MOLECULAR DETECTION OF *BRUCELLA ABORTUS* USING BSCP31 AND IS711 GENE BASED PCR ASSAY IN CATTLE AND BUFFALO

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ABSTRACT

Brucellosis has been one of the most important reproductive diseases among different livestock species as well as animal handlers and is considered a reemerging infectious disease in many areas of the world. A total of 353 clinical samples of cattle (207) and buffalo (146) were collected for molecular detection of *Brucella.* Out of 353 clinical samples, 6 samples (vaginal discharge-2, placenta-2, foetal stomach content-1, fetal lung-1) positive for *Brucella* organism by genus specific PCR based on bscp31 (B4/B5 primer, 223bp). All six genus specific positive sample also positive for *B. abortus* +IS711 (498bp) species specific PCR. All the samples which were detected positive in conventional PCR also detected positive for *Brucella* in SYBR green and TaqMan probe based real time PCR.

Keywords: *Bubalus bubalis*, buffaloes, Brucellosis, molecular detection, PCR, bscp31, B4/B5, IS711 primer

INTRODUCTION

Brucellosis is a bacterial disease caused by a gram negative, non-spore forming, facultative intracellular bacteria belonging to genus *Brucella* of family Brucellaceae (family III) with *Mycoplana* and *Ochrobactrum* of the order Rhizobiales in the class Alphaproteobacteria of the phylum Proteobacteria. The genus *Brucella* consist of seven species according to antigenic variation and primary host *viz*, *Brucella melitensis* (sheep and goats), *B. suis* (pigs), *B. abortus* (cattle), *B. ovis* (sheep), *B. canis* (dogs), *B. neotomae* (wood rats) and *B. microti, B. ceti, B. pinnipadialis* and *B. inopinata* (marine animals).

Brucellosis is a major cause of reproductive losses, abortions, placentitis, epididymitis and orchitis in animals. The symptoms and signs most commonly reported in human are fever, fatigue, malaise, chills, sweats, headaches, myalgia, arthralgia and weight loss (Kochar*et al*., 2007; Mantur*et al*., 2007; Cutler *et al*., 2005; Valderas and Roop, 2006). Humans are commonly infected through ingestion of raw milk, cheese or meat or through direct contact with infected animals, products of conception or animal discharges (e.g., among shepherds, farmers and veterinarians)

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and through inhalation of infectious aerosols by workers in abattoirs and microbiology laboratories.

Conventional bacteriology has high specificity and ability to differentiate between species and biovarieties, and is considered the gold standard technique. However, given the sensitivity and requirements of culture media, the isolation of *Brucella* spp. is laborious, requiring days to identify the agent. Moreover, it is a high-risk biological pathogen that requires laboratories with qualified staff and facilities and Class 3 personal protective equipment - PPE (Lage*et al*., 2008; Poester*et al*., 2009). Moreover, this technique of bacterial isolation exposes human to pathogens while carrying out diagnosis. Proper treatment and prevention of disease requires prompt and accurate diagnosis. Molecular diagnostics provides excellent platform for accurate and prompt diagnosis of diseases while maintaining safety of the personnel (Sola *et al*., 2014). Hence, to surmount the problems associated with serology and conventional bacteriology, nucleic acid amplification has been explored for the rapid detection and confirmation of *Brucella.*

Literature search reveals that various workers have reported the existence of Brucellosis in various parts of Gujarat, however there exist a paucity of information on epidemiology of Brucellosis in livestock and human combining with detection of *Brucella* organisms in various clinical, blood and serum samples of livestock and human employing PCR, real time PCR, speciation by species specific PCR.

MATERIALS AND METHODS

Sample collection

A total of 353 various clinical samples were collected from cattle and buffaloes in BBL broth from various places of Gujarat (Table 1). To make the tissue suspension using tissue homogenizerand centrifuged at 10,000X g for 10 minutes and the resulting supernatant was used for DNA extraction.

DNA extraction

The genomic DNA from clinical samples were extracted using DN easy blood and Tissue Kit (Qiagen, USA.) following manufacturers protocols.

