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SCIENTIFIC ARTICLE
Animal Infectious Diseases

Diagnosis of goat tuberculosis using tuberculinization and molecular techniques

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ABSTRACT

Tuberculosis is an infectious, chronic, and worldwide disease. It has been known since the beginning of humanity and still negatively influences public health and livestock, especially, in Brazil, in the northeast. Etiologic agents are the mycobacteria of the *Mycobacterium tuberculosis* complex, which is the most important in mammals' involvement. The state of Bahia has 68.7% of its territory located in the semiarid region and holds the largest goat herd in the country. Goat breeding is a social and economic activity that adds value to this region. Up to the present, data on goat tuberculosis is unknown in this state. Thus, this study seeks data on tuberculosis prevalence in goats in a semiarid region of Bahia by using the comparative tuberculin test and multiplex polymerase chain reaction (PCR). A total of 600 adult animals of both sexes were evaluated. A prevalence of 0.33% (2/600) and 33.33% (1/3) properties were found for positive animals. Each assessed property had a questionnaire to analyze the epidemiological data management and relevant aspects for the disease occurrence. To confirm the positive tuberculin test results, PCR was used to detect and identify the pathogenic mycobacteria involved in the infection. It is concluded that most of the properties performing goat breeding in the region show low technification levels and promote farming between different species. Low prevalence of the disease alerts preventive measures to avoid major proportion situations that could influence the goat breeding in the state.

Keywords: comparative tuberculin tes; infectious diseases; PCR, small ruminants; tuberculin.

INTRODUCTION

Tuberculosis is a chronic infectious disease known since the historical beginnings and is a huge problem because the advances in knowledge and technologies are taken for its control have not been enough to minimize its effects, particularly in developing countries. The disease shows granulomatous features, debilitating effect, attacking mammals and birds, causing severe problems in public health, and economic impacts by losing the animal production. It is caused by pathogenic bacteria related to human and animal diseases (LOBUE et al., 2010; CORREA W.M.; CORREA, 1992).

Mycobacterium bovis is the etiologic agent involved in the disease occurrence in cattle, goats, and humans. By behaving as the host of the infection in cattle breeding, goats are susceptible to tuberculosis, which means an obstacle for control programs and disease eradication in cattle and humans (LIÉBANA et al., 1998).

Tuberculosis in goats is detected by the comparative tuberculin test, by using bovine and bird tuberculin, to differentiate the animals truly infected with *Mycobacterium tuberculosis*, *M. bovis*, or *Mycobacterium caprae* from those sensitized by exposure to other mycobacteria (BEZOS et al., 2012; GUTIÉRREZ et al., 1998; SCHILLER et al., 2010). Despite the possibility of members' identification of *M. tuberculosis* complex by laboratory cultivation techniques, these are laborious

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and extended methods, impractical for monitoring and surveillance services (WARREN et al., 2006). Molecular techniques, such as the polymerase chain reaction (PCR), have been used to minimize the tuberculosis diagnostics obstacles in ruminants, adding agility and credibility to the results, reducing allergy occurrences, cross-reactions, and unsatisfactory or prolonged microbiological growth (SILVA et al., 2011a). Multiplex PCR (mPCR) is a quick, reliable tool, which enables the simultaneous amplification of more than one target DNA sequence in a single reaction, saving time and reagents (HERNÁNDEZ et al., 2003).

Thus, regarding the relevance of tuberculosis and its implications in public health, in socioeconomic importance of goat breeding in northeastern Brazil, its occurrence among other animal species and the need for data in the literature justified this study preparation, which purpose was to evaluate the field through a comparative cervical test, and the occurrence of goat tuberculosis in herds located in the semiarid region of Bahia.

MATERIAL AND METHODS

The state of Bahia represents 36.3% of the northeast region, 6.64% of the Brazilian territory, and 68.7% of the state is in the semiarid region. Brazil is composed of seven regions, 32 microregions, and 417 municipalities. The operating area of this study involves Capim Grosso municipality, located in the microregion of Piemonte da Diamantina, where the goat herd is composed of 4,602 animals (IBGE, 2008). The epidemiological profile analysis of herds required a questionnaire filled by each farmer when the tuberculin skin test was performed for information registration, such as water source of the property, type of food, facilities, sanitary management, breeding of other species, and the presence of wild animals.

