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Molecular typing of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) isolated from dairy goats in Brazil

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ABSTRACT

In the last decade there was a significant increase dairy goat participation in the world and Brazilian agricultural scenario. Minas Gerais state is the third largest producer of goat milk in Brazil. Paratuberculosis is a chronic intestinal disease that mostly affects ruminants and can become a public health problem due to the possible association with Crohn's disease. It is transmitted by ingesting *Mycobacterium avium* subsp. *paratuberculosis* (MAP) contaminated food or water. In recent years, with the development of molecular techniques, the disease has been identified with greater precision and more quickly. MAP strains (S-Sheep, Cattle-C and B-Bison) have also been typed. The detection of a circulating MAP strain in a herd is important to develop epidemiological studies and thus establish better strategies to control paratuberculosis. This study aimed to identify and type MAP in dairy goat farms in the Zona da Mata, the main producing region of Minas Gerais state, Brazil. Feces and milk samples of 467 animals were collected, processed, inoculated in Herrold's Egg Yolk Agar (HEYM) and submitted to PCR and REA techniques. Eleven (2.36%) animals were positive for the presence of MAP in four properties and the isolates were characterized as type C strain. It was concluded that MAP is present in dairy goats from properties in Zona da Mata, MG and that strain type C circulates in the area. This is the first report of MAP typing isolated from dairy goat in Brazil.

1. Introduction

According to the FAO (Food and Agricultural Organization of the United Nations), there were 1,005,603,003 goats worldwide in 2013, and the continent with the largest number of goats was Asia, with 59% of the animals. Europe has only 2% of world goat herd, but is responsible for 18% of the world production of goat's milk (Dubeuf, 2010). In Brazil, raising goats has increasingly become an important livestock alternative. In the last decade there was a significant increase in goat milk, meat and skin production (Da Silva et al., 2012). The Zona da Mata of Minas Gerais, Brazil, is composed of seven microregions containing approximately 20% of the herd of the Southeast (Fonseca and Bruschi, 2009). It stands out as a major goat milk producing region.

Paratuberculosis is a chronic infectious disease of the intestinal tract caused by the bacterium *Mycobacterium avium* subsp. paratuberculosis (MAP), which predominantly affects domestic and wild ruminants (Chiodini, 1984; Clarke, 1997; Ayele et al., 2005; Mota et al., 2010). The ingestion of food or water contaminated with infected animal feces is the main form of transmission. Milk has been suggested as a possible MAP transmission vehicle to humans (Grant et al., 2001).

Based on the comparison of the whole MAP genome, a biphasic evolution scheme has been proposed, distinguishing two main strains: bovine (C-cattle) and sheep (S-sheep), genotypically and phenotypically different from each other (Janagama et al., 2010). The association of each strain with the host is not exclusive and may cause disease in all types of ruminants (Biet et al., 2012). Although the insertion sequence IS*1311* is present in *M. avium* subsp. *avium* and MAP, five point mutations differentiate the sequences of the two subspecies (Whittington et al., 1998). Such mutations may be used as a target in restriction enzyme analysis (REA) to realize this differentiation. Furthermore, some copies of IS*1311* from the MAP

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Table 1
Distribution of producing properties of dairy goat in the mesoregion of Zona da Mata of Minas Gerais, Brazil, by microregions.

Microregions	Number of proprieties	Percentage (%)
Cataguases	6	14.63
Juiz de	13	31.71
Fora	8	19.51
Manhuaçu	9	21.95
Muriae	1	2.40
Ponte Nova	2	4.88
Uba	2	4.88
Viçosa TOTAL	41	100

C strain contain an additional mutation which can be used to differentiate it from S strain (Whittington et al., 1998). Whittington et al. (2001) confirmed a new type of MAP by REA: Type B (bison), originally isolated from this species, with *in vitro* growth phenotypic characteristics distinct from type C strain.

This study aimed to identify and type MAP in dairy goat farms in Zona da Mata, the main producing region of Minas Gerais state, Brazil. The detection of a circulating MAP strain in a herd is important to develop epidemiological studies and thus establish better strategies to control paratuberculosis.

2. Material and methods

A planned sampling was conducted from a list of 41 farms of dairy goats from the mesoregion Zona of Mata, Minas Gerais state, obtained from a regional association of dairy goat producers. Ten farms were selected (24.39%), according to the distribution in the different micro-regions. The regions are described in Fig. 1, and the distribution of the farms producing goat milk in the regions of Zona da Mata is shown in Table 1. The study included only farms operating for commercial purposes, with a minimum of 50 animals. Animal distribution per age was analyzed and adult animals were selected in order to include only those in milk, randomly. Farm sampling was calculated using the OpenEpi® program, considering an estimated prevalence of 5%, precision 4% and 95% confidence interval.

