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Multiple genus-specific markers in PCR assays improve the specificity and sensitivity of diagnosis of brucellosis in field animals

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Brucella-specific nucleotide sequences encoding the BCSP 31 kDa protein, Omp2 and the 16S rRNA were employed in three independent diagnostic PCR assays. Results of the three PCR assays on six reference strains of *Brucella* were in complete agreement. The results of PCR assays based on *bcsp* and *omp2* on 19 Indian field isolates (human, bovine and murine tissues) also agreed completely. However, when the 16S rRNA gene was employed as the diagnostic target in the PCR, only 14 out of these 19 isolates and 2 out of 7 bovine milk isolates were identified as the genus *Brucella*. The bovine blood samples were insensitive to 16S rRNA PCR. The antibody-detecting ELISA results of field samples (n=87) from a serologically positive herd in India were compared separately with *omp2* and *bcsp* PCRs of blood (n=62). While the *bcsp* PCR was the most sensitive, the degree of association of ELISA with *omp2* blood PCR (κ=0.37 at $P<0.05$) was similar to that with the *bcsp* blood PCR (κ =0.34 at $P<0.05$). An improvement in the correlation between ELISA and blood PCR was noticed (κ =0.5 at $P<0.05$) when a consensus result of *omp2* and *bcsp* blood PCR was considered for comparison with ELISA. The use of more than one marker-based PCR gave increased sensitivity and higher specificity and appears to be a more reliable molecular diagnostic approach for screening of field animals.

INTRODUCTION

Brucellosis causes infertility and abortion in bovines (Radostits *et al.*, 1994; Corbel, 1997) and undulant fever in humans (Corbel & Brinley-Morgan, 1984). Bovine brucellosis is usually caused by *Brucella abortus*, and less frequently by *Brucella melitensis* and *Brucella suis* (OIE, 1996). Among the six recognized species of *Brucella*, *B. abortus*, *B. melitensis*, *B. suis* and *Brucella canis* can potentially infect humans (Nicoletti, 1980) while *Brucella ovis* and *Brucella neotomae* have not been isolated from humans.

Accurate diagnosis of brucellosis requires bacteriological isolation and detection of the pathogen in the laboratory, which is impractical for regular screening of large populations (Alton *et al.*, 1988; Lulu *et al.*, 1988; Radostits *et al.*, 1994; Yagupsky, 1994). Serological tests can be nonspecific owing to cross-reaction or subsensitive or high immunity reactions, depending on subclinical or endemic prevalence of the disease (Ariza *et al.*, 1992; Weynants *et al.*, 1996; Godfroid *et al.*, 2002). Numerous PCR-based assays have been developed for the identification of the genus *Brucella* from cultures, animal/human tissues and animal products. These employ the gene encoding the 31 kDa *Brucella* cell surface salt extractable protein (BCSP), *omp2*, 16S rRNA, IS*711* and other gene targets (Baily *et al.*, 1992; Leal-Klevezas *et al.*, 1995; Da Costa *et al.*, 1996; Rijpens *et al.*, 1996; Bricker, 2002; Morata *et al.*, 2003; Bogdanovich *et al.*, 2004; Mukherjee *et al.*, 2005; O'Leary *et al.*, 2006). Real-time PCRs for high sensitivity detection (Redkar *et al.*, 2001; Probert *et al.*, 2004; Navarro *et al.*, 2006; Queipo-Ortuno *et al.*, 2005) and differential/multiplex PCRs for strain typing based on locus-specific variations (Ewalt & Bricker, 2000; Bardenstein *et al.*, 2002; Probert *et al.*, 2004; Mukherjee *et al.*, 2005; Ferrao-Beck *et al.*, 2006; Marianelli *et al.*, 2006) or variable tandem repeats (Bricker & Ewalt, 2006; Le Fleche *et al.*, 2006) of *Brucella* isolates have been reported.

