



Mycobacterium tuberculosis complex in domestic goats in Southern Spain

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ABSTRACT

Tuberculosis (TB) is a zoonotic infectious disease caused by bacteria belonging to the *Mycobacterium tuberculosis* complex (MTC), which can affect a wide variety of domestic and wild animal species. Although the role of goats as a reservoir of MTC bacteria has been evidenced, information about the circulation of MTC strains in this species is still very scarce. The aim of the present study was to determine the seroprevalence, spatial distribution, risk factors and MTC spoligotypes circulating in goats from Andalusia (Southern Spain), the Spanish region with the largest goat census and a hotspot area of TB in both cattle and wild ungulates. A total of 2155 serum samples from 80 goat flocks were analyzed by an in-house ELISA using the P22 protein complex as a coating antigen. Antibodies against MTC were detected in 473 goats (21.9%, 95% CI: 20.2–23.7) and the true seroprevalence was 22.3% (95% CI: 20.6–24.1). Seropositivity was found in 72 (90.0%) of the 80 flocks analyzed. The generalized estimating equation model showed that the management system (higher seroprevalence on intensive and semi-intensive farms), and the presence of hospital pens inside the regular stables, were risk factors potentially associated with MTC exposure in goats in Southern Spain. The spatial analysis identified a significant spatial cluster ($p < 0.001$) in Eastern Andalusia. A total of 16 different MTC spoligotypes, including five of *M. caprae* and eleven of *M. bovis*, were identified in goats between 2015 and 2022 in the study area, with SB0157 as the most frequently isolated. The results obtained indicate widespread and non-homogeneous spatial distribution of MTC in goat herds from Southern Spain. The high individual and herd-level seroprevalence values found suggest that goats could play a significant role in the maintenance and transmission of MTC in the study area. Our results highlight the importance of implementing control measures in this species.

1. Introduction

Tuberculosis (TB) is one of the most important infectious diseases affecting humans, domestic animals, and wildlife worldwide, and is a clear example of a multi-host disease without geographic boundaries (Huang and Zhao, 2022). It is caused by members of the *Mycobacterium tuberculosis* complex (MTC), with *Mycobacterium bovis* and *Mycobacterium caprae* as the main species affecting animals (Malone and Gordon,

2017).

Cattle are considered the main domestic reservoir of MTC (Vayr et al., 2018) and have been subjected to TB eradication programs in several European countries for decades (Reis et al., 2021). Other domestic species including goats, which are particularly susceptible to MTC infection and often develop cavitory lesions shedding large numbers of mycobacteria, can also play a significant role in the epidemiology of MTC in certain epidemiological scenarios (Pérez de Val et al.,

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2011; Sánchez et al., 2011; Bezos et al., 2017). Transmission of MTC from goats to other livestock species has been documented in Europe (Napp et al., 2013; Zanardi et al., 2013; Vidal et al., 2018). Moreover, recent cases of human TB caused by *M. caprae* of goat origin reported in Greece (Papaventsis et al., 2021) and Spain (Martínez-Lirola et al., 2023) highlight the importance of this small ruminant as a zoonotic source of MTC.

Due to their adaptability to different farming systems, climatic conditions, terrains, and their ability to exploit low-quality resources, goats play an important socioeconomic role worldwide, including in different regions of the European Mediterranean Basin (Lu and Miller, 2019; Morales et al., 2019). Besides the implications of caprine TB in both animal and public health, disease-associated economic losses are also important, particularly in countries such as Spain, which, with 2.6 million goats, has the second-largest population of goats in the European Union (Espinosa et al., 2021; Eurostat, 2023). In Spain, goats are included in the bovine TB eradication program only when they share pastures or are epidemiologically related to cattle herds (MAPA, 2022). Nevertheless, in the last two decades, specific mandatory or voluntary goat TB control programs have been implemented in the goat populations of some Spanish regions. However, the geographical distribution of MTC bacteria in goat farms is still unknown in many areas not subjected to specific official control of this species. Moreover, no large-scale epidemiological studies have been carried out to assess the circulation of MTC bacteria in goat populations in Europe so far. In the present study, we aimed to determine the seroprevalence, spatial distribution, risk factors and MTC spoligotypes circulating in goats from Andalusia (Southern Spain), the Spanish region with the largest goat census and a hotspot area of TB in both cattle and wild ungulates.

