



## Epidemiology of paratuberculosis in sheep and goats in southern Spain

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### ABSTRACT

Paratuberculosis is a worldwide, chronic infectious disease caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP) that mainly affects ruminant species. This disease has a significant economic impact on small ruminant production due to the costs of implementing control measures and production losses. A cross-sectional study was carried out to determine the seroprevalence, spatial distribution and risk factors associated with MAP exposure in sheep and goats in Andalusia (southern Spain). Serum samples from 4134 small ruminants (2266 sheep and 1868 goats) in 153 flocks were tested by an in-house ELISA for antibodies against MAP using paratuberculosis protoplasmic antigen 3 (PPA3) as coating antigen. Antibodies against MAP were detected in 8.1% (183/2266; 95% CI: 7.0–9.2%) of sheep and 20.0% (374/1868; 95% CI: 18.2–21.8%) of goats. The true individual seroprevalence was 8.4% (95% CI: 6.9–10.1%) in sheep and 25.2% (95% CI: 22.7–27.8%) in goats. Seropositivity was detected in 66.3% (55/83; 95% CI: 56.1–76.4%) of sheep herds and 90.0% (63/70; 95% CI: 83.0–97.0%) of goat herds. Spatial analysis identified three statistically significant clusters ( $p < 0.05$ ) associated with areas with higher seroprevalence of MAP. The main risk factors potentially associated with MAP exposure were: species (goat) and absence of perimeter livestock fencing. The results of this study show that MAP is widespread in small ruminant populations in southern Spain and suggest that goats may play a more important role than sheep in the transmission and maintenance of MAP. Because of animal health concerns and the economic consequences of paratuberculosis, appropriate surveillance and control programs are required to reduce the risk of MAP infections in small ruminant flocks in this country.

### 1. Introduction

Paratuberculosis, or Johne's disease, is a globally distributed chronic infectious disease caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP), which mainly affects domestic and wild ruminants (Windsor, 2015). MAP transmission is mainly via the fecal-oral route and occurs by ingesting fecal material from the environment, contaminated food or water, and, in young animals, through contact with MAP-contaminated

teat skin surfaces (Windsor, 2015; Stonos et al., 2017). MAP can also be transmitted through the consumption of milk or colostrum from infected animals or by intrauterine infection (Windsor, 2015).

Clinical paratuberculosis is the terminal phase of chronic subclinical infection. However, even when the clinical outcomes appear after a long asymptomatic period, infected animals can eliminate MAP in their feces from early stages of infection, which hinders the control of the disease (Windsor, 2015; Stonos et al., 2017). Once MAP has entered a herd, it

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often goes undetected until it has spread and become endemic, making it very difficult to control (Gupta et al., 2019; Whittington et al., 2019). Infection is usually milder but more insidious in sheep and goats than in cattle (Windsor and Whittington, 2010). In small ruminants, clinical paratuberculosis can result in significant weight loss, diarrhea in advanced stages of the disease, premature culling and even death (Windsor et al., 2015). The disease causes significant economic losses in small ruminant flocks as a result of reduced dairy production, decreased animal weight, the cost of diagnosing and controlling the disease and reproductive disorders (Mendes et al., 2004; Kostoulas et al., 2006; Sardaro et al., 2017; Windsor, 2015).

Spain, with 15.4 million sheep and 2.6 million goats, is the country with the largest sheep population and the second largest goat population in the European Union (EUROSTAT, 2020a, b). Small ruminant production is an important driver of Spain's economy, accounting for around 12% of total animal husbandry production (MAPA, 2021). This sector also plays a key role in preserving the natural ecosystem, as well as the sustainability of rural communities. Although MAP infection has been detected on small ruminant farms in many countries around the world (Windsor, 2015; Iarussi et al., 2019; Khamassi Khbou et al., 2020), there are very few epidemiological studies on paratuberculosis in small ruminants in Spain, and most of these have been carried out in geographically restricted areas (Table 1). The objective of this study was to assess the seroprevalence, spatial distribution and risk factors associated with MAP seropositivity in sheep and goats in southern Spain.

