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SHORT COMMUNICATIONS

Association between cattle herd *Mycobacterium avium* subsp. *paratuberculosis* (MAP) infection and infection of a hare population

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Abstract Paratuberculosis has long been considered a disease of domestic and wild ruminants only. The known host range of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) was recently extended to include non-ruminant wildlife species believed to be exposed to spillover of MAP from infected domestic cattle herds. The aim of the present study was to assess the association between cattle herd MAP infection pressure level and the infection level of a hare population in two dairy farms of southern Chile. Fifty hares from a herd A and 42 hares from herd B were captured and sampled for MAP culture. The results showed a statistically significant association between the cattle herds' infection prevalence and the hare infection prevalence.

Keywords Paratuberculosis · MAP · Hare · Cattle · Transmission · Spillover

Paratuberculosis is an infectious bacterial disease caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). It has long been recognized as a domestic and wild ruminant infection (Buergelt and Ginn 2000). More recently, the host range of MAP has been extended to include non-ruminant wildlife species, particularly rabbits (Florou et al. 2008; Nugent et al. 2011). The impact of MAP in non-ruminant wildlife is largely unknown. However, evidence from Scotland has been shown that rabbits represent a significant risk for transmitting the bacterium to domestic livestock (Judge et al. 2007; Stevenson et al. 2009). In grazing-based cattle production systems, MAP-cattle manure may heavily contaminate fields also grazed by herbivorous wildlife (Salgado et al. 2011a). This spillover of MAP is of concern to both wildlife managers and livestock keepers since infection control is almost impossible in free-ranging wildlife that may serve as infection reservoirs. It is believed that the higher the prevalence in the cattle herd, the higher also in wildlife. In Chile, the lagomorph species most frequently sharing range with cattle is the hare (*Lepus europaeus*) vs. the rabbit. A previous study by the authors (Salgado et al. 2011b) estimated a hare true prevalence of infection of 14.1 %. The aim of the present study was to assess the association between cattle herd MAP infection pressure level and the infection level of a hare population in two dairy farms of the south of Chile.

A field-study was performed using two herds (A and B) with previous known infection MAP status. Since Chile has no official control program, it is extremely difficult to select or find herds without MAP-infected animals. Herd A had an estimated individual prevalence of 20 % based on 5 years of sampling for MAP infection ELISA and MAP culture results. The veterinarian attending the herd reported that 10 clinical cases/year occurred (the diagnosis was confirmed by fecal culture) and necropsy. The herd comprised 1,300 animals grazing about 700 ha, and the grazing area was densely populated by hares. Herd B showed no history of clinical

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disease; all adult cows were negative on at least two consecutive ELISAs 1 year apart, though no infection status couldn't be stated since during the last year the owner purchased some untested but apparently healthy animals from a neighbor with a history of testing-positive animals. This herd was located in the county of Panguipulli, de Los Rios region, more than 300 km apart from herd A. Herd B comprised of 150 animals that grazed more than 120 ha also densely populated by hares. The hares were hunted by professionals in two rounds in each herd, during June and July 2009. The carcasses were transported within 2 h after hunted to the necropsy unit at the Animal Pathology Department. Ileum, mesenteric lymphatic node, and fecal samples were collected from each fresh carcass aseptically. Besides, in herd A, eight hares showed advanced pregnancy; therefore, fetal macerated samples were also taken for culture. All samples were transferred to sterile containers and then transported to the bacteriology and histopathology laboratories for subsequent processing. For histopathology, different sections of small intestine and lymph nodes were removed and fixed in 10 % buffered formalin. Sections of 5 μm were stained Ziehl Neelsen (ZN) (at least three sections per sample). Determination of gender was achieved at sampling by visual inspection and the determination of age was performed weighting the lens to estimate age (Broekhuizen and Maaskamp 1979). In addition, fecal samples from 150 animals were obtained from lactating cows older than 5-years old from each farm. To overcome the high costs of individual cow fecal culture, fecal samples were pooled (Nielsen and Toft 2008), and one aliquot of 2 g was tested from each pool. Samples were processed for bacteriological culture as previously described (Salgado et al. 2013). To obtain DNA to perform molecular confirmation from the liquid culture, a method using cethyltrimethylammonium bromide (CTAB) was followed (van Soolingen et al. 1991). A triplex real-time PCR targeting IS*900* and ISMAP02 sequences of MAP was used for confirmation of positive cultures (Maio et al. 2011). Strain characterization of confirmed positive cultures was performed by MIRU-VNTR pattern analysis. MIRU-VTNR typing was performed using seven polymorphic loci of MAP called TR 292, X3, 25, 47, 3, 7, and 10 as described (Thibault et al. 2007). The association between characteristics of hares (sex, age), location (herd A vs. B), collection round (first, second), and disease status were assessed by logistic regression (LR). The strategy for building the model consisted of obtaining unconditional models for initial screening of variables. Variables associated with the outcome ($p<0.25$) were eligible for inclusion in the conditional model that was built using a manual forward approach. Potential interactions were tested based on their biological significance. The best model was assessed by likelihood ratio test with a significance level ($p<0.05$) as a measure of goodness-of-fit.