2. Materials and methods

2.1. Study design and sample collection

A cross-sectional study was carried out to assess the circulation of MTC strains in goat farms in Andalusia (Southern Spain), just before the implementation of the current voluntary TB-eradication program in this species. The sample size was calculated assuming a herd prevalence of 20% (Espinosa et al., 2021) with a 95% confidence interval (95% CI) and an accepted error of 9%. A total of 80 farms were finally included in this study (Fig. 1). The sampling was stratified by province based on the proportion of goats in each province. Whenever possible, 30 animals were selected from each flock, to detect MTC exposure with a minimum expected seroprevalence of 10% (Espinosa et al., 2021) and a 95% CI. Within the herd animals were selected by systematic sampling (the first animal was randomly selected, and subsequent individuals were selected using a fixed sampling interval). Ultimately, a total of 2155 samples from goats were collected between 2015 and 2017. Blood samples were collected by jugular vein puncture using sterile tubes without anticoagulants (Vacutainer®, Becton-Dickinson, USA). Samples were centrifuged for 10 min at 400 g to obtain serum and stored at -20°C until serological analysis.

Epidemiological data (independent variables) related to the sampled animals and farms were gathered using a questionnaire administered to farmers. A total of 56 explanatory variables were included in the questionnaires to obtain information on exposure levels to potential risk factors associated with MTC infection in goat farms (Table S1).

2.2. Serological analysis

Antibodies against MTC bacteria were detected by an in-house indirect ELISA according to the previously described protocol (Bezos et al., 2018; Arrieta-Villegas et al., 2020) using the P22 protein complex as a coating antigen. P22 is an immunopurified subcomplex protein obtained