Detection of *Brucella* using genus specific B4/B5 primer

A PCR was standardized in a total reaction volume of 25 µl, containing 12.5 µl of 2 x PCR Master mixture, 10 pmol of forward (5'TGG CTC GGT TGC CAA TAT CAA3') and reverse (5'CGC GCT TGC CTT TCA GGT CTG3') (Bailey *et al*., 1992) primers each 1 µl, Template DNA 2 µl and nuclease free water upto 25 µl. The reaction was standardized in a thermal cycler (Eppendorf, Germany). with initial denaturation at 93ºC for 5 minutes, followed by 35 cycles at 90ºC for 60 seconds, 64ºC for 30 seconds and 72ºC for 60 seconds. Final extension was carried out at 72ºC for 10 minutes. The amplified product (223 bp) was electrophoresed in 2% agarose gel stainedwith ethidium bromide (0.5 µg/ml) and image was documented by gel documentation system (Mini BiSBioImaging System).

Detection of *Brucellaabortus* using species specific + IS711primer

A PCR was standardized in a total reaction volume of 25 µl, containing 12.5 µl of 2 x PCR Master mixture, 10 pmol of forward (5' GAC GAA CGG AAT TTT TCC AAT CCC 3') and reverse (5' TGC CGA TCA CTT AAG GGC CTT CAT 3') (Bricker and Halling, 1994) primers each 1 µl,

Template DNA 2 µl and nuclease free water upto 25 µl. The reaction was standardized in a thermal cycler (Eppendorf, Germany). with initial denaturation at 95ºC for 5 minutes, followed by 35 cycles at 95ºC for 90 seconds, 57ºC for 120 seconds and 72ºC for 120 seconds. Final extension was carried out at 72ºC for 5 minutes. The amplified product (498 bp) was electrophoresed in 2% agarose gel stained with ethidium bromide (0.5 µg/ml) and image was documented by gel documentation system (Mini BiSBioImaging System).

SYBR green based real time PCR

Prepared the PCR mix for one reaction (25 µl volume) in 0.2 ml PCR tube by adding the various components. For genus specific real time PCR quantity and concentration of various components and thermal cycling condition as per Table 2. For Species specific real time PCR quantity and concentration of various components and thermal cycling condition as per Table 3. In SYBR green based real time PCR for detection of *Brucella* genus as well as species were carried using the same primers which were used in genus (B4/B5) and species specific (+ IS711) PCR.

TaqMan probe based real time PCR

In TaqMan probe based real time PCR, Quantity and concentration of various components, Primer and thermal cycling condition as per Table 4.

Table 1. Direct detection of *Brucella* by PCR.

Type of sample	Species		No.of tested	Result
	Cattle	Buffalo		No. of sample positive in genus and species specific PCR
Blood	114	58	172	00
Vaginal swab	28	45	73	00
Vaginal discharge	08	05	13	02 (1 Cattle + 1 Buffalo)
Milk	12	08	20	00
Placenta	11	09	20	02 (1 Cattle + 1 Buffalo)
Placental fluid	05	02	07	00
Hygroma fluid	02	01	03	00
Amniotic fluid	00	02	02	00
Foetal intestine fluid	02	01	03	00
Foetal lung	06	04	10	01 (1 Buffalo)
Foetal liver	05	04	09	00
Foetal stomach content	03	01	04	01 (1 Cattle)
Foetal heart	03	00	03	00
Foetal heart blood	02	02	04	00
Cotyledon	06	04	10	00
Total	207	146	353	06 Samples

Table 2. SYBR Green based PCR (Genus specific PCR).

Quantity and concentration of various components used in PCR (Genus specific)			
Reactions	**Final concentration**		**Volume per single 25 µl reaction (µl)**
SYBR green master mix	1X		12.5 µl
B4 (*BCSP31*) forward primer	10 pg/ µl		1.0 µl
B5 (*BCSP31*) reverse primer	10 pg/ µl		1.0 µl
Template DNA	100 ng/ µl		2.0 µl
NFW	Molecular grade		8.5 µl
Total :-	---		25.0 µl
Steps and conditions of thermal cycling for different SYBR Green based primer pairs in PCR			
Steps	**Temperature**	**Incubation time**	**Number of cycle**
Initial denaturation	95°C	5 min	1 cycle
Denaturation	90°C	1 min	40 cycles
Annealing	64°C	30 sec	
Extension	72°C	1 min	
Melting curve analysis	95°C	15 sec	1 cycle
	60°C	1 min	
	95°C	30 sec	

Table 3. SYBR green based PCR(Species specific PCR).

