Epidemiological Study of Brucellosis in a dairy herd of Chattogram

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Roll No: 0120/01

Registration No: 883

Session: January-June, 2020

A thesis submitted in the partial fulfillment of the requirements for the degree of Master of Science in Public Health

One Health Institute

Chattogram Veterinary and Animal Sciences University

Chattogram-4225, Bangladesh

30 December, 2022

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This is to certify that we have examined the above Master's thesis and have found that is complete and satisfactory in all respects, and that all revisions required by the thesis examination committee have been made

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And Chairman of the Examination Committee

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30 December, 2022

Acknowledgements

All praise is dedicated to the Almighty for his graces, courage, aptitude, patience, and for allowing me to pursue higher education and finish my thesis for the Master of Science (MS) in Public Health degree. First and foremost, I would like to express my heartfelt gratitude to **Professor Dr. A.S.M. Lutful Ahsan**, (Vice-Chancellor) and **Professor Dr. Goutam Buddha Das**, Former Vice-Chancellor of Chattogram Veterinary and Animal Sciences University (CVASU) in Bangladesh, for providing this unique opportunity and research facilities.

I would like to express my deepest appreciation and gratitude to my supervisor **Dr. Sharmin Chowdhury**, Professor and Director, One Health Institute, Chattogram Veterinary and Animal Sciences University (CVASU), Bangladesh, and chairperson of the thesis evaluation committee, for her precious advice, instruction and gracious approval of my thesis. It has been an honor and a privilege to have navigated this difficult endeavor under her intellectual direction, compassionate supervision, insightful ideas, and constructive and continual inspiration throughout the whole study period.

In addition, I would want to convey my profound and sincere appreciation to both of my parents, colleagues as well as the rest of my family for their roles in shaping who I am today. Without their encouragement, moral support, kindness, and unending love, I never would have been able to make it this far.

Last but not least, I am grateful to my supportive friends, juniors, and well-wishers for their insightful talks, cheerfulness, and inspiration throughout this research. Their motivation and company kept me motivated and focused throughout the course of this research.

The Author,

December, 2022

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Abstract

Brucellosis is a highly transmissible zoonosis caused by bacteria of the genus *Brucella* that affects humans and animals.It is a serious public health and animal health problem causing reproductive complications in dairy animals; hence control measures to prevent its spread are of great importance. This study aimed to confirm probable causes of abortion with subsequent characterization of *Brucella* isolates, from a dairy herd in Chattogram district of Bangladesh.

This study was conducted on a dairy herd of 137 cattle in which two abortions were experienced within days. Samples comprising of aborted fetal tissues, uterine fluid, placental cotyledon and whole blood were collected and processed to know cause of abortions. Serum samples (n=137) were tested by Rose Bengal Plate Test (RBPT) and I-ELISA (Indirect Enzyme Linked Immune Sorbent Assay). Isolation and identification were performed following standard bacteriological and molecular techniques.

A total of 91 (66.42%, 95% CI: 58.15- 73.80) and 101 (73.72%, 95% CI: 65.75-80.40) samples were tested positive by RBPT and I-ELISA, respectively. Both the aborted samples were positive inculture characteristics along with RBPT and I-ELISA tests followed by *B. abortus* confirmation through AMOS-PCR assay. Partial genome sequencing of two PCR positive samples and subsequent phylogenetic analysis depicts close similarity with the isolates from India, Pakistan and China.

The culling of brucellosis-positive cattle is the key to control and prevent brucellosis in herds. The findings clearly highlighted the importance of brucellosis screening for optimizing both the herd health and public health.

Key words: Brucellosis**,** *Brucella abortus,* Chattogram, Bangladesh, Dairy herd

1. Introduction

Brucellosis is a common zoonosis worldwide affecting human and animal (Zhou et al., 2020). World Health Organization (WHO) classified it as one of the world's leading 'neglected zoonotic diseases' in low-income countries due to its higher disease burden (WHO, 2016). So far, four Brucella species have been identified as zoonoses: *Brucella abortus, B. canis, B. melitenis,* and *B. suis,* which have been associated with cattle, dogs, sheep with goats, and pigs, respectively, and the others are host specific (Rodríguez-Hidalgo et al., 2015). Although *Brucella abortus* is commonly thought to be the causative agent of bovine brucellosis, *Brucella melitenis* may also cause disease, particularly in areas where bovines have close contact with sheep and goats. In addition, *Brucella suis* has been reported in bovines (Ali et al., 2014).In animals, brucellosis causes serious reproductive abnormalities such as abortion, stillbirth, retained placenta, and orchitis, which results in significant productivity and economic loss (González-Espinoza, Arce-Gorvel, Mémet, & Gorvel, 2021). Brucellosis causes flu like symptoms including fever, malaise, pain and anorexia in humans (Rahman et al., 2020; Rahman et al., 2019). After being discovered in Egypt in 1929, brucellosis is now considered endemic in most countries of the world, particularly in third-world countries (Holt et al., 2011). In low- and middle-income countries of Asia and Africa, the disease are often underreported (Mcdermott, Grace, & Zinsstag, 2013). In Bangladesh, brucellosis was first reported in cattle in 1967. Since then, many studies reported sero-prevalence of brucellosis in livestock species and humans. Brucellosis can be confirmed with rapid test (milk ring, Rose Bengal plate test), serological test such as Indirect Enzyme Linked Immunosorbent Assay (iELISA), and slow agglutination test (SAT) and molecular tests (PCR, RT-PCR) (Musser, Schwartz, Srinath, &Waldrup, 2013; Rahman et al., 2020), however sensitivity varies among the diagnostic tests. AMOS-PCR is a type of multiplex PCR that can be used to easily identify and differentiate the major Brucella species (*B. abortus, B. melitensis, B. ovis, B. suis)* (Gumaa et al., 2020). Two or more tests are recommended for isolation and confirmation of the bacteria, *Brucella*. Efficient vaccines are readily available for control of *Brucella* in different countries (Shome et al., 2020). Unfortunately in Bangladesh vaccination against brucellosis is not in regular practice yet although the disease is endemic (Rahman et al., 2017).

