



## Serological diagnosis and risk factors associated with bovine paratuberculosis in the municipality of Tuta, Colombia

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### ABSTRACT

Bovine paratuberculosis or Johne's disease is caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP), which affects domestic and wild ruminants around the world. The clinical presentation of MAP infection is characterized by chronic diarrhea unresponsive to treatment. The objective of the present study was to investigate the seroprevalence and risk factors associated with bovine paratuberculosis in cattle herds of Tuta, Boyacá, Colombia. This descriptive cross-sectional study with simple random sampling was performed on 882 blood samples taken from cattle of different racial and age groups. Blood samples were processed using an indirect enzyme-linked immunosorbent assay (PARACHEK® 2 Kit, Prionics AG, Switzerland). The obtained data were analyzed by the statistical software Epi Info. In this study, a general seroprevalence of 3.1% was found, and seropositivity in females was 3.6%. The highest prevalence of antibodies against MAP was in individuals > 4 years (5%) and the Jersey breed (4.8%). Therefore, the age of over 4 years was identified as a risk factor associated with MAP. Moreover, a statistical association was found between management and biosafety variables, such as pen management ( $p = 0.012$ ), feeding with concentrate ( $p = 0.012$ ), and the presence of diarrhea on the farm ( $p = 0.048$ ). It could be concluded that the disease is present in Tuta, however, considering factors, such as the chronicity of the disease and the diagnostic method used, it is expected that the number of infected animals is much higher than presented in this research.

### Keywords

Paratuberculosis, *Mycobacterium avium* subsp. *paratuberculosis*, indirect ELISA, seroprevalence, risk Factors, DeCS

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### Abbreviations

MAP: *Mycobacterium avium* subsp. *paratuberculosis*  
PTB: Paratuberculosis  
DeCS: Descriptores en Ciencias de la Salud (Descriptors in Health

Sciences)  
ELISA: Enzyme-linked Immunosorbent Assay  
PPV: Positive Predictive Value  
NPV: Negative Predictive Value

## Introduction

Johne's disease or PTB is an intestinal infectious inflammatory disorder associated mainly with domestic and wild ruminants, which affects both animals and producers due to decreased milk production, premature slaughter, and reduced weight gain in animals infected with MAP [1].

PTB presents complex epidemiology and it has been reported that animals are infected at an early age but do not develop clinical signs until several years after the initial infection [2]. The clinical expression of MAP infection is characterized by chronic diarrhea unresponsive to treatment, leading to emaciation and ultimately, sacrifice or death [3]. PTB causes inflammation and the malfunction of the intestinal tract, with gross pathology showing thickened and edematous intestinal walls. In turn, these intestinal injuries affect the absorption of nutrients and proteins, causing muscle wasting and lower productivity [4].

In subclinical infection, adult carriers show no specific clinical signs, while may be affected by other abnormalities, such as mastitis or infertility. Milk production decreases, but vital signs are within normal limits. In advanced clinical disease, emaciation is the most obvious abnormality and is often accompanied by intermandibular edema, which tends to disappear as diarrhea develops [5].

However, PTB has not been demonstrated to be a zoonosis. Although the causative agent of the disease has occasionally been detected in some patients with Crohn's disease, its role as a human pathogen has not been fully accepted [4]. Annual losses of \$198 million, \$75 million, \$56 million, \$54 million, and \$17-28 million have been reported in the United States, Germany, France, New Zealand, and Canada Due to the disease, respectively [6].

Diagnostic tests for Johne's disease are improving, but the accurate detection of all infected animals, especially those at an early stage of infection and transmitting the organism within a herd, is not yet possible. This fact makes the test and discarding strategies ineffective [7] precisely among the indirect tests, ELISA is used more frequently and is indicated mainly for seroprevalence analysis in countries that adopt surveillance programs. The advantages of this technique include wide availability, low cost, and fast results [8].