## 2. Materials and methods

### 2.1. Study design and sampling

A cross-sectional study of small ruminant farms in Andalusia (36°N-38° 60' N, 1° 75' W-7° 25' W) (southern Spain) was carried out between 2015 and 2017. Based on the number of small ruminant farms in the study region ( $n > 10,000$ ), the sample size was calculated assuming a herd prevalence of 50%, which provides the highest sample size in studies based on unknown prevalence with a 95% confidence interval (95% CI) and accepted error of 8%, giving 151 farms to be sampled

**Table 1**

Seroprevalence of MAP by ELISA in sheep and goats in different European countries.

Species	Country	No. positive/ Overall	% Seropositive	Reference
Sheep	Cyprus	340/3429	9.9	Liapi et al. (2011)
	Germany	212/1473	14.0	Stau et al. (2012)
	Italy	129/2086	6.2	Attili et al. (2011)
	Italy	74/13,714	2.8	Iarussi et al. (2019)
	Portugal	144/3900	3.7	Coelho et al. (2007)
	Spain	261/4504	5.8	Aduriz et al. (1994)
	Spain	25/286	8.7	Tejedor, 1993
	Spain	183/2266	8.1	Present study
	Turkey	28/450	6.2	Buyuk et al. (2014)
	Turkey	72/150	48.0	Celik and Turutoglu (2017)
Goats	Cyprus	362/4582	7.9	Liapi et al. (2011)
	France	347/11,847	2.9	Mercier et al. (2010)
	Germany	28/136	21.0	Stau et al. (2012)
	Italy	20/294	6.8	Galiero et al. (2017)
	Italy	17/320	5.3	Corrias et al. (2012)
	Italy	27/269	10.0	Cecchi et al. (2019)
	Italy	134/7377	15.7	Iarussi et al. (2019)
	Spain	57/219	26.0	Falconi et al. (2010)
	Spain	51/670	7.6	Astorga-Márquez et al. (2014)
	Spain	511/3312	22.5	Barrero-Domínguez et al. (2019)
	Spain	363/1868	20.0	Present study
	Turkey	36/150	24.0	Celik and Turutoglu (2017)

(Thrusfield, 2018). A total of 153 flocks (83 sheep farms and 70 goat farms) in 98 municipalities were finally included in the study (Fig. 1). Sampling was stratified by province based on the proportion of sheep and goats in each province. Farms were selected by simple random sampling from official flock registers obtained from the Regional Government of Andalusia. Farms that had previously implemented vaccination programs against MAP were not included in this study. The herd size of the selected farms ranged from 14 to 1904 animals (mean: 442; median: 377) on goat farms and from 34 to 3214 (mean: 544; median: 391) on sheep farms. Whenever possible, thirty animals were selected from each flock, using systematic sampling to detect MAP exposure with a minimum expected prevalence of 10% and a 95% CI. The mean number of serum samples collected per farm was 27 (median: 30), ranging from 14 to 30. Finally, blood samples were collected from 4134 small ruminants, comprising 2266 sheep and 1868 goats.

Samples were obtained by puncture of the jugular vein using sterile tubes without anticoagulant (Vacutainer®, Becton-Dickinson, USA) and transported to the laboratory under refrigeration within 24 h of sampling. Samples were centrifuged at 400 g for 10 min and the serum obtained was stored at  $-20^{\circ}\text{C}$  until analysis.