Boer and Anglo-Nubian goats, their crossbreeds and mixed breeds over one year of age, of both sexes, were the goats analyzed in this study.

The minimum number of tested samples was calculated according to THRUSFIELD (2004), with a confidence level of 99% and a sampling error of 5%. The expected prevalence of 50% was used for a calculation to maximize the sample size since the estimated prevalence was unknown.

A total of 600 animals were distributed in three properties of the municipality studied, which sampling occurred from May to September 2012 (Table 1).

Table 1. Sampling distribution between the properties studied.

Property	Total of herd	Number of samples
1	1,600	400
2	60	51
3	600	149
Total	**1,860**	**600**

Source: Elaborated by the authors.

The animals were randomly selected, excluding those with physiological changes or with some disease signs, females in the peripartum process, and animals with changes in normal physiological patterns, to reduce false-negative results (MONAGHAN et al., 1994).

All properties had animal husbandry associated between goat, sheep, and cattle. Two properties showed poultry farming, and all showed the presence of birds and wild mammals in their pastures. All evaluated goats were intended for cutting and raised in a semi-extensive regime with free access to pasture during the day and in sheepfold during the night, with free contact between other species of the property. All animals receive mineralized salt for goats, *ad libitum* water, and feeding in extensive grazing with hay supplementation, cochineal cactus (*Opuntia cochenillifera*), and/or mandacaru (*Cereus jamacaru*). There was no separation of water sources between species in none of the farms visited. In addition, there is no supply of milk or colostrum for newborn goats. Goats have not shown cough, dyspnea, apathy, intolerance to exercise, or weakness signs.

The comparative cervical tuberculin test consisted of intradermal inoculation of 0.1 mL of avian tuberculin (cranially), and 0.1 mL of bovine tuberculin (caudally), on the right lateral cervical region of each animal, in two points, 7 cm far from each other, after identification, previous trichotomy, and measurement of skinfold (0 h) of the inoculation points were performed, as described by SILVA et al. (2006).

The standard inocula sensitizers (start 013/11, tuberculin bovine, and 004/11, avian tuberculin) were prepared by TECPAR Laboratory (Technology Institute of Paraná, Curitiba/PR), where the tuber protein was considered as the active ingredient, race D4 of *Mycobacterium avium* and race AN5 of *M. bovis*.

After 72 h of the post-tuberculin skin test, the thickness of the skin fold was measured, in pieces of tuberculin inoculation, by using a tuberculin caliper (Hauptner-MTV Germany – Hauptner) for evaluation of increased skin thickness due to a local immune response mediated per cells.

Alongside the comparative tuberculin test execution, a clinical examination was performed in goats, emphasizing the inspection of superficial lymph nodes and mucous membranes, cardiopulmonary auscultation, rectal temperature measurement, and body condition.

Due to the number of samples, cost for molecular techniques execution in all samples, and following the protocol of disease control programs for other species, the mPCR was held in two animals positive to tuberculin skin test compared to samples randomly collected from 28 non-reagent animals. Blood samples were collected in tubes containing ethylenediaminetetraacetic acid (EDTA) by jugular venipuncture. All samples were kept at –20 °C until the processing in the laboratory.

The Genomic Blood MiniPrep DNA (Axigen, Brazil) extraction kit was used according to the manufacturer's instructions. After purification, the DNA was excluded in 200 µL of TE (5 mmol·L^{-1} Tris-HCl; 0.1 mmol·L^{-1} EDTA; pH 8.5), purity concentration and level determined by spectrophotometry (260 and 280), and then stored at –20 °C for further use.

