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RESEARCH ARTICLE

A serosurvey of *Mycobacterium avium* subsp. *paratuberculosis* infection of goats in the North of Portugal

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ABSTRACT

To better understand the epizootiology of caprine paratuberculosis in the North of Portugal, a cross-sectional study was conducted from 2014 to 2015. The seroprevalence and risk factors for *Mycobacterium avium* subsp. *paratuberculosis* (Map) seropositivity were evaluated. Antibodies against Map were determined by a commercial ELISA. In 936 sera tested from 56 goat herds, 120 (12.8%, 95% CI: 10.8–15.1%) goats and 34 (60.7%, 95% CI: 47.6–72.4%) herds were positive. Risk factors for seropositivity were investigated by logistic regression models. The odds of Map seropositivity were found to be higher for animals with clinical signs, OR = 5.1 (95% CI: 2.7–9.6%), animals belonging to herds with previous wasting disease, OR = 2.3 (95% CI: 1.1–4.8%), and accumulation of manure in the herd, OR = 3.1 (95% CI: 1.7–5.7%). The potential risk factors identified in this study support the current recommendations for the control of paratuberculosis in these and other animals.

KEYWORDS

goat, epizootiology, *Mycobacterium avium* subspecies *paratuberculosis*, seroprevalence, ELISA, Portugal

INTRODUCTION

Caprine paratuberculosis is a chronic inflammatory disease of the intestinal tract and lymphoid organs caused by *Mycobacterium avium* subsp. *paratuberculosis* (Map). The disease provokes a progressive loss of weight, and it is responsible for high economic losses wordwide (Windsor, 2015; Bauman et al., 2016). The control is difficult and can be based on vaccination, which has limited use due to interferences with tuberculosis diagnosis and test-and-cull strategies (Juste and Perez, 2011). Clinical signs are insufficient to establish a diagnosis, but together with the epizootiological data, it is possible to establish suspicion of Map in the goat herd (Whittington et al., 2019). Therefore, estimation of the prevalence of Map infection in particular herds or regions is necessary to establish an adequate control program.

Several methods are available for estimating the prevalence of Map infection, despite the lack of a reliable test for detection. In small ruminants, serological tests have been used to

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detect the presence of antibodies against Map, such as the agar gel immunodiffusion (AGID) test, complement fixation tests and the enzyme-linked immunosorbent assay (ELISA) (Buczinski et al., 2019). The last mentioned is the most commonly used, and some commercially available methods have been developed with variable sensitivities (Nielsen and Toft, 2009).

In Portugal, Reis and Ferreira (1988) described the first cases of goat paratuberculosis in 1983. Since then, only a few epizootiological studies have evaluated Map infection in small ruminants in Portugal, and the majority of them were focused on sheep (Ferreira et al., 2002; Mendes et al., 2004; Coelho et al., 2007, 2008, 2010). We conducted a cross-sectional study to estimate the seroprevalence and risk factors of Map infection in goats in the North region of Portugal. The objectives were to expand the knowledge on the epizotiology of this disease in the country and increase the level of alertness to paratuberculosis.

MATERIALS AND METHODS

Study area

The goat herds studied were located in the region of Trás-os-Montes e Alto Douro, North of Portugal and included five sampling locations (Alfandega da Fé, Bragança, Macedo, Mirandela and Mogadouro). The total area of the region is 3,614 km^2. The maximum altitude is 1,486 m above sea level. In this region, summer is short, warm and dry, and winter is very cold with moderate rainfall (average total accumulation of 95 mm). Throughout the year, the temperature varies from 1 °C to 30 °C, and temperatures below –4 °C or above 35 °C may occur sporadically.

Herds and animals

The sample size was calculated based on the list of herds registered in the official list of the Agriculture Department of the region of Trás-os-Montes. The sample size was calculated from the population data in 2014, which was 153 goat herds. Only herds with a minimum of 14 animals per herd with goats of at least two years old were visited, so that a total of 56 goat herds were included in the study. The number of goats per herd varies from 22 to 188 heads, with an average of 78. All eligible herds agreed to participate in the study. The number of animals to be sampled was estimated using the formula $n = (1.96)^2\, p\,(1-p)/d^2$ (Thrusfield, 2013). This sample size provides a 95% confidence level for an expected prevalence of 15%. Goat herds sampled were proportionally allocated according to the number of herds in the five sampling locations under study.