Although PTB has been known for decades, research in Colombia has been insufficient to accurately reflect the epidemiological situation, economic impact, and public health impact of the disease on the country. This limits the knowledge about the magnitude of MAP circulation in animals, humans, the environment, and food in the Colombian territory [9]. Therefore, it is necessary to conduct further studies to

establish the seroprevalence and risk factors of MAP. This type of study allows knowing more about the disease behavior in the country, especially in a municipality, such as Tuta, whose economy is based on the production of bovine milk, and where the effect of this disease is not known.

In addition, these studies in the future serve to generate sufficient information for both governments and farmers, which will allow them to make sound decisions on the control and management of PTB. Currently, PTB control and management continue to be an obligation for the owner of the farms. Although this is a notifiable disease according to the World Organization for Animal Health (OIE) [10], there is very little government effort for detection and control. As mentioned before, both producers and veterinarians only get to see the repercussion of the disease in the terminal stages or at necropsy after unsuccessfully trying to treat the animals with medications, such as anthelmintics and antibiotics.

## Results

In our study, an apparent seroprevalence of 3.1% (27/882) and a true prevalence of 4.4% were found, with a PPV of 100% and an NPV of 98.6%. Females were the only seropositive with 3.6% (true prevalence: 5.1%, PPV 100% and NPV 98.4%). Animals > 4 years (5%) and the Jersey breed (4.8%) had a higher prevalence of antibodies against MAP than other groups. On the other hand, no cattle in the age group of 2-4 years and the Aryshire and Cebú breeds were seropositive to the disease (Table 1).

In the present study, disease presentation had a significant statistical association with the ages 2-4 and > 4 years, cattle gender, presence of pens in the herds, concentrate supply, and diarrhea presentation in animals ( $p \leq 0.05$ ). Moreover, it was established that the Ayrshire breed and age < 1 year were protective factors for the presentation of PTB in the evaluated cattle, while the supply of concentrate and age > 4 years were determined as possible risk factors for the presentation of PTB (Tables 2 and 3). The logistic regression model revealed that the age older than 4 years was a risk factor for the presentation of bovine PTB in the evaluated herds (Table 4).

## Discussion

The overall seroprevalence in our study was 3.1%, which is lower than the report of the Department of Boyacá by Bulla-Castañeda et al. (2020) where a prevalence of 10.9% was found [11]. Similarly, it differs from the national results in beef cattle in the

**Table 1.**

Apparent prevalence (AP) and real prevalence (RP) of bovine paratuberculosis by breed and age group in cattle from Tuta, Boyacá.

Category	number	Positives	AP %	RP %	PV + (%)	PV- (%)
<b>Age Group</b>						
< 1 year	171	2	1.2	1.7	100	99.5
1-2 years	204	5	2.5	3.6	100	98.9
2-4 years	107	0	0.0	-	-	-
> 4 years	400	20	5.0	7.1	100	97.7
<b>Breed</b>						
Holstein	498	19	3.8	5.4	100	98.3
Ayrshire	10	0	0.0	-	-	-
Jersey	21	1	4.8	6.9	100	97.8
Normando	250	5	2.0	2.9	100	99.1
Cebú	22	0	0.0	-	-	-
Cruces	81	2	2.5	3.6	100	98.9

PV +: Positive predictive value

PV -: Negative predictive value

**Table 2.**

Possible risk factors associated with bovine paratuberculosis infections.

Variable	Category	PR <sup>1</sup>	CI 95% <sup>1</sup>	p-value
<b>Breed</b>	<i>Holstein</i>	1.018	0.9951-1.0415	0.09833374
	Normando	0.9849	0.9624-1.0079	0.17619706
	Ayrshire	0.969	0.9576-0.9806	0.73159449
	Cebú	0.9686	0.957-1.0803	0.50037238
	Cruce	0.9933	0.9574-1.0306	0.54151327
	Jersey	1.0183	0.9247-1.1213	0.48342852
<b>Age</b>	< 1 year	0.9763	0.9555-0.9975	0.07933193
	1-2 years	0.9919	0.9666-1.0177	0.37884669
	2-4 years	0.9652	0.9523-1.0782	0.02878119
	>4 years	1.0373	1.0118-1.0636	0.00206385
<b>Sex</b>	-	0.964	0.9508-1.0775	0.01212846

\* Significance is denoted by a p-value <0.05.