Primers were based on the DNA sequences of different regions according to WARREN et al. (2006). The set included the primers RD1, RD4, RD9, and RD12, which detect and differentiate five members of M. tuberculosis complex, namely, *M. tuberculosis*, *M. bovis*, *M. bovis BCG*, *Mycobacterium canettii*, and *M. caprae*. The mPCR was performed through a commercial kit (QIAGEN), according to the manufacturer's instructions. The reactions were performed in a final volume of 25 µL. The Immunology and Molecular Biology Laboratory (ICS/UFBA) provided the DNA of *M. bovis* as a positive control. Amplification was performed under the following conditions: first incubation at 95 °C for 5 min, followed by 45 cycles at 94 °C for 1 min, 62 °C for 1 min, and 72 °C for 1 min. After the last cycle, a final extension was performed at 72 °C, for 10 min. Amplified products were electrophoretically separated (6 V·cm–1) in agarose gel 3% (w/v), in filler cap TBE 1X, and visualized by staining with SYBR Safe. Each procedure (pre-PCR, PCR, and post-PCR) was carried out in areas physically separated to minimize the risk of cross-contamination.

Data analysis was performed using ASSISTAT 7.6, using the significance level of 0.05. ANOVA test was applied for variance analysis.

RESULTS AND DISCUSSION

From 600 evaluated animals, 2 (0.33%) were reagent to comparative tuberculin test in property 01, and 598 (99.67%) were non-reagent. The prevalence of tuberculosis in goats, expressed in percentage, was calculated by the number of animals positive to the comparative tuberculin test, divided by the total number of evaluated goats. According to data established by SILVA et al. (2006), inconclusive reactions were not found in this study,

Although there are no control and eradication programs for goat tuberculosis in the country, it is believed that the results occurred due to management type, with animals kept in open areas and with the possibility of interaction between different species.

PIGNATA et al. (2009), in the state of Paraíba, showed similar data, where 0.48% of goats submitted to comparative tuberculin test presented positive results. These authors attributed the prevalence, among other things, to the use of management without proper technical instructions. Data similar to this study were found by BOMBONATO et al. (2010), in the state of Minas Gerais, where 233 goats were submitted to tuberculin skin test by the comparative cervical test, and a prevalence of 1.29% (3/233) was found. Low prevalence for tuberculosis in small ruminants (0.9% in sheep and 2.4% in goat), were found by JAVED et al. (2010) in a study performed in Pakistan, in which control programs are also not established for such disease. KASSA et al. (2012) found a prevalence of 0.5% of tuberculosis in small ruminants when studying 1,884 goats and 347 sheep in a pastoral region of Ethiopia.

By using the comparative cervical tuberculin test, MELO M. et al. (2005) and MELO L. et al. (2012), in the state of Pernambuco, reached a prevalence of 16.2% (11/68) and 12.2% (12/98), respectively, for dairy goats. SILVA et al. (2011b) found in the state of Pernambuco, through comparative cervical tuberculin test, a prevalence of 11.8% in herds of dairy goats. This study's different data occurred possibly because of management since the dairy goats are farmed with more intimate contact between them, favoring the spread of microorganisms. BOMBONATO et al. (2010) and PIGNATA et al. (2009) state

that the sample utilized, the geographical features, and the particular situation of each studied property could explain the differences between the prevalence observed in many studies. Besides the existence of factors related to the epidemiology of the disease affecting its development, such as the type of management, exploitation of the animals, domestic reservoirs, and climatic and/or environmental factors.

The positivity found in property 01 is assigned to the size of goat and cattle herds, which provides greater interspecies contact, implying a higher probability of goat tuberculosis occurrence. Since the practice of cattle and goats grazing together implies an increase of disease transmission risk, where the infected cattle are the main host of the infection, excreting microorganisms in-breath, feces, milk, urine, vaginal and uterine secretions, and festering lymph nodes (HIGINO et al., 2011; PIGNATA et al., 2010). Besides the size of herds, the property where the positive cases occurred has an extensive territorial area, allowing goats to walk long distances, maintaining contact with wild species, potential reservoirs for microorganisms of the *M. tuberculosis* complex. DUARTE et al. (2007), analyzing the transmissibility of *M. bovis* between domestic and wild species, from biomolecular methods, detected *M. bovis* race similar to spoligotyping technique, isolated from bovine and wild boars (*Sus scrofa*) that live in nearby regions, confirming wild species as potential reservoirs of the microorganism.

