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FIRST ISOLATION OF *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS* FROM WILD GUANACOS (*LAMA GUANICOE*) ON TIERRA DEL FUEGO ISLAND

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ABSTRACT: The aim of this study was to search for *Mycobacterium avium* subsp. *paratuberculosis* (*Map*) infection in a free-ranging wild animal species in a region where Johnes's disease has yet to be reported and to classify *Map* isolates using a genomic typing method. Fecal samples were obtained from 501 wild guanacos (*Lama guanicoe*) from Tierra del Fuego Island, Chile, in August 2006. Samples were cultured using Herrold's egg yolk medium with and without mycobactin J. After 9 mo of incubation, suspected *Map* colonies showing mycobactin dependence were confirmed by real-time polymerase chain reaction (PCR) based on IS*900* and F57. Isolates were further tested using IS*1311* PCR with restriction endonuclease analysis in order to type the guanaco *Map* strains. Twenty-one of 501 (4.2%) animals were fecal culture–positive for *Map*; identity was confirmed by real-time PCR and isolates were classified as cattle-type. Most culture-positive animals were located in four contiguous geographic areas, and the infection was most commonly found among adult animals. Prevalence was higher in females (5.9%) than males (3.1%) but the difference was not statistically significant. This represents the first isolation of *Map* from a free-ranging wildlife species in Chile. It expands the geographic range of paratuberculosis and the diversity of wildlife species that can become infected with *Map*.

Key words: Guanaco, IS*1311*, Johne's disease, *Lama guanicoe*, molecular typing, *Mycobacterium avium* subsp. *paratuberculosis*, paratuberculosis, wildlife.

INTRODUCTION

Mycobacterium avium subsp. *paratuberculosis* (*Map*) is the causative agent of Johne's disease (paratuberculosis), an infectious bacterial disease characterized by granulomatous enteritis, diarrhea, loss of body weight, and death. The disease is present worldwide and is responsible for important economic losses to livestock producers (Kennedy and Benedictus, 2001). During the last decade, an association between *Map* and Crohn's disease in humans also has been suggested (Grant, 2005; Feller, 2007). The primary hosts are domestic ruminants such as cattle, sheep, and goats, but Johne's also has been reported in wild ruminants such as bison (*Bison bison*; Buergelt and Ginn, 2000), mountain goats (*Oreamnos americnaus*; Williams et al., 1983a, b), elk (*Cervus elaphus*; Manning et al., 2003), white-tailed deer (*Odocoileus virginianus*; de Lisle et al., 1993), red deer (*Cervus elaphus*; Sharp et al., 1996), exotic deer such as axis deer (*Axis axis*; Riemann et al., 1979) and fallow deer (*Dama dama*; Riemann et al., 1979), Saiga antelope (*Saiga tatarica*; Dukes et al., 1992), and bighorn sheep (*Ovis canadensis*; Williams et al., 1983b). The infection has also been reported in some nonruminant wildlife hosts such as European rabbit (*Oryctolagus cuniculus*; Greig et al., 1999), brown hare (*Lepus europaeus*; Machackova et al., 2004), brown rat (*Rattus norvegicus*; Beard et al., 2001), and long-tailed field mouse (*Apodemus sylvaticus*; Beard et al., 2001), as well as in carnivores such as red fox (*Vulpes vulpes*), stoat (*Mustela ermine*), and weasel (*Mustela nivalis*; Beard et al., 2001). In addition, *Map* has been isolated from primates, including mandrills (*Mandrillus sphinx*; Zwick et al., 2002) and rhesus monkeys (*Macaca mulatta*; McClure et al., 1987), as well as from omnivores such as the badger (*Meles meles*; Beard et al., 2001). The broad range of hosts susceptible to *Map* infection implies both a possible wildlife reservoir and interspecies transmission.

