Investigation of brucellosis in cattle and buffaloes by conventional and molecular assays

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ABSTRACT

The present study was carried out to evaluate the conventional and molecular techniques for diagnosis of bovine brucellosis. A total of four isolates of *Brucella abortus* obtained from 100 clinical samples of foetal stomach contents, vaginal mucus and uterine discharges were characterized biochemically. The isolates were confirmed as *Brucella* spp. by PCR using B4/B5 primer pair and as *B. abortus* by Bruce Ladder multiplex PCR. By Hinic Real-time PCR, all the four isolates were confirmed as *Brucella* spp. with C values between 14-16. With DNA extracted from 40 clinical samples of foetal stomach contents, vaginal mucus and uterine discharges, Real time PCR appeared most sensitive of the three molecular and conventional techniques as it detected maximum number of positive samples.

Key words: Buffaloes, *Brucella*, Cattle, Foetal stomach contents, PCR, Uterine discharges, Vaginal mucus.

INTRODUCTION

Bovine brucellosis is an economically important emerging and reemerging disease with public health significance characterized by abortion and reduced fertility in bovines. The disease is usually caused by *Brucella abortus*, less frequently by *B. melitensis* and rarely by *B. suis* (Corbel and Brinley, 1984). *Brucella* spp. are small, Gram negative, non motile, non spore forming coccobacilli arranged singly or in pairs or short chains. Based upon the affinity for their specific natural hosts, the genus *Brucella* has been divided into six classical *Brucella* spp., namely *B. abortus* (cattle and buffaloes), *B. melitensis* (goats), *B. suis* (pigs), *B. ovis* (sheep), *B. neotomae* (desert wood rats) and *B. canis* (dogs) (Osterman and Moriyon, 2006). Though isolation of the incriminating agent remains the “gold standard” in diagnosis of brucellosis and is the confirmative test (Godfroid et al., 2010) but it requires level 3 bio-containment facilities and highly skilled technicians for handling the samples (Hinic et al., 2008), has reduced sensitivity in chronic infections and is unsuitable for use in large animal populations. Moreover, isolation and biochemical identification is not able to differentiate the various *Brucella* spp. Molecular diagnosis based on a number of genus- or species-specific conventional PCR assays using primers derived from different gene sequences from the *Brucella* genome, such as 16S rRNA, the 16S-23S intergenic spacer region, omp2 and bcspl31 have been established. The introduction of Real-time PCR offers improved sensitivity, specificity and speed of performance as compared to conventional PCR. Real-time PCR assay based on *Brucella* spp. specific multiple insertion sequence IS711 has been described for detection of *Brucella* spp. (Hinic et al., 2009). Multiplex PCR assay is a kind of PCR technique where multiple target DNA sequences can be detected in a single reaction (Richtzenhain et al. 2002). Therefore, by application of this assay we can easily do molecular typing of all *Brucella* spp. including the vaccine strains (Goni et al. 2008). For the successful eradication and control of brucellosis, identification of different species of *Brucella* circulating in the region is of great importance.

Keeping in view all the above, the present study was conducted for investigation of bovine brucellosis by conventional and molecular based assays alongwith an aim to identify whether the organisms were isolated from their natural host or not so as to investigate the prevalence patterns of various *Brucella* spp. circulating in Ludhiana distt. of Punjab.

MATERIALS AND METHODS

Collection and processing of samples for isolation of *Brucella abortus*: A total of 100 clinical samples consisting of vaginal mucus (44), uterine discharges (38), placental and foetal cotyledons (09) and foetal stomach contents (09) were collected from cattle (68) and buffaloes (32) suffering from abortions and reproductive disorders from areas in and around Ludhiana distt. of Punjab, India. Ludhiana city is located in the centre of Punjab on the Grand Trunk Road from Delhi to Amritsar at latitude 30.55 North and longitude 75.54 East in Northern India. All the samples were inoculated on Brucella agar base (Himedia) that was made selective by incorporation of *Brucella* selective supplement (Himedia) and incubated at

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37°C for 3-5 days under 5-10% CO. The suspected colonies were stained with Brucella Differential staining (modified Ziehl Nielsen’s stain) method and then biochemical characterization was carried out as per Quinn et al. (1994).