For large-scale field screening, identification of *Brucella* by genus-specific PCR tends to be simple and adequate. The diagnostic PCRs so far employed in field animals for direct screening (Fekete *et al.*, 1992; Leal-Klevezas *et al.*, 1995; Amin *et al.*, 2001; Leyla *et al.*, 2003; O'Leary *et al.*, 2006) and comparative evaluation against serology (Romero *et al.*,

Abbreviation: OPA, overall proportion of agreement.

1995b; Sreevatsan *et al.*, 2000) or isolation have relied on single gene targets. The sensitivity and specificity of diagnostic assays can influence effective prevention and control of zoonoses as well as aid in selection of animals for breeding, etc. There are few comparative studies on the specificity of the different genus-specific PCRs and their correlation with serological diagnosis. This study analyses the sensitivity and specificity of the three established genus-specific PCRs of *bcsp*, *omp2* and 16S rRNA gene sequences, and further evaluates their comparative efficiencies for the simple detection of the genus *Brucella* directly from blood samples, in a large-scale screening of individual animals from serologically positive Indian field buffaloes/cattle herds. Further, a correlation between the diagnostic specificities of PCRs and an antibody-detecting blood ELISA is assessed employing kappa statistics.

METHODS

***Brucella* isolates and blood samples.** *Brucella* strains were isolated from experimentally infected murines (n=2), field isolates of human blood (n=4) and bovine milk (n=7) and uterine discharge (n=6). Bovine blood samples (n=87) used for serology and PCR studies were from two serologically positive herds of western India.

Source and maintenance of strains. The details of the origin of the standard strains are given in Table 1. *Brucella* reference and field strains were identified and maintained as per the standard protocols (Alton *et al.*, 1988). *Escherichia coli* ATCC 3616 was propagated on nutrient agar at 37 °C; *Yersinia enterocolitica* O:3 and O:9 were maintained on brain heart infusion agar (HI Media) at 28 °C; and *Vibrio cholerae* O:1 Inaba and Ogawa strains were grown on Terrestrial Yeast Extract medium at 25 °C (Baumann *et al.*, 1984). *Mycobacterium tuberculosis* H37Rv (ATCC 25618) was maintained on glycerol-supplemented Löwenstein–Jensen medium according to the ATCC Catalogue of Bacteria and Bacteriophages.

Table 1. Bacterial strains studied by three *Brucella*-specific PCRs

Bacterial strains studied	Source*	PCR results		
		BCSP 31 kDa	*omp2*	16S rRNA
Brucella abortus 544 (23448)	ATCC	+	+	+
Brucella melitensis 16M (23546)	ATCC	+	+	+
Brucella ovis 63/290	ATCC	+	+	+
Brucella suis 1330	IVRI	+	+	+
Brucella canis	IVRI	+	+	+
Brucella neotomae (2359)	ATCC	+	+	+
Yersinia enterocolitica O:3	HAU	−	−	−
Yersinia enterocolitica O:9	HAU	−	−	−
Vibrio cholerae Inaba	NICE	−	−	−
Vibrio cholerae Ogawa	NICE	−	−	−
Escherichia coli (3616)	ATCC	−	−	−
Mycobacterium tuberculosis H37Rv (25618)	ATCC	−	−	−
B. melitensis 3/97 cattle uterine discharge	IVRI	+	+	+
B. melitensis 5/97 cattle uterine discharge	IVRI	+	+	+
B. melitensis 7/97 cattle uterine discharge	IVRI	+	+	+
B. melitensis 20/97 human male blood	IVRI	+	+	+
B. melitensis 24/97 human female blood	IVRI	+	+	+
B. melitensis 25/97 human male blood	IVRI	+	+	+
B. melitensis 5/98 human female blood	IVRI	+	+	+
B. abortus (CO_2-dependent) 10/98 cattle vaginal swab	IVRI	+	+	+
B. abortus (CO_2-dependent) 18/98 cattle uterine discharge	IVRI	+	+	+
B. abortus 86/6 cattle uterine discharge	IVRI	+	+	+
B. abortus 544 (ATCC) infected murine genital tissue	NDDB	+	+	+
B. abortus S19 (IVRI) infected liver	NDDB	+	+	+
Brucella sp. MI 4 bovine milk isolate	NDDB	+	+	−
Brucella sp. MI 19 bovine milk isolate	NDDB	+	+	−
Brucella sp. MI 22 bovine milk isolate	NDDB	+	+	+
Brucella sp.MI 23 bovine milk isolate	NDDB	+	+	−
Brucella sp. MI 42 bovine milk isolate	NDDB	+	+	−
Brucella sp. MI 43 bovine milk isolate	NDDB	+	+	−
Brucella sp. MI 46 bovine milk isolate	NDDB	+	+	−

