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SERO-MOLECULAR EPIDEMIOLOGY AND RICK FACTORS ANALYSIS OF BRUCELLOSIS IN HUMAN AND LACTATING COWS OF MILITARY DAIRY FARMS IN BANGLADESH

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ABSTRACT

Background: Brucellosis is a neglected re-emerging important zoonotic disease in the developing world. Most of the research on brucellosis was limited on the sero-epidemiology during the last 50 years and recently molecular techniques have been initiated to study brucellosis in Bangladesh.

Objectives: The objectives of this study were to determine sero-molecular prevalence, identify risk factors and detect Brucella species associated with bovine and human brucellosis in Bangladesh

Materials and Methods: Serum and milk samples from 1003 lactating dairy cows of eight military dairy farms and 715 serum samples of dairy farm workers and hospital patients were collected during the 36 months period from 2017 to 2020. All the collected sera and milk samples were tested with four different commercial diagnostic test kits to detect the prevalence of Brucella infection. The four sero-positive milkers sera and milk, and all animal samples collected from aborted cases were tested for Brucella genus-specific RT-PCR and Brucella species-specific DNA (*B. abortus* and *B. melitensis*) Multiplex PCR. Conventional PCR and sequencing were also performed. Univarient and binary logistic regression were used to identify important risk factors of brucellosis.

Results: The overall 2.39% sero-prevalence of Brucella infection was recorded with all the CFT, SAT and ELISA assay and 3.09% with RBT, whereas only 0.20% tested milks samples showed positive with MRT in the lactating dairy cows. The *B. abortus* DNA was amplified from all of the four RBT positive human serum samples tested. Phylogenetic tree of partial 16S ribosomal RNA sequences of the PCR products was closely matched with *B. abortus.* Three variables (age, parity and abortion) were found to be significantly associated with *B. abortus* infection in lactating cows.

Conclusions: *B. abortus* is the causal agent of bovine brucellosis which is identified as the first time as an etiological agent of human brucellosis in occupationally exposed dairy farm workers in Bangladesh. This study could not detect the most important zoonotic *B. melitensis* DNA either in humans or animal samples, even in any earlier studies and therefore, further studies are required to explore the occurrence of *B. melitensis* in human and animal population in Bangladesh.

Keywords: *B. abortus*, Lactating cows, Military dairy farms, Sero-tests, Genus specific RT-PCR, Species

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INTRODUCTION

Brucellosis is one of the most important ancient endemic and re-emerging zoonotic diseases that still hinder livestock productivity and human health with economic impacts attributable to human, livestock and wildlife in the world.[1-3] The major economic impact of animal brucellosis because the infection causes abortion, stillbirth and reduces fertility in herds while human brucellosis is a debilitating disease characterized by fever, sweating, pain and arthralgia.[4] This disease has been controlled or eliminated in livestock population in many developed countries[1,5,6] but it remains as a neglected endemic zoonosis in the developing world[4,7-9] with the occurrence of half million new cases per year in humans and millions of infections in animals.[1,10] A large variety of mammals (domestic, farm, wild and marine) and birds (partridges, quails) are susceptible to Brucella infection.[11] There are at least 11 species of facultative intracellular bacteria of the genus Brucella have been documented and confirmed based on their particular host, pathogenicity and genomic and phenotypic characteristic.[12,13] The 'classical' six species are *B. abortus* (cattle), *B. melitensis* (sheep and goats), *B. suis* (pig), *B. canis* (dog), *B. ovis* (sheep) and *B. neotomae* (desert wood rats).[11,13] Two species have been isolated from marine animals which include *B. ceti* (cetaceans- dolphins & whales) and *B. pinnepedialis* (seals), *B. microti* from the common vole (*Microtus arvalis*) from middle Europe,[14,15] *B. inopinata* isolated from a breast implant wound of a North American female patient[16] and more recently, *B. papionis* isolated from baboons.[17] Most of the species of Brucella can infect multiple species of animals (cross-species transmission), including humans.[18,19] In cattle, the infection is predominantly caused by *B. abortus*, less frequently by *B. melitensis* and occasionally by *B. suis*.[20] Five out of 11 known Brucella species can infect humans, of which *B. melitensis* is the main zoonotic importance followed by *B. abortus*, *B. suis* and *B. canis*.[7,8,21] The zoonotic nature of marine *B. ceti* has been documented.[21] The similarity of these species has been estimated at 100% in some parts of the genome,[22] whereas 8 (1-7,9) biovars are recognized for *B. abortus*, 3 for *B. melitensis* and 5 for *B. suis*.[23-25] However, bio-varieties of Brucella vary with respect to geographical region which include *B. abortus* biovar 1 in Egypt, Eire, Northern Ireland, New Zealand and UK, biovar 2 in Iran, biovar 3 in Iran, Turkey, UK and Bangladesh, biovar 6 in Sudan and 1, 3, 6 from Italy have been reported.[21,26-29] Serological tests are commonly used for Brucella diagnosis in ruminants especially at herd level but cross-reactions with other Gram-negative bacteria are a major problem.[30-32] Rose Bengal test (RBT), Complement fixation test (CFT) and Slow agglutination test (SAT) are widely used for the detection of sero-prevalence of brucellosis.[33] The sensitivity of RBT fulfills the requirements for surveillance of free areas at herd level but it is believed that only the combination of RBT and CFT in infected herd can obtain an accurate individual sensitivity in test-and-slaughter programs.[34] Review reports showed that 3.7% cattle, 4.0% buffaloes, 3.6% goats, 7.3% sheep, 4.8% pigs and 4.0% dogs found Brucella sero-positive in Bangladesh.[35,36] The most of the research reports on brucellosis in the developing countries including Bangladesh have been made on serological prevalence and sero-epidemiology.[35,36] An important shortcoming of brucellosis serology is the impossibility to infer which (smooth) *Brucella* spp. induced antibodies in the host.[37] There is a common practice of raising both the small and large ruminants in mixed farming in most of the developing world which has

reported to be a risk factor and a central question that has to be answered is whether cattle are infected with *B. melitensis* or with *B. abortus* or with both Brucella species.[37] Therefore, the isolation, identification and molecular characterization of *Brucella* spp. in human and the different livestock species needs to be undertaken to define a sound conceptual framework, identify the source of infection and plan appropriate control measures. Recently attempts have been made to detect Brucella species by using molecular techniques in Bangladesh but in an earlier attempt failed to detect Brucella species from aborted fetal samples of goats and then detected *B. abortus* species from sera of cattle and buffaloes but reported as surveillance of *B. abortus* and *B. melitensis* in the titles of their articles.[38,39] In addition, *Brucella* spp. detected in 14 bovine sera and all have been reported positive to *B. abortus*.[40] Then 13 sero-positive human and six animal samples have been amplified *B. abortus* DNA[41] and *Brucella* spp. have been detected in milk samples of sero-negative cows.[42] More recently, *Brucella* spp. have been isolated from uterine discharge (n = 7), milk (n = 2) and vaginal swab (n = 1) of 10 aborted dairy cows with identification of *B. abortus* biovar 3.[28] The genome sequence of *B. abortus* biovar 3 strain has also been detected from an aborted dairy cow samples of a Savar dairy farm of Bangladesh.[29] Even isolation of *B. abortus*, evaluation of humoral immune response of heat-inactivated *B. abortus* biovar 3 vaccine and hemato-biochemical and therapeutic responses of chronic bovine brucellosis in cattle have been reported from Bangladesh[43-45] The most important advantages of understanding the molecular epidemiology of brucellosis is for identification of the specific corresponding vaccinal strains to be used for the control of the disease in the specific region. The most important risk factors have been reported to be associated with brucellosis can be categorized into (a) the biology of the pathogen (pathogen factors), (b) animal management factors (age, sex, species or breed), (c) herd management (herd size, number of species, contact with wild animals or type of animal production), (d) farm management (facilities, cleaning and disinfection or veterinary support) and (e) farmers' knowledge about the disease.[11] The identification of the risk factors of brucellosis that maintain the infection in animals and/or the environment is required to achieve the cost-effective and efficient control and eradication of brucellosis in herds and country. A preliminary sero-prevalence study on brucellosis in 744 lactating cows and 347 in contact humans in eight military dairy farms by using RBT showed an average of 2.3% and 0.7% positive, respectively.[46] This paper describes the sero-epidemiology and molecular detection of Brucella species associated with lactating dairy cows and in contact humans of the military dairy farms in Bangladesh.

