Original Research

Detection of *Brucella abortus* in Cattle and Buffaloes with Spontaneous Abortion from an Organized Dairy Farm

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Abstract

Bovine brucellosis is a contagious, bacterial and economically important disease which causes late term abortions and subsequently retention of placenta in bovines. Bovine brucellosis is endemic in India affecting various domesticated animals viz. buffalo, cattle, goat and sheep. In the present study, 30(17 buffaloes and 13 crossbred cattle) animals from an organized farm with spontaneous abortion, were investigated to detect whether Brucella species was the causative agent from April 2016 to April 2017. Samples collected include fetal stomach content from the aborted fetus, placenta and blood from the aborted animal. Brucella abortus was detected by PCR using primers specific for both genus as well as species from stomach content and placenta. RBPT and c-ELISA were also conducted on the serum samples of the aborted animals. Out of the 30 animals, an overall 15 (50%) animals (11 buffaloes and 4 cattle) were positive for brucellosis by PCR, RBPT and c-ELISA. The present study may provide supportive information regarding the disease situation in Punjab, India.

Key words: Abortion, Brucellosis, Buffaloes, Cattle, PCR, ELISA

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Introduction

In dairy animals, there are several causes of abortions. Abortions may be idiopathic or may be due to either non-infectious causes like hormonal or metabolic abnormalities, nutritional deficiencies, trauma and toxicities or infectious causes which include bacteria (brucellosis, listeriosis), fungus (aspergillosis) and viruses (infectious bovine rhinotracheitis, bovine virus diarrhea) (Yaegaer, 1993). Among these, brucellosis is one of the most important causes of abortion in cattle and buffaloes.

Brucellosis is a highly contagious, zoonotic and economic important disease caused by Gram negative, facultative intracellular bacteria belonging to the Genus *Brucella*. Species of *Brucella* causing brucellosis

in domestic animals are *B. abortus* (cattle and buffaloes), *B. melitensis* (small ruminants, sheep and goats) and *B. suis* (swine). Since its first record in 1887 (IVRI, 1977), brucellosis is endemic all over India affecting animals and human. As per Singh *et al.* (2015), the prevalence of brucellosis throughout the country ranges from 6.5% to 16.4% in different species of livestock. In India, brucellosis in livestock is responsible for a median economic loss of US $ 3.4 billion where the disease in cattle and buffalo accounted for 95.6% of the total losses occurring due to brucellosis in livestock populations (Singh *et al.,* 2015). Brucellosis is an important emerging disease with a predilection for placenta and foetal membrane. The most common clinical features of brucellosis are placentitis and abortion (Silva *et al.,* 2000). In bovines, *B. abortus* is the main causative agent which causes late term abortions, placental retention, infertility and stillbirth (Gwida *et al.,* 2010). Reports are also available regarding detection of *B. melitensis* in cattle co-existing with small ruminants; sheep and goat (Samaha *et al.,* 2008, Zhang *et al.,* 2018). Human are infected with brucellosis mainly due to direct contact with infected animals or indirectly through consumption of unpasteurized milk and milk products (Radostits *et al.,* 2000). Hence, screening and diagnosis of the disease are required for proper surveillance programme.

For the diagnosis of brucellosis, there are various diagnostic techniques which includes bacterial isolation, serological [Rose Bengal Plate Test (RBPT), Indirect or Competitive enzyme-linked immunosorbent assay (ELISA)] and molecular techniques (polymerase chain reaction). Each technique has its own limitations. Though, bacterial isolation and culture is regarded as the gold standard for brucellosis diagnosis, it is laborious and requires biosafety laboratory facilities. Several serological tests are used for diagnosis with varying degree of sensitivity and specificity. Molecular techniques (PCR) are used for diagnosis of brucellosis (Cortez 2001; Richtzenhain *et al.,* 2002; Kaushik *et al.,* 2006; Mahajan *et al.,* 2017; Mittal *et al.,* 2018).

Thus, in the present study, brucellosis was diagnosed as the cause of the abortion occurring in cattle and buffaloes using RBPT and competitive ELISA on the serum of aborted animals and polymerase chain reaction (PCR) on aborted fetal stomach contents.

