# Sero-prevalence and Spatial pattern of Bovine Brucellosis of Chittagong Metropolitan Area


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Roll No.: 0214/01

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**A thesis submitted in the partial fulfillment of the requirements for the degree of Master of Science in Epidemiology**


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**JUNE 2016**

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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 aspects, and that all revisions required by the thesis examination committee have been made.**

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**JUNE 2016**

## Dedication

# Dedicated to my beloved family

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# List of abbreviations

| Abbreviations | Elaboration |
|---|---|
| **2-ME** | 2-Mercaptoethanol Test |
| **%** | Percentage |
| **α** | Alpha |
| ***B*** | *Brucella* |
| **BAU** | Bangladesh Agricultural University |
| **BPAT** | Buffered Plate Agglutination Test |
| **$^{o}$C** | Degree centigrade |
| **Cattle Prev** | Cattle level prevalence |
| **CCBDF** | Central Cattle Breeding and Dairy Farm |
| **CFT** | Complement Fixation Test |
| **cELISA** | Competitive ELISA |
| **CI** | Confidence Interval |
| **FAO** | Food and Agriculture Organization |
| **FPA** | Fluorescence Polarization Assay |
| **Herd Prev** | Herd level prevalence |
| **NA** | Not Available |
| **OIE** | World Organization for Animal Health |
| **PAT** | Plate Agglutination Test |
| **RBT** | Rose Bengal test |
| **RBPT** | Rose Bengal Plate Test |
| **SAT** | Serum Agglutination Test |
| **TAT** | Tube Agglutination Test |
| **Vs** | Versus |
| **WHO** | World Health Organization |

# Abstract

Brucellosis is the most negligible endemic zoonosis in Bangladesh having significant impact on public health, animal welfare and socio-economy of dairy farming. A cross-sectional study on *Brucella* sero-prevalence was conducted in dairy cattle of Chittagong Metropolitan Area (CMA) of Chittagong, Bangladesh from February to November, 2015, to address the current status of the presence of antibodies in serum (sero-prevalence). A total of 158 serum samples were collected from six randomly selected dairy farms of Chittagong Metropolitan Area. The Rose Bengal Plate Test (RBPT) and the Competitive ELISA (cELISA) were used for screening and as confirmatory test, respectively. Farm level and animal level demographic data and suspected risk factor data were collected through questionnaire and tested if any of these factors were contributing to the sero-prevalence by univariable and multivariable logistic regression analysis. At individual cow level, a total of 20.25% (n=32, N=158) samples were sero-positive in RBPT and 8.86% (n=14) in cELISA. Farm level prevalence were ranged from 10 to 26.3% and 5 to 20.7% in RBPT and cELISA, respectively. Among the study population, most common reproductive problem was retained placenta (7.59%) and 3.8% cows were suffering from repeat breeding. Final multivariable model revealed that lactating cows (27.54%; OR=2.59; 95% CI: 1.02-6.54; p=0.043) were significantly in high risk of being seropositive to Brucellosis. The risk of *Brucella* sero-positivity was varied significantly ($p<0.05$) according to the milk production of the population. The study demonstrates that brucellosis is endemic in the study area. However, there is probable risk of spread of the disease in the unaffected cattle population since there are no precautionary measures taken in the areas that should have been practiced by farmers. As a result, there is a need to boost up public awareness about the zoonosis and, design and implement control measures that will prevent further spread of the disease within and outside the study area.



***Key words:*** *Brucellosis, cELISA, RBPT, Retained placenta, Sero-prevalence*

# Chapter-1: Introduction

Brucellosis is one of the notable chronic infectious diseases (Špičić et al., 2010) and worldwide zoonosis affecting livestock and humans (Gwida et al., 2010). It is the second most important zoonotic disease in the world after rabies reported by World Organization for Animal Health (OIE) and most devastating trans-boundary animal diseases leading to a major obstruction to trade (Gul and Khan, 2007). Brucellosis is also known as undulant fever, Malta fever, Mediterranean fever, enzootic abortion, epizootic abortion, contagious abortion, and Bang's disease (Abubakar et al., 2012) in different species. Brucellae are small, non-motile, aerobic facultative intracellular, Gram-negative coccobacilli (Gul and Khan, 2007) that have no capsules, flagella, and endospores (Gwida et al., 2010). The genus *Brucella* has six recognized species on the basis of host specificity (Rahman et al., 2006a); *B. abortus* in cattle, *B. melitensis* in sheep and goat, *B. suis* in swine, *B. canis* in dog, *B. ovis* in sheep, (Seleem et al., 2010), and *B. neotomae* in desert rat (Godfroid and Käsbohrer, 2002). *Brucella* also can infect other ruminants and marine mammals (Lopes et al., 2010). Among six species *Brucella abortus, B. suis, B. melitensis* and *B. canis* have zoonotic importance (Young et al., 2000). *B. ceti* and *B. pinnipedialis* – have recently been discovered in marine mammals which are also pathogenic for humans (Foster et al., 2007).

*Brucella* causes reproductive losses in sexually mature animals, that makes it a major concern (Forbes and Tessaro, 1996; Wadood et al., 2009), Brucellosis is characterized by abortion or retained placenta, still birth, decreased milk production, prolonged calving interval in female and infertility, epididymitis in male (England et al., 2004; Makita et al., 2011; Megersa et al., 2011; Abubakar et al., 2012; Bertu et al., 2012; Maurice et al., 2013; Buhari et al., 2015). Irregularly hygroma and arthiritis are observed due to shedding of organisms through urine and milk (England et al., 2004; Abubakar et al., 2012). Danger continues while animals typically recover, and will be able to have live young following the early abortion, they may go on with shedding of bacteria (Lopes et al., 2010). Brucellosis is considered to be an occupational disease that mainly affects slaughter-house workers, butchers, and veterinarians and those particularly involved with handling live animals (Gul and Khan, 2007). The infection can be transmitted to humans by inhalation, animal contact (with skin abrasion), and consumption of unpasteurized dairy products and undercooked meat products (Gwida et al., 2010).

Geologically brucellosis has been reported in Asia, Africa, South and Central America, the Mediterranean Basin, Sahara (McDermott and Arimi, 2002) and World Health Organization (WHO) accounts more than 500,000 cases in humans alike, annually in the world (Pappas et al., 2006; Musallam et al., 2015). It remains as an uncontrolled major public and animal health problem in regions of highly endemic such as the Africa, Mediterranean, Middle East, parts of Asia and Latin America (Refai, 2002) while it has been eradicated from developed countries like Europe, Australia, Canada, Israel, Japan and New Zealand (Geering et al., 1995; Acha and Szyfres, 2003; Calistri et al., 2013).

The worldwide distribution of brucellosis can be summarized as 10-25% in Central American countries and Latin American countries (Memish and Balkhy, 2004). The Netherlands and England were considered to be free of bovine brucellosis by the turn of the century (Godfroid and Käsbohrer, 2002). Incidence of Brucellosis has been declining in France, Ireland and Italy but Brucellosis-positive herds were still reported (Godfroid et al., 2002). In south-east Asia sero-prevalence of bovine brucellosis can be summarized as: 5% in India (Renukaradhya et al., 2002), 4.7% in Sri Lanka (Silva et al., 2000), 3.25 to 4.4% in Pakistan (Naeem et al., 1990) and 12% in Nepal (Pandeya et al., 2013).

In Bangladesh, different aspects of *Brucella* infection in animal and human were predicted by many authors in different areas of Bangladesh including Chittagong (Islam et al., 1992; Nahar and Ahmed, 2009; Ahasan et al., 2010; Rahman et al., 2011; Rahman et al., 2012; Sikder et al., 2012a; Islam et al., 2013a; Islam et al., 2013b; Belal and Ansari, 2014; Rahman et al., 2014; Rahman, 2015). Before 1945, brucellosis was first recognized in India in 1942 when the India and Bangladesh were the same country (Renukaradhya et al., 2002) and it was first reported in the cattle of Bangladesh in 1967 (Mia and Islam, 1967). After that, several study has been performed and reported the prevalence of brucellosis from different region of Bangladesh that conclude the disease is endemic throughout the country (Nahar and Ahmed, 2009, Ahasan et al., 2010; Rahman et al., 2011; Sikder et al., 2012a; Islam et al., 2013a; Belal and Ansari, 2014; Rahman et al., 1978; Rahman, 2015). Animal-level sero-prevalence of brucellosis in cattle was recorded as 2.4%-18.4% while the herd-level sero-prevalence in cattle was estimated as 62.5% in Bangladesh (Rahman et al., 2006a). Sero-prevalence of brucellosis in different districts of Bangladesh can be recapitulated as 4.6% in Mymensingh (Nahar and

Ahmed, 2009), 5% in Chittagong (Sikder et al., 2012a), 8.6% in Sirajganj (Belal and Ansari, 2014) and 4% in Bogra (Islam et al., 1992; Rahman et al., 2012).

Prevalence of the disease could be influenced by many factors that can affect the pervasiveness of brucellosis in various species of livestock. Prevalence of brucellosis can vary according to the climatic conditions, geography, species, sex, age, breed, pregnancy etc. (Gul and Khan, 2007; Radostits et al., 2000; Nahar and Ahmed, 2009; Belal and Ansari, 2014). Although Brucellosis can be found in any season of a year but epidemic peak occurs from February to July and is closely related to the months associated with delivery and abortion in animals (Abubakar et al., 2012). Some studies found higher prevalence of the disease (39.5%) during summer season in humans (Salari et al., 2003).

Disease mapping is an important approach to describe spatial epidemiology of infectious diseases which is useful to identify areas with suspected elevations in risk, hypotheses formulation about aetiology of disease, and assessing needs for health- resource allocation. To describe the pattern of diseases and to identify regions with unusual frequency of disease, time trends or both, disease mapping is important (Meliker and Sloan, 2011; Schrödle and Held, 2011). Maps influence the recipient much more than accompanying statistics because, it produce attractive and informative maps complements by the formal analysis of spatial epidemiological data (Pfeiffer et al., 2008). In addition, space–time models can contribute to the assessment of the stability of the risk of infection, which cannot be evaluated just by spatial models (Abellan et al., 2008).

The transmission of infectious diseases is closely linked to the concepts of spatial and spatio-temporal proximity, as transmission is more likely to occur if the at- risk individuals are close in a spatial and a temporal sense. Epidemiological analyses therefore have to take both space and time into account with the basic principle being to examine the dependening observations in relation to these two dimensions (Pfeiffer et al., 2008). Methodology frequently used to explore spatial dependence includes analysis of spatial and spatiotemporal clustering and geostatistics. For control and prevention strategies of Brucellosis in cows, it is important understanding the clustering and spreading mechanisms of the disease (De La Sota et al., 2006). Exploration and evaluation of space–time clustering of Brucellosis in Chittagong, Bangladesh might be the basis

for identifying areas where more investigations can be initiated in order to explore the entrenchment and spreading sources of the bacteria.

Conventional serological tests (e.g. RBPT) are not completely specific and cannot always distinguish reactions because most serological tests rely on the unique antigenic properties of lipopolysaccharides (LPS) that are shared among the *Brucella* species and the use of LPS as antigen causes cross-reactivity with organisms such as *Vibrio* and *Yersinia enterocolitica* that share common features of the LPS (Munoz et al., 2005).

Since both the conventional serological tests and the iELISA cannot distinguish vaccinal antibody, competitive enzyme immune assays were developed which is more robust and easy to perform having higher sensitivity and specificity; and it's also can minimize the cross reaction with gram negative bacteria (Biancifiori et al., 2000; Shirima, 2005). The cELISA is a prescribed test by the OIE for international cattle trade and an alternate test for swine brucellosis (Hunter, 1998).

