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Detection of *Brucella* spp. in spiked clinical sample and bovine aborted foetal stomach contents by polymerase chain reaction

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Abstract
Present study was aimed to develop a PCR targeting insertion sequence IS711 to detect *Brucella* organism. This insertion sequence is considered as conserved and evolutionary stable nucleotide sequence among species in *Brucella* genus. This PCR assay could detect 150fg /µl *B. abortus* S99 genomic DNA, equivalent to 100 *Brucella* organism. This PCR assay did not show any cross reactivity with other related gram negative bacteria or micro-organism responsible for abortion in cattle. This PCR assay could detect *Brucella* organisms in spiked clinical sample (10^8 cfu/ml) and bovine aborted foetal stomach contents. This PCR may be used as an alternative of isolation method to detect *Brucella* organism in clinical sample.

Keywords: *Brucella* spp., spiked clinical, bovine aborted, foetal stomach, polymerase chain

Introduction
Bovine brucellosis, mainly caused by *B. abortus*, is an economically important infectious disease of domestic animals mainly affecting reproductive tract leading to abortion in last trimester of gestation period. In addition to abortion, it also causes still birth, birth of weak calf or infertility in cows and bulls (Enright *et al*., 1984) [1]. It also leads to considerable financial losses in animal husbandry due to abortion and infertility problems in cattle. It has been estimated that approximately 3.4 billion US dollar per annum economic losses occurred due to bovine brucellosis in India (Acha *et al*., 2003; Sriranganathan *et al*., 2009; Singh *et al*., 2015) [2-4].

Brucellosis, as an important zoonotic disease cause serious public health concern affected more than 500,000 human cases annually worldwide (Pappas *et al*., 2005) [5]. Brucellosis in human beings usually acquired through consumption of contaminated unpasteurized milk or milk products, inhalation or accidental inoculation of live *B. abortus* culture or *B. abortus* S19 vaccine (Young *et al*., 1983) [6]. In addition to that, this is also considered as an occupational disease acquired by Veterinarians, Animal handles, Butchers due to frequent exposure to infected animals, carcasses, uterine secretions or aborted foetuses

In India, Brucellosis is considered as an endemic disease. calfhood vaccination and Identification of infected/carrier animals followed by slaughter or segregation considering socio-political and cultural ethos is still considered best method to control/eradicate the disease. For rapid mass screening and epidemiological surveillance, serology is widely used in surveillance and control programmes for brucellosis (Al Dahouk *et al*., 2005) [7], but serological assays can give false-positive or false-negative results. In India, Rose Bengal plate agglutination test (RBPT) followed by ELISA is the most preferred method of diagnosis of brucellosis but these serodiagnostic test does not elucidate presence of organism/ antigen in clinical specimen. Due to presence of sLPS, cross-reactions between *Brucella* species and other Gram-negative bacteria, such as *Yersinia enterocolitica* O:9, *Francisella tularensis*, *Escherichia coli* O:157, *Salmonella urbana* group N, *Vibrio cholerae* and *Stenotrophomonas maltophilia*, may give false positive reaction (Munoz *et al.*, 2005) [8]

Although isolation of the etiological agent is considered gold standard and most specific test for diagnosis of brucellosis, but there are some inherent disadvantages like requiring a long incubation period and low sensitivity especially in the chronic stage of the disease. Moreover,

the culture material must be handled in Bio-safety level 3 laboratories carefully, as the *Brucella* organism is a class III pathogen and can infect livestock at very low infectious dose (Alton *et al*., 1988) [9]. Due to various reason such as low bacterial load in clinical sample or active secretion of bacteria at the time of parturition/ abortion only and localization of brucella organism in supra mammary lymph node and mammary glands, isolation can't be used as a screening test.

Molecular detection of causative agent is the other preferred alternative diagnostic method. 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 (patil *et al.,* 2014) [10], the 16S-23S intergenic spacer region(Romero *et al.,* 1995) [11], *omp2* (Leal-Klevezas *et al.,* 1995) [12] and *bcsp31* (Bailly *et al.,*1992) [13] have been established having different performances.

