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Reproductive and seminal characteristics of Pelibuey rams infected with *Mycobacterium avium* subsp. *paratuberculosis* in the subclinical stage

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ABSTRACT

Objective: To evaluate the reproductive and seminal characteristics of Pelibuey rams infected with *Mycobacterium avium* subsp. *paratuberculosis* in the subclinical phase.

Design/methodology/approach: In order to determine whether paratuberculosis (PTB) affects the reproductive variables and seminal quality in Pelibuey rams naturally infected with *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in the subclinical phase, weekly evaluations were conducted, over a period of two months, of the variables live weight, scrotal circumference, ejaculation latency and seminal characteristics: volume, pH, masal motility and progressive individual motility, concentration, live spermatozoa and dead spermatozoa, in five rams infected by MAP and a further five uninfected rams, of average weight and age 53.58 kg (±3.26) and 2.91 years (±0.59), respectively.

Results: PTB was not found to affect the reproductive and seminal variables evaluated.

Limitations of the study/implications: It would have been convenient to carry out the evaluation of the seminal characteristics for a longer time, however, the sampling was stopped in order to ensure the animal welfare of the rams as far as possible, since they began to show signs of PTB.

Findings/Conclusions: The Pelibuey rams diagnosed with PTB in subclinical phase did not present any effect on the reproductive and seminal characteristics. Meanwhile, reproductive management can be carried out with PTB-infected Pelibuey lambs in a subclinical phase.

Keywords: Subclinical paratuberculosis, live weight, seminal evaluation, rams.

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INTRODUCTION

Paratuberculosis (PTB) is a debilitating incurable disease that causes chronic granulomatous gastroenteritis with lymphangiectasia and lymphangitis. Animals infected with PTB, present chronic or intermittent diarrhea, hypoproteinemia, weight loss and eventual death; the

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etiologic agent is *Mycobacterium avium* subsp. *paratuberculosis* (MAP) and the disease mainly affects domestic and wild ruminants (Chiodini *et al.*, 1984). The main route of MAP transmission is fecal-oral (Clarke, 1997; Whittington & Windsor, 2009). Lesions caused by MAP can be presented 12 months prior to any evident clinical signs of PTB (Mcgregor *et al.*, 2015). Progression and outcome of the disease varies considerably according to the individual; clinical signs usually develop over the months or years following exposure, depending on the species (Whittington *et al.*, 2012). Likewise, it is considered that the clinical cases represent the tip of the iceberg, since it is estimated that for each clinical case detected there are at least another 25 potentially infected animals, with no apparent clinical signs (Whitlock & Buergelt, 1996). However, some factors have been described that cause stress in the ovine; such as malnutrition and parasitic, viral or bacterial infection, that can influence the transition from the subclinical to the clinical stage of PTB (Ayele *et al.*, 2001).

In Mexico, the rate of prevalence of PTB is between 5 and 30%, mainly in bovines, goats, ovines and fighting bulls (Guzmán-Ruiz *et al.*, 2016). With reference to these seroprevalence values, Stau *et al.* (2012) indicated that the most frequently used diagnostic test is the enzyme linked immunosorbent assay (ELISA), which has been used to detect antibodies for MAP since it is of low cost and the collection of samples and test procedures are easy, with little risk of contamination, and produce results faster than other diagnostic tests. A seroprevalence of 20% has been detected in bovine semen production units, raising the possibility of propagating the disease to a large number of females during artificial insemination (Abbas *et al.*, 2011). This could also occur in ovine, since the presence of MAP has been reported in infected rams (Eppleston & Whittington, 2001). In terms of the risk of transmission of MAP, Ayele *et al.* (2004) reported that the use of bovine stud bulls infected with PTB in subclinical state could present a risk for the dissemination of the mycobacteria via semen, since the presence of MAP has been reported in testicles, epididyme, seminal vesicles and semen. In the case of infected ovine, Eppleston & Whittington (2001) indicated the presence of MAP in the mesenteric lymph nodes, ileon and semen, and Velázquez-Morales *et al.* (2019) identified MAP in semen samples (42.9%) and in testicular tissue (42.8%) wearing the nested PCR technique, in rams Pelibuey with PTB in a clinical stage. In terms of the effects of PTB on semen, Khol *et al.* (2010) reported reduced motility and integrity of the membrane and morphology of the normal spermatozoid in an infected bovine during the transition from the subclinical to the clinical stage of the disease. However, no studies have shown whether PTB affects the seminal characteristics of naturally infected rams, which would represent a risk in the use of ovine semen in genetic improvement programs. Therefore, the objective of this study was to evaluate the live weight (LW), scrotal circumference (SC), ejaculation latency (EL) and seminal characteristics in rams naturally infected with MAP, in subclinical stage.