Detection and characterization of Brucella isolates by Genus specific Polymerase chain reaction (PCR), Hinic Real time PCR and Bruce Ladder multiplex PCR: Genomic DNA of B. abortus S19 and clinical isolates of B. abortus was extracted by using HipPurA bacterial genomic DNA purification kit (Himedia) as per the manufacturers recommendations and by hot cold lysis of the cultures. Genus specific PCR assay was carried out using primers B4/B5 derived from bscp31 gene of B. abortus (Baily et al., 1992). The primer sequence comprised of 5′-TGG CTC GGT TGC CAA TAT CAA-3′(F) and 5′-CGC GCT TGC CTT TCA GGT CTG-3′(R). Amplification reaction mixture was prepared in a volume of 25µl containing 1X PCR buffer, 20pmol/µl of each primer, 1.5mM MgCl₂, 1U of Taq DNA polymerase, 200µM of dNTPs, 5µl of template DNA and Nuclease free water to make 25µl. Bruce Ladder multiplex PCR was carried out by using a cocktail of eight pair primer pairs (Garcia Yoldi et al., 2006). The reaction mixture for the Bruce Ladder PCR consisted of total 25µl volume containing 1X PCR buffer, 0.4mM dNTPs, 1.5mM MgCl₂, primer cocktail containing 6.25pmol/µl of each primer, 2µl of template DNA, 1.5U of Taq DNA polymerase and PCR grade water to make volume upto 25µl. PCR cycling conditions for genus specific PCR and Bruce Ladder multiplex PCR are presented in Table 1. The amplified PCR products were analysed by electrophoresis at 79V for 1 hour in 1.5% agarose gel in 1X TBE buffer containing ethidium bromide and visualised under Alpha Imager 3400HP Gel Documentation System (Alpha Innotech) and photographed. Hinic Real-time PCR using IS711 primers and Taqman probe chemistry was employed for the detection of Brucella spp. (Hinic et al., 2009). Real time PCR was carried out using Taqman master mix (2X), primer probe (40X), DNA (2µl) and nuclease free water to make volume upto 20µl. Primer and probe sequence of Hinic Real-time is depicted in Table 2 and PCR cycling conditions are presented in Table 3. The sensitivity evaluation of Real-time PCR was performed on serial ten fold diluted genomic DNA of standard B. abortus S19 whereas specificity evaluation was carried out by screening some commonly available spp. of bacteria (E.coli, Salmonella, Staphylococcus, Proteus, Streptococcus, Pseudomonas, Klebsiella and Pasteurella) in addition to Brucella spp.

Application of PCR directly on DNA from clinical samples: DNA was extracted by bacterial genomic DNA purification kit (Himedia) directly from 40 clinical samples from cattle and buffaloes [uterine discharges (22), vaginal mucus (10) and foetal stomach contents (8)] and used for the genus specific PCR assay, Bruce Ladder PCR and Hinic Real time PCR.

RESULTS AND DISCUSSION

Brucellosis is a worldwide zoonotic disease with expansion of the existing Brucella spp. to hosts other than the natural ones and emergence of newer spp. in marine mammals. Despite the host specificity shown by various Brucella spp., there are reports of Brucella spp. infecting hosts other than its natural ones. For diagnostic purposes, as in humans, application of a genus specific PCR assay is sufficient, but for effective control and eradication programmes or for taking appropriate action for each epidemiological source of infection, it is necessary to differentiate the various Brucella spp. (Bricker, 2002). The most commonly used methods for disease diagnosis is isolation, biochemical characterisation and serological assays.