MATERIALS AND METHODS

A cross sectional study was conducted on sero-epidemiology of brucellosis in 1003 dairy lactating cows of the eight military dairy farms located in Savar, Isurdy Lalmonirhat, Jossore, Chattagram, Comilla, Shornodip and Trishal of Bangladesh during the period between 2017 and 2020. Simultaneously blood samples of 715 humans including dairy farm workers and patients of two combined military hospitals (CMH) and a civil hospital (Mymensingh Medical College Hospital) were also tested to identify the risk factors of positive cases and asses the zoonotic impact of these cases. Sero-positive sera and samples of aborted cows were tested to detect the Brucella species by using molecular methods.

Collection of blood samples

Approximately 10 ml of venous blood was collected from each of the randomly selected 1003 lactating dairy cows with disposable needles and venoject tubes, properly labeled cow's number with keeping individual animal record in registered book and after proper clotting at room temperature for three hours and then transported to the laboratory on ice within 12 hours of collection. Similarly approximately 5.0 ml of venous blood samples of each of 715 humans were collected with the cooperation of medical doctors from farms and hospitals. All the collected blood samples of both the animals and humans were kept in the refrigerator at 4 ^{0}C in the Livestock and Human Brucellosis Lab, Department of Medicine, BAU overnight. Then all tubes with blood were centrifuged at 5000 rpm for 15 minutes for serum separation. After centrifugation, the supernatants were collected in sterile duplicate Eppendrof tubes for each sample by pipettes 1.5 ml / tube and labeled properly (by keeping record on registered) to identify the individual cow and human sample accurately and finally stored at - 20 ^{0}C until tested. One aliquot was used for testing and the other was preserved in a serum bank.

Animal samples

Milk samples were collected from each of the 1003 lactating cows for detection of Brucella antibodies by using Milk Ring Test (MRT). Milk, vaginal swabs and placentas were collected from Brucella positive aborted cows within 0 to 3 days of abortion and used to isolate and detect Brucella genus and species specific DNA.

Questionnaire for data collection

A structured questionnaire was used to collect data on cattle herds' health and management and individual blood collected humans including risk factors associated with brucellosis on the sampling days.

Rose Bengal Test (RBT)

Serum samples were analyzed with RBT antigen for the detection of Brucella antibodies by using a standard procedure.[47] Briefly, sufficient antigen, test sera, positive and negative control sera for a day's testing were removed from refrigerator and kept at room temperature. For serological analysis, equal volumes (30 µl) of serum and RBT antigen (concentrated suspension of *B. abortus* biotype 1, Instituto de Salud Tropical Universidad de Navrra, Spain) were mixed and rotated on a glass plate for four minutes. If agglutination was observed after 4 minutes, samples were considered positive, otherwise they were considered negative for brucellosis.

Slow Agglutination Test (SAT)

The SAT was performed with the Protocol No. SLA-2020-RB-004 as per the instruction of OIE Reference lab of brucellosis in Germany.[48,49] Briefly, it was carried out with EDTA and *B. abortus* biotype 1 (Weybridge 99) (Synbiotics Europe, France) was used as antigen. In the first well of a 96-well micro-titer plate, 168 µl of slow seroagglutination (SAW) buffer was added and 100 µl in the second and the third wells. To obtain a 1: 6.25 dilution, 32 µl of serum was added in the first well. After mixing of serum and diluent, 100 µl from the first well was transferred to the second well to obtain a 1:12.5 dilution. Similarly, 100 µl was transferred from

the second to the third well (dilution 1:25) and 100 µl was discarded from the third. To obtain the serial serum dilutions of 1:12.5, 1:25 and 1:50, 100 µl of standardized SAW antigen was added in each well. The micro-titer plates were agitated and incubated for 20-24 hours at 37 ^{0}C. Reading of the results was done on the basis of degree of agglutination and expressed in IU. As prescribed by OIE, any serum with an antibody titer ≥ 30 IU/ ml was considered positive.[49]

Compliment-Fixation-Test (CFT)

Compliment Fixation Test (CFT) was performed with the Protocol-No. KBR-2020-RB-006 as per the instruction of OIE Reference lab of brucellosis in Germany.[49]

Milk Ring Test (MRT)

The MRT was performed as recommended by the MRT antigen manufacturer.[47] Briefly, MRT antigen (Ring test reagent, Institut Pourquier, Montpellier, France) was kept at room temperature for one hour before starting the test. The test was performed by adding 30 µl of antigen to a 1.0 ml whole milk with the highest of the milk column in the tube was at least 25 mm. The milk + antigen mixtures were incubated at 37 ^{0}C for 1 hour, together with positive and negative control samples. A strongly positive reaction is indicated by formation of a dark blue ring above a white milk column. Any blue layer at the interface of milk and cream should be considered positive as it might be significant. The test was considered to be negative if the color of the underlying milk remains homogeneously dispersed in the milk column. If the milk at the bottom of the tube becomes gradually whitened, the result was regarded as inconclusive and the test was repeated.[50]

Enzyme Linked Immunosorbent Assay (ELISA)

Level of antibody was detected by Antibody Test Kit (IDEXX Montpellier SAS, France) according to the protocol of the manufacturer and reading was performed by automated ELISA reader. Briefly, all reagents were equilibrated at room temperature (RT) and the coated plate were removed from the foil sachet and inserted into the strip holder. Four micro-wells were used for control (two positive controls and two negative controls). 190 µl of dilution buffer N.2 was dispensed into each well. 10 µl of undiluted positive and negative control solution were pipetted into the respective control wells. 10 µl of undiluted samples were dispensed into remaining wells and gently mixed after tapping. Then the plate was incubated for one hour at RT. Then each micro well was washed with washing solution for three times. 100 µl of conjugate was added to each well and sealed the plate following incubation for 30 minutes at RT and then washed with the washing solution for three times. 100 µl TMB substrates was added to each well and kept for 20 minutes at RT away from direct light. Finally 100 µl of stop solution was added to each well and OD value was read at 450 nm within 5 minutes

Molecular methods

DNA Extraction

Eight sero-positive samples were used to extract DNA. DNA was extracted from samples using the DNeasy spin columns (Qiagen Inc., Valencia, Calif, USA) according to the manufacturer's protocol. A high purity PCR template preparation kit (Roche Diagnostic,

Mannheim, Germany) was used for the extraction of DNA according to manufacturer's instructions. A ND-1000 UV visible spectrophotometer (Nano-Drop Technologies, Wilmington, DE) was used for checking the purity of DNA and its concentration. Then, the DNA samples were stored at -20 ^{0}C for further analysis.

To test the specificity of the multiplex assay, an extensive panel of well-characterized Brucella and non-Brucella strains was assembled and tested. Identification of Brucella strains was performed using partial 16S rRNA sequencing. Crude nucleic acid extracts were prepared by re-suspending a 1-µl loop of bacteria into 100-µl of TE buffer (10 mM Tris, 1 mM EDTA; pH 8), boiling the suspension for 10 min, and pelleting the cellular debris by centrifugation. The supernatant was collected as the crude DNA extract. The ability to amplify DNA from non-Brucella strains was demonstrated using a real-time PCR assay targeting the 16S rRNA gene.

Conventional PCR and sequencing

The conventional PCR was performed as per the method described earlier.[43] Briefly, the partial 16S ribosomal RNA sequences were edited in Geneious v11. The available reference nucleotide sequences for the isolates were retrieved from NCBI. Multiple alignments of the nucleotide sequences were done with reference strains using a multiple sequence alignment program MAFFT v7.2.8 in the Geneious v11 plugin. The phylogenetic tree was constructed using Fast Tree program in Geneious v11. Phylogenetic tree of partial 16S ribosomal RNA sequences were constructed.