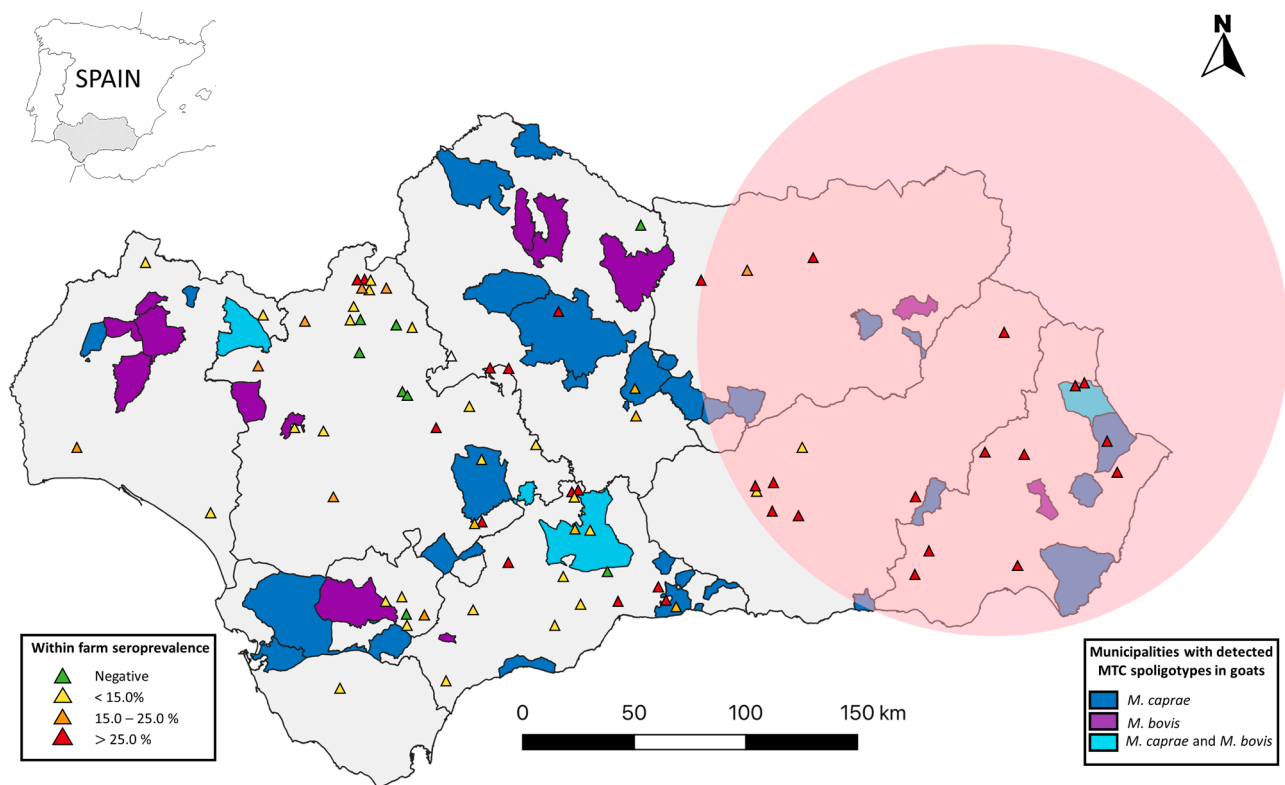


Fig. 1. Distribution of the sampled goat farms in Andalusia (Southern Spain). Color gradation shows within-farm seropositivity. The light red dots on the map indicate statistically significant spatial clusters ($p < 0.001$).

from bPPD (CZ Veterinaria SA, Porriño, Spain) (Infantes-Lorenzo et al., 2017), which has been previously used and validated as a coating antigen in ELISAs to detect the presence of anti-MTC antibodies in various domestic and wild species, including goats (e.g., Casal et al., 2017; Infantes-Lorenzo et al., 2018, 2020a; Thomas et al., 2019a,b; Arrieta-Villegas et al., 2020). The estimated sensitivity (Se) and specificity (Sp) of this ELISA in goats were 74% and 93%, respectively (Arrieta-Villegas et al., 2020).

2.3. Statistical analysis

Individual seroprevalence was estimated from the ratio of ELISA-positive samples to the total number of goats and farms examined, with 95% exact binomial CI. The seroprevalence estimate was also adjusted for P22 iELISA Se and Sp using the Rogan–Gladen estimator (Rogan and Gladen, 1978). Moreover, an intercept-only generalized estimating equation (GEE) binomial logistic regression model, with the herd as the subject variable, was used to adjust the estimated seroprevalence and 95% CI for clustering at the herd level (Dohoo et al., 2003).

Possible associations between individual seroprevalence and explanatory variables were initially analyzed with a univariable analysis using Pearson's chi-squared test or Fisher's exact test, as appropriate. For the continuous variables, the assumption of linearity was tested, and if the assumptions were not found, the variables were categorized taking the percentiles 33 and 66 as cut points in order to homogenize the scales of the explanatory variables. All explanatory variables (Table S1) were analyzed, and those with a p -value < 0.10 were selected as potential risk factors. Collinearity between pairs of variables was tested by Cramer's V coefficient. The selected variables were included in a GEE model to assess the risk factors potentially associated with MTC exposure in goats. 'Herd' was included as the subject variable, and the number of seropositive animals was assumed to follow a binomial distribution. An exchangeable correlation structure was chosen for the models and robust standard errors were used. The model was re-run until all remaining variables presented statistically significant values ($p < 0.05$). A manual forward stepwise approach was used, starting with the variable with the lowest p -value in bivariate analysis. At each step, the confounding effect of the included variable was assessed by computing change in odds ratios (OR) greater than 30%. For the choice of the best model, the quasi-likelihood under the independence model criterion (QIC) was considered. SPSS 28.0 software (Statistical Package for Social Sciences, Inc., Chicago, IL, USA) was used for statistical analyses.

2.4. Spatial analysis

A spatial cluster analysis was carried out using a Bernoulli model to detect significant clusters of MTC exposure in goat herds (Kulldorff et al., 2006). The number of Monte Carlo simulations was set to 999 for the cluster scan statistic. Analyses were performed using SaTScan™, v10.1.2. Clusters were considered to be significant at $p < 0.05$.

