Evaluation of Molecular Methods for the Detection of *Brucella* Species in Water Buffalo Milk

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ABSTRACT

Brucellosis is a highly infectious disease affecting both animals and humans. The current standard tools for the diagnosis of this bacterial infection are serological and microbiological. In this study, we evaluated the feasibility of molecular assays as diagnostic tools for the detection of *Brucella* spp. in water buffalo milk. For this purpose, we first compared different DNA extraction protocols and PCR methods on artificially spiked milk samples. The most sensitive methods were then used to examine milk from serologically positive and negative water buffaloes. Molecular results were compared with serological and bacteriological test results. Milk samples from 53 *Brucella* seropositive buffaloes (by either rose Bengal or complement fixation test) were positive by ELISA, 37 were positive by culture, 33 were positive by PCR, and 35 were positive by real-time PCR. Of the 37 culture-positive samples, a total of 25 and 26 were positive by PCR and real-time PCR, respectively. Of the 16 culture-negative samples, 8 were positive by PCR and 9 by real-time PCR. Thus, although culture showed greater sensitivity than PCR, some animals found positive by serological methods and PCR tested negative by milk culture. The combined use of bacteriological and molecular tools increased the number of positive samples to 46. In conclusion, these results suggest that the simultaneous application of these 2 direct detection methods (culture and PCR) could be more useful than one test alone for the diagnosis of *Brucella* spp. in buffalo milk.

Key words: *Brucella* spp., buffalo milk, DNA extraction, polymerase chain reaction

INTRODUCTION

Brucellosis is a zoonosis with serious implications for both humans and animals, affecting predominantly

sexually mature individuals. The main clinical symptoms of infection are abortion in females, and orchitis and epididymitis with frequent sterility in males, due to the localization of brucellae within the female and male reproductive organs. The disease is transmitted by direct contact with infectious excretions, by ingestion, by the venereal route, or, less commonly, via the conjunctiva or by inhalation. Large numbers of brucellae can be excreted in fetal fluids and mammary secretions (Enright, 1990). The causative agents of brucellosis in ruminants are *Brucella abortus* and *Brucella melitensis* (Corbel and MacMillan, 1998).

Early detection by valid diagnostic tools is crucial for the control and eradication of this disease. Currently, the diagnosis of brucellosis in live dairy cattle involves either the detection of anti-*Brucella* antibodies in serum or milk or the isolation of *Brucella* from milk samples (Alton et al., 1988).

Serological tests such as ELISA, complement fixation test (CFT), serum agglutination test, or milk ring test can be nonspecific because of cross-reaction with other common antigenically related bacteria (Perry and Bundle, 1990; Diaz-Aparicio et al., 1994), or sub-sensitive or high immunity reactions, depending on subclinical or endemic prevalence of the disease (Alton et al., 1988; Godfroid et al., 2002). A further limit of the serological tests is that they cannot discriminate between infected and exposed individuals (Blasco et al., 1994; Dobson and Meagher, 1996). In fact, animals that test seropositive may be infected animals, individuals that were infected and have recovered, or resistant animals that have merely been exposed to *Brucella*. Factors determining natural resistance to brucellosis have been reported (Price et al., 1990; Borriello et al., 2006). Bacteriological isolation of the causative organism is regarded as the gold standard for a definitive diagnosis of the disease (Alton et al., 1988; OIE, 2004). Bacteriological isolation is, however, laborious for large-scale diagnosis, time-consuming because of the long incubation time (10 to 15 d), and of high risk to laboratory personnel. In addition, the lack

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of stringent selective growth media results in frequent overgrowth of contaminants. The limitations of both of these approaches have resulted in an increased interest in developing rapid, accurate diagnostic tests for the direct detection of *Brucella* spp. in bovine samples such as milk and blood. Polymerase chain reaction has been shown to be a valuable tool for the detection of DNA from different fastidious and noncultivable pathogens, having the additional advantages of allowing the detection of small numbers of microorganisms, being reproducible and easily standardized, minimizing the risk of infection to laboratory workers, and having a total processing time of about 2 to 3 h. These characteristics can be extremely important when rapid and accurate identification of *Brucella* spp. is required. Several studies have been published on the detection of *Brucella* DNA by PCR, both from pure culture (Fekete et al., 1990; Baily et al., 1992; Herman and De Ridder, 1992) and from field samples, mostly of cattle origin (Leal-Klevezas et al., 1995; Hamdy and Amin, 2002; O'Leary et al., 2006). Despite the importance of buffalo milk as a source of human brucellosis in areas where buffalo are bred, few studies have been published on the diagnosis of brucellosis by PCR in water buffaloes (*Bubalus bubalis*; Guarino et al., 2000), and no information is available on the performance of PCR assays on buffalo milk samples. In Italy, mainly in the south, buffalo breeding is an important source of income, particularly through the production of a soft cheese called Mozzarella di Bufala.