*ATCC, American type Culture Collection, USA; IVRI, Indian Veterinary Research Institute, Izatnagar, India; HAU, Haryana Agricultural University, Hisar, India; NICE, National Institute of Cholera and Enteric Diseases, Kolkata, India; NDDB, Research & Development, National Dairy Development Board, Anand, India.

ELISA. An indirect *Brucella* antibody detection avidin–biotin ELISA (Animal Disease Monitoring and Surveillance, Indian Council for Agricultural Research, Bangalore, India) was used to assess the serological status of the two herds.

Extraction of genomic DNA from bacteria. *Brucella* grown for 72 h was washed twice in PBS (pH 6.4), pelleted by centrifugation at 3000 ***g*** for 20 min and suspended in 500 µl Tris/EDTA (pH 8.0). For the other bacteria except *M. tuberculosis* H37Rv (ATCC 25618), 24–48 h cultures were washed in PBS. The suspension was subjected to three cycles of snap freezing at −196 °C in liquid nitrogen and boiling at 95 °C for 10 min to obtain crude cell lysates. The lysates were sequentially treated with lysozyme (1 mg ml^{-1}) at 37 °C for 1 h, proteinase K (1 mg ml^{-1}) and sodium dodecyl sulfate (1 %) at 50 °C. The lysates were subsequently extracted with standard phenol/chloroform, and the genomic DNA was precipitated, dried, suspended in 50 µl TE and stored at –20 °C.

DNA was extracted from 3–4-week-old cultures of *M. tuberculosis* H37Rv according to a previously described protocol (Cousins *et al.*, 1993).

Extraction of DNA from blood samples. DNA was extracted from bovine blood samples using a slight modification of a protocol published by Leal-Klevezas *et al.* (1995). Heparinized blood (500 µl) was centrifuged at 1500 ***g*** for 3 min. Cell pellets were suspended in erythrocyte lysis buffer (155 mM ammonium chloride, 10 mM sodium bicarbonate, 100 mM disodium EDTA, pH 7.4) and centrifuged at 1500 ***g*** for 3 min; the cycle was repeated two to three times until the red colour due to the erythrocytes was minimal. The pellet was then treated with leukocyte lysis buffer (2 % Triton X-100, 1 % sodium dodecyl sulfate, 100 mM NaCl, 10 mM Tris/HCl, pH 8.0), centrifuged at 3000 ***g*** for 5 min and the pellet was digested with 10 µl proteinase K (10 mg ml^{-1}) for 40 min at 50 °C. Following enzymic digestion, the samples were extracted with phenol/chloroform and processed as above