### 2.2. Questionnaire design

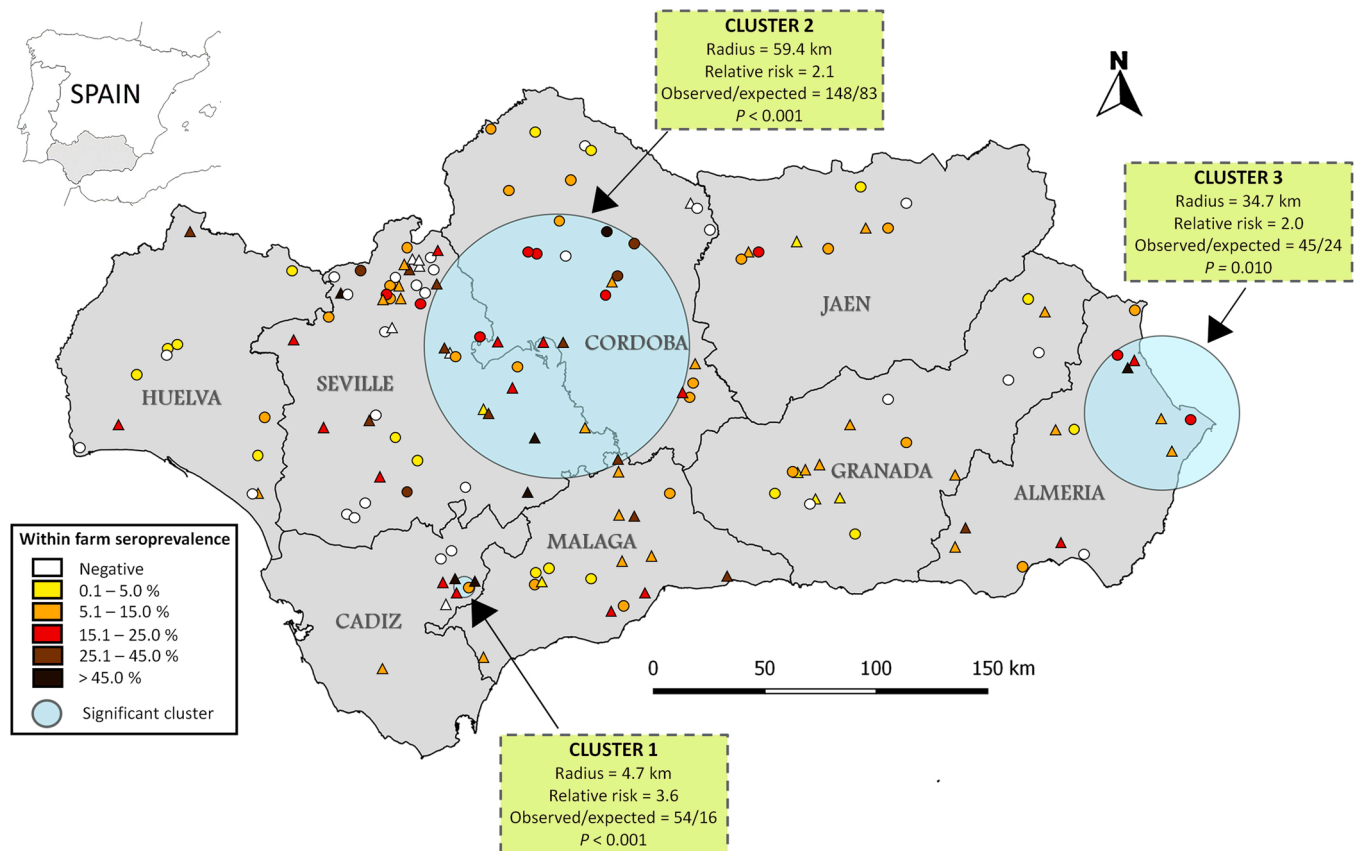
An epidemiological questionnaire was administered during sampling by means of on-farm interviews with the farmers to obtain flock and animal data. Close-ended questions were used to prevent ambiguous or vague answers. The questionnaire was first tested by members of the research group. A pilot test was then carried out, consisting of visits to five different small ruminant farms and personal interviews with farmers. A total of 59 explanatory variables were collected to obtain information on levels of exposure to possible risk factors associated with paratuberculosis on farms. The explanatory variables were grouped as follows: (a) individual data; (b) general and production data of the farm; (c) biosecurity and health measures. Climatological data (mean annual rainfall and mean annual temperatures) were also obtained from the National Meteorological Institute (Ministry for Ecological Transition and Demographic Challenge (Spain)).