Regarding the fecal pools from cattle, herd A showed 20 positive pools out of 30, compared to 12 out 30 pool cows for herd B. Fifty hares were captured from herd A and 42 from herd B. Forty five out of 50 individuals had one or more type of samples signaling culture positive in herd A, while 17/42 hares showed at least one positive culture result in herd B. Overall, herd A showed 22 and 63 fecal and tissue culture positive results, respectively. Meanwhile, herd B showed only 8 fecal and 14 tissue positive culture results. All the above differences were statistically significant ($p<0.05$). In herd A, 12 individuals presented a positive result in more than one type of sample, and 7 hares showed fecal, ileum, and lymph node samples with MAP-positive. In herd B, two hares showed positive MAP culture results for more than one sample, though only one was MAP-positive in all three types of samples. The distribution of the proportion of MAP-positive hares per age group in herds A and B are shown in Fig. 1a, b. Overall, 43 (46.8 %) of the hares were females; meanwhile, 49 (53.2 %) were males. Similar number of positive-culture results was shown for females (32) and for males (29). Only in herd A, eight pregnant female hares were found and their fetuses were aseptically sampled before maternal tissue by using separate instruments. Fetal samples were also submitted to bacteriological analysis. Three out of eight pregnant hares were older than 1 year, and two of them presented culture-positive results to all type of samples, including the fetus. The histopathology study in hares defined as infected (MAP was isolated from tissue), revealed that lesions consistent with MAP infection, such as multibacillary form and presence of Langhan's giant cells, were not observed.

The association among characteristics of hares (sex, age), location (herd A vs. B) and hunt round (first, second), and disease status were assessed by logistic regression (LR). After checking for interactions and potential confounders, the final

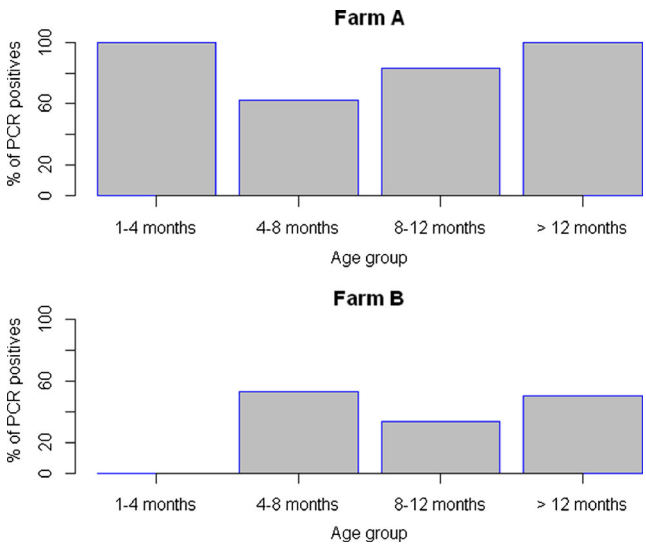

Fig. 1 Distribution of the proportion MAP positive hares per age group in herd A (high prevalence) and B (low prevalence)

