INTRODUCTION

Brucellosis is a worldwide zoonotic infection of economic and public health importance caused by bacteria from genus Brucella (Mathew et al., 2015). These are non-motile, facultative anaerobic, intracellular, Gram-negative coccobacilli and different species show strong host specificity (Pappas, Akritidis, Bosilkovski, & Tsianos, 2005). There are five species of Brucella known to cause diseases in domesticated animals: B. abortus (cattle), B. melitensis (goats), B. ovis (sheep), B. suis (pigs) and B. canis (dogs). B. abortus has been subdivided into biovars 1, 2, 3, 4, 5, 6, 7 and 9. The biovar 3 consists of two genetically disparate sub-groups 3a and 3b (Ocampo-Sosa, Aguero-Balbin,
& Garcia-Lobo, 2005). Four distinct clades of *B. abortus* have been proposed: clades A, B and C (C1 and C2) to show the intraspecies relationships among its biovars (Whatmore et al., 2016).

Brucellosis causes abortion, infertility, still birth and reduced milk production in animals. Animals get infected either through consumption of contaminated feed and water or contact with an infected animal. Routine bacteriological method and a classical biotyping scheme are used for characterization of *Brucella* both at species and subspecies levels (Alton, Jones, & Pietz, 1975; Whatmore et al., 2016). Polymerase chain reaction (PCR) assays are currently used for identification of *Brucella* at genus, species and biovar levels (Bricker & Halling, 1994; Ocampo-Sosa et al., 2005; Romero, Gamazo, & Pardo, 1995). Multi Locus Variable number tandem-repeat Analysis (MLVA) assay is used for genetic characterization of *Brucella* isolates (Le Fletch et al., 2006; Whatmore et al., 2006).

Brucellosis has been reported in humans and animals in Bangladesh (Islam, Khatun, Werre, Sriranganathan, & Boyle, 2013; Rahman et al., 2012). It is known to cause huge economic losses in livestock sector (Islam et al., 2013; Rahman, Choudhury, Rahman, & Haque, 1983). Livestock farmers, butchers, milkers and veterinarians are high risk group individuals to contract brucellosis (Rahman et al., 2012). A seroprevalence report of brucellosis in Bangladesh listed a 3.7% prevalence of brucellosis in cattle, 4% in buffalo, 3.6% in goats and 7.3% in sheep (Islam et al., 2013).

Isolation of *Brucella* at the genus level has been reported in milk sample of cattle (Islam et al., 2018). *Brucella* genus-specific DNA has been identified in the sera of humans by real time PCR assay (Rahman et al., 2012). The *B. abortus* species-specific DNA has been detected in the sera of cattle by real time PCR (Rahman et al., 2014). Isolation of *Brucella* from the infected host is considered as the gold standard for diagnosis of brucellosis (Rahman et al., 2012). However, identification of *Brucella* at species level and its biovar typing and genetic characterization of circulating *Brucella* spp. has not been reported in Bangladesh. The objectives of the present research work are: i) isolation of *Brucella* spp. from dairy cattle experiencing abortion, ii) identification of *Brucella* at species and biovar levels and iii) genetic characterization of circulating *Brucella* spp. by MLVA-16 assay.