2.5. Identification of circulating spoligotypes

A molecular epidemiological study based on the MTC spoligotypes recorded by the Spanish Ministry of Agriculture, Fisheries and Food (mycoDB.es database; Rodríguez-Campos et al., 2012), was conducted to determine circulating spoligotypes in goats in the study area during the study period 2015–2022. These spoligotypes were isolated from tissue samples of domestic goats that exhibited TB-like lesions (TBLLs) at routine inspections in slaughterhouses in the study area collected by official government veterinarians. These samples were submitted to the TB-official laboratory for microbiological and molecular analyses. Briefly, following decontamination with a final concentration of 0.37% hexadecylpyridinium chloride (Corner and Trajstman, 1988), the samples cultured in Coletsos and 0.2% (w/v) pyruvate-enriched Löwenstein-Jensen media (Dismalab, Madrid, Spain). Positive cultures

were identified as members of the MTC using PCR amplification of the *Mycobacterium* genus-specific 16 S rRNA fragment and MPB70 or IS6110 sequences (Wilton and Cousins, 1992; Lorente-Leal, 2021). Spoligotyping of isolates were performed using standardized membranes with 43 spacers, as described (Kamerbeek et al., 1997).

3. Results

The apparent individual seroprevalence of MTC was 21.9% (473/2155; 95% CI: 20.2–23.7) and the true seroprevalence was 22.3% (95% CI: 20.6–24.1). After adjusting for clustering, the estimated individual seroprevalence was 21.1% (95% CI: 17.9–24.8). At least one MTC-seropositive animal was detected in 90.0% (72/80; 95% CI: 83.4–96.6) of the farms analyzed and the within-flock seroprevalence ranged from 3.3% to 70.0% (median 20.0%; mean 23.0%).

The explanatory variables obtained from the epidemiological questionnaire are shown in Table S1. A total of 30 variables had an association ($p < 0.10$) with the seropositivity against MTC. The GEE model showed that the main risk factors associated with MTC seropositivity on goats were: the management system (higher seroprevalence in intensive and semi-intensive farms) and the presence of hospital pens inside the regular stables (Table 1).

At least one seropositive goat was detected in 56 (91.8%) of the 61 municipalities sampled. The spatial analysis identified a statistically significant spatial cluster [$p < 0.001$, Relative risk (RR): 2.6] in Eastern Andalusia. The cluster, with a radius of 138.3 km, included 21 goat farms (Fig. 1).

A total of 73 TBLLs from 51 goat flocks were positive for MTC by culture. Among these, 46 (63.0%) were identified as *M. caprae*, and the remaining 27 (37.0%) as *M. bovis*. To avoid sampling bias, only one spoligotype of each type per herd and date was considered to determine the frequency of spoligotypes. The spoligotyping analysis revealed the presence of 16 different spoligotypes. The spoligotype most frequently detected was SB0157 (54.8%), followed by SB2232 and SB0295 (6.8%); SB0134 and SB0120 (5.5%); SB0121 (4.1%), SB2560 and SB1081 (2.7%). Other spoligotypes including SB2052, SB0140, SB1053, SB1095, SB1135, SB1361, SB1610 and SB1618 were also identified (Fig. 2).

4. Discussion

Even though the role of goats in the epidemiology of TB has been pointed out in different epidemiological scenarios, this species is not yet subjected to compulsory eradication programs at the European level (Roy et al., 2020). In Spain, some regions have specific goat TB control programs, but the knowledge about the epidemiology of MTC in this species, which is a key point to implementing appropriate control measures, is still very limited.

The official in-vivo diagnostic tests used in TB eradication programs are the single and comparative intradermal tuberculin tests (SIT and SCIT, respectively), and the IFN- γ release assay (IGRA) (MAPA, 2022). Although serological assays based on humoral response have limitations

Table 1

Results of the generalized estimating equation analysis of potential risk factors associated with *Mycobacterium tuberculosis* complex seropositivity in goats in Southern Spain.

