

Full Length Research Paper

Comparative diagnosis of ovine brucellosis using single step blood-PCR with old and new serological tools

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Brucellosis is an important disease affecting mainly sheep and goats. Diagnosis based on isolation of *Brucella* organisms from the suspected animals is the golden standard but has a limited sensitivity, expensive and unpractical to apply on a large scale in control campaigns. Accordingly, the indirect diagnosis of disease based on serological tests is the method of choice in the eradication programs. In this study, a single step polymerase chain reaction (PCR) was used to diagnose brucellosis using sheep whole blood and compared its sensitivity and specificity against some of the most commonly used serological techniques and modified ones. Three hundred apparently healthy ewes were randomly chosen from different governorates of Egypt. Sera were tested against Rose Bengal test (RBT), Serum Agglutination test (SAT), ELISA using both the whole *Brucella* antigen (W-ELISA) and the periplasmic protein antigen (P-ELISA). Results showed that 39% of the blood samples were positive to the PCR test, Meanwhile 29.3, 27.0, 28.7 and 28.3% were positive to the previous serological tests respectively. We recommend the use of this blood PCR assay for accurate diagnosis of ovine brucellosis especially in the early stage of infection, which is difficult to achieve by the applied serological tests.

Key words: Ovine brucellosis, Blood PCR, RBT, SAT, ELISA.

INTRODUCTION

Brucellosis is an important disease affecting mainly cattle, sheep and goats. The disease in cattle is most commonly caused by *Brucella abortus* while brucellosis in sheep and goats is caused by *Brucella melitensis*, a very important zoonotic agent (Moriyón and Gamazo, 1998).

Isolation of *Brucella* organisms from the suspected animal is the golden standard in terms of specificity. However, this method has a limited sensitivity, expensive and cumbersome and has the added difficulty of being

unpractical to apply on a large scale in control campaigns. Accordingly, the indirect diagnosis of disease based on serological tests is the method of choice in the eradication programs (Hussein and Awad, 2007).

The standard Rose Bengal (RBT) and Complement Fixation (CFT) tests are the main serological tests used to detect antibodies against *B. abortus* and *B. melitensis*. Both tests have been used for several decades, proving to be successful for eradicating bovine brucellosis in some countries. Nevertheless, there is evidence that both tests are significantly less effective for the diagnosis of brucellosis in sheep and goats than in cattle (Nielsen, 2002).

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Indirect Enzyme-Linked Immunosorbent Assays (iELISAs) have been developed using purified smooth Lipopolysaccharides (S-LPS) as the antigen and have been reported to be at least as sensitive and specific as the combination of both RB and CF tests for the diagnosis of brucellosis in ruminants (Kittelberger et al., 1998).

Classical serological techniques rely mainly on the detection of antibodies to lipopolysaccharide (LPS), giving rise to false-positive reactions due to cross-reactivity with LPS from other bacterial species. Other drawbacks of anti-LPS antibodies have generated an increasing interest in the detection of antibodies to alternative antigens, mainly outer membrane proteins (OMPs) and cytoplasmic proteins (Cloekaert et al., 2002). Also a periplasmic protein has previously been identified as an immunodominant antigen of the cytosoluble protein extract of *Brucella*, in infected cattle, sheep, goats and humans (Cloekaert et al., 1996; Gupta et al., 2010).

Nucleic acid-based detection methods, such as PCR, are very promising tools for diagnostics. PCR assays described for *Brucella* spp. used primers derived from the 43-kDa outer membrane protein gene of *B. abortus* (Fekete et al., 1992), the 16S rRNA gene (Romero et al., 1995), insertion sequence IS711 (Casañas et al., 2001) and the OMP31 gene (Gupta et al., 2006).

This study describes the evaluation of a single step PCR for the detection of the gene encoding a 31 kDa *B. abortus* antigen of *Brucella* in whole blood of naturally infected sheep and a comparison of its performance against other conventional and modified serological tests.

