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European Hares in Chile: A Different Lagomorph Reservoir for *Mycobacterium avium* subsp. *paratuberculosis*?

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ABSTRACT: Ruminants are the principal host for infection by *Mycobacterium avium* subsp. *paratuberculosis* (*Map*), the cause of Johne's disease. Based on studies of a *Map*-infected population of European rabbits (*Oryctolagus cuniculus*) in Scotland, lagomorphs as a broad taxonomic order were proposed as potential nonruminant reservoirs for *Map*. To determine whether a different lagomorph species may serve as a wildlife reservoir, we investigated *Map* infection in European hares (*Lepus europaeus*) sharing habitat with known *Map*-infected dairy cattle in southern Chile. Fecal, mesenteric lymph node, and ileal samples were aseptically collected from 385 wild hares for liquid culture and real-time polymerase chain reaction identification of acid-fast isolates. All tissue samples were also acid-fast stained and examined microscopically. We isolated *Map* from at least one tissue from 48 hares (12.6%) and fecal samples from 16 hares (4.2%). No *Map* was found in tissues of eight of the fecal-culture–positive hares. Histologically, all tissues from all hares were within normal limits, and no acid-fast organisms were observed in any sample. Active infection, implying amplification of the organism secondary to resultant disease, was not evident. With this report *Map* isolations on a population versus incidental detection have now been made from two lagomorph species. However, although the rabbit population studied in Scotland appears to function as a *Map* reservoir, the hares studied in Chile appear to be a dead-end host, serving only as potential mechanical vectors for the organism.

Key words: Hares, Johne's disease, *Mycobacterium avium* ss. *paratuberculosis*, reservoir, spillover, transmission, vector.

The intracellular pathogen *Mycobacterium avium* subsp. *paratuberculosis* (*Map*) causes paratuberculosis (Johne's disease) in adult ruminants. Infection occurs primarily in juveniles, but clinical signs of disease usually do not emerge until maturity. Disease is characterized in ruminants by granulomatous enteritis accompanied by weight loss and, in some species, diarrhea. Significant financial losses result from this disease through diminished milk production, reduced slaughter weights, and shortened productive life spans (Ott et al., 1999). The zoonotic potential of *Map* continues to be debated in the literature.

Although primarily considered a ruminant infection, incidental isolates of *Map* have been made in numerous nonruminant species, including several individual wild rabbits and hares captured near infected cattle herds (Machackova et al., 2004; Corn et al., 2005; Florou et al., 2008; Kopecna et al., 2008). In 1997 in Scotland, however, a "hot spot" (a cluster of numerous *Map* infections within a temporally and spatially defined population) in European rabbits (*Oryctolagus cuniculus*) was described (Greig et al., 1997, 1999; Beard et al., 2001; Judge et al., 2005). Based on epidemiologic studies of these rabbits sympatric with *Map*-infected dairy cattle, Beard et al. (2001) concluded that although *Map* may be capable of infecting many wildlife species, only ruminants and lagomorphs regularly show evidence of resultant disease, as determined by the presence of gross or microscopic lesions with associated acid-fast bacteria. This important distinction between active infection and resultant pathology versus the

simple presence of the organism in unaffected nonruminant tissue was made by another investigation assessing *Map* strains obtained from ruminants and nonruminants from seven EU countries (Stevenson et al., 2009).

To expand our understanding of potential nonruminant *Map* reservoirs, we wished to investigate a population of a different species of *Lagomorpha*, especially since the extensive infection and ensuing disease seen in Scottish rabbits have not been reported elsewhere. There are distinct ecologic and behavioral differences between the European rabbit and the European hare (*Lepus europaeus*) that could affect exposure to *Map* deposited by infected cattle into the environment. The hare's offspring are precocial, while the rabbit's are altricial, and hares are primarily solitary whereas rabbits form colonies congregating underground in burrows, resulting in young rabbits' repeated exposure to feces from numerous adults (Broekhuizen and Maaskamp, 1982; Zörner, 1996). To augment our understanding of the susceptibility to and maintenance of *Map* infection in *Lagomorpha*, we conducted an investigation of a population of European hares sharing habitat with *Map*-infected dairy cattle in Chile.