Moreover, it is worth mentioning that very few studies were conducted for the molecular characterization of the organism in Bangladesh. Therefore, more studies focusing isolation and characterization of Brucella species is required in Bangladesh. This study reports the occurrence of *Brucella abortus* in a dairy herd where multiple late abortions were experienced. Molecular detection and phylogenetic analysis were performed for the aborted cases. Screening performed for all the individual animals subsequently. Molecular diagnosis through PCR and partial sequence data generated from this study was the very first in the Chattogram region of Bangladesh, one of the important dairy belts of her agricultural dependent economy.

Objectives:

- To determine prevalence of *Brucella spp* in dairy herds
- To determine subsequent characterization of *Brucella spp*

2. Literature Review

2.1 Etiology

The etiologic agent of bovine brucellosis is *Brucella abortus*, which has been identified as having at least nine biotypes and several strain variations (Radostits et al., 2000). The majority of animals that are susceptible to *Brucella abortus* infection are cattle, however it can also occasionally infect sheep, swine, dogs, and horses. When cattle share pasture or facilities with diseased pigs, goats, or sheep, they can also contract *B. suis* and *B. melitensis*. When compared to *B.* abortus infections, those brought on by heterologous species of Brucella in cattle typically last less time.

2.2 Risk factors for infection

The risk factors that influence the initiation, spread, maintenance and control of bovine brucellosis are related to the animal population, management and to biology of the disease (Radostits et al., 2000)

2.2.1 Agent risk factors

A facultative intracellular pathogen that can multiply and survive inside of host phagocytes is called *Brucella abortus* (Joint et al., 1986). Polymorphonuclear leukocytes phagocytoze the organisms, some of which survive and proliferate. These are then sent to the unborn placenta and lymphoid tissues. The spread of the infection to local lymph nodes, other locations like the reticuloendothelial system, and organs like the uterus and udder is largely due to the leukocytes' inability to eliminate virulent Br. abortus at the initial site of infection. Because it can survive phagolysosomes, the organism can also endure inside macrophages. Neutrophils and macrophages are two immune cells that Brucellae can use to fend off humoral and cellular bactericidal responses (Abela, 1999).

2.2.2 Host risk factors

Age, sex, breed, and the animal's reproductive status all affect a cow's susceptibility to *B. abortus* infection. Cattle of all ages can become infected, but illness often lasts longer in sexually mature animals (Megersa et al., 2011). Although latent infections may occur, younger animals tend to be more resistant to infection and commonly clear infections (Walker, 1999). Only 2.6% of animals who were infected at birth still carry the infection as adults. In comparison to sexually immature cattle of either sex,

pregnant and sexually mature cattle are more susceptible to infection with the bacterium (Radostits et al., 2000). Pregnancy and a rise in gestational age increase susceptibility (Bertu et al., 2010). Natural exposure to field strains mostly happens when infected cows give birth. The exposure risk to the other cattle in the herd increases as more sick cows calve or abort, depending on the situation (Radostits et al., 2000). The sensitivity to brucellosis of all cattle breeds seems to be similar, and it appears that no particular breed has developed a resistance to the disease (Radostits et al., 2000). The opposite is true, according to a report in the near future, it will be able to choose livestock with brucellosis genetic resistance (Belal & Ansari, 2013).

2.3 Management risk factors

Most often, the passage of an affected animal from an infected herd into a non-exposed herd is what causes the disease to spread from one herd to another and from one location to another (Mitiku & Desa, 2020). A herd's chance of introduction into the herd is influenced by whether it breeds its own replacement animals or buys replacement animals (Kreutzer et al., 1979). The primary reason for the failure of brucellosis eradication operations is the uncontrolled movement of cattle from contaminated herds or areas to herds or areas that are free of the disease. Large herd sizes, active abortion, and loose housing all lengthen the period needed for the herds to recover from the infection and become brucellosis-free (Rahman et al., 2011).

