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Isolation of *Brucella spp* from milk of brucellosis positive cows in São Paulo and Minas Gerais states*

Isolamento de *Brucella spp* do leite de vacas positivas para brucelose nos estados de São Paulo e Minas Gerais

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SUMMARY

Brucellosis is a chronic zoonosis that plays an important role in Public Health. Considering the lack of data in Brazil regarding its presence in raw milk and non-pasteurized dairy products, we determined the presence of brucellae in milk from brucellosis positive animals. The slide agglutination test (SAT), tube agglutination test (TAT) and TAT treated with 2-mercaptoethanol were used to identify positive animals in studied herds. For 3 days, 300 ml milk samples/cow (75 ml/teat) were collected from all productive quarters of the positive animals. These were mixed and centrifuged. Part of the pellet and of the supernatant were inoculated in Farrel and Brodie-Sinton (BS) media supplemented with antimicrobial agents. The inoculated plates and tubes were incubated at 37ºC for 7 days, with 10 per cent CO_2 atmosphere. The suspected bacterial growth in BS media was immediately cultivated in agar Brucella media, under the same conditions. Colonies were submitted to identification procedures including Gram stain, CO_2 requirement, H_2S production, urease activity and growth in the presence of thionin and fuchsin. Of the 49 analysed samples, 15 (30.61%) contained *Brucella abortus*. The distribution was as follows: biotype 1 in one sample (2.04%), biotype 2 in eight (16.32%) and biotype 3 in six samples (12.25%).

UNITERMS: Milk; *Brucella abortus*; Cows; Brucellosis.

INTRODUCTION

Brucellosis occurs worldwide except in many European and Asian countries from which it has been erradicated[21]. As a herd disease, bovine brucellosis represents an economic problem; animals seldom eliminate infection without presenting symptoms and sequelae that directly affect the productive and reproductive aspects. The estimated economic loss exceeds 600 thousand dollars[1]. It also has an enormous impact on public health because brucellosis can be transmitted to man either direct or by indirect contact with animal products[5].

The prevalence of bovine brucellosis is variable in cattle but is generally higher among dairy cattle than range cattle due to the intensive farming practices to which these animals are submitted. In Brazil, the prevalence of the disease in bovine indicates that 2.49% of cattle seropositive and 2.04% show suspicious results[2].

Brucellic mastitis is chronic and often clinically unapparent. Because infected females excrete large numbers of viable brucellae in milk for months to years, apparently normal glands represent important sources of infection not only to other lactating cows but also to calves and humans who consume raw milk[12,15,21]. This was demonstrated by Pedrix and Chirol[18] who isolated *Brucella* spp. from milk samples collected during several lactations from serologically positive females that had recently aborted. Dafni *et al.*[9] recovered *B. melitensis* from milk samples from brucellic animals and demonstrated that microorganisms can remain in latency, most commonly in udder tissues and in suprammamary lymph nodes. Zowghi *et al.*[23] recovered *B. abortus* from 26.32% of samples collected from positive animals and from 2.09% of 5,686 milk samples collected from serologically negative bovine females. *B. abortus* was found in 29.8% of milk samples obtained from cows with positive results to card test[13].

Guercio *et al.*[11] observed a significant rise in

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prevalence of human brucellosis associated with the consumption of raw milk. Cooper[7] extended this observation by showing that the main source of infection for the general population is not only contaminated raw milk but also non-pasteurized dairy products. Brucellae can survive food processing depending on maturation and acidification periods to which each product is submitted[19].

Considering the importance of milk in the epidemiology of brucellosis as a zoonosis, we carried out this study to investigate the presence of brucellae organisms in milk from cows seropositive for brucellosis in São Paulo and Minas Gerais states.

MATERIAL AND METHOD

Milk samples were collected from Holstein, Gir and crossbred cows belonging to 10 dairy farms from São Paulo and Minas Gerais states with different milking management such as manual and mechanical, with milk yields ranging from 200 to 1,200 liters/day, all of the farms were deficient in milking hygiene procedures. These cows were in different lactation stages and were not vaccinated against brucellosis.

To identify animals with brucellosis, blood samples were initially collected from each bovine female, either from jugular or mammary vein with 40 x 20 needles, after careful local disinfection, and submitted to the slide-agglutination test (Huddleson test), tube-agglutination test (Wright-Bang test) and tube-agglutination test with 2-mercapthoethanol using *B. abortus* antigen[17]. Forty-nine animals with positive reactions to anyone of these diagnostics tests were submitted to clinical examination, which consisted in careful palpation of udder and suprammamary lymph nodes and inspection of milk secretion for presence of clots, flakes, discoloration and wateriness. Milk from each quarter was also examined using California Mastitis Test (CMT)[22] for detection of subclinical mastitis.