Adult hares were collected by professional hunters in the Chilean dairy farming provinces of Valdivia and Osorno (May–July 2009). We chose this region because Johne's disease is common in cattle grazed throughout the year. Although detailed studies are lacking, one study estimated dairy herd prevalences approaching 40% (van Shaik et al., 2007). The hare sample size was 385 (based on an assumed prevalence of 50%, 95% confidence interval). The hares were examined at a hunter check station where samples (ileum, mesenteric lymph node, fecal, lenses) and statistics (time of death, location, length, sex, and weight) were collected. The lens from each eye was processed to determine hare age (Broekhuizen and Maaskamp, 1979). Fecal samples were processed per the manufacturer's protocols (ParaTB MGIT, Becton Dickinson, Sparks, Maryland, USA). Tissues were processed as described by Godfroid et al. (2000) for ParaTB MGIT liquid culture and fixed in 10% formalin for histopathologic analysis. Cultures were incubated at 37 C for 49 days in a MGIT 960 machine after which any tube producing a positive signal at any time was inverted three times before 500 µl was removed for *Map* DNA extraction via cell lysis (Salgado et al., 2011). The sample was then treated with ethanol and differential centrifugation to remove potential inhibitors. The primers and probes used for real-time polymerase chain reaction (rRT-PCR) confirmation test were previously described (Herthnek and Bölske, 2006). Cultures of the ileum, lymph node, and feces, and age determination were completed for all but five animals. If *Map* was isolated from any sample, the hare was considered infected. Infection prevalence was estimated using a Bayesian approach (Branscum et al., 2004). The associations among hare characteristics (gender, age), location (Valdivia vs. Osorno province), and infection status were assessed by logistic regression.

All samples positive by MGIT culture were also acid-fast positive and confirmed as *Map* by rRT-PCR. A *Map* isolate was obtained from at least one tissue sample from 48/380 hares (12.6%). For 11 hares, more than one sample yielded a *Map* isolate. Fecal samples were *Map* positive for 16 hares (4.2%), eight of which were tissue-culture negative. Isolates were obtained in 22 (5.7%) ileum, 22 (5.7%) mesenteric lymph node, and 16 (4.2%) fecal samples (Table 1). For none of the sampled animals were all three sites *Map* positive. Ziehl-Neelsen staining of tissues did not reveal acid-fast organisms in any animal. All tissues were microscopically normal with neither granulomatous infiltrates nor lesions consistent with *Map* infection as seen in ruminants or as described in rabbits (Beard et al., 2001).

TABLE 1. Distribution of 60 *Mycobacterium avium* subsp. *paratuberculosis* (*Map*) isolates from European hares (*Lepus europaeus*) in southern Chile by age of hare and origin of sample.

	Sample type[a]					
Age (mo)	F	F+IL	F+MLN	IL	IL+MLN	MLN
1–4	1	1	1	2	0	5
4–8	4	2	1	7	2	3
8–12	2	0	1	2	0	3
>12	1	1	1	4	1	3
Total	8	4	4	15	3	14

[a] F=fecal; IL=ileum; MLN=mesentric lymph node.

The median of the estimated posterior for the true prevalence was estimated to be 14.1% (95% credibility interval 0.1; 19.9) (Table 2).The age ranges included juveniles (1–4 mo old), subadults (4–8 mo), adults (8–12 mo), and older adults (>12 mo). *Map* was 1.42 times more likely to be isolated from hares 1–4 mo old in this study than from older animals (confidence interval 90% 1.02; 1.96). The hares captured in Osorno province were 1.68 times (confidence interval 90% 1.01; 2.83) more likely to test positive than those in Valdivia.

The *Map* isolation rate in this hare population was lower than that for rabbits in Scotland (12.6% vs. 19.4–39.7%). A seasonality effect was noted in the rabbit population, with higher detection rates in the spring months in Scotland (Beard et al., 2001; Judge et al., 2006). The hares for this study were killed during the Chilean autumn.