The direct detection of pathogenic bacteria in food samples is hampered by the presence of PCR-inhibitory substances frequently associated with the food matrix itself (Rossen et al., 1992). In milk, components such as Ca^{2+} , proteinase, fats, polysaccharides, and milk proteins may act as inhibitors of nucleic acid amplification by shielding DNA from polymerase access (Wilson, 1997). Compared with cow's milk, buffalo milk contains less water, and more solids (fat and proteins) and lactose. This underscores the need for effective protocols for the extraction of high-quality DNA from buffalo milk samples in preparation for PCR amplification. In this study, we assess the feasibility of PCR and real-time PCR assays as diagnostic tools for the detection of *Brucella* spp. in milk from seropositive and seronegative water buffaloes.

MATERIALS AND METHODS

Laboratory Inoculated Samples

We used the *B. abortus* RB51 vaccine (CZ Veterinaria S.A., Porrino, Spain) to inoculate pooled milk from a *Brucella*-free herd in the nonendemic area of Grosseto

(Italy). The milk was microbiologically confirmed to be negative to brucellosis, and was used to evaluate the sensitivity of 5 different DNA extraction methods used in combination with 2 PCR primer pairs.

Field Samples

Blood and milk samples were collected from 60 water buffaloes. Fifty-three of the animals were from 3 farms in the endemic area of Salerno (Italy), and were classified as seropositive based on a positive reaction to one or both of the following tests: 1) rose Bengal test (RBT); and 2) CFT, as described below. Seven animals were from a *Brucella*-free dairy herd in Grosseto, a nonendemic area of Italy, and used as negative controls. Samples were classified as negative by RBT and CFT. All milk samples were obtained from animals during their routine milking time. Samples from each animal were obtained from all 4 quarters of the mammary gland. Five hundred microliters of milk was used for the molecular detection of *Brucella* spp.

Serological Testing of Field Samples

Serum samples of 60 water buffaloes were tested by RBT and CFT according to Alton et al. (1988). Sera were considered positive when showing any degree of agglutination in the RBT, or 50% or less hemolysis at a dilution of 1/4 or greater in the CFT (i.e., ≥ 20 IU/mL). Milk samples were tested by bovine brucellosis milk ELISA test using *Brucella* S-LPS as antigen (Institut Pourquier, Montpellier, France). The ELISA results were classified as negative or positive using the cut-off values recommended by the manufacturer.

Bacteriological Examination of Field Samples

The cream and sediment mixture from each of the 60 samples was collected after 20 mL of milk from each sample was centrifuged at $2,000 \times g$ for 20 min at 4°C. Cream and sediment were then spread onto Brucella agar plates with added Brucella supplement (Oxoid Ltd., Hampshire, UK) and 5% horse serum. The plates were then incubated at 37°C in 10% CO_2 for 5 to 10 d and examined daily for the presence of colonies, following the identification methods adopted by Quinn et al. (1994).

Sensitivity Studies on Laboratory Inoculated Samples

Raw milk samples obtained from water buffaloes with no history of brucellosis were used for sensitivity studies. These samples were used in all spiking experi-

ments and, in the entire study, as negative controls for PCR. The sensitivity of 5 DNA extraction protocols and 2 pairs of PCR primers (F4/R2 and B4/B5) were evaluated. Five-hundred-microliter samples of raw, *Brucella*-free milk were spiked with 10-fold serial dilutions of strain RB51, starting from 4×10^9 cfu/mL to 0.4 cfu/mL. One aliquot of milk was not spiked and was used as a negative control. For each DNA extraction protocol, we thus prepared a 12-tube series: 11 tubes each containing 500 μ L of serially diluted spiked milk and 1 tube containing the same amount of control (unspiked) milk; DNA was extracted from these dilutions using 5 protocols as described below.