<sup>1</sup> The results are shown as prevalence ratio (PR) and 95% confidence interval (95% CI).

sistent with what was established by Doria-Ramos et al. (2020) who indicated that female cows were affected with PTB more than males (OR = 4.37) [22]. According to Hole & Maclay (1959), there is a certain degree of susceptibility in certain bovine genetic lines. Furthermore, various reproductive states of females, such as childbirth and lactation, generate immunological alterations that can make females more prone to acquiring the infection [23–26].

In the present study, the highest seroprevalences were determined in Jersey (4.8%) and Holstein (3.8%) breeds, which coincided with the significant differences in positivity in different dairy breeds. Females of the

Department of Antioquia [12], in which the presence of bovine PTB was 33.8% and 17% in dairy biotype breeds from the same department [13]. The difference in the reported results mainly results from the response of the ELISA technique to MAP due to the stage of infection. Subclinical cases are usually seronegative, while animals with a high bacterial load are seropositive. Therefore, in the early stages of infection in most female cattle, when fecal excretion is low, the humoral antibody response is below the detection limit of serological tests [14, 15].

Comparing the results of this study with other investigations in Africa, Europe, and Latin America reveals that the values obtained in the present research are similar to those reported by various authors. Elmagzoub et al. (2020) and Ozsvári et al. (2020) observed a seroprevalence of 5.5% in dairy herds in Hungary, while a seroprevalence of 6.3% was found in Sudan [16, 17]. In Latin America, a seroprevalence of 2.1% was reported in dairy cattle and 9% in beef cattle in Argentina, and the first value was close to what was found in Tuta [18]. On the other hand, seropositivity values ranging from 6.3% to 10.7% have been established in Brazil, Chile, and Argentina [19–21].

Regarding the gender of the specimen, in our study, there was a significant statistical association between females and disease presentation, which is con-

**Table 3.**

Possible risk factors associated with bovine paratuberculosis infections, according to the management and biosafety variables of the farms.

Variable	Category	PR <sup>1</sup>	CI 95% <sup>1</sup>	p-value
Management of animals in pen	-	0.964	0.9508-1.0775	0.01212846
Natural service	-	1.0283	1.0069-1.0502	0.01673262
Artificial insemination	-	1.0088	0.9856-1.0325	0.30021614
Presence of other species in the farm	-	1.007	0.9836-1.0309	0.36282154
Own animals grazing in leased land	-	0.033	1.0204-1.0457	0.31903584
No presence of fence / inadequate or damaged fences	-	0.9961	0.972-1.0209	0.47205467
Hand milking	-	1.0126	0.9874-1.0384	0.20646591
Mechanical milking	-	0.9909	0.9674-1.0149	0.2846629
Silo feeding	-	1.0235	0.996-1.0518	0.05268572
Hay feeding	-	1.006	0.9723-1.0408	0.43831931
Feed with concentrate	-	1.0383	0.9793-1.1009	0.08539856
Quarantine upon entry of new animals	-	0.9833	0.9498-1.0181	0.1970303347
Presence of diarrhea	-	1.0240	0.9975-1.0513	0.0485341893
Herd size	Small herd (< 10 animals)	1.0106	0.9773-1.0451	0.3153541107
	Large herd (> 10 animals)	0.8873	0.8193-1.1031	0.1779873347

\* Significance is denoted by a *p*-value <0.05.

<sup>1</sup> The results are shown as prevalence ratio (PR) and 95% confidence interval (95% CI).

**Table 4.**

Analysis of the variables as possible factors of paratuberculosis in cattle from Tuta, Boyacá.