Quantity and concentration of various components used in PCR (Species specific)			
Reactions	**Final concentration**	**Volume per single 25 µl reaction (µl)**	
SYBR green master mix	1X	12.5 µl	
IS711 forward primer	10 pg/ µl	1.0 µl	
IS711 reverse primer	10 pg/ µl	1.0 µl	
Template DNA	100 ng/ µl	2.0 µl	
NFW	Molecular grade	8.5 µl	
Total : -	---	25.0 µl	
Steps and conditions of thermal cycling for different SYBR green based primer pairs in PCR			
Steps	**Temperature**	**Incubation time**	**Number of cycle**
Initial denaturation	95°C	5 min	1 cycle
Denaturation	90°C	90 sec	40 cycles
Annealing	57°C	2 min	
Extension	72°C	2 min	
Melting curve analysis	95°C	15 sec	1 cycle
	60°C	1 min	
	95°C	30 sec	

Table 4. Taqman Probe based Real Time PCR.

List of Taqman probe based Real Time PCR primers and probe (Hinic*et al.*, 2008)			
Target sequence	**Oligonucleotides 5`-3`**		**Reference**
IS711 Primer	GCTTGAAGCTTGCGGACAGT		Hinic*et al.* (2008)
	GGCCTACCGCTGCGAAT		
Probe	FAM-AAGCCAACACCCGGCCATTATGGT-TAMRA		
Quantity and concentration of various components used in PCR			
Reagents	**Final concentration**	**Volume per single 25 µl reaction (µl)**	
TaqMan universal PCR master mix, no AmpErase UNG	1X	12.5 µl	
Forward primer	10 pg/ µl	1.0 µl	
reverse primer	10 pg/ µl	1.0 µl	
Probe	200 Nm	2.5 µl	
TaqMan exogenous internal positive control mix	0.5x	2.5 µl	
Exogenous internal positive control	0.5x	0.5 µl	
Bacterial lysate	100 ng/ µl	2.5 µl	
NFW	Molecular grade	2.5 µl	
Total volume	----	25 µl	
Steps and conditions of thermal cycling for different primer pairs in Taqman probe based Real Time PCR			
Steps	**Temperature**	**Incubation time**	**Number of cycle**
Initial denaturation	60°C	1 min	1 cycle
Denaturation	95°C	10 min	40 cycles
Annealing	95°C	30 sec	
Extension	60°C	50 sec	

RESULTS AND DISCUSSION

A total of 353 clinical samples *viz*., vaginal swab; vaginal discharge, placenta, milk, foetal stomach content, foetal liver, foetal spleen, foetal lung, foetal heart blood, serum, cotyledon, were screened for detection of *Brucella* using bcsp31 genus specific PCR. Of these, total 06 samples (Table 1) were detected positive for presence of *Brucella* yielding 223 bp when electrophoresed through 2% agarose gel (Figure 1) and same samples found positive in IS711 species specific PCR yielding 498 bp when electrophoresed through 2% agarose gel (Figure 2). Primers targeting for the