Materials and Methods

The study includes 30 cases of spontaneous abortions in 13 cattle and 17 buffaloes from an organized dairy farm located at Ludhiana, Punjab. The affected animals did not have the history of vaccination. Fetal stomach content from aborted fetus and blood samples from aborted animals were collected. DNA was isolated from placenta and fetal stomach content and performed PCR using both genus and species specific primers. The serum samples from aborted animals were screened for brucellosis using Rose Bengal Plate Test (RBPT) and competitive enzyme-linked immunosorbent assay (cELISA) using commercially available ELISA kit. Kappa value which measures the agreement between the two tests was also calculated. The

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result of Kappa value was interpreted as poor agreement = kappa value less than 0.20, fair agreement = 0.20 to 0.40, moderate agreement = 0.40 to 0.60, good agreement = 0.60 to 0.80, very good agreement = 0.80 to 1.00.

DNA Isolation and Polymerase Chain Reaction (PCR)

DNA was isolated from the fetal stomach content by using QIAamp Blood and Tissue Kit (Qiagen) as per the manufacturer's protocol. DNA was eluted and stored at -80°C. PCR reactions were performed as per Leal-Klevezas *et al.* (1995) with genus specific primers (JPF/JPR) and Bricker and Halling (1994) for species specific primers. A known positive control for both *Brucella abortus* and *Brucella melitensis* and a non-template control were also used in each reaction. PCR reaction for genus specific primers was carried out with slight modification in a reaction mixture (25 µl) containing 12.5 µl of Taq PCR master mix (Qiagen), 5 µl of DNA template and 0.2 µM of each primers using primer for Omp2 gene target JPF (forward) 5'-GCGCTCAGGCTGCCGACGCAA-3' and JPR (Reverse) 5'-ACC AGC CAT TGC GGT CGG TA-3'targeting an amplified regions of 193 bp.

Further, another in-house *Brucella* genus specific primer was also designed and employed to detect *Brucella* targeting the OMP 25gene. The primer sequence are VM (Forward) 5'-CGATTAACACAACCTGAAGA-3' and VM (Reverse) 5'-GGTGTAGCGATATTCCAGA-3'. All the samples which were positive for *Brucella* genus were screened further by using species specific PCR targeting the IS711 gene. PCR reaction for species specific primers was carried out which consist of a reaction mixture (25 µl) containing 12.5 µl of Taq PCR master mix (Qiagen), 5 µl of DNA template and 0.25 µM of each primers for *B. abortus* specific (forward) (20pmol/ µl) 5'-GACGAACGGAATTTTTCCAATCCC-3' and *B. melitensis* specific (forward) (20pmol/ µl) 5'-AAATCGCGTCCTTGCTGGTCTGA-3' and IS711 specific primer (reverse)(20pmol/ µl) 5'-TGCCGATCACTTAAGGGCCTTCAT-3'.Thermal cycling was performed in T Gradient Thermocycler (Biometra, Germany) for genus specific PCR (Table 1).The PCR products were analyzed by 1.5% agarose gel electrophoresis. The DNA bands were visualized with a UV transilluminator.

Table 1: Thermal cycling parameters

Primers	Thermal cycling parameters					Product Size (bp)
	Initial Denaturation	Denaturation	Annealing	Extension	Final Extension	
		35 cycles				
JPF/R	94^0C (4 minutes)	94^0C (60 sec)	60^0C (60 sec)	72^0C (60 sec)	72^0 C (10 min.)	193 bp
VMF/R	95^0C (3 minutes)	95^0C (45 sec)	50^0C (45 sec)	72^0C (30 sec)	72^0C (10 min.)	298 bp
Species specific	94^0C (5 minutes)	95^0C (75 sec)	55.5^0C (120 sec)	72^0C (120 sec)	72^0C (5 min.)	498 bp (*B. abortus*); 731 bp (B. melitensis)

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Rose Bengal Plate Test (RBPT)

All the serum samples collected were tested for the presence of antibodies against bovine brucellosis as per protocol of the OIE (OIE, 2009). After mixing of 40 µl each of test and control sera with the antigen (Punjab Veterinary Vaccine Institute, Ludhiana) the plates were gently shaken by hand for about 3-5 minutes. The results were interpreted as negative with no agglutination and positive with perceptible to fine agglutination or coarse clumping.