All hares were apparently unaffected by *Map* at the time they were sampled; each tissue was histologically within normal limits. The detection of the organism in these hares is not, by itself, evidence that the population serves as a wildlife reservoir (Corner, 2006). In fact, multiplication of *Map* (i.e., *Map* replication in tissues and subsequent heavy shedding in manure or milk) is not characteristic of species (whether ruminant or nonruminant) whose tissues are free of pathologic lesions (Cleland et al., 2010). The lack of gross and microscopic lesions may indicate that the fecal sample isolates were due only to pass-through from a contaminated environment versus shedding secondary to progressive mycobacterial infections. Another factor arguing against hares as a *Map* reservoir is that young hares are less exposed to nonparental adult fecal pellets than are young rabbits. Rabbit dens may be contaminated by multiple infected adults resulting in pseudovertical or horizontal *Map* infection; this denning pattern does not occur for hares. For these reasons, hare populations exposed to *Map* may be less likely to sustain the infection than *Map*-exposed rabbit populations. Even if they are only dead-end hosts, however, hares may still serve as

TABLE 2. Number of European hares (*Lepus europaeus*) yielding *Mycobacterium avium* subsp. *paratuberculosis* (*Map*) isolates by age range, southern Chile, 2009.

Age (mo)	No. hares evaluated	No. hares *Map* positive	Prevalence (95% CI)
1–4	50	10	0.208 (0.113–0.332)
4–8	128	19	0.152 (0.097–0.220)
8–12	84	8	0.101 (0.050–0.177)
>12	118	11	0.098 (0.054–0.159)
Total	380	48	

mechanical vectors for *Map* by taking up and passively excreting the organism into the environment (Nielsen and Toft, 2008).

Numerous ruminant and now two lagomorph species have now been described as capable of becoming infected by *Map* on a population basis, although the pathologic impact of the infection differs. It is interesting to consider the potential influence on *Map* transmission of the ruminant ingesta recycling system and the analogous cecotrophic habits of lagomorphs. Lagomorphs produce cecotropes in the cecum that are reingested by the animal and eaten by its offspring before weaning. If the forage of rabbits and hares is contaminated with *Map*, the organism may be concentrated in the cecotrope. Reswallowing the ingesta may increase the frequency of *Map* exposure and opportunity of uptake, as may occur with the cud of ruminating animals.

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LITERATURE CITED

Beard, P., M. J. Daniels, D. Henderson, A. Pirie, K. Rudge, D. Buxton, S. Rhind, A. Greig, M. R. Hutchings, I. McKendrick, K. Stevenson, and J. M. Sharp. 2001. Paratuberculosis infection of nonruminant wildlife in Scotland. Journal of Clinical Microbiology 39: 1517–1521.

Branscum, A. J., I. A. Gardner, and W. O. Johnson. 2004. Bayesian modeling of animal- and herd-level prevalences. Preventive Veterinary Medicine 66: 101–112.

Broekhuizen, S., and F. Maaskamp. 1979. Age determination in the European hare (*Lepus europaeus* Pallas) in the Netherlands. Journal of Mammalian Biology (Zeitschrift für Saugetierkunde) 44: 162–175.

———, and ———. 1982. Movement, home range and clustering in the European hare (*Lepus europaeus* Pallas) in the Netherlands. Journal of Mammalian Biology (Zeitschrift für Saugetierkunde) 47: 22–32.

Cleland, P. C., D. R. Lehmann, P. H. Phillips, D. V. Cousins, L. A. Reddacliff, and R. J. Whittington. 2010. A survey to detect the presence of *Mycobacterium avium* subspecies *paratuberculosis* in Kangaroo Island macropods. Veterinary Microbiology 145: 339–346.

Corn, J. L., E. J. Manning, S. Sreevatsan, and J. R. Fischer. 2005. Isolation of *Mycobacterium avium* subsp. *paratuberculosis* from free-ranging birds and mammals on livestock premises. Journal of Applied Environmental Microbiology 71: 6963–6967.

Corner, L. A. 2006. The role of wild animal populations in the epidemiology of tuberculosis in domestic animals: How to assess the risk. Veterinary Microbiology 112: 303–312.

Florou, M., L. Leontides, P. Kostoulas, C. Billinis, M. Sofia, I. Kyriazakis, and F. Lykotrafitis. 2008. Isolation of *Mycobacterium avium* subspecies *paratuberculosis* from non-ruminant wildlife living in the sheds and on the pastures of Greek sheep and goats. Epidemiology and Infection 136: 644–652.

