

Prevalence of *Mycobacterium avium* subsp. *paratuberculosis* infection in sheep flocks from three regions of Antioquia, Colombia

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ABSTRACT. Paratuberculosis or Johne's disease is a slow-developing infectious disease caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP) affecting mainly domestic ruminants and producing a significant economic threat to livestock production systems. Although reports on paratuberculosis in small ruminants in Colombia are very scarce, the Colombian sheep industry has identified paratuberculosis as one of the causes of its low development. There have been reports of MAP infection in sheep flocks, mainly in the Cundiboyacense Plateau and the Bogotá savannah, but the prevalence of MAP infection in sheep and goat populations in Colombia is yet unknown. Therefore, the present study aimed to accurately estimate the prevalence of MAP infection at flock level in a sheep population of 24 flocks located in three regions of the province of Antioquia, Colombia. ELISA test as well as culture and direct qPCR were used as diagnostic tools. Overall, 456 blood serum samples were analysed and at least one seropositive animal was found in 17 (70% IC: 51.2-90) out of the 24 study flocks and, in total, 37 animals showed positive ELISA results (8% IC: 5.5-10.5). Regarding MAP direct detection, 90 faecal pools from the 24 flocks were cultured and subjected to qPCR diagnosis. Both direct qPCR and culture detected 25 (27.7%) and 64 (71.1%) faecal pools as MAP positive, respectively. More specifically, MAP positive pools were detected in 45.8% (IC: 24.3-67.3) and 83.3% (IC: 67.3-99.3) of the flocks by direct qPCR and culture, respectively. MAP infection is widespread in sheep flocks in the study regions and the combination of several diagnostic tests was necessary to achieve a more accurate and precise infection detection of this important pathogen.

Key words: Paratuberculosis, prevalence, Johne's disease, small ruminant.

INTRODUCTION

Mycobacterium avium subsp. *paratuberculosis* (MAP) is one of the most fastidious members of the *Mycobacterium* genus. It is the causal agent of Johne's disease (also known as paratuberculosis) which is an untreatable disease characterized by granulomatous enteritis, diarrhoea, loss of body weight and death (Chioldini 1993).

Although it is generally assumed that this infection occurs similarly in all domestic ruminant species, there is sufficient evidence to suggest that MAP infection in small ruminants is different to that in cattle, both in the clinical form as well as the MAP strains involved (Clarke 1997).

The disease is responsible for significant economic losses to livestock production worldwide (Sweeney 2011, Garcia and Shalloo 2015). Additionally, a zoonotic potential has been proposed since MAP has been consistently found in humans with Crohn's disease (Zarei-Kordshouli *et al* 2019). Furthermore, an increasing number of diseases such as Blau syndrome, type 1 diabetes, Hashimoto thyroiditis and multiple sclerosis have also been associated to MAP presence, reinforcing the zoonotic potential of this pathogen (Lee *et al* 2011, Sechi and Dow 2015).

Some research on MAP infection in Colombia has been reported for small ruminants (Mogollón *et al* 1983, Mancipe *et al* 2009, Hernández *et al* 2017), but information on the presence and distribution of this infection is still scarce and the true prevalence (TP) of MAP infection in sheep populations in Colombia is clearly unknown. However, the Colombian sheep industry has identified paratuberculosis as one of the causes of the low development of the industry, limiting meat commercialization at national and international levels (Castellanos *et al* 2010). Unfortunately, there are currently no programs regarding the prevention and control of MAP infection for sheep populations in Colombia.

The lack of a prevalence estimate not only limits the capacity to assess the real impact of this important infectious disease, but also limits the capacity to allocate sufficient resources for its control precluding an adequate monitoring of the effectiveness of potential control measures. Since neither the Colombian sheep industry nor the Colombian government have estimates on the TP of MAP infected flocks, we aimed to accurately estimate the flock level prevalence of MAP infection and also to explore flock level risk factor associated with the presence of MAP antibodies in these sheep flocks located in the Antioquia province, Colombia.

