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Abortions in an organized dairy farm from North India reveal the possibility of breed susceptibility to Bovine Brucellosis

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ABSTRACT

The present study was undertaken over a three year period (2012–2014) in an organized dairy farm located in North India to ascertain *Brucella abortus* as the putative cause of abortion. The dairy farm maintained cattle of Frieswal, Crossbred and Sahiwal breeds and followed calf-hood vaccination with *Brucella abortus* Strain 19 live vaccine in all the heifers. Even with the recommended vaccination schedule and good managemental practices in place, 88 cases of abortions clinically suspected of bovine brucellosis (40 from Frieswal breed, 17 from Crossbred cattle and 31 from Sahiwal breed) were reported from this farm. From these abortion cases, bacteriological isolation was possible in only four dams while 16 dams were found to be serologically positive in Serum Tube Agglutination Test (STAT). Molecular screening by PCR assay (specific for the *bcsp31* gene of *B. abortus*) revealed that 24 dams were positive, out of which 20 were from Frieswal breed and rest four were from Crossbred herd. Prominently, all Sahiwal dams were found to be negative in bacteriological isolation and also in PCR assay. These results thus indicate towards the possibility of breed predisposition to abortions due to *B. abortus* infection. Statistical analysis by Fischer exact test ($p < 0.01$) too substantiated that breed susceptibility exists among these PCR positive cases. This study is novel as breed variation in abortions due to *B. abortus* in cattle is being documented for the first time. Seven representative PCR amplicons generated during the study were also sequenced and submitted to NCBI GenBank. Moreover, this study also accentuates the importance of PCR screening especially in vaccinated herd and raises concerns on over-dependence of serological assays when intensive vaccination is practised without any concomitant DIVA strategy. Thus, besides assisting in planning pragmatic control strategies against bovine brucellosis these findings are also imperative from 'One Health' context, also.

1. Introduction

Brucellosis an economically important disease known to human civilization remains a major threat for all major livestock species and poses a significant public health concern. This disease is considered by OIE as the most widespread zoonosis with an estimate of five million new cases every year [1,2]. Brucellosis is endemic globally, with exception of countries from where it has been already eradicated. Global distribution of this disease is continuously changing with emergence and re-emergence of new foci [3]. The etiological agents of the disease, *Brucella* spp. are facultative intracellular gram negative cocco-bacilli, non-spore forming, non-capsulated bacteria of α2-Proteobacteriacea family [4]. The members of the *Brucella* family exhibit host specificity and affects different livestock species including wild animals. *Brucella abortus* is usually considered the causative agent of bovine brucellosis or contagious bovine abortion. The disease is associated with abortion during the last trimester of pregnancy, stillbirths or weak newborn calves and infertility among cows and bulls [5]. Thus, screening for the pathogen in dairy herds and breeder bulls, is pivotal for adopting effective control strategies.

Bacteriological isolation of the causal organism in specific media remains the gold standard method for the diagnosis of brucellosis [6]. The isolation procedure is tedious, time consuming and involves high risk of transmission to laboratory personnel. Serological diagnosis of

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brucellosis is based on the ability of test serum to agglutinate a standardized quantity of killed *B. abortus*. The common serological tests used for routine diagnosis are Rose Bengal Plate Agglutination Test (RBPT), Serum Tube Agglutination Test (STAT), Milk Ring Test (MRT), Complement Fixation Test (CFT) and ELISA. Though these traditional serological assays are easy to perform, faster and reduce risk of laboratory acquired infection; they suffer from issues of lower sensitivity and specificity in diagnosing the disease [7]. Hence, nucleic acid based amplification techniques provide a rapid and sensitive alternative to these conventional tests. PCR based on different genes of *B. abortus* have been used for the diagnosis of brucellosis from clinical specimens [8].