However several studies were conducted in different areas of Chittagong region of Bangladesh, had several limitations like, none of them used epidemiologically structured study design, probability sampling scheme, and thorough statistical analysis of the data to identify risk factors. We performed cELISA; was unique for this region. Moreover, previous studies provided inadequate information about risk factors. Therefore, we aimed to estimate the sero-prevalence and associated risk factors following standard epidemiological study design to facilitate effective prevention strategies. We set our objectives as:

**Objectives:**

- To estimate the sero-prevalence of brucellosis in dairy cattle of Chittagong Metropolitan Area, Bangladesh
- To evaluate the effect of different farm management related and animal level risk factors on sero-prevalence of brucellosis in dairy cattle of Chittagong Metropolitan Area
- To evaluate the agreement between different diagnostic tests to estimate Brucella sero-prevalence

# Chapter-2: Review of Literature

## 2.1 Overview of Chittagong

### 2.1.1 Topography and climate

Chittagong holds human population of 7,616,352 (BSB, 2011). Located in between 21°54' and 22°59' north latitudes and in between 91°17' and 92°13' east longitudes. It is bounded by Khagrachhari and Rangamati districts and Tripura state of India on the north, Cox's bazar district on the south, Bandarban, Rangamati and Khagrachhari districts on the east and Noakhali district and the Bay of Bengal on the west. Chittagong district is quite different from other districts for its unique natural beauty characterized by hills, rivers, sea, forests and valleys. The average elevation of Chittagong, Bangladesh is 15 meters having tropical monsoon climate. Administratively, the district is divided into 14 upazilla (sub-district).

### 2.1.2 Cattle population and management practice in Bangladesh

Livestock population in Bangladesh is currently estimated to 25.7 million cattle, 0.83 million buffaloes, 14.8 million goats, 1.9 million sheep, 118.7 million chicken and 34.1 million ducks (Banglapedia, 2012; Begum et al., 2016). The density of livestock population per acre of cultivable land is 7.37. This density has been increasing every year in the country. The country has a relative density of livestock population well above the averages for many other countries of the world. In spite of a high density of livestock population, the country suffers from an acute shortage of livestock products like milk, meat and eggs. The shortage accounts for 85.9%, 88.1% and 70.7% for milk, meat and eggs, respectively. In Bangladesh, 83.9 percent of total households own livestock (animals or poultry or both). About 45.9 percent households possess bovine stack, and 76.3 percent possess poultry. On average, each household owns 1.52 bovine animals, 0.9 goat and sheep and 6.8 chicken and ducks (Banglapedia, 2012; Rahman et al., 2014). Cattle reared in Bangladesh are mainly indigenous zebu, some exotic breeds and their crosses predominantly Holstein-Friesian, Jersey, Sahiwal and Sindhi. Indigenous cattle are relatively small and give less milk as compared to crossbred cattle (Khan et al., 2009). To improve milk production of the native cow, crossbreeding of indigenous cattle with Holstein-Friesian and Sahiwal is common. Commercial goats, beef and dairy farms are found in small towns, cities and rural areas and are sufficient to meet the local demands of meat and milk. Small farms raise cattle, buffalo, sheep and goats with smaller numbers of animals in contrast with the large herds

of commercial farms. Traditionally, marginal and poor farmers keep a few head of livestock such as cattle, buffalo, sheep and goats maintained under a scavenging system with little or no housing, feeding or health care (Saadullah, 2012). Livestock in the rural areas are maintained on communal grazing land. They are allowed to graze during the day on natural pasture, homestead forest and fallow land. Men play a major role in raising large animals, while women play a vital role in sheep and goat production activities. Furthermore, children also play a significant role in raising livestock in Bangladesh (Paul and Saadullah, 1991; Islam et al., 2013a).

Chittagong Metropolitan Area is situated in the center of Chittagong division. The animals reared in urban area intensively where animals depend on concentrate feeding and fewer amounts of green grass. The health status, disease susceptibility and reproductive performance depends on husbandry practice. There is no comprehensive study on urban husbandry practice and their role in *Brucella* prevalence in Chittagong district.

**2.2 History of Brucellosis**

In 1861, Brucellosis was first suspected in human with malaise, anorexia, fever and profound muscular weakness as presenting sign; and the condition was called "gastric remittent fever" (Shirima, 2005). British scientist, Sir David Bruce isolated the causative agents named it *Micrococcus melitensis* in 1987 from the liver of diseased soldiers in the Mediterranean island of Malta (Wyatt, 2005). The genus *Micrococcus* was resultant from its morphology and "Melita", the species name from the Roman name for the 'Isle of Malta' where the, disease was first recognized (Shirima, 2005; Wyatt, 2009). The soldiers got infection of Malta fever through consumption of contaminated milk of goats (Wyatt, 2005). The Danish researcher Bernhard Bang recognized "*Bacillus abortus*", after ten years of "*Micrococcus melitensis*" discovery, (i.e. *Brucella abortus*) in bovine aborted fetuses (Rahman, 2015). *M melitensis* and Abortus Bacillus of Bang has close bacteriological and serological relationship and the genus name is changed to *Brucella* in honour of Sir David Bruce by Alice Evans (Shirima, 2005). In 1914, the third member of the genus called *Brucella suis* was isolated from an aborted pigs in United States by Traum (Shirima, 2005; Rahman, 2015). BuddIe and Simmons recognized the fourth genus of Brucella named *Brucella ovis* in sheep of Australia and New Zealand in 1953 (Shirima, 2005). Another *Brucella* organism was isolated in 1957 from desert wood rats in USA by Stoenner and Lackman and called *Brucella neotomae* (Shirima, 2005). In 1968, *Brucella canis* was reported in

the USA by Carmichael and Brunner in dogs. In 1994 however, an unofficially designated *Brucella maris* was isolated from marine mammals (Shirima, 2005).

### 2.3 What is Brucellosis?

Brucellosis is world's most widespread (Shirima, 2005; Rahman, 2015) neglected infectious zoonotic diseases infecting both human and animals caused by bacteria of the genus *Brucella,* primarily affects cattle, sheep, goats, swine, and dogs, and is characterized by abortion or infertility and also affects people and other animal species (Ray, 1979; Tun, 2007) and animals develop orchitis, hygromas and sometimes inflammation of the seminal vesicles in male (Tesfaye et al., 2011).

Brucellosis is caused by Gram-negative, aerobic, non-spore-forming, non-motile and non-capsulated bacteria of the genus *Brucella* spp. are coccobacilli (Shirima, 2005; von Bargen et al., 2012; Rahman, 2015) but coccal and bacillary forms also occur (Tun, 2007) who are able to multiply in life-less media. *Brucella* organisms are facultative extracellular intracellular parasites (Moreno and Moriyón, 2002; Rahman, 2015) of Brucellaceae family within the order Rhizobiales of the class $\alpha$2-Proteobacteriaceae (Garrity et al., 2001; Smit, 2013). The first member of the group, *Brucella melitensis*, affects sheep and goats, the second member of the group, *Brucella abortus*, affects cattle, the third member of the group, *Brucella suis*, affects pigs, the fourth member of the group, *Brucella ovis*, affects rams and ewes, the fifth member of the group, *Brucella neotomae*, affects the desert wood rats, and the sixth member of the group, *Brucella canis*, affects male dogs and bitch (Tun, 2007).

#### 2.3.1 Virulence and pathogenicity of *Brucella*

*B. abortus*, *B. melitensis*, and *B. suis* are not host-specific, cross transmission of brucellosis can occur between cattle, swine, sheep and goats and other species including dogs, horses, feral swine, bison, rein deer and camels (Robinson and Production, 2003). Sexually mature female animals are most susceptible to infection. It is usually detected in pregnant females through abortions (England et al., 2004). In all host species *Brucella* grows intra-cellularly, producing a variable bacteraemic phase followed by localization in the tissues of the genital organs and in the mammary gland. Abortion; and orchitis and epididymitis are typical clinical sign of the female and male animals, respectively (Tun, 2007).

### 2.3.2 Pathogenesis of *Brucella*

*Brucella* can infect both non-phagocytic and phagocytic cells but the mechanism of invasion of non-phagocytic cells is not yet undoubtedly time-honored. In non-phagocytic cells, Brucellae tend to localize in the rough endoplasmic reticulum (Zhan et al., 1995).

The Brucellae are swallowed by various local phagocytic cells after invasion and they localized temporarily in the lymph nodes of invasion site results in hyperplasia and acute inflammation of lymph nodes. This cycle is repeated by the multiplying of the Brucellae in the cytoplasm of the phagocytes, rupturing and ingested by new phagocytes (Tun, 2007). From the nodes spreading occurs via the blood to other lymph nodes and the reticuloendothelial cells which leads to bacteraemia that may last for several months, and -may either resolve or be recurrent (Shirima, 2005). In this period, *Brucella* organisms are carried intra-cellularly or free in the plasma and localize in various organs such as the gravid uterus, udder, supra-mammary lymph nodes, spleen, testes and male accessory sex glands and in synovial structures (Radostits et al., 2000). Different nutrient of gravid uterus like sugar alcohol, erythritol act as growth stimulant of *B. abortus*, thus accounting for its localization in the gravid uterus (Tun, 2007). In case of non-pregnant cows, localization occurs in the udder and uterus. Infected udders don't show clinical signs but important source of infection of the uterus and also a source of infection in calves and humans by drinking the milk (Tun, 2007). As the infection assumes a chronic form, bacteraemia becomes intermittent and tends to occur around parturition (Shirima, 2005). *Brucella* can survive in an aborted fetuses in sheds and in liquid manure for up to eight months, three to four months in feces, and two to three months in wet soil and one to two months in dry soil (Bishop et al., 1994). Favorable environmental conditions that enhance survival could, therefore, perpetuate transmission of the organisms (Shirima, 2005).

### 2.3.3 Mode of transmission

Transmission of brucellosis in a clean herd or flock may occur through introduction of *Brucella* infected pregnant animals, aborted or recently delivered (Shirima, 2005) and animals to animal via ingestion of *Brucella* contaminated feed or water or licking an infected placenta, calf or fetus, or the genitalia of an infected animal soon after it has aborted or gave birth (Rahman, 2015) and transmission of infection to other cattle may also occurs through contact with contaminated birthing materials (Rahman, 2015). Young animals may got infection through drinking of

contaminated milk or colostrum (Shirima, 2005). Transmission via inhalation and conjunctiva is also reported (Shirima, 2005).

Male animal may get infection at early life and retain for whole life but they are rarely responsible for introduction or spread of infection to female animal by natural service. Transmission from male to female take place via using of infected semen in artificial insemination. Usually semen are collected from bull's free *Brucella* infection (Shirima, 2005).

### 2.3.4 Public health importance

It is the second most important zoonotic disease in the world after rabies reported by World Organization for Animal Health (OIE) (Hunter, 1998) that infect approximately 500,000 people worldwide annually (He, 2012). Brucellosis is most common regionally neglected (Pappas et al., 2006) zoonotic disease results in significant human morbidity, particularly in few endemic countries (Hunter, 1998; Boschiroli et al., 2001). Recurrent fever is the characteristic symptoms in human with other different manifestations led to the description of the disease as undulant fever, Malta fever or Mediterranean fever (Cutler et al., 2005; Smit, 2013). Acute, subacute or chronic course is found with the incubation period is usually one to three weeks, however occasionally, it may be several months (Shirima, 2005). Among six *Brucella* organism, *Brucella melitensis, B. abortus, B. suis* and *B. canis* are pathogenic to humans, where *Brucella melitensis* infection is most common and infections with *B. canis* are rare (He, 2012) (Figure 2.1).

Transmission of diseases from animal to human by consumption of contaminated non-pasteurised milk and cheese or as an accidental or occupational acquaintance to infected animals or carcasses, aborted foetuses or uterine secretions and sometimes due to, manipulation of live vaccine strains or virulent *Brucella* in the laboratory (Young, 1995; Corbel, 1997).

Farmers, dairy workers, slaughterhouse personnel, veterinarians, and laboratory personnel are most susceptible to animal brucellosis (Rahman et al., 2012) resulting from raw milk consumption, close intimacy with animals and low awareness on zoonosis facilitate transmission of the disease to men (Megersa et al., 2012). *Brucella melitensis, B. suis* and *B. abortus* strains are also used as bioweapons (Pappas et al., 2006).