Initial hygiene milking was conducted prior to milk collection, according to the management of each property, followed by the cleaning of the teats with 70% ethanol and drying with paper towels. The first three milk jets were discarded, and 40 mL of milk were collected, 20 mL of each teat. Feces samples were collected individually, from rectum, using disposable gloves and subsequently transferred into sterilized vials and identified. Feces samples processing followed the method described by Stabel (1997) using hexadecylpyridinium (HPC) 0.9% and antimicrobial solution containing nalidixic acid (50 mg/L), vancomycin hydrochloride (50 mg/L) and amphotericin B (150 mg/L). Milk samples processing followed the method described by Pillai and Jayarao, (2002), using HPC 0.75% and the same anti-microbial solution mentioned above. 100 μL of the processed material were added to tubes containing Herold's Egg Yolk Medium medium (HEYM medium) with or without mycobactin *J*, then incubated at 37 °C and monitored weekly for 18 weeks. This project was approved by the ethics committee on animal use of Universidade Federal de Viçosa, CEUA/UFV with number 12/2013.

Milk samples were also submitted to PCR, as well as suspect colonies in tubes. The DNA was extracted using the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA) and the protocol followed the manufacturer's recommendations. Conventional IS*900* PCR reactions were performed using primers BN1 (5′-GTTATTAACGACGACGCGGAGC-3′) and BN2 (5′-ACGATGCTGTGTTGGGCGTTAG-3′) (Sivakumar et al., 2005). The positive samples were sequenced in the Laboratory of Molecular Biology, LANAGRO/MG- MAPA, Brazil. Amplicons were sequenced in a genetic analyzer 3500 (Life Technologies, USA) and the sequences obtained were submitted to Blast for identification.

Table 2
Products generated by enzymatic restriction with *Mse*I enzymes and *Hinf*I enzymes (Whittington et al., 1998).

Species	Restriction enzyme	Restriction pattern generated (pb)
MAP *sheep* (S)	*Hinf*I	285, 323
MAP *cattle* (C)	*Hinf*I	67, 218, 285, 323
MAP *bison* (B)	*Hinf*I	67, 218, 323
MAP	*Mse*I	608
Mycobacterium avium	*Hinf*I	608
Mycobacterium avium	*Mse*I	134, 189, 235

MAP: *Mycobacterium avium* subsp. *paratuberculosis*

Table 3
Number of selected properties and animals, per microregion of Zona da Mata of Minas Gerais, Brazil.

Microregions	Number of selected properties	Number of sampled animals
Viçosa	01	90
Ubá	01	32
Juiz de Fora	03	174
Manhuaçu	02	82
Muriaé	02	76
Cataguases	01	13
[a]Ponte Nova	0	0
TOTAL	10	467

[a] It was not sampled because insufficient number of animals.

The positive samples were submitted to PCR-IS*1311* using M56 (5′-GCGTGAGGCTCTGTGGTGAA-3′) and M119 (5′-ATGACGACCGCTTGGGAGAC-3′) primers and to subsequent restriction enzyme analysis (REA) according to Marsh et al. (1999). Enzymes *Hinf*I (Promega) and *Mse*I (NEB) were used to differentiate MAP from *Mycobacterium avium* subsp *avium*. Strain MAP-K10 and ultrapure water were used as positive and negative controls, respectively. Restriction pattern generated by *Hinf*I-digestion differentiates strains type S (sheep) from type C (catlle), while restriction pattern generated by *Mse*I differentiates MAP strains from *Mycobacterium avium*.

Table 2 shows the products (pb) generated by enzymatic restriction with *Mse*I and *Hinf*I enzymes (Whittington et al., 1998).

3. Results

A total of 467 dairy goats from seven microregions were sampled (Table 3), and 467 feces samples and 464 milk samples (two were lost in transportation) were obtained.

In two tubes (0.43%) with mycobactin *J* containing feces samples from animals of different properties, typical colonies of MAP were observed between 13 and 16 weeks. However, no colonies were found in the tubes containing milk samples. Nine (1.94%) milk samples from four properties were positive in PCR. The identification of all isolates was confirmed also by sequencing, with 94–99% similarity with Map K-10 strain. No digestion was observed with *Mse*I enzyme confirmed more one time to be MAP (Fig. 2). The percentage of MAP occurrence in dairy goats of Zona da Mata, MG, was 2.36% (11/467). The Fig. 3 shows the result provided by the Restriction enzyme analysis (REA). All isolates were typed as type C.

4. Discussion

In Brazil, data on paratuberculosis in goat herds are scarce, which highlights the need for epidemiological studies to investi-