Molecular detection and epidemiology of *Brucella* in dairy cattle of Bangladesh

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**ABSTRACT**
*Brucella*, a Gram – (ve) bacterium, causes late abortion in dairy cattle. The genus *Brucella* has six recognized species on the basis of host specificity but the greatest economic impact results from *Brucella abortus*. *Brucellosis* is considered as one of the most widespread zoonoses in the world by the Food and Agriculture Organization (FAO), the World Health Organization (WHO) and the Office International des Epizooties (The World Organization for Animal Health, OIE). It is an important zoonotic disease which causes significant losses in reproduction of sexually mature animals. In pregnant cows, brucellosis is characterized by late abortion, birth of weak calves, stillbirths and infertility. Therefore, a cross-sectional study was conducted in commercial dairy cattle in Bangladesh to describe the epidemiological scenario of *Brucella*. Nineteen upazillas (sub-districts) from 12 districts of 7 divisions were randomly chosen for the study. A total 44 dairy cattle farm, 1–6 farms per upazilla was recruited based on the presence of abortion history within past six months. A pretested questionnaire was used to collect epidemiological information through face to face interview and direct observation. Blood samples and aborted fetuses were collected for laboratory evaluation. Aborted fetuses were collected only from Chattogram Metro. Sero–prevalence for *Brucella abortus* was evaluated on the samples obtained using OIE protocol based iELISA technique. Aborted fetuses were evaluated through bacteriological culturing followed by PCR. The overall sero–prevalence of *Brucella* was 1.50 in dairy cattle of Bangladesh (Herd level and individual level). In this study, iELISA is more specific and sensitive for the detecting the *Brucella* antibody. PCR gives more accurate and precious results for detecting *Brucella abortus*.


**1. INTRODUCTION**

*Brucella* is a Gram negative, facultative intracellular cocco – bacillus, or short rod bacteria, within the family of *Brucellaceae* (Baek et al., 2003; Kakoma et al., 2003). According to the host specificity, *Brucella* has six recognized species: *Brucella abortus*, *B. melitensis*, *B. suis*, *B. ovis*, *B. canis* and *B. Neotomae* (Foster et al., 2007). *B. abortus* has seven biovars (1–6 and 9) and usually causes brucellosis in cattle, bison and buffalo (Acha and Szyfres, 2003). Wildlife reservoirs carry the largest risk to cattle herd re-infection. *B. abortus* causes abortion and reduces milk production in dairy cattle as well as decrease the survival rate of newborns (Bricker and Halling, 1994; Roth et al., 2003).
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Brucella–prevalence and Brucella sero – prevalence was reported to be higher in dairy cattle, cross-bred cattle, adults, pregnant cattle and late stage fetuses in cattle living with other livestock or those supplied contaminated water or were serviced by infected bull (Mayer, 1980; Shuaibi, 1999; Amin et al., 2005; Gul and Khan, 2007; Jittapalapong et al., 2008; Dinka and Chala, 2009; Munir et al., 2011; Rahman et al., 2011; Abubakar et al., 2012).

Commonly used serological tests for Brucella spp. includes Rose Bengal Plate Test (RBPT) (Amin et al., 2005; Uzal et al., 1995), Indirect – Enzyme Linked Immuno Sorbent Assay (iELISA) (Rojas and Alonso, 1994; Shafee et al., 2012; Uzal et al., 1995), Complement Fixation Test (CFT) (Alton et al., 1975, 1988; MacMillan, 1990), Tube Agglutination Test (TAT) (Rahman, 2004). Frequently used diagnostic tests to isolate or identify Brucella are bacteriological culture ((Dehkordi and Taghizadeh, 2012) and Polymerase Chain Reaction (PCR) (Dehkordi and Taghizadeh, 2012; Moshkelani et al., 2011; Yazdi et al., 2008). As there are variable level of sensitivity and specificity of Brucella diagnostic tests, the present study therefore used iELISA to determine Brucella antibody and bacteriological culture and PCR to identify Brucella. The specific objectives of the present study were: to estimate sero – prevalence of Brucella in dairy cattle in Bangladesh, to determine Brucella prevalence in aborted fetuses of dairy cattle in Chattogram, Bangladesh, Molecular detection of Brucella through PCR.