The present study was focused on detecting bovine brucellosis in an organized dairy farm located in North India (Uttar Pradesh) where high incidences of abortions, retention of placenta and stillbirths were reported. A detailed study was conducted encompassing following objectives, to estimate the risk of abortion due to brucellosis among pregnant dairy cows on this large dairy farm, to compare the risk of abortion due to brucellosis among different breeds and to compare the performance of different diagnostic assays i.e. by bacteriological isolation, conventional serological test (Serum Tube Agglutination Test-STAT) and by molecular techniques (PCR based on the *Brucella* cell surface 31 kDa - *bscp31* protein gene segment and sequencing of the PCR amplicons) were employed in the study.

2. Materials and methods

2.1. Clinical specimens

Samples were collected from an organized dairy farm located in North India (Uttar Pradesh) which reported cases of abortions from cattle in their last trimester of pregnancy over a three year period from 2012 to 2014. The farm maintained > 1000 adult animals comprising of 730 dams of Frieswal (FSL) breed [Frieswal breed of cows have 57.5% - 67.5% of Holstein Friesian (HF) and 32.5% - 42.5% of Sahiwal (Sah) inheritance], 180 dams of Crossbred (XB) [cows with less or more than the range of 57.5% - 67.5% of Friesian inheritance were considered to be crossbred] and 90 dams of Sahiwal breed only.

Abortions were reported from dams of all the three breeds (40 from Frieswal; 17 from Crossbred and 31 from Sahiwal) at different gestation period, calving and age groups from this farm. Clinical specimens' viz., foetal heart blood, foetal stomach contents from the aborted foetuses, placental tissues from aborted dams were aseptically collected and transported on ice to the laboratory for bacterial isolation and nucleic acid isolation for PCR. Blood was collected aseptically from these aborted dams in BD® Vacutainer (Becton, Dickinson and Company, USA) containing clot activator and was transported to the laboratory on ice. Serum was separated from the blood samples by centrifugation at 2000*g* for 15 min and stored at − 20 °C until further use. Bacteriological, serological and molecular analyses were carried out in Central Military Veterinary Laboratory (CMVL), Meerut.

2.2. Bacterial isolation

Isolation was attempted by inoculation of morbid materials/swabs on 5% sheep blood agar and selective media. The selective media contained a nutritive Brucella agar base with 5% sterile inactivated horse serum. Inactivation of horse serum was done by heating at 56 °C for 30 min. To this medium selective antibiotic supplement was added to prevent the growth of contaminating bacteria. The supplement contained Polymixin B sulphate, Bacitracin, Nystatin, Cycloheximide, Nalidixic acid and Vancomycin. These plates were incubated both aerobically and micro-aerophilically in a CO_2 incubator at 37 °C for upto 15 days. The isolates were identified on the basis of cultural, morphological characteristics, and biochemical tests [4].

2.3. Serum tube agglutination test

Serum Tube Agglutination Test (STAT) was performed in clean grease free glass tubes (12 mm × 75 mm) according to the method described by Alton et al. [6]. Calf hood vaccination in female calves (at six to nine months of age) was practiced stringently in this farm with *Brucella abortus* strain 19 (S19) live vaccine and all the dams under study have undergone S19 vaccination during their calf hood stage. Hence, as per the guidelines of Third report of Joint FAO/WHO Expert Committee on Brucellosis [9] agglutination at 1:80 dilution (160 IU) was considered as positive and 1:40 dilution (80 IU) was considered as doubtful for brucellosis.

2.4. PCR amplification of bscp31 gene

2.4.1. Nucleic acid isolation

DNA was extracted from foetal blood and placental tissues using DNeasy® Blood & Tissue Kit (Qiagen, Hilden, Germany) while DNA from foetal stomach contents was extracted with DNeasy® Stool Kit (Qiagen, Hilden, Germany). All the DNA samples were extracted as per manufacturer's recommendations. The isolated DNA was checked for purity and quantity using Nanodrop Spectrophotometer (ND-1000, Thermo Fisher Scientific, Wilmington, USA) and stored at − 20 °C until further use.