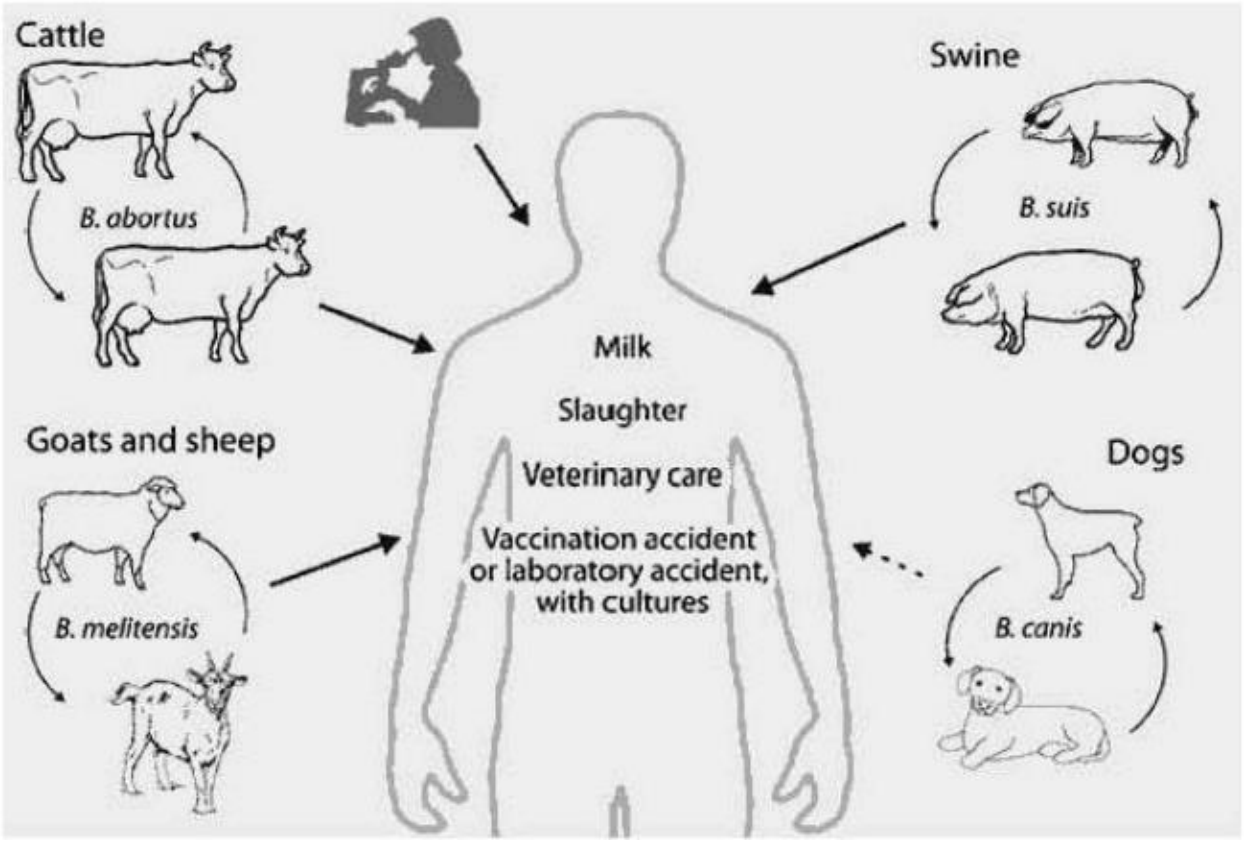


**Figure 2.1: Transmission *Brucella* to human (Smit, 2013).**

### 2.4 Epidemiology of Brucellosis in cattle

Brucellosis occurs in most parts of the world (Chukwu, 1985; Corbel, 1997). It was once an important disease in developed countries but has been eradicated in several countries through test and slaughter, vaccination and restriction of animal movements (Meldrum, 1995).

#### 2.4.1 The global overview

Although the incidence of brucellosis has been reduced to a low level or eradicated in developed countries (Minas et al., 2005), in other parts of the world such as the Mediterranean region, the Middle East, Western Asia, and parts of Africa and Latin America, its magnitude has increased (Rahman, 2015) due to increased animal production, intensive keeping of animals under poor hygienic conditions, in addition to social-economic and behavioural factors (Salehi et al., 2006). In many of these areas, the prevalence of animal brucellosis is high (Amato-Gauci, 1995).

**Table 2.1 Overview of prevalence of bovine Brucellosis in some endemic countries**

| Country | Sample size (Herd/animal) | Study level | Test used | Herd Prev. (95% CI) | Cattle Prev. (95% CI) | Reference |
|---|---|---|---|---|---|---|
| Argentina | NA | National | BPAT, SAT, 2-ME, cELISA, FPA, CFT | 12.4% (10.89–14.0) | 2.10% (1.90–2.40 | De La Sota et al., 2006 |
| Brazil | 921/10170 | Sub-national | RBT and 2-ME | 15.9% (13.6-18.5) | 2.32% (2.04-2.63) | Borba et al., 2013; Chiebao et al., 2015 |
| Georgia | 5673 | Sub-national | RBT | - | 8.5% (7.8-9.3) | Mamisashvili et al., 2013 |
| Algeria | 95/1032 | Sub-national | RBT | 26.3% (17.8-35.4) | 8.2% (6.6-10.1) | Aggad and Boukraa, 2006) |
| Cameroon | 146/1377 | Sub-national | cELISA | 20.3% (4.2-77.6) | 3.1% (1.8-4.4) | (Scolamacchia et al., 2010 |
| Egypt | 1966 | National | RBT | - | 4.98% (4.1-6.0) | Samaha et al., 2008 |
| Ethiopia | 903/7196 | National | RBT, CFT | 20.4% (17.8-23.2) | 4.3% (3.6-4.5) | Ibrahim et al., 2010; Mekonnen et al., 2010 |
| Libya | 42 | Sub-national | - | - | 42.1% (20.3-66.5) | Ahmed et al., 2010a |
| Niger | | Sub-national | iELISA | 14.9% (12.4-17.8) | 3.2% (2.7-3.9) | Boukary et al., 2013 |
| Nigeria | 271/4745 | Sub-national | cELISA | 77.5% (68.6-84.5) | 26.3% (22.1-31.0) | Mai et al., 2012 |
| Zambia | 179/2537 | Sub-national | RBT, cELISA | 56.4% (48.8-63.8) | 16.3% (14.9-17.8) | Muma et al., 2013 |
| Iran | 600 | Sub-national | RBT | - | 3.7% (2.3-5.5) | Akbarmehr and Ghiyamirad, 2011 |
| Jordan | 62/671 | National | RBT, iELISA | 25.8% (15.5-38.5) | 10.1% (7.9-12.7) | Al-Majali et al., 2009 |
| Kyrgyzstan | 1818 | National | RBT, iELISA, FPA | - | 12.0% (7.0-23.0) | Dürr et al., 2013 |
| Tajikistan | 443/904 | Sub-national | iELISA | 4.1% (2.1-6.3) | 2.0% (1.2-3.1) | Lindahl et al., 2014 |
| Turkey | 626 | Sub-national | RBT | - | 35.3% (31.6-39.2) | Şahin et al., 2008 |
| India | 6813 | Sub-national | iELISA | - | 13.6% (12.8-14.4) | Islam et al., 2014 |
| Pakistan | 3699 | Sub-national | RBT, iELISA, cELISA | - | 14.1% (12.9-15.2) | - |
| Mayanmar | 82 | Sub-national | MRT | - | 13.41 (7.21-23.15) | Tun, 2007 |

Legend: CI: Confidence Interval; Herd Prev.: Herd level Prevalence; Cattle Prev.: Cattle level prevalence; RBT: Rose Bengal test; CFT: Complement Fixation Test; BPAT: Buffered Plate Agglutination Test; SAT: Serum Agglutination Test; 2-ME: 2-Mercaptoethanol Test; cELISA:

Competitive ELISA; FPA: Fluorescence Polarization Assay; PAT: Plate Agglutination Test; NA: not available.

### 2.4.2 In Bangladesh

In Bangladesh, brucellosis is endemic (Rahman et al., 2011). Brucellosis in cattle in Bangladesh was first reported in 1967 (Rahman et al., 2011). A serological investigation of brucellosis was performed in cattle on the dairy farm of Bangladesh Agricultural University (BAU) by Rahman and Mia in 1970 (Islam et al., 2013a) that demonstrated an 18.4% prevalence of brucellosis. Milk samples collected from dairy farms were tested by milk ring test (MRT) that showed 11.4%, 11.7% and 4.2% prevalence of brucellosis in Savar, Tangail and BAU dairy farms, respectively (Rahman et al., 1978). Milk samples of cattle on the BAU dairy farm, central cattle breeding and dairy farm (CCBDF) Savar, Dhaka tested by MRT found 5.5% and 11.4% prevalence rates of brucellosis in BAU dairy farm and CCBDF, respectively (Rahman and Rahman, 1981). Prevalence of brucellosis in cows on dairy farms of Pabna and Faridpur districts as well as cows reared in domestic holdings of some villages of Bogra district were studied (Rahman and Rahman, 1981). The prevalence of brucellosis by MRT was 11.5% in Pabna and 2.9% in Faridpur. The prevalence rate was 2.0% in cows reared at the domestic holding of Bogra. Islam et al. (1992) recorded a 15% prevalence of brucellosis in exotic breed of cows and 9% in local cattle breed after screening 760 sera of cows from Avoynagar, Puthia, Hazirhat, Comilla, Manikgonj and Moshurikhola of Bangladesh by rapid screening test and tube agglutination test (TAT). Ahmed et al. (1992) reported 5% prevalence of brucellosis in dairy farms and 2.8% prevalence in rural cows by plate agglutination test (PAT) and TAT cited by Rahman et al. (2014). This study recorded 3.2% prevalence of brucellosis in pregnant cows and 3.1% prevalence in non-pregnant cows. Prevalence of brucellosis was higher in cows above 3 years age (4.8%) than cows less than 3 years (0.7%). The prevalence of brucellosis was 9.1% in cows with a history of previous abortion. During the period of 2004–2012 a total of 1487 serum or milk samples obtained from cattle in six districts of Bangladesh such as Mymensingh (n = 717), Dinajpur (n = 50), Bogra (n = 60), Gaibandha (n = 70), Bagherhat (n = 90) and Chittagong (n = 500) were tested to measure the prevalence of brucellosis (Ahasan et al., 2010; Rahman et al., 2012; Sikder et al., 2012b). The overall prevalence of brucellosis was 4.2% in Mymensingh, 8% in Dinajpur, 1.1% in Bagherhat and 5% in Chittagong. The overall prevalence of brucellosis in

cattle in all six districts estimated by meta-analysis was 3.7% (95% confidence intervals 2.1–66). From the above literature review, it can be calculated that none of the study has conducted solely on urban commercial dairy cattle to know the sero-prevalence of bovine brucellosis with cELISA.

**Table 2.2 Dynamic prevalence of bovine Brucellosis in different areas of in Bangladesh**

| Year | Area (Serology) | Sample size (Positive) | Tests used | Prevalence (95%CI) | References |
|---|---|---|---|---|---|
| 2015 | Mymensingh, CCBDF | 1060 | RBT, SAT, iELISA | 18.2% (14.3-22.8) | Rahman, 2015 |
| 2013 | Mymensingh, Tangail, Sherpur, Sirajgonj | 150(23);270(23); 190 (2): 610 (71) | RBT; Rapid *Brucella* Ab test kit, iELISA | 11.6% (9.2-14.5) | Belal and Ansari, 2014 |
| 2012 | Bagerhat, Bogra, Gaibandha, Mymensingh, Sirajgonj | 465 (4) | iELISA, RBT, cELISA and FPA (performed in South Korea | 0.9% (0.4-2.2) | Rahman et al., 2012 |
| 2011 | Bagherhatt, Bogra, Gaibandha, Mymensingh | 188 (4) | RBT, iELISA | 2.1% (0.6-5.4) | Rahman et al., 2011 |
| 2010 | Dinajpur, Mymensingh | 182 (6) | RBT, iELISA, cELISA | 3.3% (1.2-7.0) | Ahasan et al., 2010 |
| 2009 | Mymensingh | 200 (9); 200 (10) | RBT | 4.8% (2.9-7.3) | Nahar and Ahmed, 2009 |
| 2006 | Mymensingh, Sherpur | 300 (7) | TAT | 2.3% (0.9-4.7) | Sikder et al., 2012a |
| 2004 | Mymensingh | 120 (4) | RBT, PAT, TAT | 3.3% (0.9-8.3) | Amin et al., 2005 |
| 1992 | Chittagonj, Comilla, Jessore, Manikgonj | 350 (17) | RBT, PAT, TAT | 4.9% (2.9-7.7) | Ahmed et al., 1992 |
| 1970 | Mymensingh | 412 (76) | TAT | 18.4% (14.8-22.5) | Rahman and Mia, 1970 |
| | Overall | 3127 (167) | | 5.3% (4.8-6.2) | |

Legend: RBT: Rose Bengal Test; iELISA: indirect ELISA; cELISA: Competitive ELISA; FPA: Fluorescence Polarization Assay; PAT: Plate Agglutination Test; TAT: Tube Agglutination Test. CCBDF: Central cattle breeding and dairy farm.