PCR assays. Three genus-specific PCR assays were performed for the identification of *Brucella*. (i) The PCR for the genus-specific *Brucella* cell surface salt extractable (BCSP) 31 kDa protein gene (Bricker *et al.*, 1988; Mayfield *et al.*, 1988) was performed on bacterial lysates and DNA extracts of isolates from experimentally infected mouse spleen, bovine blood and milk isolate samples, employing forward primer B4 (5′ TGG CTC GGT TGC CAA TAT CAA 3′) and reverse primer B5 (5′ CGC GCT TGC CTT TCA GGT CTG 3′) as described by Baily *et al.* (1992). (ii) The *omp2* gene (GenBank accession no. M26034) (Fitch *et al.*, 1989) was amplified from reference and field strains of *Brucella* in a 25 µl reaction mixture using primers JPF (5′ GCG CTC AGG CTG CCG ACG CAA 3′) and JPR (5′ ACC AGC CAT TGC GGT CGG TA 3′) as per Leal-Klevezas *et al.* (1995). However, for amplification of DNA from field blood samples, the above protocol was modified by using 100 pmol of each primer and 4 mM Mg^{2+} in the PCR. (iii) The 16S rRNA gene (EMBL accession no. X13695) (Dorsch *et al.*, 1989) was amplified from reference and field strains of *Brucella* by modifying the PCR protocol of Romero *et al.* (1995b). The PCR employed 0.5 µM each of a forward F4 (5′ TCG AGC GCC CGC AAG GG 3′) and a reverse R2 (5′ AAC CAT AGT GTC TCC ACT AA 3′) primer (Romero *et al.*, 1995a). The 25 µl reaction mixture consisted of IX PCR reaction buffer (Promega), 200 µM dNTPs and 0.5 units *Taq* polymerase. The PCR cycling parameters were: 30 cycles of denaturation at 95 °C for 30 s, annealing at 54 °C for 90 s and extension at 72 °C for 90 s, preceded by heating at 95 °C for 5 min and followed by a final extension at 72 °C for 6 min. For amplification of the 16S rRNA from blood samples from the field, elevated magnesium ion concentrations at an increment of 2.5, 3.0 and 3.5 mM and a primer concentration of 1.0 µM were used.

The PCR products of the *bcsp* and *omp2* gene targets were electrophoresed on 1.5 % agarose while the amplicons from the 16S rRNA gene targets were electrophoresed on a 2 % agarose gel, stained with ethidium bromide and visualized under UV light.

Comparison of results and statistical analysis. Comparisons were made within the results of: (1) the three different PCRs on reference and 19 field isolates of *Brucella*; (2) the blood/serum ELISA and the PCR based on the *bcsp*/*omp2* gene on blood from 87 field samples; (3) the consensus of blood PCR based on the *bcsp* and *omp2* gene with the antibody detection ELISA on 62 blood samples from field. The overall proportion of agreement (OPA) and the proportion of agreement beyond chance (κ value) between different blood PCR protocols and antibody detection ELISA were analysed employing the software WinEpiscope 1.0 (EPIDECON) at 95 % confidence intervals.

RESULTS AND DISCUSSION

Although serological tests are the major diagnostic tools for screening of animal brucellosis in the field, they are neither fully sensitive nor specific due to insufficient immunity or serological cross-reactivity. Bacteriological isolation of *Brucella* on the other hand is regarded, because of its specificity, as the gold standard for diagnosis. Since this procedure is laborious for large-scale diagnosis and since detection of specific DNA is a true indication of the presence of a pathogen, we wanted to compare the applicability of different established PCRs against serology for rapid, sensitive and specific detection of *Brucella* in animals from a large population under endemic situations. Few reports have described the application of diagnostic PCR on field samples (Amin *et al.*, 2001; Fekete *et al.*, 1992; Romero *et al.*, 1995b; Sreevatsan *et al.*, 2000) and there are no studies correlating results of PCR from multiple gene targets with those of serological diagnosis.

The study was designed in two components: (1) evaluation of the specificity of the genus-specific *bcsp* PCR and its comparison with other established diagnostic PCRs on the *omp2* (Leal-Klevezas *et al.*, 1995) and 16S rRNA (Romero *et al.*, 1995b) genes using standard *Brucella* strains, serologically related non-*Brucella* organisms as well as *Brucella* isolates from human and bovine infections; (2) PCR with confirmed specificity was then used as a positive indicator of infection to screen bovine blood samples from sero-positive herds and compare with ELISA.