MATERIAL AND METHODS

STATEMENT OF ANIMAL RIGHTS

The authors declare that the present study does not contain clinical studies or patient data. Informed consent

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was obtained from all individual participants included in the study. The study was conducted according to the current law of animal protection in Colombia and was approved by the Ethics Committee for Animal Experimentation of the Universidad de Antioquia, Colombia (Act 111, May 2017).

ANIMAL POPULATION AND STUDY DESIGN

A cross sectional study was carried out using ELISA test as well as faecal culture and direct qPCR as diagnostic tools to assess MAP infection prevalence in this animal population and to explore the influence of multiple flock management practices associated with MAP seroprevalence (figure 1).

To avoid a possible selection bias and taking into account that in the province of Antioquia there were no official records of sheep flocks, a census to identify each sheep farm located in the study region was carried out before selecting the participants. According to this, the whole sheep population located in the Metropolitan Area, the Northern, and the Eastern region of the Province of Antioquia, Colombia, were the sample frame of the present study (n=2,479).

As a result of this search, 25 sheep farms were identified but one refused to participate in the study. All remaining sheep farmers agreed to participate, permitting the sampling

and the interview (n=24) to take place. The Metropolitan Area region, the Northern region, and the Eastern region of the Province of Antioquia, Colombia were selected as the study regions due to their increase in sheep production in recent years and proximity to Medellín city, which is the nearest and main sheep consumption market.

The only inclusion criterion for sampling was that animals must be over one year of age. A representative sample of this animal population was taken following a multistage sampling procedure, in which a constant proportion of animals was taken from each flock (Dohoo *et al* 2010). The sample size calculation allowed an error of 5%, 95% confidence, and expected prevalence of 50%. According to this, the sample size was estimated at 384 animals. In each study participant flock, 20% of the animals over one year of age was randomly sampled. In those flocks with less than 20 animals over one year of age, only five animals were sampled in order to complete at least one single faecal pool. In total, 456 animals were sampled in selected flocks.

DATA AND SAMPLE COLLECTION

Between August and September 2017, each of the 24 sheep flocks was visited once for data and sample collection. Overall, blood and faecal samples were collected from