Real-time multiplex PCR

The PCR method described here used heat to inactivate the organisms which greatly reduced the risk of laboratory-acquired infection with Brucella. Finally, the multiplex format of the assay will reduce reagent cost and staff time required to perform testing for brucellosis but in a multiplex format a real-time triplex assay that permits rapid confirmation of *Brucella spp., B. abortus,* and *B. melitensis* isolates in a single test. The primers and TaqMan probes (Qiagen, Alameda, Calif.) utilized for the multiplex assay are shown in Table 1. All primers and TaqMan probes were designed using the multiplex TaqMan design feature of Beacon Designer software (Premier BioSoft International, Palo Alto, Calif.). For *Brucella* spp. identification, the primers and probe target the *bcsp31* gene (Gen Bank accession number M20404). The nucleic acid targets for *B. abortus* and *B. melitensis* identification are similar to those described.[51,52] However, the primers and probes to these targets were redesigned for the multiplex TaqMan format. The *B. abortus* primers and probe set targets the specific insertion of an IS711 element downstream of the alkB gene (GenBank accession number AF148682), whereas the *B. melitensis* primers and probe set targets the insertion of an IS711 element downstream of BMEI1162 (Gen Bank accession number NC_003317). Both targets share the same IS711 reverse primer, while the forward primers target either alkB (*B. abortus*) or BMEI1162 (*B. melitensis*). The *B. abortus* and *B. melitensis* TaqMan probes target the alkB and BMEI1162 gene, respectively. The 50-µl multiplex PCR mixture consisted of 1 × AmpliTaq Gold buffer (Applied Biosystems, Foster City, Calif.), 6 mM $MgCl_2$, 2 mM of deoxynucleoside triphosphate blend (Applied Biosystems), a 200 nM concentration of each primer, a 100 nM

Table 1. Oligonucleotide primers and probes used in the real-time multiplex PCR assay for the detection of *Brucella* spp., *B. abortus* and *B. melitensis*

PCR identification	Forward primer[a]	Reverse primer[a]	Probe[a]	5′ Fluorophore/3′ quencher[b]
Brucella spp.	G**CTCGGTTGCCAATATCAATGC**	**GGGTAAAGCGTCGCCAGAAG**	**AAATCTTCCACCTTGCCCTTGCCATC**A	6-FAM/BHQ1
B. abortus	**GCGGCTTTTCTATCACGGTATTC**	**CATGCGCTATGATCTGGTTACG**	**CGCTCATGCTCGCCAGACTTCAATG**	HEX/BHQ1
B. melitensis	**AACAAGCGGCACCCCTAAAA**	**CATGCGCTATGATCTGGTTACG**	**CAGGAGTGTTTCGGCTCAGAATAATCCACA**	TexasRed/BHQ2

[a]Oligonucleotide sequence provided in5′ to 3′ orientation.
[b]6-FAM - 6-carboxy fluorescein; HEX- 6-hexachlorofluorescein;BHQ1 – BlackHoleQuencher1;BHQ2 –Black Hole Quencher 2

concentration of each probe, 2.5 U of AmpliTaq Gold DNA polymerase (Applied Biosystems) and 5 µl of a DNA extract. Amplification and real-time fluorescence detection was performed on the iCycler real-time PCR detection system (Bio-Rad Laboratories, Hercules, Calif.) using the following parameters: 10-min denaturation and polymerase activation step at 95 °C followed by 45 cycles of 95 °C for 15 second and 57 °C for 1 minute. A sample with a fluorescence signal 30 times greater than the mean standard deviation in all wells over cycles 2 through 10 was considered a positive result, whereas a sample yielding a fluorescence signal less than this threshold value was considered a negative result.

Statistical analysis

All data were entered in Microsoft Excel. Age and parity were converted to categorical variables. Months were converted to four seasons.[53] The data were entered in R 3.5.0 (The R foundation for Statistical Computing, 2018) for further analysis. The serologic test status (Yes/No) of brucellosis were summarized by using the "tabpct" function of the R package "epicalc"[54] for different categories of the explanatory variables.

Uni-variable mixed-effect logistic regression analyses

Initially, a uni-variable logistic regression analysis was performed using a mixed-effect logistic regression model by including farm / herd as random intercept (R package "lme4"[55] If an animal was tested positive in at least one serologic test then it was considered as positive. The model used brucellosis status (Yes / No) as the response and each risk indicator variable in turn as an explanatory variable. Any explanatory variable associated with brucellosis status with a p-value of ≤ 0.10 was selected for multiple mixed-effect logistic regression analysis. Collinearity among explanatory variables was assessed by calculating a Cramer's phi-prime statistic (R package "vcd," "assocstats" function.[56] A pair of variables was considered collinear if Cramer's phi-prime statistic was > 0.70.[57]

Multivariable mixed-effect logistic regression analyses

A manual forward stepwise mixed-effect multiple logistic regression analysis was performed to identify risk factors for brucellosis. Initially, the best uni-variate model was selected based on the lowest Akaike's information criterion (AIC) value. The remaining variables were then added in turn using a stepwise algorithm, based on AIC. The final selected model had the lowest AIC. Confounding was checked by observing the change in the estimated coefficients of

the variables that remained in the final model by adding each non-selected variable to the model. If the inclusion of this non-significant variable led to a change of > 25% of any parameter estimate, that variable was considered to be a confounder and retained in the model.[58] The two-way interactions of all variables remaining in the final model were assessed for significance based on AIC values.[58]

RESULTS

Sero-prevalence of brucellosis was carried out in a total of 1003 lactating dairy cows of the eight military dairy farms in Bangladesh by using four different serological tests to compare their efficacy. Similar sero-prevalence rates of 2.39% was recorded with ELISA, SAT (Fig.1) and CFT (Fig. 2) whereas RBT (Fig. 3) detected higher rate of 3.09% sero-positive cases (Table 2).

Table 2. Comparison of the efficacy of different tests for the detection Brucella infection in serum and milk samples of the military cattle dairy farms (MCDF) in Bangladesh

S/N	MCDF: Places	No. of cows tested	Tests used with No. of positive results						
			RBT	ELISA	SAT	CFT	MRT	C-PCR	MRT-PCR*
1	Ishurdy	105	4	3	3	3	0	1	0
2	Lalmonirhat	108	5	4	3	3	0	1	0
3	Savar	141	4	3	4	4	0	1	1
4	Jossore	145	9	9	7	7	2	2	1
5	Chattagram	143	5	3	3	3	0	1	0
6	Comilla	178	2	1	2	2	0	1	0
7	Trishal	100	2	1	2	2	0	1	0
8	Sornodip	083	0	0	0	0	0	0	0
	Overall (%)	1003	31 (3.09)	24 (2.39)	24 (2.39)	24 (2.39)	2 (0.20)	8 (0.80)	2 (6.45)

RBPT = Rose Bengal plate test; ELISA = Enzyme linked immunosorbent assay; RTKS = Rapid test kit for serum
SAT = Slow agglutination test; CFT = Complement fixation test; MRT = Milk ring test
C-PCR = Conventional PCR (C-PCR); MRT-PCR = Multiplex RT-PCR; *31 samples tested, of which 2 (6.45%) positive

Examination of milk samples showed on only two (0.20%) positive cases with MRT (Fig 4) in the Jessore military farm (Table 2).

Conventional PCR (Fig. 5) and phylogenetic tree of partial 16S ribosomal RNA sequences of the PCR products was closely matched with *B. abortus* (Fig. 6). No *B. melitensis* DNA could be amplified either from human or animal samples. Only two (6.45%) of 31 animal samples investigated were positive in the genus-specific BCSP31 real-time Multiplex PCR assay (Table 2). None of 02 positive MRT sample were positive in the *B. abortus* specific RT-PCR.