### 2.3. Laboratory analysis

Serum samples were tested by an in-house indirect enzyme-linked assay (ELISA) to determine the presence of MAP-specific antibodies using the paratuberculosis protoplasmic 3 antigen (PPA3) as coating antigen (Allied Monitor®, Fayette, MO, USA). This ELISA has been described previously for different species, with minor modifications (Reyes-García et al., 2008; Boadella et al., 2010, 2011). Briefly, 96-well plates (NUNC® MicroWell™, Roskilde, Denmark) were coated with 10 µg/ml PPA3 complex diluted in carbonate buffer (Sigma®, Barcelona, Spain), and stored overnight at 4°C. The wells were subsequently washed with phosphate buffer saline (PBS) with 0.05% Tween-20 (PBST). Blocking solution (BS) (5% skim milk powder in PBS) was added, then incubated for 60 min at room temperature (RT). The plates were subsequently emptied, the sera were added at a dilution of 1/100 in BS and incubated for 60 min at 37°C. After three washes with PBST, the plates were incubated in the dark at RT for 30 min with horseradish peroxidase (HRP)-conjugated rabbit anti-sheep IgG (H+L) (SouthernBiotech®, Birmingham, USA) at 1/2000 in BS. After four washes with PBST, the plates were incubated in the dark at RT for 20 min with substrate solution (Fast OPD, Sigma®, Barcelona, Spain). The reaction was stopped with H<sub>2</sub>SO<sub>4</sub> (3 N) and optical density (OD) was measured at 450 nm using a spectrophotometer.

Positive and negative control sera from goats and sheep positive and negative respectively for MAP culture were validated by PPA3-ELISA (OD > 1 for positive and OD ≤ 0.2 for negative) and added in quadruplicate to each plate.

The OD values for each serum were converted to a blocking



**Fig. 1.** Distribution of the sampled small ruminant farms. Triangles and circles represent goat and sheep flocks sampled, respectively. The color grading shows within-farm seropositivity. Light blue dots represent the three significant spatial clusters observed in the study area ( $p < 0.05$ ).

percentage (E%) using the formula:  $\text{sample E\%} = [\text{OD sample} / (2 \times \text{mean OD negative control}) \times 100]$ . The cut-off value was defined as the value maximizing the sum of Se and Sp, and was calculated using a ROC analysis based on the Clopper-Pearson method (Graphpad prism 5.0, USA). The presence or absence of PTB lesions combined with PCR detection of MAP in intestinal tissues was employed as a reference standard (Delgado et al., 2012). The cut-off value was defined as the ratio of the mean sample OD to twice the mean OD of the negative control (Boadella et al., 2010). A cut-off value set at 94 E% had both higher Se (73.3%) and higher Sp (97.9%) and was chosen as the cut-off. Samples with an E% greater than 94 were considered positive and those between 80% and 94% as doubtful. All serum samples with positive or doubtful results were retested twice. A sample was considered positive only when it was positive in two tests.

#### 2.4. Statistical analysis

The individual apparent prevalence of antibodies against paratuberculosis was estimated from the proportion of seropositive samples to the total number of animals examined with a 95% CI. Coefficients and standard error values generated by an intercept only generalized estimating equation (GEE) binomial logistic regression model, with herd as subject variable, were used to adjust the estimated seroprevalence and 95% CI for clustering at herd level (Dohoo et al., 2003). The seroprevalence estimate was also adjusted for PPA3 ELISA Se and Sp using the Rogan–Gladden estimator (Rogan and Gladen, 1978). In order to detect nonlinear relationships and to homogenize the scales of the explanatory variables, continuous variables were transformed into qualitative variables with three categories, considering the 33rd and 66th percentiles as cut-off points.