In order to detect *Brucella* spp., milk samples of approximately 300 ml/cow (75 ml/teat) were collected from the first milking of the day during three consecutive days and were stored at 4ºC until laboratory procedures started. Before sampling, each teat was carefully washed, dried and the surface of the teat ends was sterilized by wiping with clean cotton dipped in 70º alcohol. Samples were collected in sterile vials.

For routine aerobic microbiological examination, 0.1 ml of each sample was inoculated in plates with 10% bovine blood agar and in plates with MacConkey agar, and incubated at 37ºC for 72 hours. Growth characteristics were observed at 24, 48 and 72 hours by macroscopic analysis of colonies. Microscopic study of smears treated with Gram stain followed. The isolated microorganisms were analysed by biochemical tests and identified according to Carter and Cole Jr[6].

For brucellae investigation, the three milk samples collected from each animal was homogenized and centrifuged for 15 minutes at 6,000 rpm. A portion of the sediment was mixed with an equal volume of sterile saline and anphotericin B (4 µg/ml), nistatin (100 U/ml), nalidixic acid (5 µg/ml), vancomicin (20 µg/ml) and bacitracin (20 U/ml), were added. This mixture was left for 60 minutes at 4 to 8ºC to eliminate undesirable microorganisms. Two guinea pigs (*Cavia porcellus*) were inoculated intraperitoneally for each cow sample[4] with 1.0 ml of the suspended material. These were observed daily. If symptoms or death were seen, a detailed necropsy was performed. A histopathological study of smears of samples from abnormal organs, specially lymph nodes efferent to inoculation point and spleen, was made. Tissue was stained with hematoxilin-eosin[14] and by the Köster method as modified by Costa *et al.*[8]. A microbiological examination was also carried out on animals with no symptoms that were sacrificed at week three and six. Before sacrifice a blood sample was obtained to perform the described serological tests[16].

The isolation of *Brucella* spp was performed on Farrel Medium plates[10] inoculated with 0.1 ml of the sediment (SE) and with 0.1 ml of supernatant (SU) and incubated at 37ºC, for 7 days, under aerophlic condition (10% CO_2). In addition two tubes containing 4 ml of Brodie Sinton[3] medium was inoculated with 0.5 ml of sediment and with 0.5 ml of supernatant and incubated as for Farrel Medium. Seven days later, to recover brucellae, 0.1 ml of this enriched culture was inoculated on to Brucella agar and reincubated.

Plates were observed daily for bacterial growth. Pinpoint, smooth, glistening and translucent colonies, resembling *Brucella* spp., were smeared and Gram stained. Morphological studies were performed by the modified Köster method[8]. Isolated organisms were submitted to the following tests to identify the biotype[20]:

CO_2 requirement for primary isolation: plates were streaked and incubated aerobically and under CO_2.

H_2S production: the isolate was grown on a trypticase soy agar with the test strip suspended over the slant. Blackening of the strip indicated a positive test.

Urease activity: a Christensen urea slope was inoculated with a loopful of a culture and incubated at room temperature. The test was regarded as negative if there was no reaction after 24 hours.

Growth in the presence of thionin and fuchsin: the test was carried out by incorporating the dyes thionin and basic fuchsin separately in trypticase soy agar at the concentration of 20 µg/ml (1:50,000) or 40 µg/ml (1:25,000). The medium was prepared by heating a 0.1 per cent solution of either dye in a boiling water bath for 20 minutes and then adding it to the required amount of autoclaved agar. The dye was mixed with the agar and poured into Petri dishes. A sterile swab was used to inoculate the dye media with a suspension of the test strain. The inoculated plates were

incubated at 37ºC under 5-10 per cent CO_2 for 3-4 days and then examined for growth.

RESULTS AND DISCUSSION

Microbiological examination resulted in the recovery of several types of bacteria from 10% bovine blood agar and MacConkey agar. Seven (14.30%) milk samples with microbiological negative results, under routine conditions of aerobic culture, were positive for isolation of *Brucella abortus* biotype 3. This fact reinforces the necessity of using conditions to isolate species that are rarely involved in cases of mammary infections for accurate diagnosis.

Only three animals reacted positively to CMT, or had clinical mastitis (Tab. 1). The serological titers against *Brucella abortus* ranging between 1:100 UI and 1:1,600 UI, with 6.7% of 1:800 UI reactions, 13.3% of 1:200 UI, 26.7% of 1:100 UI and 1:1600 UI. *Brucella abortus* biotype 1, 2 and 3 were isolated in one or both differential media. When the isolation of *Brucella* from the sediment and the supernatant were compared, it was observed that the rates of isolation were higher when the sediment was inoculated on Farrel media than on Brucella Agar. In summary, Farrel media was better for the isolation of this microorganism with 40.0% and 46.6% positive using the supernatant and the sediment, respectively.