Large numbers of organisms are shed from the reproductive tract when infected cows abort. In cows which lactate following abortion, milk, including colostrum, is an important source of infection, and bacteria are excreted intermittently in milk throughout the lactation period. The fluid in hygromas caused by *B. abortus* infection may contain large numbers of organisms, but because of being restricted to the lesion they do not seem to be important in the spread of the disease (Fyumagwa et al., 2009).

2.4 Transmission

2.4.1 Sources of infection

The risk associated with exposure of susceptible animals to the disease following parturition or abortion of infected cattle depends on three factors: the number of organisms excreted, the survival of these organisms under the existing environmental condition, and the probability of susceptible animals being exposed to enough

organisms to establish infection. *Brucella abortus* achieves its greatest concentration in the contents of the pregnant uterus, the fetus and the fetal membranes after birth (Radostits et al., 2000). In addition, vaginal discharge and to a lesser extent, farm areas contaminated by fecal matter of calves fed on contaminated milk could be considered as main source of infection (Khan & Zahoor, 2018). Infected animals also shed organisms in the milk. Therefore, raw milk or raw milk products of bovine origin are ready sources for infections in humans (Tolosa, 2004). There can be also accidental self-inoculation with live *Brucella* vaccine strains that result in the disease.

2.4.2. Mode of transmission and route of infection

When contaminated pasture, feed, fodder, or water is consumed, the gastrointestinal system becomes the most prevalent route of transmission (Fig. 1). Additionally, cows frequently lick their newborn calves, fetuses, and postpartum cows, all of which may contain a significant number of the organisms and serve as a significant source of infection. Bulls typically don't mechanically spread virus from sick cows to non-infected cows. Since the virus can spread to numerous herds, using infected bulls for artificial insemination poses a significant risk (Joint et al., 1986). Humans become infected by consuming raw or unpasteurized infected milk, by coming into contact with infected discharges, or by handling diseased tissues (Tolosa, 2004).

2.5 Pathogenesis

In cells of the reticulo-endothelial system, including the bone marrow, lymph nodes, liver, spleen, and kidney, Brucella survives and multiplies after exposure (Tolosa, 2004). In this situation, 5–10% of animals may have prolonged multiplication of the organisms, resolution of the situation itself, or recurrence for at least two years. Recurrence is especially common during parturition. Bacteria are conveyed intracellularly by neutrophils and macrophages or are present free in the plasma during bacteremia and concentrate in a variety of organs, particularly the gravid uterus, udder, and supra mammary lymph nodes.

Localization may also occur in other lymph nodes and the spleen, testes, and male accessory sex glands. Occasionally bacterial localization occurs in synovial structures causing a purulent tendovaginitis, arthritis, or bursitis (Fyumagwa et al., 2009).

The preferential localization to the reproductive tract of the pregnant animals is due to the presence of unknown factors in the gravid uterus. These are collectively referred to as allantoic fluid factors that would stimulate the growth of *Brucella*. Erythritol, a four-carbon alcohol, is considered to be one of these factors (Tolosa, 2004) which are elevated in the placenta and fetal fluid from about the fifth month of gestation (Fyumagwa et al., 2009). The preferential replication of *B. abortus* in the extraplacentomal site within trophoblasts of the chorioallantoic membrane results in rupture of the cells and ulceration of the fetal membrane. The damage to placental tissue together with fetal infection and fetal stress will induce maternal hormonal changes. As a result, abortion occurs principally in the last three months of pregnancy, the incubation period being inversely proportional to the stage of development of the fetus at the time of infection (Radostits et al., 2000).

2.6 Clinical features

Between 14 and 120 days make up the incubation phase (Radostits et al., 2000). The reproductive system is involved in the primary clinical symptoms of brucellosis. Abortion after the fifth month of pregnancy is the disease's signature symptom in extremely sensitive, pregnant non-vaccinated cattle(Radostits et al., 2000). The frequent aftereffects of abortion includemetritis and placentaretention (Tolosa, 2004).

Because of acquired immunity, females often only have one miscarriage. A longer calving interval and lifelong infertility are potential effects of abortion with placenta retention and the ensuing metritis. The epididymis and accessory sexual glands in male animals are typically impacted, with painful necrotic tissue degradation and a decline in the quality of the semen (Kassahun et al., 2010).

2.7 Diagnosis

There are basically two main groups of diagnostic methods for detecting brucellosis: Identification of the agent and serological tests.

2.8 Identification of the agent

2.8.1 Microscopic examination

This is a useful procedure for examination of abortion materials. Smears of placental cotyledon, fetal stomach contents or uterine exudates should be heat fixed and stained by a Stamp's modification of the Zeihl-Neelsen stain. It is a small, Gram-negative coccobacilli or short rod measuring 0.6 to 1.5µm by 0.5 to 0.7µm(Radostits et al., 2000). No capsules, flagella, or spores are produced; however, an external envelope has been demonstrated by electron microscopy around *B. abortus*, *B. melitensis*, and *B. suis*(Tolosa, 2004). Brucellae are also non-motile (Verger & others, 1994).