Amplification of *bcsp*, *omp2* and the 16S rRNA from reference strains and Indian field strains

The three independent PCR assays resulted in the amplification of 223, 193 and 905 bp amplicons, respectively, from the *bcsp*, o*mp2* and 16S rRNA gene PCRs from all six *Brucella* reference strains but not from other serologically related strains (Table 1). All PCRs were repeated twice. All the three categories of PCR products from the field isolates were confirmed for specificity by Southern hybridizations using non-radioactive probes prepared by labelling of corresponding amplicons from

standard strains (data not shown). The 905 bp amplicon from the 16S rRNA gene in the *Brucella* reference strain was obtained after modification of the protocol of Romero *et al.* (1995b) by changing the Mg^{2+} concentration from the recommended 1.0 mM to 1.5 mM and the annealing temperature from 54 to 50 °C.

All 19 Indian field strains were identified as belonging to the genus *Brucella* by the PCR assays based on both *bcsp* and the *omp2* gene (Fig. 1). These were therefore concluded as specific for *Brucella* and taken further for analysis of blood samples. However, 16S rRNA gene specific amplification was obtained only in 14 of the 19 isolates. Of the 19, 7 were bovine milk isolates of which only 2 were identified as *Brucella* by the 16S rRNA PCR (Fig. 2).

Isolation of *B. abortus* biovars 1, 3, 4, 6 and 9, *B. melitensis* biovars 1, 2 and 3, *B. suis* biovar 1 and *B. canis* has been reported from India (Sen & Sharma, 1975) and there is enough evidence from previous work by others (Leal-Klevezas *et al.*, 1995; Romero *et al.*, 1995b; Da Costa *et al.*, 1996; Sifuentes-Rincon *et al.*, 1997; Lopez-Goni, 2001; Bricker, 2002; Probert *et al.*, 2004) that PCR assays based on *bcsp*, *omp2* and the 16S rRNA are able to detect all species and biovars of the genus *Brucella*. However, even with modifications only two out of seven bovine milk isolates yielded 16S rRNA PCR products. All *Brucella* 16S rRNA gene sequences have been reported to be identical (Gee *et al.*, 2004). However, our subsequent multiple alignment analysis of 16S rRNA sequences of various *Brucella* spp. to the primers used (Romero *et al.*, 1995a) showed alignment of the primer F4 with 100 % identity to the sequence of a single *B. abortus* (Dorsch *et al.*, 1989; accession no. X13695), while with other species and other *B. abortus*-like sequence (Gee *et al.*, 2004), alignment was seen only for the first 10 of the 17 nucleotides in the primer owing to the presence of an additional base 'C' in these strains that was absent in the said *B. abortus* (Fig. 3). This lack of alignment beyond 10 bp would have resulted in defective annealing at the temperature used yielding no PCR products. The reverse primer too showed an identity of 18/20 for strains other than *B. abortus*. These do not, however, explain why the standard strains yielded expected PCR products albeit at a lower annealing temperature than

