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Lack of Exposure to *Mycobacterium bovis* and *Mycobacterium avium* subsp. *paratuberculosis* in Chilean Cervids, and Evidence of a New *Mycobacterium*-like Sequence

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ABSTRACT: Screening of serum and fecal samples from huemul (*Hippocamelus bisulcus*) and pudu (*Pudu puda*) from southern Chile for *Mycobacterium bovis* and *Mycobacterium avium paratuberculosis* (MAP) found all but four samples *Mycobacterium*-negative. The positive sequences showed only 92–93% similarity with MAP and were from remote Isla Riesco populations.

Bovine tuberculosis and paratuberculosis are globally distributed mycobacterial diseases affecting domestic and wild ruminant populations and causing major economic losses in livestock production (Carta et al. 2013; Bernitz et al. 2021). Both *Mycobacterium bovis* and *Mycobacterium avium paratuberculosis* (MAP) can be transmitted from livestock to wild ruminants, which could become sources of infection or be affected by these diseases (Roller et al. 2020; Bernitz et al. 2021).

In South America, bovine tuberculosis is associated with larger intensive dairy farms, and the epidemiological situation among countries is quite different. Chile uses a surveillance system, but prevalence is still high (Ferreira-Neto 2019). Information about *M. bovis* and MAP in South American cervids is scarce, hampering prevention and control of these diseases (Sanchez-Vazquez et al. 2021). Reports of MAP in southern pudu (*Pudu puda*) and huemul (*Hippocamelus bisulcus*) in Chile suggest it might be a conservation threat for both species (González-Acuña et al. 2011; Salgado et al. 2015, 2017; Corti et al. 2020). Our survey aimed to provide information about exposure to or infection with *M. bovis* and MAP in free-living pudu and huemul.

Antibodies against *M. bovis* were investigated in 34 free-living pudu at admission to two wildlife rescue centers in the Los Lagos region (41°50′22″S, 73°56′16″W and 58°81′57″E, 53°67′52″N) in 2015–18 (Table 1). Blood samples were collected from the cephalic or saphenous vein using an evacuated tube system (Vacutainer, Beckon, Dickson, and Company, Franklin Lakes, New Jersey, USA); serum was extracted by centrifugation and stored at –20 C until analyzed with the indirect in-house P22 enzyme-linked immunoassay. Briefly, we covered wells of a 96-well plate with an antigen derived from *M. bovis* (P22), added positive and negative controls each in quadruplicate, and added sera being analyzed at a 1:100 dilution (in a solution of 5% skim milk powder in phosphate buffered saline) to the remaining wells in duplicate.

TABLE 1. Current and past samples of wild Chilean cervids (southern pudu [*Pudu puda*] and huemul [*Hippocamelus bisulcus*]) analyzed for detection of *Mycobacterium bovis* and *M. avium* subsp. *paratuberculosis* (MAP).

Source	Species	Sample	Sample size	Collecting region	Date	Positive *M. bovis*	Positive MAP	Positive MAP-like
This study	*Pudu puda*	Serum	34	Los Lagos	2015–18	0	0	0
		Feces	72	Los Lagos	2009–18	0	0	0
			4	Aysén				
	Hippocamelus bisulcus	Feces	45	Magallanes	2017–18	0	0	4
Salgado et al. 2015	*Pudu puda*	Feces	3	Los Ríos	2009	0	3	0
Salgado et al. 2017	*Hippocamelus bisulcus*	Feces	14	Magallanes	2017	0	1	5
Corti et al. 2020	*Hippocamelus bisulcus*	Feces	3	Magallanes	2016	0	2	1

The plate was incubated for 1 h at 37 C. After washing, a 1:2000 dilution of protein G-horseradish peroxidase (Sigma-Aldrich, St. Louis, Missouri, USA) was added to each well followed by 1 h incubation at room temperature. After washing, o-phenylenediamine dihydrochloride substrate (FAST OPD, Sigma-Aldrich) solution was added, and the plate was incubated for 20 min at room temperature in dark conditions. The reaction was stopped with 2 M sulphuric acid solution, and then the optical density was measured with a spectrophotometer (Multiskan GO, Thermo Scientific, Waltham, Massachusetts, USA) as described previously (Thomas et al. 2019).

Molecular detection of *M. bovis* and MAP was carried out in 76 fecal samples from pudu picked up across the Los Lagos and Aysen regions in 2009–18 (Table 1) and in 45 fecal samples from huemul from populations at Isla Riesco (53°00′S, 73°00′W) and Torres del Paine National Park (53°3′22.82″S, 72°35′3.62″W), Magallanes Region, collected in 2017–18 (Table 1). Feces were collected fresh, as described by Corti et al. (2021). All the pudu fecal samples and all but three huemul samples were frozen at –20 C within 1–10 d after collection, until their laboratory processing in 2019. Three huemul samples from Isla Riesco were kept at 4 C for 35 d before being frozen at –20 C.

We extracted DNA from the feces with a pressure filtration method (QuickGene DNA tissue kit S, Fujifilm-Kurabo, Tokyo, Japan). For *M. bovis* detection a TaqMan real-time PCR assay was conducted followed Sweeney et al. (2007). For detection of MAP, we performed both a nested PCR and a TaqMan real-time PCR, targeting ISMap02 and IS900, respectively (Park et al. 2016). We considered a sample positive when it was positive in at least one of the PCRs. Sequencing of amplicons obtained from the ISMap02 nested PCR was used to confirm results. Sequences were compared with the GenBank using a Blast search (NCBI 2019), and alignment was carried out using the ClustalW algorithm in MEGA X Software (Kumar et al. 2018). A maximum likelihood phylogram was generated using 1000 bootstrap replicates. All the phylogenetic analyses were performed using the MEGA X software.

