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MICROBIOLOGY

Ring test in milk samples preserved with Bronopol®

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ABSTRACT: *This study evaluated if the milk ring test (MRT) used to the brucellosis diagnostic at dairy herd level can be done in milk samples with conservative (Bronopol®) collected to the somatic cell count (SCC) that is used to mastitis monitoring. It were analyzed 38 bulk tank milk samples (BTMS) from 19 dairy herds artisanal cheese producers and 36 BTMS from a brucellosis free dairy herd, used as negative control (NC) and positive control (PC), when was added serum from vaccinated animals. The milk samples were collected in two different bottles (with or without Bronopol®). All the 38 BTMS from artisanal cheese producers (samples with and without Bronopol®) were non-reagent to MRT, as all NC. All PC were reagent to MRT, without interference of Bronopol®. Results showed that the milk sample used to SCC presented the proportion of agreement of results in all milk samples (100%), that is, false positive and false negative results were not observed. Results indicated that Bronopol® did not interfere with the color in the time of MRT reading suggesting that the same milk samples could be used to monitoring mastitis and brucellosis.*
Key words: *Brucella abortus, raw dairy products, monitoring, milk ring test.*

Teste do anel em amostras de leite conservadas com Bronopol®

RESUMO: *O objetivo deste estudo foi avaliar se o teste do anel do leite (TAL), utilizado para o diagnóstico da brucelose em nível de rebanho leiteiro, pode ser realizado em amostras de leite com conservador (Bronopol®) coletado para contagem de células somáticas (CCS) que é utilizado para monitoramento de mastite. Foram analisadas 38 amostras de leite de tanques de expansão (ALTE) de 19 rebanhos leiteiros produtores de queijo artesanal e 36 ALTE de rebanho leiteiro livre de brucelose, utilizadas como controle negativo (CN) e controle positivo (CP), quando foi adicionado soro de animais vacinados. As amostras de leite foram coletadas em dois frascos diferentes (com e sem Bronopol®). Todos as 38 ALTE de produtores artesanais de queijo (amostras com e sem Bronopol®) foram não reagentes ao TAL, como todos os CN. Todos os CP foram reagentes ao TAL, sem interferência do Bronopol®. Os resultados mostraram que a amostra de leite utilizada para CCS apresentaram a proporção de concordância dos resultados em todas as amostras de leite (100%), ou seja, não foram observados resultados falso-positivos e falso-negativos. Os resultados indicam que o Bronopol® não interferiu na cor no momento da leitura do TAL, sugerindo que as mesmas amostras de leite poderiam ser usadas para monitorar mastite e brucelose.*
Palavras-chave: *Brucella abortus, produtos de leite cru, monitoramento, teste do anel do leite.*

INTRODUCTION

Brucellosis is a re-emergent widespread zoonosis and *Brucella abortus* is one of the main causes of human brucellosis, having the bovine as the main species affected (DADAR, SHAHALI & WHATMORE, 2019; SELEEM, BOYLE & SRIRANGANATHAN, 2010). It is known that the risk of human brucellosis is greater in endemic areas of animal brucellosis and mainly when unpasteurized milk and its dairy products are consumed (GODFROID et al., 2013; PAPPAS et al., 2006).

In Brazil, bovine brucellosis is still endemic in many areas of the country (NETO et al., 2016). In Minas Gerais State, the largest state in milk production and dairy herds that produce cheese from unpasteurized milk (artisanal cheese), the prevalence of herds seropositive for *Brucella abortus* in seven regions of the state vary from 2,02% to 5,06% (OLIVEIRA et al., 2016). Once *B. abortus* is mainly transmitted to humans by consumption of unpasteurized milk and dairy products or by the contact with infected animals, the surveillance epidemiologic system based on One Health concept

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for bovine brucellosis is extremely important (CORBEL, 1997).

Since the consumption of artisanal cheese has been increasing in Minas Gerais State and current legislation allows manufacturing and trade (BRASIL, 2019; BRASIL, 2006), the concern about transmission of *B. abortus* to humans has also increased. In this sense, the concept of "One Health" becomes fundamental, once the most effective way to prevent human brucellosis is the control of the disease in animals, since there is no vaccine available to humans.

Programs of control and eradication of animal brucellosis worldwide establish that the main approach to control brucellosis in animals should be focused on vaccination of susceptible animals and in the test-and-slaughter strategy (PEREZ-SANCHO et al., 2015). Still, it is important to highlight that the success of bovine brucellosis control programs depends on monitoring the disease in herds in a regional level (PEREZ-SANCHO et al., 2015).