## 2 | MATERIALS AND METHODS

### 2.1 | Study areas and samples

The study was conducted in 1,285 dairy cattle on 22 farms located in the following geographical areas of Bangladesh: Mymensingh Sadar (24.7500°N 90.4167°E), Dhaka Savar (23.8583°N 90.2667°E), Gazipur Sadar (24.0000°N 90.4250°E), Jamalpur Sadar (24.9167°N 88.9583°E) and Dinajpur Sadar (25.5856°N 88.6531°E) (Figure 1). Each of the dairy farm consisted of 20–100 cattle which were indigenous breed and crossbreed of Friesian, Sahiwal and Red Chittagong. The cattle of the study farms were not vaccinated against brucellosis. A total of 240 samples consisted of uterine discharges (*n* = 45), milk (*n* = 115), vaginal swabs (*n* = 71), placenta (*n* = 7) and aborted fetuses (*n* = 2) were collected from dairy cattle with the clinical sign of abortion (*n* = 55) or without the history of abortion (*n* = 185) during the period from August 2016 to December 2017. Samples were collected after 1 to 3 days of abortion. The abortion was occurred at third trimester of gestation from first to third pregnancy. Uterine discharge (10 ml) was collected by inserting a disposable artificial insemination pipette into the uterus. Midstream milk sample (20 ml) was collected from each quarter of the udder into a sterile 50 ml falcon tube. Vaginal swab was collected using a sterile applicator stick (HiMedia, Mumbai, India). Placental cotedyons were collected from aborted cattle aseptically using sterile forceps and scissors and kept in a sterile plastic container. The aspirate of stomach content (50ml) was collected from the aborted fetus. The samples were transported to the Department of Microbiology and Hygiene, Bangladesh Agricultural University, using an ice box and kept at 4°C and cultured within 3 days.

### 2.2 | Isolation and biotyping of bacteria

Uterine discharge, vaginal swab and aspirate of fetal stomach content were streaked duplicate onto the Brucella selective agar supplemented with antibiotics (polymyxin B sulphate, bacitracin, nystatin, cycloheximide, nalidixic acid, vancomycin) (HiMedia, Mumbai, India) that inhibit growth of bacteria other than *Brucella* (Alton, Jones, Angus, & Veger, 1988). Milk was centrifuged at 3500rpm for 15 min. The cream and sediment were inoculated onto the Brucella selective agar (HiMedia, Mumbai, India) using a sterile cotton swab. Placental cotyledons were cut into small pieces and placed in a sterile plastic bag with equal volume of phosphate-buffered saline. The cotyledons were macerated by a stomacher for 5 min and tissue homogenate was inoculated onto the Brucella selective agar (HiMedia, Mumbai, India) using a sterile...
cotton swab. Inoculated plates were placed in an incubator supplied with 5% CO₂ at 37°C. The plates were observed daily up to 14 days for Brucella like colonies (smooth, small, translucent, glistening, dew drop like round and convex colony). Identification of bacteria in pure culture was performed by colony morphology, Gram’s staining reaction, catalase, oxidase, H₂S and urease tests (Alton et al., 1975).

_Brucella_ spp. were subjected to classical biotyping described by Alton et al. (1988). A panel of biotyping tests such as CO₂ requirement for growth, H₂S production and growth in presence of thionine and basic fuchsin were performed.

### 2.3 Molecular identification and genotyping of _B. abortus_

The genomic DNA was extracted from suspect _Brucella_ colonies by a genomic DNA extraction kit using manufacturer’s protocol (GeneJet Genomic DNA Purification Kit, Thermo Fisher Scientific, Vilnius, Lithuania).

To confirm _Brucella_ spp. at molecular level a genus specific PCR assay targeting 905 bp fragment of the 16S rRNA gene was performed (Romero et al., 1995). Identification of _B. abortus_ biovar 1, 2 and 4 was performed by AMOS PCR assay with oligonucleotide primers and PCR conditions described by Bricker and Halling (1994). Enhanced AMOS- ERY PCR assay was performed for the detection of _B. abortus_ biovar 3b, 5, 6 and 9 (Ocampo-Sosa et al., 2005), with modifications (Al Dahouk et al., 2007). Loci considered were Bruce 06, Bruce 08, Bruce 11, Bruce 12, Bruce 42, Bruce 43, Bruce 45, Bruce 55, Bruce 18, Bruce 19, Bruce 21, Bruce 04, Bruce 07, Bruce 09, Bruce 16 and Bruce 30.