<table>
<thead>
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<th>Stage</th>
<th>Steps</th>
<th>Temperature (°C)</th>
<th>Duration</th>
<th>No. of cycles</th>
<th>Temperature (°C)</th>
<th>Duration</th>
<th>No. of cycles</th>
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<td>1</td>
<td>95</td>
<td>7 min</td>
<td>1</td>
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<tr>
<td>2</td>
<td>Denaturation</td>
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<td>60s</td>
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<td>Annealing</td>
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Table 2: Hinic Real-time PCR primer and probe sequence.

<table>
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<tr>
<th>PCR Target Sequence</th>
<th>Forward primer/ reverse primer (5′→3′)</th>
<th>Fluorophore−→3′ Quencher</th>
<th>Quencher</th>
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<tr>
<td>IS711</td>
<td>GCTTGAAGCTTTGCAGGA</td>
<td>GAGCCAACACCC</td>
<td>GCGGAAT</td>
</tr>
<tr>
<td>Probe</td>
<td>(5′Fluorophore−→3′ Quencher)</td>
<td>GAGCCAACACCC</td>
<td>MRA</td>
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</table>

Table 1: Genus specific PCR and Multiplex PCR (Bruce ladder) cycling conditions.

Table 3: Cycling conditions used in diagnosis of brucellosis by Real-time PCR using Taqman chemistry.
In our study, out of 100 samples (68 from cattle and 32 from buffaloes) processed for isolation of *B. abortus*, the organism could be isolated from four of these samples (one from vaginal mucus, one from uterine discharge and two from foetal stomach contents). Morphological and cultural characteristics revealed pinpoint, smooth, glistening, bluish, translucent colonies. All the isolates were Gram negative cocobacilli. Modified Ziehl Nielsen’s stain positive, non motile and failed to grow on MacConkey’s lactose agar. Isolation and biochemical characterization of *Brucella* spp. from clinical samples has been reported by various other researchers (Abbas and Talei, 2010; Ghodasra *et al*., 2010; Priyantha, 2011; Al Saadi *et al*., 2012; Ica *et al*., 2012; Jabbar *et al*., 2012; Jain *et al*., 2013; Sanjrani *et al*., 2013; Shahzad *et al*., 2014 and Geresu *et al*., 2016). All the isolates were oxidase, catalase, nitrate and urease positive, indole negative, produced H$_2$S and the reaction of all isolates was alkaline on triple sugar iron (TSI) medium. In slide agglutination test performed with the standard antiserum obtained from IVRI, all the isolates were agglutinated. All the four isolates of *B. abortus* grew in the presence of basic fuchsia (20 µg/ml) but not in the presence of thionin (20µg/ml) and were thus biotyped as biotype 1. Biotyping of *Brucella abortus* has been reported by various researchers (Shahzad *et al*., 2014; Jain *et al*., 2013 and Priyantha, 2011).

Though isolation and identification of the organism is considered as the gold standard for diagnosis of Brucellosis, but, the success rate in isolation is affected by a number of factors such as type of the sample, time of sampling and isolation techniques alongwith the requirement of a expert person for interpretation of the results. Hence, molecular techniques capable of differentiating various species of *Brucella* are being currently used for diagnosis. Various studies have demonstrated that these assays in comparison to culture and serological tests have higher sensitivity and specificity. By PCR using B4/B5 primer pairs, amplicon size of 223 bp was obtained in all the four isolates (Fig. 1). With Real-time PCR, all the four isolates showed C$_t$ values between 14-16 thus confirming the isolates to be that of *Brucella* spp. (Fig. 2). The sensitivity evaluation of Real-time PCR was performed using the genomic DNA of standard *B. abortus* S19 and it was found that Real-time PCR could detect upto 0.35fg of DNA of *Brucella* (Fig. 3) The specificity evaluation of Real-time PCR was carried out by screening some commonly available spp. of bacteria (*E.coli, Salmonella, Staphylococcus, Proteus, Streptococcus, Pseudomonas, Klebsiella* and *Pasteurella*) and no amplification was observed (Fig. 4). By Bruce Ladder PCR, all the isolates were confirmed to be of *B. abortus* with amplified product size of 1682 bp, 794 bp, 587 bp, 450 bp and 152 bp. With reference *B. melitensis* DNA, an additional 1,071 bp amplified fragment was obtained whereas reference *B. suis* was confirmed by the presence of two additional fragments of 1,071 b and 272
Fig 4: Specificity evaluation of Real-time PCR.

