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 37°C for 7 days, with 10 per cent CO₂ 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, CO₃ requirement, H₂S 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 eradicated. 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. 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.

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.

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

Considering the importance of milk in the epidemiology of brucellosis as a zoonosis, we carried out this study to investigate the presence of brucelae 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 sampling, each teat was carefully washed, dried and the surface 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. The isolation of Brucella spp was performed on Farrel Medium plates inoculated with 0.1 ml of the sediment (SE) and with 0.1 ml of supernatant (SU) and incubated at 37°C, for 7 days, under aerobic condition (10% CO₂). In addition, two tubes containing 4 ml of Brodie Sinton 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. Isolated organisms were submitted to the following tests to identify the biotype:

- CO₂ requirement for primary isolation: plates were streaked and incubated aerobically and under CO₂.
- 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 fuchsins: the test was carried out by incorporating the dyes thionin and basic fuchsins 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 collected from each animal and sent to the laboratory for further examination.
incubated at 37°C under 5-10 per cent CO₂ 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, 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.

<table>
<thead>
<tr>
<th>Animal</th>
<th>CMT</th>
<th>Titer*</th>
<th>Farrel SU</th>
<th>Farrel SE</th>
<th>BS-Brucella Agar SU</th>
<th>BS-Brucella Agar SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>negative</td>
<td>100</td>
<td>negative</td>
<td>B.abortus b. 3</td>
<td>negative</td>
<td>negative</td>
</tr>
<tr>
<td>4</td>
<td>negative</td>
<td>100</td>
<td>negative</td>
<td>negative</td>
<td>B.abortus b. 3</td>
<td>B.abortus b. 3</td>
</tr>
<tr>
<td>5</td>
<td>negative</td>
<td>200</td>
<td>negative</td>
<td>negative</td>
<td>B.abortus b. 3</td>
<td>B.abortus b. 3</td>
</tr>
<tr>
<td>10</td>
<td>negative</td>
<td>400</td>
<td>negative</td>
<td>negative</td>
<td>B.abortus b. 3</td>
<td>B.abortus b. 3</td>
</tr>
<tr>
<td>18</td>
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<td>800</td>
<td>negative</td>
<td>B.abortus b. 3</td>
<td>negative</td>
<td>negative</td>
</tr>
<tr>
<td>24</td>
<td>negative</td>
<td>100</td>
<td>negative</td>
<td>negative</td>
<td>B.abortus b. 3</td>
<td>B.abortus b. 3</td>
</tr>
<tr>
<td>30</td>
<td>negative</td>
<td>1600</td>
<td>negative</td>
<td>B.abortus b. 2</td>
<td>negative</td>
<td>negative</td>
</tr>
<tr>
<td>33</td>
<td>negative</td>
<td>1600</td>
<td>negative</td>
<td>B.abortus b. 2</td>
<td>negative</td>
<td>negative</td>
</tr>
<tr>
<td>34</td>
<td>negative</td>
<td>400</td>
<td>B.abortus b. 2</td>
<td>negative</td>
<td>negative</td>
<td>negative</td>
</tr>
<tr>
<td>35</td>
<td>2 tests ++</td>
<td>1600</td>
<td>B.abortus b. 2</td>
<td>negative</td>
<td>negative</td>
<td>negative</td>
</tr>
<tr>
<td>36</td>
<td>2 tests ++</td>
<td>400</td>
<td>B.abortus b. 2</td>
<td>negative</td>
<td>negative</td>
<td>negative</td>
</tr>
<tr>
<td>39</td>
<td>negative</td>
<td>1600</td>
<td>negative</td>
<td>B.abortus b. 2</td>
<td>B.abortus b. 3</td>
<td>negative</td>
</tr>
<tr>
<td>44</td>
<td>negative</td>
<td>200</td>
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<td>negative</td>
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</tr>
<tr>
<td>47</td>
<td>negative</td>
<td>100</td>
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<td>negative</td>
<td>negative</td>
</tr>
<tr>
<td>48</td>
<td>2 tests ++</td>
<td>400</td>
<td>B.abortus b. 2</td>
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<td>negative</td>
<td>negative</td>
</tr>
</tbody>
</table>

* serological titers of cows.
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. and Zowghi et al. who isolated B. abortus from 29.8% and 26.32% respectively, from milk samples, and Zowghi et al. 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. As amostras diurnas 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 37°C, em microaerofilia. As colônias suspeitas no meio BS foram imediatamente repicadas para agar-Brucella, e cultivadas sob a mesma condição. Os microrganismos isolados foram submetidos a procedimentos de identificação, incluindo a coloração de Gram, requerimento de CO₂, produção de H₂S, atividade da urease e crescimento na presença de tironina e fuciana. 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%).

UNINTERMOS: Leite; Brucella abortus; Vacas; Brucelose.

REFERENCES


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