Amplification of MLVA-16 loci was performed using multiplex PCRs as described previously (Garofolo, Ancora, & Giannatale, 2013). PCR amplifications were performed in a total volume of 10 μl containing 1.50 ng DNA, 1 x Type-it microsatellite PCR Master Mix (QiagenSrl, Milan, Italy), and proper concentration of each fluorescent primer pairs (Garofolo et al., 2013). Thermal cycling was conducted on a GeneAmp 9700 thermal cycler (Applied Biosystems) following thermal reaction profiles: initial heating at 95°C for 5 min, 30 cycles denaturation at 95°C for 30 s, annealing at 60°C for 90 s and extension at 72°C for 30 s. A final extension step at 60°C for 45 min and 20°C for 120 min was run to reduce artefacts such as stutter and non-templated 3’ A nucleotide additions. Fragments were then separated through capillary electrophoresis on an ABI 3500 instrument with POP 7 polymer. Data analysis was done using Genemapper 4.1 (Applied Biosystems) to assign for each VNTRs the actual allele. Clustering analyses were conducted with BioNumerics 6.6 (Applied-Maths) accessing additional data from the international MLVA Database (http://mlva.u-psud.fr/mlvav4/genotyping/) and treating as a character dataset with the categorical distance coefficient and UPGMA (Unweighted Pair-Group Method Arithmetic Average) and MST (Minimum Spanning Tree) methods. The MST for single clade retrieved the clonal complexes with the most stringent (conservative) definition, where all members assigned to the same group differ only at one locus format least one other member of the group.

### 3 RESULTS

#### 3.1 Isolation and biotyping characteristics

Ten _Brucella_ spp. were isolated from uterine discharge (n = 7, sample ID no. 21/S-4023, 46/S-5083, 49/G-7, 66/G-12, 106/G-5213, 107/S-55 and 109/S-1978), milk (n = 2, sample ID no. 72/ G-22 and 84/S-756) and vaginal swab (n = 1, sample ID no. 61/S-1000) of 10 dairy cows that suffered an abortion at the third trimester of pregnancy. They grew in a 5% CO₂ atmosphere after 3–14 days incubation at 37°C. Bacterial colonies were small, convex and regular with smooth surface, honey coloured, shiny and translucent. The organisms appeared to be Gram negative, small coccobacilli arranged singly or in pairs. The isolates were catalase, oxidase, H₂S and urease positive. The isolates grew in the presence of thionin and basic fuchsin dyes suggesting that all isolates belonged to the biovar 3 (Table 1).

#### 3.2 Molecular identification by PCR

_Brucella_ genus specific PCR targeting 16S rRNA gene amplified 905 bp size of PCR amplicons. It confirmed the identity of the 10 isolates as _Brucella_. The enhanced AMOS- ERY PCR reconfirmed all isolates as _B. abortus_ with the production of 1,700 bp size PCR amplicons.

### TABLE 1 Biotyping results of _Brucella abortus_ isolated from dairy cattle

| No. of _B. abortus_ isolates tested | Urease activity | CO₂ requirement | H₂S production | Serum requirement | Thionin blue* | Basic fuchsin** | Interpretation |
|------------------------------------|----------------|----------------|----------------|------------------|---------------|----------------|---------------|
|                                    | +             | +              | +              | -                | + b c         | + b c          | + B. abortus biovar 3 |

Note: + = Positive, - = Negative, *Concentration of thionin blue (a = 1:25,000, b = 1:50,000, c = 1:10,0000), **Concentration of basic fuchsin (b = 1:50,000, c = 1:10,0000)
3.3 | MLVA genotyping

The dendrogram analysis revealed the 10 isolates from Bangladesh shared the same genotype 40, based on Panel 1 MLVA 8 and were identical to three isolates from Brazil and one isolate from France (Figure 2). A further seven isolates from Brazil clustered closely, differing only by one highly polymorphic minisatellite from Panel 2B, (Bruce 04). Two isolates from China were also found within the same branch, differing by one locus from Panel 1, (Bruce 06), where known, all closely related isolates were reported as members of biovar 3 or biovar 6. The 10 isolates from Bangladesh fell within Clade C1 (Figure 3). All MLVA profiles have been submitted to the MLVA bank (http://microbesgenotyping.i2bc.paris-saclay.fr/).

