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Seroepidemiology of tuberculosis in sheep in southern Spain

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ABSTRACT

Tuberculosis (TB) is a multi-host infectious disease caused by members of the *Mycobacterium tuberculosis* complex (MTC). In Mediterranean ecosystems, where multiple animal hosts of TB are present, identifying the role of the different species involved in the epidemiology of TB is a key point to be able to implement proper control measures. Sheep are susceptible to MTC infection but have traditionally been considered a spillover host. However, the occurrence of outbreaks involving sheep in recent years evidences the need to better understand the role of this small ruminant species in the epidemiology of the disease. Here, we aimed to determine the seroprevalence and risk factors associated with MTC seropositivity in sheep in Andalusia (southern Spain), a region with one of the highest prevalence of MTC infection in both cattle and wild ungulates. A total of 2266 sheep from 83 flocks were tested for antibodies against MTC using an in-house indirect ELISA. Anti-MTC antibodies were detected in 16 (0.7%) of the 2266 sheep (adjusted true prevalence 0.29%, 95% posterior probability interval 0.01–1.05). Seropositivity was found in 14.5% (12/83; 95%CI: 6.9–22.0) of the sheep farms analyzed. A semi-extensive management system was identified as a risk factor associated with MTC seropositivity in sheep farms (OR = 3.7; $p < 0.038$; 95%CI: 1.1–12.4) in the study area. To the best of the authors' knowledge, this is the first active TB surveillance study carried out to assess MTC exposure in sheep. Our results indicate MTC circulation in sheep farms in southern Spain. However, the low individual seroprevalence obtained suggests that sheep may play a limited role in the epidemiology of TB in this region. Serosurveillance programs could be a valuable tool to detect MTC circulation in sheep in risk scenarios or target farms, in order to optimize control measures on TB animal in multi-host Mediterranean ecosystems.

1. Introduction

Animal tuberculosis (TB) is a chronic infectious disease caused by members of the *Mycobacterium tuberculosis* complex (MTC), mainly *M. bovis* and *M. caprae* (Rodríguez-Campos et al., 2014; Pesciaroli et al., 2014; Malone and Gordon, 2017). The disease affects a wide variety of wild and domestic mammal species worldwide, including humans (Pesciaroli et al., 2014). Due to its zoonotic nature, as well as its high economic impact in livestock husbandry, European Union (EU) countries have worked towards the elimination of TB through the implementation of eradication programs during the last decades (Reviriego-Gordejo and Vermeersch, 2006; Reis et al., 2021). In this context, TB control programs co-funded by the EU are underway in Portugal, Spain, Ireland, Malta and Italy (E.C., 2020).

In the Iberian Peninsula, TB is maintained in a multi-host epidemiological cycle that represents a major obstacle to its control and

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eradication (Gortázar et al., 2015; Sun et al., 2021). To reach disease eradication, a holistic approach must be taken, considering all the potential TB reservoir species (Santos et al., 2020). Despite sheep being susceptible to MTC infection (Muñoz-Mendoza et al., 2012), this ruminant species has traditionally been considered a spillover host. Nevertheless, in recent years the occurrence of outbreaks involving sheep in different countries, including Spain (Muñoz-Mendoza et al., 2012; Van der Burgt et al., 2013; Vidal et al., 2018; Konold et al., 2020), have led to reconsider this species as MTC reservoir in certain epidemiological scenarios (Konold et al., 2020). Moreover, recent studies have suggested zoonotic transmission from sheep in northeastern Spain (Pérez de Val et al., 2021) and northern Africa (Tunisia) (Saad et al., 2020).

Spain, with 15.4 million animals, has the largest sheep population in the EU (Eurostat, 2022). Nevertheless, epidemiological information on MTC in sheep is still very limited because it is not included in the TB eradication programs in the EU and, therefore, is not routinely subjected to TB testing. In this sense, only two targeted survey studies in sheep have been carried out in Europe, which demonstrated a high circulation of MTC in this small ruminant species in specific epidemiological scenarios of northern Atlantic Spain, in particular, in sheep cohabiting with TB-infected cattle (Muñoz-Mendoza et al., 2016) and in TB hotspot areas (Infantes-Lorenzo et al., 2020). However, no active surveillance studies or programs have been carried out anywhere in the world. In the present study, we aimed to determine the seroprevalence and risk factors associated with MTC seropositivity in sheep farms in southern Spain, a region with one of the highest prevalence of MTC infection in both cattle and wild ungulates (Gortázar et al., 2012; Barasona et al., 2017; MAPA, 2017; Ferreras-Colino et al., 2022).

