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Milk-enzyme linked immunosorbent assay for serological diagnosis of *Brucella melitensis* infection in goats

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ABSTRACT

The milk- ELISA (m-ELISA) was performed with milk samples (n, 980) taken from organized farms and field goats from northern India. Milk samples were obtained in parallel with serum samples. The assays used *B. melitensis* recombinant outer membrane protein 31 (OMP31) as antigen. The sensitivity of m-ELISA, serum-ELISA, SAT and RBPT was 95.45%, 90.1%, 54.54% and 68.18% respectively. The specificity of m-ELISA, serum-ELISA, SAT and RBPT was 95.5, 90.9, 77.28 and 81.81% respectively. However, the results obtained with the m-ELISA were in good agreement with those of the serum-ELISA, SAT and RBPT under field and farm conditions. The m-ELISA can be used as an alternative to the serum-ELISA for diagnosing *B. melitensis* infection in goats.

Key words: *Brucella melitensis*, Goat, milk-ELISA

Caprine brucellosis due to *Brucella melitensis* is also responsible for serious infections in humans. Owing to the zoonotic implications of *B. melitensis,* more emphasis should be given to improve diagnostic methods for brucellosis in sheep and goats with special reference to application and evaluation of enzyme immunoassay (Neilson *et al*. 1988). Classical serological techniques rely mainly on the detection of antibodies to lipopolysaccharide (LPS), giving rise to false-positive reactions. Because of drawbacks of anti-LPS antibodies, more attention was given to detection of antibodies to outer membrane proteins (OMPs) and cytoplasmic proteins. Major OMPs from *Brucella* are classified in groups 2 and 3 (Cloeckaert *et al*. 2002). Predicted amino acid sequence of Omp31 revealed a significant homology (34% identity) with *Brucella* Omp25 (Vizcaíno *et al*. 1996). Omp31 is expressed in all *Brucella* species, except *Brucella abortus* (Cherwonogrodzky *et al*. 1988).

Brucella abortus antigen is mostly used for brucellosis test reagents although there is serologic cross-reactivity between *B. abortus* and *B. melitensis*, and the validity of assays that use *B. abortus* test antigens in goats is unknown. Most ELISA studies for detection of antibodies against *B. melitensis* in sheep and goats were conducted on a limited number of animals (Marin *et al*. 1999, Biancifiori *et al*. 2000, Nielsen and Gall 2001, Nielsen *et al*. 2004, 2005). However, a large portion of testing cost consists of taking of blood sample which is mostly restricted to veterinarians. This raises the questions if milk can be used as alternative to blood (serum) (Bartels *et al.*2005, van Weering *et al.* 2007, Kramps *et al.* 2008) and if flock monitoring on the basis of testing bulk milk is feasible. The ELISA can be applied for detecting *Brucella* antibodies in milk of lactating goats (Biancifiori *et al*. 1996, Vanjini *et al*. 1998). Chand *et al*. (2004) reported that milk-ELISA is comparatively more sensitive and superior than milk ring test in determining *Brucella* antibodies in ewe's milk. In this study the possibility of using a recombinant omp31 based ELISA for the diagnosis of caprine brucellosis in milk (m-ELISA) was investigated.

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MATERIALS AND METHODS

Samples: Milk samples were collected from 980 goats of different breedes (field goats 670, organized farm goats 310) located in the western Uttar Pradesh (Table 1). These goats were having a history of abortions. Milk samples were obtained in parallel with serum samples. Milk and serum samples were collected in sterile vials and kept frozen till use.

Bacteria: The *Brucella melitensis* 16 M was isolated in our laboratory from stomach contents of the aborted fetus of goat (Rana *et al*. 2002). Classical biotyping of brucella was done by biochemical, growth characteristic, antigenic and phage typing as per Alton *et al.* (1988). For molecular typing of *Brucella* isolates PCR-RFLP was used, which utilizes DNA polymorphism. We used the omp2 gene of *Brucella*

sp. as a locus of 2 nearly homologous repeated copies that differ slightly among *Brucella* species and biotypes in presence or absence of the *Pst* I site to differentiate between them. The *omp 2* gene exists as a locus of 2 nearly homologous repeated copies (*omp2a* and *omp2b*) that differ slightly among *Brucella* sp.

Positive and negative reference sera: The negative reference sera used for determining the appropriate cut-off for the ELISA were collected from 22 goats, which were culture-negative for *Brucella*. To obtain a representative panel of positive reference sera, 22 naturally infected with *B. melitensis* and culture positive goats were randomly selected from different places. All serum samples were tested positive by m- ELISA, serum-ELISA RBPT and SAT.