## 2.5 Associated risk factors of Brucellosis in Cattle

Previous studies evaluated several potential risk factors for brucellosis in cattle. These factors included age (>4 years vs. <4 years), sex, breed (exotic vs. local), management system (commercial vs. backyard; free grazing vs. stall grazing), pregnancy status, and reproductive disorders (abortion vs. retained placenta vs. repeat breeding) (Al-Majali et al., 2009; Chand and Chhabra, 2013; Patel et al., 2014b) and the herd level risk factors of bovine brucellosis identified are large herd size, mixed farming, agroecological zones, contact with wildlife, new entry in the herd, artificial insemination, etc. (Muma et al., 2007; Al-Majali et al., 2009; Chand and Chhabra, 2013; Patel et al., 2014a). Meta-analysis showed that the prevalence odds of brucellosis in cattle under commercial farming were 3 times greater than the prevalence odds of brucellosis for cattle under backyard farming (odds ratio 3.3, 95% confidence intervals 1.3–8.5) ($p = 0.011$). Additionally, the prevalence odds of brucellosis in cows that had had an abortion were 7 times greater than the prevalence odds of brucellosis in cows that had had repeat breeding (odds ratio 7.4, 95% confidence intervals 1.8– 30.3) ($p = 0.005$).

Bovine brucellosis is associated with abortion during the last trimester of gestation, and production of weak newborn calves, and infertility in cows and bulls (Xavier et al., 2009). Bovine brucellosis may also be responsible for retention of placenta and metritis and results in 25% reduction in milk production in infected cows (Acha and Szyfres, 2003; FAO, 2006).

### 2.5.1 Risk factors associated with bovine brucellosis in Bangladesh

The severity and prevalence of the disease may vary with the type of diagnostic test, geographic location, breed, husbandry and environmental factors (Amin et al., 2005). The sero-prevalence is significantly higher in animals with previous abortion reported, compared to animals with no abortion record. In addition, in females a relatively higher prevalence is found than in male cattle, sheep and goats although in the case of buffaloes, this is the other way around (Rahman et al., 2012). Furthermore, significant association is reported between age and sero-prevalence of brucellosis. In cattle and buffalo, the highest sero-prevalence was found in the age group above 48 months of age (Rahman et al., 2011). This may be because the bacteria can remain latent or chronic for an unspecified period of time before manifesting as clinical disease. Alternatively, the higher sero-prevalence among older cows may be related to aged animals having a greater chance of coming into contact with other animals and becoming infected (Rahman et al., 2011).

Because vaccination has never been practiced in Bangladesh, sero-positivity is considered to be due to natural infection (Amin et al., 2005).

### 2.6 Diagnosis of Brucellosis in cattle

Diagnostic methods, including direct and indirect tests, are essential. Direct tests involve microbiological analysis or polymerase chain reaction (PCR)-based methods detecting DNA. On the other hand, indirect tests, either applied in vitro on blood or milk or in vivo (skin test), are based on the detection of immune responses induced by infection (Godfroid et al., 2010). In developing countries direct diagnosis is usually difficult to perform due to the requirement of sophisticated laboratory facilities with high level of safety containment and experienced personnel. Diagnostic methods for brucellosis have therefore primarily been based on serology. All serological tests have limitations, no single serological test is appropriate in all epidemiological situations (Nielsen et al., 2006). The use of at least two tests is recommended; samples that are positive in a screening test should be assessed in a confirmatory test and/or complementary strategy (Gwida et al., 2010).

#### 2.6.1 Serology

Brucellosis is often diagnosed by serology. Serological tests are not completely specific and cannot always distinguish reactions because most serological tests rely on the unique antigenic properties of lipopolysaccharides (LPS) that are shared among the *Brucella* species and the use of LPS as antigen causes cross-reactivity with organisms such as *Vibrio* and *Yersinia enterocolitica* that share common features of the LPS (Munoz et al., 2005). In cattle, sheep and goats, serology can be used for a presumptive diagnosis of brucellosis, or to screen herds. Serological tests commonly used to test individual cattle or herds include the buffered *Brucella* antigen tests (Rose Bengal test and buffered plate agglutination test), complement fixation, indirect or competitive enzyme-linked immunosorbent assays (ELISAs) and the fluorescence polarization assay. The classical Rose Bengal test (RB) is often used as a rapid screening test (Ruiz-Mesa et al., 2005). For confirmation of RB the Wright or serum agglutination test (SAT) or in more sophisticated equipped laboratories enzyme linked immunosorbent assay (ELISA) may be used (Munoz et al., 2005). Rivanol precipitation, acidified antigen procedures and the serum agglutination test (tube or microtiter test) are also available. Supplemental tests such as complement fixation or rivanol precipitation are often used to clarify the results from plate or

card agglutination tests. ELISAs or the *Brucella* milk ring test (BRT) can be used to screen herds by detecting antibodies in milk. In USA, the two primary methods of testing of brucellosis in cattle are: the *Brucella* ring test (detect antibody in pooled milk samples from dairy herds) and the market cattle identification blood test (to test serum antibodies in blood samples). In vaccinated cattle, the native hapten-based gel precipitation tests (gel diffusion or radial immunodiffusion tests) are sometimes used to distinguish vaccination from infection. In sheep and goats, *B. melitensis* can be diagnosed with the buffered *Brucella* antigen tests, complement fixation or ELISAs. Native hapten-based gel precipitation tests are also used in vaccinated sheep and goats. The bulk milk ring test is not used in small ruminants. Serological tests used to detect *B. ovis* include ELISAs, agar gel immunodiffusion (AGID) and complement fixation. Other tests including hemagglutination inhibition and indirect agglutination have also been described. Serological tests used to detect *B. canis* in dogs include rapid slide agglutination (card or RSAT) tests, tube agglutination, an indirect fluorescent antibody (IFA) test, AGID and ELISA.

### 2.6.2 **Importance of cELISA**

Since both the conventional serological tests and the iELISA cannot distinguish vaccinal antibody, competitive enzyme immune assays were developed. The main rationale for these assays was that vaccines induced antibody of lower affinity due to the shorter exposure to antigen due to immune elimination compared to field infection in which antigen persisted, resulting in increased antibody affinity (Nielsen et al., 1989; MacMillan et al., 1990). Thus a competing antibody could be selected to inhibit binding of vaccinal but not field strain induced antibody. Because of their inherent supply and uniformity advantages, monoclonal antibodies were selected as competing antibodies, however, a similar assay, the particle concentration fluorescence immunoassay (PCFIA) used a polyclonal antibody for competition. The latter assay was used exclusively in the USA as a rapid screening procedure (Snyder et al., 1990; Nicoletti and Tanya, 1993). The selected monoclonal antibody should be specific for a common epitope of the OPS molecule, allowing its use for *B. abortus*, *B. melitensis* and *B. suis* serology (Nielsen et al., 1995; Biancifiori et al., 2000; Paulo et al., 2000). The most commonly used format of the cELISA utilizes SLPS from *B. abortus* as antigen, passively attached to a polystyrene matrix, followed by incubation with competing antibody and appropriately diluted test serum. After mixing and incubation, a reagent for detecting bound monoclonal antibody, labeled with an

enzyme, usually horse radish peroxidase or alkaline phosphatase, is added followed by substrate/chromogen after a suitable incubation period. A wash procedure is performed between each step. A series of controls, including a strongly positive, a weakly positive, a negative serum as well as a buffer (no serum) controls must be included. Results are calculated as percent inhibition against the buffer control (0% inhibition). The cELISA is a prescribed test by the OIE for international cattle trade and an alternate test for swine brucellosis (Hunter, 1998).

### 2.7 Spatial epidemiology

Spatial epidemiology is the sub-discipline of epidemiology (Durr and Gatrell, 2004) where the geographical location of the events is a fundamental component (Sáez and Saurina, 2007) with the primary purpose is to describe and explain spatial pattern of diseases (Durr and Gatrell, 2004).

Up to the 1980s, it is difficult to find examples in the veterinary literature; the exceptions are works undertaken by parasitologists, interested in the interaction between climate and disease via its effect on vectors and intermediate hosts. One of the first works was conducted by Robson et al. (1961) who showed that the East Coast fever in Tanzania was confined to areas where tsetse flies were absent and cattle was present. Also in Tanzania (Lake Victoria) by carefully mapping disease outbreaks in relation to the cattle population, draw a line separating enzootic and epizootic areas and map the spatial development of the disease (Kaiser et al., 1988). In human medicine, there are studies dating from the beginning of the 1800s in which maps were employed to demonstrate the distribution of disease (Lawson et al., 2001). Possibly the most famous use of mapping in epidemiology in this period were the studies by John Snow of the cholera epidemics in London in 1854 through observation of the addresses of the people who die. Snow was among the first to show clearly that cholera could be spread through a contaminated water supply (Lawson et al., 2001) (Figure 2.2).


**Figure 2.2: John Snow map of cholera deaths and water supply in London**

Nevertheless, it was not until the 1980s, thanks to the technical breakthroughs in computing, that spatial epidemiology was really developed. Advances in geographic information systems (GIS), statistical epidemiology and availability of high-resolution, geographically referenced health and environment data, created new opportunities to investigate geographic variations in disease occurrence (Elliott and Wartenberg, 2004). GIS, spatial analysis and remote sensing are the main tools employed in spatial epidemiology (Durr and Gatrell, 2004). GIS are powerful tools for displaying and querying spatial information and in the last years they have been built more user-friendly and powerful software packages. The fundamental ingredient of these packages is the use of map layers which contain different information about the mapped area. Each layer can be manipulated interactively (edited) to provide a composite map (Lawson et al., 2001). Spatial analysis techniques have been developed in parallel, but largely independently. As a result, modern GIS software still has fairly limited spatial analysis functionality Pfeiffer, 2004) .

### 2.7.1 **Application of spatial epidemiology in veterinary field of Bangladesh**

In Bangladesh no previous report on spatial epidemiology of large and small animal diseases. In case of poultry, first spatial epidemiology of avian influenza outbreak in poultry reported by (Ahmed et al., 2010) in January 2010. In the same year Loth et al. (2010) applied spatial epidemiology technique to identify the cluster of avian influenza outbreak cluster in Bangladesh.

Spatial analysis deals with the exploration, description and analysis of data taking into account their geographical distribution (Sáez and Saurina, 2007). Spatial data are defined as geographical features and the attributes of these features, each feature will often have multiple attributes (Pfeiffer and Hugh-Jones, 2002).

### 2.7.2 Disease mapping

Disease mapping is an approach to summarize spatial variation in disease risk, in order to assess and quantify the amount of true spatial heterogeneity and the associated patterns, to highlight areas of elevated or lowered risk and to obtain clues as to the disease aetiology (Best et al., 2005). The detection of disease clusters has typically been come up to as a hypothesis testing problem; whether the geographical distribution of disease or any event is random or not, adjusting for the geographical distribution of the population (Ugarte et al., 2005). Disease mapping methods are most useful for apprehending gradual regional changes in disease rates, and are less useful in detecting abrupt localized changes indicative of clustering (Gangnon and Clayton, 2000). The objectives of presenting the data in map are to identify locations with unusually high or low disease levels, a communal parameter represented is the ratio between the observed and expected cases Elliott and Wartenberg, 2004).

### 2.7.3 Data visualization

The results of the statistical procedures are represented visually in mapped form. Hence, some consideration must be given to the purely cartographic issues that affect the representation of geographical information (Lawson et al., 2003). The type of map presentation depends on the type of data available, either the actual event locations (such as the x-y coordinates) or aggregate data (Pfeiffer and Hugh-Jones, 2002),

#### 2.7.3.1 Point data

To visualize ***point data,*** the oldest and most frequently-used method is to plot the locations of the study subjects using their Cartesian coordinates. Whereas plots of point events provide a general impression of the spatial characteristics of the process under investigation, they present problems where there are multiple events at the same location since no indication of event density can be appreciated. Because of this, point maps are best suited for displaying location information for small number of events (Stevenson, 2003). If a continuous surface is to be mapped based on a discrete set of observation points, then interpolation techniques, based on geostatistical methods, must be used ( Lawson et al., 2001) . Interpolation techniques enable the construction of *isopleth* maps. These maps show the distribution of spatially continuous phenomena by a logical sequence of tones colour that symbolizes equal values. Isolines are often overlaid on top of an isopleth map to indicate threshold value.