To simplify the sample collection process, the number of samples taken per herd was 14–18. This sample size provides a 95% confidence level for an expected prevalence of 1% per herd. In this way, a compromise between cost and precision of the estimates was achieved. Samples in the herd were randomly collected with aleatory numbers taken for a list of animals in each herd. Herds with previous vaccination history were excluded. The blood samples from goats of at least two years of age were collected by official veterinarians of the local health units. The sampling procedures and the laboratory tests were performed from February 2014 to January 2015. A goat herd was defined to be Map seropositive if at least one seropositive adult goat was present. In all goat herds, risk factors and health management protocols were recorded in a questionnaire adapted from Coelho et al. (2010) and are shown in Table 1.

Enzyme-linked immunosorbent assay (ELISA) procedure

Blood samples (10 mL) were collected from each animal by jugular venipuncture into 10-mL tubes (Vacutainer®, Becton Dickinson, Plymouth, UK) with clot activator. Blood samples were allowed to clot at room temperature. Then, the serum was obtained by centrifugation at 200×g for 10 min and stored at −20 °C until analysis. Serological testing for the presence of antibodies (Ab) against Map was determined in all animals using an indirect ELISA validated for goats, ID Screen® Paratuberculosis Indirect (IDVet, Grabels 34790, France). The test was a *Mycobacterium phlei* absorbed ELISA detecting anti-Map immunoglobulin G. The technique was performed according to the manufacturer's instructions for caprine samples. On each 96-well plate, 94 serum samples were tested in single wells with negative and positive controls provided by the manufacturer. The absorbance was measured at 450 nm using an automated spectrophotometer. Following the manufacturer's instructions, results were reported as the sample to positive control ratio (S/P ratio) calculated using the formula S/P ratio = $[(OD_{Sample} - OD_{Positive\ control}) \div (OD_{Positive\ control} - OD_{Negative\ control}) \times 100]$. Goats were assigned a Map infection status, as positive or negative, with those with a result of S/P ≥ 70% classified as positive. This ELISA kit has a reported sensitivity of 41.5% and a specificity of 99.42% (Fry et al., 2008).

Statistical analysis

The true prevalence (TP) was calculated from the apparent prevalence (AP) using the Rogan and Gladen (1978) equation. The formula for the calculation is:

$$TP = (AP + Sp - 1)/(Se + Sp - 1).$$

The TP and Blaker's 95% confidence intervals (CI) (Blaker, 2000) were calculated using the Epitools epidemiological calculator (Sergeant, 2016).

The χ^2 test and Fisher's exact test were used to test for associations between the seropositivity and the demographic, husbandry and environmental variables under study. Multivariate logistic regression was used to model the odds ratio (OR) and its confidence interval (95%) of being seropositive related to the variables. The outcome variable was dichotomised as positive versus not positive to identify any risk factor associated with the seropositivity. Significant potential risk factors at $P < 0.05$ (two-tailed; alpha = 0.05) were then evaluated using stepwise regression to construct a

Table 1. Individual *Mycobacterium avium* subsp. *paratuberculosis* seroprevalence and potential risk factors