MATERIAL AND METHODS

Accommodation and animals

The experiment was conducted in the observation and isolation corrals of the ovine flock of the Córdoba Campus of the Colegio de Postgraduados (ColPos), a research and

study unit with a background of prevalence of PTB. The rams were kept in different facilities in separate corrals in order to avoid possible infection, but under the same conditions of intensive management, which allowed the practice of protocols of control and prevention of naturally infected ovines. The experiment was designed under the criteria of the Mexican Official Norm (NOM-062-ZOO-1999) covering technical specifications for the production, care and use of laboratory animals (SAGARPA, 2001), in concordance with the regulations for the use and care of research animals (ColPos, 2019). The Campus is located at Córdoba-Veracruz federal highway (18° 51′20″ N; 96° 51′37″ W, and 720 meters above sea level). The climate is warm subhumid, with a mean temperature of 18 °C and mean annual precipitation of 1807.3 mm (García, 2004). Ten rams of the Pelibuey breed were used: five infected with PTB in subclinical stage and five non-infected, with mean weight and age of 53.58±3.26 kg and 2.91±0.59 years, respectively. Each ram was given 2.0 kg with alfalfa hay (18% PC) per day, with 200 g of concentrate (14% PC; 1.7 DS/kg) and water was provided freely.

Diagnosis of PTB

To diagnose PTB in the Pelibuey rams, the serological ELISA test was performed at the beginning and end of the experimental phase. This test presents a sensitivity of 79.31% and specificity of 82.25%, according to Martínez-Covarrubias *et al.* (2012). The test was conducted with blood samples taken from the jugular vein. The blood serum was recovered by centrifugation at 1000 x g for 10 min, and stored at −20 °C until subsequent processing in the CENID (Centro Nacional de Investigación Disciplinaria)-Microbiology laboratory of the Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias (INIFAP).

Measurement of reproductive variables and collection of ejaculate

During the reproductive season (october and november), eight measurements were taken weekly in the mornings. The parameters measured were live weight (LW), scrotal circumference (SC) and ejaculation latency (EL), and semen was collected. The measurements of LW and SC were conducted prior to initiating the semen collection. For measurement of LW, an electronic balance of capacity 250 kg ± 100 g (Braunker YP200S) was used; for SC, a flexible metallic scrotometer was used, following the methodology indicated by Benmoula *et al.* (2017). The EL measurement was conducted with each collection of semen, following that proposed by Swelum *et al.* (2017). Prior to beginning semen collection, the preputial hair was cut, and the prepuce was washed with antiseptic liquid soap and disinfectant (Dermocleen^{MR}). Seminal samples were then obtained from each individual using the artificial vagina method in accordance with that established by Williams *et al.* (2001).

Semen evaluation

Immediately after semen collection, the seminal quality was evaluated with macro and microscopic tests. The variables of the seminal evaluation were: volume (V, mL), masal motility (MM, classification from 0 to 5), individual progressive motility (IPM, %), spermatic concentration (C; value × 10^6 spermatozoids mL^{-1}), live spermatozoids