One of the tests recommended by OIE for monitoring the occurrence of bovine brucellosis at herd level is the Milk Ring Test (MRT). This test consists in a screening of specifics antibodies against *B. abortus* in bulk milk tanks (PEREZ-SANCHO et al., 2015) and it is used in Brazil according to National Program of Control and Eradication of Animal Brucellosis and Tuberculosis (PNCEBT) (BRASIL, 2006; BRASIL, 2017).

Brazilians artisanal cheese producers must certify their dairy herds as bovine brucellosis-free and should establish a control program of mastitis and evaluate its efficacy through monitoring of somatic cell counts (SCC) (BRASIL, 2006). These SCC samples are collected in bottles containing the conservative Bronopol® and sent to laboratories of the Brazilian Milk Quality Control Laboratories Network (RBQL) to be analyzed. Currently, milk sample from more than 400,000 dairy herds are analyzed monthly in nine laboratories from RBQL. This study evaluated if MRT can be done in milk samples collected to SCC and analyzed by RBQL without the interference of Bronopol®.

It was collected 38 bulk tank milk samples from 19 dairy herds artisanal cheese producers located on Minas Gerais State, Brazil. Samples of each bulk tank (n=2) were collected in two different bottles (with or without Bronopol®) intended to be analyzed through MRT.

Bulk tank milk samples (n=36) from a certified brucellosis-free dairy herd by the Brazilian Ministry of Agriculture, Livestock and Supply (MAPA), were collected to be used as negative and positive controls. The negative controls (NC; n=6) were divided in two groups: with and without Bronopol®. The positive ones (PC; n=30) were prepared by inoculating different volumes (20, 50, 100, 150 and 200 µL) of three vaccinated calf serum between 3 and 8 months with vaccine B19 and positive for the Rosa Bengal Test (RBT) in 1 mL of milk. The RBT was performed from animals that had more than 14 days and less than 30 days from vaccination and immediately before the inoculations of the serum into the milk samples. Samples inoculated were, then, divided in two groups: with or without Bronopol®.

MRT were performed according to the PNCEBT guide [9]. Briefly, the samples and the antigen were left at room temperature for 60 minutes. Then, 1 mL of each milk sample (with and without Bronopol®) was transferred to a glass tube and 30µL of standard antigen for MRT, provided by National Agriculture Laboratory in Minas Gerais state (Lanagro MG), were added. After inversion-homogenization, the samples were incubated at 37 °C for 60 minutes and, then, the results were analyzed. If there was a blue ring at the top and the column of milk was white or bluish, the herd was classified as reagent (R). Conversely, if the ring was white and the milk column was blue, the herd was classified as non-reagent (NR) (BRASIL, 2006). As MRT is a qualitative test, a blind evaluation was carried out by three enable persons in order to eliminate divergences and the results were compared later.

All the 38 bulk tank milk samples from artisanal cheese producers were NR to MRT, showing 100% of concordance between samples with and without Bronopol® (Table 1). In the milk samples from a brucellosis free herd, the NC presents the same result as for samples with and without Bronopol® (Table 1). Of the 30 samples used as PC, all of them showed the same result without interference of Bronopol® and regardless of the volume of serum inoculated, with a clear blue ring formation on the milk column (Table 1 and Figure 1).

The antigen used to perform MRT is stained with hematoxylin, which gives the bluish color on the milk column (if the herd is NR) or on the ring formed on the top of the column (if the herd is R). Because the conservative Bronopol® stains the milk sample of salmon color, the question is if it interferes on the interpretation of the MRT results. It showed that the conservative Bronopol® did not interfere in the reading of the MRT, being possible to visualize

Table 1 - Results of the milk ring test (MRT) from bulk tank milk samples of artisanal cheese producers and negative and positive controls according to the appraiser.

Samples	Bronopol®	N	MRT AP1	MRT AP2	MRT AP3	Agreement(%)
BMT	Yes	19	NR	NR	NR	100
BTM	No	19	NR	NR	NR	100
NC	Yes	3	NR	NR	NR	100
NC	No	3	NR	NR	NR	100
PC_20	Yes	3	R	R	R	100
PC_20	No	3	R	R	R	100
PC_50	Yes	3	R	R	R	100
PC_50	No	3	R	R	R	100
PC_100	Yes	3	R	R	R	100
PC_100	No	3	R	R	R	100
PC_150	Yes	3	R	R	R	100
PC_150	No	3	R	R	R	100
PC_200	Yes	3	R	R	R	100
PC_200	No	3	R	R	R	100

BMT = bulk milk tank; NC = negative control; PC_20 = positive control with 20 µL of vaccinated calf serum; PC_50 = positive control with 50 µL of vaccinated calf serum; PC_100 = positive control with 100 µL of vaccinated calf serum; PC_150 = positive control with 150 µL of vaccinated calf serum; PC_200 = positive control with 200 µL of vaccinated calf serum; N = number of evaluated samples; MRT AP 1 = test reading by appraiser 1; MRT AP 2 = test reading by appraiser 2; MRT AP 3 = test reading by appraiser 3.

clearly the blue on the milk samples used as PC and NC. So, the milk samples collected for SCC analysis could be used for monitoring bovine brucellosis at regional, state and national levels.