2.4.2. Oligonucleotide primers

Primers as described earlier [10,11,12,13,14,15] targeting a 443 bp fragment in the *Brucella* cell surface 31 kDa (*bscp31*) protein gene were used in the present study. Primers sequences 5′-GGGCAAGGTGGAAG ATTT-3′ for the forward and 5′-CGGCAAGGGTCGGGTGTTT-3′ for the reverse one were custom synthesized from Bio-Serve Biotechnologies (India) Pvt. Ltd., Hyderabad, India.

2.4.3. PCR controls

DNA extracted from *Brucella abortus* (S19) vaccine vial(s) was used as a positive control, which showed a similar amplified product corresponding to 443 bp on agarose gel and was confirmed by sequencing to be specific gene fragment of *bscp31* of *Brucella abortus*. Negative control, positive template control and non-template control were also put up each time when PCR was run. For confirmation of PCR results, each clinical sample was put up at three separate times and duplicate/repeat testing of samples was conducted during the course of study.

2.4.4. PCR amplification

PCR amplification was performed in thin walled 0.2 ml PCR tubes using approximately 50–100 ng of genomic DNA, 5 µl 10 × PCR buffer, 2 mM $MgSO_4$, 1 µl of 10 mM dNTPs, 10 µM of forward and reverse primer, 2.5 U of Dream Taq DNA Polymerase (Thermo-Scientific, USA) and the volume was made up to 50 µl with Nuclease Free water (NFW). The PCR amplification was performed using thermo-cycler (Master Cycler®, Eppondorf, Hamburg, Germany) and consisted of initial denaturation at 95 °C for 3 mins followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 53 °C for 30 s, and extension at 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. The PCR amplified products (Suppl Fig. 1) were resolved on 1.5% agarose gel in Tris acetate EDTA (TAE) buffer (1 ×). The agarose gel was stained with ethidium bromide and documented under UV light in a gel documentation system (Alpha ImagerA® EP, Alpha Innotech, San Leandro, CA, USA).

2.5. Sequencing of the PCR products

The PCR products were excised from the gel using QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) following manufacturer's instructions. The purified products were assessed for quality and quantity and were forwarded for sequencing to Bio-Serve

Table 1
Table showing animal number, breed, age, clinical presentation, STAT titre, source material for PCR and the accession no. submitted to NCBI GenBank. (FSL- Frieswal; XB- Crossbred; P-Positive; N- Negative; D- Doubtful).

Animal no.	Breed	Age	Clinical presentation	STAT titre	Tissue material for PCR	Accession no.
CMVL-1/Rani	FSL	07 yrs	Abortion at 7.5 months of pregnancy	1:40 D	Placenta piece	KF564031
CMVL-2/Ceat	FSL	07 yrs	Abortion at 8 months of pregnancy with retention of placenta	1:20 N	Placenta piece	KF564032
CMVL-3/Nagin	XB	04 yrs	Retention of placenta	1:40 D	Placenta piece	KF564033
CMVL-4/Ketan	FSL	08 yrs	Retention of placenta; aborted twice before in 2006 and 2012	1:80 P	Placenta piece	KF564034
CMVL-5/Maria	FSL	05 yrs	Abortion at 7 months of pregnancy	1:40 D	Foetal stomach content and placenta piece	KF564035
CMVL-6/Lime	FSL	03 yrs	Abortion at 7.5 months of pregnancy	1:40 D	Placenta piece	KF564036
CMVL-7/Sirka	XB	05 yrs	Abortion at 5.5 months of pregnancy	1:20 N	Placenta piece	KF564037

Biotechnologies (India) Pvt. Ltd., Hyderabad, India. The sequencing was performed using Big dye terminator v3.1 Cycle Sequencing Kit chemistry (Applied Biosystems Inc., CA, USA) on an automated DNA sequencer (Applied Biosystems 3130 Genetic Analyzer, Applied Biosystems, CA, USA). The sequence chromatogram was visualized in BioEdit Sequence Alignment Editor version 7.0.5 analysis software (Isis Therapeutics, Carlsbad, CA, USA). Mega Blast was performed with the deduced sequence within the non-redundant nucleotide database (http://www.ncbi.nlm.nih.gov/Blast) to confirm the presence of *bscp31* gene specific to *Brucella abortus*. The annotated sequences were submitted to NCBI GenBank and were assigned the Accession numbers which have been summarized in Table 1.