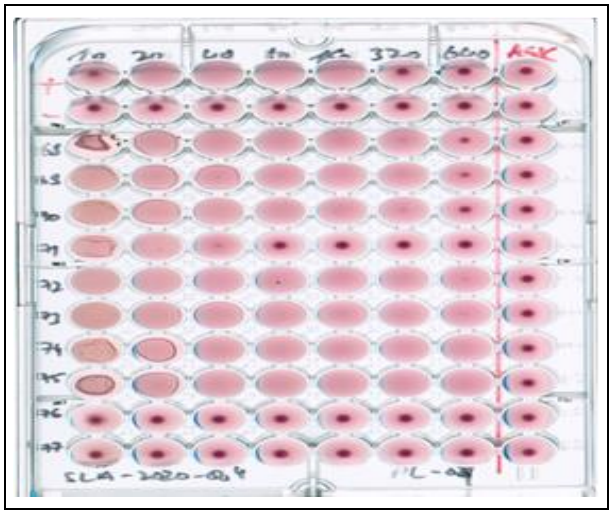

Plate 1: 67,76,77 no series of hole indicate negative control sera; 68,69,70,71,72,73,74 no series of hole indicate positive control sera

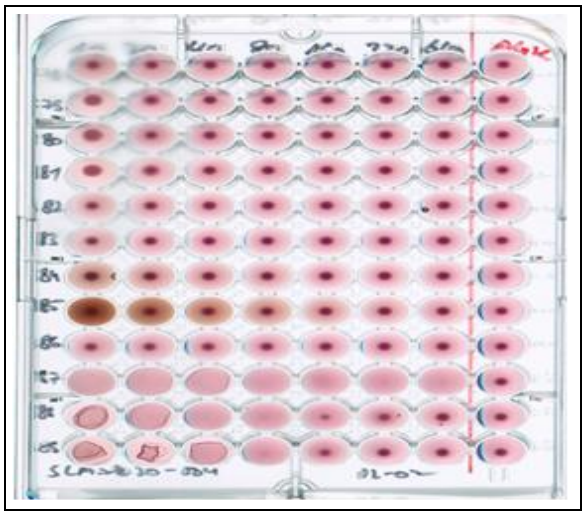

Plate 2: 78,79,82,83,84,85,86 no series of hole indicate negative control sera; 80,81,86,87,88,89 no series of hole indicate positive control sera

Fig 1: Slow agglutination test (SAT) [Plate- 1 on left and Plate -2 on right]

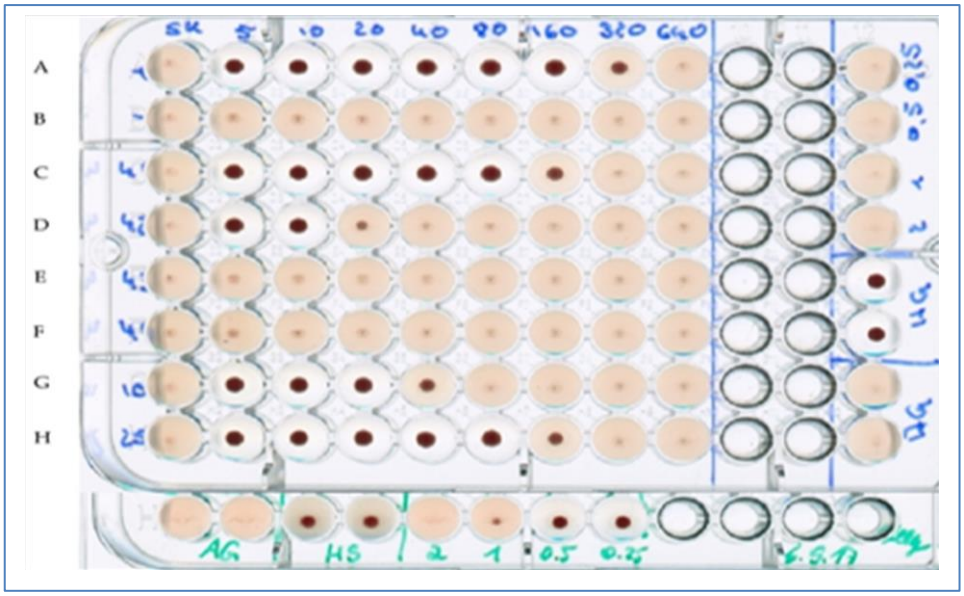

Fig 2 : Complement fixation test- the degree of hemolysis at different dilution (1:5 to 1:640). Row A = Positive control, B = Negative control, C-D & G-H Positive field samples, E-F = Negative field samples. Last row- Complement control button in 0.5 and 0.25 dilutions.

Fig. 3: Rose Bengal Test (left) reaction during RBT (No. 68-75 & 87-90 are positive and No. 76-86 are negative for brucellosis). Rose Bengal antigens used for diagnosis of brucellosis (right).

Fig. 4 : Milk Ring Test (MRT) positive reaction showing ring of cream more colored than underlying milk.

The *B. abortus* DNA was amplified from all of the four RBT positive human serum samples tested (Fig. 7 & 8). Among brucellosis-infected patients, 75 %, 50 %, 25 % and 75 % had fever, arthralgia, backache and sweating, respectively (Table 3).

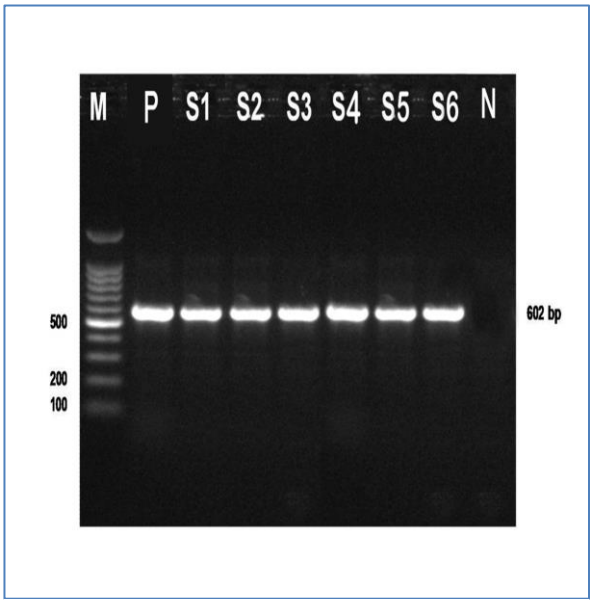

Fig.5: Conventional PCR Assay- P = positive control, Lane S1 to S5 = Cow sera, S6 = Human sera, Lane N = Negative control

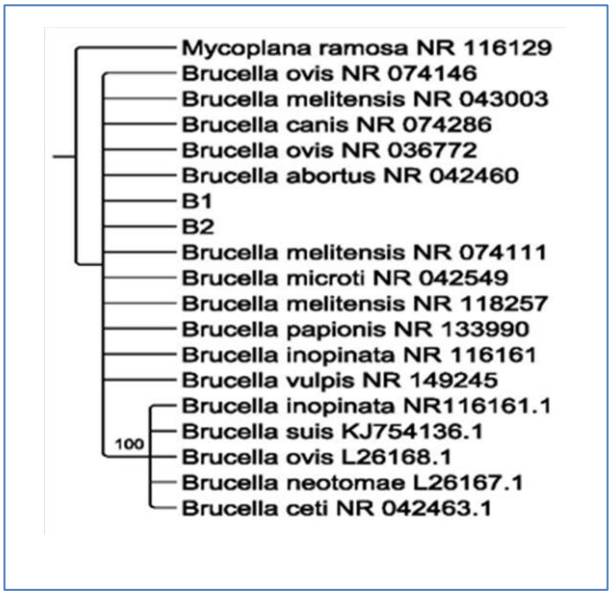

Fig. 6: Phylogenetic tree of partial 16S ribosomal RNA sequences of the PCR products closely matched with *Brucella abortus*

