

# Comparison of conventional and non-conventional techniques for the diagnosis of bovine brucellosis in Sudan

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**Abstract** The objective of the present study was to detect brucellosis in suspected dairy cattle in Khartoum State, Sudan using the conventional serological tests and tests done on milk in comparison to a PCR-based technique. Milk and blood samples collected simultaneously from suspected brucellosis cows ( $n=147$ ) in 12 different dairy farms around Khartoum State were used in the study. Overall, 54 (36.7%) of the total milk samples were positive according to the milk ring test (MRT), while 29 (19.7%) of the serum samples were positive according to the Rose Bengal test (RBT); microscopy on modified Ziehl–Neelsen-stained slides detected 13.6% of the cases, and recovery of *Brucella* species on both *Brucella* medium and tryptic soya agar was 7.5%. Thirty-three (22.4%) samples were found positive on PCR-amplified IS711 which were then taken as positive brucellosis cases. The differences of RBT and PCR-IS711 from MRT were highly significant ( $P<0.05$ ). MRT detected more cases of bovine brucellosis compared to RBT, PCR, microscopy, and culture. MRT is recommended as a noninvasive test compared to RBT, and it is less expensive compared to PCR and culture.

**Keywords** Milk ring test · Rose Bengal test · PCR · IS711 element · Sudan

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## Introduction

Brucellosis is one of the most important zoonotic diseases which affects a variety of domestic and non-domestic animals and humans, causing high economic loss and public health burden especially in countries with no effective control programs. It is caused by bacteria in the genus *Brucella*, which contains several species that are defined mainly on the basis of animal host specificity (Boschioli et al. 2001; Joint WHO/FAO/OIE 2004). Six species according to the primary host, *Brucella abortus* (cattle), *Brucella melitensis* (sheep and goats), *Brucella suis* (swine), *Brucella ovis* (desert wood rat) are validly described with many biotypes and strain variants (Mantur et al. 2007; Christopher et al. 2010).

Diagnosis of brucellosis is the cornerstone of any control program and is based on bacteriological and immunological findings. These methods are not wholly satisfactory since the bacteriological isolation is a time-consuming procedure. Moreover, the handling of the microorganisms is hazardous, and the use of serological test is recommended as a mean of indirectly diagnosing the disease. However, many current serological tests have proven to be either too sensitive, giving false positive results, or too specific giving false negative results (Mangen et al. 2002). Milk ring test (MRT) and Rose Bengal test (RBT) are first-line screening tests for brucellosis in cows in some countries; their lack of specificity is of concern. Therefore, the requirement for other confirmatory tests that are more specific should be considered for control and eradication of the disease, especially in countries such as Sudan. In Sudan, animal brucellosis was reported as early as 1904. Subsequently, many studies on the disease and its etiological agents have been carried out (Khalafalla et al. 1987; Sixl et al. 1988; Musa et al. 1990).

Numerous polymerase chain reaction (PCR)-based assays have been developed for the identification of *Brucella* to

improve diagnostic capabilities. Some of the major advancements in molecular diagnostics for *Brucella* including the development of procedures designed for the direct analysis of a variety of clinical samples have been reviewed (Bricker 2002; Christopher et al. 2010). These techniques include several strategies to differentiate *Brucella* species and strains, including locus-specific multiplexing (e.g., AMOS-PCR based on IS711), PCR-RFLP (e.g., the *omp2* locus), arbitrary-primed PCR, and ERIC-PCR (Brikenmeyer and Mushahwar 1991; Hamdy and Amin 2002).

Conventional *Brucella* typing and diagnostic tests notably RBT, MRT, CFT, and ELISA remain standard tests and are available. But no single test is applicable for all purposes of brucellosis diagnosis. Molecular tests including PCR-based procedures are at present under development but are still relatively expensive (Joint FAO-APHC/OIE 2009). The objective of the present study was to detect brucellosis in suspected dairy cattle in Khartoum State, Sudan using conventional tests in comparison to PCR-amplified IS711.