DNA Extraction Protocol A

Protocol A was as described by Romero and Lopez-Goni (1999). Briefly, 500- μ L samples were mixed with 100 μ L of NET buffer [50 mM NaCl, 125 mM EDTA, 50 mM Tris-HCl (pH 7.6)] and 100 μ L of 24% SDS. The mixtures were cooled on ice after incubation at 80°C for 10 min. Digestion with proteinase K and RNase (final concentrations of 325 and 75 μ g/mL, respectively) was carried out at 50°C for 2 h. The DNA was extracted with phenol-chloroform-isoamyl alcohol using phase Lock Gel Heavy tubes (Eppendorf AG, Hamburg, Germany) and precipitated with 1/10 volume of 3 M NaOAc, pH 5.2, 0.8 μ L of glycogen (10 μ g/mL), and 1 volume of isopropanol, and then washed with 75% ethanol and dried. The DNA pellet was dissolved in 100 μ L of nuclease-free water and stored at -20°C until further use. Two microliters of extracted DNA was used for PCR.

DNA Extraction Protocol B

Protocol B followed the Leal-Klevezas method (Leal-Klevezas et al., 1995); briefly, 400 μ L of lysis solution (2% Triton-X 100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl, pH 8.0) and 5 μ L of proteinase K (20 mg/mL) were added to the samples, thoroughly mixed, and incubated for 30 min at 50°C. Thereafter, DNA was extracted and precipitated as described in protocol A. Two microliters of extracted DNA was used for PCR.

DNA Extraction Protocol C

Three hundred microliters of lysis buffer (3 M guanidine thiocyanate, 20 mM EDTA, 10 mM Tris-HCl pH 6.8, 40 mg/mL of Triton X-100, 10 mg/mL DL-dithiothreitol), described previously (Cremonesi et al., 2006), was added to the samples, which were then incubated for 10 min at room temperature. Digestion with 100 μ L of 10% SDS and 3 μ L of proteinase K (20 mg/mL) were

carried out at 50°C for 1 h. Three microliters of RNase (4 mg/mL) was then added, and samples were incubated at 37°C for 1 h. Thereafter, DNA was extracted and precipitated as described in protocol A. Two microliters of extracted DNA was used for PCR.

DNA Extraction Protocol D

Samples were centrifuged at $9,700 \times g$ for 15 min for bacterial concentration. The fatty top layer and supernatant were discarded and the remaining pellet resuspended in 500 μ L of 0.9% saline. Bacterial DNA was extracted following protocol A. Two microliters of extracted DNA was used for PCR.

DNA Extraction Protocol E

The commercial kit QIAamp DNA Mini Kit (Qiagen S.p.A., Milan, Italy) was used to extract DNA from milk samples according to the manufacturer's instructions. The DNA was eluted from the columns in 200 μ L of elution buffer. Four microliters of extracted DNA was used for PCR.

PCR and Real-Time PCR

Two pairs of primers amplifying different regions of the *Brucella* genome were used: 1) primers B4 (5'-TGG CTC GGT TGC CAA TAT CAA-3') and B5 (5'-CGC GCT TGC CTT TCA GGT CTG-3'), which amplify a 223-bp fragment of the 31-kDa outer membrane protein (Baily et al., 1992); and 2) primers F4 (5'-TCG AGC GCC CGC AAG GGG-3') and R2 (5'-AAC CAT AGT GTC TCC ACT AA-3'), which amplify a 905-bp fragment of the 16S rRNA sequence (Romero et al., 1995a). All amplifications were performed in a total volume of 25 μ L using GoTaq Green Master Mix (Promega Corp., Madison, WI). Reactions with primers B4/B5 were performed at a denaturation temperature of 95°C for 2 min. This was followed by 40 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s and one final extension at 72°C for 10 min. Reactions with primers F4/R2 were performed at a denaturation temperature of 95°C for 2 min. This was followed by 30 cycles at 95°C for 30 s, 54°C for 30 s, and 72°C for 90 s and one final extension at 72°C for 10 min. After amplification, all reaction mixtures were analyzed by electrophoresis in a 2% agarose gel, stained with ethidium bromide, and photographed.