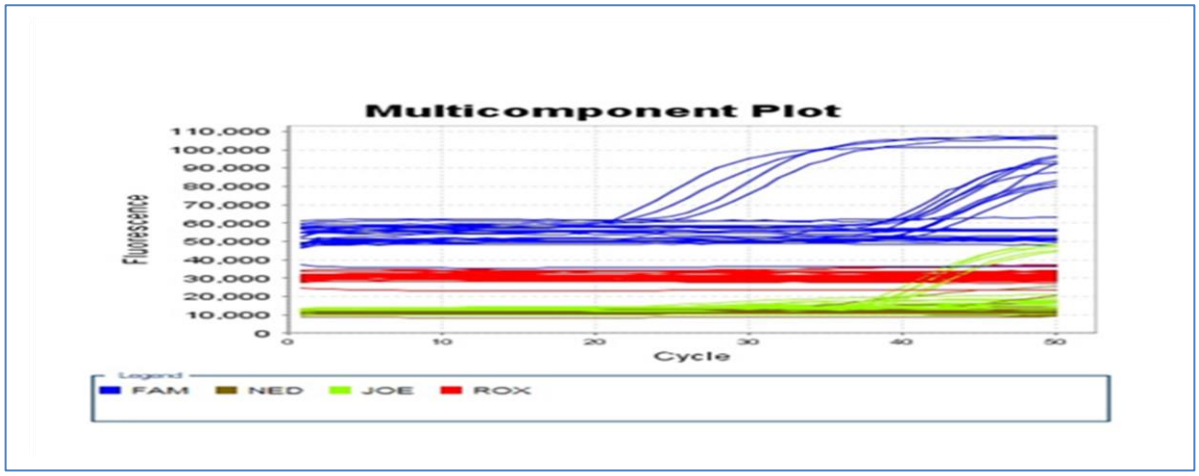

Fig. 7: Real-time multiplex PCR multicomponent plot

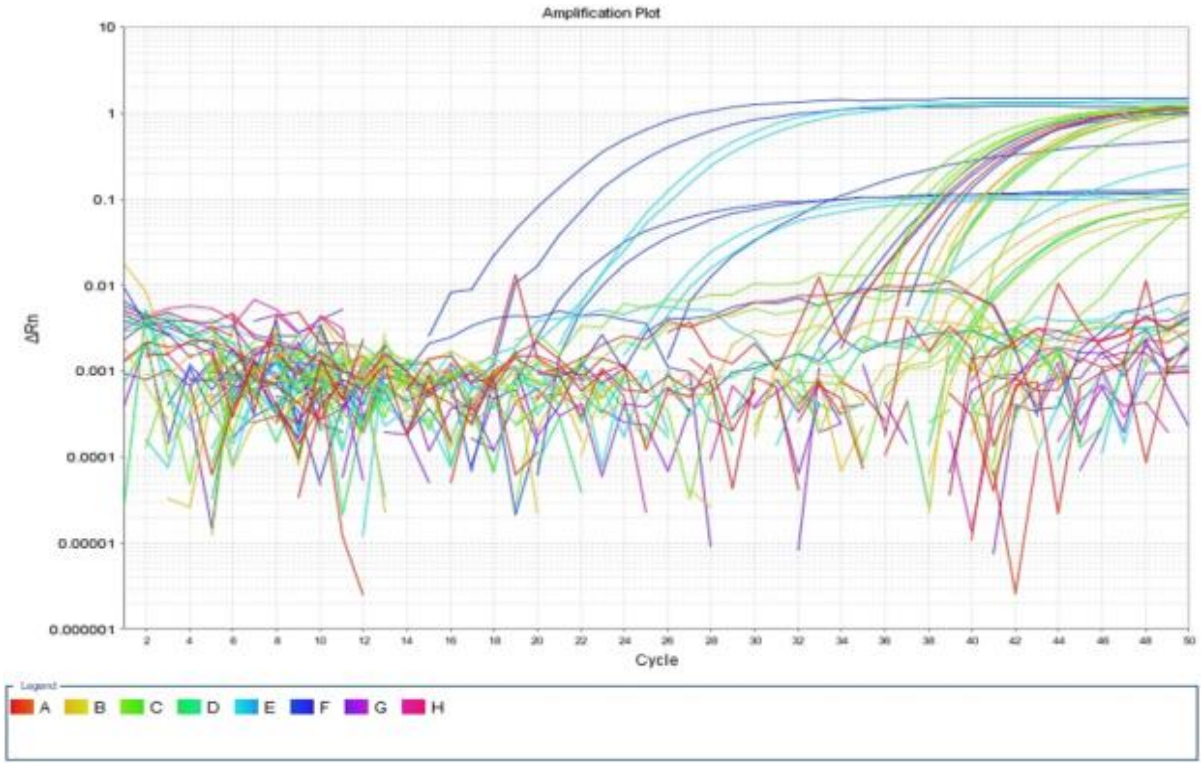

Fig. 8: Real-time multiplex PCR amplification plot

Three variables were found to be significantly associated with bovine brucellosis in military dairy farms (Table 4). The odds of brucellosis was 4.3 times (95% confidence interval [CI]: 1.2-10.58) higher in cows aged more than 6 years than those aged 4-6 years. Brucellosis was

Table 3. Risk factors and symptoms associated with the four Brucella RBPT positive milkers of the military cattle dairy farms in Bangladesh*

Patient/ Milker No.	Risk factors				Symptoms			
	Age (years)	Duration of service (years)	In farm living	Out farm living	Fever/ Pyrexia	Arthralgia	Backache	Sweating
1	23	4.0	Yes	No	Yes	Yes	No	Yes
2	25	5.0	Yes	No	No	No	Yes	No
3	25	5.0	No	Yes	Yes	No	No	Yes
4	28	2.0	No	Yes	Yes	Yes	No	Yes
Av & %	25.2	3.75	50.0	50.0	75.0	50.0	25.0	75.0

*Out of 715 tested human sera, only 4 (0.5%) milkers sera positive to RBTwith 95% confidence interval at 0.2 to 1.5

also significantly higher in cows those calved more than three times (odds ratio [OR] =3.7; (95% confidence interval [CI]: 1.5-9.1) than those calved one to two times. The odds of brucellosis was about 43 times higher (OR = 42.9; (95% confidence interval [CI]: 10.7-100.107) in aborted cows than non-aborted cows (Table 4).

Table 4. Univariable logistic regression analyses to evaluate the association between explanatory variables and brucellosis status in military cattle dairy farms in Bangladesh

S/N	Risk factors	Categories	Tested / Positive	Prevalence (95% CI)	p-value
1	Breed	Jersey	080 / 00	00.0 (00.0 - 05.7)	0.99
		Holstein-Friesian cross	923 / 31	03.5 (02.3 - 04.8)	
2	Age (years)	4 to 6	942 / 26	02.7 (01.8 - 04.1)	0.03
		> 6	061 / 05	08.2 (03.1 - 18.8)	
3	Parity	1 to 2	598 / 11	01.8 (00.9 - 04.4)	0.009
		3 to 4	405 / 20	04.9 (03.1 - 07.6)	
4	Abortion	No	895 / 07	00.8 (00.3 - 01.7)	< 0.001
		Yes	108 / 24	22.2 (15.0 - 31.4)	
5	Repeat breeding	No	171 / 10	05.8 (02.9 - 10.8)	0.03
		Yes	832 / 21	02.5 (01.6 - 03.9)	
6	Retention of placenta	No	898 / 29	03.2 (02.2 - 04.7)	0.41
		Yes	105 / 02	01.9 (00.3 - 07.4)	
7.	Seasons	Pre-monsoon (March-May)	316 / 13	04.1 (02.3 - 07.1)	0.49
		Monsoon (June-August)	238 / 06	02.5 (01.0 - 05.7)	
		Post-monsoon (Sept-Nov)	233 / 08	03.4 (01.6 - 06.9)	
		Winter (Dec-Feb)	216 / 04	01.9 (00.6 - 04.9)	

The univariable logistic regression analyses was used to evaluate the associated between sero-prevalence of bovine brucellosis with breeds, age, parity, abortion, repeat breeding, retention of placenta and seasons in lactating dairy cows in military farms (Table 4). The logistic regression reveals that breed, age, parity and abortion had significant association with seropositivity of brucellosis in dairy lactating cows (Table 4 & 5). However, there was no significant difference between the sero-prevalence and repeat breeding, retained placenta and seasons of the years in lactating dairy cows (Table 4).