None of the antibody-positive pudu showed clinical signs of mycobacterial infection, such as poor body condition, weight loss, emaciation, or diarrhea. All fecal samples were negative for MAP and *M. bovis*. Four huemul fecal samples from Isla Riesco showed compatible bands in the ISMap02 PCR, but the obtained sequence was highly divergent from MAP sequences in GenBank (identities 92.1–93.0%; Fig. 1); therefore we classified it as MAP-like (Table 1).

Despite previous reports, we detected no evidence of infection by MAP in huemul and pudu. González-Acuña et al. (2011) found lesions characteristic of paratuberculosis at necropsy of one pudu from Concepción, Bío-Bío Region, in 2005; however, no ancillary tests were performed to confirm MAP as the

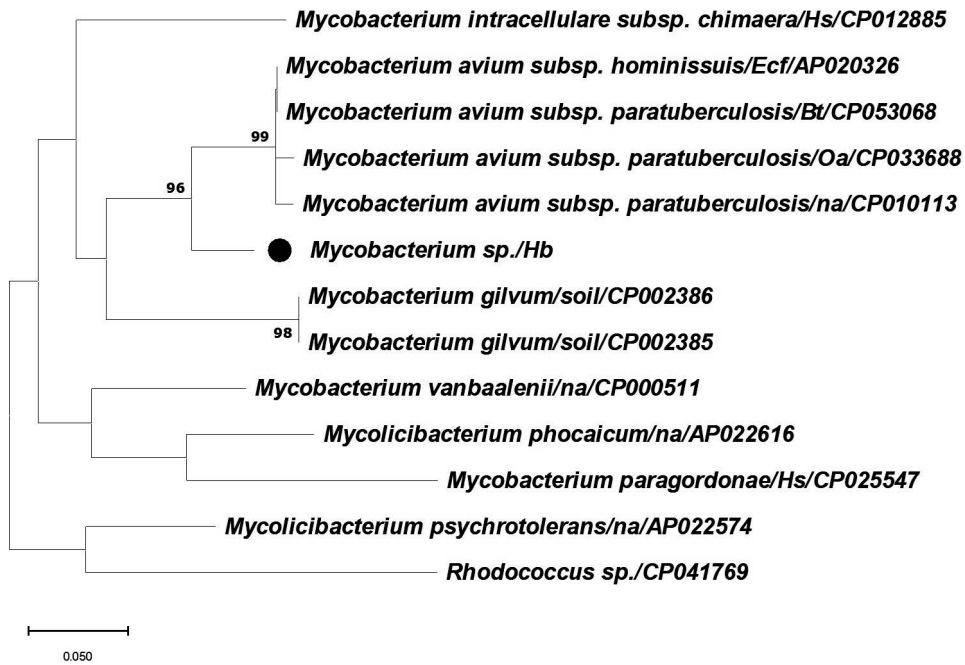

FIGURE 1. Maximum-likelihood phylogenetic tree of *Mycobacterium* spp. and related sequences, with a bootstrap value of 1000 replications. A bootstrap value less than 70 is omitted. The sequences are named by the bacterial species, followed by the source of origin and the GenBank accession number. Sequences obtained in this study are marked with a large dot. Bt=*Bos taurus*; Ecf=*Equus caballus ferus*; Hb=*Hippocamelus bisulcus*; Hs=*Homo sapiens*; Oa=*Ovis aries*; na=not available.

cause. According to Roller et al. (2020) histopathological examinations provide limited insight into disease occurrence in wildlife disease surveillance, being insufficient to enable identification and classification of the etiological agents of the clinical and pathological findings. Additional diagnostic techniques such as microbiological and genomic methods should complement gross necropsy and histologic studies (Artois et al. 2009; Ryser-Degiorgis 2013). Our results showed nothing to suggest that pudu and huemul are maintenance hosts for MAP or *M. bovis*; therefore previous reports are probably spillover events.

The lack of evidence of exposure or infection by *M. bovis* in pudu and huemul may be attributed to the low infection prevalence of *M. bovis* (0.3%) in livestock, the main reservoir in the sampled areas (Raffo et al. 2019). Studies are needed for the detection of *M. bovis* in pudu and huemul populations inhabiting regions of high prevalence in livestock, such as Maule, Ñuble, and Bio-Bio, as well as in other wild species prone to be reservoirs, such as the wild boar (*Sus scrofa*).

Regarding the new sequence of *Mycobacterium* sp., we can only speculate about its origin and pathological significance. Considering that it was found only in huemul from Isla Riesco, this sequence could be endemic to this deer population and be classified as *Mycobacterium avium* subsp. *paratuberculosis*-like. The presence of endemic sequences of MAP-like is supported also by a previous study describing genotypes specific from huemul from Torres del Paine using Tandem Repeat Analysis and one Short Sequence Repeat Analysis (Corti et al. 2020). That study shows three fecal samples of huemul were positive for MAP, of which two were 100% identical to each other but different from those described in cattle herds in Chile. Although we tested the samples only with culture-independent nested PCR, and we therefore could not attempt to classify the

genotypes found, our results could be in concordance with those of Corti et al. (2020). Additionally, our results point out the need for sequencing the products of PCR to confirm the sequences, especially in wildlife, where previously undescribed specific microorganisms may be present.

Our results do not support that MAP is a threat for these species in Chilean Patagonia, as claimed by other authors. Further investigation is warranted to determine if *M. bovis* or MAP is present in cervids and wild boars in the Chilean Central-South region.

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