2. MATERIALS AND METHODS

Study Period: June, 2013 to June, 2014

Study Area: 44 selected dairy farms from 73 sero – positive farms under the study periods by I –ELISA (OIE, 2009).

A cross-sectional study was carried out in commercial dairy cattle in Bangladesh during June – July 2013. The study covered 7 divisions of 7, 12 districts of 64 and 19 upazillas (sub–districts), or Thanas of 467. A total 44 dairy cattle farms were recruited with the consultation of local practicing cattle veterinarians of respective sub-districts based on abortion history of farms within the past six months. Accordingly, 1 – 6 farms were randomly chosen from each upazilla, or Thana, for the study. A total of 592 cattle (4 – 66 lactating or non-lactating cattle or both per farm) were randomly selected for the study. The age of the selected animals ranged from 3 – 6 years.

Three interviewers (two MS students and one PhD student from Chattogram Veterinary and Animal Sciences University, CVASU, Bangladesh were thoroughly trained in surveying and interviewing techniques at CVASU. A questionnaire was developed in English. The questionnaire contained closed (yes/no/multiple choice) and open questions. It explored farm characteristics and individual animal level information i.e., farm location, farmers’ education, number of employees, farm size, source of animals, source of semen, AI inseminator, abortion history, still birth history and age, breed, body condition score, parity, lactation stage and physiological status of individual animals. The questionnaire was pre–tested with three dairy cattle farms in Chattogram. None of these farms were included in the main study. Questions were updated following the pilot testing with ambiguous questions being eliminated or modified. Comments and suggestions made by the respondents in the pilot-testing were also addressed to improve the questionnaire. Farmers were interviewed on their own premises. Immediately before the interview and verbal consent of the respondent was acquired. Interviewers interviewed 2 – 3 farmers per day. Interview length lasted approximately 45 minutes.

Age was assessed based on farm records or dentition (Degefu et al., 2011). Breed was confirmed by phenotypic characteristics (Amin et al., 2005). Body condition score was evaluated as per criteria described by Abubakar et al. (2012).

Samples: Sera (592) from 7 Divisions; and aborted fetuses (25) from Chittagong Division

Experimental Design: Samples collected from aborted fetuses (lungs, kidneys, liver and eyes) were inoculated on Columbia Agar Base and Brucella Selective Supplement plus Horse Serum specific to Brucella for growing and isolating. The inoculated plates were then incubated at 37°C and 5% CO₂ for 7 days. After growth, several colonies were transferred into 50% glycerin and kept at – 20°C for molecular testing. DNA from cultured samples of Brucella spp. was extracted using the FABGK001 (50 preps) DNA extraction kit and its extraction protocol
A published PCR protocol was used for \textit{Brucella} evaluation (Dehkordi et al., 2012). The primers derived from the \textit{rrs} (16S) gene of \textit{B. abortus}, primer A, 5'-GCCGCAGGCTGGAGGCAAC-3' and primer B, 5'-ACCAAGATGCTCGGCGGTGTA-3' (Dehkordi et al., 2012) and 5 ml of template sample DNA.

An iELISA developed by SVANOVAR \textit{Brucella}–Ab I–ELISA (Article Number 10 – 2700 – 10) was used to detect IgG antibodies against acute infection of \textit{Brucella abortus}. The OIE protocol was followed for testing serum samples (OIE, 2009). The ratio of the Percent Positivity (PP) value of test sample and positive control of equal or more than 40 was considered as \textit{Brucella abortus} sero–positive.