2.6. Statistical tool

Based on the results of these three diagnostic assays, an online statistical tool (http://in-silico.net/tools/statistics/fisher_exact_test), Fischer exact test was used in the study for analysing the breed susceptibility among these abortions.

3. Results

3.1. Bacterial isolation

Brucella abortus could be isolated from only four clinical cases out of the total 88 abortions screened (4.54%). *B. abortus* on 5% sheep blood agar formed small, glistening, non-haemolytic colonies which became opaque with passage of time (Suppl Fig. 2). Modified Ziehl-Neelsen technique [16] stained smears revealed red staining cocco-bacilli. The isolates were non-motile, catalase positive, oxidase positive, reduced nitrate and were indole negative. All the three isolates were CO_2 dependent and showed unequivocal urease activity within 1–2 h.

3.2. Serum tube agglutination test

STAT screening of all the total 88 dams serum samples revealed that 16 dams (18.18%) had a titre of 1:80 (160 IU) and were positive; 15 dams (17.05%) had a titre of 1:40 (80 IU) i.e. were doubtful while the remaining samples (64.77%) as had a titre of 1:20 (20 IU) or less and were negative. On PCR screening of clinical specimens' viz., placental tissues, foetal heart blood and foetal stomach contents from all these abortion revealed that 24 (27.27%) samples were positive while controls depicted the desired results. A comparative table showing result of screening of serum samples by STAT and PCR is enumerated in Table 2. Of the four positive cases in which isolation was possible, all of them were also positive in PCR but serologically only two were positive while the remaining two were doubtful.

3.3. PCR amplification

Out of the 24 samples positive in PCR, 20 samples were from Frieswal breed and only 4 samples from Crossbred dams. *B. abortus* could not be detected in any of the 31 Sahiwal dams which showed abortion. Seven representative PCR positive samples from animals of these two breeds were excised from the gel and sequenced in an Automated DNA sequencer. Sequence chromatogram was visualized with BioEdit Sequence Alingment Editor Software version 7.0.5 and thereafter annotation BLAST was performed using NCBI database. A sequence homology of 99% at the nucleotide level was observed with other *Brucella abortus* strains specific for the *bscp31* gene fragment. The animal number, breed, age, clinical presentation, STAT titre along with the NCBI GenBank Accession number of these seven isolates has been enlisted in Table 1.

3.4. Statistical analysis

Among 40 aborted cattle in Frieswal herd 50% (20/40) were PCR positive for *B. abortus*, while it was 23.52% (4/17) for Crossbred and none in the Sahiwal herd. Statistical analysis by Fischer exact test on these PCR positive results also revealed that there exists significant breed wise difference in abortion due to *B. abortus* infection ($p < 0.01$). However, when the Fischer exact test was employed to analysis of the STAT positive cases ($\geq$ 1:80 or $\geq$ 160 IU) and positive bacteriological isolation cases, it did not revealed any significant difference between the abortive dams of the three breeds. The *p* values of Fischer exact test for the three diagnostic assays are mentioned in the Table 3.

4. Discussion

India is considered to be endemic for brucellosis as observed from serological surveys for screening of Brucella antibodies in bovine and small ruminants by various research workers [17,18,19,20]. They all have reported varying prevalence of brucellosis in dairy herds from different states of India. Brucellosis causes significant impediment in all livestock production systems and causes huge economic losses estimated to Rs. 350 million every year to this country [21]. Though, there exists close interaction between animal and animal handlers in dairy farms but this disease remains a neglected zoonosis. In view of 'One Health' approach, it is assumed that control of brucellosis in animals will definitely reduce the number of human brucellosis cases. In the absence of any pathognomic lesions and symptoms clinical diagnosis is difficult while confirmatory diagnosis of brucellosis can only be inferred by specific diagnostic assays. Brucellosis mass control and eradication programme can only be successful if it is based upon reliable screening assay best suited to socio-economic situation of the country. PCR is considered to be a reliable, sensitive and specific method for the diagnosis of bovine brucellosis [8,12]. This assay has excellent reproducibility if it is performed under good laboratory practices (GLP). PCR targeting the *Brucella* cell surface 31 kDa (*bscp31*) protein gene segment have been routinely used for the diagnosis of *Brucella* from different clinical samples [10,11,12,13,14,15].

