Original Article

Serological diagnostic for Bovine Paratuberculosis in the Municipality of Sotaquirá, Colombia

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Abstract

Bovine Paratuberculosis (BPT) or Johne's disease is a pathology caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). The parasite causes chronic granulomatous enteritis in domestic and wild grazers. Oral-fecal transmission is the most common form of transmission with calves less than six months old being the most susceptible but not presenting clinical symptoms until five years later. It is a disease that spreads during dairy production generating economic loss due to low productive yield and the early discarding of infected animals. The objective of our study was to perform a serological diagnostic of BPT in herds from the municipality of Sotaquirá, Boyacá. The study was designed as an observational, cross-sectional study with simple random sampling. Blood samples were taken from 1000 individuals and were analyzed via a commercial indirect ELISA assay kit (PARACHEK® 2 KIT, Prionics, Switzerland). The assay had a sensitivity of 70% and a specificity of 100%. Data was analyzed using EpiInfo® software. We found a low prevalence of MAP in Sotaquirá (3.5% PA and 5.0%PR), where we found that the age group with highest prevalence of MAP were individuals older than four years old (5.6%). We also found that Jersey (4.8%) and Holstein (4.5%) cattle breeds also had a higher prevalence of the disease. We also established cattle were aged more than four years and we similarly found a statistically significant association with handling practices (p<0.05). Determination of seroprevalence and principal risk factors within livestock farms allows for establishment of prevention and control programs through implementation of biosecurity measures.

Keywords: Paratuberculosis, Enzyme linked immunosorbent assay, prevalence, risk factors, diagnostic

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Received May 17, 2021
Accepted April 4, 2022
https://doi.org/10.14456/tjvm.2022.31

Introduction

Bovine paratuberculosis or Johne's disease is a chronic, debilitating, intestinal bacterial disease caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). It is of global interest as it negatively impacts the health and well-being of an ample variety of animals, especially domesticated ruminants (Retamal *et al.*, 2016; Schwalm *et al.*, 2019). Additionally, it generates significant economic loss for the cattle industry and is of concern due to possible zoonosis due to it and is of concern because of its zoonotic potential and the postulated role in Crohn's disease (Navarro *et al.*, 2019).

MAP is a slow-growing, intracellular, acid and alcohol resistant facultative bacterium. However, it survives *in vivo* in mononuclear phagocytic cells of ruminants under conditions of individual susceptibility, virulence of the infecting strain and immune status of the affected individual (Lavers *et al.*, 2013; Ramírez and Maldonado, 2013). In domesticated ruminants and wild animals, MAP is transmitted horizontally via oral-fecal contact, either through consumption of colostrum, contaminated water or by insect bites. Vertical transmission of the disease can happen via transplacental route (Hernández *et al.*, 2014). Infection generally occurs in the first year of life with newborn animals being the most susceptible (Windsor and Whittington, 2010).

MAP infection is characterized by being subclinical in presentation in the majority of dairy cows causing chronic granulomatous enteritis. Dairy producers begin to show clinical signs of infection like chronic diarrhea, emaciation, weakness and spontaneous death in cattle, which is more common in older individuals (McKenna *et al.*, 2006; Collins *et al.*, 2010; Gilardoni *et al.*, 2016). The subclinical form of the disease produces progressive weight loss, reduction in milk production, loss of its basic components, negative impacts on fertility, udder health, as well as premature sacrifice leading to reduction of value at time of sacrifice. This undoubtedly leads to economic loss for cow herders (McKenna *et al.*, 2006; Beaunée *et al.*, 2015; Britton *et al.*, 2016; Wiszniewska *et al.*, 2020).

In public health, MAP is of great relevance due to the slow and progressive propagation of the disease, the technical limitations of diagnosis, the absence of a therapeutic focus and the difficulty of control strategies in farms to prevent infection (Whittington *et al.*, 2012). Additionally, it is well understood that there is a apparent relation between MAP and Crohn's disease, as well as the fact that milk as well as its derivatives are a source of transmission for the microorganism, requiring the development of surveillance strategies for monitoring milk derived from livestock farms (Caraballo *et al.*, 2018).

In Colombia, the Colombian Agricultural Institute (ICA) considers BPT as a notifiable disease common to several animal species (ICA, 2015). Added to this, presently there are also insufficient studies on the prevalence of this disease for herds at a national or even departmental level (Jaramillo *et al.*, 2017). The municipality of Sotaquirá in Boyacá belongs to the milk belt of the department (Dehaquiz and Zambrano, 2012), giving way to the importance of being able to thus facilitate MAP diagnosis. The objective of this study was then to complete a serological diagnosis of BPT in herds from Sotaquirá, Colombia.