Associations between serological results (dependent variable) and

the different explanatory variables in the epidemiological questionnaire were analyzed as follows. First, a bivariate GEE analysis was performed. The number of seropositive animals was assumed to follow a binomial distribution and herd was used as the subject variable. An exchangeable correlation structure was chosen for the models and robust standard errors were used. Variables with a  $p$ -value  $< 0.05$  in bivariate analysis were selected as potential risk factors. Second, Cramer's V coefficient was calculated between pairs of variables to prevent collinearity. When collinearity was detected (Cramer's V coefficient  $\geq 0.60$ ) the variable with the strongest *priori* biological association with MAP was retained. Given the large number of explanatory variables, the four data subsets (A. Individual data; B. General production data of the farm; C. Biosecurity and health parameters; D. Climatological variables) were analyzed separately. Finally, multivariable analysis was carried out using a GEE model to study the effect of potential variables selected from bivariate analysis. Forward selection of variables 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 calculating the change in odds ratio (OR). The model was re-run until all remaining variables showed statistically significant values (likelihood-ratio Wald test,  $p < 0.05$ ) and a potential relationship with the response variable. For the choice of the best model, the quasi-likelihood under the independence model criterion (QIC) was considered. All statistical analyses were performed using SPSS v25.0 software (IBM Corp., Armonk, NY, USA).

#### 2.5. Spatial cluster analysis

The Bernoulli spatial scan statistic was used to detect the presence of aggregations of small ruminant flocks with significantly higher MAP seropositivity (Kulldorff et al., 2006). The maximum spatial window size

was set at 50% of the surface of the study region and the number of Monte Carlo simulations was set at 999. Analyses were run using SaTScan™, v9.6. Clusters were considered to be significant at  $p < 0.05$ .

### 3. Results

Overall apparent individual seroprevalence was 13.5% (557/4134; 95% CI: 12.4–14.5%). Antibodies against MAP were found in 8.1% (183/2266; 95% CI: 7.0–9.2%) of the sheep and 20.0% (374/1868; 95% CI: 18.2–21.8%) of the goats. After adjustment for clustering, the estimated individual seroprevalence was 7.9% (95% CI: 5.8 – 10.7%) in sheep and 19.4% (95% CI: 15.7–24.7%) in goats. Finally, Se and Sp values were used to calculate the true seroprevalence in sheep (8.4%; 95% CI: 6.9 – 10.1%) and goats (25.2%; 95% CI: 22.7 – 27.8%).

A total of 118 out of 153 farms (77.1%; 95% CI: 70.5–83.8%) were MAP-seropositive. Seropositivity was detected in 66.3% (55/83; 95% CI: 56.1–76.4%) of sheep flocks and 90.0% (63/70; 95% CI: 83.0–97.0%) of goat flocks. Within-flock seroprevalence ranged from 3.3% to 71.0% (median 7.1%; mean 11.9%) in sheep and from 3.3% to 90.0% (median 13.3%; mean 20.3%) in goats. Significantly higher seropositivity was observed in goats than in sheep, both at individual ( $p < 0.001$ ) and farm ( $p < 0.001$ ) levels.

Seropositive animals were detected in 86 (87.8%) of the 98 municipalities included in the study. The Bernoulli model identified three statistically significant clusters in the study area ( $p < 0.05$ ) (Fig. 1). The first cluster, with a radius of 4.7 km, was located in the Grazalema Natural Park (Cadiz province) and included four farms (Relative Risk (RR) = 3.6;  $p < 0.001$ ). The second cluster, with a radius of 59.4 km, was located in central Andalusia, between the provinces of Cordoba and Seville, and contained 25 farms (RR = 2.1;  $p < 0.001$ ). Finally, the third cluster, with a radius of 34.7 km, included six farms (RR = 2.0;  $p = 0.010$ ) located in eastern Andalusia (Almeria province).