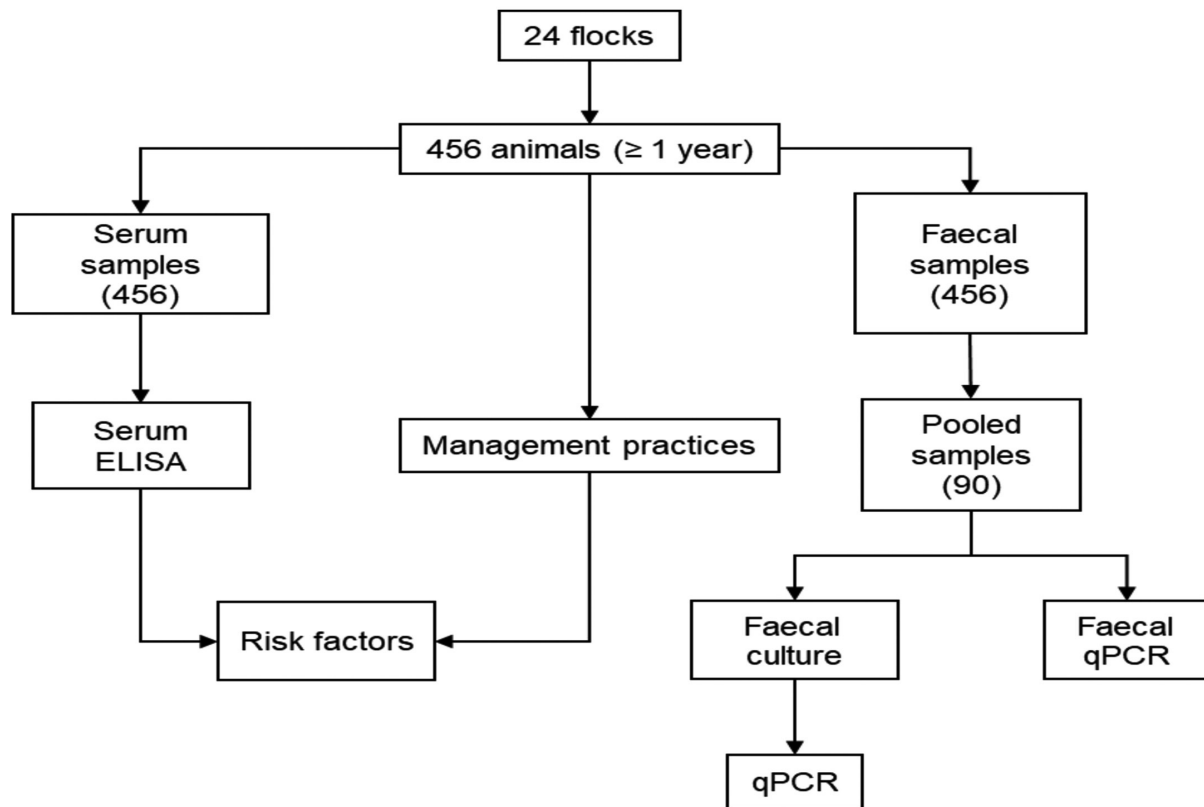


Figure 1. Overview of the study design for the determination of MAP prevalence in 24 sheep flocks of three regions of Antioquia, Colombia.

456 sheep of the 24 participating flocks. Available data regarding flock management practices were collected using a questionnaire, the same day in which blood and faecal samples were collected. The information was obtained from the flock manager or flock owner. All questionnaires included an introductory paragraph explaining the rationale and importance of the questions, how data was going to be used, and a confidentiality agreement. The questions were divided into three sections: 1) general information of the herd, 2) herd management practices, and 3) knowledge about the disease. Questions were read out to the farmer and answers were selected from multiple closed responses or otherwise written down. The questions searched for MAP infection transmission risk factors in ruminants, for example: shared roads between neighbouring flocks (Dhand 2007), presence of different species of ruminants in the same flock (Al-Majali *et al* 2008), co-grazing between cattle and goats or sheep (Çetinkaya *et al* 1997), community grazing, poor control of intestinal parasites (Angelidou *et al* 2014) and animal trade between related flocks (Marquetoux 2016).

DIAGNOSTIC TESTS

Enzyme-linked Immunosorbent Assay (ELISA). Blood samples were taken from the jugular vein using Vacuette® tubes of 7 mL without anticoagulant (Greiner Bio-one, Kremsmünster, Austria) and a single 21G x 1½" needle per animal, after local cleaning and disinfection with antiseptic alcohol. After collection, the blood samples were left to stand at room temperature to allow clot retraction. Subsequently, each sample was centrifuged at 2000-2500 rpm for 3-5 minutes to ease the serum extraction. The serum obtained was kept refrigerated until arrival at the Diagnostic Unit of the Facultad de Ciencias Agrarias, Universidad de Antioquia in Medellín, Colombia, where it was frozen at -20 °C until analysis by ELISA in October 2017. The presence of antibodies against MAP in the blood serum samples was determined by ELISA using the commercial diagnostic kit CATTLETYPE® MAP Ab (Qiagen, Leipzig, Germany) according to manufacturer guidelines.

The test characteristics of the ELISA assays used in this study, which are licensed in Germany for the detection of antibodies against *Mycobacterium avium* subsp. *paratuberculosis* in cattle, are sensitivity 59.1% and specificity 98.6%, determined by the Friederich Loeffler Institut, National Reference Laboratory for Paratuberculosis, using the reference panels for serum and milk of the NRL for paratuberculosis.