Variable	Odds Ratio (OR)	Lower confidence interval (LCI 95%)	Upper confidence interval (UCI 95%)	<i>p</i> -value *
Concentrate	5.8932	0.7631	45.51	0.089
> 4 years	3.5707	1.4942	8.5329	0.0042

\* Significance is denoted by a *p*-value <0.05.

Channel Island breeds (Jersey and Guernsey) were more likely to be seropositive for MAP than the females of other breeds [27, 28]. Genome-wide association analysis in Jersey cattle by Kiser et al. (2017) concluded that some variables contribute to the diversification in the reported heritability estimates, including the level of exposure to MAP in a given herd, precision of diagnostic tests and the samples used, handling of the specimens, and sample size [29]. In addition, a genetic predisposition of certain bovine breeds to MAP infection has been reported [1, 30, 31].

We found that the age of > 4 years was a risk fac-

tor for PTB presentation, which might be related to the nature of the disease because the incubation period of the disease is 5 years [32]. According to Fecteau (2018), cattle develop resistance with age and are usually infected when they are calves. Consequently, in a few animals, the infection evolves before 2 years of age, but from 2 to 6 years of age [1]. A large proportion of infected animals seem to develop anti-inflammatory immune responses characterized by IgG antibodies, which would explain why in this study the older individuals were the ones with the highest seroprevalence [33].

Another important factor to take into account for



the diagnosis of PTB is the test used for its identification. The capacity of a diagnostic test is determined by the age of an animal due to the chronic nature of MAP infection [34, 35]. Sweeney et al. (2016) reported a sensitivity of 15% for disease detection by ELISA in young subclinical cows and a sensitivity of 87% for detecting clinical cases that normally occur in older than 2 years by the same ELISA [36]. Therefore, the high seropositivity by ELISA in animals older than 4 years of age and its consideration as a risk factor for PTB in our study is in line with the reports of other investigations [20, 22, 37–41].

Regarding the management and biosecurity measures, our results showed that the presence of diarrhea had statistically significant associations with the variable feeding with concentrate and pen management. Eisenberg et al. (2012) reported significantly less frequent detection of MAP outside the stable compared to inside the stable because the high dilution of airborne contamination contained MAP mainly inside the barn, which would explain the association of the mentioned variables [42]. If adequate sanitary measures are not followed, for example, the MAP-contaminated manure is carried on the shoes when passing from the pen of adult cows to the pen of young calves, the disease spreads more easily. Therefore, some authors recommend separating newborns from adults, even when there is enough space between the pens [43]. In addition, the dust that is continuously produced in the animal housing by the movement of animals is made up of skin, hair, dry fecal matter, as well as feed and bedding material that can be spread throughout the barn by air and carry MAP particles [44].

Storing concentrate in places close to the management area of the adult cattle without the frequent cleaning of these sites, makes the feed contaminated more easily. As a result, the management of the concentrate in farms is considered important for the prevention of PTB. On the other hand, there is research that reports an association between feeding with concentrate and seropositivity for the disease [11]. Therefore, it is necessary to disinfect the storage area and leave the barn empty for two weeks to effectively reduce the presence of MAP on surfaces and in the environment as mentioned by Eisenberg et al. (2011) [42].

We also found a statistical association between the positive animals and those who presented diarrhea, which is very important because diarrhea is considered a clinical sign of the disease. The latter association has already been revealed in some studies. For example, in China, cows from two dairy farms in Tai'an City, Shandong Province, that exhibited this clinical sign and were resistant to antibiotic treatment

were found to be positive for PTB based on clinical inspection and histopathological examination [45]. In Turkey, in a study on animals with chronic diarrhea, the disease was detected more in animals with this clinical sign [46].

In the current research, the size of the herd did not have an association with the disease. On the other hand, some investigations observed changes in disease behavior depending on the size of the herd, showing that an increase in the number of animals rises the chance of having positive subjects [47, 48]. However, further research is required, especially because the average size of dairy herds in the area continues to increase [49]. Currently, the size of the herds is not large enough to make a statistically significant difference.

