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Research Article

ISOLATION AND IDENTIFICATION OF *BRUCELLA* SPECIES FROM ABORTED FETUSES OF CROSSBRED CATTLE

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ABSTRACT

Bovine brucellosis is an endemic disease in India, caused by *Brucella abortus* and had been reported almost all part of the country. The present study was conducted to isolate and identify the biovar of *Brucella abortus* from aborted fetuses of crossbred cattle from an organized farm of Fatehabad district of Haryana, India. Out of 12 pregnant animals, four animals aborted in duration of two months. Biological samples such as uterine discharge, placental tissue, fetal liver, lungs and fetal stomach contents were collected. Isolation of bacteria was attempted from stomach contents of all four fetuses using conventional bacteriological methods and colonies of *B. abortus* were successfully isolated from one fetal stomach content. Further, isolated colonies were typed as *B. abortus* using TaqMan probe based qPCR assay specific to *B. abortus*. This information is important for adoption of control measures. Further studies are needed from different geographical areas of the state to execute control strategies against bovine brucellosis.

Key words: Bovine, Brucellosis, qPCR, TaqMan probe, *B. abortus.*

Bovine brucellosis is endemic in many developing countries including India and is mainly caused by *Brucella abortus* (Geresu *et al*., 2016). It is one of the most contagious reproductive diseases and highly prevalent among Indian dairy animals and is responsible for annual economic loses to the tune of US$ 58.8 million (Kollannur *et al.,* 2007). Abortions, stillbirths, reduced milk production, infertility and revenue losses due to international trade impediment for animals and their products contribute to the great economic losses (Kollannur *et al*., 2007). After rabies, brucellosis is the second most important zoonotic disease and has gained importance over the years since its discovery on the island of Malta (Abubakar *et al*., 2012*). Brucella* species are gram negative coccobacilli. There are six "classical" recognized species; *B. abortus, B. melitensis, B. suis, B. ovis, B. canis,* and *B. neotomae* (Hadush and Pal, 2013). Recently, four new *Brucella* species have been recognized and classified, namely, *B. pinnipedialis, B. ceti, B. microti and B. inopinata* (Scholz *et al*., 2009).

The diagnosis of brucellosis is based on serological, bacteriological, allergic skin reaction, and molecular methods (Simsek *et al*., 2004). The most important confirmatory method of *Brucella* infection is bacteriological diagnosis since its specificity is much higher than that of other diagnostic methods and it is used as a gold standard diagnostic method. The existence of different *Brucella* biotypes among the *Brucella* spp. and their identification is important to confirm the infection and trace the source of the infection (Guler *et al*., 2003). Because of the complications involved in the diagnosis of the disease, including the difficulties in differentiation between infected and vaccinated animals by conventional serological tests, bacteriological isolation and identification of biotypes of the etiological agent are necessary steps in the design of epidemiological and eradication programmes (Zinstag *et al*., 2005). Molecular diagnostic methods are also currently being used for the detection and identification of *Brucella* spp. from diverse biological samples (Sahin *et al*., 2008).

Conventional serological tests are unable to differentiate infected and vaccinated animals hence bacteriological isolation and identification of biotypes of the etiological agent are necessary steps in the design of epidemiological and eradication programs (Refai, 2002; Zinstag *et al*., 2005). Molecular diagnostic methods are also currently being used for the detection of *Brucella* spp. in various samples (Sahin *et al*., 2008). The present study aimed to isolate *Brucella* spp. from dairy cattle with a history of abortion by using standard bacteriological isolation and molecular methods in order to improve knowledge of the epidemiology of the disease for the control and prevention of brucellosis in the state of Haryana.

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MATERIALS AND METHODS

Collection of Biological Samples: A total of 32 biological samples i.e., peripheral blood, uterine discharge of dams (n=4) and stomach contents, placenta, liver, spleen, lung and heart of aborted fetuses (n=4) were collected from an organized livestock farm of Fatehabad district of Haryana where out of 12 pregnant animal, four animals aborted in two months of duration. All aborted dams were in last trimester of pregnancy.

Bacterial Isolation: Culture isolation was attempted only from stomach contents of aborted fetuses according to Alton *et al*. (1988) protocol. In brief, the specimens (0.5ml in duplicate) were inoculated onto trypticase soya agar (TSA) supplemented with antibiotics (Farrell, 1974). The inoculated plates were incubated at 37°C in candle jar with 5–10 % CO_2 tension for a period of 10 days. The plates were first inspected after 3 days of incubation for the development of growth of smooth, entire, circular, raised, honey-colored, and translucent colonies. Preliminary examination of suspected colonies with respect to growth characteristics, Gram's staining of the isolates, and biochemical tests, were carried out as described by Alton *et al*. (1988).

Extraction of DNA: DNA was extracted from all 32 biological samples using PureLink genomic DNA Mini kit (Thermo Fisher Scientific). The quantities and purities of the extracted DNA from all samples were determined using NanoDrop Spectrophotometer (Thermo Scientific).