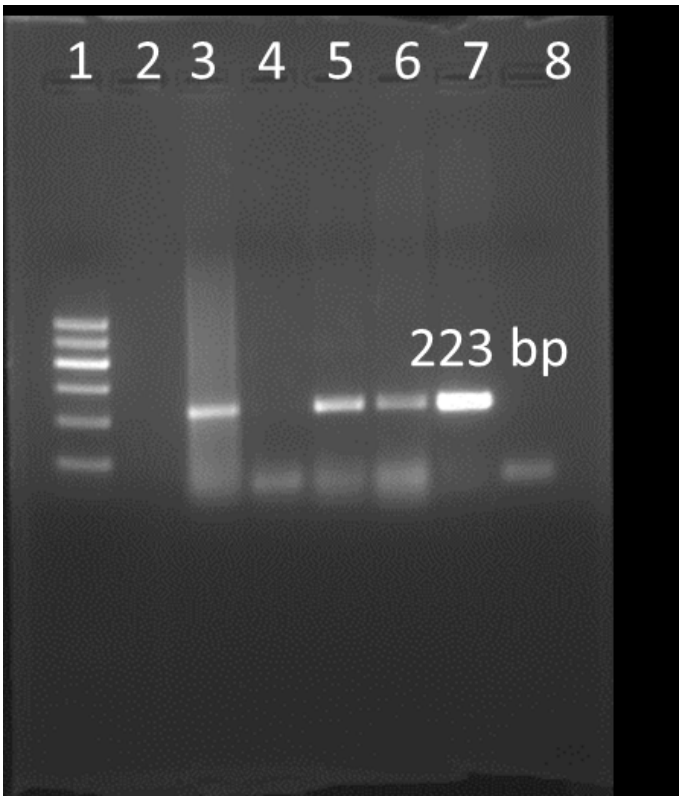

Figure 1. Genus specific PCR 223bp PCR products with B4B5 primer.

1- Ladder
2- Negative control
3- Positive control
4- Sample (negative)
5- Sample (positive) placenta
6- Sample (positive) vaginal discharge
7- Sample (positive) foetal stomach content
8- Sample (negative)

bcsp31 gene were used for *Brucella* genus specific PCR (Bailey *et al*. 1992). This bcsp31 gene based primer had also been successfully used by Jung *et al*. (1998); Kanani (2007) for detection of *Brucella* DNA in semen of bulls. Matar*et al*. (1996) also used this primer pair for diagnosis of human brucellosis directly from whole blood and in their study PCR assay was found to be rapid and specific.

Similarly Patel *et al*. (2015) undertaken 56 clinical samples of cattle (23) and buffaloes (33) in north Gujarat. 03 samples of cattle (vaginal swab-2 and placenta-1) and 7 sample of buffalo (placenta-1, foetal stomach content-1, foetal liver-1, spleen-1, foetal lung-1, foetal heart blood-1 and cotyledon-1) were positive for *Brucella* in species specific PCR yielding 498 bp band when electrophoresed through 2% agarose gel. Same study was also performed by various workers in different place either from

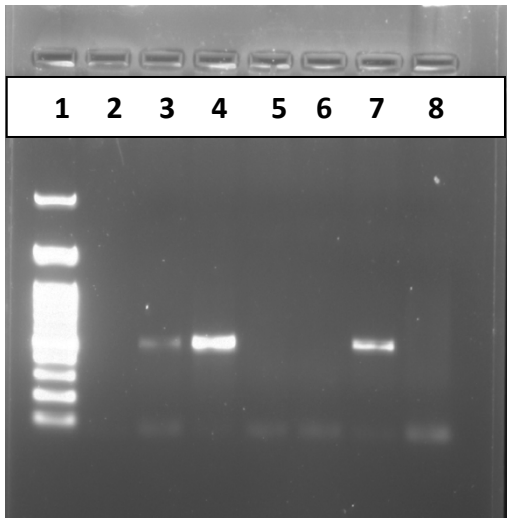

Figure 2. Species specific PCR 498 bp PCR product with primer IS711.

1- Ladder
2- Negative control
3- Positive control
4- Sample (positive) foetal lung
5- Sample (negative)
6- Sample (negative)
7- Sample (positive) placenta
8- Sample (negative)

blood or vaginal secretions independently for direct detection of *Brucella* by PCR (Morata *et al*., 2001; Zerva*et al*., 2001; Varasada, 2003). In present study, SYBR green based real time PCR complete cycling parameters as described in Material and methods data analysis was done based on amplification curves obtained (Figure 3). The specificity of the amplified PCR products was assessed by performing a melting curve analysis. The samples which were detected positive in conventional genus and species specific PCR also detected positive for *Brucella* as it is matching the tm values of the control sample.