## Material and methods

### Samples

A total of 294 samples consisting of 147 milk and 147 sera were collected from dairy farms with known history of bovine brucellosis. The cattle were of different ages and breeds (cross between local Butana breed and the foreign Friesian breed). The farms were from different localities in Khartoum State, namely Omdurman and Khartoum north. Milk and serum samples were collected following a standard method (Quinn et al. 1999).

### Culture

Tryptic soy agar (TSA, Difco) supplied with bacitracin (25 µg/mL), cyclohexamide (100 µg/mL), naladixic acid (5 µg/mL), nystatin (12.5 µg/mL), polymyxin B (5 µg/mL), and vancomycin (20 µg/mL) and *Brucella* medium (Mast Diagnostics, UK) were used. The medium was prepared by reconstitution of *Brucella* medium base or tryptic soy agar (Difco), and then sterilized by autoclave at 121°C for 15 min. The basal medium was left to cool to 56°C, then sterile horse serum (5–7%) was added for enrichment. The mixture was distributed into sterile Petri dishes (20 ml) and left to solidify. Slants were made by placing 5 ml of the medium into sterile McCartney bottles and left to solidify in a sloping position.

Milk was centrifuged at 3,000×g for 15 min, and then the layer between the cream and sediment was discarded to obtain the mixture of sediment and cream which was used for culture (Alton et al. 1988). A loopful of the mixture was

streaked onto both media; duplicate plates were also inoculated with another set of sterile loops.

Plates were incubated at 37°C aerobically and in the presence of 5–10% CO<sub>2</sub>. Plates were examined every 3 days for growth. Typical and well-isolated *Brucella*-like colonies from the primary culture were streaked on fresh plates of the corresponding medium. Pure cultures were obtained by replating the subcultures on TSA, and then identified macroscopically for the presence of small, transparent, raised, and convex, with an entire edge and smooth, glistening surface along the streak lines. Microscopically, the growth was examined by Gram's and modified Ziehl–Neelsen's stain (Quinn et al. 1999).

### Identification of isolates

Purified isolates from primary or from subcultured plates were identified to the species level according to the criteria outlined by Barrow and Feltham (1993). The following tests were done: motility, CO<sub>2</sub> requirement, oxidase test, catalase test, urease production, and H<sub>2</sub>S production.

### Milk ring test

MRT was done according to the procedure described in the OIE Terrestrial Manual (2009). The antigen used for MRT was supplied by the Central Veterinary Laboratory, Khartoum, Sudan. It is a suspension of *Brucella* cells stained with hematoxylin blue. The test was done by adding 0.03 mL of stained milk ring test antigen to 1 mL of suspected milk. Both were mixed well and incubated at 37°C for 3 h; then, the test was observed for a blue ring formation which was taken as a positive reaction.

### Modified Ziehl–Neelsen stain

Thin film smears were prepared from each milk sample, dried, and fixed by heat. Dilute carbol fuchsin was applied for 15 min and washed under running water. The smear was decolorized with 0.5% acetic acid for 15 s, washed, and methylene blue is applied for 2 min as a counterstain, washed, dried, and then examined under oil immersion (Quinn et al. 1999).

### Rose Bengal test

The antigen used in the RBT was obtained from Central Veterinary Laboratory, Khartoum; it was prepared and standardized as per OIE procedure (2009). Serum samples and antigens were removed from the refrigerator and placed at room temperature for an hour. The test was done by dispensing 0.03 mL of each serum to be tested to an enamel plate. The same amount of Rose Bengal antigen was added

to each serum and mixed and rocked by hand for 4 min, and the test was then read. Agglutination appeared as weak positive, positive, strong positive, or very strong positive.

#### Amplification of IS711 element by PCR

##### DNA extraction

Extraction of DNA from milk samples was adapted locally. Mixture of the cream and sediment obtained from the centrifugation of 20 mL of the milk sample at 3,000×g for 15 min was taken and then diluted to 1:80 by using deionized distilled water in a screw-capped microcentrifuged tube. The diluted mixture was vortexed and then placed on water bath at 100°C for 5 min, and then the supernatant was gently aspirated and centrifuged for 2 min; then, the aspirated supernatant was used directly for PCR amplification without any further processing.