Rose Bengal plate tests (RBPT) and standard tube agglutination terst (SAT): The antigens for RBPT and SAT were procured from the IVRI, Izatnagar, and the tests were performed according to Alton *et al*. (1975). Serum samples exhibiting any degree of clumping of coloured antigen in RBPT and 50% agglutination reaction at a dilution of 1: 40 or above in SAT were considered positive.

Recombinant Omp31 (rOmp31): A 720 bp *B. melitensis* DNA fragment encoding Omp31 was cloned in pTargeT mammalian expression system vector (Gupta *et al.* 2007). The resultant plasmid (pTargeT-Omp31) contained the Omp31 gene. Competent *Escherichia coli* JM109 was transformed with pTargeT-Omp31. Ampicillin-resistant colonies were grown in luria-bertani medium containing 100 µg/ml of ampicillin at 37°C with agitation (225 rpm). Protein expression was induced by adding 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and incubating transformed cells for 4 h. Bacteria were pelleted by centrifugation (15 000 × *g*, 20 min, 4°C) and frozen at –20°C. Bacterial cells were suspended in a solution consisting of 50 mM Tris, 5 mM EDTA, and 1% Triton X-100 (pH 8.0) (suspension solution) and sonicated for thrice, 1 min cycles at 4°C. Inclusion bodies were pelleted at 20 000 × *g* for 30 min at 4°C and washed twice with suspension solution without Triton X-100. Inclusion bodies were solubilized in a solution containing 50 mM Tris, 5 mM EDTA, and 8 M urea (pH 8.0) at room temperature overnight with agitation. After centrifugation (20 000 × g, 30 min, 4°C), soluble protein was purified by chromatography through Ni-agarose. The presence of rOmp31 in eluates was checked by western blotting with specific MAb A59/10F09/G010 (Cloeckaert *et al*. 1996). Purity was assessed by SDS-polyacrylamide gel electrophoresis and coomassie blue staining. rOmp31 was adsorbed with sepharose-polymyxin B to eliminate LPS contamination.

Enzyme-linked immunosorbent assay: Polystyrene plates (96 wells) were coated with rOmp31 (0.1ug/well) diluted in phosphate-buffered saline (PBS). Following one washing with phosphate buffer saline (PBS: pH 7.2; 0.01 M) containing 0.05% Tween-20 (PBS-T) and blocking for 1 h with 5% bovine serum albumin (dissolved in PBS-T), milk samples (diluted 1: 2) or serum (diluted 1: 50), diluted in PBS-T containing 1% BSA and dispensed to the wells. Specific antibodies were detected with rabbit antigoat HRPO IgG conjugate. The reaction was developed by adding *ortho*-phenylenediamine (2 µg/ul) in 0.1 M citrate-phosphate buffer containing 0.03% H_2O_2. To establish the cutoff values of the assays, serum samples from noninfected controls were tested under the same conditions. The cutoff value of each enzyme-linked immunosorbent assay (ELISA) system was calculated as the mean specific OD of control sera plus 2 standard deviations (SD). The samples were tested in duplicate and results were expressed as percentage positivity (%P) using the following equation:

$$\%P = (\text{mean } OD_{\text{sample}} \times 100)/(\text{mean } OD_{\text{positive control}})$$

Specificity and sensitivity of serological tests: The sensitivity and specificity of the tests were calculated with respect to the infected and brucella-free groups (Jones *et al*. 1980). Sensitivity of milk-ELISA, serum-ELISA, RBPT, SAT and PCR in milk/serum of 22 goats from a *Brucella* positive flock with culture positive history. Likewise, specificity of m-ELISA, serum-ELISA, RBPT, SAT and PCR was evaluated on the above mentioned 22 milk/serum samples from *Brucella*-free flocks (culture negative).

RESULTS AND DISCUSSION

The aim of this study was to investigate the diagnostic performance of the test under field and farm conditions by establishing whether the specificity and sensitivity of a milk-ELISA would be high enough to detect low levels of *Brucella* antibodies in milk from *Brucella* infected goats.

The cutoff of the milk ELISA was determined with sera from healthy goats (culture negative). The cutoff value of each enzyme-linked immunosorbent assay (ELISA) system was calculated as the mean specific OD of control sera plus 2 standard deviations (SD). Similarly, the cutoff of the serum-ELISA was determined with sera from healthy goats (culture negative).