In our study, serological screening by STAT detected, 16 positive cases (18.18%) and 15 doubtful cases (17.05%). This may be due to fact that not all infected animals produce detectable level of circulating antibodies and factors such as cross-reacting organism, calf-hood

Table 2
Comparative table showing summary of diagnostic assay results viz., Serum Tube Agglutination Test (STAT), PCR for *bscp31* gene fragment and bacteriological isolation on clinical specimens from total of 88 abortion cases observed during the study. Breed wise risk of abortion due to *B. abortus* is shown as percentage and the ratio of *Brucella* positive cases to the total number of abortions (88) are shown in the parenthesis.

SNo	Breeds	Abortions reported	PCR			STAT				Bacteriological isolation		
			Positive	Negative	Abortion percentage	Positive (≥ 1:80 or ≥ 160 IU)	Doubtful (1:40 or 80 IU)	Negative (≤ 1:20 or 40 IU)	Abortion percentage	Positive[a]	Negative	Abortion percentage
1	Frieswal (FSL)	40	20	20	22.73% (20/88)	10	8	22	11.36% (10/88)	3	37	3.41% (3/88)
2	Crossbred (XB)	17	4	13	4.55% (4/88)	4	6	7	4.55% (4/88)	1	16	1.14% (1/88)
3	Sahiwal (SAH)	31	0	31	0 (0/88)	2	1	28	2.27% (2/88)	0	31	0 (0/88)
Total		88	24	64	27.27% (24/88)	16	15	57	18.18% (16/88)	4	84	4.55% (4/88)

[a] All the four positive cases in bacteriological isolation were also found positive in PCR assay.

vaccination and endemic condition of the brucellosis in this country further impairs the serological diagnosis. Bacterial isolation was possible from only four out of 88 cases of abortions observed during the study. The main reasons for the low percent of isolation may be due to quantum of the bacteria in the clinical sample or the use of antibiotics in treatment of clinical cases and inherent difficulty of *B. abortus* to adapt to the culture milieu. Further, the contaminating bacteria present in some of the samples might have hindered in the successful isolation. Kaushik et al. [12] and Probert et al. [22] also observed that the isolation is not a sensitive method as bacteria could not be cultured from some known positive cases. PCR detected 24/88 samples (27.27%) as positive for *B. abortus*. Among 24 PCR positive cases, 20 samples were from the Frieswal breed and four were from Crossbred cows. None of the abortions among the Sahiwal dams were found positive for *B. abortus* by PCR. During the study, PCR positive was observed more from the placental tissue; rather than foetal heart blood or foetal stomach contents. The reduced positivity from the foetal tissues may be due to the presence of PCR inhibitors in the samples. Hence, for nucleic acid amplification techniques, placental tissue is the preferred clinical sample while bacterial isolation was found to be better from the foetal tissues.

