Isolation of *Mycobacterium* spp. in milk from cows suspected or positive to tuberculosis

Isolamento de *Mycobacterium* spp. do leite de vacas suspeitas e positivas para tuberculose

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SUMMARY

This study was performed considering the public health hazards related to the elimination of mycobacteria through milk of dairy cows suspected or positive for tuberculosis presenting no clinical alterations. A total of 780 milk samples from 52 animals, positive or suspected for tuberculosis, according to Stormont's test, were analysed to detect *Mycobacterium* spp. The samples consisted of 300 ml/cow, collected in the first milking of the day, during 15 days. Frozen samples were sent to the laboratory, inoculated in Löwenstein-Jensen with reduced glicerol (0.5%) and Stonebrink media and kept under 37ºC for at least 90 days. The genus of each observed colony was initially confirmed by Ziehl-Neelsen and auramin staining methods. The isolation of *Mycobacterium* spps was confirmed in 78 (10%) samples collected from 19 (36.54%) animals. According to thin layer chromatography, time and temperature growth characteristics and colonies aspects, the 19 animals eliminated: *M. avium* (5.26%), *M. fortuitum* (10.52%), *M. bovis* (5.26%) and *Mycobacterium* spp. (78.95%).

UNITERMS: Tuberculosis; Cattle; Milk; Public health; Mycobacterium.

INTRODUCTION

According to World Health Organization, Latin America and Caribean countries present a 300 million bovine herd, from which only 80 million can be found in countries where the prevalence of animal tuberculosis is considered low or even nule. The remaining 220 million are distributed in countries where there is no recorded data about this occurrence[16]. Brazil is among these countries with a herd composed of approximately 150 million animals and where only 40% of performed tests have their results notified[23].

Negative effects of tuberculosis can not be exactly evaluated because infected animals disseminate bacteria for a non-determined period, but Smolyaninov and Martynov[28] and Dhinakaran et al.[8] observed that milk production of infected animals was reduced in 11.6%. Nevertheless, tuberculous animals have not only a sanitary or economical relevance, but infected cows can also disseminate pathogens through their milk, which, according to World Health Organization, is responsible for transmission of another 15 bacterial diseases[3,4].

Although respiratory route is considered the most important for transmission of tuberculous bacilli[12,21], the alimentary route of infection plays an important role in spreading this disease because milk carries microorganisms farther into urban areas if the population still drink raw milk[20]. Supporting the participation of milk in the transmission of bovine tuberculosis and other mycobacterioses, Hosty and McDurmont[15] isolated *Mycobacterium marinum*, *M. scrofulaceum*, *M. gordonae*, *M. flavescens* and *M. fortuitum* from 68.8% out of 51 raw milk samples. Appuswami et al.[1] observed human, bovine and atypical mycobacteria in 4.3% of 209 samples collected from dairy herds. In another study, Sanchez and Rosell[26] recovered saprophyte mycobacteria from 13% of 285 milk samples. Batish et al.[2] isolated *Mycobacterium* spp. from raw milk samples collected from clinically normal cows.

Demonstrating that the large number of bacteria excreted by a single cow is generally enough to render pooled milk infective, Roosevelt et al.[25] recovered opportunistic mycobacteria in more than 55% of milk plants samples and Guindi et al.[13] isolated *M. tuberculosis* and *M. bovis* from 24 samples all collected from bulk tanks.

Bovine tuberculous mastitis is characterized by progressive induration and hypertrophy of the mammary gland until the infected quarter becomes fibrous and pendulous. In earlier stages, milk is quite normal, then thin clots and amber secretion become evident and somatic cell count is the same observed in chronic mastitis[19,30]. Heide et al.[14] studied milk secretion of 79 cows presenting chronic mastitis from which 31% excreted *M. smegmatis*. Thomson et al.[29] also isolated this agent in 16 out of 83 bovine females. *M. avium, M. fortuitum* and Runyon's group III mycobacteria were found by Grieger et al.[11] in 36% of 252 cows with chronic mastitis. During approximately seven months, Wetzstein and Greenfield[31] identified 18 cases of mycobacterial mastitis from which 17 were caused by *M. fortuitum* and one by *M. chelonei*.

This study was performed considering: (1) the possibility of elimination of this agent through milk of infected cows; (2) the association of microbiologically positive cases with lactation stage of the studied animal; (3) the occurrence of mastitis in such animals.

MATERIAL AND METHOD

A total of 780 milk samples were collected from 52 cows proceeding from six dairy farms in São Paulo State. These were Holstein, Girolando and crossbred, primiparous and pluriparous bovine females, that presented suspected or positive results to tuberculosis according to Stormont's test[17].