Culture. From each of the 456 study animals, a faecal sample (2-5 g) was taken with a new clean glove directly from the rectum. No animals had been vaccinated against MAP infection and researchers were unaware of their historical infectious status or the status at the time of sampling. The samples were kept refrigerated until arrival

at the laboratory. To overcome the high costs of faecal culture, faecal samples of individual animals were pooled at the laboratory. Therefore, individual faecal samples of five animals from the same flock were pooled in a new sterile container (Fiorentino *et al* 2012, Mita 2016). Briefly, each faecal sample (2-5 g) from the study animals was homogenised in a sterile container. The pools (n=90) were frozen at -80°C, then shipped to the Laboratorio de Enfermedades Infecciosas, Instituto de Medicina Preventiva Veterinaria, Universidad Austral de Chile, Valdivia, Chile at 4°C allowing the slow defrosting of samples. On arrival, samples were immediately refrigerated and processed by both culture and direct MAP qPCR detection within the following hours. Pool faecal samples were processed in the BACTEC™ MGIT™ Para TB System (BD Diagnostic Systems, Franklin, NJ, USA), according to the manufacturer's instructions. Each inoculated MGIT tube was inserted into an MGIT 960 instrument (BD Diagnostic Systems, Franklin, NJ, USA) and incubated at 37°C for 49 days. Tubes signalling positive by day 49 were removed and confirmed for the presence of MAP by IS900 qPCR. Tubes not signalling positive by that time were considered negative.

DNA extraction. A simple, efficient and low-cost method of harvesting MAP DNA based on mechanical cell disruption was used to extract DNA from direct faecal pool samples as well as from a positive MGIT culture tube (Salgado *et al* 2014). Briefly, a 200 µL aliquot of a bacterial suspension from pooled faecal samples or the medium of the MGIT tube was aseptically transferred to 1.5 mL centrifuge tubes, which were then centrifuged at 5,000 g for 5 min. The supernatant of each tube was discarded, and the opening of the tube was briefly touched with a clean soft paper tissue to remove the remaining liquid. The pellet was disrupted by pipetting with a mixture of 500 µL lysis buffer (2 mM EDTA, 400 mM NaCl, 10 mM Tris-HCL pH 8.0, 0.6% SDS) and 2 µL proteinase K (10 mg/mL), and then it was transferred to a bead beating tube (BioSpec Products Inc., Bartlesville, OK, USA) containing 200 µL of beads (0.1 mm zirconia/silica beads; BioSpec Products Inc., Bartlesville, OK, USA). The tubes were incubated at 56 °C for 2 h with shaking at 600 rpm. The tubes were then shaken in a cell Disrupter (MiniBeadbeater-8; Biospec Products) at 3,200 g for 60 sec and incubated on ice for 10 min. To remove foam and beads from the inner walls, the tubes were centrifuged at 5,000 × g for 30 sec. The samples were briefly vortexed to ensure that any DNA adhering to small solid particles was not lost when the lysate was transferred. All liquid contents from the bead-beating tube were transferred to 1.5-mL microcentrifuge tubes (Eppendorf tubes; Sigma-Aldrich) and 500 µL of 100% ethanol was added. The tubes were left standing for 2 min at room temperature before being vortexed for 5 seconds and centrifuged at 18,000 × g for 5 min. at 18 °C. The supernatant was discarded and the pellet was

washed once in 200 µL 70% ethanol by resuspension and centrifugation under the same conditions as mentioned above. Next, the pellet was resuspended in 50 µL of sterile distilled water. The tubes were placed in a dry heating block (Eppendorf; Germany) at 100 °C for 5 min. The solution was briefly centrifuged at full speed (16,000 × g for 30 sec) to remove any contaminating material. Finally, a 25-µL aliquot of the supernatant was placed into a new Eppendorf tube (Eppendorf tubes; Sigma-Aldrich) to be used as a template for qPCR.