Table 5. Multivariable logistic regression model of the risk of bovine brucellosis in military cattle dairy farms in Bangladesh

SN	Risk factor	Categories	Estimate	SE	Odds ratio (95% CI)	p-value
1	Age (years)	4 to 6	1	Reference	-	-
		> 6	1.5	0.66	04.3 (01.2 - 10.58)	0.02
2	Parity	1 to 2	1	Reference	-	-
		3 to 4	1.3	0.46	03.7 (01.5 - 09.1)	0.005
3	Abortion	No	1	Reference	-	-
		Yes	3.8	0.48	42.9 (10.7-100.1.07)	<0.001

SE = Standard error CI = Confidence interval

DISCUSSION

Bovine brucellosis is a chronic infectious disease caused by *B. abortus* which is prevalent in both sub-clinical and clinical forms. Clinical disease is characterized by late abortion, neonatal mortality, reduced fertility and decreased in milk production in female cattle.[5,59,60] The Brucella life cycle contains two phases: (a) a chronic infection of phagocytic macrophage, which results in bacterial survival and replication for prolonged periods of time and (b) an acute infection leading to reproductive tract pathology and abortion when the bacteria infect non-phagocytic epithelial cells.[61] The Brucella organisms which have developed mechanism to live intra-cellularly are able to infect cattle for long periods of time.[62,63] Some animals are asymptomatic, having latent infection without exhibiting clinical signs, thus maintaining the reservoir infection in a herd.[64,65] Tissue tropism of Brucella organism includes pregnant uteri, male genital organs, mammary glands and associated supra-mammary lymph nodes.[9] At pregnancy, release of erythritol from the placenta into the circulation causes translocation of Brucella organism out of lymph nodes and migration to the pregnant fetus, where replicate to a very high level @ 10^3 bacteria / g of tissue that ultimately causes abortion in infected animals.[9] However, most Brucella infected pregnant animals abort only once in their lifetime but may remain infected their entire life.[18] Brucella organisms are usually excreted with the fetus, placenta and uterine discharges during abortion and it also excreted through milk of infected cows for prolonged period representing a major risk for public health.

Brucella organisms can be transmitted either by direct contact with infected animals and animal excreta or indirect contact through ingestion of contaminated food and water containing large quantities of bacteria.[66] Mucosal contact with the discharges of aborted fetuses, fetal membranes and uterus is an important means of Brucella transmission in cattle. The vertical transmission and higher transmission rate from Brucella infected bull through natural service (4.0%) than AI with infected semen (2.0%) have also been reported.[67] Contact with soil contaminated with abortion secrets is also source for infection. Brucellae can survive up to 15 to 25 days on pastures[68] and can survive 20 to 120 days in soil, 70 to 150 days in water and 60

days in milk and meat.[13] However, it is being inactivated within few hours by high temperature and direct sunlight.[66]

Human brucellosis and its prevalence are directly correlated with the presence of Brucella infection in animals. The prevalence of Brucella infection in animals and practices that expose humans in infected animals or their products can significantly increase the risk of the occurrence of this infection in humans. Humans are readily acquire Brucella infection through consumption of unpasteurized milk and dairy products, direct contact with infected animals, placentas or aborted fetuses or inhalation of aerosols.[1,4,5] It is considered to be an occupational hazard in animal farmers, animal caretakers, abattoir workers, veterinary medical practitioners, workers in the dairy industry and bacteriological laboratory personnel.[4] Brucellosis in humans may be acute or insidious and typically manifests as a range of non-specific symptoms including malaise, fatigue, arthritis, osteo-articular disease and fever with chronicity and recurring febrile conditions with joint pain are common sequelae[1,8,19,69,70] and often misdiagnosed as other febrile syndromes like malaria and typhoid fever resulting mistreatments and underreporting.[71,72] Economic losses caused by brucellosis in humans are a consequence of long-term hospital treatment, cost of drugs and loss of work or income due to illness.[73]

The serological tests which have been used for the diagnosis of brucellosis by using either whole-cell antigen or prepared by chemical extraction of lipopolysaccharides (LPS) antigen.[74] The primary immune-determinant and virulence factor for Brucella species is the cell wall surface lipopolysaccharide (LPS) which is antigenically similar to the LPS of other Gram-negative bacteria. While *B. ovis* and *B. canis* have a rough type LPS (R-LPS) and other Brucella spp. have a smooth (S-LPS) with an o-polysaccharide linked to the oligosaccharide.[75,76] Brucella o-polysaccharides create three basic epitope: A, C and M and they are distributed in various proportions among S Brucella species and biovars so that neither A nor M is characteristic of *B. abortus* and *B. melitensis*, respectively. In addition to the S-LPS, S Brucella produces a free polysaccharide called native hapten (NH).[75,77] Most of the serological tests for brucellosis utilize *B. abortus* antigen because common epitopes are present in *B. melitensis*, *B. suis* and *B. abortus*.[78] False-positive Bacteria antibody test results can be caused by cross-reactivity of antibodies to *Yersinia enterocoilitica* O:9, *Vibrio cholera* O1, *Ochrobacterum anthropic, Salmonella enterica serotype urbana* (group N, O:30), *Francisella tularensis*, *Stenotrophomonas maltophilia*, *Escherichia coli* O157, *Moraxella phenylpyruvica* and some *Escherichia hermani* strains.[30-32,79,80] Moreover, the serological response of Brucella infection in cattle is influenced by several factors, include the time of exposure, the stage of gestation, vaccination status, variable and long incubation period during which sero-test results are negative.[81]

The first inception of the serologic assays for brucellosis was in 1897,[82] since then different types of sero-methods have been developed and used and these tests can be grouped into three general classes: (a) Agglutination tests e.g. Slow agglutination test (SAT), Rose Bengal test (RBT), Milk ring test (MRT), (b) Complement fixation test and (c) Primary binding assays e.g. iELISA, cELISA, Fluorescence polarization assay (FPA).[9,74] The most commonly used serological tests for diagnosis of brucellosis are Rose Bengal Test (RBT), Slow agglutination

test (SAT), Complement fixation test, Fluorescence polarization assay (PA), competitive (c-ELISA) and indirect (i-ELISA) enzyme-linked immunosorbent assay.[65,83] The milk ring test (MRT) detects milk Brucella antibodies and tests only possible on lactating animals.[84-86]

With exception of c-ELISA which measure specific antibodies against the immune-dominant O-polysaccharide section of lipopolysaccharide (LPS), all these tests use as antigens whole bacteria or bacterial extract enriched in smooth or rough LPS, which are composed of a complex mixture of antigens.[74,87] Therefore, current serological tests suffer from false-positive reactions due to cross-reactivity with other antigens and/or common epitopes present in the lipid A and core sections of LPS.[88,89] Finally, a problem still unsolved in the sero-diagnosis of brucellosis is the lack of a standardized reference antigen for diagnosis of the disease.[90] In addition, the Brucella is a facultative intracellular bacterium and the infected animals can only be clinically diagnosed at the abortion at the end of gestation whereas it is difficult to diagnose in male animals. During abortion, a huge number of bacteria set free and it usually up to 1 billion of bacteria per gram of aborted materials[91] that material could be used for bacteriological culture. However, it is again difficult to get bacteriological samples from slaughtered animals because outside the gestation time, *B. abortus* has no organ of predilection. Therefore, review of literature reveals two points on diagnosis of brucellosis which include (a) the focus on serological tests that reduce or eliminate wrongly positive reactions and (b) the focus on molecular tests that allow fast detection and typing of the bacteria. A molecular characterization is essential to identify a relation between two or more strains and possibly source of infection.

This study recorded overall sero-prevalence of brucellosis in 3.09% with RBT and 2.39% with ELISA, SAT and CFT in lactating dairy cows in eight military dairy farms in Bangladesh. This prevalence rate (2.39-3.09%) appears to be more or less similar to the reviewed reports (3.7%) [35,36] but lower than the recent reports of 5.3%[39] and 6.6%.[42] These differences might be due to difference of breeds, test used, animal husbandry practices of the herds, status of pregnancy, lactation, breeding methods and selection of samples.[36]

The higher sero-prevalence of brucellosis was found in cows more than 6 years old (8.2%) than younger cows aged between 4 to 6 years (2.7%) supports the earlier reports of 8.18% in cows more than 4 years and 4.29% in cows aged between 3 to 4 years.[42] These results are also in conformity with 6.97% prevalence in cows over 4 years in comparison to 3.64-5.88% in cows aged between 2 to 4 years[92] and 4.0% in cows over 4 years and 2.3% cows less than 4 years of age[93] have been reported from Bangladesh. Susceptibility to brucellosis has been reported with the increased of age and increased parity of cows.