The guanaco (*Lama guanicoe*) is the largest South American camelid, naturally distributed between 8°S latitude in Peru and 55°S on Tierra del Fuego Island (Patagonian Region) at altitudes from the sea level to 4,600 m. The guanaco population in Chile is about 86,000 and most are concentrated in the Patagonian Region. It is estimated that approximately 45,000 guanacos live in the southern part of Tierra del Fuego Island. The guanaco is the only wild ungulate species widely distributed across the Patagonian steppe and shares grazing land with approximately one million sheep and about 20,000 cattle (Chile, 2007). It is considered a broad-range herbivore, eating grass on pastures or bush according to seasonal food availability. Guanaco prefer open habitats occupying steppe and prairies, although they also make use of forest habitat. Its behavior can be described as seasonally territorial but during harsh and changing environmental conditions social group composition and size may change (Puig and Videla, 1995). In wild guanaco populations three different basic structures are recognized: family groups, groups of young males, and solitary individuals. There is no published information on *Map* in this species.

In order to control guanaco overpopulation on the Chilean island of Tierra del Fuego, and in order to protect the land from the deleterious effect of these animals on the native forest, the Regional Agriculture and Livestock Department of the Ministry of Agriculture of Chile authorized a supervised yearly hunting quota of 2,000 animals, respecting preservation of the species. The aims of this study were to test these animals for *Map* and to characterize isolates by IS*1311* polymerase chain reaction (PCR) with restriction endonuclease analysis (REA).

MATERIALS AND METHODS

Fecal and serum samples were collected simultaneously from 501 wild guanacos populating different areas of the Timmaukel County, Tierra del Fuego Island, Chile, in August 2006. Sampling was synchronized with a hunting activity carried out by a private company under the supervision of the Ministry of Agriculture. Hunting was restricted to a small group of adult animals (2,000) in order to control population expansion. At the time of sampling, body condition, sex, and age of each animal, determined by dental chronometry (Skewes et al., 2000), were recorded.

Carcasses were processed in a portable slaughterhouse. Fecal samples (approximately 5 g) were aseptically collected directly from the rectum of each animal using individual gloves and sterile plastic screw-cap bottles. Fecal samples were kept at room temperature prior to submission to the Microbiology Department, Faculty of Sciences, Universidad Austral de Chile, Valdivia, Chile, and were cultured within 72 hr after collection.

For culture, fecal samples were decontaminated with hexadecylpyridinium chloride (Sigma/Aldrich, St. Louis, Missouri, USA) and an antibiotic mixture (amphotericin B, vancomycin, and nalidixic acid; Sigma/Aldrich) according to the procedure described by Shin et al. (1990). A 0.2-ml aliquot of each final fecal suspension was used to inoculate three tubes of Herrold's egg yolk medium (HEYM, made in the laboratory) with mycobactin J (Allied Monitor, Inc., Fayette, Missouri, USA) and one HEYM tube without mycobactin. The inoculated HEYM tubes were incubated for 9 mo at 37 C and examined weekly. Colonies resembling *Map* and showing mycobactin dependence and a Ziehl Neelsen positive reaction were presumptively identified as *Map* and were confirmed by IS*900* and F57 PCR.

For DNA extraction, single presumptive *Map* colonies grown on HEYM with mycobactin were suspended in a buffer solution and were boiled for 10 min to 15 min. DNA of the three first isolates were freeze-dried and submitted to the Bacteriology Department at the National Veterinary Institute, Uppsala, Sweden, where they were tested by real-time PCR based on the IS*900* and F57 genes; the PCR protocols included an internal control to detect PCR inhibition (Herthnek et al., 2006). With the exception of excluding glycerol from the PCR mix, PCR protocols were run as described by Herthnek et al. (2006). The remaining isolates were confirmed by PCR with only IS*900* marker at the Molecular Biology Laboratory, Microbiology Department, Universidad Austral del Chile, according to the protocol described by Green et al. (1989).

To distinguish subtypes of *Map*, the guanaco isolates were assayed by IS*1311* PCR-REA