The study did not reveal any case of abortion in pure Sahiwal (*Bos indicus*) breed attributable to *Brucella abortus* infection. The PCR procedure was repeated thrice to confirm this finding. Statistical analysis by Fischer exact test on PCR positive cases also revealed that there exist significant breed wise differences in abortions due to *B. abortus* infection ($p < 0.01$). PCR being ten times more sensitive assay than bacteriological isolation [12] is more suitable assay for determining possible cases of brucellosis during abortions in cattle. Hence, this assay can be used to determine breed wise differences in brucellosis related abortion among bovines. However, genetic basis of disease resistance to brucellosis has been studied sparingly in buffalo and some cattle breeds. The natural resistance to intracellular pathogen *Brucella* has been associated with the macrophage protein 1 (NRAMP1) gene polymorphism in the 3′ untranslated region- 3'UTR [23]. Recently, polymorphisms in microsatellites at the 3′UTR of the *SLC11A1* (solute carrier family 11 member A1) gene have been associated with natural resistance to *Brucella abortus* and *Mycobacterium bovis* infection in livestock species. Hasenauer et al. [24] have investigated the role of this particular gene polymorphism in *Bos taurus*, *Bos indicus* or Crossbred animals. Previous studies from the same farm carried out in our laboratory (CMVL) found that none of the animals from the native breed (Sahiwal) were infected with bovine tuberculosis while the Frieswal and Crossbred animals were susceptible [25]. The possible inherent resistance of the Sahiwal dams to brucellosis related abortions may be attributed to the polymorphism at these loci. Though, this study has been done from a single farm, breed susceptibility of cattle to brucellosis need to be studied on a larger population size from different indigenous (*B. indicus*) breeds, so as to potentiate the findings from current study.

Immunoprophylactic vaccination with *B. abortus* strain 19 vaccine was being routinely and stringently carried out at six to nine months of age in the heifers in this particular farm so as to prevent the interference of maternal antibodies which may prevent the production of effective immune response. There have been previous reports by various researchers that there are abortions of the pregnant animals also in the vaccinated herds [26,27,28]. The probable reasons for the failure may be due to the failure of the animals to generate protective antibody titre against the quantum of bacterial infection. There are also possibilities of breed difference in the antibody response to the vaccine [28].

5. Conclusion

Novelty of present study lies in variation observed among three different breeds (Frieswal, Crossbred and Sahiwal), in susceptibility to abortions caused by *B. abortus*. Findings from the study pertinently emphasizes that results from serology are not often conclusive due to interference from vaccination antibody titres. Large scale calf-hood vaccination is being followed in this country as part of the control program of brucellosis. However, without companion DIVA strategy it is very difficult to confirm the presence of infection based on serological

Table 3
The table depicts the breed wise association between *Brucella* positive abortion cases tested by three diagnostic assays; PCR, serum tube agglutination test (STAT positive titres ≥ 1:80 or ≥ 160 IU) and bacteriological isolation. Dissimilar superscripts a, b, c indicates significant difference between the number of *Brucella* positive cases from three different breeds (Frieswal, Crossbred and Sahiwal) when tested with PCR. Bacteriological isolation and STAT (positive titres ≥ 1:80 or ≥ 160 IU) did not reveal any significant difference between the number of *Brucella* positive cases from three breeds. *P*-values of Fischer exact test applied to different tests have been provided (http://in-silico.net/tools/statistics/fisher_exact_test).

S no	Breeds	Total no of abortions	PCR positive for *Brucella* spp.	Fisher exact test (*p*-value)	STAT positive for *Brucella* spp.	Fisher exact test (p-value)	Bacteriology isolation for *Brucella* spp.	Fisher exact test (p-value)
1	Frieswal (FSL)	40	20[a]	$p < 0.01$	10	$p = 0.093$	3	$p = 0.2518$
2	Crossbred (XB)	17	4[b]		4		1	
3	Sahiwal (SAH)	31	0[c]		2		0	
Total		88	24		16		4	

a, b, c- are dissimilar superscripts, indicating significant difference between cases of abortion found positive for *Brucella* by PCR ($p < 0.01$)

assays. In such scenario, molecular tools viz., PCR and sequencing can be relied upon for conclusively proving the presence of the organism and the cause of abortion in the vaccinated herds. Moreover, this study also reveals the possibility of genetic susceptibility and of vaccination failure in exotic germplasm. Thus, there is need for undertaking studies on the genetic resistance of different cattle breeds to brucellosis. These pertinent points will definitely help in control of bovine brucellosis, which in turn is directly linked to control of human brucellosis in developing countries like India.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.onehlt.2017.11.001.

Conflict of interest statement

We declare that all the authors have no conflict of interest.

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