The milk-ELISA was compared with the serum-ELISA, RBPT and SAT in terms of specificity and sensitivity. From the testing of 22 milk and serum samples from individual goats in *Brucella-free* flocks, the milk-ELISA demonstrated a 100% concordance with all the 3 tests. Relative specificities of milk-ELISA, ELISA, RBPT and SAT were 95.5, 90.9, 77.28 and 81.81 respectively (Table 2). The results of testing of 22 serum and milk samples from individual goats in *Brucella-infected* flocks are depicted in Table 2. Relative sensitivities of milk-ELISA, ELISA, RBPT and SAT were 95.45, 90.1, 54.54 and 68.18 respectively.

Out of 980 samples of serum and milk tested milk-ELISA, ELISA, RBPT and SAT detected 83, 78, 62 and 37 positive reactors. Relative to ELISA (with serum and milk both), SAT and RBPT demonstrated a lower sensitivity. The observed higher sensitivity of the ELISA may be accounted for by the

Table 1. Detection of *B. melitensis* antibodies by serology in goat samples

Location of goats	Number of goats tested	Number of goats positive in			
		m-ELISA	serum-ELISA	SAT	RBPT
Field	670	53	49	28	42
Organized farm	310	30	29	09	20
Total	980	83	78	37	62

physiological dominance of IgG_1 over IgA and IgM in milk as well as by the higher analytical sensitivity of the test (Caffin *et al.* 1983, 1988 and Nielsen *et al.*1996b).The results reported indicated that the m-ELISA used for testing milk samples was more sensitive than the other serological tests. With similar assay procedures Kerkhofs *et al.* (1990) and Nielsen *et al.* (1996a), Bercovich and Taaijke (1990) observed similar results.

In rural areas, humans often consume milk in the form of raw milk and cheese prepared under Indian conditions, therefore the pathogen could be easily acquired by consumers. Therefore, there is no doubt that control of this disease in goats will have an immediate effect on the incidence of this disease in humans. Although goats are crucial in the economy of developing countries and *B. melitensis* is a common cause of human infection, the brucellosis of goats has received comparatively little attention. An important problem affecting the sensitivity of the tests used earlier concerns the standardization of antigens. These conditions, which seem to be suitable for diagnosis of *B.abortus* infection in cattle, are not adequate for the diagnosis of *B.melitensis* infection in goats (Blasco *et al*. 1994a, 1994b). The low sensitivity of SAT and RBPT in culture proven cases is also well documented (Alton *et al.* 1975, Cargill *et al*. 1985). New tests, including the iELISA, cELISA and FPA were developed to facilitate diagnosis of *B. melitensis* infection in small ruminants. However, these studies were not performed with sera from goats shown to be actually infected or free of brucellosis, and therefore, the actual value of those tests is unknown.

The specificity, the m-ELISA was very satisfactory in that the cut-off values derived with reference standard sera were truly representative of the negative goats under study. The m-ELISA did not produce much false-positive results compared to other conventional screening tests used in the study. Relative specificities of milk-ELISA, ELISA, and SAT were 95.5, 90.9, 77.28 and 81.81% respectively (Table 2). Testing milk samples has some advantages over testing serum samples. Sampling is not invasive; therefore, the accidental transmission of diseases by needle and reduction of production due to stress can be reduced. Based on our results, where m-ELISA was applied to goat flocks with high disease

Table 2. Comparative result of serology in infected (culture positive) and non- infected (culture negative goats)

Goats	m-ELISA			Serum-ELISA			SAT			RBPT		
	No. of goats	Sensitivity %	Specificity %	No. of goats	Sensitivity %	Specificity %	No. of goats	Sensitivity %	Specificity %	No. of goats	Sensitivity %	Specificity %
Naturally infected goats *B.melitensis* (infected)	21/22	95.45	–	20/22	90.9	–	12/22	54.54	–	15/22	68.18	–
Brucella- free goats (non-infected)	1/22	–	95.5	2/22	–	90.9	5/22	–	77.28	4/22	–	81.82

prevalence, our test proved to be sensitive enough for the detection of infected goats by random testing of milk. An ELISA based on recombinant proteins would permit a better standardization of the assay compared to the more complex whole cell antigen preparations currently in use and hence to overcome the limitations associated with the use of LPS based antigens an antigen based on recombinant protein is used for the present work.

Although the performance of the m-ELISA in this study was not much superior to that of the serum ELISA or other described assays like FPA, but it was concluded that the m-ELISA can be used as an alternative to the serum-ELISA without reducing the diagnostic effectiveness.

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