both at the Bacteriology Department at the National Veterinary Institute, Uppsala, Sweden (the first three strains), and at the Microbiology Department, Universidad Austral de Chile (the remaining strains) according to the protocol described by Marsh et al. (1999). The previously established primers M56 (5′-GCGTGAGGCTCTGTGGTGAA-3′) and M94 (5′-CAGCGATCGTCGACAGTGTG-3′; Whittington et al., 1998; Marsh et al., 1999) were used to amplify a region of the IS*1311* insertion sequence. Briefly, a reaction volume of 50 µl containing 5 µl of the DNA sample, 4 µl of each primer (10 pmol/µl), 22.6 µl of water, 4 µl dNTPs (10 mM), 5 µl MgCl2 (25 mM), 5 µl PCR buffer II (10×), and 0.4 µl *Taq* Gold polymerase (5 U/µl) was used. The PCR procedure was performed under the following conditions: one cycle of denaturation at 94 C for 3 min followed by 37 cycles of denaturation at 94 C for 30 sec, annealing at 62 C for 15 sec, and extension at 72 C for 1 min. Polymerase chain reaction results were assessed by electrophoresis in 2% agarose gels stained with ethidium bromide. The IS*1311* PCR products were gel-purified using the Qiaquick Gel extraction kit (Qiagen GMBH, Hilden, Germany). Restriction endonuclease analysis was prepared by adding 4–12 µl of purified PCR product, 2 U of *Hinf*I restriction endonuclease (Promega Corp., Madison, Wisconsin, USA), 1.6 µl of buffer (supplied with restriction endonuclease), and made up to 16 µl with sterile purified water. Restriction digests were incubated for 2 hr at 37 C and were assessed by electrophoresis in 4% agarose gels stained with ethidium bromide.

Chi-square analysis to determine association of *Map* infection with animal location, age, and sex was performed using GraphPad Prism® version 5.00 for Windows (GraphPad Sotware, San Diego, California, USA).

RESULTS

Gross examination indicated no signs of Johne's disease or any other pathologic condition in any of the sampled guanacos; *Map* was isolated from fecal samples of 21 (4.2%) guanacos. The rate of culture contamination was low (<5%). In the 21 culture-positive samples, of the three HEYM tubes inoculated, no more than two showed colonies and each had <20 colonies per tube indicating low-level shedding of *Maps*. Colonies grown on HEYM with mycobactin were confirmed by real-time PCR for both the IS*900* (all 21 isolates) and F57 (only the first three isolates). All 21 *Map* isolates were positive for IS1311 and corresponded to the cattle-type (C-type) strain. Most infected animals were concentrated in four adjacent geographic areas that were characterized as forested (Table 1; $P=0.05$). Although not statistically significant, *Map* prevalence was slightly higher in females (5.9% culture-positive) than in males (3.1% culture-positive) and in adult (>4 yr old) than in younger animals (Tables 2 and 3).

TABLE 1. Fecal culture test results of 501 guanacos sampled for paratuberculosis from different geographic areas in Tierra del Fuego, Chile.

Geographic areas	Positive results	Negative results	Total
Forest	18	313	331
Nonforest	3	167	170
Total	21	480	501

TABLE 2. Distribution of fecal culture test results by age of 501 guanacos sampled for paratuberculosis in Tierra del Fuego, Chile.

Age	Positive results	Negative results	Total
<1 yr	2	23	25
2–3 yr	8	132	140
>4 yr	11	325	336
Total	21	480	501

TABLE 3. Distribution of fecal culture test results by sex of 501 guanacos sampled for paratuberculosis in Tierra del Fuego, Chile.

Sex	Positive results	Negative results	Total
Male	8	258	266
Female	13	222	235
Total	21	480	501

DISCUSSION

The hunting activity represented a unique opportunity to check the *Map* in this wild animal population in a region where Johne's disease has not previously

been reported. This study confirmed the presence of *Map* in 4.2% of guanaco and represents the first isolation of *Map* from a free-ranging wild animal in the Chilean Patagonia. The combined use of the IS*900* and F57 genetic markers for *Map* assured no false-positive results (Englund et al., 2002; Tasara and Stephan, 2005; Herthnek and Bölske, 2006).

Very little information has been published about Johne's disease in the guanaco species. Only Karesh et al. (1998), evaluating the health status of free-ranging guanacos in the Argentinean Patagonia, investigated the serum antibody response to *Map* by enzyme-linked immunosorbent assay (ELISA) and found no ELISA-positive animals. Our findings add a new wild-ranging species, an ungulate classified as a pseudoruminant, to the list of wild and domestic animals that can be infected with *Map*. In addition, the detection of *Map* infection in a free-ranging wild animal species is an important finding in a region where livestock production is the basis of the regional economy and where Johne's disease has not previously been reported in either cattle or in sheep.