(LS, %) dead spermatozoids (DS, %) and pH (values from 0 to 14). Examination of the V and IPM and estimation of MM were conducted following the protocol of Benmoula *et al.* (2017). Determination of V was conducted in graduated collection tubes of 15 mL, at a temperature of 37 °C. The IPM was determined under a microscope (objective 400 x), with values expressed as percentages from 0 to 100. To determine MM, the semen was observed under a microscope (objective 10 x) (Carl ZEISS, Primo Star, CP11406, Microimaging GmbH 37081, Gottingen, Germany, Series-Nr: 3125001511), with values assigned from 0 (null motility) to 5 (vigorous motility). The percentage values of LS, DS and C were determined following the methodology of Ogundele *et al.* (2016); LS and DS were calculated from a smear stained with the eosin-nigrosin technique, in which the live and dead spermatozoids were quantified using bright-field microscopy (400 x). The value of C was determined using the hemocytometer method, visualizing the semen (objective 40 x) in a NeuBauer chamber (Marienfeld®). The pH was measured using paper pH test strips (Macherey-Nagel GgmbH and Co. KG) with a pH interval of 6.5 to 14 (El'Tohamy *et al.*, 2012).

Statistical analysis

The experimental design was a completely randomized model with repeated measures over time. The infected animal was used as a fixed effect and each animal nested in the treatment with PTB used as a random effect. For analysis of all variables, a Mixed procedure was used in the statistical package SAS® (SAS Institute 2006). Comparison of means was conducted with a Tukey test, employing a significance level of $P \leq 0.05$.

RESULTS AND DISCUSSION

Live weight, scrotal circumference and ejaculation latency

The presence of MAP in the rams infected with PTB in subclinical stage did not affect LW, SC and EL ($p > 0.05$) over a period of two months (Table 1).

The results of this study indicated that PTB in subclinical stage had no effect on LW. Despite the fact that PTB is a disease characterized by causing weight loss, dehydration and profuse diarrhea in adult bovines, this diarrhea is not a constant in ovines (Verin *et al.*, 2016). Likewise, the presence of diarrhea was not reported in this study and no effect was found on LW. In contrast, Mcgregor *et al.* (2015) reported an effect on LW when they conducted a three-year longitudinal study in PTB infected Merino sheep of up to three years old, which could involve a weight loss of up to 5 kg. That study stated that

Table 1. Live weight, scrotal circumference and ejaculation latency in Pelibuey rams with and without PTB in subclinical stage, over a period of two months.

Animals	Variables		
	Live weight (LW, kg)	Scrotal circumference (SC, cm)	Ejaculation latency (EL, s)
With PTB	54.3±3.2	28.6±1.47	48.8±8.09
Without PTB	54.5±3.2	30.4±1.47	40.7±8.02

No differences were found between treatments (Tukey; $p > 0.05$). Mean ± standard error.

the weight loss was the result of the effects of intestinal lesions caused by MAP, which generally occur at two years after exposure. It should be noted that, unlike in the males, the females more habitually present the effects of protein metabolism, catabolism and mobilization of fats, which can be particularly notable in ewes at the end of pregnancy or in early lactation when the demands of synthesis of energy and proteins are increased (Allen *et al.*, 1974). This supports that stated by Clarke (1997) and Jaimes *et al.* (2008), whom indicate that in small ruminants, PTB is manifested at between two and three years old, due to the diffuse hypertrophy caused by MAP in the mucous membrane of the jejunum and ileon; these lesions typical of MAP (granulomatous enteritis and thickening of the intestinal mucous membrane) cause poor absorption of nutrients with enteropathy, and thus generate a loss in corporal condition. However, it has not been determined whether these lesions present in subclinical stage reduce the intestinal function to a degree that is sufficient to produce a negative energetic balance (Kostoulas *et al.*, 2006). In relation to SC, no differences were found between treatments in this study; further, it was demonstrated that PTB did not generate a reduction in SC. To date, there is no literature that reports alterations in the SC of rams infected with MAP; however, Khol *et al.* (2010) took measurements of the epididyme over a period of one year in a bovine infected with MAP and reported a diameter decrease of 3 cm. Smith *et al.* (2010) indicated the likelihood that PTB acted to reduce fertility in dairy cows, due to the fact that the infection generates a negative balance of energy and protein, but not because of any direct effect of MAP at the reproductive level. This coincides with that found in the present study, since no modifications were found in the values produced by the weekly evaluations conducted, demonstrating that the variables LW, SC and EL presented no changes, at least during the sampling period.

Seminal quality

The quality of the ejaculate showed no variation among the rams with and without PTB infection ($p>0.05$; Table 2) over the two-month period of the test.