It is evident from the above review that brucellosis is endemic in Chittagong district of Bangladesh. Several studies on brucellosis sero-prevalence in animals and its risk factors in cattle are available. The previous studies used non-random sampling techniques (non-probability) and the sample size was remarkably small. Prevalence of a disease is a population parameter, if it is not estimated from a random and representative sample, it will not reflect the disease status in that population due to selection bias. The tests used for the identification of brucellosis antigen or antibody were not sensitive enough and their performance was not evaluated in Chittagong, Bangladesh context. Indeed, when diagnostic tests are used without evaluating their performance in a specific context usually generate unreliable results, which in turn may lead to wrong epidemiological inferences (Godfroid et al., 2013). The prevalence of brucellosis and risk factors of bovine brucellosis along with spatial distribution were not studied in Chittagong. Moreover, the epidemiological understanding of bovine brucellosis in Chittagong, Bangladesh is incomplete and sometimes misleading for the decision makers to initiate a control strategy.

# Chapter-3: Materials and Methods

## 3.1 Study site and study period

The present study was conducted to investigate the epidemiology of brucellosis in dairy cattle in Chittagong metropolitan area (CMA), Bangladesh from February to November 2015. Chittagong metropolitan city is located 22°22'N and 91°48'E, and is 29 m up from the sea level. This city is situated in the tropical zone and characterized by annual average range temperature of 13°C to 32°C, rain fall of 5.6 mm to 727.0 mm and humidity of 70 to 85% (Anon, 2016). Chittagong metropolitan has 14 distinct areas. However, the study area were divided into five sub-sites like north, south, east, west and centre and included for the study and they were Chadgoan (North), Bayezid (West), Bakulia (East), Halishahar (South) and Panchlaish (Central). The sub-sites of the study were selected based on cattle population density.

## 3.2 Reference and study population

All commercial dairy cattle in Chittagong district, consisting of 14 upazila (sub-districts) was considered as reference population (N=450 commercial dairy farms; n=7000 cattle population) where the commercial dairy cattle in Chittagong metropolitan area (covering aforementioned study sub-sites) was taken as study population. The number of total dairy commercial farms and population, having at least 2 cattle per farm, in CMA is 297 and 6054, respectively (Figure 3.1), whereas the number of commercial dairy farms and population, having at least 25 cattle per farm, in selected study areas of CMA is 57 and 3507, respectively. The distribution of farms and cattle population by study sub-sites is presented in Table 3.1.

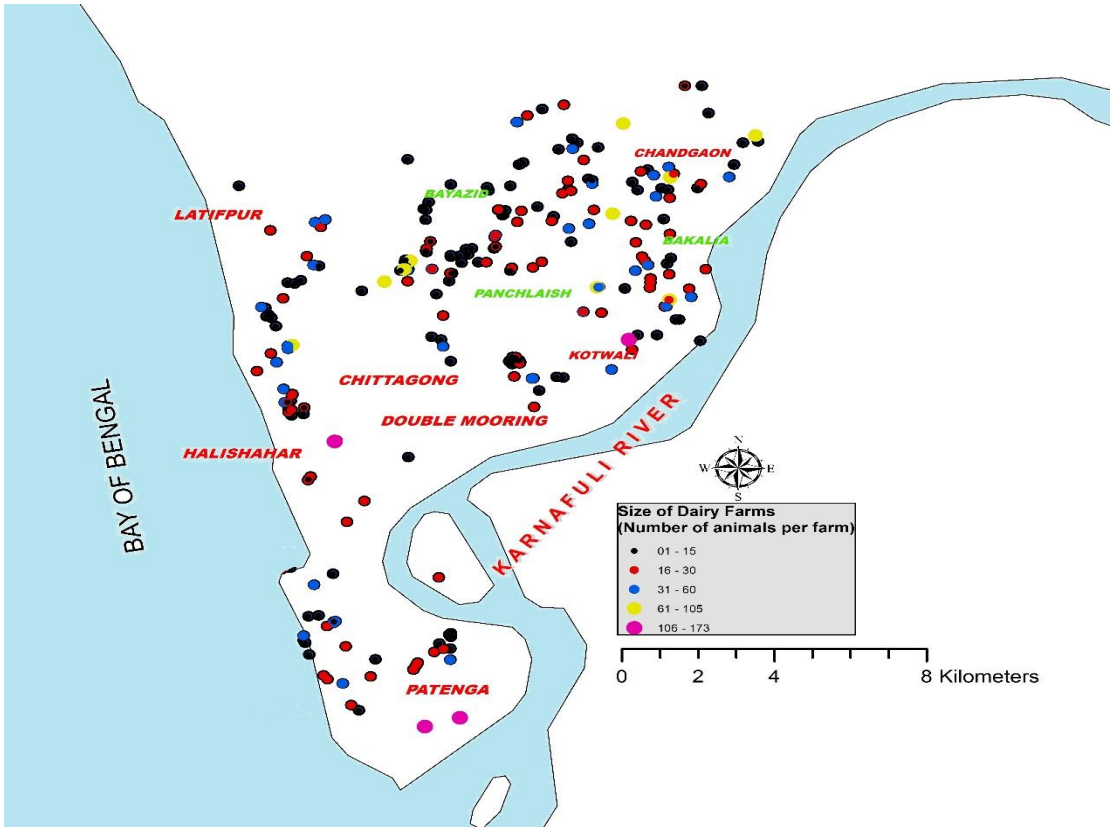


**Figure 3.1: Distribution of cattle farms in Chittagong Metropolitan Area (N=293)**

**Table 3.1 Distribution of farms and cattle population by study sub-sites in Chittagong Metropolitan areas (N=144), Chittagong**

| Study sub-sites | Total number of farms | Total population | Range population per farm |
|---|---|---|---|
| Chadgoan (North) | 21 | 748 | 3-93 |
| Bayezid (West) | 29 | 263 | 5-43 |
| Bakulia (East) | 40 | 683 | 11-135 |
| Halishahar (South) | 15 | 806 | 4-80 |
| Panchlaish (Centre) | 39 | 584 | 4-45 |

### 3.3 Study design

A cross sectional study was conducted to estimate the sero-prevalence of brucellosis in dairy cattle and associated risk factors.

### 3.4 Sample size determination

Animal-level sero-prevalence of brucellosis in cattle was recorded as 2.4%-18.4% while the herd-level sero-prevalence in cattle was estimated as 62.5% in Bangladesh (Rahman et al., 2006a). Sero-prevalence of brucellosis in Chittagong was estimated as 5% (Sikder et al., 2012b).

Assuming total number of dairy cattle population (i.e., cows) at the study area as 7000 (based on an initial pilot survey), with 10% expected prevalence , precision ± 5% and 95% confidence interval and design effect 1% the estimated sample size was 136 dairy cattle. For safety, another 24 animals (approximately 15% of the estimated sample size) were additionally considered to reach the sample size of 158 dairy cows (Table 3.1).

### 3.5 Sampling strategy and distribution of sampled farms and cattle population

Six farms were randomly selected, one from each study sub-site except Panchlaish. Two farms were chosen from Panchlaish. Afterwards all eligible cattle (158) belonging to the selected farms were sampled and these sampled numbers were almost matched up with the estimated sample size (160) for the study. The distribution of sampled farms and population are presented in Table 3.2.

**Table 3.2 Distribution of sampled farms and eligible population (only matured dairy cattle) by study sub-sites in Chittagong Metropolitan areas, Chittagong (N=6 farms, n=158 cattle)**

| Study sub-sites | No. of selected farm | Total eligible population |
|---|---|---|
| Chadgoan (North) | 1 | 29 |
| Biozid (West) | 1 | 39 |
| Bakulia (East) | 1 | 29 |
| Halishahar (South) | 1 | 20 |
| Panchlaish (Central) | 2 | 19 |
| | | 22 |

### 3.6 Collection of blood samples and recording epidemiological data

Blood samples from the selected cows were collected for serological evaluation in the present study. The individual animals were identified by their respective identification numbers or names. None of the animals was vaccinated against *B. abortus* confirmed by farm recording system and farmers' responses. The animals were held back in the controlling crate and bled by puncturing the jugular vein. About 5 - 10 ml of blood was collected in plain tubes without EDTA (Ethylenediaminetetraacetic acid). The blood sample was labeled using the tag number assigned to each individual animal. It was essential to avoid the shaking of the tubes (which contain blood) during the transport to prevent the distraction of the blood cells and hemolysis. The tubes were placed vertically at room temperature for 1 hour and were then refrigerated at 4°C for overnight before spinning at 3000 rpm for 10 minutes. The separated serum samples were taken into sterile epindroff tubes and kept in a refrigerator when used after short periods or in a freezer (-20ºC) for longer periods. A standard questionnaire was administered to each farmer through face to face method by a technical person. This questionnaire was designed for an active survey to collect animal level risk factors associated with the sero-prevalence of brucellosis (age, parity, breed, lactation stage, BCS, reproductive problem, milk yield). In addition, general characteristics of the farm, such as the management system, recording system, the issue of biosecurity and reproductive problems were recorded. Information of the questionnaire was recorded by different means: by visual examination of the farm, from record book and by asking questions to the farmer. Some animal level data were verified by physical examination of the animal (e.g., age of the animal, pregnancy period of the animal etc.). To validate data regarding milk yield per animal, farms were visited during the time of milking. Body Condition Score were assessed by using the following characteristics as described by Wildman et al. (1982) and Stádník and Atasever (2015): the feeling of amount of muscling and fat deposition over and around the vertebrae in the loin region of the cows. The score was scaled from 1 to 5 (1=emaciated; 2=thin; 3=average; 4=fatty and 5=obese cows).

The interview was conducted with one member of the farm who was knowledgeable about the herd and/or flock. The information collected included retrospective information over a period. Each interview took about 30-40 minutes. The geographic location of each farm was recorded

using a hand-held Garmin® Global Positioning Systems (GPS). The questionnaire used in the study is given in the **Appendix 1**.

### 3.7 Laboratory evaluation

Two serological tests were used to evaluate serum samples for brucellosis in cattle. They were Rose Bengal Plate Test (RBPT) (Se and SP: 96.1% and 63.6% respectively (Padilla et al., 1999)) and Competitive Enzyme Linked Immunosorbent Assay (cELISA) (Se and SP: 97.5% and 98.3%, respectively (Padilla et al., 2010).

#### 3.7.1 **Rose Bengal Plate Test**

The RBT is a rapid agglutination test based on mixing a drop of serum and a drop of antigen together. Atlas *Brucella* test® (Atlas Medical, Cambridge) reagent was used for this test. One drop (30 µl) of RBPT reagent was added to equal volume of serum sample on a glass slide to produce a zone approximately 2 cm in diameter. After that, both drops were mixed by the disposable stirring stick, spreading them over the full surface of the circle. Afterwards the serum and the antigen were mixed thoroughly. The mixture plate was rotated for 4 minutes at an ambient temperature. The plate was then observed for the presence or absence of any degree of agglutination. The assessment was carried out exactly 4 minutes after the beginning of the shaking. Any visible reaction was considered to be positive.

#### 3.7.2 Enzyme Linked Immunosorbent Assay

The SVANOVIR *Brucella*-Ab cELISA, which was used for the present study for confirmatory diagnosis, is designed to detect antibodies to *B. abortus* and *B. melitensis* in serum. It is a multi-species assay allowing detection of *Brucella* specific anti-bodies in various species. In cattle, the assay is capable of distinguishing between *Brucella* infected animals, *Brucella* strain 19 vaccinated animals and animals infected with cross-reacting Gram negative bacteria.

The kit procedure (SVANOVIR® *Brucella*-Ab cELISA by Svanova, Sweden) is based on a solid phase cELISA. In this procedure, the samples were exposed to *B. abortus* smooth lipopolysaccharide (S-LPS) coated well on 96 wells microtiter plate together with a mouse

monoclonal antibody (mAb) specific for an epitope on the o-poly-saccharide portion of the S-LPS antigen.