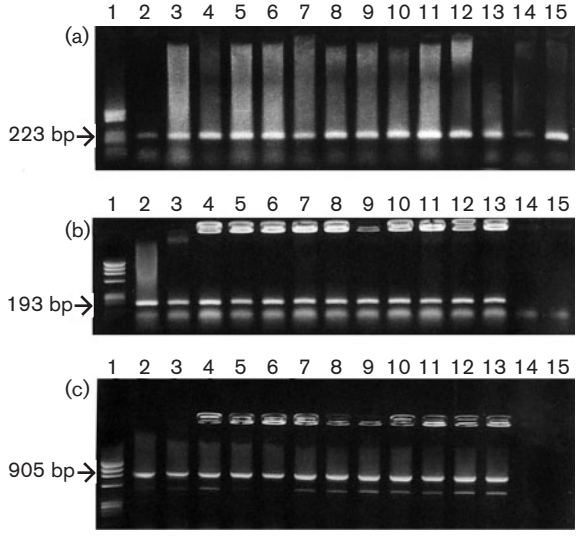

Fig. 1. *Brucella*-specific BCSP 31 kDa gene (Baily *et al.*, 1992) (a), *omp2* (Leal-Klevezas *et al.*, 1995) (b) and 16S rRNA gene (Romero *et al.*, 1995b) (c) PCRs on isolates from field cattle from the Indian Veterinary Research Institute (IVRI), Izzatnagar. Lanes: 1, DNA molecular size markers [marker V (Boehringer Mannheim) in (a); phiX174 *Hae*III digest (Promega) in (b) and (c)]; 2 and 3, *Brucella abortus* 544 (ATCC) and *Brucella melitensis* (ATCC), respectively; 4–13, human and bovine isolates from IVRI (10/98, 24/97, 3/97, 5/97, 20/97,6/86, 5/98, 25/97 and 7/97, respectively); 14 and 15, *B. abortus* isolates from mice liver and genital tissue (a) and *M. tuberculosis* H37Rv and water (b, c). Ten microlitres of amplicon was separated by electrophoresis, treated with ethidium bromide, and visualized under UV light.

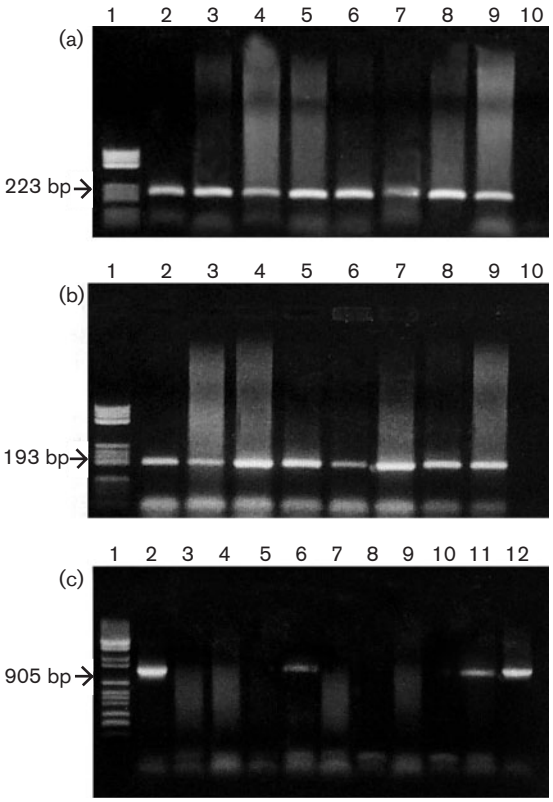

Fig. 2. *Brucella*-specific PCR on isolates from bovine milk. (a) BCSP 31 kDa PCR; (b) *omp2* PCR; (c) 16S rRNA PCR. Lanes: 1, DNA molecular size markers [marker V (Boehringer Mannheim) in (a); phiX174 *Hae*III digest (Promega) in (b) and (c)]; 2, *Brucella abortus* 544 (ATCC); 3–9, isolates from bovine milk; 10, water control; 11 and 12 in (c), *B. abortus* isolates from mice liver and genital tissue. Ten microlitres of amplicon was separated by electrophoresis, treated with ethidium bromide, and visualized under UV light.

Fig. 3. Multiple alignment of 16S rRNA sequences of various *Brucella* spp. indicating differences between *B. abortus* (X13695) and other *Brucella* spp. in sequence conservation corresponding to the forward primer F4 (a) and reverse primer R2 (b) used for PCR as per Romero *et al.* (1995a). The source, accession number and strain name for each sequence are indicated on the left.

recommended. A new primer that is fully and specifically conserved in all *Brucella* species is required for genus-specific 16S rRNA PCR. In a study using 16S rRNA target (Lopez-Goni, 2001), milk culture and PCR results have been negatively correlated with each other where 26 out of 31 *B. melitensis* biovar 3 isolates (from bovine milk) could be identified as *Brucella*.

Results of 16S rRNA PCR on blood samples

Although Mg^{2+} was elevated and the primer concentration increased to 1 μM, the 905 bp specific amplicon could not be detected directly from any of the blood samples tested on repeated attempts under various PCR conditions, probably owing to the reasons described above. Hence the 16S rRNA was not used for comparison with *bcsp* and *omp2* PCRs. The sensitivity of 16S rRNA primers has been previously reported to be affected by the presence of human DNA (Navarro *et al.*, 2002).