Variable	Category	B	OR	95% CI	p-value
Management system	Intensive	1.376	4.0	1.6–9.5	0.002
	Semi-intensive	1.196	3.3	1.4–7.8	0.006
	Extensive	^a	^a	^a	^a
Hospital pen inside the regular stables	Yes	0.527	1.7	1.1–2.7	0.027
	No	^a	^a	^a	^a

^a Reference category.

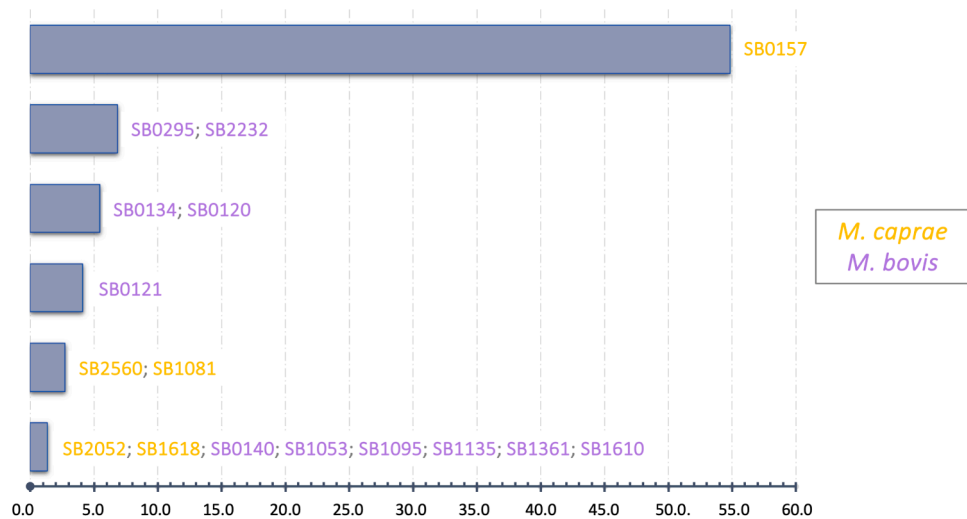


Fig. 2. Frequencies of spoligotypes of *Mycobacterium tuberculosis* complex isolated in goats in Andalusia (Southern Spain) during the period 2015–2022. The spoligotypes represented in yellow and purple are identified as *M. caprae* and *M. bovis*, respectively.

for the detection of anti-MTC antibodies in recently infected animals (Pollock and Neill, 2002), ELISA has been shown to be a useful TB diagnostic tool for surveillance studies in different wildlife and domestic species such as wild boar (*Sus scrofa*), red deer (*Cervus elaphus*), alpaca (*Vicugna pacos*), llama (*Lama glama*), pig, sheep or goats (Infantes-Lorenzo et al., 2018, 2019; Thomas et al., 2019a,b; Arrieta-Villegas et al., 2020; Infantes-Lorenzo et al., 2020a). Moreover, serology may improve the detection of infected animals, specifically when there is an inadequate cellular immune response (Bezós et al., 2018). Another factor to consider in the diagnosis of TB is the use of vaccination against *Mycobacterium avium* subsp. *paratuberculosis* (MAP), a common practice in small ruminants to prevent the clinical course of the disease (Bastida and Juste, 2022). In this regard, the in-house ELISA used in the present study, based on a multiprotein complex named P22, shows high levels of Sp, with no significant cross-reactivity with other mycobacteria (Roy et al., 2018; Arrieta-Villegas et al., 2020). This is further substantiated by the absence of statistically significant differences ($p = 0.448$) in seroprevalence between the animals vaccinated against MAP (20.9%; 47/178) and those non-vaccinated (21.6%; 402/1467). In summary, considering that ELISA is a fast and cost-effective method (Infantes-Lorenzo et al., 2019), conducting serosurveillance studies is useful for understanding the epidemiological situation of this disease in species not subjected to eradication programs.