Materials and Methods

Study area: The study was performed in the municipality of Sotaquirá, belonging to the central province of the department of Boyacá that is an important part of the milk belt of the department (Gutierrez, 2017). The municipality is 2,860 meters above sea level with an average temperature of 14°C and has an extension of 288,650 km. The livestock sector of Sotaquirá occupies 10,582 hectares with the production of managed and natural pastures destined for livestock exploitation at an intensive and extensive level. However, the land tenure is characterized by small holdings (Alcaldía Municipal de Sotaquirá, 2016).

***Sample size*:** 19,333 heads of cattle are registered in the municipality of Sotaquirá, a statistic obtained in the National Livestock Census performed by ICA (2019). Based on this data, a sample size of 1,000 individuals with a sampling fraction of 5.17%, was considered an accepted error of 3.1%, a 95% confidence interval and an expected prevalence rate of 50% since no studies have ever been done in this region. We then applied the following formula.

$$n = \left(\frac{Z\alpha/_{2\sqrt{p(1-p)}}}{E} \right) = \frac{Z^2\alpha/2 \cdot p(1-p)}{E^2}$$

Where: n= sample size, Z_α= constant at 95% confidence interval, p= expected prevalence, E= acceptable margin of error.

Evaluated Variables: The variables evaluated in this study are classified in two categories, variables related to the animals and those related to the farm management practices of the animals. In the first category, we specifically looked at age group, sex, breed and clinical signs evidenced in animals. In the second category, we identified the following variables: attendance at livestock shows, presence of damaged fences and the source of the water.

Sample Collection and Processing: Samples were taken from males and females of different ages and breeds, distributed in 65 herds. The breeds used in this study were Ayrshire, Holstein, Jersey, Normande, Zebu and crossbreeds. Blood samples were taken via coccygeal venipuncture, with 7 ml of blood extracted from each animal. Samples were stored in Vacutainer tubes for storage, subsequent refrigeration and transport which were refrigerated during transport to the Veterinary Parasitology laboratory at the Universidad Pedagógica y Tecnológica de Colombia (UPTC). At the laboratory, the samples were then centrifuged (Hermle, Z206A, Germany) at 2500 rpm for 10 minutes to obtain serum. The serum was then transferred to an Eppendorf tube for storage at -20°C. The serum samples were then processed by indirect ELISA assay using PARACHEK® 2 KIT commercial kit (Prionics, Switzerland) with a sensitivity of 70% and a specificity of 100%, following the manufacturer's protocol. Those samples whose percentage of positivity was ≥ 15% of the cut-off point were determined as positive.

Statistical Analysis: This was a descriptive cross-sectional study which employed simple random sampling. Using the consolidated and refined database, the results were processed with the statistical program EpiInfo®. The determining factors were then defined by calculating the Prevalence Ratio (PR). The dependent variable (Y) were the obtained serological results, while the independent variables (X) were all determining factors established in the structured epidemiological survey that was implemented during the sampling. Once these factors were established, a final model was built using logistic regression analysis.

***Ethical approval*:** The study was performed under consideration of and in compliance with Law 576 576 of 2000 and Law 84 of 1989 of the Republic of Colombia. Informed consent was obtained from cattle owners prior to collecting samples for the study. The study was approved by the Ethics Committee of UPTC.

Results

Of the 1000 bovine samples in Sotaquirá, Boyacá, 869 were female and 131 were male. We determined an apparent seroprevalence of 3.5%, in which 35 females (4.0%) were found to be seropositive presenting with antibodies against MAP; we also found that no males were seropositive. Likewise, we established that the real seroprevalence rate was 5.0% with a positive predictive value (+PV) of 100%, meaning that a positive result had a 100% probability of identifying a sick individual. We also found that we had a negative predictive value (-PV) of 98.4%, in which a negative result had a 98.4% probability of being a healthy individual.

In terms of the breeds studied, Zebu (0% AP; 0 PR%) and Ayrshire (0.0% AP; 0 PR%) did not test positive for the presence of antibodies against MAP. Jersey (4.8% AP; 6.9 PR%), crossbreeds (2.3% AP; 3.3 PR%) and Normande (1.9% AP; 2.7 PR%) showed the lowest seroprevalence rates while the Holstein breed had the highest seroprevalence (4.5% AP; 6.4 PR%) (Table 1). In evaluating cattle age groups, the greatest prevalence was in cattle more than four years old (5.6% AP; 8.0 PR%), while individuals two to four years old did not present as seropositive for MAP (Table 2).