In the present study, we aimed to optimize PCR targeting *IS711* genomic region using in house designed primers, as a rapid, sensitive and specific molecular test to detect presence of Brucella antigens in various clinical specimen which can be used in association with other test for diagnosis of brucellosis.

Materials and Methods

Bacteria

Standard *B. abortus* S99 cultures procured from, Division of Veterinary Public Health, IVRI, Izatnagar and revived on Potato infusion agar (PIA) media by incubating at 37 ^{0}C for 5-7 days. For testing specificity of PCR assay, reference strains of *B. abortus (*S99, S19, field isolates), as well as other non-Brucella species including *Escheria coli, Pasteurella multocida, Campylobacter spp.* Listeria monocytogenes and Infectious bovine rhinotracheitis (IBR) virus DNA were used.

Bacterial DNA extraction

DNA of the all the bacterial isolates was extracted by boiling broth culture in a microcentrifuge tube for 5 minute followed by snap chilling on ice, which was centrifuged at 10000 rpm for 10 min and supernatant was stored at -20 ^{0}C till further use as DNA template in PCR.

Genomic DNA extraction from Spiked Sample and clinical sample (bovine aborted foetal stomach contents)

Clinical samples (Foetal stomach contents from Brucellosis negative aborted cattle, milk and vaginal swab suspended in PBS) were spiked with heat inactivated 10^8-10^{10} cfu/ml of *B. abortus* strain S99. Foetal stomach contents (n=25) (clinical sample) from naturally bovine abortion cases were heat inactivated at 80 ^{0}C for 30 min. Genomic DNA was extracted from these spiked ad clinical specimen using Fermantas genomic DNA extraction kit following manufacturer's guidelines, which was used as template for PCR.

Quantification and Quality determination of DNA

Quality and purity of extracted DNA was determined by taking absorbance at 260 and 280 nm in UV spectrophotometer (Eppendorf, Germany). The purity of DNA was assessed by the ratio of OD260/ OD280 in UV spectrophotometer. Quality of DNA sample was analyzed by running the DNA sample in 1% agarose gel electrophoresis and single sharp band without any smearing was considered as of satisfactory quality.

Designing of PCR Primer

PCR primer was designed targeting *IS711* genomic region of *Brucella* organism using primer 3 and oligoanalyzer software considering absence of any hairpin formation, primer-dimer formation and absence of any cross matching with nucleotide sequence of other related organism (table 1).

Optimization of PCR reaction

For choosing optimum annealing temperature, gradient PCR was applied with annealing temperature ranging from 57 ^{0}C to 70 ^{0}C. The temperature range was considered on the basis of T_m value of both forward and reverse primer (+/- 5 ^{o}C to T_m value). After ascertaining optimum annealing temperature, the temperature and time combination for denaturation and extension in each PCR cycle was optimized. In next step, concentration of different PCR reaction component such as PCR primer concentration, DNA template concentration was also optimized to amplify the desired targeted nucleotide sequence without compromising sensitivity or specificity of PCR assay. The amplification was carried out in 25 µl of the PCR mixture containing 12.5 µl of 2x PCR master mix (Fermantas, UK), 10 Picomol of each primer (desalt purified, Sigma, India), 2 µl of genomic DNA as template DNA and added Nuclease free water(Fermantas, UK) to make it 25 µl. The optimized PCR conditions are mentioned in table 2.

Agarose gel electrophoresis

The PCR products were analyzed in 2.0% Agarose gel (Purified Agarose, Sigma, USA) in a submarine horizontal electrophoresis unit at 70 V/cm for 45-60 minutes. The size of PCR amplicon was measured by relative mobility of PCR amplicon to 100 bp/50bp DNA marker (Fermantas, Thermo scientific, UK) in UV transillumination and gel image was captured in gel documented system.

Analytical sensitivity of PCR

Analytical sensitivity of PCR was determined as minimum amount of DNA that can be detected by PCR. It was determined by serially 10 fold dilution of genomic DNA extracted from *B. abortus* S99 bacterial culture and PCR was carried out in 25µl of reaction volume using 2µl (300ng of genomic DNA) of genomic DNA as template from each dilution. The detection limit was determined as the highest dilution of DNA showing a clear distinct PCR amplicon of ~162bp.