Godfroid, J., F. Boelaert, A. Heier, C. Clavareau, V. Wellemans, M. Desmecht, S. Roels, and K. Walravens. 2000. First evidence of Johne's disease in farmed red deer (*Cervus elaphus*) in Belgium. Veterinary Microbiology 77: 283–90.

Greig, A., K. Stevenson, D. Henderson, V. Perez, V. Hughes, I. Pavlik, I. M. E. Hines, I. McKendrick, and J. M. Sharp. 1999. Epidemiological study of paratuberculosis in wild rabbits in Scotland. Journal of Clinical Microbiology 37: 1746–1751.

———, ———, V. Perez, A. Pirie, J. M. Grant, and J. M. Sharp. 1997. Paratuberculosis in wild rabbits (*Oryctolagus cuniculus*). Veterinary Record 140: 141–143.

Herthnek, D., and G. Bölske. 2006. New PCR systems to confirm real-time PCR detection of *Mycobacterium avium* subsp. *paratuberculosis*. BMC Microbiology 6: 87.

Judge, J., I. Kyriazakis, A. Greig, D. J. Allcroft, and M. R. Hutchings. 2005. Clustering of *Mycobacterium avium* subsp. *paratuberculosis* in rabbits and the environment: How hot is a hot spot? Journal of Applied Environmental Microbiology 71: 6033–6038.

———, ———, ———, ———, and ———. 2006. Routes of intraspecies transmission of *Mycobacterium avium* subsp. *paratuberculosis* in rabbits (*Oryctolagus cuniculus*): A field study. Journal of Applied Environmental Microbiology 72: 398–403.

Kopecna, M., I. Parmova, L. Dvorska-Bartosova, M. Moravkova, V. Babak, and I. Pavlik. 2008. Distribution and transmission of *Mycobacterium avium* subspecies *paratuberculosis* in farmed red deer (*Cervus elaphus*) studied by faecal culture, serology and IS*900* RFLP examinations. Veterinaria Medicina 53: 510–523.

Machackova, M., P. Svastova, J. Lamka, I. Parmova, V. Liska, J. Smolik, O. A. Fisher, and I. Pavlik. 2004. Paratuberculosis in farmed and free-living wild ruminants in the Czech Republic in the years 1999–2001. Veterinary Microbiology 101: 225–234.

Nielsen, S. S., and N. Toft. 2008. Ante mortem diagnosis of paratuberculosis: A review of

accuracies of ELISA, interferon-γ assay and faecal culture technique. Veterinary Microbiology 129: 217–235.

OTT, S. L., S. J. WELLS, AND B. A. WAGNER. 1999. Herd-level economic losses associated with Johne's disease on US dairy operations. Preventive Veterinary Medicine 40: 179–92.

SALGADO, M., M. T. COLLINS, F. SALAZAR, J. KRUZE, G. BÖLSKE, R. SÖDERLUND, R. JUSTE, I. A. SEVILLA, F. BIET, F. TRONCOSO, AND M. ALFARO. 2011. Fate of *Mycobacterium avium* subsp. *paratuberculosis* after application of contaminated dairy cattle manure to agricultural soils. Journal of Applied Environmental Microbiology 77: 2122–2129.

STEVENSON, K., J. ALVAREZ, D. BAKKER, F. BIET, L. DE JUAN, S. DENHAM, Z. DIMARELIM, K. DOHMANN, G. F. GERLACH, I. HERON, M. KOPECNA, L. MAY, I. PAVLIK, J. M. SHARP, V. C. THIBAULT, P. WILLEMSEN, R. N. ZADOKS, AND A. GREIG. 2009. Occurrence of *Mycobacterium avium* subspecies *paratuberculosis* across host species and European countries with evidence for transmission between wildlife and domestic ruminants. BMC Microbiology 9: 212.

VAN SCHAIK, G., F. HARO, A. MELLA, AND J. KRUZE. 2007. Bayesian analysis to validate a commercial ELISA to detect paratuberculosis in dairy herds of southern Chile. Preventive Veterinary Medicine 79: 59–69.

ZÖRNER, H. 1996. Der Feldhase. 2nd Edition. Westarp Wissenschaften, Magdeburg, Germany, 172 pp.

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