Real-time PCR reactions were performed in a total volume of 25 μ L using 2 $\times$ SensiMix DNA Kit (Quantace Ltd., London, UK), 0.5 μ L of 50 $\times$ SYBR Green I solution, 2 μ L of template DNA, and primers B4/B5 according to the manufacturer's instructions. Samples were amplified using an ABI Prism 7000 Sequence De-

Table 1. Serological, microbiological, and molecular results on field samples of milk

Buffaloes	ELISA	Culture	PCR		Real-time PCR	
			+	–	+	–
Seropositive, n = 53	53 (+)	37 (+)	25	12	26	11
		16 (–)	8	8	9	7
Seronegative, n = 7	7 (–)	7 (–)	0	7	0	7

tection System (Applied Biosystems, Foster City, CA) under the following conditions: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min, concluding with a dissociation curve. All samples were processed in triplicate.

Sequencing

The PCR products were sequenced with an ABI PRISM 310 Genetic Analyzer, using the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems); PCR primers were used for sequencing. A BLAST search was then conducted to compare the resulting nucleotide sequences to those published in the database (<http://www.ncbi.nlm.nih.gov/BLAST/>).

RESULTS

Analytical Sensitivity Estimates

Brucellosis-negative buffalo milk was spiked with a known number of *B. abortus* RB51, from 4×10^9 to 0.4 cfu/mL, processed using 5 different DNA extraction methods, and subsequently tested with different PCR and real-time PCR assays. The results of the sensitivity studies are shown in Figure 1.

The different combinations of extraction methods and PCR essays yielded different limits of RB51 detection: the limit of detection was 4×10^3 cfu/mL using DNA extraction protocol A followed by the B4/B5 PCR assay, and 4×10^5 cfu/mL using the same DNA extraction protocol with the F4/R2 PCR assay (Figure 1, panels A and B, respectively). The limit of detection was 4×10^4 cfu/mL using DNA extraction protocol B, C, or D followed by the B4/B5 PCR assay, and 4×10^6 cfu/mL using 1 of these 3 DNA extraction protocols with the F4/R2 PCR assay (Figure 1, panels C and D, respectively). The limit of detection was 4×10^5 cfu/mL using DNA extraction protocol E with the B4/B5 PCR assay, and 4×10^7 cfu/mL using the same DNA extraction protocol with the F4/R2 PCR assay (Figure 1, panels E and F, respectively). The most sensitive PCR method, yielding the lowest limit of detection (4×10^3 cfu/mL), was the B4/B5 PCR assay on spiked milk samples processed by DNA extraction protocol A.

Spiked milk samples extracted following the most sensitive method (protocol A) were also evaluated by real-time PCR assay using SYBR Green and primers B4/B5. The limit of detection was lower than for conventional PCR (4×10^2 cfu/mL). Bacterial DNA extraction protocol A with B4/B5 PCR and real-time B4/B5 PCR were used to examine the 60 field samples for direct detection of *Brucella* spp.

Comparison of Serological, Microbiological, and Molecular Tests on Field Samples

Blood and milk samples were collected from 60 water buffaloes, of which 53 were classified as seropositive and 7 as seronegative by RBT/CFT tests, as described above. Milk samples were examined by ELISA for the detection of *Brucella* antibodies, and then by culture and PCR techniques for the detection of *Brucella* spp. (Table 1). Results obtained through *Brucella* antibody detection in both sera and milk concurred. As expected, milk samples from all seropositive buffaloes were positive by ELISA, 37 were positive by culture, 33 were positive by PCR, and 35 were positive by real-time PCR. Assuming that the culture test is 100% accurate, the sensitivity (% of true positives) and specificity (% of true negatives) of serology tests were on the order of 100 and 30%, respectively. The matching rate between serology and culture was 73%.

Of the 37 culture-positive samples, a total of 25 and 26 were also positive by PCR and real-time PCR tests, respectively. However, of the 16 culture-negative samples, 8 were positive by PCR and 9 by real-time PCR. Combining bacteriological and molecular methods, the number of positive samples identified increased to 46. To identify the amplified fragments, PCR products were sequenced and the presence of the 31-kDa outer membrane protein of *Brucella* confirmed. All samples from *Brucella*-free buffaloes were confirmed negative by serology, culture, and PCR assays.