Competitive Enzyme-Linked Immunosorbent Assay (cELISA)

Further confirmation of the RBPT-positive samples was carried out by competitive ELISA using commercially available SVANOVIR®Brucella-Ab C-ELISA kit. The procedure was performed as per the manufacturer's protocol. The optical density (OD) was measured at 450 nm in an ELISA reader.

Results

The overall detection frequencies of brucellosis in cattle and buffaloes using PCR, RPBT and cELISA is shown in table 2&3. Out of the 30 animals, an overall 15 (50%) animals (11 buffaloes and 4 cattle) were positive by *Brucella* genus specific PCR as indicated by product size of 193 bp and 298bp. The product size detected using species specific primers was 498bp indicating that *B. abortus* was the causative agent of this abortion. None of the animals were positive for *B. melitensis*. Agarose gel electrophoresis of the products of genomic DNA from samples using primers specific for *Brucella* genus and *B. abortus* species are shown in Figs. 1-3.

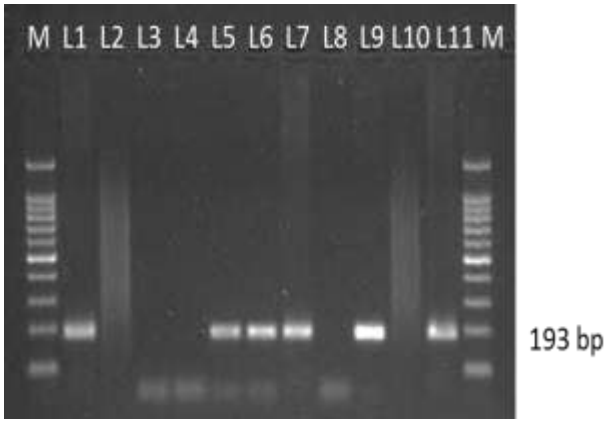

Fig. 1: Agarose gel electrophoresis of the amplified product (193bp) OMP gene with primers JPF/JPR specific for *Brucella* genus. M: 100bp ladder DNA Marker, L1: positive control, L2: negative control, L3-L11: samples.

Fig. 2: Agarose gel electrophoresis of the amplified product (298bp) OMP gene with in-house VMF/VMR primers specific for *Brucella* genus. M: 100bp ladder DNA Marker, L9: positive control, L10: negative control, L1-L8: samples.

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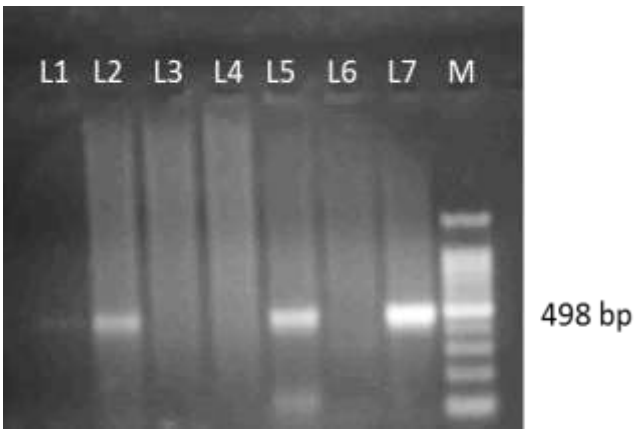

Fig. 3: Agarose gel electrophoresis of the amplified product (498bp) with primers specific for *Brucella abortus*. M: 100bp ladder DNA Marker, L7: positive control, L6: negative control, L1-L5: samples.

Further, serological analysis showed that out of 30 samples, 9 (6 cattle and 3 buffaloes) were positive by RBPT which was furthered confirmed by c-ELISA. These animals were also found positive by PCR. There was moderate degree of agreement between PCR and RBPT/cELISA as indicated by kappa value of 0.6.