There are other tests that can be performed on milk samples for diagnosing brucellosis in animals, like ELISA and Fluorescence Polarization Test (FPT). However, the MRT is the screening test recommended by the PNCEBT for herds. The others are recommended for individual diagnosis. This study offered a practical tool to the production units of milk and artisanal cheese to monitor brucellosis in their herds using samples that are already collected in the routine for monitoring mastitis.

In Brazil, this monitoring is especially important for the artisanal cheese producers who use raw milk since the main form of human infection is through direct consumption and its dairy products (DADAR et al., 2019; SELEEM, BOYLE & SRIRANGANATHAN, 2010; GODFROID et al., 2013; PEREZ-SANCHO et al., 2015). However, this monitoring is also recommended for the other dairy herds, once bovine brucellosis is highly prevalent in many areas of the country (NETO et al., 2016). In this sense, the MAPA could use the samples sent to the RBQL laboratories for this monitoring and, then, to develop a surveillance epidemiological system based on the One Health concept (ROBINSON, 2003). Milk samples are sent monthly to the RBQL laboratories for SSC as part of the mastitis monitoring program, so the use of these samples for MRT would be advantageous. The scope of the monitoring would be very wide if all the country's herds had their samples collected and analyzed. The knowledge of this information would help to identify the critical regions and to make decisions about the animal brucellosis control policy at regional, state and national levels.

Moreover, Brazil has recently entered into trade agreements with other countries aiming for the exportation of Brazilian dairy products, mainly cheeses and powder milk, which further reinforces the need for the sanitary quality control of raw milk and dairy herds.

Results indicate that the same milk sample used for monitoring mastitis through SCC in dairy herds linked to artisanal cheese production can also be used for bovine brucellosis monitoring and, furthermore, as screening diagnosis for a surveillance epidemiological system. However, new assessments must be conducted with a larger number of dairy herds in order to evaluate the results at the population level. Also include positive animal serum as control in the next step of the research.

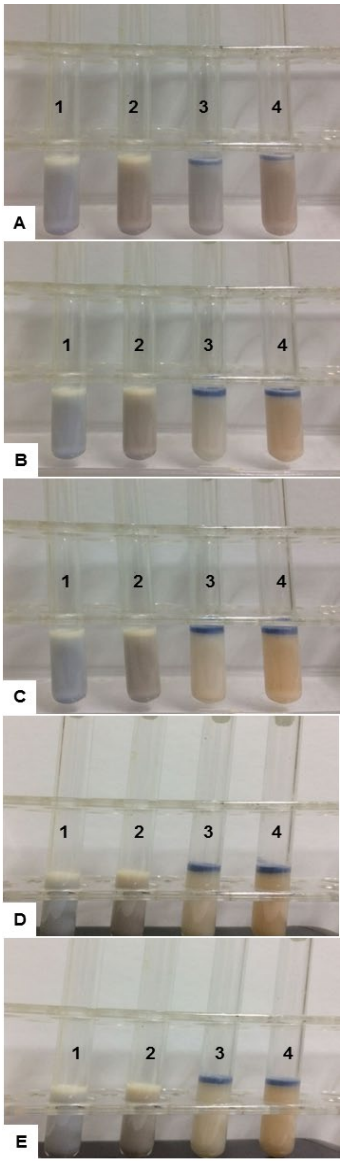

Figure 1 - Results of negative and positive controls according to the volume of vaccinated calf serum between 3 and 8 months with inoculated vaccine B19 in milk from brucellosis free dairy herd. A = 20 µL of serum; B = 50 µL of serum; C = 100 µL of serum; D = 150 µL of serum; E = 200 µL of serum. 1 = negative control without Bronopol®; 2 = negative control with Bronopol®; 3 = positive control without Bronopol®; 4 = positive control with Bronopol®.

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DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTIONS

Conceptualization and study design: GNS and MRS. Milk samples collect: JFMM, MFAP, NMV and FFAC. Laboratory analysis: JFMM, MHCA and JBR. Database organization: FFAC. JFMM and GNS prepared the draft of the manuscript. All authors critically revised the manuscript and approved of the final version.

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