Bacterial DNA Isolation: Genomic DNA (gDNA) isolation from cultured *Brucella* spp. was also carried out using PureLink genomic DNA isolation kit (Invitrogen). Then loop-full colonies from suspected TSA plate were harvested and suspended in 400 µl of lysis buffer of DNA isolation kit. Suspension was mixed by vortexing and then centrifuged at 10000 rpm for 10 minutes. Supernatant was used further in extraction protocol of kit and finally DNA was eluted in 50 µl of elution buffer

qPCR for the DNA Extraction Control: A bovine growth hormone (bGH) gene specific TaqMan probe based real-time PCR assay was performed using universal master mix (Applied Biosystem). This was conducted to rule out the DNA extraction failure and also act as internal positive control for target specific qPCR. The primers and probe are given in the Table 1. For the 20 µl of reaction mixture, 10µl of 2x Universal qPCR mastermix, 0.25 µM of each primer and 0.125 µM of probe, 2 µl of extracted DNA and 6.5µl of NFW were mixed. The amplification was carried out at 50ºC for 2 min (activation of UDG), 95ºC for 10 min followed by 40 cycles of 95ºC for 15 s, and 60ºC for 1 min.

Identification of *Brucella* spp. using real-time PCR (qPCR): Extracted nucleic acid (DNA) from bacterial field isolate as well as from collected biological samples were analyzed using TaqMan probe based qPCR assays as described by Hinic *et al*. (2008). The oligonucleotides used are listed in Table 1. The reaction volume and cycling parameters used in this study were the same as those of *bGH* gene specific qPCR assay except for the replacement of each primer and probe for *B. abortus* and *B. melitensis.*

RESULTS AND DISCUSSION

Bacterial Isolation: Out of four aborted fetuses, *Brucella* spp was successfully isolated from stomach content of one fetus. The colonies obtained were characterized as *Brucella* spp. by colonies morphology, Gram staining, microscopic examination and biochemical tests. The isolate was oxidase positive, urease positive and produced hydrogen sulphide. Based on these characters, the isolate was suspected as being biovar 1 of *B. abortus.*

Gulhan *et al*. (2011) isolated *Brucella* spp. from aborted cattle fetuses with the 26.7% success of isolation from different regions of Turkey. In another study, Celebi *et al*. (2011) have reported *Brucella* isolation rate from milk and vaginal samples from cattle as 4.4% and 6.4%, respectively. Earlier Chand *et al.* (1987) isolated 15 (71%) *Brucella* isolates from stomach contents of 21 bovine fetuses. Since isolation of *Brucella* is cumbersome and labour intensive hence it depends upon several factors e.g. climatic conditions, quality of fetal samples etc. and might be the reason of low percentage of isolation.

qPCR: The extracted DNA of all 32 biological samples collected from dams and fetuses were analyzed in bGH, *B. abortus* and *B.melitensis* specific qPCR assays. The results of qPCR assays are given in the Table 2.

All the 32 samples were found positive in bGH specific qPCR assay with Ct (cycle threshold) in the range of 24 to 31 (Fig. 1). The results of bGH specific qPCR assay confirmed successful DNA extraction and

Table 1
List of primers and probes of qPCR assay

Oligo Name	Bacteria	Sequence 5' to 3' (include modification codes if applicable)	References
Bab-qF	*Brucella abortus*	GCACACTCACCTTCCACAACAA	Hinic *et al.*, 2008
Bab-qP		**JOE**-TGGAACGACCTTTGCAGGCGAGATC-**BHQ**	
Bab-qF		CCCCGTTCTGCACCAGACT	
Bme-qF	*Brucella melitensis*	TCGCATCGGCAGTTTCAA	
Bme-qP		**FAM** -CCTCGGCATGGCCCGCAA-**BHQ**	
Bme-qF		CCAGCTTTTGGCCTTTTCC	
bGH-F	*bGH gene specific*	CCTTCGGCCTCTCTGTCTCTC	Wang *et al*., 2007
Probe (bGH)		**FAM**-TCCCTTGGCAGGAGC-**TAMRA**	
bGH-R		TTGTCATAGGTCTGCTTGAGGATCT	

excluded the probability of PCR inhibitors in the template. In *B. abortus* specific qPCR only stomach contents and placental tissue samples of fetuses and uterine discharge of dams were found positive (Fig. 2). The results of this assay revealed that the average bacterial load was highest in placental tissue as compared to stomach content because of lower Ct value. Lack of amplification in *B. melitensis* specific qPCR assay in all tested samples confirmed that the abortions were because of *B. abortus* (Fig. 3). The findings of *Brucella* specific assays in these studies are in close agreement with the findings of Chand *et al.* (2013) who had also reported *B. abortus* specific abortions in bovines in Haryana. The advantage of molecular method (qPCR) is that the results can be obtained within a day as compared to seven days by classical microbiological technique. Out of four cases of abortion only one was found *Brucella* positive by isolation, however, all four were found positive by molecular method indicating that the sensitivity of qPCR assay was higher as compared to convention isolation technique. The molecular approaches appeared to be faster and more sensitive than traditional bacteriological tests.