**Procedure**

1. All reagents were equilibrate to room temperature 18-25ºC before used.
2. At first 45 µl of sample dilution buffer was added into each well that were used for serum samples, serum controls and conjugate controls.
3. 5µl of positive, weak positive and negative serum controls were added, into each of the appropriate well, respectively. For confirmation purposes, it was duplicated the controls.
4. 5µl of sample dilution buffer was added into two appropriate well designated as conjugate controls.
5. 5µl of test serum samples were added to each of the appropriate wells.
6. 50µl of mAb solution were added into all wells used for controls and samples.
7. Then sealing and thorough mixing was done for 5 minutes using plate shaker.
8. Then plate was incubated at 37ºC for 30 minutes.
9. Following incubation, plate was rinsed 4 times with PBS-tween buffer.
10. 100µl of conjugate solution was added into each well and sealed the pate followed by incubating the plate at room temperature (18-25ºC) for 30 minutes.
11. Step 9 was repeated.
12. 100µl of substrate solution was added to each of the wells and incubated for 10 minutes at room temperature (Timing was counted from first well was filled).The optical density (OD) was measured by micro-plate photometer at 450 nm. The percentage of OD of the conjugate (% OD) was calculated as the average OD of the paired sample wells were divided by the average OD of the conjugate wells on the plate. The standard %OD cut-off of 70% was used for interpretation of results.

### 3.8 Data entry and statistical analysis

Field and laboratory data were stored in Microsoft Excel 2007 spread sheet. Data were cleaned, coded and checked for integrity in MS Excel 2007 before exporting to STATA-13*(StataCrop, 4905, Lakeway Drive, College station, Texas 77845, USA)* for performing epidemiological analysis.

#### 3.8.1 **Descriptive analysis**

1. Overall sero-prevalence (Animal level) (%, 95% CI, N)

2. Sero-prevalence in cattle within farm (%, 95% CI, N)

3. Management characteristics (%, N)

Descriptive statistics (frequency number and percentages) were calculated to express Brucellosis status of cattle and different factors.

Categorical variables were summarized as frequency and percentages; continuous variables were summarized as mean ± standard deviation (SD). An animal was considered seropositive if it is tested positive to either RBPT or cELISA (parallel interpretation). A herd, defined as the total number of cattle belonging to the same household, was considered seropositive if it included at least one seropositive animal. Animal and herd apparent sero-prevalence was calculated by dividing the number of positive test results by the total number of animals and herds sampled, respectively. The within-herd sero-prevalence was calculated by dividing the number of seropositive animals in the herd by the total number of animals tested in that herd.

#### 3.8.2. **Spatial analysis**

##### *3.8.2.1* **Sero-prevalance in cattle according to space**

Dot maps were created to show the location of the study population and the sampled farms. Herd level sero-prevalence of brucellosis tested by cELISA and RBPT was shown in dot maps. To create spatial maps of farm distribution and herd level sero-prevalence, ArcGIS version 10.2.1 (ESRI, Redlands, California, USA) was used.

### 3.8.3 **Risk factor analysis**

#### *3.8.3.1* **Univariate analysis**

The associations between sero-positivity and categorical risk factors were tested using Chi Square test or Fisher's exact test. To select variables, univariable random effects (RE) logistic regression model (farms as random effect) was fitted to assess associations of predictors with the dependent variable (sero-positivity).

#### *3.8.3.2* **Multivariate analysis**

Independent variables that were significantly associated with sero-positivity in the univariable analysis ($p < 0.20$) were included in to the multivariable RE logistic regression model. In multivariable analysis, a backward elimination procedure was used applying the maximum likelihood estimation procedure and statistical significance of contribution of individual predictors (or group of predictors) to the models tested using the Wald's test and likelihood ratio test as described by Dohoo et al. (2003). The interactions between variables were assessed by constructing two way interaction product terms for the significant main effect variables in the model, forcing them into the model and examining changes in the odds ratio (OR) and p values of the main effects. Confounding effect of the explanatory variables was evaluated by observing the change of parameter estimates before and after removal of a variable from the model. If the parameter estimate of a variable increased or decreased $\geq 15\%$ after removing a variable from the model then these two explanatory variables were considered to have confounding effect on the outcome variable. All biologically meaningful interactions were also checked.

# Chapter- 4: Result

### 4.1 Overall sero-prevalence of *Brucella* in cattle

On RBPT the sero-prevalence of *Brucella* was 20.3% (95% CI: 14-27; N=158) in dairy cattle of CMA. However, the sero-prevalence of Brucella in cELISA testing was 8.7% (95% CI: 5-14; N=158) in dairy cattle of CMA. The results between diagnostic tests were significantly varied ($\chi 2$ test value 40.75; p=0.000).

### 4.2 Sero-prevalence of *Brucella* in farms and within farm

In RBPT, all 6 farms had Brucella Ab positive, whereas 5 farms had *Brucella* Ab positive in cELISA. Within the farm, the sero-prevalence ranged from 10%- 26.3% and 5%-20.7% by RBPT and cELISA, respectively (Table 4.1).

**Table 4.1: Sero-prevalence of *Brucella* in dairy cattle farms and within farms in Chittagong Metropolitan area**

| Study sub-sites | No of cattle tested | RBPT % positive (95% CI) | cELISA % positive (95% CI) |
|---|---|---|---|
| Chadgoan (North) | 29 | 24,1 (10-43 ) | 20.7 ( 8-40) |
| Biozid (West) | 39 | 20.5 (9-36 ) | 5.1 (6-17 ) |
| Bakulia (East) | 29 | 20.7 (8-40 ) | 6.9 (1-22 ) |
| Halishshahar (South) | 20 | 10.0 ( 1-32) | 5.0 (0.1-25 ) |
| Panchlaish (Centre) | 19 | 26.3 (9-51 ) | 0 ( ) |
| | 22 | 18.2 (5-40) | 13,6 (3-35) |

### 4.3 Spatial distribution of sampled farm along with their infection status

The sero-positivity of the farm based on cELISA along with the sampled farm locations are presented in Figure 4.1. Sampled farms were found randomly distributed throughout the study area. The only one negative farm (none of the cows showed positive result in cELISA from the farm) was located at the middle of the study area.

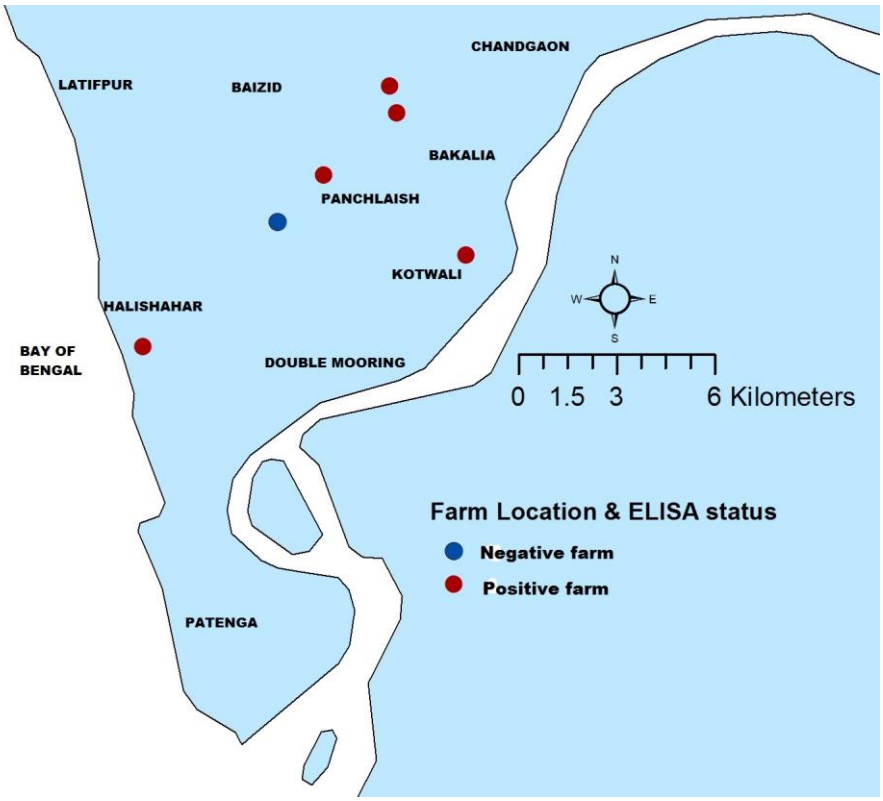


**Figure 4.1: Geographical location and infection status of the farms by cELISA**

### 4.4 Risk factor analysis between *Brucella* prevalence in dairy cattle (based on RBPT) and the selected risk factors

#### 4.4.1. Univariate analysis

Univariate Chi-square and logistic regression analysis determined statistically significant effect of lactation, anestrous, over all reproductive disorders, milk yield, lactation number, trimester and abortion on the individual animal sero-prevalence ($p \leq 0.2$) (Table 4.2).

Lactating cows had significantly higher (27.5%) sero-prevalence than non-lactating cows ($p=0.04$). The odds ratio (OR) indicated that lactating cows were 2.5 time more (95% CI: 1-4.9) likely to be sero-positive to *Brucella*. In respect to cows with any kinds of reproductive disorders, cows with reproductive problems had significantly more chance becoming sero-positive to *Brucella* (37.5%) than cows without any problems (17%). Cows with reproductive problems were about 3 times (OR=2.9; 95%CI: 1.1-7.5) more likely to become sero-positive than other cows. Cows which had history of anestrous had higher prevalence (66.67%) than cows which had no such history ($p=0.04$). When the cows had a history of abortion, the risk of being sero-positive to *Brucella* was 4 times higher (95% CI: 06-30.5) compared to cows with no abortion history ($p=0.13$). Pregnant cows in 2$^{nd}$ to 3rd trimester were less (OR=0.48; 95% CI: 0.18-1.2) prone to Brucella positive serologically than cows in other trimester of pregnancy period ($p=0.53$) (Table-4.2).

**Table 4.2: Univariate associations between potential risk factors and Brucella spp. Serological status tested by RBPT in dairy cattle of CMA, Bangladesh**

| Variables | Category | $\chi$2 test | | | Univariate logistic regression | | |
|---|---|---|---|---|---|---|---|
| | | + (%) | - | p | OR | 95% CI | p |
| Age (Years) | 1-4 | 10 (18.5) | 44 | 0.477 | 1 | | |
| | 4.1-6 | 7 (14.9) | 40 | | 6.3 | 0.79-50 | 0.082 |
| | 6.1-7 | 6 (30) | 14 | | 4 | 0.41-39.35 | 0.235 |
| | 7.1-14 | 9 (24.3) | 28 | | 3.7 | 0.41-33.52 | 0.241 |
| BCS | 2 | 2 (33.3) | 4 | 0.298 | 1 | | |
| | 3 | 26 (22.2) | 91 | | 0.51 | 0.1-3.33 | 0.531 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | 4 | 4 (11.8) | 30 | | 0.27 | 0.4-2 | 1.94 |
| Lactating | Yes | 19 (27.5) | 50 | 0.049 | 1 | | 0.05 |
| | No | 13 (14.8) | 75 | | 2.19 | 0.99-4.8 | |
| Heifer | Yes | 2 (10) | 18 | 0.22 | 1 | | 0.23 |
| | No (Cows) | 30 (21.9) | 107 | | 0.4 | 0.08-1.8 | |
| Pregnancy | Yes | 13 (16.9) | 64 | 0.30 | 1 | | 0.306 |
| | No | 19 (23.5) | 62 | | 0.66 | 0.30-1.40 | |
| Milk yield (Liter) | 0-2 | 10 (25) | 30 | 0.17 | 1 | | |
| | 2.1-12 | 12 (24.5) | 37 | | 0.97 | 0.37-2.6 | 0.96 |
| | 12.1-15 | 2 (6.3) | 30 | | 0.2 | 0.04-1 | 0.04 |
| | 15.1-28 | 8 (21.6) | 29 | | 0.83 | 0.29-2.4 | 0.73 |
| Parity | No | 4 (21.05) | 15 | 0.686 | 1 | | |
| | 1 | 4 (14.29) | 24 | | 0.625 | 0.14-2.9 | 0.547 |
| | 2 | 24 (21.62) | 87 | | 1.03 | 0.31-34 | 0.956 |
| Lactation no. | 0-3 | 20 (17.4) | 95 | 0.143 | 1 | | 0.147 |
| | 4-12 | 12 (27.9) | 31 | | 1.84 | 0.81-4.86 | |
| Trimester | 1st | 26 (23.4) | 85 | 0.128 | 1 | | 0.133 |
| | 2nd-3rd | 6 (12.8) | 41 | | 0.48 | 0.18-1.2 | |
| Duration of last calving (Months ago) | 1-2 | 10 (17.5) | 47 | 0.524 | 1 | | 0525 |
| | 2.1-24 | 22 (21.8) | 79 | | 1.3 | 0.57-3 | |
| Reproductive disorders | Yes | 9 (37.5) | 15 | 0.022 | 1 | | 0.03 |
| | No | 23 (17.2) | 111 | | 2.9 | 1.13-7.42 | |
| Anestrous | Present | 2 (66.7) | 1 | 0.043 | 1 | | 0.088 |
| | Absent | 30 (19.5) | 125 | | 8.33 | 0.73-94.9 | |
| Abortion | Present | 2 (50) | 2 | 0.134 | 1 | | 0.164 |
| | Absent | 30 (19.5) | 124 | | 4.13 | 056-30.5 | |
| Retained placenta | Present | 3 (25) | 9 | 0.670 | 1 | | 0.671 |
| | Absent | 29 (19.9) | 117 | | 1.34 | 0.34-5.28 | |
| Repeat breeding | Present | 2 (33.3) | 4 | 0.416 | 1 | | 0425 |
| | Absent | 30 (19.7) | 122 | | 1.81 | 0.36-11.62 | |