Analytical specificity of PCR

PCR was carried out in 25µl of reaction volume using 2µl of genomic DNA as template from each micro-organism using optimized amplification thermal profile as mentioned above. Positive (*B. abortus* S99 genomic DNA) and negative control (No DNA template) was also included in each PCR reaction.

Screening of clinical sample

Twenty Five (n=25) aborted foetal stomach contents, which were tested with other diagnostic tests like isolation were screened with optimized PCR.

Results

Morphological and Cultural Characteristics

The revived cultures of *B. abortus* strain S99 on PIA media produced typical translucent, small, round, smooth margined colonies of 2-4 mm in diameter having pale honey color. The microscopic examination of the Gram stained cultures revealed as Gram negative, coccobacilli, short rods, usually arranged singly or pair or in small groups. The organisms were non-motile, non-sporing and non-capsulated. The bacterial cultures were found positive in catalase, oxidase,

urease, H_2S production and nitrate reduction test. Smoothness of the culture was confirmed with absence of agglutination with acriflavin dye.

Quantification and quality determination of extracted genomic DNA

The concentration of extracted DNA from *Brucella abortus* strain S99 was ~150 ng/µL and The purity of extracted DNA was analyzed by measuring the absorbance ratio at 260 nm and 280 nm by UV spectrophometer (Eppendorf, Germany). The ratio of A260/A280 was observed as 1.8 that indicated absence of any protein or RNA contamination.

Optimized PCR assay

Although PCR could amplify the targeted *IS711* genomic region up to highest 66ºC annealing temperature but 64ºC was considered as the optimum annealing temperature with maximum sensitivity without producing any non specific PCR products or any Primer- dimer formation. (Fig.1 and 1a). The targeted *IS711* genomic region of *Brucella* organism could be amplified using optimized thermal reaction condition profile (table 2) and produced ~162bp amplicon without producing any non specific amplicon.

Analytical sensitivity of PCR assay

The PCR assay could detect as low as 150 fg/µl DNA equivalent to 100 bacilli/ml, producing amplicon of ~162bp (Fig.2).

Analytical specificity of PCR assay

The PCR did not produce any amplification with genomic DNA of other selected micro-organism (mentioned in material and method). This showed absence of any cross reactivity with these micro-organisms. (Fig.3)

PCR amplification of spiked and bovine aborted foetal stomach contents

The PCR assay could detect up to 10^8 cfu/ml *Brucella* organism in spiked milk, vaginal swab and foetal stomach content sample.

Screening of clinical sample

Five out of twenty five bovine aborted foetal content samples (clinical sample) were found positive in PCR, produced ~162bp amplicon without showing any non-specific product**.** (Fig.4)

Discussion

For confirmation of brucellosis, isolation of causative agent from clinical sample is considered as gold standard but considering zoonotic nature of organism, there is always a risk of infection to laboratory personnel handling the infected clinical sample. The suspected clinical specimen or bacterial culture should be handled in BSL-3 containment facilities. As an alternative to isolation, PCR may be a choice of diagnostic method because PCR may use heat inactivated clinical sample. Heat inactivation may eliminate or reduce the infectivity of infectious micro-organism that minimizes the chance of infection to laboratory personnel. PCR or other molecular techniques have been successfully used in detection of zoonotic pathogen such as *Mycobacterium bovis*, *Mycobacterium tuberculosis* and *Burkholdeia mallei* in clinical sample or spiked samples (Prakash *et al*., 2014, 2015, Dolker *et al*., 2012, Shukla *et al.,* 2016a,b) [14-18]. These PCR based diagnosis can further be used for molecular characterization of the organism that may help in determining genetic determinants of virulence, phylogenetic analysis (Prakash *et al*., 2014, Shukla *et al.,* 2017) [19-20]. In addition to that, it may help us in determining the point source of infection as well.

The present study was designed to develop PCR based detection of Brucella organism. In this study, insertion sequence *IS711* was targeted because insertion sequence is considered as evolutionary stable genomic region having minimum polymorphism among different species across the genus. This insertion sequence *IS711* based PCR can be used to detect all the virulent brucella species causing abortion or reproductive disorders in different domestic animals.