Most (85%) of the fecal culture-positive guanacos were harvested from one of four contiguous geographic areas. These areas comprise hundreds of hectares of "lenga" trees (*Nothofagus pumilio*) managed for forestry purposes. Replanting of young trees creates a highly palatable source of fodder for guanacos and hampers reforestation efforts (the primary reason for the controlled hunting activity). This situation also artificially increases guanaco concentrations, in turn potentially increasing the odds of *Map* transmission among guanacos. Additionally, these areas contain a large sheep farm which annually gathers thousands of animals in its facilities for management purposes, such as shearing, potentially increasing the risk of disease transmission from sheep to guanaco.

Two *Map* strain types have been described based on restriction fragment length polymorphism analysis (RFLP): sheep (S) and cattle (C) strains (Collins et al., 1990). Because RFLP requires large amounts of DNA, it is complex, time-consuming, and expensive. Marsh et al. (1999) developed an easier, faster, and less costly method, a PCR-REA based on polymorphisms in IS*1311*, an insertion sequence present both in *Map* and in *Mycobacterium avium* subsp. *avium*. This alternative method makes it possible to differentiate *Map* from *M. avium* subsp. *avium*, and detection of a stable variation at base pair 223 in the insertion element IS*1311* also allows recognition of S and C strains.

Because specific differences in culture characteristics have been reported between different genotypes of *Map*, the rate of *Map* isolation obtained (4.2%) could have been higher if two or more culture media had been used in parallel, such as HEYM and Lowenstein-Jensen. According to Juste et al. (1991), Lowenstein-Jensen medium improves the primary isolation rate of S-type strains by about 90%, but could decrease the isolation of C-type strains by approximately 37% in relation to HEYM. Whittington et al. (2001), in their typing of IS*1311* polymorphisms, confirmed that infected bison carried a strain of *Map* distinct from that occurring in cattle and other domesticated livestock, called type B strain. These authors also reported that *Map* B strains appear to have different nutritional preferences regarding carbon source for growth, and that HEYM without pyruvate was more suitable for isolation of the type B strain.

Older animals were more likely to be *Map* fecal culture-positive; 91% of the 21 fecal culture-positive guanacos were >1 yr old (Table 2). It is well known that Johne's disease is a chronic disease and that fecal shedding of *Map* by infected animals increases with age as the disease progresses (Whitlock and Buerguelt, 1996). Detection of *Map* infection in this type of wild animal population may

improve if older animals are targeted during sampling.

A higher prevalence of *Map* infection was observed in female rather than male guanacos (Table 3). This may be related to increased stress in females due to pregnancy, lactation, and nursing, which could favor the progress of the infection.

Interpretation of *Map* isolation results from guanaco, or any other wild animal species, must be made with caution. There is a tendency to label such results as evidence of a wild animal "reservoir" of infection, implying that infected wild animals are sources of infection for domestic animal populations. It is equally plausible that the reverse is true; domestic animals are the reservoir that threatens the health of wild animal population (Ris et al., 1987, 1988; Mokresh and Butler, 1990). It is also conceivable that the *Map* recovered from guanaco feces are merely environmental contaminants originating from domestic animals and merely passively passing through the guanaco without actually causing infection with attendant pathology and immune response.

Isolation of C-type *Map* suggests cattle as the primary source of the organisms. However, C-type strains are also found in sheep (Kennedy and Benedictus, 2001; Muskens et al., 2001). Moreover, the use of HEYM potentially biased isolation efforts in favor of C-type *M. paratuberculosis* strains and against S-type strains. Thus, the local sheep flocks can not be eliminated as possible sources of the *Map* found in guanaco feces.

Future Johne's disease studies in this region, either of domestic or wild animals, must include consideration of the use of multiple culture media in order to improve the isolation rate of all types of *Map*. This is also crucial for molecular epidemiologic studies aimed at identifying infection sources, because choice of culture media may affect the genotypes isolated (Cernicchiaro et al., 2008). Additionally, future studies should perform full genotyping of all isolates for more effective interpretation of the molecular epidemiology of Johne's disease (Motiwala et al., 2004). Finally, tissue culture and histopathology should complement the abovementioned bacteriology in order to improve the understanding of *Map* infection in wild guanaco.

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