The average skin thickness, measured by tuberculin caliper Hauptner for avian proventricular dilatation disease (PPD), was 3.41 ± 0.75 mm, at 0 h, and 5.69 ± 1.19 mm at 72 h. Regarding bovine PPD, we found 3.38 ± 0.72 mm at 0 h and 5.27 ± 1.01 mm at 72 h. The difference between ΔB and ΔA was –0.39 ± 0.92 mm. The average skin thickness increase related to avian PPD occurred probably due to unspecific reactions caused by the previous contact with nonpathogenic mycobacteria present in the ground. Positive samples (Fig. 1) presented an average of 3.06 ± 1.22 mm at 0 h, and 5.26 ± 1.33 mm at 72 h, 2.88 ± 0.57 at 0 h, and 9.01 ± 1.97 at 72 h, for avian and bovine tuberculin, respectively. The difference between ΔB and ΔA was 3.93 mm, representing a positive reaction according to data published by SILVA et al. (2006), determining positive values for comparative tuberculin test, when bovine tuberculin reaction is greater than the avian, at least in 2.5 mm. Two animals diagnosed as positive to allergic immune test did not show any characteristic clinical signs for tuberculosis or another disease; however, because it is a reaction of delayed hypersensitivity of type IV, they showed inflammatory tissue reactions in tuberculin inoculation places, with the well-defined area, hyperemia, severe pain on palpation, swelling and heat, besides the increase of pre-scapular right lymph nodes (TIZARD, 2009).

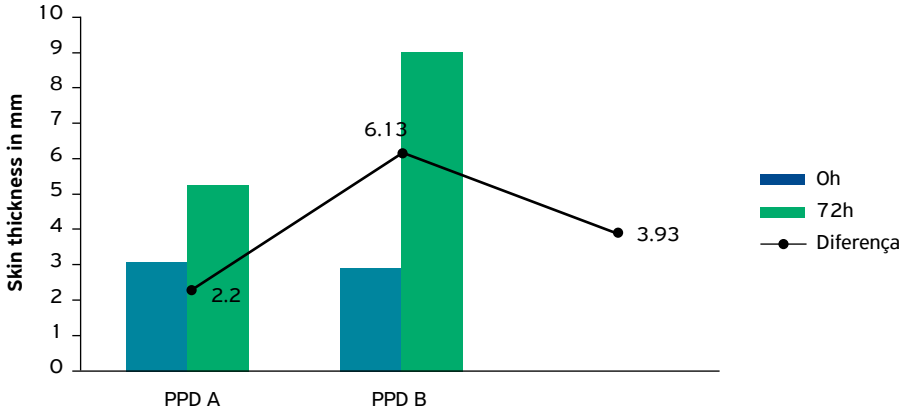

Figure 1. Tuberculin caliper Hauptner results of positive samples, at 0 and 72 h, indicate the difference between ΔB and ΔA.
Source: Elaborated by the authors.

MELO L. et al. (2012) found in animals positive to tuberculin comparative allergy test an average avian PPD of 3.8 ± 0.2 mm, at 0 h, and 9.7 ± 3.5 mm at 72 h. As for bovine PPD, the average was 3.4 ± 0.4mm, at 0 h, and 11.8 ± 4.0mm at 72 h, with a difference between ΔB and ΔA of 5.9 ± 2.2mm. They differ from the results found in this study with the averages and clinical signs occurrence. Such authors observed emaciation, anemia, lymphadenopathy, and bilateral nasal discharge in the evaluated animals.

The occurrence of positive goats for tuberculosis in this study warns for the risk of spread to other species and as a potential source of infection for humans, meaning obstacles to the success of bovine tuberculosis control programs (HIGINO et al., 2011). There is also a strong concern on the goat tuberculosis issue, because the animals detected as positive may continue spreading the disease to other animals and herds, hindering control and eradication actions since the farmers are not indemnified, preferring to sell or keep the animals on the property. Another aspect of being considered is specific

legislation to ensure the sacrifice of animals with two inconclusive or positive results, as established for cattle in the National Program for Control and Eradication of Brucellosis and Tuberculosis (NPCEBT) (PIGNATA et al., 2009).

Table 2 shows that there was no statistically significant difference between negative and positive samples at 0 h; however, at 72 h, there was a statistically significant difference between avian and bovine tuberculin, which was already expected because the positivity was associated with higher amplitude of cutaneous increase in the inoculation place of bovine tuberculin ($p < 0.05$).