DISCUSSION

In this study, we evaluated the feasibility of molecular assays as diagnostic tools for the detection of *Brucella* spp. in water buffalo milk. For this purpose, we first

compared different DNA extraction protocols and PCR methods on artificially spiked milk samples. The most sensitive molecular methods were chosen as potential diagnostic tools for the direct detection of *Brucella* spp. in milk from serologically positive water buffaloes. Results were then compared with those obtained by bacteriological methods. An indirect ELISA for the detection of *Brucella*-specific antibodies in milk was also evaluated. Our findings indicated that the sensitivity of the ELISA test surpassed that of both culture

and PCR assays in buffalo milk. We also found that bacteriological methods identified a greater number of brucellosis-infected buffaloes than molecular methods. Neither culture nor molecular assays detected *Brucella* organisms in any of the seronegative animals.

As described in previous studies (Fekete et al., 1990; Bailly et al., 1992; Herman and De Ridder, 1992), PCR-based assays can be extremely useful for analyzing pure microbial cultures. However, when applied directly to food samples, their efficiency can be markedly reduced

Figure 1. Agarose gel showing the results of 5 different DNA extraction methods used in combination with 2 PCR assays for detection of *Brucella abortus* RB51 in artificially spiked buffalo milk samples. A) DNA extraction protocol A and B4/B5 PCR assay; B) DNA extraction protocol A and F4/R2 PCR assay; C) DNA extraction protocol B, C, or D and B4/B5 PCR assay; D) DNA extraction protocol B, C, or D and F4/R2 PCR assay; E) DNA extraction protocol E and B4/B5 PCR assay; F) DNA extraction protocol E and F4/R2 PCR assay. Lane 1 = 100-bp DNA ladder; lanes 2 to 12 = milk samples artificially spiked with serial dilutions of RB51 from 4×10^9 to 0.4 cfu/mL; lane 13 = unspiked milk samples, used as a negative control.

by poor sample preparation, which may inadvertently introduce inhibitory substances precluding DNA amplification. Fats, enzymes, polysaccharides, proteins, and high concentrations of Ca^{2+} have been proposed as potential inhibitors of PCR by interfering with nucleic acid degradation or capture, and inhibiting polymerase activity for amplification (Rossen et al., 1992; Rijpens et al., 1996; Wilson, 1997). To the best of our knowledge, no information has yet been published on the performance of PCR assays on buffalo milk, which contains more fat and more protein than cow's milk.

The efficient extraction of bacterial DNA from a matrix is a critical step in PCR because the sensitivity of the test can be hindered by the method used to isolate the nucleic acid target. In the present study, we therefore evaluated different bacterial DNA extraction protocols to identify the method that would achieve the strongest and most reproducible amplifications possible.

The sensitivity of 2 different PCR methods for the detection of *Brucella* spp. was also compared. We chose 2 pairs of primers: B4/B5, the excellent sensitivity of which was previously reported (Matar et al., 1996; Morata et al., 1998), and F4/R2, which is commonly used for the detection of *Brucella* spp. in milk (Romero et al., 1995a,b; Romero and Lopez-Goni, 1999). Amplification conditions were based on those used in a previous study, which evaluated the sensitivity of PCR assays using the same primer pairs (Navarro et al., 2002), and on the Taq polymerase manufacturer's instructions. The greatest sensitivities were obtained using DNA extraction protocol A and PCR primers B4/B5 (detection limits were 4×10^3 cfu/mL and 4×10^2 cfu/mL for PCR and real-time PCR, respectively). This occurred despite the fact that primers F4/R2 amplify a region of the 16S rRNA gene present in several copies in the bacterial genome, whereas primers B4/B5 amplify one copy of the gene encoding the 31-kDa *B. abortus* antigen. *Brucella* detection limits in cow's milk as reported in the published literature vary greatly, ranging from 2.8×10^4 cfu/mL (Rijpens et al., 1996) through 2×10^3 cfu/mL (Sreevatsan et al., 2000), to between 5 and 50 cfu/mL (Romero and Lopez-Goni, 1999). In addition, different limits were reported for different species. In a study on milk samples artificially spiked with either *B. abortus* or *B. melitensis*, for example, detection limits were 100 cfu/mL for *B. abortus* and 1,000 cfu/mL for *B. melitensis* (Hamdy and Amin, 2002). Romero et al. (1995b) reported similar results. Thus, different factors may affect the sensitivity of PCR. Among these are the effectiveness of the DNA extraction protocol, the size of the processed sample, the molecular assay used, and the *Brucella* species tested for.