2. Materials and methods

2.1. Study design and sampling

A cross-sectional study was conducted to determine the prevalence of antibodies against MTC in sheep in Andalusia (southern Spain). The number of farms sampled was estimated assuming an expected prevalence of 15% (Infantes-Lorenzo et al., 2020) with a 95% confidence level and an accepted error of $\pm$ 8%. A stratified proportional sampling design was performed based on the number of farms in each province of the study region. Within each province, farms were randomly selected. Thirty animals were randomly selected in flocks with more than 30 animals, while all sheep were sampled when flock size was $< =30$. This sample size would allow the detection of exposure to MTC with a probability of 95% assuming a minimum within-farm prevalence in infected flocks of 10%. A total of 2266 sheep, not vaccinated against *Mycobacterium avium* subsp. *paratuberculosis* (MAP), from 83 farms were sampled between 2015 and 2017. Anti-MAP vaccination programs were not implemented on any of the sampled farms.

Blood was obtained by jugular vein puncture using a sterile collection system (Vacutainer®, Becton-Dickinson, USA). Samples were centrifuged at 400 *g* for 10 min and the serum obtained was frozen in aliquots at - 20 °C until analysis. Epidemiological data related to animals and farms sampled were collected through personal interviews with the farmers during the sampling using a standardized questionnaire. Data collected included: individual animal data, general production data of the farm, and biosecurity and health parameters (Table S1 and supplementary information). Within the study region, bovine TB high-risk areas were defined according to Spanish TB eradication program results (MAPA, 2017) and the seroprevalence of MTC in wild boar at the province level were obtained from the Epidemiological Surveillance Program in Wildlife (Regional Government of Andalusia) (CAGPDS, 2022).

2.2. Laboratory analyses

The presence of anti-MTC antibodies was assessed by an indirect in-

house ELISA (iELISA) using P22 as a coating antigen and following the protocol previously used in domestic small ruminants (Arrieta-Villegas et al., 2020; Infantes-Lorenzo et al., 2020). Briefly, the plates (Nunc MaxiSorp™, Denmark) were coated with P22 at 10 µg/ml and incubated overnight at 4 °C. Plates were subsequently washed with phosphate buffer saline (PBS) solution containing 0.05% Tween 20 (PBST). Wells were blocked with 5% skimmed milk solution (BS) for 60 min at room temperature (RT). The plates were subsequently emptied, and sera was added at 1:100 dilution in BS and incubated for 60 min at 37 °C. After three washes with PBST, the secondary antibody (rabbit anti-sheep IgG (H/L)-HRP) (SouthernBiotech®, Birmingham, USA) was added at 1:1500 in PBS and incubated for 30 min at RT. Plates were washed four times with PBST and then substrate solution (Fast OPD, Sigma®, Barcelona, Spain) was added. The reaction was stopped after 6 min in darkness and RT conditions, with 3 N H_2SO_4, and the optical density (OD) was measured at 450 nm in a spectrophotometer. The ELISA results were expressed as percentage (E%), that was calculated as: sample E% = [mean sample OD/ (2 × mean of negative control OD)] × 100. Serum samples with E% values greater than 100 were considered positive. Positive and negative sheep sera controls were tested in quadruplicate in each plate. Positive control sera were obtained from animals with gross and microscopic TB compatible lesions and previously confirmed by culture to MTC, while that negative control sera were obtained from animals with absence of TB compatible lesions of historical TB-free herd (Infantes-Lorenzo et al., 2020). The estimated sensitivity and specificity of this iELISA in small ruminants are 74.0% and 93.0%, respectively (Arrieta-Villegas et al., 2020). This ELISA has also shown high specificity in the detection of antibodies against MTC in MAP-vaccinated/infected small ruminants (Arrieta-Villegas et al., 2020; Infantes-Lorenzo et al., 2019, 2020).

2.3. Statistical analysis

The apparent prevalence of exposure was calculated as the proportion of positives out of the total number of samples analyzed. True prevalence was then estimated taking into account the expected sensitivity and specificity of the test through a Bayesian latent class model for one test and one population as previously described (Branscum et al., 2005). Beta distributions were used as priors for the sensitivity, specificity and true prevalence based on available information and expert opinion (Table S2). To obtain the posterior estimates for the true prevalence, three chains were run for 7500 iterations after a burn-in period of 2500 iterations. To avoid autocorrelation in the posterior values, chains were thinned by selecting one in every 10 consecutive samples. Convergence was assessed visually by checking the trace plots and more formally using the Gelman-Rubin statistic (Gelman and Rubin, 1992). The model was fitted in OpenBUGS (Lunn et al., 2009) using the package 'R2OpenBUGS' (Sturtz et al., 2005) in R (R Core Team, 2020).