Table 1 Conditional logistic regression model estimates adjusted by diagnostic test performance

Variable	Category	OR	95 %CI
Herd*	B	Ref.	
	A	33.15	(2.9; 377.3)
Hunting round	Second	Ref	
	First	363.1	(0.1; 1267440,0)
Age	Less than 12 months	Ref	
	More than 12 months	5.35	(0.46; 62.6)

*$p<0.05$

model contained three variables (Table 1). However, only one variable (herd where hare was shot) was statistically associated with the outcome and indicates that hares hunted from herd A were more likely to be infected than those from herd B. Two different MIRU-VNTR genotypes were found in cattle and hares regardless of their herd of origin. These two strain types were consistent with patterns INMV 1 and nine being both types grouped into the MAP cattle type.

The association of hare infection in the infected cattle herd A may be due to a number of reasons. The close interaction between hare and cattle populations could favor MAP transmission through the fecal-oral route. As has been mentioned, both herd A and B, and in general all dairy systems in southern Chile, are based on grazing management of the animals, ensuring that MAP could contaminate grass that may be ingested by hares. Besides, pastures used in dairy production in Chile are often established on steeply sloping soils, which means that infected cattle grazing in these paddocks, or manure application management, could constitute a primary source for MAP dispersion to surface water by water runoff after heavy rains (Salgado et al. 2013), coming in contact with wildlife, especially herbivores, such as the hare. Although this association could not be proven to be causal, it is at least one potential plausible path to link interspecies transmission in addition to grazing contaminated fields.

In the present study, hare age distribution provides clues about MAP transmission, suggesting how transmission from the infected host ruminants to the wild potentially can occur. In the case of herd A, the distribution of affected ages might be due to a higher exposition to the pathogen compared to herd B and a longer exposure time given that herd A is known to be infected some years ago; on top of this, the odds to get infected were randomly distributed. In contrast, in herd B, the lower number and percentage of infected hares and the similar proportions in each age category could reflect a lighter bacterial load in the pasture environment and/or exposure to a more recent introduction.

There is a strong evidence that hares in this study met the definition as an infected host (Nielsen and Toft 2008), however since there was a lack of gross pathological and histopathological evidence, we can confirm that hares are affected. An interpretation of the latter could be a different pathogenesis than that in domestic ruminants may be suggested in this wildlife species. Livestock husbandry, with mainly crowded populations, tends to facilitate infectious transmission, but the management system also serves as a stressor of the host immune system, activating a latent intracellular replication of MAP, which may be negligible in hares. It might also be hypothesized that the macrophage as the main bacterial target may be thought of as a different environment from the infectious point of view in a non-ruminant species (such us the hare) than in a ruminant species, and the paucibacillary, or tuberculoid, form which has a diffuse lymphocytic infiltrates in the lamina propria, with few or no visible mycobacteria may be the result of MAP infection in hares (Pérez et al. 1996). The condition "passive shedders of the bacterium" might more precisely coincide with reality, where their "infectious status" is considered a transient step (Sweeney et al. 1992), especially under the epidemiological circumstances where these hares were captured. However, the presence of three pregnant hares with positive-culture samples, both from the dams and from the fetuses, could represent the opposite suggesting an active infection, since the bacterium could colonize the intestinal epithelium. This again raises the question of whether at this age, individuals of the hare species can develop an active MAP infection, or whether they only act as a transient passive infected carriers due to the high environment MAP load their mothers were exposed to. It can be hypothesized according to the results of the present study, that herd A could provide an important infection-transmission pressure where MAP could find another host niche to parasite. Finally, other evidence that confirms that both populations are connected is the fact that molecular characterization of the isolated MAP either in cattle and hare showed the same patterns. The latter could be indicative that both species shares the same strains and by this contribute with a piece of evidence to think of interspecies transmission.

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Conflict of interest The authors declare that they have no conflict of interest.

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