This study recorded significantly ($p < 0.001$) higher sero-prevalence of brucellosis in aborted cows (22.2%) in comparison to non-aborted cows (0.8%) which supports the earlier reports of 28.07%[42] and 15.05% [94] in Bangladesh and 14.2%[95] in Sudan with the history of abortion in cows.

The four RBT test-positive human sera and all animal samples collected from aborted, retained placenta and repeat breeder cows including milk, vaginal swabs and placentas were screened by Brucella genus-specific real-time Multiplex PCR. Positive samples were then tested by IS711 RT-PCR to detect *B. abortus* and *B. melitensis* DNA. The partial 16S

ribosomal RNA sequences were edited in Geneious v11. The available reference nucleotide sequences for the isolates were retrieved from NCBI. Multiple alignments of the nucleotide sequences were done with reference strains using a multiple sequence alignment program MAFFT v7.2.8 in the Geneious v11 plugin. The phylogenetic tree was constructed using Fast Tree program in Geneious v11. Phylogenetic tree of partial 16S ribosomal RNA sequences of the PCR products was closely matched with *Brucella abortus*. However, B. *melitensis* DNA could not be detected in this study but this species is mostly associated with zoonotic human brucellosis worldwide. This result also supports the earlier report in which also failed to detect *B. melitensis* from Bangladesh.[39]

The successful amplification of *B. abortus* DNA in sera of four milkers of military dairy farms supports the report of Pakistan[96] where the animals and their products were the sole source of human infection. Consequently, the presence of *B. abortus* DNA in human samples together with their history of contact demonstrates that dairy animal populations of military farms appear to be a source of brucellosis in humans and that *B. abortus* is the most common causative agent of human brucellosis in Bangladesh. The presence of *B. abortus* DNA in serum supports this conclusion, and moreover, the recent detection of *B. abortus* DNA from bovine sera in Bangladesh[40] provides further evidence of the causal role of *B. abortus*. In addition, patient of various Combined Military Hospital (CMH) and Mymensingh Medical College Hospital suggests that infection in dairy cattle of military farms is widespread. However, due to small size and non-randomness of the sample our result does not represent the whole figure of Bangladesh.

Brucella spp was detected from milk samples of one Military Farm (Jossore) of Bangladesh. Possible source of Brucella of Holstein Friesian bull semen originating from one of the government farms included in the current study and found to be Brucella infected has been used for this cross-breeding purpose.[97]

Abortion is the most common clinical sign of brucellosis in female domestic ruminants, and usually aborted fetuses, fetal membranes and fluids contain high bacterial loads contaminating the environment and causing a high risk of infection to other animals.[98] In this study, none of the 26 fetal membranes and vaginal swabs originating from cattle contained Brucella DNA. Although the sample size is small, it indicates that Brucella may not be a major cause of abortion in domestic cattle in military dairy farm of Bangladesh. However, *Brucella spp.* has been detected from some of the MRT-positive and culture negative milk samples.[42] The possible reason for unsuccessful recovery of isolates may be that the samples had been stored for more than two years after collection prior to shipment to The OIE Reference Laboratory for Brucellosis, Germany for isolation and molecular detection. Brucellae are rarely isolated from samples with a competing microflora.[99] The presence of competing organisms (observed during culture) may be another potential reason for isolation failure.[38]

This study further confirms that *B. abortus* is one of the cause of human brucellosis in Bangladesh and that infection is likely endemic in cattle population. Contaminated milk represents a potential source of infection.[42] Testing representative number of dairy cattle fetal membranes from different military dairy farms of the country will help understanding the presence of *B. melitensis* in Bangladesh. The strength of this study is that for the first time the

presence of *B. abortus* in occupationally exposed humans in military dairy farms of Bangladesh has been described. This study has also some limitations including small size and non-randomness of sample selection.

Risk factors for brucellosis

The epidemiology of brucellosis is considered a complex due to its wide hosts, the variable status of infection (latent, carrier, sub-clinical and clinical) at both the individual and population levels and influenced by several risk factors. Infected animals can remain infectious after their first abortion and spread the infection.[100] Brucellosis directly affects large ruminants which are the main reservoirs of *B. abortus*, although other domestic and wild animals can also act as reservoir.[37,100,101] Large ruminants are usually infected with *B. abortus* through ingestion of contaminated food, water and grazing forage, close contact with infected animals, contact with uterine secretions or aborted fetuses and through vertical and sexual transmission.[102,103] Transmission within and between farms has been associated with different risk factors: the maintenance of positive animals in the herds, large farms, communal pastures, semi-intensive production systems and adult animals.[104] Several researchers have extensively studied and reviewed the risk factors associated with Brucella infections of animals and humans and they have classified into three to five categories which include: (a) Pathogen risk factors, (b) Host risk factors, (c) Occupational risk factors, (d) Management risk factors and (e) Agro-ecological risk factors.[11,105-107]

Pathogen risk factors

This study recorded 2.39% sero-positive to Brucella infection in lactating dairy cows by using SAT, CFT and ELISA assay, whereas all the four sero-positive milkers of the dairy farm showed positive to *B. abortus* infection by using molecular assay. This indicates that the *B. abortus* is mainly associated with brucellosis in both animals and contact people in the investigated dairy farms. However, the risk factors associated with Brucella organism which is facultative intracellular coccobacilli capable of invading epithelial cells, placental trophoblasts, dendritic cells and macrophages and able to survive and replicate within phagocytic cells (phagosome). The organisms are phagocytized by polymorphonuclear leucocytes in which some survive and multiply. The organism is able to survive within macrophages because it has the ability to survive phagolysosome. The bacterium possesses an unconventional non-endotoxin lipopolysaccharide which confers resistance to anti-microbial attacks and modulates the host immune system. These properties make lipopolysaccharide an important virulence factor for Brucella survival and replication in the host.[21] In addition Brucella species has been reported to pass through milk of sero-negative lactating cows in Bangladesh.[42] It can persist on fetal tissues and soil or vegetation for 21 to 81 days depending upon environmental conditions.[108]

Host risk factors

The sero-prevalence of brucellosis have been reported in different domestic animals of Bangladesh including cattle (3.7%), buffaloes (4.0%), sheep (7.3%), goats (3.6%), pig (4.8%),

horse (1.79%), dogs (4.0%) and humans (2.5 to 18.6%).[35,36,109] These results suggest the endemic nature of brucellosis in animals and humans in Bangladesh. However species variation of sero-prevalence of brucellosis has also been reported elsewhere.[11] This study recorded serological and molecular prevalence of *B. abortus* infection in dairy cows and associated human workers. Brucella organism infects a variety of domestic and wild animals and marine mammals including humans representing an important risk for the maintenance of the agent in the different animal species causing incapacitating disease. The susceptibility of animal to Brucella infection has been reported to be influenced by the age, breed, sex, pregnancy status, parity, history of retained placenta and abortion, and milking method.[63,110-112] This study recorded an overall 3.5% sero-prevalence of brucellosis in lactating Holstein-Friesian cross cows whereas all the 80 Jersey cross cows found negative to any used sero-tests. This result could not be compared due to lack of similar reports however higher sero-prevalence of brucellosis has been reported in cross-bred (22.7%) than local breed (13.8%) cattle.[107]

Significantly higher sero-prevalence of brucellosis was recorded in lactating cows older than six years (8.25%) of age than cows aged between 4 to 6 years (2.7%) group. This result supports the significantly higher sero-prevalence of brucellosis in cows older than 4 years of age than younger cows,[113] older than 3 years than younger than 3 years cattle.[107,114] This observation suggests that the older animals have been exposed earlier on and are probably immune and perhaps persistent carriers.[107,114] However, the higher sero-prevalence of brucellosis is observed in older animals since susceptibility increases after sexual maturity and pregnancy. It could be associated with the tropism of Brucella organism to erythritol, a 4-carbon sugar produced in the fetal tissues of ruminants that stimulates the growth of Brucella organism. This fact may explain the higher prevalence of brucellosis in adult than in young, female than male, sexually mature than immature, pregnant than non-pregnant, late than early gestation animals.[11,63,115]