Fig 5: Gel electrophoresis of PCR amplified fragments from Brucella spp. by Bruce Ladder Multiplex PCR assay (Lane 1:100 bp plus DNA ladder, Lane 2: B. abortus S19, Lane 3, 4, 5, 6: B. abortus field isolates, Lane 7: B. suis (standard positive), Lane 8: B. melitensis (standard positive).

bp. However, PCR with B. abortus S19 DNA did not produce the 587 bp fragment (Fig. 5).

A number of genus and species specific conventional PCR assays have been described and reviewed by Yu and Nielsen (2010). Real time PCR using Taqman probe chemistry has been evaluated by other researchers (Bounaadja et al., 2009; Hinic et al., 2009; Surucuoglu et al., 2009; Doosti and Ghasemi, 2011). Multiplex PCR assay is a kind of PCR technique where multiple target DNA sequences can be detected in a single reaction. AMOS PCR for differentiation of B. abortus bv 1, 2 and 4, B. melitensis, B. ovis bv 1 and B. suis bv 1 using five primers was developed by Bricker and Halling (1994). This PCR assay was modified by the addition of 3 primer pairs and the modified AMOS-PCR was able to differentiate Brucella field strain isolates and the vaccine strains, S19 and RB51 (Bricker and Halling, 1994; Ewalt and Bricker, 2000). Advancement of multiplex PCR which could detect B. microti has also been reported (Mayer-Scholl et al., 2010). Lopez Goni et al. (2011) described a new multiplex PCR assay, Suis-ladder, for fast and accurate identification of B. suis at the biovar level and for the differentiation of B. suis, B. canis and B. microti. In general, Garcia-Yoldi et al. (2006) opined that Bruce ladder PCR was capable of detecting almost all the species and vaccine strains of Brucella. This assay is also capable of differentiation of Brucella strains from different geographical origin (Goni et al., 2008).

When applied on DNA extracted directly from clinical samples, out of 40 samples, only two samples (foetal stomach contents) were positive by genus specific PCR, three (two foetal stomach contents and one uterine discharge) by Hinic Real time PCR and none by Bruce Ladder PCR (Fig. 6 and Fig. 7). Of these, only one sample (foetal stomach content) was culture positive. Thus Real time PCR appeared most sensitive of the three molecular and conventional techniques as it detected maximum number of positive samples. Higher sensitivity of Real-time PCR as compared to culture methods has been reported by various other researchers (Surucuoglu et al., 2009; Hinic et al., 2009; Doosti and Ghasemi, 2011). Few studies have been performed with field samples to evaluate multiplex PCR as a diagnostic method for brucellosis.
Negative results obtained for multiplex PCR in the present study could be due to a lower diagnostic sensitivity of multiplex PCR than culture methods (Mirnejad et al., 2013). It is also relevant to mention that the PCR performance with the Brucella DNA extracted from clinical samples is very often compromised by the presence of PCR inhibitors and further complicated because Brucella is an intracellular pathogen (Leal et al., 1995). So, in summary, the protocol of isolation followed by biochemical characterisation, genus specific PCR, Real time PCR and multiplex PCR can be an approach for diagnosis of disease and for studying the epidemiological patterns so that proper control strategy can be adopted.

REFERENCES