### 4.4.2 Multivariate analysis

Risk factors that had significant effect ($p \leq 0.2$) in univariable chi-square and logistic regression were fitted in a model of multivariable logistic regression. Seven out of fifteen variables examined were significantly associated with the serological status of individual cattle against *Brucella* spp. in the univariable analysis ($p<0.20$) (Table 4.2).

In multivariable logistic regression model, no two way interaction was found between the variables in the data set. Regression coefficients were converted into odds ratios (ORs) and their 95% confidence intervals (CIs). After the backward elimination of insignificant variables, the final logistic model was constructed with two significant variables: lactating cows had a ($p<0.05$) higher odds (OR: 2.59; 95%CI: 1.02-6.55) of being positive and cows with medium to high milk yield showed protective effect compared to low yielding cows (Table 4.3). The Likelihood Ratio test (LRT) of goodness of fit was not significant ($p=0.14$) and the area under the Receiver Operating Curve (ROC) was 0.76, indicating that the model fitted the data well and had a high predictive ability to discriminate sero-positive and sero-negative animals.

**Table 4.3: Results of multivariable logistic regression on serological status of cows against Brucella spp.**

| Variables | Categories | Odds ratio | 95% CI | p |
|---|---|---|---|---|
| Lactation | No | 1 | | |
| | Yes | 2.6 | 1.0 – 6.6 | 0.043 |
| Milk yield (liter) | 0 - 2 | 1 | | |
| | 2.1 - 12 | 0.3 | 0.1 – 1.0 | 0.051 |
| | 12.1 - 15 | 0.1 | 0.01 – 0.4 | 0.003 |
| | 15.1-28 | 0.2 | 0.1 – 0.9 | 0.029 |

### 4.5 Farm management practice

All six farms followed regular deworming, artificial insemination (AI) for breeding and did not practice grazing of animals at all. Five farms had cemented floors with no maternity pens and did not test the animals for Brucellosis prior to introduction into the herd. Three farmers collected their cattle from both 'raised cattle within the farm' and 'other sources (Market, neighbor etc.)', two farmers collected their cattle only from their own farms and the rest one farmer collected cattle from other sources (Table 4.4).

**Table 4.4: Existing farm management practices in dairy cattle in Chittagong Metropolitan City, Bangladesh (N=6, n=158)**

| Factors | Categories | Frequency | % |
|---|---|---|---|
| Types of farm | Cattle | 5 | 83.3 |
| | Mixed (cattle with goat/sheep) | 1 | 16.7 |
| Farm size | 25 - 41 | 3 | 50.0 |
| | 52 - 56 | 3 | 50.0 |
| Floor | Brick | 1 | 16.7 |
| | Cement | 5 | 83.3 |
| Maternity pen | No | 5 | 83.3 |
| | Yes | 1 | 16.7 |
| Replacement of animal | Own | 2 | 33.3 |
| | Others (market, neighbor etc.) | 1 | 16.7 |
| | Both | 3 | 50.0 |
| Replacement with prior testing | No | 5 | 83.3 |
| | Yes | 1 | 16.7 |
| Breeding system | AI | 6 | 100 |
| Bio-security condition of farms | Good | 2 | 33.33 |
| | Moderate | 4 | 66.67 |
| Deworming | Yes | 6 | 100 |
| | No | 0 | 0 |
| Vaccination against Bruclla | Yes | 1 | 16.67 |
| | No | 3 | 50 |

| | | | |
|---|---|---|---|
| | Don't know | 2 | 33.33 |
| Grazing | No | 6 | 100 |
| | Yes | 0 | 0 |
| Consultancy by | Veterinarian | 4 | 66.67 |
| | Others (Local veterinary practioner ) | 2 | 34.33 |
| Presence of pet animal | No | 6 | 100 |
| | Yes | 0 | 0 |
| Fate of aborted calf | Throwing in open place or offer dog | 5 | 83.33 |
| | Buried | 1 | 16.67 |
| Knowledge about Brucellosis | No | 4 | 66.67 |
| | Yes | 2 | 33.33 |

### 4.6 Comparison of the serological test results

The sero-prevalence of bovine brucellosis was compared between RBPT and cELISA by Kappa statistics to identify the tests agreement and the test characteristics of RBPT were calculated considering cELISA as gold standard. The relative sensitivity and specificity of the RBPT was found 85.7 and 60%, respectively. The Kappa statistics value was 86% suggesting a very good agreement between the tests ($p<0.001$) (Table 4.5).

**Table 4.5: Outputs of Kappa statistics to assess the agreement between RBPT and cELISA and the test characteristics of RBT**

| **RBPT** | **cELISA** | | | **Kappa statistic** |
|---|---|---|---|---|
| | + | - | Total | |
| + | 12 | 20 | 32 | Agreement=86% P<0.000 |
| - | 02 | 124 | 126 | |
| Total | 14 | 144 | 158 | |
| Relative Sensitivity | 85.7% | | | |
| Relative Specificity | 60.0% | | | |

# Chapter-5: Discussion

An overall animal level sero–prevalence of 20.25% was observed in the present study using RBPT and 8.86% using cELISA. The cELISA result was in agreement with Belal and Ansari (2014), where 8.51% prevalence of brucellosis was recorded using rapid *Brucella* antibody test kit in Sirajgonj district of Bangladesh. Moreover, in a previous study, 7.6% prevalence was recorded using indirect ELISA (iELISA) in commercial dairy cattle of Chittagong district (Sikder et al., 2012a). Findings of the present study were close to the result from Rahman et al. (2006b) where an animal-level sero-prevalence of brucellosis in cattle was reported as 2.4–8.4% in Bangladesh using iELISA. Overall prevalence recorded using RBPT in the present study was in agreement with Smit (2013) who reported 18.2% prevalence in the government dairy farm of Dhaka, Bangladesh. Rahman and Mia (1970) also reported 18.4% (95% CI: 14.8-22.5%) prevalence of Brucellosis in cattle using Tube Agglutination Test (TAT) at Mymensingh region of Bangladesh and 20.5% (95% CI: 16.4-26.3%) in the government dairy farm of Dhaka, Bangladesh (Rahman, 2015).The present sero-prevalence was higher than 2.66% reported from Bagerhat, Bogra, Gaibangha, Mymensingh and Sirajgonj of Bangladesh (Rahman et al., 2011); 5% reported from Mymensingh and Patuakhali of Bangladesh and 4.5% recorded in Mymenshingh region by Nahar and Ahmed (2009) using conventional serological tests. In last year, investigation on bovine brucellosis in Mymensingh region carried out by Rahman et al. (2015) recorded only 0.3% prevalence in cattle. Higher sero-prevalence (14.8% in Mymenshingh and 33.5% in government dairy farm) of brucellosis using iELISA was observed by Smit (2013).

In south-east Asian context, higher prevalence of Brucellosis was reported from different districts of India (21.36% in cattle of Punjab) (Islam et al., 2013b), and Nepal (32%in Kailali district) (Pandeya et al., 2013); and 14.1% in Pakistan (Hamidullah et al., 2009) compared to Bangladesh.

From the above discussion, it can be noted that sero-prevalence of *Brucella* is varied from region to region within and outside Bangladesh. Different factors might influence the regional variation of the level of occurrence of an infectious disease; variation in climate and management system certainly some of them. Moreover, studies varied remarkably regarding study design, sampling methods and diagnostic tests. Considering the contagious nature of Brucella species, sex, breed,

drinking water points (Mekonnen et al., 2010), geographical variation and sensitivity of the test may have contributed to the current observed sero-prevalence (Alhaji et al., 2016). Since cELISA is based on the specific epitopes of the (O-polysaccharide) OPS and can therefor eliminates some of the cross-reaction and false negative problems seen in other serological tests. In our study only female and cross breed animals were studied that might be the cause of higher sero-prevalence because female animals are kept for longer in a particular herd and are stocked together compared to male animals which are usually individually housed, thereby increasing chances of exposure in females (Mekonnen et al., 2010). Higher sero–prevalence of brucellosis in female and cross breed animals has also been reported by various studies (Tolosa et al., 2008; Asfaw, 1998; Muma et al., 2007; Bayemi et al., 2009).

In the present study we compared RBPT test results with cELISA. In some previous studies RBPT was compared with cELISA in different countries (Ahasan et al., 2010; Rahman et al., 2012; Rahman, 2015). The present study revealed that both tests agreed for 85% results. By taking the cELISA as gold standard test, the relative sensitivity and specificity of RBPT was estimated as 86% and 60%, respectively. The sensitivity was within the range (70.6-97.7%) reported by Rahman (2015) by meta-analysis, however specificity was lower than the range (84.3 – 99.9%) reported in that study for RBPT. RBPT is the most commonly used conventional screening and rapid serological test for Brucellosis in animals all over the world (Rahman et al., 2012; Maurice et al., 2013; Musallam et al., 2015; Rahman, 2015b), though the reliability of the test was not found absolute (Kanani, 2007). RBPT, which was first officially introduced in Britain in 1970, is a rapid, simple and sensitive test but it has moderate specificity (Falade, 1983). RBPT and Serum Agglutination Test are the quantitative measurements of antibodies and are affected by many factors. By this test, there is chance of false positive results as it cannot differentiate antibodies originated from vaccination, or from infection with some other organism such as *Yersinia enterocolitica* (Godfroid and Käsbohrer, 2002) and it can give false negative results in early stage of infection, or immediately after abortion (Mohammed, 2016). Thus, the positive predictive value of this test is low and a positive result is required to be confirmed by some other more specific test like ELISA. However, the negative predictive value of RBPT is high as it excludes active brucellosis with a high degree of certainty (Gul and Khan, 2007).

In this study, 32 samples were positive in RBPT, 12 of them were positive in cELISA. On the other hand 2 samples that were negative in RBPT, showed also positive result. The differences in serological response to RBPT and cELISA may be due to the differences in antibody type (IgG or IgM), or may be due to the stage of infection (early or late stage), the titer of antibody, time after abortion, or vaccination (Waghela et al., 1980). These results were in alignment with Salem et al. (1977); Hosie et al. (1985); Alton, (1987); Morgan et al. (1978). ELISA is considered as one of the highly preferred confirmatory serological test in diagnosis of *Brucella* in farm animals because of its high sensitivity (100%) and specificity (99.7%) (Mohammed, 2016). To overcome the problems of RBPT; to minimize false positive and false negative test result, complement fixation test (CFT) or cELISA could be used (Mohammed, 2016).