These animals were submitted on day 0 of the study to clinical examination of each mammary quarter and of supramammary lymphnodes to detect signs of inflammatory reaction and inspection of milk secretion in order to find clots, flakes, discoloration and wateriness. Subsequently, California Mastitis Test[27] was performed in each producing quarter to identify subclinical mastitis.

In order to detect *Mycobacterium* spp., milk samples of approximately 300 ml/cow were collected in the first milking of the day during 15 consecutive days and were stored at -18ºC until laboratory procedures started. In sampling procedures, each teat was initially washed, dried and the surfaces of the teat ends were cleaned by wiping with clean cotton dipped in 70º alcohol.

The samples were initially centrifuged for 20 minutes at 1,610 g and 2 ml of sediment were submitted to a decontamination process. The sodium laurilsulphate decontamination method was initially performed according to Kantor[16], but sodium hydroxide and sulphuric acid concentrations previously established were not able to destroy contaminating microorganisms and thus they were modified, according to Quinn et al.[24] and Corner et al.[5], to final concentrations of 2% sodium hydroxide and 1.8% sulphuric acid, maintaining the respective volumes, time and temperature formerly established.

In sequence, the samples were centrifuged for 30 minutes at 1,610 g, and three to five drops of the decontaminated sediment were inoculated in two tubes containing Stonebrink[10,16,22], and in other two with Löwenstein-Jensen media with reduced concentration of glycerol (0.5%)[7]. Inoculated tubes were sealed and incubated at 37ºC for at least 90 days with daily and then weekly observation[6].

The identification of isolated mycobacteria was initially based on acid-fastness and microscopical morphology, observed in smears submitted to Ziehl-Neelsen and auramin methods, and in growth characteristics as time, temperature and colony features. Then, the suspected colonies were submitted to thin layer chromatography[18].

The results obtained in the present study were submitted to a statistical test of 95% confidence interval.

RESULTS AND DISCUSSION

Due to the lack of information on Brazilian literature about isolation of mycobacteria from milk of animals positive or suspected for tuberculosis, the present discussion was based on data proceeding from non-Brazilian authors.

A 10% frequency of isolation among 780 samples studied is similar to the results observed by Sanchez and Rosell[26], in Cuba, who isolated atypical mycobacteria in 13% of 285 milk samples. Otherwise, they differ from results obtained by Hosty and

McDurmont[15] and by Appuswami et al.[1] in 51 and in 209 milk samples collected, respectively from dairy cows in North America and in India.

From 52 animals studied, 19 (36.54%) presented *Mycobacterium* spp. in their milk, one value that is quite close to that observed by Heide et al.[14] in Germany, and higher than the result found by Thomson et al.[29] in United States. Nevertheless, this result is not different from that of Grieger et al.[11], also in Germany.

Just as Batish et al.[2], *Mycobacterium* spp. was isolated from raw milk samples collected from clinically normal cows, even though they presented positive or suspected reactions to Stormont's test, which is considered by Gottschalk et al.[9] and Kerr et al.[17] as one of the most specific and sensitive to identify tuberculous cattle. Thus, the risks of maintaining such animals in a dairy herd is reinforced by our results, once 78.95% of 19 animals presenting mycobacteria in their milk showed positive reaction to this test.

The evaluation of elimination pattern of mycobacteria during the 15 days of sample collection showed an intermittent and irregular character. There was also no influence of lactation stages on microorganisms' recovery. Even though reviewed literature do not offer information regarding such observations, if one considers that the ascending infections of mammary quarters are more frequent in the initial and final stages of lactation, in the present study only 5.26% and 21.05% of the animals were, respectively, recently calved and in dry period.

Regarding to the types of mastitis observed, once more our results confirm those from Batish et al.[2]: all 19 cows were clinically normal. On the other hand, they completely differ from the observations of Heide et al.[14], Grieger et al.[11], Thomson et al.[29] and Wetzstein and Greenfield[31] who recovered mycobacteria only from cases of chronic mastitis that did not respond to any treatment. The occurrence of subclinical cases were relatively high, once 78.95% of animals eliminating *Mycobacterium* spp. presented at least one mammary quarter with positive CMT.