One to three colonies from grown cultures and from reference strain were picked up into 100-μl deionized distilled water in a 1.5-mL screw-capped microcentrifuged tube and then placed on boiling water bath for 10 min. The supernatant was taken and used directly for amplification.

##### Oligonucleotide primers

The primers designed by Bricker and Halling (1995) were used. They were obtained from MWG Biotech (AG 32-1074-1/6). The sequences of the primer were as follows: AB 5' GAC, GAA, CGG, AAT, TTT, TCC, AAT, CCC 3' and IS711 5'-TGC, CGA, TCA, CTT, AAG, GGC, CTT, CAT-3. The primers were designed to amplify species-specific-sized products which hybridize to the IS711 element and to *B. abortus* species-specific regions adjacent to the IS711 element (498 bp)

##### PCR amplification

PCR assay was done by DNA thermocycler following the instructions of the manufacturer (Bioline) with some modifications. PCR amplification was carried out in a 50-μl reaction

mixture consisting of 5 μl of a sample (or genomic DNA) containing template DNA, 1.5 U of Tag DNA polymerase, 5 μl of 10× PCR amplification buffer, 20 pmol/μl each primer, 200 mM deoxynucleoside triphosphate, 25 mM MgCl<sub>2</sub>, and double-distilled water to a final volume of 50 μl. To minimize evaporation, 25 μl of mineral oil was added to the reaction mixture on top of each PCR tube. The amplification was done by the program described by Bricker and Halling (1995). So, the thermal cycler device (Techne) was programmed to provide the following thermal profiles: first denaturation at 95°C for 5 min, and cycling condition consists of 35 cycles. DNA denaturation at 95°C for 1.15 min, primers annealing at 55.5°C for 2 min, and extension of the two strands at 72°C for 2 min. After the final cycle, the reaction was terminated by an extra run at 72°C for 10 min for final extension.

##### Electrophoresis

Amplified products from the samples were confirmed on electrophoresis on ethidium bromide-stained 1.5% agarose gel. Amplicons (498 bp) were visualized under ultraviolet illumination and photographed afterwards using gel documentations system. The product fragments were identified by comparing the product bands with the band of the positive control and with the DNA size marker.

##### Statistical analysis

Differences between each of the conventional and non-conventional tests were assessed by the  $\chi^2$  test. Significant differences were considered when the probability (*P*) was <0.05.

## Results

The results of the different methods used for the diagnosis of bovine brucellosis are shown in Table 1. Reactions of cattle to various tests in Table 1 were given as categories (1 to 6) and summed up as subtotal in the last column.

**Table 1** Assay results and (percentage) for conventional and non-conventional tests for cattle brucellosis conducted on 147 suspected cases in Khartoum, Sudan

Cases which gave positive results to PCR-amplified IS711 were considered positive for brucellosis

Category	Rose Bengal test	Milk ring test	Modified ZN	Culture	PCR-amplified IS711	Number (%) of cattle in category
1.	+	+	+	+	+	11 (7.5)
2.	+	+	+	–	+	9 (6.1)
3.	+	+	–	–	+	9 (6.1)
4.	–	+	+	–	+	4 (2.7)
5.	–	+	–	–	–	21 (14.3)
6.	–	–	–	–	–	114 (77.6)
Total	29 (19.7)	54 (36.7)	20 (13.6)	11 (7.5)	3 (22.4)	147 (100)

Out of the 147 milk samples inoculated onto *Brucella* and TSA media for the growth of *Brucella* species, 11 samples (7.5%) revealed growth indicative of *Brucella* spp. According to colonial morphology, motility, microscopic appearance with Gram and modified Ziehl–Neelsen (MZN) stains, and biochemical tests, the 11 isolates were identified as *B. abortus*.

Using MZN, 20 (13.6%) out of the 147 milk samples showed the presence of acid fast mixture of small coccoid and coccobacilli indicative of *Brucella* spp.