Variable[a]	Animals (*n*)	Positive (*n*)	Seroprevalence (%)	CI[b] 95 (%)	*P* value
Sex					0.735
Female	908	117	12.9	10.9–15.2	
Male	28	3	10.71	3.7–27.2	
Age					0.008
2–4 years	571	81	14.2	11.6–17.3	
5–7 years	249	32	12.9	9.3–17.6	
8–10 years	82	7	8.5	4.2–16.6	
>10 years	34	0	0.0	0.0–10.2	
Breed					0.000
Alpina	34	0	0.0	0.0–10.2	
Mixed	190	43	22.6	17.3–29.1	
Sannen	18	7	38.9	20.3–61.4	
Serrana	694	70	10.1	8.1–16.6	
Animals with clinical signs					0.000
Yes	73	31	42.5	31.8–53.9	
No	863	89	10.3	8.5–12.5	
Type of production					0.010
Meat	142	8	5.6	2.9–10.7	
Dairy	52	7	13.5	6.7–25.3	
Mix	742	105	14.2	11.8–16.8	
Milking type					0.008
Not performed	110	5	4.6	2.0–10.2	
Manual	773	108	14.0	11.7–16.6	
Mechanic	53	7	13.2	6.6–24.8	
Source of animals for replacement					0.024
Not performed	85	4	4.7	1.9–11.5	
Own herd	590	77	13.1	10.6–16.0	
Other herds	261	39	15.0	11.1–19.8	
Replacement, %					0.000
0%	17	3	17.7	6.2–41.0	
5%	124	7	5.7	2.8–11.2	
10%	407	62	15.2	12.1–19.1	
15%	81	1	1.2	0.2–6.7	
20%	258	28	10.9	7.6–15.2	
30%	35	7	20.0	10.0–35.9	
50%	14	2	4.9	13.5–16.1	
Type of husbandry					0.000
Intensive	60	21	35.0	24.2–47.6	
Semi-extensive	778	91	11.7	9.6–14.2	
Outside	98	8	8.2	4.2–15.3	
Type of shelter					0.002
Shared with other animals	198	39	19.7	14.8–25.8	
Goats only	738	81	11.0	8.9–13.4	
Shelter duration					0.000
Half-day	234	54	23.1	18.1–28.9	
Night	672	64	9.5	7.5–12.0	
Always	30	2	6.7	1.9–21.3	
Humidity of the bedding					0.007
Very moist	65	10	15.4	8.6–26.06	
Slightly moist	412	37	9.0	6.59–12.1	
Normal	459	73	15.9	12.8–19.5	
Culling rate					0.007
0–5%	615	59	9.6	7.5–12.2	
>5%	321	61	19.0	15.1–23.7	
Age at culling					0.000
0–3 years	14	2	14.3	4.2–39.9	
>3–4 years	220	48	21.8	16.9–27.7	
>4–5 years	233	19	8.15	5.3–12.4	
>5–6 years	341	40	11.7	8.7–15.6	

(continued)

Table 1. Continued

Variable[a]	Animals (*n*)	Positive (*n*)	Seroprevalence (%)	CI[b] 95 (%)	*P* value
>6 years	128	11	8.6	4.9–14.7	
Time of (transitory) clinical recovery					0.002
Days	250	24	9.6	6.5–13.9	
Weeks	351	33	9.4	6.8–12.9	
Months	190	38	20.0	14.9–26.3	
Never	109	20	18.4	12.2–26.7	
Do not know	36	5	13.9	6.1–28.7	
Animals from herds with previous wasting disease					0.000
Yes	598	97	16.2	13.5–19.4	
No	338	23	6.8	4.6–10.0	
Manure accumulation					0.000
Yes	409	79	19.3	15.8–23.4	
No	527	41	7.8	5.8–10.4	
Contamination of utensils with manure					0.007
Yes	765	108	14.1	11.8–16.8	
No	171	12	7.0	4.1–11.9	

[a]variables that were significant ($P < 0.05$) on screening and offered to the logistic model; [b]CI: confidence intervals.

multivariate model (Wald test stepwise p-Wald value to enter $P < 0.05$). The multivariate logistic model was developed using a stepwise approach. Backward elimination followed by a forward selection for each variable at a time was done using a likelihood ratio test at each step with 0.05 (two-tailed; alpha = 0.05) as a significant level for removal or entry. The fit of the models was assessed using the Hosmer and Lemeshow goodness-of-fit test (Hosmer and Lemeshow, 2000). The model was rerun until all remaining variables presented statistically significant values ($P < 0.05$). All statistical analyses were performed using SPSS™S 24.0 software for Windows.

RESULTS

A total of 936 animals from 56 herds were tested. One hundred and twenty (12.8%, 95% CI: 10.8–15.1%) samples were positive by the ELISA test. Map seropositive animals (one or more) were detected in 34 herds (60.7%, 95% CI: 47.6–72.4%). When the estimated prevalence was adjusted to the sensitivity (41.5%) and specificity (99.42%) that were identified in previous studies (Fry et al., 2008), the expected true prevalence of paratuberculosis was 29.9% (95% CI: 25.1–35.5%).

Seventeen variables were associated ($P < 0.05$) with seropositivity in goats. Seropositivity significantly correlated with the following factors (Table 1): age, breed, animals with clinical signs of paratuberculosis, type of production, milking type, source of animals to replace); percentage of replacement, type of husbandry, type of shelter, shelter duration, humidity of the bedding, culling rate, age at culling, time of transitory clinical recovery in animals with compatible signs, animals from herds with previous wasting disease, manure accumulation in the herd, contamination of utensils with manure. These variables were included in the multivariate model. A backward stepwise conditional logistic regression was employed using all of the statistically significant variables mentioned above. Multivariate logistic regression analysis of the odds ratio (OR) for being seropositive to the potential risk factors listed above is presented in Table 2. At the individual level, the odds of Map seropositivity were found to be higher for animals with clinical signs, OR = 5.1 (95% CI: 2.7–9.6%), animals from herds with previous wasting diseases, OR = 2.3 (95% CI: 1.1–4.8%), and accumulation of manure in the herd, OR = 3.1 (95% CI: 1.7–5.7%).