Correlation studies between *bcsp* and *omp2* gene based blood PCR on field samples

The observed proportion of agreement (OPA=0.71 at $P<0.05$) was good and the degree of association ($\kappa=0.45$ at $P<0.05$) was moderate between the two diagnostic blood PCR assays. The two PCR assays disagreed in 28.7 % of cases (Table 2). The *bcsp* was more sensitive as it could detect 24 % more samples (21/87) as positive than *omp2*. Such a difference was not seen in PCR carried out on bacterial isolates. The present studies seemed to indicate that the presence of host DNA could affect the sensitivity of primers for the detection of *Brucella* in bovine blood as observed previously (Navarro *et al.*, 2002). The sensitivity of the detection system could also be affected by modification of the original blood PCR protocol (Romero *et al.*, 1995a, b; Leal-Klevezas *et al.*, 1995) in our hands. Finally it cannot be ruled out that the *bcsp* gene sequence is better conserved than the *omp2* sequence in the genus *Brucella*. Variation in the *omp2* sequence has been used as a basis for typing strains (Bardenstein *et al.*, 2002; Ferrao-Beck *et al.*, 2006).

Correlation studies between antibody-detecting ELISA and blood PCRs

ELISA versus *bcsp* PCR. Correlation studies on 87 bovine/buffalo blood samples from the field showed that although the overall proportion of agreement between the two tests (0.66 at $P<0.05$) was strong, the degree of association was weak ($\kappa=0.34$ at $P<0.05$) (Table 2). The PCR could detect 20.6 % more blood samples as positive compared to ELISA. *Brucella* genus specific sequence signatures were detected from 47 % (24/51) of serologically negative animals. Also this PCR was unable to detect *Brucella* in 6.8 % (6/87) of samples that were ELISA-positive.

Increased sensitivity of BCSP PCR over serology has been observed by Queipo-Ortuno *et al.* (2006), where seroagglutination was inconclusive in 30 % of cases whereas real-time PCR assay was positive in 90 % of samples of human brucellosis.

ELISA versus *omp2*-based PCR. The *omp2* gene could be amplified from blood samples by a modified PCR protocol. The 193 bp amplicon was obtained only after increasing the concentration of Mg^{2+} from 3.0 to 4.0 mM and that of the primers from 50 to 100 pmol in the reaction. The overall proportion of agreement (OPA=0.70 at $P<0.05$) and the degree of association ($\kappa=0.38$ at $P<0.05$) between

Table 2. Correlation of blood PCRs and their comparison with antibody-detecting ELISA

Blood PCRs	BCSP-positive	BCSP-negative	Total	OPA and proportion of agreement beyond chance (κ value)*
Omp2+	30	4	34	OPA=0.71, κ=0.45
Omp2−	21	32	53	
Total	51	36	87	
ELISA versus BCSP/Omp2 PCRs	**ELISA-positive**	**ELISA-negative**		
BCSP+	30	24	54	
BCSP−	6	27	33	OPA=0.66, κ=0.34
Total	36	51	87	
Omp2+	21	11	32	
Omp2−	15	40	55	OPA=0.70, κ=0.38
Total	36	51	87	
BCSP+ Omp2+	20	10	30	
BCSP− Omp2−	6	26	32	OPA=0.74, κ=0.5
Total	26	36	62	

*Statistical features were calculated from category-wise comparison of test results employing WinEpiscope 1 software (EPIDECON) with a 95 % confidence level.

the two tests were similar to those in ELISA versus *bcsp* PCR (Table 2). The ELISA identified 41.4 % (36/87) of samples as positive for brucellosis; in comparison, the *omp2* blood PCR detected *Brucella* in 38 % (32/87) of samples. From serologically negative samples 21.6 % (11/51) were PCR-positive while 17.2 % (15/87) of ELISA-positive samples yielded negative results for *omp2* blood PCR.

omp2-based PCR has been used previously (Leal-Klevezas *et al.*, 1995) for identification of *Brucella* from blood of naturally infected caprines where 86.3 % (19/22) were identified as positive by PCR in comparison to 63.6 % (14/22) by serology. The reduced sensitivity of the *omp2* PCR against ELISA on blood samples might be a mere reflection of the high immune response in the bovine system as compared to the caprine system.