2.8.2 Isolation of Brucella

Culturing of the organism: from milk sample, tissue sample, and genital discharges, fluid from hygromas, fetal stomach contents and semen, etc.; is possible and can be cultured directly or after centrifugation where appropriate, and the use of selective medium is recommended.

Inoculation: Into Guinea pig and mouse is the technique that has value for the isolation of *Brucella* when specimens are derived from potentially contaminated sources such as milk, cheese, semen, or genital discharges. Inoculation should be made subcutaneously into Guinea pig or intravenously (0.1ml), or subcutaneously if the material is heavily contaminated, into mice. A guinea pig is killed 3 weeks post infection and the second 6 weeks after inoculation. A blood sample for serological examination is taken at the time of killing; macroscopic lesions are recorded and the spleen is cultured. The mice are killed 7 days after inoculation and the spleen and liver removed for culture on nutrient medium.

Most strains are fastidious and slow growing, and require CO2 (5-10%) supplementation for primary isolation at an optimal growth temperature of 20-400C. Complex medium containing serum is required on sheep blood agar, the colonies not be as distinctive as when grown on serum dextrose agar(Mai et al., 2012). The optimum PH is 6.6 to 7.4 (Tolosa, 2004). Colonies have smooth or non-smooth morphology. Non-smooth colonies have intermediate, rough or mucoid forms. Smooth forms are often markedly pathogenic, whereas the rough variants are usually less so(Khan & Zahoor, 2018). The mucoid colonies are similar to the rough colonies except for having a glutinous texture (Tolosa, 2004).

The metabolism of *B. abortus* is oxidative and *Brucella* culture shows no ability to acidify carbohydrate in conventional tests. They are catalase positive and usually oxidase positive and reduce nitrate to nitrites. The production of H2S from sulfur containing amino acids also varies, showing some correlation with nomen-species and biovars urease activity varies from fast to very slow (Verger & others, 1994).

2.8.3 Serological tests

When bacteriological diagnosis is not practicable diagnosis has to be based on serological methods, e.g. in surveys or eradication programs.

2.9 Economic importance

On an average, outbreak of bovine brucellosis resulted in a loss of milk production of the herd as much as a 20% and this can reach 40-50% in early abortion (Fyumagwa et al., 2009). In addition to the loss of milk production, there is the loss of calves and interference with the breeding programs. This is of greater importance in beef herds where calves represent the sole source of income (Radostits et al., 2000). The common sequel of infertility increases the period between lactations, and in an infected herd the average inter calving period may be prolonged by several months.

Losses in animal production due to the disease can be of major importance, primarily because of the decreased milk production by aborting cows(Radostits et al., 2000) and this is often associated with retained placenta, metritis and a subsequent period of infertility (Dobrean et al., 2002). In general, economic losses due to brucellosis are usually caused byChukwu, (1987):

- Losses due to abortion,
- Diminished milk production,
- Cull and condemnation of animals due to breeding failure,
- Endangering animals export trade of a nation,
- Human brucellosis causing loss of some hours and medical costs,
- Government costs on research and eradication schemes

2.10 Public health importance

Brucellosis is a disease of animals in which man is infected as terminal host. The incidence of brucellosis in man is clearly correlated to the degree of incidence in the domestic animals around him (Fyumagwa et al., 2009). In developing countries, brucellosis is a relatively common disease among animals and man, and in these countries, it constitutes a large and uncontrolled public health problem(Joint et al., 1986). According to world health organization, about half a million cases of human brucellosis occur each year (Joint et al., 1986).

Man becomes infected when there is indirect contact with cows at abortion, parturition, or in the post parturition period from splashing of infected droplets into the eyes (Sewell and Brocklesby, 1990) or drinking unpasteurized milk or milk products (Roberts, 1971). Brucellosis is an occupational disease, occurring most often in veterinarians, farmers, stock inspectors, abattoir workers, laboratory personnel, butchers (Sewell and Brocklesby, 1990; Bishop et al., 1994). The disease in humans is characterized by a multitude of somatic complaints, including fever, sweat, anorexia, malaise, weight loss, depression, headache and joint pains (WHO, 1997) and is easily confused with malaria and influenza (Sewell and Brocklesby, 1990).

2.11 Control and eradication

2.11.1 Chemoprophylaxis

An effective treatment for animals with brucellosis is not known to date (Fyumagwa et al., 2009). The treatment of brucellosis in the cow has generally been unsuccessful because of the intracellular sequestration of the organisms in lymph nodes, the mammary gland, and reproductive organs and the bacteria are facultative intracellular which survive and multiply within the cells (Radostits et al., 2000). Generally, treatment of infected livestock is not attempted because of the high treatment failure

rate, cost, and potential problems related to maintaining infected animals in the face of ongoing eradication programs (Tolosa, 2004). Man can be treated with antibiotics (doxycycline with rifampicin), however, relapses are not impossible (Fyumagwa et al., 2009).

