Indian Journal of Animal Sciences **84** (3): 251–253, March 2014/Article

Omp31 gene based molecular detection of *Brucella melitensis* from serum samples of goats

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Received: 27 September 2013; Accepted: 30 November 2013

ABSTRACT

The present study analyses the sensitivity and specificity of OMP31 gene based amplification directly from sera samples. Sero-positive animals (73) in rOmp31 i-ELISA were subjected to Omp31 gene amplification from sera samples and only 40 samples amplified 723kb product suggesting these as true positive animals. When similar test was applied with the sera samples of 16 infected (culture positive) and 20 non-infected (culture negative) goats, all the culture positive animals revealed 723 kb product while none of the culture negative goats revealed amplification. Hence serum based PCR appeared to be 100% sensitive and specific in truly infected animals. Thus serum is the optimal specimen for the diagnosis of brucellosis and leads to simplification of assay and shortens turnaround time.

Key words: Brucellosis, Goat, Omp31gene, Serum PCR

The diagnosis of brucellosis is usually based on serological tests being rapid, sensitive and easy to perform, but these lack specificity due to cross-reactions with other bacteria, particularly *Yersinia enterocolitica* 0: 9, that result from O-chains antigenic similarity (Nielsen *et al*. 2004). As the *Brucella* species is of zoonotic nature, it is a potential hazard for laboratory personnel and cultures must be performed in well-equipped laboratories with highly skilled personnel. The process from clinical samples to final diagnosis requires a minimum amount of viable *Brucella* in the specimen (Manish *et al*. 2013). PCR is a promising alternative for the problematic culturing and identification of *Brucella* spp. by conventional techniques for *Brucella* detection (Bricker 2002). PCR-based assays were developed for the identification of *Brucella* (Vizcaino *et al*. 1996, Mukherjee *et al*. 2007). In spite of diagnostic tests of brucellosis it is frequently difficult to establish its presence in goats (Gupta *et al*. 2006 a, b). 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. Hence the present study analyses sensitivity and specificity of OMP31 gene based amplification directly from sera samples, in a large-scale screening of individual animals from serologically positive Indian field goat flocks.

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MATERIALS AND METHODS

Sample collection: Out of 300 sera samples of goats with the history of abortions from the different parts of the country, 73 positive samples at rOmp 31 based i-ELISA test (Gupta *et al*. 2002) were used for Omp 31 gene amplification (Vizcaino *et al*. 1996).

Positive and negative samples: Recently aborted 16 goats from the Barbari unit, CIRG, Makhdoom, Mathura and found positive by serology, culture and 16S rRNA PCR were taken as positive control for study. Non infected goats (20), confirmed negative for brucellosis by serology, culture and 16S rRNA PCR, were taken as negative control.

Extraction of DNA from serum samples: Isolation of DNA from serum samples was performed according to Gupta *et al.* (2006 a). In the first step, 10 ml of the serum sample was combined with an equal volume of the lysis buffer (containing 100 mM KCl, 20 mM Tris-HCl (pH 8.3), 5 mM $MgCl_2$, 0.2 mg of gelatin/ml, and 0.9% polysorbate 20 solution). Then proteinase K was added to a final concentration of 20 mg/ml. The mixture was incubated for 60 min at 55°C. Proteinase K was then inactivated by heating the mixture to 95°C for 10 min. The supernatant was collected for PCR amplification following centrifugation at 12,000 × g for 10 min at 4°C.

Polymerase chain reaction: In the present study DNA isolated from sera samples were used for polymerase chain reaction for the amplification of Omp 31 genes for the

confirmatory identification of *Brucella melitensis* by using Taq PCR mater mix kit applying a set of forward (5'-TGACAGACTTTTTCGCCGAA -3') and backward (5'-TATGGATTGCAGCACCG-3') primers (Vizcaino *et al*.1996). The 25 µl of PCR reaction was prepared with 12.5 µl Taq PCR master mix (2×); 1µl forward primer (10 pmol/µl); 1µl reverse primer (10 pmol/µl); 2µl template DNA and 8.5µl nuclease free water. The final reaction volume of 25 µl for each sample was used in thermal cycler. The amplification of 16SrRNA gene was conducted with initial denaturation at 95°C for 5min, denaturation at 95°C for 30 sec, annealing at 54°C for 1.5 min, extension at 72°C, 1.5 min and finally the final extension at 72°C for 10 min. The Omp 31 gene amplification was performed with initial denaturation at 95°C for 5 min, denaturation at 95°C for 1min, annealing at 58°C for 1min, extension at 72°C, 1min and finally the final extension at 72°C for 10 min.

Quantitation and quality assessment of DNA of PCR products by agarose gel electrophoresis: For the electrophoresis of PCR products 1% agarose gel was prepared in TAE buffer as per Gupta *et al.* (2006 b). The gel was photographed under the UV illuminator. The size of the amplicon was assessed on the basis of co-migration of standard DNA ladder of molecular weight in the range of 100–1,000Kb.

RESULTS AND DISCUSSION

Sero-surveillance with rOmp31ELISA: Out of 300 sera samples of goats only 73 samples were found positive in recombinant Omp31 based-enzyme linked immunosorbent assay (ELISA) with the sero-positivity of 24.33%. These 73 sera samples were further subjected for the omp31 gene based PCR amplification.

PCR amplification of omp31 gene: PCR amplification was carried out using the omp31 gene specific primers and genomic DNA of *B. melitensis* act as template for omp31 primers (Vizcaino *et al*.1996). On PCR amplification, an amplified product of about 723 bp size was found in *B. melitensis* which was analyzed by agarose gel electrophoresis (Fig. 1). However, PCR amplification from 73 ELISA positive sera samples genomic DNA revealed amplified product of about 723 bp size in 40 samples only.