During optimization of annealing temperature, although PCR could amplify the targeted nucleotide sequence from 57 ºC to 66ºC but at the lower annealing temperature, there is higher possibility of annealing the primer with some non targeted site in the bacterial genome that may produce some non specific amplicon or Primer-dimer formation. Beyond 64ºC annealing temperature, reduction in the intensity of amplicon band was observed due to lower annealing capacity of primer to the targeted sequence.

Detection limit of this PCR assay was up to 150 fg DNA/µl or 300fg DNA/ reaction. This detection limit is equivalent to 100 cfu/ml of *Brucella* organism that is comparable to detection limit of 60-100 fg DNA detection limit (Baily *et al.,* 1992, Fekte *et al*., 1990a) [13, 21]. The PCR assay could detect 10^8 cfu/ml of brucella organism in spiked sample. Although the detection limit in spiked sample is lower that may be due to poor DNA extraction efficiency in complex clinical sample matrix, yet it is sufficient to detect Brucella organism in aborted clinical sample because Brucella organism secreted in the range of $2.4X\ 10^8$ to $1.4\ X10^{13}$ CFU/gm that is much higher to detection limit (Hopper *et al*., 1992) [22]. This PCR could detect *Brucella* spp. in clinical bovine aborted foetal stomach content sample, also emphasizes that detection limit of PCR is not a limiting factor in direct detection of brucella organism from clinical sample.

The PCR did not show any cross reactivity with selected bacterial or viral pathogen that establish its high specificity. This minimize possibility of false positive results and it may help us to prevent elimination of healthy productive (false Sero-positive) animals. This sero-positivity may be due to cross reactivity of antibodies with shared epitope among non brucella saprophytic micro-organisms.

This PCR assay may be used as a choice of molecular diagnostic test to detect brucella organism in aborted foetal stomach content or tissue sample. This may be an alternative to isolation of brucella organism with rapidity and accuracy.

Table 1: Primer sequence used in PCR to detect *Brucella sp.*

	Nucleotide Sequence (5'-3')	Amplicon size
Forward primer	F- 5' GGCCATTATGGTGACTGTCC 3'	162bp
Reverse Primer	R- 5' GGAACGTGTTGGATTGACCT 3'	

Table 2: Amplification reaction condition of PCR

Step 1	Initial denaturation	94 ^{0}C for 5 min
Step 2	Cyclic denaturation	94 ^{0}C for 1 min
Step 3	Annealing	64 ^{0}C for 45 sec.
Step 4	extension	72 ^{0}C for 45 sec
Step 5	Repeat step 2 to 4 for 35 cycles	
Step 6	Final Extension	72 ^{0}C for 7 min.
Step 7	Hold	4 ^{0}C

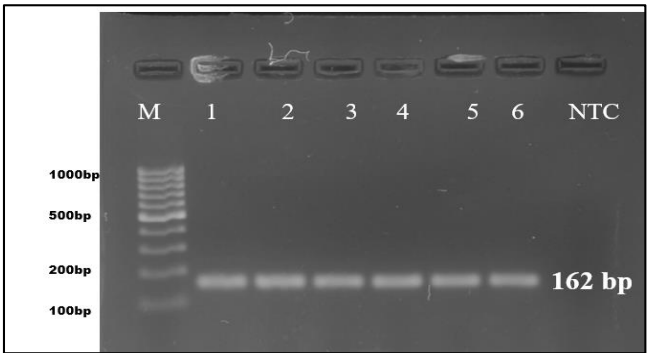

Fig 1: Optimization of annealing temperature (transformed image)

M: 100 bp DNA marker
L1: 57 ^{0}C, L2: 59 ^{0}C, L3: 60 ^{0}C
L4: 61 ^{0}C, L5: 62 ^{0}C, L6: 63 ^{0}C
L7: NTC

Fig 1a: Optimization of annealing temperature for PCR (transformed image)

M: 100 bp DNA marker
L1: 64 ^{0}C, L2: 65 ^{0}C, L3: 66 ^{0}C
L4: 67 ^{0}C, L5: 68 ^{0}C, L6: 69 ^{0}C
L7: NTC