MATERIAL AND METHODS

Samples

A total of 300 ewe's blood samples were collected from areas where brucellosis is known to be endemic in Kafr El-Sheikh and Gharbia governorates of Egypt. Sera used for serological examinations were separated from whole blood samples (5 ml without anticoagulant) collected by jugular venipuncture. Another 5 ml sample whole blood sample from each animal was collected with EDTA as coagulate for the PCR analysis.

Serological tests

All antigens were kindly provided by Serum and Vaccine Institute, Abbasia, Egypt. RBT was performed using *Brucella abortus* S-99 antigen (Alton et al., 1988), while Serum Agglutination Test (SAT) was done using *Brucella abortus* S-99 white antigen that diluted in hypertonic phenol saline solution 5% NaCl as described by Alton et al. (1988). Indirect ELISA was performed using both *Brucella* whole

(W-ELISA) and periplasmic antigens (P-ELISA).

Extraction of periplasmic antigen

Periplasmic antigen of *B. abortus* S-99 was prepared with modified method from Yifan et al. (1993). The antigen was pelleted by centrifugation at 3000 x g/5 min. The pellet was resuspended to the original volume using sterile sodium chloride solution (0.15 M). Centrifuged at 12,000 xg / 30 min., then supernatant was collected, for each 10ml of solution, ammonium sulphate (3.13 gm) was added and dissolved. Centrifuged at 3000 x g/15 min., the pellet was resuspended in 1X phosphate buffer saline (PBS). Periplasmic antigen preparation was dialyzed at 4°C against 1X PBS to remove ammonium sulphate and stored at -20°C.

Whole killed *Brucella* antigen was prepared from *Brucella abortus* S-99 according to Berman et al. (1980).

Indirect ELISA

Checker board titration was done according to Narayanan et al. (1983) to optimize each of periplasmic and whole *Brucella* antigen concentration against positive serum dilution. Indirect ELISA was performed according to Bassiri et al. (1993). Results were recorded on an ELISA reader (verse max) at 492 nm wave length. Samples showing double the optical density (O.D.) of the mean control negative were considered positive.

Polymerase chain reaction (PCR)

Isolation of DNA from blood samples

DNA was isolated from blood samples (150 µl each) using Invisorb® Spin Blood Mini Kit (Invitex GmbH, Berlin) following the instruction of the manufacturer.

Synthetic oligonucleotide design

PCR assay for the detection of *Brucella* spp. was carried out as described by Baily et al. (1992) using primers B4 (5'-TGGCTCGGTTGCCAATATCAA-3') and B5 (3'-CGCGCTTGCCCTTTCAAGGTCTG-5) that were chosen from within the coding sequence of a gene encoding a 31 kDa *B. abortus* antigen (Figure 1).

DNA amplification by PCR

Single step PCR assay was carried out in 200 µl microtubes using 13 µl of Milli-Q water, 25 µl Taq PCR Master Mix (Cat.no. 201445, Qiagen Co.), 1 µl of each primer B4, B5, and 10 µl of extracted DNA.

Temperature cycling for the amplification was performed in a PTC-200 thermocycler (MJ Research, Watertown, MA, USA) as follows: initial denaturation at 94°C for 5 min; 39 cycles comprising denaturation at 94°C for 60 s, annealing at 60°C for 60 s, extension at 72°C for 60s, and a final extension at 72°C for 10 min (Baily et al., 1992). The size of the amplified DNA (223bp) was determined

Table 1. Evaluation of RBT, SAT, W-ELISA, P-ELISA and PCR for diagnosis of ovine Brucellosis.

PCR (%)	P-ELISA (%)	W-ELISA (%)	SAT (%)	RBT (%)	
117(39)	85 (28.3)	86 (28.7)	81(27)	88 (29.3)	Positive
183 (61)	215 (71.7)	214 (71.3)	219 (73)	212(70.7)	Negative

Table 2. Comparison of blood PCR with RBT, SAT, W-ELISA and P-ELISA.