4 | DISCUSSION

Brucellosis causes abortion in the third trimester of bovine pregnancy (Megid, Mathias, & Robles, 2010). The Brucella are known to be shed in the aborted materials of cattle such as: uterine discharge, vaginal swab, placenta and fetus. In the present study, B. abortus was isolated from the aborted materials of dairy cattle at the third trimester of gestation. B. abortus was not isolated from the stomach content of aborted fetus. This may be either due to absence of bacteria in the stomach content or low number of samples tested. Similar results were also reported by Geresu, Ameni, Wubete, Arenas-Gamboa, and Kassa (2016). The current research work also isolated B. abortus from the milk of dairy cattle that had aborted. Brucella is known to shed from the milk of infected cattle (Capparelli et al., 2009). In
brucellosis endemic areas, transmission of *Brucella* to humans can occur through consumption of unpasteurized milk (Deshmukh et al., 2015). In Bangladesh milk ring tests are not routinely practiced for screening *B. abortus* specific antibodies in milk (Islam et al., 2018). The presence of *B. abortus* in cattle milk constitutes a public health hazard as people in Bangladesh mostly purchase unpasteurized milk.

In the study, AMOS PCR assay failed to amplify a 498 bp PCR amplicon (data not shown) indicating none of the *Brucella* isolates belonged to the *B. abortus* biovar 1, 2 and 4 (Bricker & Halling, 1994). The AMOS- ERY assay identifies *B. abortus* biovar 3b, 5, 6 and 9 (Ocampo-Sosa et al., 2005) suggesting that the *B. abortus* isolates from cattle might be any one of these four biovars.

In the present study, all *B. abortus* isolates of cattle belonged to the biovar 3, indicating this biovar is being transmitted in the dairy cattle in the study areas. In this study, *B. abortus* was isolated from dairy farms located in two neighbouring districts of Bangladesh; Dhaka (Savar) and Gazipur. Therefore, it is very difficult to draw a conclusion that the *B. abortus* biovar 3 is predominately circulating in dairy population in Bangladesh as this study screened only a small number of samples obtained from aborted cows in a limited geographical area of Bangladesh. All isolates were designated MLVA panel-1 genotype 40 and had full MLVA-16 profiles identical to three isolates from Brazil (Minharro et al., 2013) and one isolate from France (Vergnaud et al., 2018). While Bangladesh and Brazil are clearly geographically well separated, it has previously been reported that most of the cattle imported into Brazil are from Europe or India (Minharro et al., 2013). This provides a plausible explanation for the sharing of MLVA-16 profiles between Brazil and Bangladesh, which borders India. The MLVA data, compared against the multilocus sequence typing (MLST) clades proposed by Whatmore et al. (2016), showed the isolates fell within Clade C1. In contrast with clade B, associated almost exclusively with isolates from Africa, this clade has a global distribution. Within clade C1, the most common biovar association is with biovar 3, which is consistent with the results obtained in this study. Biovar 3 is known to consist of at least two major genetically disparate groups (Ocampo-Sosa et al., 2005; Whatmore et al., 2016) and the isolates described here correspond to sub-group 3b of *B. abortus* biovar 3, more commonly of European origin than sub-group 3a associated with African origins.

Similarly, while all isolates shared an identical MLVA profile, much more sampling is required to understand the local diversity of *Brucella*, and whether sufficient diversity exists such that MLVA

![Figure 3](image-url)
may be a useful tool to inform understanding of epidemiological linkages locally. Isolates were identical to some previously reported from Brazil and closely related to others from China (Jiang et al., 2019) whether these represent true epidemiological linkages or simply homoplasy remains unclear. Further analysis with approaches such as whole genome sequencing would help to more categorically establish the relationship of these isolates with the global population. Characterization of the species and biovars of Brucella from field outbreak and to trace back the source of new strain in a particular geographic area is important to undertake effective control measures against brucellosis (De Massis et al., 2019). The data of species and biovar identification and genetic characterization of Brucella field isolates of the present work may be useful to formulate policy and strategies for the control of bovine brucellosis in Bangladesh.

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CONFLICT OF INTEREST

The authors declare no competing interest.

ETHICAL STATEMENT

The protocol for field studies and collection of animal sample was approved by Animal Welfare and Ethical Committee, Bangladesh Agricultural University, Mymensingh-2202. Farmers were informed and their verbal consent was taken previously for the collection of samples from their animals.

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