Continuous variables (census of sheep, pre-breeding census and distance to nearest ruminant farm) were categorized considering percentiles 33 and 66 as cut-off points. Associations between serological results at farm-level (positive/negative) and independent variables (extracted from the epidemiological questionnaire) were analyzed by Pearson's chi-square or Fisher's test (for variables with less than six observations in any of the categories). Variables with $p \leq 0.15$ in the univariable analysis were selected for further analysis. Collinearity between pairs of variables was tested by Cramer's V coefficient. Finally, a Generalized Estimating Equations (GEE) model was carried out. The number of positive farms was assumed to follow a binomial distribution, and the municipality was included as a random factor. Differences were considered statistically significant when *p*-value < 0.05. Statistical analyses were performed using SPSS 25.0 software (IBM Corp., Armonk, NY, USA).
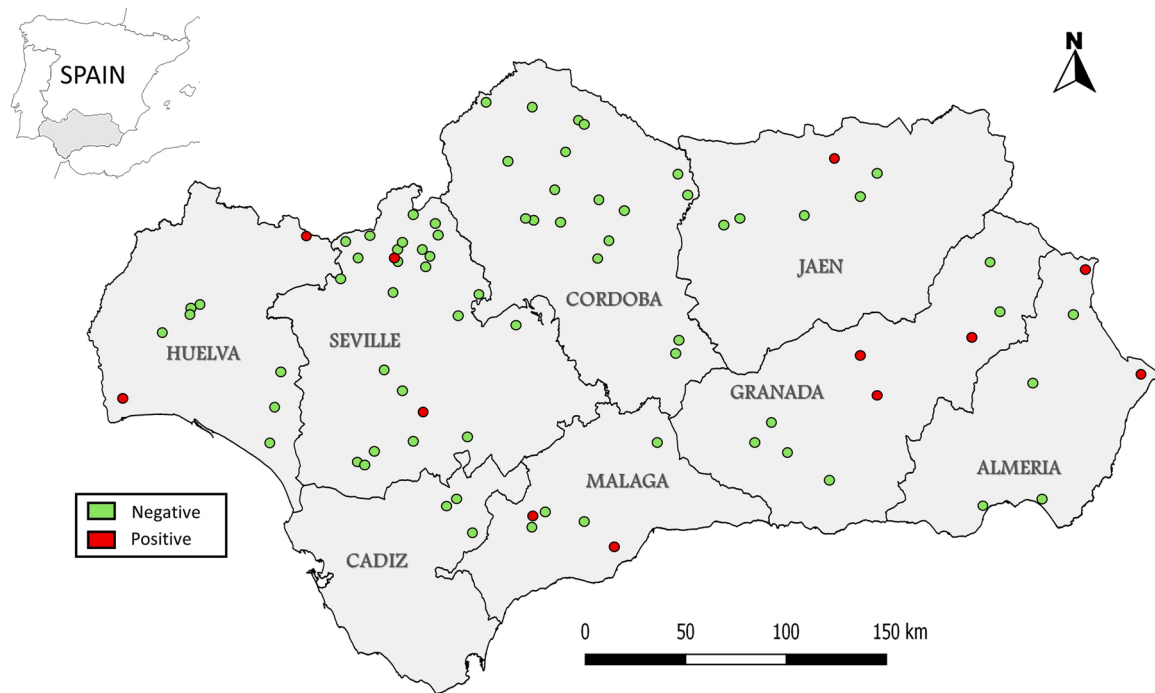
Fig. 1. Distribution of the sampled sheep farms in southern Spain. Red and green dots indicate MTC-positive and MTC-negative farms, respectively.

3. Results and discussion

TB is one of the most important infections shared at the livestock-wildlife interface in Iberian Mediterranean ecosystems (Jiménez-Ruiz et al., 2022). Members of the MTC are maintained in epidemiologically related communities involving both domestic (Napp et al., 2013; Cano-Terriza et al., 2018; Muñoz-Mendoza et al., 2016; Infantes-Lorenzo et al., 2020) and wildlife (Vicente et al., 2006; Gortázar et al., 2011; Gortazar and Boadella, 2014; Blanco-Vázquez et al., 2021) species.