The microbiological results are in Tab. 1. The animals 3 and 4; 18 and 24; 30, 33, 34, 35 and 36; 39, 44 and 47 belonged, respectively, to same herd. The table shows the isolation of *Brucella abortus* biotype 1 in one sample (2.04%), biotype 2 in eight samples (16.32%) and biotype 3 in six samples (12.25%), making a total of 30.61% positive samples.

None of the sediment samples inoculated intraperitoneally resulted in the recovery of the agent from the organs examined. The guinea pig serum samples were negative to serum agglutination test. This probably occurred because of the small amount of brucellae in the samples. The amount being insufficient to cause the infection or the disease in the intraperitoneally inoculated animals. The histopathology of the spleen, lymphnodes and liver from all the animals inoculated showed no alterations. The cytology, using Köster modified method[8], was normal, indicating that the animals were not infected after the intraperitoneal inoculation.

Table 1

Microbiological results isolated from supernatant (SU) and sediment (SE) from milk samples submitted to CMT of positive cows for brucellosis. Botucatu, 2000.

			Farrel		BS-Brucella Agar	
Animal	CMT	Titer*	SU	SE	SU	SE
3	negative	100	negative	B.abortus b. 3	negative	negative
4	negative	100	negative	negative	negative	B.abortus b. 3
5	negative	200	negative	negative	B.abortus b. 3	B.abortus b. 3
10	negative	400	negative	negative	B.abortus b. 3	B.abortus b. 3
18	negative	800	negative	B.abortus b. 3	negative	negative
24	negative	100	negative	negative	negative	B.abortus b. 3
30	negative	1600	negative	B.abortus b. 2	negative	B.abortus b. 2
33	negative	1600	negative	B.abortus b. 2	negative	negative
34	negative	400	B.abortus b. 2	negative	negative	negative
35	2 teats ++ 2 teats +	1600	B.abortus b. 2	negative	negative	negative
36	2 teats ++	400	B.abortus b. 2	negative	negative	negative
39	negative	1600	negative	B.abortus b. 2	B.abortus b. 3	negative
44	negative	200	B.abortus b. 1	negative	negative	negative
47	negative	100	B.abortus b. 2	B.abortus b.2	negative	negative
48	2 teats	400	B.abortus b. 2	B.abortus b. 2	negative	negative
ISOLATION			40.0%	46.6%	20.0%	33.33%

* serological titers of cows.

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Considering that many people in our country consume raw milk, the isolation of *Brucella* spp. from 30.61% of the 49 samples studied shows the potential importance of the milk as a vehicle of this agent to men. A similar result was found by Huber *et al.*[13] and Zowghi *et al.*[23] who isolated *B. abortus* from 29.8% and 26.32% respectively, from milk samples, and Zowghi *et al.*[23] also isolated this agent from serologic negative cows. Milk improperly pasteurized continues to be a potential vehicle of *B. abortus* for humans in Sao Paulo and Minas Gerais States.

RESUMO

A brucelose é uma zoonose crônica de importância para a Saúde Pública. Considerando o pequeno número de dados brasileiros sobre a sua presença em leite cru e derivados não-pasteurizados, estudamos a presença de brucelas em leite de animais sorologicamente positivos. A soroaglutinação rápida (SAR), a soroaglutinação lenta (SAL) e a soroaglutinação lenta com tratamento do soro com 2-mercaptoetanol foram utilizados para identificar os animais positivos nas propriedades estudadas. Amostras diárias de 300 ml de leite foram colhidas por três dias de todos os quartos mamários produtivos (75 ml/teto). As amostras eram misturadas e centrifugadas. Parte do sedimento e do sobrenadante foi inoculada em meios de Farrel e Brodie-Sinton (BS) suplementados com agentes antimicrobianos. As placas e tubos foram cultivados por sete dias a 37ºC, em microaerofilia. As colônias suspeitas no meio BS foram imediatamente repicadas para ágar-Brucella, e cultivadas sob a mesma condição. Os microrganismos isolados foram submetidos a procedimentos de identificação, incluindo a coloração de Gram, requerimento de CO_2, produção de H_2S, atividade da urease e crescimento na presença de tionina e fucsina. Das 49 amostras examinadas, isolou-se *Brucella abortus* de 15 (30,61%). Os biótipos isolados foram: biótipo 1 em uma amostra (2,04%), biótipo 2 em oito (16,32%) e biótipo 3 em seis amostras (12,25%)

UNITERMOS: Leite; *Brucella abortus*; Vacas; Brucelose.

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