DISCUSSION

The present study investigated the seroprevalence and risk factors of Map in goats from different sampling locations in the North of Portugal. The disease was first described in small ruminant herds in Portugal in 1983 (Reis and Ferreira, 1988). Until now, and as far as the authors are aware, no report has evaluated and described the current situation of goat paratuberculosis prevalence in the North of Portugal. This is the first cross-sectional survey on paratuberculosis among goats in Portugal.

The seroprevalence of paratuberculosis, in the sampled animals, was found to be 12.8%, while in 60.7% of the herds at least one goat proved positive. These values should be considered high, bearing in mind that the animals were randomly selected and positive ELISA results increase considerably when clinical cases are present in the herd (Clarke, 1997; Pérez et al., 1997; Vazquez et al., 2013; Windsor, 2015). Because serological methods, particularly ELISA, have not been widely used to assess the prevalence of goat paratuberculosis, the results obtained in this survey are difficult to compare with previous studies. Moreover, there is a limited amount of information in the literature about the

Table 2. Risk factors associated with *Mycobacterium avium* subsp. *paratuberculosis* infection of goats in the North of Portugal

Risk factor	β[a]	S.E.β[b]	*P*	OR[c] 95%	95% CI[d] (OR)
Animals with clinical signs	1.743	0.295	0.000		
No				1.00	
Yes				5.1	2.7–9.5
Animals from herds with previous wasting disease	0.832	0.372	0.025		
No				1.00	
Yes				2.3	1.1–4.8
Manure accumulation	1.133	0.314	0.000		
No				1.00	
Yes				3.1	1.7–5.7

[a]β: logistic regression coefficients; [b]S.E.β: standard error; [c]OR: odds ratio; [d]CI: confidence intervals.

prevalence of paratuberculosis in goats. Other studies carried out in small ruminants in Portugal, using a different ELISA technique, reported an individual apparent seroprevalence of 7.8–10.2% and a 6–18% of positivity among goat herds (Ferreira et al., 2002). Epizootiological studies performed in the Centre of Portugal identified a 27% herd prevalence (Mendes et al., 2004). In the studied region, an individual seroprevalence of 3.7% and a herd seroprevalence of 46.7% were found in sheep (Coelho et al., 2007). The individual and herd seroprevalence found in goats in this study was much higher than that previously reported in sheep. However, the seroprevalence reported in the present study was lower than expected considering the recent results in Southern Spain (Andalucía), which found 87.5% of the herds (n = 42) and 22.5% of goats (n = 551) as reactors in the ELISA test (Barrero-Domínguez et al., 2019).

In Korea, the seroprevalence of Map in goats was estimated to be 18.2–38.2% and 4.6–15.3% for herds and individual goats, respectively (Lee et al., 2006). In French dairy goats, in a study of seroprevalence using ELISA, apparent and estimated true prevalences proved to be 55.2% and 62.9% at herd level, and 2.9% and 6.6% at individual level, respectively (Mercier et al., 2010). These values are markedly lower than those found in our study.

In other regions and countries, such as Missouri (USA), true animal, within-herd, and between-herd prevalences were 1.4%, 3%, and 54.7%, respectively (Pithua and Kollias, 2012). Seroprevalence studies in goats in Latin American and Caribbean countries revealed an overall prevalence of 4.3% at the animal level and 3.7% at the herd level (Fernández-Silva et al., 2014). In Tanzania, seroprevalence in goats was 10.9% (Mpenda and Buza, 2014). In Brazil, seropositivity in goat herds ranged between 5.3% and 16.6% (Theonys Freitas et al., 2015). In Medina, Saudi Arabia, 13.8% of goats were serologically positive (Shabana and Aljohani, 2019). In the Netherlands, the percentage of positively tested herds was 78.2% (Luttikholt et al., 2019). A recent study performed in Apulia, Southern Italy has revealed a true seroprevalence of 66.2% for sheep flocks and goat herds and 9.7% at the animal level (Iarussi et al., 2019).