The explanatory variables obtained from the epidemiological questionnaire and the results of the bivariate analysis are summarized in Table S1. A total of 11 explanatory variables were selected in the bivariate analysis ( $p < 0.10$ ). The final GEE model identified two potential risk factors associated with MAP infection in small ruminants in Andalusia: species and the absence of livestock perimeter fencing. Significantly higher seropositivity was observed in goats than in sheep. Similarly, the prevalence of MAP antibodies was significantly increased in animals from farms without livestock perimeter fencing compared to those did have livestock perimeter fencing (Table 2; Table S1).

### 4. Discussion

Paratuberculosis is an OIE-listed disease and must be reported to the OIE as indicated in the Terrestrial Animal Health Code (OIE, 2021a). Nevertheless, at country level, the obligation to notify depends on the species, and cases in small ruminants are not always systematically reported. Whittington et al. (2019) found that paratuberculosis was underreported in 26 of the 35 countries surveyed where it was notifiable. This could have been due to the farmers' lack of knowledge, their concerns about the consequences of reporting or the veterinary challenge of diagnosing non-clinical cases. Among other reasons, this would explain why only 64 cases of paratuberculosis in small ruminants (61 in

sheep and three in goats) have been reported to the OIE in Andalusia over the last decade, the last notification being in 2013 (OIE, 2021b).

To the best of the author's knowledge, this is the first large-scale study to jointly assess MAP exposure in two small ruminant species in Spain. The individual true seroprevalences found in sheep (8.4%) and goats (25.2%) are within the ranges previously reported in other European countries (Table 1). The last studies on MAP carried out in sheep in the study country were developed two decades ago, so that there is no up-to-date information available. Nevertheless, the values are very similar to those observed in central Spain between 1990 and 1991 (Tejedor, 1993) and slightly higher than those found in northern Spain in 1994 (Aduriz et al., 1994). In goats, the individual seroprevalence detected in the present study was similar to those found previously by Falconi et al. (2010) in northern Spain (26.0%) and also to the 22.5% observed on goat dairy farms in southern Spain (Barrero-Domínguez et al., 2019). However, Astorga-Márquez et al. (2014) found a lower individual seroprevalence (7.6%) in goats from Malaga province (southern Spain), which contrasts with the seropositivity of 19.4% (64/330) detected in the same province in our study. These findings suggest endemic circulation of MAP in sheep and goat populations in Spain.

The high herd seroprevalences detected in sheep (66.3%) and goat (90.0%) flocks are higher than those previously reported in these species (20–40% in sheep and >40% in goats) in Spain (Whittington et al., 2019) and indicate that MAP is widespread in small ruminant farms in this country. In connection with this, given the Se of the ELISA used in the present study, and the fact that it was not possible to reach a sufficient sample size in some herds due to logistic constraints during sampling or to the limited size of some farms, the herd prevalence obtained in the present study may be underestimated. The spatial analysis showed that MAP was not homogeneously distributed in small ruminant populations in southern Spain, since three clusters were identified at farm level with significantly higher positivity ( $p < 0.05$ ) (Fig. 1). The level of environmental contamination would determine the risk of MAP infection (Fredriksen et al., 2004; Angelidou et al., 2014). Environmental and climatic factors favoring the persistence of MAP in the environment are possible explanations for the geographical differences observed (Whittington et al., 2004, 2005; Boadella et al., 2010). In this context, the spatial cluster of high seropositivity detected in the Grazalema Natural Park (cluster 1) could be associated with the fact that this particular location has one of the highest mean annual rainfalls in the Iberian Peninsula (Naranjo-Barea et al., 2017; AEMET, 2021). Standing water has been shown to increase the survival of MAP in the environment and can be a source of infection for livestock (Singh et al., 2013). Possible factors associated with the higher prevalences detected in the second and third clusters are the presence of large agricultural areas, which have been associated with higher MAP seroprevalence in sheep (Dhand et al., 2009; Morales-Pablos et al., 2020), and the significant presence of communal pastures (CAGPDS, 2013, 2015), which enable livestock interactions, respectively. Future studies are required to establish the factors involved in the increased circulation of MAP in the identified risk areas.