Molecular detection and culture confirmation of MAP by qPCR. To detect MAP, either directly from faecal pools or confirmation of positive MGIT tubes, a qPCR protocol previously reported by Salgado *et al* (2013) was used. Briefly, the target was the insertion element IS900. The qPCR mixture included 5 µL DNA template, 10 µL TaqMan Universal Master Mix (Roche, Indianapolis, IN), 0.2 µM IS900 primers, 0.1 µM probe (Roche, Indianapolis, IN), and water for a total volume of 20 µL. Primer sequences for IS900, which amplified a 63-nucleotide fragment of the IS900 gene target, were 5'-GACGCGATGATCGAGGAG-3' (left) and 5'-GGGCATGCTCAGGATGAT-3' (right). The probe sequence was TCGCCGCC. The reactions were carried out in a Roche LightCycler System version 2.0 (Roche, Indianapolis, IN, USA) under the following standard conditions: one cycle at 95 °C for 10 min; 45 cycles with three steps of 95 °C for 10 s, 60 °C for 30 s, and 72 °C for 1 s; and a final cooling step at 40 °C for 30 s. Negative (Mix and Water PCR-grade) and positive (*Mycobacterium avium* subsp. *paratuberculosis* ATCC 19698) PCR controls were included.

STATISTICAL ANALYSIS

The information collected through the questionnaire and the ELISA test was first analysed descriptively and then analytically. These analyses were carried out using a bivariable and multivariable logistic regression, to explore the influence of multiple flock management practices to MAP seroprevalence. The calculation of the 95% confidence interval for the prevalence results was performed. Also, the calculation of the true prevalence was made using the WinEpi platform, available at www.winepi.net. An unconditional mixed-effects logistic regression analysis, grouped by flock to account for clustering, was also performed. The criteria of Hosmer-Lemeshow ($P < 0.25$) was used to retain variables for the multivariable model. Statistical analysis was carried out using the Stata 12.0 software (StataCorp LP, College Station, Texas, USA). For the exploration of the influence of multiple flock managements practices to MAP seroprevalence, the unit of analysis was the individual and the case definition of a positive flock was the one that had at least one positive result to the ELISA test, culture or qPCR.

RESULTS

FLOCK CHARACTERISTICS

Flock characteristics and management practices explored and then considered as predictors for the risk factor assessment are presented in table 1. The study flocks were all pasture-based and mostly had an area of less than 2 hectares and the flock average population (> 1 year) was 155 animals. The flock distribution was composed mainly of small flocks with limited access to veterinary assistance. Katahdin, Dorper and Santa Ines were the predominant breeds. Most of the farms managed other ruminant species in their facilities, mainly cattle, and in 40.3% of the cases these species shared paddocks. A 61.1% of the flocks shared roads with neighbouring flocks and 52.6% spread slurry onto pastures. Purchase of animals was highly frequent in the study flocks. Most of the interviewed owners/managers reported not having heard about the disease before and not having seen paratuberculosis-symptomatic animals in their flocks in the last 2 years.

SEROPREVALENCE

Eight per cent (37/456, IC: 5.5-10.5) of the serum samples were positive, and at least one or more seropositive animals were detected in 70.8% (17/24, IC: 51.2-90.0) of the flocks. Positive ELISA results among regions were 70, 100, and 63.6% in the Metropolitan Area, Northern and Eastern region of Antioquia, respectively (table 2). The intra-flock seroprevalence ranged from 0 to 21.4% (data not shown).

FAECAL CULTURE

Overall, 78.8% (71/90) of faecal pools showed positive results after confirmation by qPCR. MAP positive pools (one or more) were detected in 83.3% (IC: 67.3-99.3) of the flocks. The apparent prevalence values among different regions were 100, 67 and 72.7% in the Metropolitan Area, Northern and Eastern region, respectively (table 3).

qPCR OF FAECAL POOLS

qPCR informed 25 (27,7%) positive pools out of 90 faecal pools tested. Forty-six per cent (IC: 24.3-67.3) of the flocks showed MAP positive results in one or more of the analysed pools. The apparent prevalence among regions based on qPCR results of pools were 40, 100 and 36,4% in the Metropolitan Area, Northern and Eastern regions, respectively (table 3).

Table 1. Unconditional analysis of factors associated with the *Mycobacterium avium* subsp. *paratuberculosis* ELISA status in 24 flocks of three regions of Antioquia, Colombia.