TaqMan probe based real time PCR with the target sequence of IS711 was carried out with the probe of *Brucella* Hinic Probe (IS711) (Hinic *et al*., 2009). The filter used was FAM 465 to 510 nm filter. By using these protocols, we were able to detect all the 06 samples which were positive (Figure 4) in the species specific conventional PCR well as SYBR green base real time PCR.

The use of the Polymerase Chain Reaction (PCR) to identify *Brucella* DNA at genus, species and even biovar levels has becoming extended to improve diagnostic tests and a diversity of methods have been developed. Applications for PCR methods range from the diagnosis of the disease to characterization of field isolates for epidemiological purposes including taxonomicstudies.

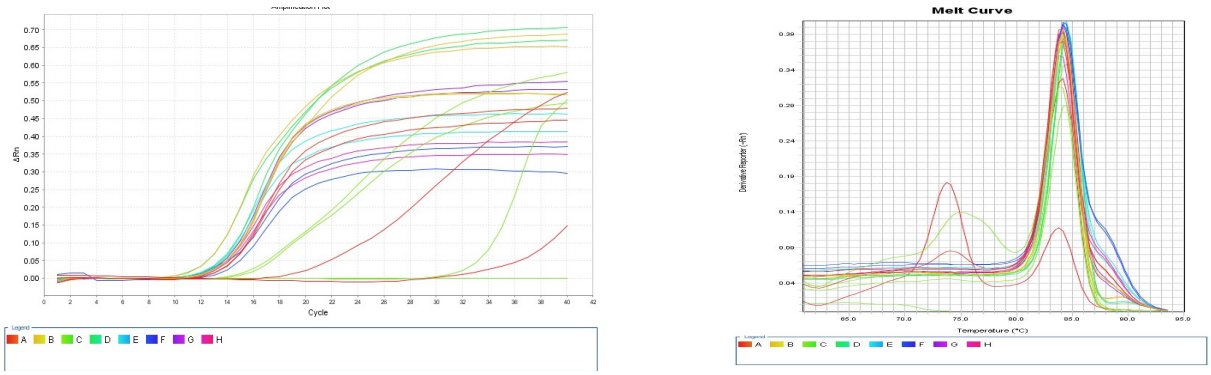

Figure 3. SYBR green based Real time PCR amplification plot and Melt curve.

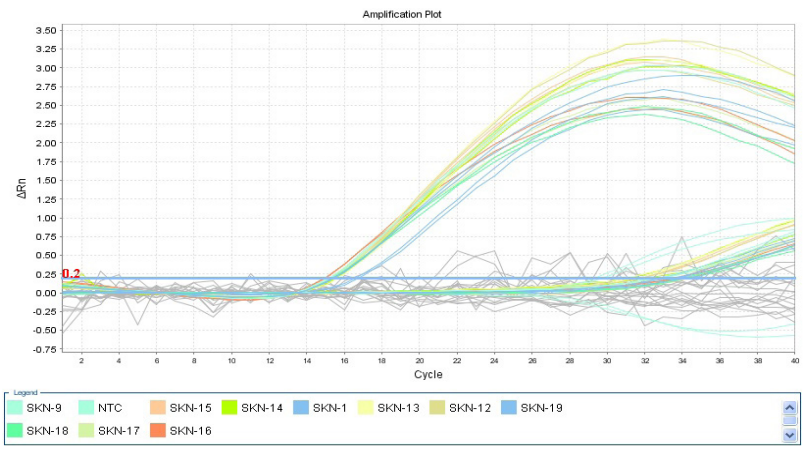

Figure 4. *Taqman* Probes based Real time PCR.

CONCLUSION

The present study indicates direct detection of *Brucella* by genus specific PCR, species specific PCR, Real time PCR and TaqMan base real time PCR. This method is specific, reliable and simple to perform require less time than the cultural method. Prevalent of brucellosis in Gujarat which is of public health importance because it is zoonotic disease. There is need to educate about how to prevent and control of brucellosis due to it cause high socioeconomic loss to the farmer.

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