2.11.2 Immunoprophylaxis

The strategies for preventing brucellosis have to be adapted to the animal production system (Bertu et al., 2010). The successful prevention of this disease, which is so difficult in cattle production in the tropics, requires that, as far as possible, all available steps be taken to combat it (Fyumagwa et al., 2009). Failures of disease control are mostly due to the application of a scheme for which neither the veterinary infrastructure, nor the required reliable serological laboratories exist and the animal holder does not have the socio- economic prerequisites. Principally two alternatives exist (Mitiku & Desa, 2020): involves recognition of all animals which have responded immunologically to a Brucella infection and subsequent culling of the reactors According to (Mitiku & Desa, 2020) this method could be achieved when the rate of infection is reduced to an acceptable level (about 1-2%). Part of the scheme has to be a careful control of all animals which will be newly added to the herd as well as a production system which prevents contact with infected neighboring farms and/or contaminated feed or pasture.

Vaccination of exposed herds with inactivated or live vaccines.

- Calf hood vaccination- only performed on heifer calves between ages of 4-10 months. Vaccinated calves must be identified by a tattoo and ear tag.
- Adult vaccination the whole herd is vaccinated whenever there are certain problem herds. Herds have to be maintained in quarantines until all vaccinated animals have been removed from the herd.

The following are some of the vaccinations available against brucellosis**:**

- **Live *Brucella abortus* strain 19 vaccines**

  A single dose at 3 to 7 months of age is required with *B. abortus* strain 19. Adult animals vaccinated with strain 19 develop a better immunity than calves. However, due to the danger of abortion in pregnant animals, vaccination has thus so far been performed, above all, in calves, resulting in an

average protection from infection of about 70 % (Fyumagwa et al., 2009). Bulls should not be vaccinated because orchitis can develop (Oloffs et al., 1998).

- **Killed Br. abortus 45/20 vaccines**

Two doses administered 6 weeks apart in animals over 6 months of age are required with *B. abortus* 45/20. Adult cow vaccination is sometimes performed as a regulatory effort to control infection in a herd(Edmonds et al., 1999).

- **Brucella abortus strain RB51 vaccines**

This is a recently developed vaccine and has replaced *B. abortus* strain 19 in a number of countries as the approved calf hood vaccine because it does not interfere with serological evaluation (Edmonds et al., 1999). *Brucella abortus* strain RB51 is a live stable rough mutant of *B. abortus* strain 2308, which lacks much of the lipo-polysaccharide O-side chain and has been investigated as an alternative to strain 19 vaccines(Radostits et al., 2000). Adult vaccinations with *B. abortus* strain RB51 only rarely cause abortion.

2.11.3 Hygienic Prophylaxis

Experience shows that vaccination alone cannot bring about the eradication of the disease (Mitiku & Desa, 2020). From the epidemiology of the disease, important steps were derived at an early stage as hygienic prophylactic measures. These include:

- The isolation of calving animals in separate calving pens which are subsequently disinfected with 2.5 % formalin (Fyumagwa et al., 2009).
- Wet and well-grassed calving camps should be avoided, and vehicles used for transporting infected animals should be disinfected after use (Kassahun et al., 2010).
- Aborted fetuses, placentas, and uterine discharges must be disposed of, preferably by incineration (Radostits et al., 2000).
- All cattle, horses, and pigs brought to the farm should be tested, isolated for 30 days, and retested (Kassahun et al., 2010)
- Cows, which are in advanced pregnancy, should be kept in isolation until after parturition, since occasional infected cows may not show a positive serum

reaction until after calving or abortion(Radostits et al., 2000)

- Replacement stock should be purchased from herd free of brucellosis (Bishop et al., 1994).

  Chlorhexidine gluconate is an effective antiseptic against *B. abortus* and is recommended for washing the arms and hands of animals attendants and veterinarians who are exposed to contaminated tissues and materials (Tolosa, 2004)

3. Materials and Method

3.1 Study farm and sampling

The farm is located in Anwara sub-district of Chattogram, the southeastern part of Bangladesh (22°13'52.7"N, 91°52'19.7"E). The farm had a total of 137 crossbreed cattle at the time of sampling categorized as calf, heifer, bull and dairy cows according to published article(Hasib et al., 2021). There has been no history of vaccination against brucellosis in the study farm. Samples included blood from all individuals, two aborted fetus including fetal membrane, placental cotyledons and uterine discharge were collected to reveal the cause of abortion. About 5 ml whole blood sample was collected from jugular vein from all animals (n=137) aseptically into sterile plain vacutainer tube and immediately transferred to the laboratory maintaining proper cold chain. To allow serum separation, blood samples were held at room temperature for around 4 hours. After that, the serum was pipetted into sterile tubes and stored at -20^{o}C. The fetus and fetal membrane was investigated externally to evaluate the gross lesions. The fetus was found to be fresh, and its gestation stage was believed to be more than 6 months, according to the record kept in the farm. All the samples including fetal membranes were collected in tightly sealed sterile plastic bag and preserved at -20^{o}C for bacterial culture and isolation.