To the best of the authors' knowledge, this is the first active TB surveillance study carried out worldwide to assess MTC exposure in sheep. Antibodies against MTC were detected in 16 (0.7%; 95%CI: 0.4–1.1) of the 2266 sheep tested. When the sensitivity and specificity of the test were considered in the analysis, the estimated true prevalence was even lower (0.29%, 95% posterior probability interval 0.01–1.05%), indicating a very low level of circulation of MTC members in sheep in the study region. Information available regarding MTC circulation in sheep farms is very limited. In European countries, most of the previous studies on TB in sheep have been focused on suspected TB-infected animals or farms (Malone et al., 2003; Marianelli et al., 2010; Muñoz-Mendoza et al., 2012; Van der Burgt et al., 2013; Vidal et al., 2018; Pérez de Val et al., 2021; Konold et al., 2020). In Spain, the previous serosurvey studies were conducted on farms or areas with high prevalence of MTC in cattle. Thus, Muñoz-Mendoza et al. (2016) detected antibodies against MTC in 41 (59.4%) of the 69 sheep that cohabited with TB-infected cattle or goats in flocks located in northwestern Spain. In addition, Infantes-Lorenzo et al. (2020) observed a seropositivity of 17.9% (698/3998) in sheep from cattle TB-hotspots in northern Spain, using the same iELISA employed in the present study. Although these results suggest a potential role of sheep as a reservoir of MTC in these particular epidemiological scenarios, our results indicate that in southern Spain sheep do not seem to have a significant role in the epidemiology of TB.

Anti-MTC antibodies were detected in animals from twelve (14.5%; 95%CI: 6.9–22.0) of the 83 sampled farms (Fig. 1). Moreover, at least one MTC-positive farm was found in six of the eight (75.0%) provinces and twelve of the 59 (20.3%) municipalities of the study region, indicating a widespread distribution of MTC on sheep farms in southern

Spain. It has been suggested that sheep may play a significant role in the epidemiology of TB when they share resources with other domestic species and the opportunities for interspecies interactions increase (Pesciaroli et al., 2014; Muñoz-Mendoza et al., 2016; Vidal et al., 2018; Infantes-Lorenzo et al., 2020). Of note, in nine of the 12 TB-positive farms detected in our study, the sheep cohabited or shared habitats with domestic goats, wild boar or red deer, which are well-known reservoirs of MTC in Spanish Mediterranean ecosystems (Gortázar et al., 2011; Napp et al., 2013; Pesciaroli et al., 2014). However, in our study, positive sheep farms were found spread across the study area and were not significantly associated with the presence of TB-hotspots in cattle (p = 0.505) or wild boar (p = 0.485). Only three of the 35 farms (8.6%) of Sierra Morena Mountains (north of the provinces of Cordoba, Seville, Huelva and Jaen), one of the highest TB-prevalence areas in both cattle and wild ungulates in Andalusia (MAPA, 2017, 2021; Cano-Terriza et al., 2018), were positive. Interestingly, seropositivity was not detected in any of the 10 mixed sheep-cattle farms analyzed, even though six of these farms had positive cattle during the sampling period. This result suggests that there was no MTC transmission from cattle to sheep, and supports the hypothesis that this small ruminant species is naturally more resistant to MTC infection than cattle, as previously proposed (Gelalcha et al., 2019). Nevertheless, further epidemiological and molecular studies on mixed farms are needed to support these hypotheses and ruled out the potential influence of other factors.

The GEE model identified the management system as a potential risk factor associated with MTC seropositivity. Thus, the risk of MTC exposure was 3.7 times higher (95%CI =1.1–12.42; p = 0.038,) in semi-extensive raised sheep farms than in extensive ones. Sheep managed under semi-extensive conditions remain grazing during the day, with a similar infection risk to those kept in strictly extensive systems, but also, they are usually housed overnight in sheds. Farming practices related to housing could explain the difference observed. In this regard, several deficiencies in biosecurity practices including storing of manure indoor or unproper feed storage have been identified as risk factors associated with MTC infection in livestock (Kaneene et al., 2002; Ramírez-Villaescusa et al., 2010; Walter et al., 2012). In fact, changes on farming practices to improve biosecurity have shown to be key measures to reduce the risk of TB breakdowns in cattle (Ciaravino et al., 2021). In

any case, further studies are needed to assess differences in the risk of MTC exposure between different management conditions in sheep farms.

In conclusion, our results confirm the circulation of MTC in sheep farms in southern Spain. The low individual and moderate farm seroprevalences suggest that sheep may play a limited role in the epidemiology of TB in the multi-host community of this region. The detection of seropositive sheep in areas with low TB prevalence in both cattle and wild boar, points out the need to carry out epidemiological and molecular studies to determine the origin of the infection in these epidemiological scenarios. Serosurveillance programs can be useful tools to identify where MTC is circulating so that control measures can be aimed at these risk scenarios or target farms, thereby optimizing animal TB control programs in multi-host Mediterranean ecosystems.

Ethical statement

The collection of blood samples analyzed in the present study was part of the official Animal Health Campaigns of the Regional Government of Andalusia, Spain. Therefore, no ethical approval was necessary.

Declaration of competing interest

The authors declare that they have no conflict of interest.

Data availability

The data that support the findings of this study are available from the authors upon reasonable request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.prevetmed.2023.105920.

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