ELISA versus consensus of PCR based on *bcsp* and *omp2*. Only 62 out of 87 samples were screened by ELISA and *bcsp* and *omp2* PCR. The consensus data of the *bcsp* and *omp2* blood PCR showed the best overall proportion of agreement (OPA=0.74 at P <0.05) and a fair improvement in the degree of association (κ=0.5 at P <0.05) with ELISA as compared to ELISA versus *bcsp* or ELISA versus *omp2*-based PCR (Table 2). The consensus of two PCRs identified 6 % more samples (30/62) than ELISA (26/62) as positive and detected *Brucella* infection in 27.7 % (10/36) of animals that were serologically negative, but eliminated 9.7 % (6/62) animals that were ELISA-positive.

In the absence of the availability of an antigen-detecting ELISA we had used the antibody detection ELISA, widely used for serological monitoring programmes in India, despite the fact that antibody status did not always indicate disease under the chronic endemic situation that exists in the country. Evidence from human brucellosis indicates that the expression of anti-brucella antibodies does not correlate with the status of the disease (Elfaki *et al.*, 2005). Therefore, we wanted to study how the degree of association (κ value) between two tests systems was affected when the diagnostic gene targets in the PCR assays were altered. As observed in this study, the change in the gene target did not affect the nature or degree of association between ELISA and blood PCR. However, the closest and the best degree of statistical association (κ=0.5 at P <0.05) was achieved when consensus results of *bcsp* and *omp2* PCR were compared with those from ELISA. Therefore, we believe that consensus PCR is a more reliable diagnostic approach.

In an overall analysis of differential detection rates by ELISA and the two blood PCRs, the *bcsp* PCR was the most sensitive (92.72 %) followed by o*mp2* PCR (61.81 %) and ELISA (55.55 %). These values were calculated taking into consideration that, in the absence of pathogen isolation, a positive PCR for *bcsp* or *omp2* from the blood samples was regarded as a true indication of infection, as their specificities were confirmed by the PCRs on field isolates. Thus *bcsp* and *omp2* PCRs together indicated 55 true infections (Table 2), wherein *bcsp* showed maximum

sensitivity (51/55). This assumption did not permit any consideration of false positive by PCR. Moreover, neither PCR gave false positives with non-brucella cultures. Thus both PCRs exhibited a specificity and positive predictive value of 100 % while ELISA showed 81.8 % specificity and 83.3 % positive predictive value. *bcsp* PCR also gave a higher negative predictive value (88.88 %) than the *omp2* PCR (61.81 %) and ELISA (55.55 %). Since anti-brucella antibodies do not always indicate disease, evaluation of ELISA was done using *bcsp* PCR results as a reference (Table 2). ELISA was positive for 30 out of 54 *bcsp*-positive samples, making it the least sensitive test. The consensus of PCRs detected 6 % more samples as positive than ELISA and perhaps it should be considered most specific, as it seemed to be neither under- nor over-detecting *Brucella* infection. The consensus of PCRs also seemed to show a low degree of failure since it was unable to detect infection on 9.7 % of occasions from ELISA-positive animals. Compared to the PCR consensus the *omp2* system failed on 17.2 % occasions. The most favourable factor regarding the *omp2* PCR was that it had the best agreement of 78 % (40/51) with the ELISA-negative samples in comparison to other PCR systems. We finally conclude that the use of more than one marker-based PCR gave increased sensitivity and higher specificity and appears to be a more reliable molecular diagnostic approach for screening of field animals.

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