The presence of the disease in the municipality of Tuta is confirmed. However, the seroprevalence was not high due to the chronic course of the infection. In cattle > 4 years the seroprevalence was significant and was considered a risk factor. Therefore, it is presumed that the number of infected animals is much higher than that presented in this work considering the number of subclinically infected animals that do not show a strong immune response diagnosed as seropositive.

Feeding with concentrate presented a statistical association as well as management in the pen, showing the importance of taking different sanitary measures that will notably influence disease control. Moreover, a statistical association was identified with diarrhea, which could be taken as a biosecurity measure on farms because animals with this clinical sign can be separated and prevent the spread of the disease.

Further studies using different diagnostic methods in the area that specify the impact of the disease on the cattle herds of the municipality of Tuta are recommended. These investigations may serve as a basis for an adequate control plan and prevention of bovine PTB.

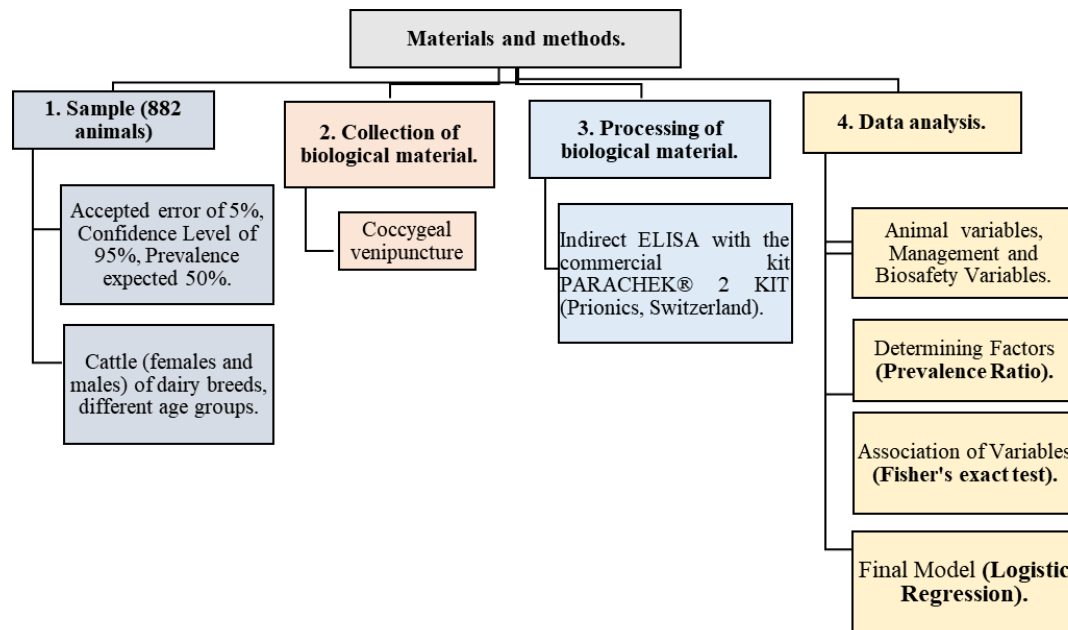
## Materials & Methods

### Geographic location

Tuta is located 26 km from the city of Tunja on the Briceño-Sogamoso double carriageway. It is part of the central province of the Department of Boyacá and is located in the green valleys of the Tuta River, which is part of Alto Chicamocha. The urban area is located at north latitude 05° 41' 36" and west longitude 73° 13' 51" at 2600 meters above sea level with an average temperature of 14 °C [50]. The relative humidity throughout the year is 75%. In the municipality of Tuta, there are two rainy periods during the year, the first of which is between April and June and the second rainy period is between September and November [51].

### Sample size

According to the Livestock Census of the Instituto Colombiano Agropecuario (Colombian Agricultural Institute) (ICA), carried out during the two annual vaccination cycles against foot-and-mouth disease and brucellosis, there are 26,411 cattle in the municipality of Tuta [52].