Risk factor analysis showed that species and the absence of perimeter livestock fencing were potential risk factors associated with MAP exposure on small ruminant farms in southern Spain. A significantly higher seroprevalence was found in goats than in sheep. This finding is in agreement with the small number of previous studies that have assessed both species together (Stau et al., 2012; Iarussi et al., 2019) and indicates that goats may play a more important role in the transmission and maintenance of MAP. It has been observed that goats are naturally more susceptible to MAP infection than sheep and cattle (Stewart et al., 2007). The higher seropositivity in goats may also be related to ethological and morphological differences between the species. Goats are generally more active and selective than sheep during grazing and tend to cover greater distances in search of more palatable food (Lu, 1988; Devendra, 1989; Lovreglio et al., 2014) and could therefore be more

**Table 2**

Results of the generalized estimating equation (GEE) model of risk factors associated with MAP exposure in small ruminants in southern Spain.

Variable	Categories	$\beta$	p-value	OR (95% CI)
Species	Goat	1.08	< 0.001	2.9 (1.9-4.6)
	Sheep	<sup>a</sup>	<sup>a</sup>	<sup>a</sup>
Perimeter fencing	No	0.77	0.013	2.2 (1.2-3.9)
	Yes	<sup>a</sup>	<sup>a</sup>	<sup>a</sup>

<sup>a</sup> Reference category

likely to ingest MAP-contaminated pastures. There are also morphological differences in the femoral head of the two species that affect their movement capability, which is more limited in sheep than in goats (Zedda et al., 2017). The greater freedom of movement of goats, linked to the morphological and ethological differences between the species, often results in goats climbing into or placing their feet in feed and water troughs, stepping into or defecating in feed or water, which could also favor contamination with MAP-positive feces.

Biosecurity measures are considered to be one of the most effective management strategies to prevent the entry of MAP into farms (Windsor, 2015). Multivariate analysis showed that the risk of seropositivity to MAP was 2.2 times higher in small ruminants on farms without perimeter livestock fencing than in those on farms where this biosecurity measure was in place. Perimeter fencing has been shown to be an effective biosecurity measure that limits contact between farm animals and off-farm personnel, foreign vehicles, and other wild or domestic species, and could therefore minimize the risk of the entry of transmissible pathogens, including MAP (Wells and Wagner, 2000; Robertson, 2020). It has been suggested that wild species, including wild ungulates and wild rabbits (*Oryctolagus cuniculus*), have a limited role in the epidemiology of paratuberculosis in Spanish Mediterranean ecosystems (Maio et al., 2011; Sobrino et al., 2011; Carta et al., 2012; Gómez-Guillamón et al., 2020), so that, on farms without perimeter fencing, the main risk of transmission of MAP in the flock could come from contact with other domestic animals.

## 5. Conclusions

Our results show the widespread, but not homogenous, distribution of MAP among small ruminant farms in southern Spain and suggest that goats may play a more important role than sheep in the transmission and maintenance of MAP. We provide information that could be helpful in the selection of risk-based strategies for the control of MAP on small ruminant farms in Spain. Focusing control measures on goats and promoting biosecurity measures such as perimeter fencing, especially in areas detected to be at higher risk, could be particularly useful for reducing MAP exposure on small ruminant farms.

## Ethical approval

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

## Data availability

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

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## Conflict of interest

The authors report no declarations of interest.

## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.pvetmed.2022.105637.

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