The detailed iELISA procedure is given as below:

1. Conjugate was prepared by adding 11.5ml sample diluent buffer to each one of five vials. For preparation of sample and control, a dummy plate was taken.
2. A total of 196µl sample diluents were added to each well of this dummy plate. Then 4µl negative control (NC) was added in 1st well (A1) and 2nd well (B1,) and 4µl positive control (PC) was added in 3rd well (C1) and 4th well (D1). A total of 4µl sample was added in rest of the wells and mixed them properly.
3. Then an ELISA plate was taken. A total of 100µl diluted sample was loaded from the dummy plate. This was incubated at 37°C for 1 hour in shaking incubator. It was then washed 4 times by 200µl washing buffer in each well.
4. A total of 100µl conjugate was added in each well and then incubated at 37°C for 1 hour in shaking incubator. It was washed 4 times by 200µl washing buffer in each well.
5. A total of 100µl substrate was added in each well and incubated 15 minutes at 37°C. A total of 100µl of stop solution was added to each well.
6. Then it was read at 405 nano–meter (nm) with a corrected optical density (OD) using a reference filter. Calculation of results was done as described below:

\[
PP = \frac{OD_{Sample \ or \ NC}}{OD_{PC}} \times 100
\]

(Here, OD = Optical Density; NC = Negative Control; PC = Positive Control)

PP of ≥40 was considered as positive.

Data analysis: Descriptive and summative statics were used on the results of Bacteriological and PCR test results. Statistical analysis was carried out on field and laboratory data as required by using STATA Software. A \( p \) – value of <0.05 was considered statistically significant.

3. RESULTS

Sero-prevalence of \textit{Brucella}

The overall sero–prevalence of \textit{Brucella abortus} 1.5% in dairy cattle of Bangladesh (Table 1)

Table 1. Overall sero–prevalence of \textit{Brucella} in dairy cattle of Bangladesh accounting different cluster variables (N=592).

Univariate association

\textit{Univariate association between factors and the sero–prevalence of \textit{Brucella}}

Table 2. Univariate analysis of the bacteriological result of \textit{Brucella} in Cattle, Chattogram.

Cattle of older age group (≥4.6 years) had higher sero– prevalence than cattle of younger age.
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group (≤4.5 years) (*p*=0.74). Fetus age group had similar findings as cow age group (*p*=0.69).

**Laboratory identification and prevalence of Brucella**

**Bacteriological identification of Brucella in aborted fetus**

Five (20%, all specimens of individual fetus were positive) of aborted fetuses (N=25) were determined as *Brucella abortus* positive on bacterial evaluation. Out of 100 individual specimens (4 specimens per fetus: eye – ball, liver, lung and kidney), 20 specimens were turned out as the positive of *Brucella abortus*. The distribution of *Brucella abortus* by specimens as follows: 5 in eye – ball, 5 in liver, 5 in lung and 5 in kidney. Age of cow and age of abortion of fetuses had no effect on the occurrence of *Brucella abortus*.

**Molecular identification of Brucella**

All 20 individual specimens of 5 *Brucella abortus* positive aborted fetuses (based on bacterial culture) were also PCR *Brucella abortus* positive. The results of PCR of *Brucella abortus* are presented in Table 3 and Figure 1.

### Table 3. PCR result for *Brucella abortus* from aborted fetuses.

<table>
<thead>
<tr>
<th>Fetus ID</th>
<th>Eyeball</th>
<th>Liver</th>
<th>Lung</th>
<th>Kidney</th>
<th>Pooled sample</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fet 1</td>
<td>+ve</td>
<td>+ve</td>
<td>+ve</td>
<td>+ve</td>
<td>+ve</td>
<td>(113bp)</td>
</tr>
<tr>
<td>Fet 11</td>
<td>+ve</td>
<td>+ve</td>
<td>-ve</td>
<td>+ve</td>
<td>-ve</td>
<td>+ve</td>
</tr>
<tr>
<td>Fet 14</td>
<td>+ve</td>
<td>+ve</td>
<td>-ve</td>
<td>-ve</td>
<td>+ve</td>
<td>(113bp)</td>
</tr>
<tr>
<td>Fet 24</td>
<td>+ve</td>
<td>-ve</td>
<td>+ve</td>
<td>+ve</td>
<td>-ve</td>
<td>+ve</td>
</tr>
<tr>
<td>Fet 25</td>
<td>+ve</td>
<td>+ve</td>
<td>+ve</td>
<td>-ve</td>
<td>+ve</td>
<td>(113bp)</td>
</tr>
</tbody>
</table>

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Figure 1. An ethidium bromide-stained agarose gel of PCR products was showing the sensitivity of the assay where DNA marker 50bp and band at 113bp.

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