The individual seroprevalence obtained in the present study (21.9%) indicates high MTC exposure in goats in Southern Spain, a hotspot area of TB in both cattle and wild ungulates. The health status of goats regarding TB remains largely unknown worldwide, and only a few studies have been carried out to assess the MTC circulation in goat populations so far (Tafess et al., 2011; De Cardona et al., 2016). A lower individual prevalence (0.4%) compared to our study was found in Castilla y León (CyL; Northwest Spain) using single intradermal tuberculin test (Espinosa et al., 2021). Differences between our study area and CyL could be related to the epidemiological context or the fact that CyL is implementing a caprine TB eradication program since 2002 (BOCyL, 2010). It is noteworthy that during the study period, the average individual and herd TB prevalences of cattle in CyL (0.1% and 1.6%, respectively) were 14 and 10 times lower compared to Andalusia (1.8% and 15.6%, respectively) (MAPA, 2015, 2016, 2017). A voluntary caprine TB control program in Andalusia is implemented since 2018 (BOJA, 2018), just after the serosurvey study period (2015–2017). Future studies are warranted to assess the effectiveness of the caprine TB control program in Andalusia.

Goats raised under intensive and semi-intensive management

systems had significantly higher risk of MTC exposure than those extensively-reared (Table 1). Direct transmission of MTC through respiratory droplets expelled during coughing, sneezing, or nose-to-nose contact, is one of the main infection routes in goats as the presence of cavitory lesions, common in this species, significantly increases the shedding of mycobacteria (Sánchez et al., 2011; Deresa et al., 2013; Zanardi et al., 2013; Roy et al., 2020). When animals are housed indoors, the direct transmission of MTC could increase due to the higher concentration of animals and infected aerosols (Ameni et al., 2006). In this sense, adequate ventilation in indoor pens is a key point for preventing of airborne pathogens transmission in intensive and semi-intensive management systems (Roland et al., 2016; Quintana et al., 2020).

The isolation of sick animals in hospital pens is considered a useful biosecurity measure to reduce the transmission of infectious pathogens in livestock (Edwards, 2010; Bellini, 2018). However, this measure must be complemented by other additional measures to prevent direct and indirect transmission from the hospital pen to other facilities (Renault et al., 2018; Damiaans et al., 2019). In our study, goats from farms with the hospital pen inside the regular stables had 1.7 times higher risk of being MTC seropositive than those from herds with the hospital pen located outside this facility. Since isolation in the hospital pen usually involves permeable physical barriers, such as fencing, there is still a risk of transmission of airborne pathogens, such as MTC. It should be noted that goats infected with MTC are usually asymptomatic or have non-specific clinical symptoms (Daniel et al., 2009; Romha et al., 2018). Therefore, goats are likely to be housed in hospital pens due to clinical signs caused by other pathogens, but given the seroprevalence of MTC found in goats in our study, a significant proportion of them will also be infected by MTC bacteria. Co-infections with other pathogens may lead to immunosuppression in the animals, resulting in increased shedding of mycobacteria, and if those animals are housed indoors, favor the transmission within the herd. Further investigations should be conducted to assess biosecurity measures on those herds with the hospital pen inside the regular stables to identify improvement possibilities.

The high herd seroprevalence (90.0%) found in the present study indicates a widespread distribution of MTC on goat farms in Southern Spain. Positive farms were detected across the study area, but their spatial distribution was not homogeneous. The spatial analysis showed a significant cluster in Eastern Andalusia (Fig. 1). It should be noted that the density of both cattle and wild ungulates as well as the prevalence of TB in these species in Eastern Andalusia are lower than in the Western area (MAPA, 2017; CSMEA, 2022). The high within-flock seroprevalence (37.0%) found within the cluster suggests that goats can play a

potential role as reservoir of MTC in this area. Additional studies are needed to better understand the factors that contribute to the increased MTC exposure in goats in the risk area identified in the present study.