**Figure 1.** Summary of the instruments used and description of the steps taken in this research

A sample size of 882 animals with a sampling fraction of 3.44% was determined by the following formula:

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

Where n is the sample size, Za represents constant at the 95% confidence interval, p denotes expected prevalence, and E is the acceptable margin of error. An accepted error of 5%, a confidence level of 95%, and an expected prevalence of 50% were considered because no studies of this type have been carried out in the municipality.

### Collection of biological material

Blood samples were collected from the female and male cattle of dairy breeds in different age groups. The blood specimens were obtained by coccygeal venipuncture using needles of 16 g × 3, 18 g × 3, and 16 g × 1 gauge. Before taking the blood samples, the area was depilated and disinfected with alcohol to facilitate taking the sample and avoid contamination. For blood collection, the vacuum tube system (Vacutainer®) was used, which provides an aseptic condition and preserves the samples due to being a closed system [53].

### Processing blood samples

The samples were centrifuged at 2500 rpm for 10 min to separate the cells from serum. Subsequently, using a Pasteur pipette, the serum or supernatant was separated and transferred to a storage tube to perform the test [53]. Serum samples were assessed using indirect ELISA by commercial PARACHEK® 2 KIT (Prionics, Switzerland) (sensitivity 70% and specificity 100%) following the manufacturer's instructions. Those samples whose percentage of positivity was ≥ 15% of the cut-off point were determined as positive.

### Variables

The evaluated variables were classified into two categories of animals

and management. The management and biosafety variables included management in the pen, the use of artificial insemination or natural service, presence of other species on the farm, grazing on rented land, fence general condition, manual or mechanical milking, feed supplementation with silo, hay, or concentrate, quarantine upon the entry of new animals, presence of diarrhea, and herd size. The variables related to the animal were gender, age group, and breed.

### Statistical analysis

The samples of this descriptive cross-sectional study were selected through a simple random sampling method, in which each member of the study population had the same probability of being selected. The apparent prevalence and the real prevalence were determined utilizing the statistical software WinEpi. With the epidemiological database consolidated in Excel, the results were processed by the statistical software EpiInfo®. The determining factors were established by calculating the prevalence ratio, where the dependent variable was the serological results and the independent variables were all the determining factors established in the structured epidemiological applied survey. The association between the variables and the obtained results was examined by Fisher's exact test. Once these factors were established, a final model was built using logistic regression analysis. The size of animals and herds affected by PTB that were exposed to a factor was compared with the same proportion of a population not exposed to that factor to estimate the prevalence ratios. Prevalence ratio was used because this test is recommended for cross-sectional studies, and is a conservative and consistent measure of association with better interpretation. The prevalence ratio was used to evaluate the association between PTB and the determinants, as well as the importance of these associations by the means of Fisher's exact test [54]. Prevalence ratios greater than 1 (95% LCI lower confidence interval < 1) and with p < 0.05 were considered risk factors, while prevalence ratios less than 1 (95% UCI upper confidence interval < 1) and with p < 0.05 were considered protective factors. The dependent variable was the MAP ELISA results, and the independent variables included all the determinants established in the epidemiological survey applied during the sampling, such as age group, breed, gender, and the

management and biosecurity variables of the farm. Once these factors were established, a stratified logistic regression was performed to test for confounding and identify simultaneous interactions between variables significantly associated with MAP [55] (Figure 1).

### Ethical considerations

The study was carried out under the regulations of laws 576, 2000, and 84 of 1989 of the Republic of Colombia. Informed consent was obtained from animal owners prior to sample collection.

### Authors' Contributions

S.E.C.E conceived and designed the experiment and interpreted the data, D.J.L.B. collaborated with the experiment and critically revised the manuscript. D.M.B.C collaborated with the experiment and analyzed the data. D.J.G.C. critically revised the manuscript. M.O.P.M. supervised the experiment and critically revised the manuscript.

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### Competing Interests

The authors declare that there is no conflict of interest.

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