Table 2: Detection frequencies of brucellosis in cattle and buffaloes using PCR, RPBT and cELISA

Test	Positive		
	Cattle	Buffaloes	Total
PCR	5/13 (38.46%)	10/17 (58.82%)	15/30 (50%
RBPT &cELISA	6/13 (46.15%)	3/17 (17.64%)	6/30 (20%)

Table 3: Cross tabulation of PCR and RBPT/cELISA results

Test	PCR (Positive)	PCR (Negative)	Total
RBPT/cELISA Positive	9	0	9
RBPT/cELISA Negative	6	15	21
Total	15	15	30

Kappa value = 0.6

Discussion

In the present study, we report the diagnosis of bovine brucellosis in spontaneous cases of abortion in an organized dairy farm in Ludhiana, Punjab. The prevalence of brucellosis among livestock animals varies extensively throughout India's diverse agro ecological landscape, differing from region to region and farm to farm (Mathur and Amarnath, 2008). In the presents study, detection of *Brucella* genus was performed using both published (JPF/JPR) primers and in-house primer (VM1/VM2) in the placenta and fetal stomach content and further confirmed as *B. abortus* using species specific primers. None of the animals were positive for *B. melitensis*. PCR targeting *Brucella* omp-2 gene has been used by other researcher in different clinical samples (Solmaz *et al.*, 2014; Mahajan *et al.*, 2017; Kala *et al.*, 2018). Out of 30 animals, 15 (50%) were positive for *B. abortus* by PCR. Kaur *et al* (2017) investigated brucellosis in cattle and buffaloes

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suffering from abortions and reproductive disorders from areas in and around Ludhiana, Punjab by conventional and molecular assays. PCR is considered to be a reliable, sensitive and specific method for the diagnosis of bovine brucellosis (Bricker, 2002). Moreover, for identification of *B. abortus* as the causative agent of bovine abortion, various PCR protocols have been developed (Cortez *et al.*, 2001; Richtzenhain *et al.*, 2002; Aslan *et al.*, 2016; Chisi *et al.*, 2017).

Among 15 PCR positive cases, 10(58.8%) were from buffaloes and 5 (38.46%) from cattle. The numbers of buffaloes affected were more as compared to that of cattle. This may be due to the fact that buffaloes have behavioral characteristics of wallowing for long period in mud which aids them expose to *Brucella* which can survive in water, urine, feces, moist soil, and manure for weeks or months under favorable humidity and temperature states (Borriello *et al.*, 2013).The infected animals expel the bacterium during abortion, or excretion via placenta, foetal fluids, vaginal discharge and milk which may serve as a source of infection for herd mates (Schlafer *et al.*, 2007). Various sero-prevalence studies on bovine brucellosis has been conducted in India (Trangadia *et al.*, 2016; Gogoi *et al.*, 2017; Kylla *et al.*, 2017; Mahajan *et al.*, 2017, Islam *et al.*, 2018) by using various serological test (RBPT, indirect-ELISA, c-ELISA, etc). In the present study, by RBPT only nine (30%) animals were seropositive which was further confirmed by c-ELISA. Competitive ELISA which we used in the present study could differentiate *Brucella*-infected animals from vaccinated animals. Thus the seropositive animals were mainly due to natural *Brucella* infections and not the vaccine strain. The lower number of seropositive animals may be due to low sensitivity of RBPT. Therefore RBPT cannot be a sole test for diagnosis of brucellosis in cattle and buffaloes.

The present study showed that PCR is more suitable for diagnosis of brucellosis in affected animals. PCR can be valuable laboratory diagnostic techniques of either chronic infections or very early stage when antibodies could not be detected (Ghorbani *et al.*, 2013). Further, the remaining 15 *Brucella* negative animals may have aborted due to other causes like metabolic or hormonal abnormalities, nutritional deficiencies, trauma, toxicities, or other infectious agents (Yaeger *et al.*, 1993). In the present study, the cases were reported from an organized dairy farm. As per (Smits and Kadri, 2005), the disease incidence is usually high in organized farms compared to the marginal herds and this primarily associated with intensive farming practices in large organized animal farms. Further, occurrence of brucellosis among livestock in India may be due to various factors such as lack of a proper control policy, failure to vaccinate young calves, non-application of test and slaughter method, and lack of proper therapeutic measures and usual practice of selling positive reactor animals to other farmers (Singh *et al.*, 2015). Thus, diagnosis of the disease and segregation from the herd; vaccination of young calves may be accomplished to control the disease in livestock and human.

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Conclusion

The present study report cases of brucellosis causing abortion in cattle and buffaloes. PCR proved as the most valuable tools for rapid diagnosis of brucellosis in cases of abortion. This study will provide additional knowledge in the field of brucellosis and its diagnostic measures. This may further provide supportive information regarding the disease situation in Punjab.

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