3.2 Serological Examination

3.2.1 Rose Bengal plate test (RBPT)

All serum samples from cattle were tested for presence of *Brucella* antibodies using RBPT antigen following the manufacturer's instructions (IDvet®, 310, rue Louis Pasteur, Grables, France). Briefly, an equal volume of serum (50µl) and dye (50µl) were mixed on a microscopic slide homogeneously and was stranded 5 minutes for visible changes **(Figure 2).** The RBPT was performed within an 2 hours of the serum collection (Khan et al., 2018; Mohamand, Gunaseelan, Sukumar, & Porteen, 2014; Sikder, Das, & Varyasyonlar, 2012). *Brucella* positive serum was used as a positive control in the test.

3.2.2 Indirect ELISA

All sera from cattle were analyzed for the presence of *Brucella spp* specific antibodies using ID Screen® Brucellosis Serum Indirect Multispecies ELISA kit following manufacturer's instructions (IDvet®, 310, rue Louis Pasteur, Grables, France). In case of I-ELISA the procedure ends after mixed with stop solution and optical density (OD) measured in automatic digital ELISA reader. Sample to positive ratio (S/P ratio) greater than or equal 120% was considered as positive for Brucellosis.Positive control serum was always included to monitor inter-assay variations.

3.3 Isolation, identification of *Brucella spp*

Homogenized cotyledon and uterine fluid were from two aborted animal were subjected to bacterial culture. The samples were inoculated on blood agar (Oxoid™ Ltd, UK) and incubated in anaerobic jar (Oxoid™ AnaeroJar™ 2.5L) under microaerophilic condition with CO_2 sachet (ThermoScientific^{TM}OxoidAnaeroGen 2.5L sachet) (10% CO_2, 95% humidity) in 37° C for 3 days (Islam et al., 2019; Percin, 2013). After incubation, small translucent, non-hemolytic and convex colonies were observed and further confirmed by the Gram's staining propertiesand followed by several biochemical test includingcatalase test, oxidase test, indole test, urease test, methyl red test, H_2S test, citrate test (Emy Koestanti, Misaco, Chusniati, & Maslachah, 2018; Geresu, Ameni, Wubete, Arenas-Gamboa, & Kassa, 2016; Kutlu et al., 2016).

3.4 Molecular identification of *Brucella spp*

Genomic DNA extraction was performed directly from aborted samples (fetal tissues, placental cotyledon and uterine discharge) as well as the bacterial colonies from the agar plate using Addprep Genomic DNA extraction Kit (ADDBio®, Korea) following manufacturer's guidelines.The extracted DNA was stored at -20°C for subsequent analysis.DNA amplification was performed using AMOS-PCR targeting IS*711* gene described as previous article (Gumaa et al., 2020) to identify genera and species respectively. The oligonucleotide primers for DNA amplification are presented in Table 1. AMOS-PCR was performed in 25 µL reaction mixture having 1 µL DNA template (average 4.18 ng/mL DNA), 1 µL forward and 1 µL reverse primer (20

picomole/mL), 9.5 µL nuclease-free water, and 12.5 µL master mix (Thermo Fisher Scientific®). PCR reaction was carried out at 93°C for 5 minutes followed by 35 cycles of denaturation at 95°C for 1.15 minutes, annealing at 55.5°C for 2 minutes, extension at 72°C for 2 minutes with final extension at 72°C for 10 minutes. After amplification in a thermocycler (2720 Thermal cycler; Applied Biosystems®), the PCR product(5 µL) was analyzed by electrophoresis in 1% agarose gel stained with ethidium bromide (0.5 mg/mL) (Sigma-Aldrich®) and visualized by a UV trans-illuminator (BDA digital, Biometra® GmbH, Germany).

Table 1: List of oligonucleotide primer used for AMOS-PCR (Gumaa et al., 2020)

PCR Type	Primer type	Sequence (5'-3')	Target gene	Amplicon size
AMOS	BA (F)	GACGAACGGAATTTTTCCAATCCC	IS711	498
	BA (R)	TGCCGATCACTTAAGGGCCTTCA		
	BM (F)	AAATCGCGTCCTTGCTGGTCTGA	IS711	730
	BM (R)	TGCCGATCACTTAAGGGCCTTCA		
	BO (F)	CGGGTTCTGGCACCATCGTCG	IS711	976
	BO (R)	TGCCGATCACTTAAGGGCCTTCA		
	BS (F)	TGCCGATCACTTAAGGGCCTTCA	IS711	285
	BS (R)	TGCCGATCACTTAAGGGCCTTCA		