Different molecular typing tools have been employed for TB epidemiological studies, including spoligotyping (Reis et al., 2021). This technique identifies polymorphism within the genomic direct repeat locus, comprising multiple sequences and non-repetitive spacer sequences (spacers). MTC strains can be grouped into distinct spoligotypes hinging on variances in the existence or absence of spacers (Kamerbeek et al., 1997). Although spoligotyping has some limitations compared to other molecular techniques, this technique is considered a powerful method to study the molecular epidemiology of MTC being a useful tool for decision-making within TB control programs (Gupta and Anupurba, 2012; García-Jiménez et al., 2016; Price-Carter et al., 2018). In the present study, a total of 16 different MTC spoligotypes from 73 TBLLs were detected in goats from Southern Spain; 63.0% of them were identified as *M. caprae* and 37.0% as *M. bovis* (Fig. 2). The high diversity of spoligotypes found in this species may be associated with the movement of animals between different regions, as previously suggested (Rodríguez et al., 2011). In this context, compulsory pre-movement TB diagnosis in goats within the EU was not carried out until it was specified in the Animal Health Law (Regulation EU 2016/429), which came into effect in April 2021. Of note, the most prevalent spoligotype identified in the present study was SB0157, which is also the most common spoligotype of *M. caprae* in Spain (Rodríguez et al., 2011; Espinosa et al., 2021), and one of the most frequent MTC-spoligotypes detected in wild boar in the study area (CSMEA, 2022). In Spain, SB0157 has also been isolated in other domestic and wildlife species, including domestic pig, sheep, dromedary camel (*Camelus dromedarius*) and red deer (Rodríguez et al., 2011; Cano-Terriza et al., 2018; Infantes-Lorenzo et al., 2020b). These findings highlight the importance in the TB-multi host community of Southern Spain of this and other *M. caprae* spoligotypes, such as SB1081, also detected in goats in the present study but previously found in wild and other domestic animals in Spain (Rodríguez et al., 2011; García-Jiménez et al., 2013; Varela-Castro et al., 2021). Of note, 11 different spoligotypes of *M. bovis* were also identified in goat farms from Southern Spain. This result is in accordance with that previously reported in this species (Shanahan et al., 2011; Zanardi et al., 2013; Di Marco et al., 2022), and highlights the importance of the bovine-goat interface in the epidemiology of TB in southern Spain.

This study has some limitations that should be considered when interpreting the results obtained. First, moderate to low correlation has been previously observed among cellular-based MTC diagnostic techniques (such as SIT or IGRA) and humoral-based ones (ELISA) (Casal et al., 2014; Bezos et al., 2018). Hence, discrepancies among studies should be compared carefully. And second, as the identification of spoligotypes circulating in goats in the study area was based on data recorded in the Spanish Database of Animal Mycobacteriosis during the period 2015–2022, the relatively low number of spoligotypes officially reported from this species could bias the diversity of circulating spoligotypes. Collectively, further investigations incorporating culture, spoligotyping, and complementary molecular techniques with a larger sample of TB-positive goat herds, are warranted for better understanding the MTC genetic diversity in this species.

In conclusion, the serological and molecular results obtained in the present study indicate that goats can play a role in the maintenance of MTC in Southern Spain. Moreover, our results increase the knowledge of TB epidemiology in Europe and highlight the importance of implementing control measures for this species, particularly in the identified high-risk areas. On-farm biosecurity measures may prove effective in reducing TB transmission on goat farms.

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 by an Institutional Animal Care and Use Committee was deemed necessary.

CRedit authorship contribution statement

David Cano-Terriza: Writing – review & editing, Supervision, Project administration, Methodology, Conceptualization. **Remigio Martínez:** Writing – review & editing, Investigation, Data curation. **Mercedes Domínguez:** Writing – review & editing, Validation, Methodology. **Sebastián Napp:** Writing – review & editing, Supervision, Formal analysis. **Inmaculada Moreno:** Writing – review & editing, Validation, Methodology. **Beatriz Romero:** Writing – review & editing, Validation, Methodology, Investigation. **Déborá Jiménez-Martín:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Data curation. **María A. Risalde:** Writing – review & editing, Validation, Supervision, Methodology, Conceptualization. **Ignacio García Bocanegra:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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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.2024.106204.

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