Test	RBT		SAT		W-ELISA		P-ELISA		Total	
	(+)	(-)	(+)	(-)	(+)	(-)	(+)	(-)		
PCR	(+)	82	35	80	37	86	31	85	32	117
	(-)	6	177	1	182	0	183	0	183	183
	Total	88	212	81	219	86	214	85	215	300

by electrophoresis on 1.5% agarose gels.

RESULTS

Serum samples from 300 ewes at Kafr El-Sheikh and Gharbia governorates of Egypt were used. In this study both RBT and SAT detected 88 (29.3%) and 81 (27%) positive cases, respectively. While, ELISA using *Brucella abortus* periplasmic antigen detected 85 (28.3%) positive cases and failed to diagnose one ovine serum in comparison to ELISA using whole *Brucella* antigen (28.7%) as shown in Table 1.

PCR testing was able to detect higher percentage of positive samples (39%) than all examined serological assays (Table 1). Moreover, PCR was able to identify an additional number of positive samples which were serologically negative. On the contrary, although, PCR amplification was able to detect all serologically positive samples, it failed to identify six samples in the RBT and one sample in the SAT (Table 2).

DISCUSSION

Detection of ovine brucellosis can be proven problematic due to the sharp decline in serum titers of some infected animals after infection, or due to lack of reactivity in the commonly used serological tests (Morgan, 1984). Since diagnostic errors occur, these criteria can only be implemented if a gold standard test is used to detect the true disease status of the animals. Isolation of brucellae could be the gold standard as it unequivocally establishes the cause of infection. However, bacteriological isolation cannot always be relied on to prove the presence or

absence of the disease in individual animals especially in the blood of live animals (Corbel et al., 1980).

The presumptive diagnosis provided by the serological tests, is usually accepted as indication of brucellosis. Although it can only detect IgM and IgG2 and fails to detect IgG1 (Rice and Boyes, 1971), SAT assay is approved by the veterinary authority organization in Egypt. However, chronic carriers produce mainly IgG1 that block the agglutinating activities of IgG2 (Farina, 1985) which may result in lower detection rates. This may explain the lower number of positive samples detected by SAT (27%) in comparison to other serological tests used.

On the other hand, as RBT assay can detect antibodies of classes IgG1 and IgM against surface antigen lipopolysaccharides (LPS) of smooth *Brucella* (Davies, 1971), it was able to detect higher number of positive samples (29.3%). Indeed, this test is internationally acknowledged as the choice for the screening of brucellosis in small ruminants (Garin-Bastuji and Blasco, 2004). However, due to cross reactivity between these antigen with other bacterial species including *Yersinia enterocolitica* 0:9 and *E. coli* serotype O:57 (Chukwu, 1985; Kittelberger et al., 1995), RBT may suffer higher rates of false positive results than other serological tests. ELISA has been shown to be highly specific and of equal or greater sensitivity than CFT and RBT and suitable test for large scale screening for Bovine Brucellosis (Saravi et al., 1995; Hermoon et al., 2001). Besides latent infection could be detected earlier by ELISA than other serological tests as it detect all classes of antibodies (Reynolds et al., 1985).

This study aimed to compare indirect ELISA using periplasmic protein and whole killed *Brucella* antigens with other conventional serological tests (RBT and SAT).