Lactating animals were significantly associated with higher risk of being sero-positive to *Brucella* antibody both in univariable logistic regression and multivariable logistic regression analyses. In previous studies, sero-positivity was found in lactating and pregnant cows only (Adugna et al., 2013). Omer et al. (2000) also reported similar findings. In present study, the lactating animal might have conceived again and became pregnant or they were older than the non-lactating cows. In non-lactating group, there were some heifers. Sexually mature and pregnant cows are usually more susceptible to *Brucella* infection than sexually immature cattle of either sex (Adugna et al., 2013). This pattern might have been attributed to the affinity of this bacteria to the pregnant uterus and to erythritol in fetal tissue, possibly also to steroid hormones (Radostits et al., 2000).

In the present study, the cow producing less amount of milk were more sero-positive to *Brucella* and had a significant association in multivariable logistic regression analysis with the outcome variable of interest. Usually diseased cows produce less amount of milk, particularly those suffering from different reproductive problems. Therefore the sero-positive cows might also have been suffering from different reproductive diseases like metritis or endometritis from the last parturition that led to physical problems, resulting in lower milk production (Aulakh et al., 2008; Tebug, 2013; Patel et al., 2014a). During last trimester of gestation when the cow reached at her last stage of lactation (i.e. less milk production) is suitable period for *Brucella* organism to infect the host. There are several reports on last trimester as one of the most important risk factor for high *Brucella* antibody (Radostits et al., 2000; Islam et al., 2013b).

Reproductive disorders like abortion, and anestrous were found significantly associated with *Brucella* sero-positivity in univariable analysis in the present study and when all reproductive disorders were investigated together as a single variable (i.e. presence/absence of any reproductive disorders), resulted OR indicated that cows having reproductive disorders were nearly 3 times more (OR=2.90) likely to become seropositive. Similar result was also stated in some studies (Islam et al., 2013a; Patel et al., 2014a; Rahman, 2015). However, in multivariable analysis, variables related to cow's reproductive disorder (abortion, anestrous and presence of any reproductive disorder etc.) were not found significantly varying between positive and negative animals. However, when different reproductive problems were tested separately in multivariable analysis, lower number of positive cases for different reproductive problems might have decreased the study power to find out a significant relationship.

With respect to age, the sero–prevalence of brucellosis was higher when the cows were more than years old; however the relationship was not significant by statistical test. Similar result was stated in the study concluded in Shirajgonj (Belal and Ansari, 2014); and Mymensingh, Gaibandha, Bogra and Bagerhat districts of Bangladesh (Rahman et al., 2012). Lower prevalence of brucellosis in young ones could be due to resistance of young animals to infection (Paul, 1980). Kumar et al. (2005) suggested that with passage of time animals become more likely to be exposed to the bacteria and contract the disease. However, Amin et al. (2005) reported that high prevalence of brucellosis among old animals might be related to maturity with advancing age, thereby the organism may propagate to remain as latent infection or it may cause disease. Although susceptibility to brucellosis increases with age, it seems to be commonly associated with sexual maturity than age (Islam et al., 2013b).

During this study it was observed that, some livestock keepers (16.67%) practice multiple livestock herding particularly mixing cattle with goats and sheep. Among 32 sero-positive cattle, 7 (24.14%) were reared with multiple livestock. Several studies have shown that, herds with multiple livestock species have high odds of sero-positivity to *Brucella*, suggesting possibility of cross species transmission of *Brucella* (Megersa et al., 2011; Alhaji et al., 2016). Furthermore, introduction of new cattle bought from cattle market into herds, and socio-cultural factor of cattle gifts or using cattle to pay for dowries might be predisposing determinants of bovine brucellosis in the dairy cattle herds (Alhaji et al., 2016). Purchase of infected cattle has been reported to be

associated with *Brucella* infection in cattle herds in previous studies (Diaz, 2013; Asmare et al., 2013).

In the course of sample collection, we collected information about previous knowledge about brucellosis infection, transmission; and control and prevention. Study showed that most of the farm owner and/or workers were not aware about brucellosis. Alike result was also testified by Sikder et al. (2012a). Knowledge of a disease is a crucial step in the development of prevention and control measures (Gumi et al., 2011). In the present study majority of livestock keepers (83.33%) are not knowledgeable and aware about brucellosis and its zoonotic potential (Table 4.4). Lack of knowledge and awareness about the disease and information on the zoonotic potential of brucellosis signify that farmers do not take required precautions when handling *Brucella* infected animals; and products and by products from infected animals thus threatening their health. Moreover, with these results it is obvious that no precaution was taken to prevent spread of the disease to other herds within or outside the study area. The perception that brucellosis can be cured and the habit of selling diseased animals either to the market or other livestock keepers as it was observed during this study can lead to propagation of the disease to other areas or herds which are not infected. Holt et al (2011) indicated that, selling infected animals at the market may increase transmission of brucellosis not only between households in the same village but also between villages and even larger geographical areas. Bruner et al. (1966) pointed out that; animals are not usually given antimicrobials for prophylaxis or therapy against Brucella infection as they cause L-transformation on the cell wall thereby possibly creating carrier animals. In the study area, livestock keepers also do not separate animal (s) that abort. The workers were not however aware of any potential modes of transmission to humans other than direct contact with aborted calves and aborted material. As a result of this lack of awareness, workers continue high-risk practices including home slaughter of cattle and subsequent meat preparation (Kagumba and Nandokha, 1978). This underestimation of disease severity may also play a role in the workers ignorance regarding high-risk practices such as assisting parturition or handling of aborted materials from ewes without gloves or masks (Kagunya, 1977; Kadohira et al., 1997). Uncontrolled movement of livestock is another practice which was noted in the study area. Haphazard animal movement can lead to dissemination of a disease to other places where disease is not present.

The farmer should be sensitized about the economic and public health impact of Brucellosis that will help to control and prevent the infection in developing countries. Kadohira et al. (1997) mentioned that the people are more easily convinced to receive message about the diseases which have high economic impact and risks of human infection. This lack of awareness concerning signs of human brucellosis and modes of transmission may be attributed to inadequate communication by the public health authorities, shortage of awareness campaigns usually associated with the underreporting of disease and inadequate surveillance (Msanga et al., 1986; Kadohira et al., 1997).

The majority of participants reported that they fed aborted fetuses to stray dogs or throw aborted materials into water canals used by sheep and other livestock for drinking or bathing (Table 4.4). Such types of practice attribute increase transmission and tenacity of infection in the herd. Dogs play as mechanical vector due to dragging of aborted materials in the ground which is responsible for transmission of the infection (Díaz, 2013) for the reason that (Forbes, 1990) reported naturally acquired infection of dogs with *B. abortus* from infected cattle and demonstrated horizontal transfer of infection like dog to dog, cattle to dog, dog to cattle and dog to human etc. and that dogs with naturally acquired infections with *B. abortus* play important role in the epidemiology of bovine brucellosis. The practice of discarding aborted materials into watercourses is a likely cause of water contamination and increases the risk of disease transmission to humans and other animal populations in the region because the farmers, workers and villagers come regular contact with such types of contaminated waters (Wael et al., 2010). The relationship of dogs and outbreak of brucellosis in cattle has also been demonstrated earlier (Prior, 1976; Forbes, 1990).

Six farms of the study area were examined thoroughly to investigate the presence of *Brucella* antibody in serum of dairy cows and heifers in the present study. Inter farm transmission factors and farm level variables (usual management practices in aborted cases, rearing other animals in to the farm etc.) were not possible to be investigated by statistical models due to small number of observations (6 farms). Therefore, to identify farm level risk factors of the intra and inter farm transmission of brucellosis, further studies including more farms is highly advised. We have included only female animals into our study although male cattle can also be infected with *Brucella* and can play an active role in transmission of this pathogen. During blood collection,

sometimes we missed to collect blood from every single cattle within the selected farms due to difficulties in restraining and handling (lack of facilities at farms). Besides these limitations, since all 158 samples were subjected to two diagnostic tests, it can be concluded with some certainty that the infection is persisting in the study area as an endemic state. As its impact on the society renders as economic losses via abortion, anestrous in animals etc. and via diseases in humans, appropriate awareness regarding control and prevention measures is highly recommended for the farmers of the endemic areas.

# Chapter-6: Conclusion

This study revealed that the Brucellosis is present in the cattle of the study area and the sero-prevalence ranged from 8-20% tested by cELISA and RBPT. In addition, lactating and low milk producing cows are in higher risk of being positive to the infection. The higher prevalence of the disease might significantly impact on public health. So, emphasis should be given to control Brucellosis in animal in order to protect human health, increase animal welfare and to minimize the economic losses.

# Chapter-7: Recommendation

The following recommendations are forwarded to curb further spread of the disease in both cattle and human populations:

- Isolation of aborted animals and proper disposal of aborted fetuses and fetal membranes, preferably, by incineration.
- Isolation of calving animals in separate calving pens.
- Replacement stock should be purchased from herd known to be free from brucellosis.
- Strict movement control of animal from one area to another in order to prevent the spread and transmission of the disease from infected cattle to the non-infected ones.
- Proper hygienic practices and good husbandry management should be exercised and these could in many situations, minimize the spread of disease in the herd.
- Awareness creation among farmers, butchers men, abattoir workers and animal health workers about the nature and effect of the disease through informal educational channels is required.
- Unless and otherwise the reactor animals are removed from infected herds, greater percentage of the remaining animals in an infected herd and increasing number of herds in the population could acquire the infection.

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# Appendix 1

## Questionnaire for Sero-prevalence and Spatial pattern of Bovine Brucellosis of Chittagong Metropolitan Area, Bangladesh

### Farm data

Date: Sl. No.:

Farm ID: ……..……...
Latitude: Longitude:
Cattle ID: ……..…….
Total no.: (Milking……./dry……./calf……/heifer……)
Feed supplied: 1. Roughage: straw/green grass (napier /para /german / road side/……..……)
2. Concentrate: Rice polish /wheat bran /pea husk/molasses/oil cake/ready mix/…….….

Animal grazing : Y / N. If yes (where): high land / low land / plain land /…….
Housing: Brick / cemented / clay /……..
Deworming : Y / N Vaccination: Y / N. Types:
How you manage problems? Vet / VFA / Self.
How many **cows died last 12 months**?
Fate of aborted cow : Kept/sold/slaughtered /through to open place/Offer to dog……
**How disposed** aborted materials? Burial / burning /……
Any disinfectants used in farm area after abortion: Y / N. If yes (type): ……
Maternity pen : Y / N.
Replacement : Own / market / both.
Replacement with Prior testing: Yes/No
Type of farm: Only cattle/ Mixed

### Individual Animal Data

**Age** : **Breed**: RCC / local / cross.

**BCS** : 1 / 2 / 3 / 4 / 5.

Types of cows: Heifer/ Pregnant/ Lactating

**Pregnancy** : Y / N. Duration (Months): ……..

**Lactation no.** : Last calving date:

**Milk yield** (daily) :

**Breeding system** : NS / AI / Both. **Who** do it? VFA / AIT (DLS / BRAC)

Any **reproductive disorder** last year? Abortion / still birth / retained placenta

**Samples** taken : Milk / blood / feces.

**RBPT** : +ve / -ve**.** **ELISA:** +ve / -ve.

**Workers data**

Animals as pet : Y / N. If yes type:

No. of attendants work :

Any **knowledge of Brucellosis** : Y /N

Any p**rotection** (hand gloves) weared : Y / N.

**Milk consumption** : Raw / boiled / pasteurized.

Pooled milk sample to be collected: Only for milk ring test. If possible, ELISA will be done.

RBT: Yes/ No SAT: Yes/ No

**Signature**

## Brief Biography

Shariful Islam passed the Secondary School Certificate Examination in 2005 followed by Higher Secondary Certificate Examination in 2007. He obtained his Doctor of Veterinary Medicine Degree in 2012 (held in 2014) from Chittagong Veterinary and Animal Sciences University (CVASU), Bangladesh. Now, he is a Candidate for the degree of MS in Epidemiology under the Department of Medicine and Surgery, Faculty of Veterinary Medicine, CVASU. He has been working as a Wildlife Veterinarian in EcoHealth Alliance, New York, USA, attached in Institute of Epidemiology, Diseases Control and Research (IEDCR) since June 2015. He published scientific articles in national and international peer- reviewed journals. He has immense interest to work as Wildlife epidemiologist to prevent the next pandemic zoonotic disease outbreaks.

Anon, 2016. Bangladesh Meteorological Department, retrived from http://bmd.gov.bd/?/home/ on 5th April, 2016.