Variable	Unit/Category	No of sampled animals	Distribution (%) [*]	No ELISA positive animals		P
				n	%	
Flock population (animals > 1 year)	≤70	107	23.4	14	13	0.086 ^a
	71-140	71	15.5	4	5.6	
	141-210	57	12.5	4	7	
	211-280	90	19.7	7	7.7	
	>280	131	28.7	8	6.1	
Flock size (hectares)	< 2 has	226	49.5	16	7	0.791
	≥ 3 ≤ 5 has	151	33.1	16	10.5	
	> 6 has	79	17.3	5	6.3	
Presence of other ruminants	No	148	32.4	15	10.1	0.365
	Yes	308	67.5	22	7.1	
Sharing paddocks	No	272	59.6	17	6.2	0.128 ^a
	Yes	184	40.3	20	10.8	
Sharing roads	No	177	38.8	11	6.2	0.196 ^a
	Yes	279	61.1	26	9.3	
Spreading of manure on pastures	No	216	47.3	14	6.4	0.260
	Yes	240	52.6	23	9.5	
Use of dewormer	No	100	22	5	5	0.195 ^a
	Yes	346	78	32	9.2	
Mobilization between flocks	No	239	52.4	21	8.7	0.480
	Yes	217	47.5	16	7.3	
Sheep purchase	No	240	52.6	17	7	0.515
	Yes	216	47.3	20	9.5	

^aVariables used for the multivariable analysis ($P < 0.25$).

^{*}The distribution refers to the percentage that each value represents.

Table 2. Sheep-level seroprevalence of ovine paratuberculosis infection by ELISA test in three regions of the Province of Antioquia, Colombia.

Region	Flocks	Number of animals ^a	Tested animals	Positive animals by ELISA	Individual seroprevalence (%)	Positive flocks by ELISA	Flock seroprevalence (%)
Metropolitan Area	10	1012	194	13	6.7	7	70
Northern	3	323	62	5	8	3	100
Eastern	11	1144	200	19	9.5	7	63.6
Total	24	2479	456	37	8.11	17	70.8

^aAnimal over one year of age.

FACTORS ASSOCIATED WITH THE MAP SEROLOGICAL STATUS

Among the nine risk factors explored in the univariable analysis, four (flock population, sharing paddocks, sharing roads, use of dewormer) were associated with the seropositivity to MAP infection ($P \leq 0.25$) and were eligible for their inclusion in the final model (table 1).

However, no significant variables were obtained in the final model when the multivariable logistic regression analysis was carried out.

DISCUSSION

This study was carried out using a combination of direct and indirect diagnostic methods to detect MAP

Table 3. Flock-level prevalence of MAP infection in sheep by faecal culture and qPCR in three regions of Antioquia, Colombia.

Region	Flocks	Pools	Faecal culture			qPCR		
			Positive pools	Flock positive result ^a	Flock apparent prevalence	Positive pools	Flock Positive result ^a	Flock apparent Prevalence
Metropolitan Area	10	38	37	10	100	10	4	40
Northern	3	13	6	2	67	5	3	100
Eastern	11	39	28	8	72.7	10	4	36.4
Total	24	90	71	20	83.3	25	11	45.8

^aRefers to flocks in which at least one pool resulted MAP positive by culture or PCR.

infection in sheep in the Metropolitan Area, Northern and the Eastern regions of the province of Antioquia, Colombia. The combination of ELISA, direct qPCR and faecal culture assured an accurate MAP infection detection in the study regions. To the author's knowledge, this is the first epidemiological report on sheep MAP infection in Colombia, and one of the few studies in Latin America performed in small ruminants to estimate MAP prevalence and to explore management practices associated with MAP seropositivity.

The results show that antibodies against MAP are widespread in the study regions, which in general means that MAP infection had progressed significantly in this animal population, and the serological results indicate a significant proportion of individuals are in the late phase of this infection and, as such, efficient MAP shedders. The latter finding agrees with the results reported elsewhere in the same ruminant species (Attili *et al* 2011, Stau 2012, Moron-Cedillo *et al* 2013, Bauman *et al* 2016^a, Morales-Pablos *et al* 2020). Additionally, the observed flock-level prevalence of MAP infection in sheep flocks could be considered as high, in comparison with what has been reported by Bauman *et al* (2016^b) in sheep flocks in Canada, using direct and indirect diagnostic methods (ELISA, bacterial culture and qPCR). According to a systematic review for Latin America and the Caribbean, the prevalence of this infection in sheep is 16% (7.9-24.1%) at an individual level, and the prevalence in sheep at the flock level was not reported due to the lack of studies that met the inclusion criteria; the high heterogeneity detected in overall prevalence estimations could be attributed to the high diversity in study design, the variable quality of measures, or the test used (Fernández-Silva *et al* 2014).