Analyzing this data, given the cattle sampled, we found that Holstein (p=0.024) and Normande (p=0.079) breeds, cattle aged less than one year (p=0.014), more than four years (p=.0002) and the gender of the cattle (p=0.0067) showed a significant association to the presence of antibodies against MAP, indicating that presentation with BPT is associated with breed, gender and age of the cattle sampled (Table 3).

Relating to the farm management practices evaluated, attending cattle expositions (p=0.003), the presence of broken fencing (p=.049) and the use of gulches and ravines as sources of fresh water (p=0.032) showed a significant statistical association with the presence of antibodies against MAP (Table 4).

Similarly, we found that retention of the placenta (p=0.003), the presence of agalactia (p=.044), fever (p=0.015) and diarrhea (p=0.035) in cattle were significantly associated with the seropositivity of the disease (Table 5).

The prevalence ratio (PR) allowed for the identification of the variables identified in Table 6 as possible risk factors for the presentation of BPT. After performing a logistical regression analysis on these variables, we found that the only variable that could be identified as a true risk factor was cattle aged greater than four years having a 1.0445 times greater risk of having BPT (Table 3).

Table 1 AP and PR of BPT by breed in cattle from the municipality of Sotaquirá, Boyacá.

Breed	n	Positive	AP (%)	PR (%)	+PV (%)	-PV (%)
Holstein	601	27	4.5	6.4	100	98
Ayrshire	11	0	0.0	0	0	0
Jersey	21	1	4.8	6.9	100	97.8
Normande	257	5	1.9	2.7	100	99.2
Zebu	22	0	0.0	0	0	0
Crossbreed	88	2	2.3	3.3	100	99

RP= ((AP - (1- Specificity)) / (1-[(1- specificity) + (1- sensibility)])
+VP= (RP*sensibility) / ((RP*sensibility) + ((1-RP)*(1- specificity)))
-PV= ((1 - AP)*specificity) / (((1 - RP)*specificity) + (PR x (1 - sensibility)))

Table 2 AP and PR of BPT in cattle of different age groups in the municipality of Sotaquirá, Boyacá.

Age	n	Positive	AP (%)	PR (%)	+PV (%)	-PV (%)
< 1 yr.	179	2	1.1	1.6	100	99.5
1-2 yrs.	209	5	2.4	3.4	100	98.9
2-4 yrs.	112	0	0.0	0	0	0
>4 yrs.	500	28	5.6	8	100	97.5

Table 3 Analysis of the breed, age and gender of cattle as possible risk factors associated with infection of BPT, results displayed as prevalence ration (PR) and 95% Confidence Interval. Bolded values are shown to be statistically significant ($p \leq 0.05$).

Variable	Category	PR	95% CI	*p*-value
Breed	**Holstein**	1.026	1.0034-1.0492	**0.02466299**
	Normande	0.9787	0.9567-1.001	**0.07904983**
	Ayrshire	-	-	-
	Zebu	-	-	-
	Crossbreed	0.9862	0.953-1.0206	0.39060493
	Jersey	1.0135	0.9204-1.1161	0.53040985
Age	**< 1 yr.**	0.9606	0.9479-0.9735	**0.01448914**
	1-2 yrs.	0.9857	0.961-1.0109	0.22626577
	2-4 yrs.	-	-	-
	>4 yrs.	1.0445	1.02-1.0696	**0.00020329**
Gender	-	0.9597	0.9467-0.9729	**0.00669494**

Table 4 Analysis of farm management practices as possible risk factors associated with infection of BPT. Results are presented as prevalence ratio (PR) and 95% Confidence Interval (CI). Bold values indicate statistically significant association ($p \leq 0.05$).

Variable	PR	95% CI	*p*-value
Cattle Expositions	1.1261	0.9978-1.2708	**0.00317006**
Broken Fencing	1.0237	0.9969-1.0511	**0.04864841**
Gulches and Ravines as water source	1.0265	0.9991-1.0548	**0.03195296**

Table 5 Analysis of clinical manifestations as possible risk factors for infection MAP. Results are presented as prevalence ratio (PR) and 95% Confidence Interval (CI). Bold values indicate statistically significant association ($p \leq 0.05$).