Assessment of sensitivity and specificity of omp31 gene based serum PCR: Omp31 gene based serum PCR amplified a product of 723 bp size in all the 16 infected (culture positive) goats whereas none of the 20 non-infected (culture negative) goats revealed amplification. Hence serum based PCR appeared to be 100% sensitive and specific. Still the diagnosis of goat brucellosis depends primarily on isolation of *Brucella* spp. (Manish *et al*. 2013), followed by performing a set of bacteriological tests that allows for reliable identification to the subspecies (biotype) level but that requires up to 7 days for completion. Another diagnostic option is the use of serologic assays; however, the specificity of these tests is inconsistent because of cross-reactivity with bacteria closely related to the genus *Brucella* (Nielsen *et al*. 2004). Hence, a PCR based assay for the rapid and specific laboratory diagnosis of brucellosis directly from tissue and milk using specific primers (Vizcaino *et al.* 1996) for the PCR amplification of a 723 bp region on the sequence encoding the 31kDa immunogenic *B. melitensis* protein (omp31) developed by Gupta *et al.* (2006 b) was applied for the confirmation of *Brucella melitensis* from sera samples of suspected animals.

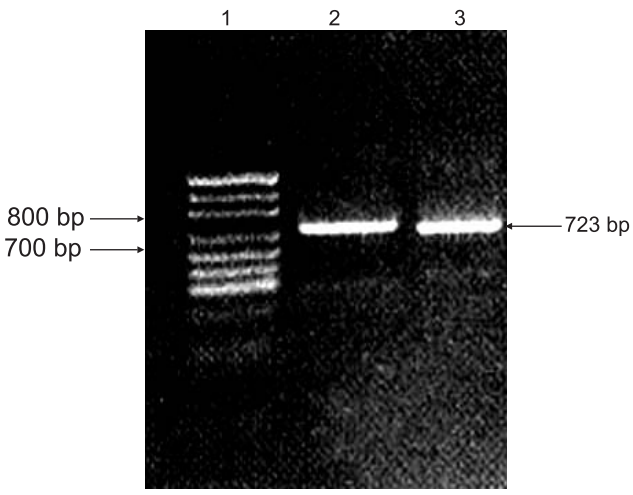

Fig. 1. Agarose gel electrophoresis PCR-amplified omp31 gene of *Brucella melitensis.* Lane 1: standard DNA marker; lane 2: positive control; lane 3: Omp 31 amplified product of 723bp from serum sample.

PCR amplification of omp31 gene (723 bp) from previously extracted genomic DNA using specific oligonucleotide primers confirmed the presence of this gene in *B. melitensis* and its absence in *B. abortus* as reported previously (Ashrafzadeh *et al*. 2009, Whatmore 2009). Consequently, the amplified product obtained as single band of 723 bp in *B. melitensis*, was used for the downstream characterization of the gene. None of these previous studies examined the possibility of amplifying *Brucella* DNA in serum samples of suspected goats.

Al-Garadia *et al.* (2011) used serum to detect IS711 region of *B. melitensis* in suspected goats and only 29.5% samples were found positive. However, the use of serum instead of whole-blood samples offers several advantages for nucleic acid amplification methods including easier extraction in comparison to blood and tissues (Zerva *et al.* 2001, Al-Garadia *et al.* 2011). Inhibition by anticoagulants, hemoglobin, human DNA, or any other substance present in whole blood but not in serum is circumvented. Red blood cell lysis, washings by centrifugation, and measurement and adjustment of isolated DNA concentrations are not required. Overall, the procedure is simplified and turnaround time is shorter, while sensitivity is higher (Zerva *et al.* 2001, Bricker 2002). Regarding the origin of pathogen nucleic acids in serum samples, most probably they are released into the circulation as breakdown products during bacterium. Several studies documented presence of circulating pathogen DNA in serum samples (Kawamura *et al.* 1999). Thus similar to PCR from genomic DNA from bacterial colonies, serum PCR was also applied from the sera samples

of ELISA positive sera samples with genus specific (Weisburg *et al.* 1991) and *B. melitensis* species specific PCR (Vizcaino *et al.* 2000). In the present study, out of 73 sera samples positive in ELISA only 40 revealed positive samples (54.8%) by serum PCR. Moreover, the serum PCR described in the present study appeared to be highly specific (100% specificity) as none of the 20 culture negative animals in a separate controlled experiment was positive. In another study the sensitivity of serum PCR was also higher (90%) in comparison to blood PCR (80%). These findings revealed better amplification in suspected sera samples in comparison to earlier findings of Al-Garadia *et al.* (2011) and that might be due to amplification of different gene loci. Thus, on the basis of previous studies and the findings of the present study, it is inferred that the optimal clinical specimen for this test is not whole blood but serum, which leads to assay simplification and also indicates that goat brucellosis is characterized by some degree of bacterial DNAemia.

Hence, on the basis of findings of the present study it can be concluded that serum is the optimal specimen for the diagnosis of brucellosis in goats by PCR, a choice that leads to assay simplification and shortens turn around time. Moreover, the hazard in isolation can be avoided.

ACKNOWLEDGEMENTS

The authors are thankful to Director, Central Institute for Research on Goats (CIRG), Makhdoom, Mathura, India. Dean, College of Biotechnology, Vice Chancellor of UP Pt. Deen Dayal Uphadhaya Veterinary University and Gau Anusandhan Sansthan (DUVASU), Mathura; for providing the requisite facilities and support to carry out this investigation.

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