Although no seropositive animals were identified in some flocks (data not shown) in this study. These animals were likely to be found in the early stages of infection and the levels of antibodies may have not been detectable (Nielsen 2010). The location and flock management practices also may limit the contact between neighbouring flocks and different animal species in these regions since the paddocks

are distant to each other or the flocks are isolated, and the entry of other animals is not allowed. Nevertheless, MAP infection shows similar behaviour in the study regions, e.g. few seropositive animals being observed within each flock, but many flocks with at least one seropositive animals (Coelho *et al* 2007, Stau *et al* 2012, Morales-Pablos *et al* 2020, Khamassi Khbou *et al* 2020).

The lower rate of positive results obtained by qPCR in comparison to culture was expected as previously reported (Alinovi *et al* 2009, Plain *et al* 2014) for sheep. The different diagnostic sensitivity between qPCR and culture is mainly explained by the fact that the direct qPCR tool has to deal with faecal sample inhibitors for polymerase activity (Monteiro *et al* 1997, Thornton and Passen 2004) as well as intermittent MAP shedding (Whitlock *et al* 2000). On the other hand, culture can address these difficulties along the culture period due to the bacteria replication rate (Harris and Barletta 2001). It is well known that liquid media based on Middlebrook 7H9 Broth base such as BACTEC MGIT system has shown better results to grow several strains of *Mycobacterium avium* subsp. *paratuberculosis* than conventional media (HEYM) (Gumber and Whittington, 2007). However, the combination of both MAP detection tools improves the capacity of detection of infected flocks.

Even though some flocks showed only negative qPCR and culture results, it is also likely that the results for the tests did not necessarily mean that the animals were not infected, and it may be that the shedding phase has not started (infected animal in a noninfectious phase) yet (McKenna *et al* 2006, Nielsen 2010). The high MAP prevalence obtained in this study is consistent with what has been previously confirmed for cattle in the same region (Fernández-Silva *et al* 2011, Ramírez *et al* 2011, Correa-Valencia *et al* 2016, Correa-Valencia *et al* 2019). In addition, the absence of strategies, programs, or projects to prevent or control the infection and the historic management practices that allow the entrance and spread of the infection in the different animal productions help to explain the findings.

The small number of observations and the fit for several factors may explain the lack of association in

the multivariate linear or logistic regression analysis (Hackshaw 2008, Figueiredo *et al* 2013). Also, the number of positive and negative outcomes in the observed data influence the precision of the estimates of coefficients in the model. It has been suggested that the dataset should contain a minimum of $10(k+1)$ positive outcomes where k is the number of predictors in the model in order to adequately fit the model (Dohoo *et al* 2010). These reasons can explain why no significant variables were obtained in the final model.

In the last decade, the arrival of sheep in the study regions has occurred for purposes of restocking and genetic improvement due to the increase of lamb consumption in the region. However, these animals are purchased without quarantine or any diagnostic test. Most of these animals come from different provinces of Colombia where the prevalence of paratuberculosis infections is unknown. In the absence of biosecurity practices, a single animal (clinically or subclinically sick) is enough to infect a flock and disseminate MAP among other flocks. The paratuberculosis prevalence may increase as the biosecurity practices intended for the prevention and control of infection are not being implemented or executed in the flocks (Morales-Pablos *et al* 2020).

In conclusion, MAP infection is widespread in sheep flocks of the study regions and the combination of several diagnostic tests were necessary to achieve a more accurate diagnosis of MAP infection. Further studies including a larger sample size are needed to identify the risk factors associated with MAP infection in sheep in Colombia.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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