Reports on the ability of PCR to detect *Brucella* spp. in milk samples from infected animals compared with that of standard serological and bacteriological methods may seem somewhat inconsistent. The sensitivity of PCR detection has been shown to exceed that of serological and bacteriological methods in both experimentally and naturally infected animals (Leal-Klevezas et al., 1995; Hamdy and Amin, 2002). This is in agreement with the findings reported by Hamdy and Amin (2002) on bovine milk samples, who found the sensitivity of PCR to be greater than that of bacterial culture. Yet in sheep samples, the same authors found that bacteriological methods detected more positive cases of brucellosis than PCR. Others working on bovine milk samples found bacterial culture (Romero et al., 1995b; O'Leary et al., 2006) and serological methods (ELISA; Romero et al., 1995b) to yield more sensitive results than PCR. Interestingly, according to the findings described by O'Leary et al. (2006), tissue samples from lymph glands appeared to be the most promising sample type for *B. abortus* detection by PCR, whereas whole blood seemed to yield unsatisfactory results.

Several possible reasons have been proposed for the relatively inconsistent performance of the PCR assay at *Brucella* detection compared with that of serological or bacteriological methods: 1) the stage of infection may influence the number and location of bacteria (Morgan and MacKinnon, 1979; Alton et al., 1988); 2) the sample type used for diagnostic purposes may affect the results (O'Leary et al., 2006); 3) the presence of large amounts of host genomic DNA may inhibit the PCR reaction (Navarro et al., 2002); and 4) the DNA extraction method used may be crucial in determining the ability of the PCR assay to detect the bacterium (Romero and Lopez-Goni, 1999).

Serological tests indicate the presence of *Brucella* antibodies in animals, but it is only possible to positively identify an animal as infected if positive cultures are grown from tissue collected from that animal (Dobson and Meagher, 1996). In our study, the performance of serological tests in diagnosing brucellosis in a population of naturally infected buffaloes was evaluated. *Brucella* antibody detection in milk by ELISA concurred with RBT/CFT tests on field samples. Comparing bacteriological and serological methods, 37 out of 53 seropositive buffaloes were infected (culture-positive subjects) and 16 individuals (culture-negative and seropositive subjects) were potentially exposed but not necessarily infected animals. Therefore, estimates of *Brucella* prevalence based on sero-reactors may give an overestimate of the true level of infection. Although serological tests are the major diagnostic tools for screening of animal brucellosis in the field, they

are unable to distinguish between truly infected and noninfected animals as previously reported (Blasco et al., 1994; Dobson and Meagher, 1996). The ability to classify animals into truly infected and noninfected is essential for a correct diagnosis.

Because detection of specific DNA, as well as bacteriological isolation of *Brucella*, is a true indication of the presence of the pathogen, we wanted to evaluate the feasibility of molecular assays as diagnostic tools for brucellosis. Not all bacteriologically positive samples were also positive by PCR assay. Bacteriological and molecular tests agreed (both positive) in 25 to 26 cases. Factors that may compromise DNA recovery include difficulties in disrupting bacterial cell walls, loss of DNA template through extraction procedures, or the presence of potential polymerase inhibitors. In addition, the amount of milk used for PCR is much smaller than that required for bacteriological methods, and the number of organisms contained in a sample may thus not reach PCR detection limits. On the other hand, some culture-negative samples were positive by molecular methods. These results may indicate the presence of dead organisms in the milk. When an animal is negative to the bacteriological test, but positive to a molecular test, the culture test should be repeated 1 or 2 wk later to exclude intermittent shedding of the pathogen or a typing mistake. The sensitivity of the real-time PCR assay was slightly greater than that of conventional PCR in *Brucella* DNA detection in field samples.

It is noteworthy that all milk samples collected from *Brucella*-free buffaloes and diagnosed as brucellosis negative by serological and bacteriological methods tested negative by PCR assays as well, a finding pointing to the specificity of molecular assays.

In conclusion, our findings indicate that 1) estimates of incidence of *Brucella* based on sero-reactors should be treated with extreme caution unless accompanied by data from tissue culture, thus avoiding overestimates of the true level of infection; 2) although the bacteriological assay was more sensitive than PCR in detecting *Brucella* spp. in the milk of seropositive buffaloes, molecular tools revealed false-negatives in the culture results. The simultaneous application of both direct methods may be advisable, because it increases the performance of diagnostic tools for *Brucella* detection.

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