Table 2. Arithmetic average and standard deviation (mm) of the increases in the skin thickness of goats submitted to comparative tuberculin test, positive and negative samples.

Negative samples		
PPD	**Oh (a.i.)**	**72 h (p.i.)**
A	3.41 ± 0.75 aA	5.69 ± 1.19 aB
B	3.38 ± 0.72 aA	5.27 ± 1.01 aB
Positive samples		
PPD	**Oh (a.i.)**	**72 h (p.i.)**
A	3.06 ± 1.22 aA	5.26 ± 1.33 aB
B	2.88 ± 0.57 aA	9.01 ± 1.97 bB

*In the same column, averages with different lowercase letters indicate a statistical difference between the groups within each moment of reading ($p < 0.05$). Averages with different uppercase letters in the same line indicate a statistical difference between reading times within each group ($p < 0.05$). a.i.: cutaneous measure before inoculation (in mm). p.i.: cutaneous measure after inoculation (in mm).
Source: Elaborated by the authors.

The samples, product of DNA extraction from total blood, showed an appropriate concentration of DNA in the spectrophotometry. After using the mPCR technique, only negative samples were found for the presence of mycobacteria or any other pathogenic microorganism since any genomic fragment in agarose gel was not visualized. Fact attributed to the absence of microorganisms in blood circulation because of intracellular characteristics of the etiologic agent, which ceased the multiplication process in two to three weeks postinfection, occurring an immune response mediated by cells with granulomas formation (VERONESI; FOCACCIA, 2010). The tubercle bacillus survives inside the granuloma throughout the animal's life, special environment with mechanisms to ensure the inhibition of host immune response.

The mPCR used is adequate because it is able to detect and identify pathogenic microorganisms constituent of *M. tuberculosis* complex. This technique amplifies two or three different targets, being able to differentiate the *M. tuberculosis* from nontuberculous mycobacteria (POROCA et al., 2009) because the primers correspond to different regions (RD1, RD4, RD9, and RD12) that are absent and/or present in pathogenic mycobacteria, allowing the differentiation of *M. canettii*, *M. tuberculosis*, *M. caprae*, *M. bovis*, and *M. bovis BCG* (WARREN et al., 2006).

SILVA et al. (2011a), using as a template for direct product reaction of DNA extraction from specimens, found results of the conventional PCR, and in unproductive real-time, since none genomic fragment of *M. tuberculosis* and *M. avium* complexes was visualized in agarose gel, corroborating with this study. POROCA et al. (2009) attributed the failure of techniques used for DNA extraction of clinical samples of ruminants infected by *M. bovis* to a small number of bacilli, unlike the clinical sputum samples of human beings that have a large number of bacilli of *M. tuberculosis*, facilitating the DNA extraction.

CONCLUSIONS

Only one property among the three studied showed positive animals, despite being the only one to have adequate sanitary management and veterinary care. Two properties presented none or low level of technification in their spaces; however, they do not use cow milk for goat feeding, which shows an important management aspect in goat tuberculosis prevention. The results found for goat tuberculosis in this study showed the need for further epidemiological studies of the disease for a better understanding of the natural foci, the forms of agent movement between the diverse species of animals, and the behavior of the disease in the assessed region, to support preventive actions by veterinarians, as well

as extension, health protection, and public health services. It is observed from molecular results that the total blood samples are still an option for detection for in vivo evaluation, although they are not the best options for *Mycobacterium* spp. detection through biomolecular techniques.

AUTHORS' CONTRIBUTIONS

Conceptualization: Bahia, R.C. **Data curation:** Lopes, C.V.S; Fernandes, B.P.; Rosa, M.G.; Cunha, D.P.; Bahia, R.C. **Formal analysis:** Lopes, C.V.S; Fernandes, B.P.; Rosa, M.G.; Cunha, D.P.; Bahia, R.C.

AVAILABILITY OF DATA AND MATERIAL

All data generated or analyzed during this study are included in this published article.

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CONFLICTS OF INTEREST

The authors declare there is no conflict of interests.

ETHICAL APPROVAL

Not applicable.

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