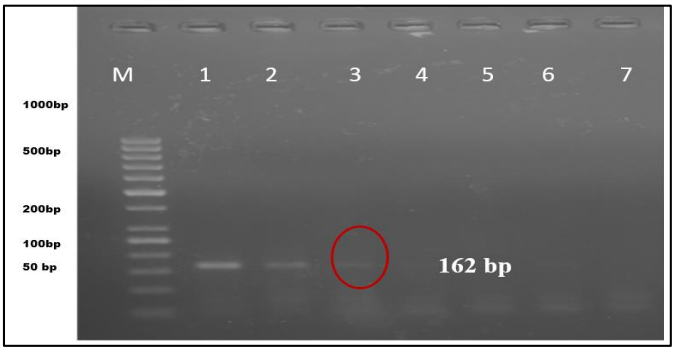

Fig 2: Analytical sensitivity of PCR (transformed image)

Lane M: 50 bp DNA marker
L 1: 15 pg, L 2: 1.5pg, L 3: 150fg
L 4: 15fg, L5: 1.5 fg, L6: 0.15 fg
L 7: NTC

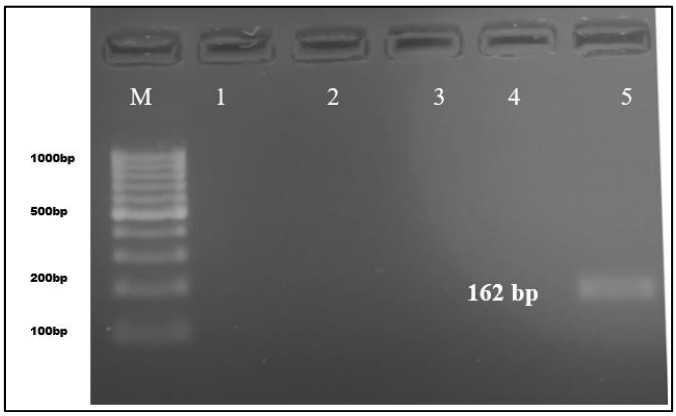

Fig 3: Analytical specificity of PCR (transformed image)

M: 100 bp DNA marker
L1*: L. monocytogenes*
L2: *P. multocida*
L3: *Camylobacter spp.*
L4: IBR virus
L5: *B. abortus* S99

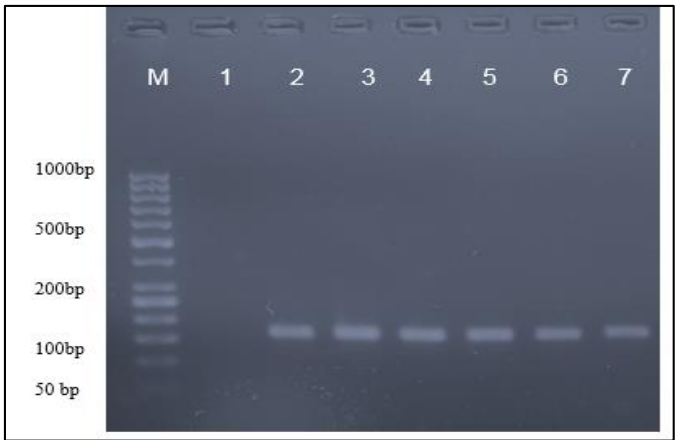

Fig 4: Detection of *Brucella* spp. in spiked and clinical samples

M: 50 bp DNA marker, L1: Negative control (NTC), L2: Milk (spiked), L3: Foetal stomach content, (spiked) L4: Vaginal swab (spiked), L5- L6: bovine aborted foetal stomach contents (clinical sample), L7: Positive control (*B. abortus* S99)

Conclusion

This PCR assay could detect 150 fg *Brucella* DNA equivalent to 100 brucella bacilli and it detected 10^8 cfu/ml in spiked sample. This PCR assay did not any show any cross reactivity with selected common contaminant bacteria. As this PCR assay use heat inactivated clinical sample, it minimize chance of infection to laboratory personnel and it can provide diagnosis within 3-4 hrs. This PCR assay may be successfully used to detect *Brucellla* sp. as an alternative to isolation method.

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