There was no significant difference in seroprevalence between male and female goats. The results of previous studies about the influence of sex are contradictory. A recent study by Shabana and Aljohani (2019) has shown that female goats (10.7%) had a higher prevalence than males (3.1%), but another work found that male goats had a higher frequency than females (Singh et al., 2017), while no differences were seen in our study.

The seroprevalence among breeds was significantly different. The influence of the breed on paratuberculosis prevalence is widely reported (Juste et al., 2018; Iarussi et al., 2019) and might be explained by differences related to husbandry factors (Chiodini et al., 1984; Theonys Freitas et al., 2015).

In this survey, we found seropositive goats in herds from all studied locations. The herd seroprevalence was high, which suggests that paratuberculosis is endemic in the studied region. The contact between the neighbouring herds could be very frequent in this territory since the pasture lands are communal or contiguous. Therefore, the transmission of the infection is easy. On the other hand, the results of our study suggest that goat management and housing practices might affect seroprevalence, in agreement with previous studies carried out also in goats (Angelidou et al., 2014; Bauman et al., 2016). Previous studies found that paratuberculosis is more prevalent in dairy animals than in those devoted to meat production (Chiodini et al., 1984; Mendes et al., 2004). In this study, we found a higher prevalence in mixed-type extensive production systems than in dairy or meat goat farms in the region.

We additionally studied other risk factors for seropositivity. Contrary to extensive cattle and sheep studies (Lugton, 2004; Coelho et al., 2010; Windsor, 2015), the information in goats is scarce and less species-specific (Mainar-Jaime and Vázquez-Boland, 1998; Robbe-Austerman, 2011; Angelidou et al., 2014; Bauman et al., 2016). In our study, seventeen variables were significantly correlated with seropositivity ($P < 0.05$) in the logistic regression model. The factors selected for the questionnaire were included taking into account individual and global practices previously thought to affect Map transmission.

In this study, an association between the presence of clinical signs and seropositivity was found. The odds ratio of Map seropositivity was found to be 5.1 for animals with clinical signs. This finding is consistent with previous studies as serological tests detect Map antibodies in the clinical stage (Elmagzoub et al., 2020). The diagnostic power of ELISA increases with the advancement of the disease because the antibody response is highly dependent on the total number of Map. The most advanced cases have detectable antibody responses due to the increased antibody production (Juste et al., 1994; Sweeney et al., 1995; Pourmahdi Borujeni et al., 2021).

The odds ratio of being positive was significantly higher in animals from herds with previous wasting diseases. In

paratuberculosis, certain clinical signs related to Map infection can guide the veterinarian to a presumptive diagnosis. These signs are the presence of some adult goats with dramatic emaciation due to progressive weight loss during several weeks/months without change in appetite, and with marked diarrhoea and depression in the terminal stages of the disease (Djønne, 2010).

Accumulation of manure, which permits the contact between animals and Map-containing faecal material, was previously determined to be associated with paratuberculosis (Cashman et al., 2008; Dieguez et al., 2008). This fact reinforces and supports the findings of several studies on the economic benefits of appropriate biosecurity protocols (Sardaro et al., 2017). Among others, limiting the exposure of animals to contaminated manure is an important measure, since Map is transmitted horizontally via the faecal-oral route (Mainar-Jaime and Vázquez-Boland, 1998; Bastida and Juste, 2011).

This work has some limitations, such as its design, since it was a cross-sectional study, with a small sample size of herds and wide confidence intervals of the generated odds ratios and self-selection bias. The study might also have a type-1 error due to the high number of variables included in the model and the number of statistical tests performed. Due to the limitations imposed by design, these results need to be interpreted carefully, given that it was not possible to identify a cause-and-effect relationship clearly.

The results of this ELISA-based survey indicate that Map infection is widely distributed among goats in the region of Trás-os-Montes e Alto Douro in Portugal. Considering the paucity of other similar studies, our results could contribute to the effective control of goat paratuberculosis. These results also confirm that the seroprevalence of Map infection in goats was underestimated in Portugal. The presence of clinical signs in goats, animals from herds with previous wasting disease, and accumulation of manure in the herd were associated with a higher odds of Map infection. The results of this study emphasise the need for farmers to regularly screen and eliminate seropositive goats from the herds. The results of the present study should be considered in the development of an efficient caprine paratuberculosis control program in Portugal.

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