Related to the identification of isolated mycobacteria, we observed that despite the culture media, the decontamination method, and the time/temperature of incubation were applied according to the literature indications, only the colonies isolated from 4 (21.05%) out of 19 animals showed eugonic growth of convex, depigmented and visible colonies. The remaining 15 (78.95%) females presented dysgonic growth of point-like and colorless colonies and due to this fact each tube was carefully examined with magnifying glasses before discarding. Nevertheless, microscopic evaluation of smears from all colonies, stained with Ziehl-Neelsen and auramin methods, showed acid-fast and fluorescent bacilli, respectively.

As shown in Tab. 1, only the colonies with abundant growth were submitted to thin layer chromatography and these results in association with other characteristics classified them as *M. bovis* (5.26%), *M. fortuitum* (10.52%) and *M. avium* (5.26%). Classification of dysgonic colonies was impossible either by biochemical tests or by thin layer chromatography. Thus, they were classified as *Mycobacterium* spp. in spite of their characteristics resembled *M. avium* and *M. intracellulare*, according to Grange[10]

Table 1

Characterization of agents from genus *Mycobacterium* spp. isolated from milk samples collected from positive and suspected cows to tuberculosis according to Stormont's test. Botucatu, SP, Brazil. 1998.

Animals	Tlc*	Growth Time	Species	N. Animals	%
1	I.IV.VI	30-60 days	*M.avium*	1	5.26
2	I.V	4-5 days	*M.fortuitum*	1	5.26
3	I.III.IV	60-120 days	*M.bovis*	1	5.26
4	I.V	3 7 days	*M.fortuitum*	1	5.26
Other animals	**	30 180 days	*Mycobacterium* spp.	15	78.95

* Thin Layer Chromatography; ** not submitted to TLC.

and Corrêa and Corrêa[7] or resembled dysgonic *M. bovis*, according to World Health Organization Technical Manual[22]. The fact that 12 (80%) out of 15 animals with dysgonic colonies had reacted positively to Stormont's test reinforces the possibility of being *M. bovis*, once it is considered specific and sensitive to diagnose bovine tuberculosis. Another fact that justify this classification was pointed out by Corrêa and Corrêa[6] who said that approximately 10% of *M. bovis* samples require at least 60 days of incubation to develop colonies and in the cases of these dysgonic colonies, 66.67% appeared after 60 days and 33.33%, after 30 days of incubation at 37ºC.

CONCLUSIONS

This study was performed considering: (1) the possibility of elimination of this agent through milk of infected cows; (2) the association of microbiologically positive cases with lactation stage of the studied animal; and (3) the occurrence of mastitis in such animals. According to the results observed in this study, we concluded that:

- 36,54% out of 52 studied cows, positive or suspected for tuberculosis by Stormont Test, clinically normal and maintained among the producing cows eliminated mycobacteria through their milk;
- There was no influence of lactation stages on pattern of mycobacteria elimination;
- The rate of clinical mastitis on cows shedding mycobacteria through their milk was nule; and the occurrence of animals with subclinical cases was relatively high, for 78.95% of the 19 cows with positive mycobacteria isolation presented at least one affected quarter.

The results also reinforce the participation of milk in transmission of mycobacteria to several animal species and human beings, particularly in Brazil, where Brandão[4] evidenced that approximately 47% of milk sold in urban and rural areas are *in natura*.

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RESUMO

Considerando-se o risco à saúde pública representado pela eliminação de micobactérias pelo leite, foram colhidas 780 amostras de 52 fêmeas bovinas leiteiras consideradas suspeitas ou positivas para tuberculose ao teste de Stormont. As amostras foram submetidas à pesquisa de *Mycobacterium* spp**.** e consistiam de 300 ml de leite/vaca, colhidos na primeira ordenha do dia, durante 15 dias. As amostras congeladas foram enviadas ao laboratório, processadas pelas técnicas preconizadas por Kantor[16], Quinn et al.[24] e Corner et al.[5], inoculadas em meios de Löwenstein-Jensen com teor reduzido de glicerol (0,5%) e de Stonebrink, e incubadas a 37ºC por um período mínimo de 90 dias. O gênero de cada colônia isolada foi inicialmente confirmado pelos métodos de coloração de Ziehl-Neelsen e da auramina. O isolamento de *Mycobacterium* spp. foi confirmado em 78 (10%) amostras provenientes de 19 (36.54%) animais. De acordo com a cromatografia em camada delgada, as características de tempo e temperatura de crescimento e os aspectos coloniais, os 19 animais eliminaram: *M. avium* (5.26%), *M. fortuitum* (10.52%), *M. bovis* (5.26%), and *Mycobacterium* spp. (78.95%).

UNITERMOS: Tuberculose; Leite; Bovinos; Saúde pública; Micobactéria.

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