Variable	PR	95% CI	*p*-value
Retention of Placenta	1.0349	1.0127-1.0576	**0.00307463**
Agalactia	1.0228	0.9997-1.0465	**0.04391521**
Fever	1.0296	1.0035-1.0564	**0.01545914**
Diarrhea	1.0247	1.0005-1.0494	**0.03460512**

Table 6 Analysis of variables as possible risk factors associated with infection of BPT.

Variable	Odds Ratio	LCI (95%)	UCI (95%)	*p*-value
Holstein	0.6229	0.2389	1.6245	0.3331
Normande	0.4716	0.1810	1.2286	0.1239
1-2 yrs.	1.3616	0.6332	2.9282	0.4295
> 4 yrs.	4.1742	1.8066	9.645	**0.0008**
Cattle exhibitions	2.2529	0.7183	7.0654	0.1637
Broken fencing	1.4412	0.6831	3.0405	0.3373
Gulches and ravines as water sources	1.5559	0.7374	3.2830	0.2459
Retention of Placenta	2.5569	0.9213	7.0958	0.0714
Agalactia	1.1298	0.4965	2.5710	0.7710
Fever	1.3582	0.6153	2.9980	0.4486
Diarrhea	1.6203	0.7724	3.3989	0.2017

Discussion

In Colombia, there have been various reports of seroprevalences of BPT. Different dairy herds in the country have reported a value up to 2.2% (Fernández *et al*., 2011), 8% in Nariño (Benavides *et al*., 2015), 17% in the municipality of San Pedro de los Milagros, Antioquia (Jaramillo et al., 2017), 4.1% in the department of Antioquia (Correa *et a*l., 2019), 17.2% in the departments of Sucre and Córdoba (Correa *et al*., 2020) and 10.9% en Boyacá (Bulla-Castañeda *et al*., 2020).

MAP has been reported to be resistant to different environmental factors and presents a survival duration range between 1 to 55 weeks (Whittington *et al*., 2004; Eppleston *et al*., 2014), however, low seroprevalences reported nationally are possibly due to exposure of the pathogen to the sun, as the provision of 70% of the shade trees is an important factor in extending their survival (Whittington *et al*., 2004; Eppleston *et al*., 2014), and the infrared wavelength emitted by the sun that drives diurnal temperature may be the detrimental component that correlates with lack of shade (Whittington *et al*., 2004). Therefore, it is considered that a climate with high temperatures and pastures with a presence of few trees or infrastructure that prevent direct exposure to the sun may contribute within the range of reported seroprevalences.

Similarly, seroprevalence of antibodies against MAP varies globally and is also variable. In Peru, seroprevalence rates have been established at 36.7% (Bustamante *et al*., 2011), in Brazil at 2.7% (Sá *et al*., 2013), in Mexico at 5% (Milián *et al*., 2015), in Chile at 6.3% (Verdugo *et al*., 2018), 18.8% in Canada (Corbett *et al*., 2018), between 2.3% and 26% in New Zealand (Bates *et al*., 2019), and 25% in Ecuador (Echeverr *et al*., 2020). The variation in these reported seroprevalence rates can be due to the diagnostic implemented, the protocol and manufacturers of the different ELISA, the type of sample collection design or the type of sanitary management adopted in the different herds (Sá *et al*., 2013).

In discussing breed, we found the highest seroprevalence in individuals from the Jersey and Holstein cattle breeds, while we also found that crossbreeds and Normande breeds had the lowest seroprevalence. These findings are in agreement with the findings from Benavides *et al.* (2015), which found that the Jersey breed had more animals presenting with antibodies against MAP (50%), followed by Normande (22.7%) and crossbreeds (12.2%). However, they note that although the largest population were Holstein, they could not determine relevant data on seroprevalence, which this was not what was found in the current Sotaquirá study. Likewise, it has been established that susceptibility to MAP infection is a hereditary trait in the Holstein breed that can be modified through genetic selection (Kirkpatrick and Lett, 2018).

On the other hand, we found that animals aged more than four years showed the highest seroprevalence, while cattle aged between two and four years did not test as seropositive for BPT. This differs from findings from Benavides *et al.* (2015), who found that the highest seroprevalence in 3 year old cattle (11.2%), which they suggest may be due to the fact that during the subclinical phase of infection, antibody response is low in infected individuals, being more likely to develop BPT in cattle aged more than five years (Mallikarjunappa *et al*., 2019; Schwalm *et al*., 2019).