BA=*B. abortus*, BM=*B. melitensis*, BO=*B. ovis*, BS=*B. suis*, F= forward, R= reverse

Figure 1: Aborted fetus collected from suspected farm

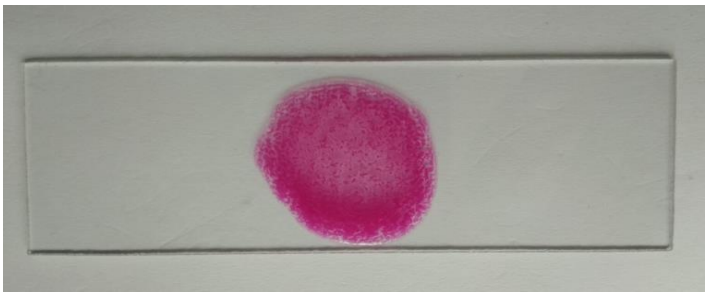

Figure 2: Clotted appearance indicated positive RBPT test

3.5 Partial sequence and phylogenetic analysis

Two samples have sequenced through Sanger sequencing technique by the Macrogen® (Korea) followed by data submission in the NCBI GenBank and acquired accession number was MW940712 and MW940713. MEGA-7 software used for the phylogenetic tree preparation using the Maximum Likelihood (ML) method comparing isolates chooses after BLAST search.

3.6 Data analysis

Descriptive statistics, prevalence and 95% confidence intervalwere estimated using the Modified Wald method in Graph Pad software Quick Calcs (www.graphpad.com/quickcalcs/).

4. Results

4.1 Serological findings

Out of 137 serum samples 91 from cattle were positive in RBPT giving a within-herd prevalence of 66.42% (95% CI: 58.15%-73.80%) while 101 of 137 serum samples delineates positive with I-ELISA resulting prevalence of 73.72% (95% CI: 65.75%-80.40%). All RBPT positive samples (n=91) showed positive results in I-ELISA test. The results of serological test areillustrated in Figure 3.

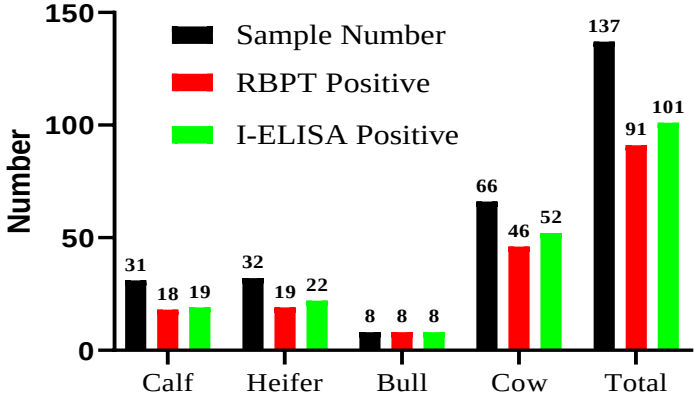

Figure 3: Comparison of serological tests results among the animals of the herd

4.2 Isolation of *Brucella abortus*

Two isolates were obtained after culturing showed common phenotypic characteristics typical for the genus *Brucella*. Bacterial colonies were small, convex, and regular in shape, with a smooth surface that was honey colored, shiny, and translucent. The organisms were gram negative, single coccobacilli, urease positive, oxidase and H_2S positive, and produced urease after 24 hours but not before. The isolates showed negative results in indole, methyl red and citrate test.

4.3 Molecular confirmation and phylogenetic analysis

Brucella abortus was identified in all extracted DNA samples showed 498 fragment sizes in gel electrophoresis after specific multiplex AMOS-PCR. The phylogenetic analysis showed that one sequence (MW 940712) had close similarities to other *Brucella abortus* strains isolated from India, Pakistan and China in different sources (Figure 4). Interestingly, other sequence of *Brucella abortus* strain (MW 940713) indicated a distinctive origin which showed close similarities with another strain from china.