This study has also recorded significantly higher sero-prevalence of brucellosis in lactating cows with three to four parity (4.9%) than one to two parity (1.8%) cattle. These findings are in conformity with earlier reports in which higher prevalence of brucellosis has been reported in higher parity than lower parity herd mates.[116,117]

This study recorded abortion as a risk factor of brucellosis in cows with significantly ($p < 0.001$) higher prevalence of Brucella infection in aborted (22.2%) than non-aborted cows (0.8%). This finding supports that the herds with a history of last trimester abortion reported to be more likely to be positive than herds without such history.[118] This study recorded that the sero-positivity of Brucella infection was found lower in lactating cows with history of retained placenta (1.9%) and repeat breeding syndrome (2.5%) in comparison to without any history of retained placenta (3.2%) and repeat breeding syndrome (5.8%). Most of the published reports have identified history of abortion and retained placenta as a significant risk factors for brucellosis[112,119-121] whereas some authors did not find any significant association between Brucella sero-positivity and abortion and retention of placenta.[107,122,123]

Occupational risk factors

This study recorded *B. abortus* infection in four (0.5%) milkers as an occupational risk factor by using multiplex real-time PCR in the military dairy farms in Bangladesh. However, an overall 2.0% sero-prevalence of *B. abortus* in pyretic patients has also been reported by RT-PCR with 8.9 times higher who handled goats than cattle in Bangladesh.[41] Of the four milkers affected with *B. abortus* of which 75% had fever, 50% arthralgia, 25% backache and 75% sweating in military dairy farms. These findings also support the 16.7% arthralgia and 25.0% backache recorded in *B. abortus* affected pyretic patients.[41] The seroprevalence of brucellosis in humans has been reviewed in Bangladesh as 2.6 to 21.6% in livestock farmers, 18.6% milkers, 2.5% butchers and 5.3 to 11.1% in veterinarians who had direct contact with animal and its products or who consume raw milk.[35,36] Human brucellosis has also been reported to be highly associated with (a) animal exposure (animal exposure at home 95.1%, animal exposure at work 9.0%, handling aborted animals 51.4%, slaughtering and butchering 68.8% and milking animals 23.6%) and (b) Food exposure (processing raw milk products 36.1%, consuming raw milk 13.9%, consuming milk products made with raw milk 15.3%) elsewhere.[121,124] However, sero-negative result of 350 human sera tested with RBT, SAT, CFT, iELISA, BCSP and IS711 assay have also been reported from Bangladesh.[39] Brucella infection through direct contact is a potential health threat to occupational groups of people such as veterinarians, dairy farmers, ranchers, slaughter house workers, hunters, laboratory workers, bacteriologists, milkers and inseminators and farmers have been reported elsewhere.[125-127] Handling aborted materials or attending retained placenta or dystocia without protective gear is a common practice to most field veterinary assistants, abattoir workers and in many rural pastoral settings.[128] Laboratory workers handling Brucella cultures are at high risk of acquiring Brucella through accidents, aerosolization and/or in adequate laboratory procedures.[129] In BD, 83.9% of total households own livestock and 45.9% households possesses bovine stock[130] and it indicates that a huge percentage of population in Bangladesh are at great risk to brucellosis if it is not eradicated.

Management risk factors

Although brucellosis is worldwide distributed, it is more common in low income developing countries with poor standardized animal and public health program. This study detected *B. abortus* in milk as well as four milkers with some clinical symptoms of the military dairy farms due to faulty dairy farm management and hand milking. This finding supports the presence of Brucella organism in both the RBT positive (n = 11) and negative (n = 6) cows in Bangladesh.[42] However, the zoonotic transmission of brucellosis by improper milking procedures has been reported with skin lesions in hands.[131] The persistence of infection in the udder and supramammary lymph nodes leads to a constant or intermittent excretion in milk in successive lactations.[132,133] This fact constitutes an important source of infection for humans and for the suckling animals.

Management risk factors for brucellosis include production system (intensive or extensive), screening of new arrivals, hygiene, awareness of the disease, vaccination, herd size and breeding practice.[63,134] Large herd size has been reported to be higher risk for brucellosis[112,113,134,135] because it provides more opportunities for infection, especially

following abortions, through increased contact between the animals and common feeding and watering points promoting transmission of Brucella organism.[107] Bull exchange for mating, natural breeding services and introduction of new animals to the herd have been reported to be the major risk factors for brucellosis.[60,121,136] Farming several species in the same herd has also been described as a risk factor.[100,135,137] Cattle infections have originated from small ruminants since *B. melitensis* biovar 3 was isolated from cow's milk.[11] The significant risk factors have been reported the replacement of animals from farms not certified as brucellosis-free and introducing an infected newly purchased animal into a herd.[35,138]

Agro-ecological risk factors

Geographical location, climate and presence of susceptible wildlife have been reported as agro-ecological risk factors.[139] The influence of the agro-ecological zone has also been referred to as a brucellosis risk factor, and some authors reported a higher prevalence and some reported lower prevalence of brucellosis in dry zone.[106] Higher prevalence of brucellosis in dry zone might be due to scarcity of pasture areas caused unrestricted animal-to-animal contact, whereas lower prevalence has explained due to lower survival of Brucella organism in aborted material in dry zones. Some non-significant seasonal influence was recorded on the seroprevalence of brucellosis in lactating dairy cows with higher prevalence at pre-monsoon and post-monsoon period than monsoon period and lower during winter months. This observation is in conformity that there is no any relationship between seasons and prevalence of brucellosis in animals.[140] However, this finding correlates with the observation that the season has an impact on herd management and animal nutrition, mainly in production systems involving transhumance or nomadic practices.

CONCLUSIONS

Brucellosis affects a wide species of animals including humans, causing both subclinical and clinical illness, remains undiagnosed in most cases in low-income countries including Bangladesh. Methods of diagnosis of brucellosis remain challenge, and control and eradication program by using vaccination and 'test and slaughter' in animals is still under discussion especially in developing world. Moreover, bovine brucellosis is a re-emerging disease in low income countries but still neglected by both the veterinary medical and human medical and government as well. Under this circumstance, it appears that the brucellosis caused by *B. abortus* is an endemic disease associated with reproductive disorders in cattle and occupational health hazard in humans in Bangladesh. The serological, cultural and molecular assay have been used to detect the Brucella infection in both in animals and humans and it reveals that the true prevalence rate of Brucella infection is still within the application of eradication method of 'test and slaughter' stage especially in the lactating dairy cows in military dairy farms where only prevalence is 2.39%. However, this study has led to a better understanding of the risk factors associated with bovine brucellosis in Bangladesh especially both sero-positive and sero-negative animals may shed organism in the milk of lactating animals, purchase of replacement animals without knowing Brucella infection, natural breeding services and absence of Brucella monitoring and eradication program at national level. These risk factors would help to prevent, control and eradication of brucellosis not only in military dairy farms but also in dairy industry

in Bangladesh. Prevention and control of zoonotic human brucellosis is dependent upon the control and eradication of Brucella infection in animals. However, an interdisciplinary and collaborative, 'One Health' approach that consists of public education, the development of an infrastructure for disease surveillance and reporting in both veterinary medical, human medical and wildlife professionals to collaboratively develop, adopt and promulgate a brucellosis 'One Health' paradigm for the prevention, control and eradication in livestock, humans and wildlife species. However, further studies are required to explore the cross-species transmission of *B. abortus* and *B. melitensis* in animals and humans to detect accurate status in animal and humans in Bangladesh.

ETHICAL APPROVAL

The study protocol of Ethical statement was peer reviewed and approved by the Ethical Review Committee of appropriate authority and individual respective all personnel and informed written consent prior to the collection of blood. Animal research was approved by the Faculty of Veterinary Science of Bangladesh Agricultural University and concern Military authority of Bangladesh Army.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this article.

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