We found a statistically significant association between the Holstein and Normande and the presence of antibodies against MAP. Mortier *et al.* (2013), indicate genetic susceptibility markers have been identified in these breeds and it can be speculated that genetic variation related to the regulation of the cellular immune response could be responsible for the differences observed in host responses. However, Benavides *et al*. (2015), observed that breed susceptibility could be confused with popularity of the breed, leading to a greater number of these animals being found in the farms studied.

Likewise, we identified cattle in the age range greater than four years old as a risk factor for BPT infection through a statistically significant association. This concurs with the natural history of the disease, considering that the incubation period of the disease is approximately five years. However, the disease can convert from a subclinical to a clinical state when an animal is subjected to stressors such as nutritional factors, gestation, lactation, environmental disease or other concurrent disease, which could trigger clinical manifestation of the disease at an early age (Singh *et al.*, 2016).

In terms of the gender of the animals studied in this work, females were identified as the only ones to be seropositive and a statistically significant association could be formed between the presence of antibodies against the bacteria and the gender of the studied cattle. This concurs with the findings from Caraballo *et al.* (2018), who reported that 98.9% of cattle testing positive for the pathology were female. However, Veléz *et al*. (2016), points out that this difference between genders may be due to the low number of males that were sampled used in the study, which concurs with the present study in the municipality of Sotaquirá.

Although diarrhea is considered an important clinical sign of BPT it can be an erroneous indicator since this symptom is associated with other general syndromes and common diseases. On the other hand, a long asymptomatic phase is characteristic of BPT. (Sánchez *et al*., 2009). Hence, a significant statistical association has been found between the disease and the presentation of diarrhea in the individuals evaluated.

In contrast, Picó (2015) reported that there are no specific reproductive parameters in bovines that correlate with presented seropositivity. . In the municipality of Sotaquirá we found a statistically significant association between presentation of the placenta and positive cases of BPT. However, Mato (2017) signaled that the probability of having difficulties during labor was 2.74 times higher for seropositive females. Also, it has been reported that there is an alteration in the performance of milk production, where it can vary from four to up to fourteen percent of milk produced, depending on the lactation number in which the individual is found (Villamar, 2012), but there are no existent reports associated with the presentation of agalactia in cattle.

Discussing evaluated farm management practices, the presence of gulches as a main water source for cattle presented a statistically significant association with seropositivity of BPT. According to Aboagye y Rowe (2011), it can result when infected animals excrete the pathogen in stools which then contaminate local water sources. Therefore, the infection of water supplies is coupled with the long survival period for MAP in the environment. Additionally, Kennedy *et al.* (2014), points out that within the biosecurity measures on milk farms, access to water that passes through neighboring farms should be prevented to reduce between-herd transmission (Lombard, 2011).

The presence of damaged fences in the studied herd presented a statistically significant association in the municipality of Sotaquirá, since the presence of these could allow animals to encounter fecal matter from contaminated cattle or with other seropositive animals from other farms. This is in agreement with results from the study reported by Sayers *et al.* (2014), and Kennedy *et al*. (2014), highlighting the importance of maintaining farm boundaries to prevent the transmission of BPT. Likewise, cattle expositions are considered spaces where animals can come into contact with cattle from other farms increasing the risk of MAP infection (Künzler *et al*., 2014). This matches with reports from Kennedy *et al*. (2014), who point out that preventing nose to nose contact between different management age groups within and outside production, can prevent transmission of MAP.

Finally, early diagnosis of BPT is difficult due to the extended incubation period and its slow progression (Ramirez *et al*., 2011). Early detection of antibodies against MAP is an important preventative measure, however, diagnostic tests available today currently lack sufficient sensitivity to detect animals with the disease during early phases of infection (Gilardoni et al., 2016).

In conclusion, we found a relatively low MAP seroprevalence rate in the municipality of Sotaquirá. However, compared to the data found at a national level, it is within the average values reported. This survey provides an important benchmark fora department that will allow the establishment of disease control and prevention programs for this disease in milk producers. We identified cattle over the age of four as a risk factor for presentation of BPT and there were also statistically significant associations with other farm management practices. This will provide information to implement biosecurity and protection measures for cattle, especially those considered a susceptible population to infection with MAP, preventing the propagation of the disease within productions.

Acknowledgements

This study was funded by Vecol. The authors would like to thank the support received from the producers that participated making this study possible.

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