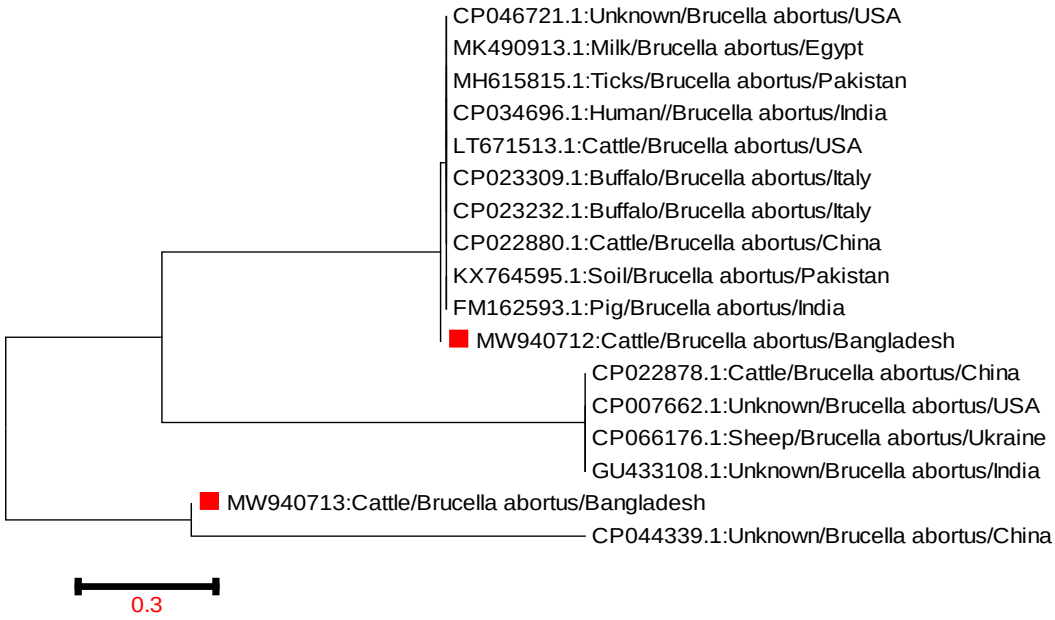

Figure 4: Phylogenetic tree with partial sequence of IS*711* gene of *Brucella abortus* strain. The sequences from cattle in Bangladesh (Red marked) were compared with representative sequence obtained from NCBI gene bank. Maximum likelihood method was performed to infer the evolutionary relationship.

4.4 Comparison of the diagnostic tests

Comparison between RBPT and I-ELISA tests showed in the table below (Table 2). The Kappa value was 82% which indicates strong agreement between the diagnostic tests (p=0.0044) (Gardner, Stryhn, Lind, & Collins, 2000)

Table 2:Comparison between the diagnostic tests

	I-ELISA Positive	I-ELISA Negative	Total
RBPT Positive	91	10	101
RBPT Negative	0	36	36
Total	91	46	137

5. Discussion

Brucellosis is one of the significant bacterial zoonosis with at least half a million human cases worldwide (Figueiredo, Ficht, …, & 2015, 2015). In Bangladesh, this disease remains under reported in most of the occasions. The confirmation of the presence of *B. abortus* in the study area was similar with the surrounding regional findings. Unauthorized cattle trade is a common phenomenon of the border area of Bangladesh and that might have resulted the observed relationship with the isolates of India and China (Hasib et al., 2021; Khalil, Sarker, Hasib, & Chowdhury, 2021). As limited sequences data of *Brucella* organism in Bangladesh was documented, this study investigates the plausible sources of the organism with limited evolutionary data.

In this study a remarkably high prevalence of brucellosis was observed using both the diagnostic tests. Previous published reports found out 2-10% herd level prevalence in Bangladesh with either RBPT or ELISA test (M. S. Islam et al., 2018; S. Islam et al., 2020; M. et al., 2017; Munsi et al., 2018).The discrepancy might be due to management system of the studied farm where all animals were kept in three open shed and animals can easily be contacted with each other. Moreover, bulls were used for natural insemination without prior diagnosis. History of the herd also revealed that subsequent abortion was a common problem along with repeat breeding in the cows. Proper disposal of the aborted materials and isolation of the infected animal was not practiced in the farm and that might be the reason behind the severe dissemination of brucellosis inside the herd (Terefe, Girma, Mekonnen, & Asrade, 2017). The studied farm practiced integrated farming, and we observe weak bio-security and animal movement regulation in place. Moreover, newly introduced animals were kept without screening. The epidemiology of many diseases is influenced by a combination of management factors including a lack of bio-security within the herd and inadequate monitoring of animal movements. In the case of brucellosis, an extremely contagious disease, the infection can easily be spread from animal to animal following an episode of abortion through contaminated pasture or feed, conjunctiva inoculation, skin contamination, or contaminated utensils used to carry infected colostrum for new born calves (Carbonero et al., 2018; Cotterill et al., 2020). Unplanned breeding, which is

common in this type of production system, may facilitate the sexual transmission of the disease (Anka et al., 2014).

This study also explored that I-ELISA was relatively sensitive diagnostic tool for the detection of brucellosis. Though multiple diagnostic tests are recommended for diagnostic purpose due to variable sensitivity and specificity of the tests, we observed alike pattern for both the rapid test and serological test. This study reveals that about 90% of the samples were detected positive by both the diagnostic tests having similarity with the previous published article (Ahasan, Rahman, Rahman, & Berkvens, 2017). Disagreement between the diagnostic tests might be due to samples from the early stage of infections.

In the research area, Brucellosis is endemic in cattle at high levels, and the population might be at risk of infection through direct contact. Appropriate management techniques are required to limit livestock production losses and avoid infections in individuals who are exposed to these animals. The importance of the screening of animals for brucellosis was indicated here in this research. Moreover, host and route of infection are needed to take account in control measures for this zoonotic disease in Bangladesh to mitigate the economic losses from the reproductive failures.

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