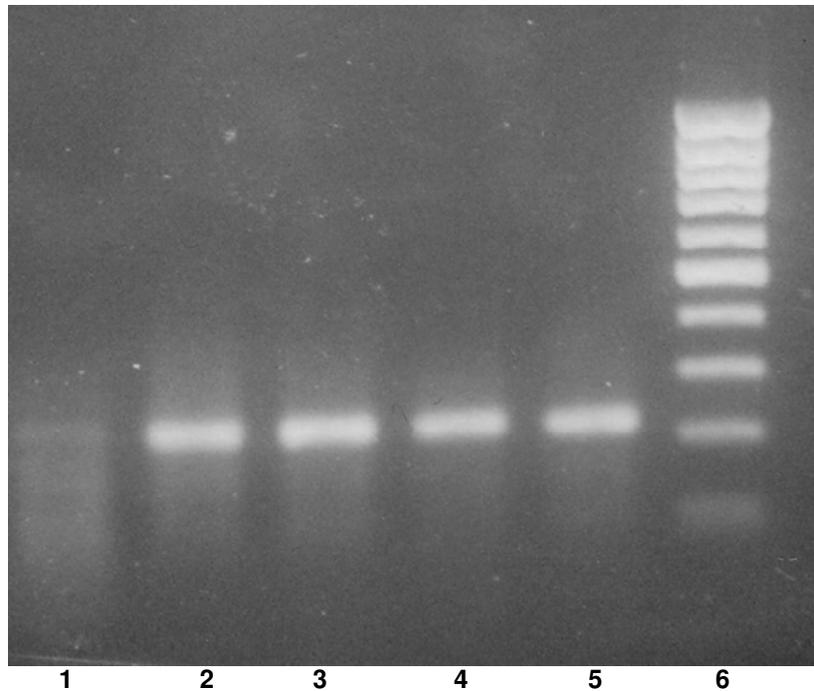


Figure 1. PCR assay for detection of *Brucella* Spp in ovine blood. Lane 6: 100bp DNA marker; lanes 1–4: positive blood sample DNA PCR; lane5: positive control; the 223-bp PCR product is indicated.

ELISA using periplasmic antigen detect 85 cases (28.3%) and failed to detect one case that was detected using whole *Brucella* antigen. The relatively higher results obtained from whole killed *Brucella* antigen (28.7%) may be due to the detection of external and internal antigens especially LPS that evoke antibody response of all classes of antibodies, than periplasmic protein antigen which is considered as an internal enzymatic protein as reported by Clockaert et al. (1991) and Diaz-Aparicio et al. (1994). This also was agreed by Shringi et al. (2002) who confirmed that the use of periplasmic antigen resulted in lower detection rates than sonicated and whole *Brucella* antigen preparations. This may indicate also that P-ELISA is relatively more specific than W-ELISA.

Amplification of microbial DNA from clinical samples offers the potential for rapid, sensitive and specific identification of pathogens, either directly from tissues or body fluids or after culture of such samples (Gupta et al., 2006). Indeed, from our results PCR amplification was able to identify several other positive samples in addition to all the samples detected by the serological assays. This may be due to that these animals were in an early

stage of infection (12-16 days after infection), while antibodies titers produced are still low to detect. On the other hand, PCR detects the causal agent itself found in the bloodstream as previously reported by Leal-Klevezas et al. (2000). These results indicate the higher sensitivity of PCR which could be especially useful during early infection.

The failure of PCR amplification to detect a few number of samples (6 samples in RBT and one sample in SAT) could be explained by the presence of PCR-inhibitory compounds, such as EDTA or heparin used as anticoagulants in blood samples or heme compounds from hemolyzed erythrocytes which often copurify with DNA in the extraction process as reported by Leal-Klevezas et al. (1995) and Morata et al. (1998). Another possible explanation is the presence of a problem in the specificity of these serological tests (false positive) such as cross reactivity with other Gram-negative bacteria, mainly, *Y. enterocolitica* O:9 as mentioned by Garin-Bastuji et al. (2006).

This agrees with the results of Gupta et al. (2006) in goats' blood and milk where PCRs showed higher sensitivity and specificity of 93 and 100% respectively

than that of both SAT and dot-ELISA (71 and 86% respectively).

In conclusion, it appears that PCR detection of the gene encoding a 31 kDa *B. abortus* antigen of *Brucella* in ovine blood provides a simple to perform and rapid diagnostic test which has a high sensitivity and specificity, versatility in sample handling and reduced risk for laboratory personnel compared with other serological tests. This makes it a valuable tool in the diagnosis of ovine brucellosis especially in the early stages of infection.

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