The repeated measurements of V and C did not present variation over time as a result of the effect of MAP ($p>0.05$). Again, no effect was found in the measurements of pH,

Table 2. Seminal characteristics in Pelibuey rams with and without PTB in subclinical stage, over a period of two months.

Variables	Rams	
	With PTB	**Without PTB**
Volume (V, mL)	0.9±0.09	0.9±0.09
pH	6.9±0.13	6.9±0.13
Masal motility (MM, 0-5)	3.8±0.21	4.2±0.20
Individual progressive motility (%)	82.7±2.83	85.5±2.76
Concentration (C, 10^6 spermatozoa mL^{-1})	2,420±425.4	2,634±425.4
Live spermatozoa (LS, %)	83.2±5.8	81.3±5.8
Dead spermatozoa (DS, %)	16.7±5.8	18.7±5.8

No differences were found between treatments (Tukey; $p>0.05$). Mean±standard error.

LS and DS (Table 2) taken over the two-month period of testing ($p>0.05$). The presence of MAP also had no influence on the values of the repeated measurements over time in the variables MM and IPM ($p>0.05$). Khol *et al.* (2010) reported the case of a bovine in which seminal evaluations were conducted over the period of one year, which showed variations in V and pH of between 4 and 12 mL and between 6 and 7.4, respectively; the MM decreased from 72 to 49%, and C never exceeded 2.8×10^6 per mL. For this reason, the semen was considered to be of poor quality, but these authors indicated that this low seminal quality was due mainly to malnutrition and the poor corporal condition of the animal and was not a consequence of infection with MAP. In addition, Caldeira *et al.* (2021) described that Bulls Nelore semen inoculated with MAP (10^3 to 10^8 CFU/mL) generates a decrease in sperm motility and vigor, which is possibly due to the adhesion of MAP with the intermediate part (tail) of the sperm, region containing a large amount of fibronectin in the plasma membrane.

Since the infection in the rams of this study was acquired naturally, it should be noted that the duration of the period of this infection is unknown. For this reason, there is uncertainty regarding the number of granulamatomas present in the intestine. These are lesions that take years to produce changes in the corporal condition and health of ruminants (de Silva *et al.*, 2018). Clinical signs are generally presented at between two and five years old in bovines and ovines (Verin *et al.*, 2016). Since the age of the rams in this study was less than three years, it is possible that this prevented the progression of these lesions, since PTB is a chronic disease that presents a prolonged preclinical phase (Subharat *et al.*, 2012). It is also important to mention that the two month duration of the study is a relatively short period, which precluded recording of the progression and effects in the rams with PTB in subclinical stage.

For their part, de Silva *et al.* (2018) indicated that a chronic disease such as PTB requires years to develop under natural conditions, and that an experiment lasting 12 months therefore represents a short period. For this reason, the duration of the present study (two months) could not demonstrate whether PTB in subclinical stage affects the rams in terms of productive and reproductive variables. Nevertheless, it is important to highlight the risk presented by the use of naturally infected stud rams, even in the absence of clinical signs and with no apparent change in seminal variables during their productive lives, as shown in the results of this study with Pelibuey rams. For their part, Eppleston & Whittington (2001) indicated that infected rams can carry MAP in the semen, and can thus transmit the infection horizontally. For this reason, any possibility of the propagation of PTB is of great concern due to the associated considerable economic losses and risks to public health, since it has been related to Crohn's disease in humans (Ghadiali *et al.*, 2004; Garcia & Shalloo, 2015). In this regard, Jaimes *et al.* (2008) indicate that control of the disease in domestic ruminants depends on the early detection and elimination of infected animals, although this is limited by the current lack of an adequate diagnostic. It is therefore necessary to implement strategies of vigilance, detection and control of MAP, despite the fact that rams infected with PTB in subclinical stage present reproductive capacity and are phsyiologically fit.

CONCLUSIONS

The Pelibuey rams diagnosed with PTB in subclinical stage did not present any effect on the variables LW, SC and EL attributable to the presence of the MAP. Equally, the seminal variables V, pH, MM, IPM, C, LS and DS presented no significant differences as a result of the infection with MAP.

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