a previously identified *Map* bison isolate from the United States did not demonstrate the unique nucleotide sequence found in samples from the northern Canadian herds.

This report demonstrates the existence of *Map* DNA in wild, northern Canadian bison. Isolation of bacteria from fecal samples and the DNA extraction protocol were chosen after comparison to other commonly used extraction methods in which amplification was not observed. The presence of *Map* DNA was detected with the use of a nested *Map*-specific PCR assay and a nested *Map* strain-typing protocol. It was important to modify the PCR assay and the PCR-REA from previous publications to improve the detection limit from less than satisfactory sample specimens.

The unsuccessful cultivation of *Map* organisms from the fecal samples may have resulted from the use of unsuitable laboratory growth conditions for these particular isolates. Previous evidence of toxicity associated with growth elements (sodium pyruvate) in *Map* culture media has been described (Juste et al., 1991). PCR may be more sensitive than culture for the detection of mycobacteria (Saboor et al., 1992); therefore, low levels of viable *Map* shed in the feces may be undetected by culture. Reduced viability of organisms at culture due to frozen storage or repeated freeze-thawing of samples is also possible, and therefore the percentage of northern Canadian bison positive for *Map* DNA could potentially be higher than fecal culture would indicate.

The practical significance of finding *Map* DNA in northern Canadian bison herds is uncertain because the bison in and around WBNP have never and do not now manifest the clinical signs of *Map* infection (Johne's disease). It is possible but unlikely that wild bison can become infected with *Map* and eventually clear the infection, or that northern Canadian bison are in fact dying of Johne's disease but are not discovered, perhaps due to the remoteness of the herds and the efficient work of scavenging wildlife. Alternatively, the apparent lack of disease in northern Canadian bison could be due to the recovery of DNA from a less virulent strain of *Map*. Our study suggests that the strains infecting northern Canadian bison are distinct from those previously described. Perhaps these strains are not capable of creating clinical disease. Although the sequences obtained only differ by three nucleotides in a region of the gene, which appears to be variable, this is significant considering that 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.

The samples 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.

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