In conclusion, the TaqMan real-time PCR assay was found to be useful as a rapid, easy and discriminative method as compared to conventional bacteriological cultural technique for the diagnosis of brucellosis from aborted fetuses as well as from dam. The epidemiological data generated confirms the previous findings that *Brucella abortus* is the species infecting cattle in Haryana. To control brucellosis, the results of this study and further more on identification

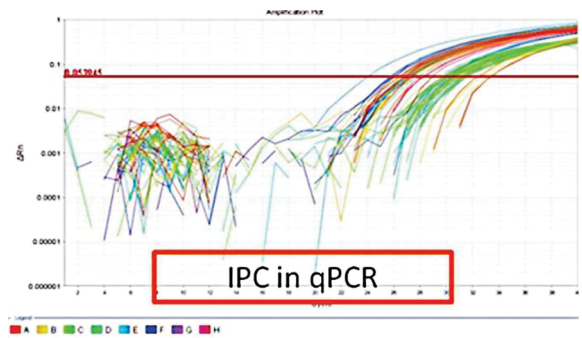

Fig. 1 : Log Amplification plot of bGH specific qPCR assay (IPC-Internal positive control)

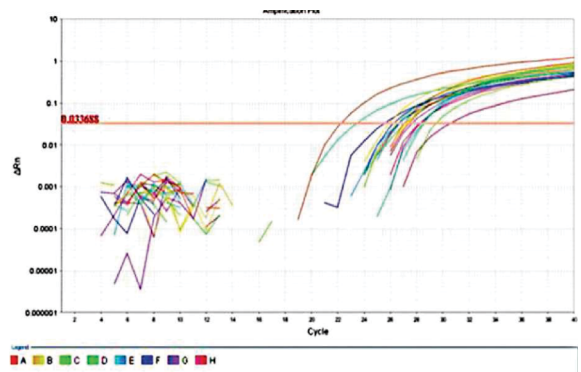

Fig. 2: Log Amplification plot of *Brucella abortus* specific qPCR assay

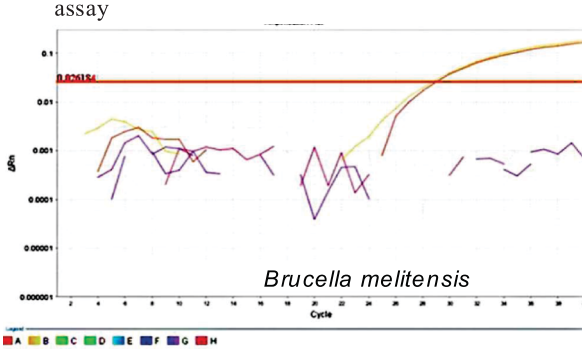

Fig. 3: Log amplification plot of *Brucella melitensis* specific qPCR assay

Table 2
Results of qPCR assays

S.N.	Animal ID	Sample type	bGH-qPCR(Ct)	B.ab-qPCR(Ct)	B.me-qPCR(Ct)
1.	CB-27-HSR-ABT	Stomach contents	26.31	32.43	Undetected
		Placenta	25.23	31.20	Undetected
		Spleen	27.89	Undetected	Undetected
		Liver	26.22	Undetected	Undetected
		Lung	27.34	Undetected	Undetected
		Heart	27.21	Undetected	Undetected
		Blood	26.39	Undetected	Undetected
		Uterine discharge	27.27	33.27	Undetected
2.	CB-28-HSR-ABT	Stomach contents	25.23	27.31	Undetected
		Placenta	24.32	28.43	Undetected
		Spleen	30.22	Undetected	Undetected
		Liver	27.12	Undetected	Undetected
		Lung	28.12	Undetected	Undetected
		Heart	26.47	Undetected	Undetected
		Blood	28.11	Undetected	Undetected
		Uterine discharge	26.78	30.11	Undetected
3.	CB-29-HSR-ABT	Stomach contents	28.11	34.12	Undetected
		Placenta	24.93	30.70	Undetected
		Spleen	28.82	Undetected	Undetected
		Liver	26.12	Undetected	Undetected
		Lung	27.34	Undetected	Undetected
		Heart	29.22	Undetected	Undetected
		Blood	29.37	Undetected	Undetected
		Uterine discharge	29.12	31.15	Undetected
4.	CB-30-HSR-ABT	Stomach contents	26.67	32.43	Undetected
		Placenta	28.11	31.20	Undetected
		Spleen	27.22	Undetected	Undetected
		Liver	26.78	Undetected	Undetected
		Lung	30.12	Undetected	Undetected
		Heart	27.61	Undetected	Undetected
		Blood	26.52	Undetected	Undetected
		Uterine discharge	27.11	31.71	Undetected
5.	CB-28-HSR-ABT(Foetus)	Bacterial culture	NOT USED	18.89	Undetected

and characterization of dominant strains throughout the state are considered to be helpful, especially for vaccine development studies.

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