Out of the 147 milk samples, 54 (36.7%) showed positive reactions for MRT (Table 1). Positive samples showed formation of a clear blue ring at the top column of the milk in the test tube. When using RBT, 29 (19.7%) serum samples showed agglutinations to *Brucella* antigens. The 29 positive samples showed varied degrees of agglutination from + to +++++. Examination of the milk samples with PCR to amplify IS711 element revealed *B. abortus* species-specific regions (498 bp) in 33 (20.6%) of the samples (Table 1).

## Discussion

In low-income countries such as Sudan, surveillance using conventional tests done on serum and tests done on milk can be used for screening and plays an important role in campaigns to eliminate the disease. Also, they are used for testing animals for trade purposes. Countries should educate farmers and animal health workers in wildlife, zoo, or at local human and animal health facilities about the importance of reporting events of potential zoonoses such as brucellosis (Joint WHO/FAO/OIE 2004).

The present study aimed to compare the utility of conventional tests for the diagnosis of brucellosis in cattle in comparison to a nucleic acid-based technique. MRT in this study detected more cases (36.7%) among the studied population than RBT (19.7%) and than other assays used. Practicality and feasible utility of the MRT over other tests have been acknowledged in many developing countries including Nigeria, Ethiopia, and Syria (Cadmus et al. 2008, 2010; Mekonnen et al. 2010; Al-Mariri et al. 2011). Cadmus et al. (2008) recorded a highly significant difference ( $P < 0.05$ ) between MRT (18.61%) compared to RBT (9.77%). One disadvantage of MRT is that it may give false positive reactions since test results are influenced by factors such as presence of mastitis coinfection, mechanical agitation, and previous vaccination (Morgan et al. 1978). This is evident in our results as shown in Table 1 compared to PCR-amplified IS711.

Although the Rose Bengal plate test is considered a valuable screening test (Mangen et al. 2002) and recommended by many due to its reasonable sensitivity, it has, however, provided low sensitivity (19.7%) in the present study compared to MRT (36.7%). This may be attributed to

the fact that efficiency of the test is affected by cell concentration and the standardization procedure of the antigen (Emmerzaal et al. 2002).

The diagnosis of a cow as positive for brucellosis was considered in this paper if it gave a positive reaction to IS711 (Table 1). This is in accordance with published literature which demonstrates that PCR-based methods are reliable methods for detecting DNA from different clinical samples including milk (Brikenmeyer and Mushahwar 1991; Leal-Klevenzas et al. 1995; Hamdy and Amin 2002; Hinić et al. 2009). PCR detects living and dead organisms, while culture detects only living organisms. PCR limit of detection was found to be below 1 cfu/g with excellent reproducibility, sensitivity (96.7%), and specificity (100%) (Di Giannatale et al. 2009). These results could be reproduced in a good laboratory setting. In the present study, PCR-amplified IS711 detected 22.4% of the cases from the milk samples exceeding the culture method (7.5%).

It was evident from our results that *Brucella* was not detected by culture or PCR from some seropositive animals (Table 1), and this was expected as the excretion of the organisms in milk is intermittent (Morgan et al. 1978; Alton et al. 1988) and is more common during late lactation and can persist for several years. Although the sensitivity of the serological tests is better than the culture, the specificities are low and false positive reactions may occur.

The present study demonstrated that isolation of *Brucella* sp. on synthetic media (7.5%) as well as the microscopic detection of *Brucella* spp. was difficult. Smears made from milk samples yielded poor results (13.6%). If laboratory setting is improved, PCR has the potential to be a useful method for diagnosis of brucellosis from milk samples. In the veterinary field, routine diagnosis of brucellosis is dependent on MRT and/or RBT (Mantur et al. 2007; Christopher et al. 2010). Improvement of culture methods is required as well, but this improvement has to put in consideration the state of test animals with regard to history of vaccination, exposure to infections, and records of abortion.

The study recommended MRT assay for rapid large-scale screening as it detected more brucellosis cases. It is noninvasive and feasible compared to RBT and less expensive compared to PCR.

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