Abnormal 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 allconstantly. 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 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-Proteobacteria 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 stan-
dardized 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 la-
boratory 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 re-
ported. A detailed study was conducted encompassing following ob-
jectives, 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 iso-
lation, 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 con-
sidered 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
2000g 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 CO2 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’-GGGCAAGGTCGGGTGTTT
ATT-3’ for the forward and 5’-CGGCAAGGTGCGGTGTTT-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 corre-
sponding to 443 bp on agarose gel and was confirmed by sequencing to
be specific gene fragment of bscp31 of Brucella abortus. Negative con-
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 MgSO4, 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®, Eppendorf, Hamburg, Germany) and consisted of initial de-
naturation 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 am-
plified 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 doc-
umentation system (Alpha Imager® 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
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 for \textit{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

\textit{Brucella abortus} could be isolated from only four clinical cases out of the total 88 abortions screened (4.54%). \textit{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 cocaco-bacilli. The isolates were non-motive, 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. \textit{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 Alignment 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 \textit{Brucella abortus} strains specific for the bscp31 gene fragment. The animal number, breed, age, clinical presentation, STAT titre along with accession no. submitted to NCBI GenBank 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 \textit{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 \textit{B. abortus} infection (p < 0.01). However, when the Fischer exact test was employed to analysis of the STAT positive cases (≥ 1:80 or ≥ 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 \textit{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 pathogonomic 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 \textit{Brucella} cell surface 31 kDa (bscp31) protein gene segment have been routinely used for the diagnosis of \textit{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

| 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 7 months of pregnancy | 1:20 N     | Placenta piece          | KF564037      |
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 foetal 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 nine 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 2

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

*All the four positive cases in bacteriological isolation were also found positive in PCR assay.*

### Table 3

| 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) | Bacteriological isolation for Brucella spp. | Fisher exact test (p-value) |
|------|-------------|-----------------------|-------------------------------|-----------------------------|---------------------------------|-------------------------------|---------------------------------------------|-----------------------------|
| 1    | Frieswal (FSL) | 40                  | 20                            | p < 0.01                    | 10                             | p = 0.093                     | 3                                           | p = 0.2518                   |
| 2    | Crossbred (XB) | 17                  | 4                             | 1                           | 4                              | 2                             | 0                                           | 4                           |
| 3    | Sahiwal (SAH) | 31                  | 0                             | 2                           | 2                              | 4                             | 4                                           | 4                           |
| Total| 88          | 24                  | 16                            |                             | 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.

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Conflict of interest statement

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

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