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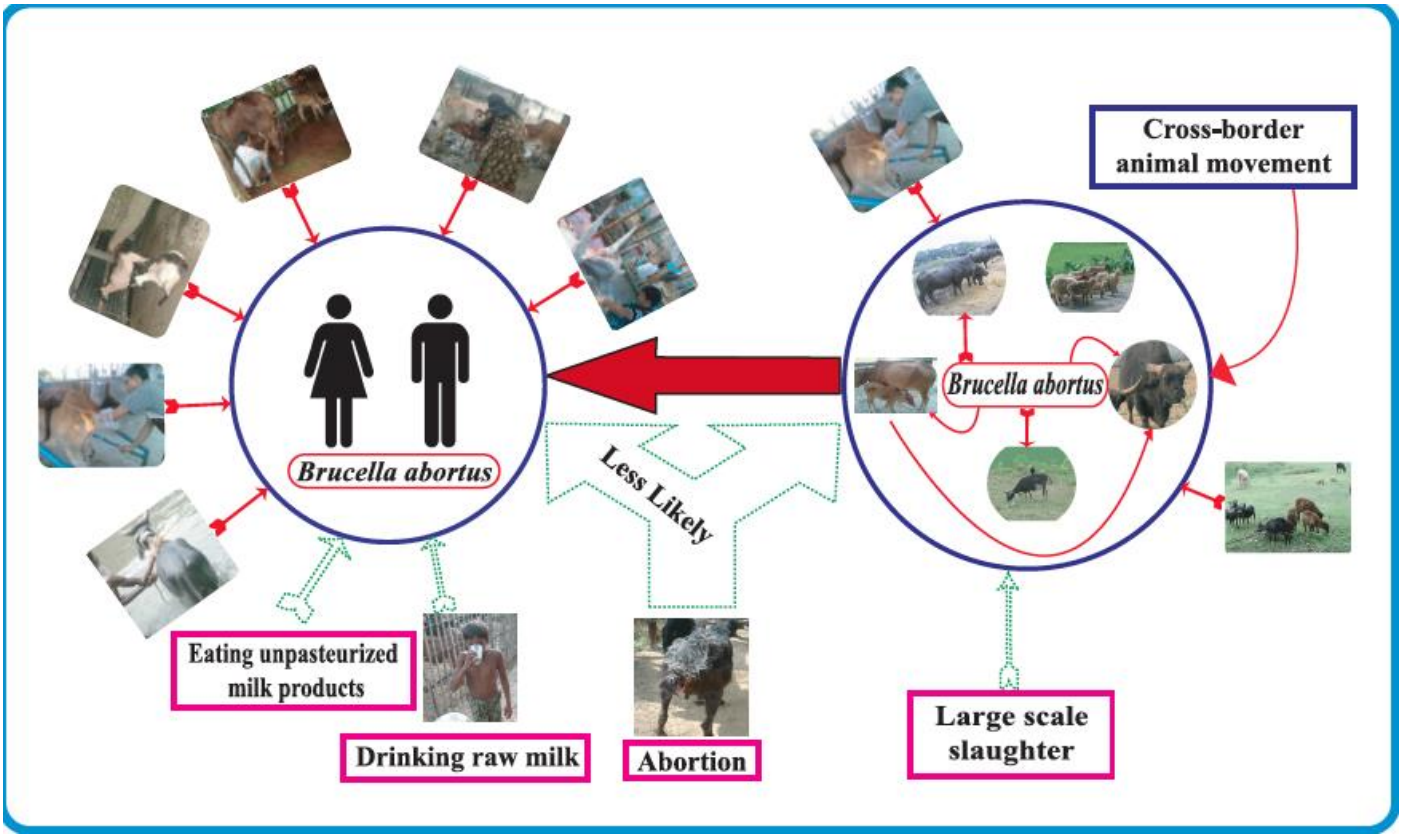
Epidemiology of brucellosis in humans and domestic ruminants in Bangladesh



Epidémiologie de la brucellose bovine chez les ruminants domestiques et chez l'homme au Bangladesh

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Thèse présentée en vue de l'obtention du grade de
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List of Abbreviations

A.D.	*Anno domini*
AI	Artificial insemination
Bayes-p	Bayesian p value
B.C.	Before Christ
BCSP	*Brucella* cell surface protein
BLRI	Bangladesh livestock research institute
BPAT	Buffered plate antigen test
CCBDF	Central cattle breeding and dairy farming
CDC	Center for disease control
cELISA	Competitive ELISA
CFU	Colony forming unit
CFT	Complement fixation test
Cgs	Cyclic glucan synthase
CI	Confidence/Credible Interval
DIC	Deviance information criterion
DLS	Department of livestock services
ECTAD	Emergency centre for transboundary animal diseases
EDTA	Ethylenediaminetetraacetic acid
FAO	Food and agriculture organization
FMD	Foot and mouth disease
FPA	Fluorescence polarization assay
FPSR	False positive serological reactions
GDP	Gross domestic product
Govt.	Government
GPS	Global Positioning System
HPAI	Highly pathogenic avian influenza
HROG	High Risk Occupational Group
i-ELISA	Indirect ELISA
Ig	Immunoglobulin
LFA	Lateral Flow Assay
LRI	Livestock research institute
MET/MET	2-mercaptoethanol
MMC	Mymensingh Medical College

MoFL	Ministry of fisheries and livestock
MRT	Milk ring test
mRBT	Modified RBT
NPV	Negative Predictive Value
OPS	O-polysaccharide
p_D	The effective number of estimated parameters
PPR	Peste des Petits Ruminants
PPV	Positive Predictive Value
PUO	Pyrexia of unknown origin
PRR	Pattern recognition receptors
RBT	Rose Bengal test
R-LPS	Rough lipopolysaccharide
rt PCR	Real time polymerase chain reaction
SAT	Serum agglutination Test
Se	Sensitivity
S-LPS	Smooth lipopolysaccharide
Sp	Specificity
SPAT	Serum plate agglutination test
STAT	Standard tube agglutination test
TAT	Tube agglutination test
TLR	Toll like receptor
ULDC	Upazilla livestock development center
USAID	United States agency for international development
VNTR	Variable number of tandem repeats

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Chapter 1

Summary

Background

Brucellosis is an ancient and one of the world's most widespread zoonotic diseases affecting both, public health and animal production. It is endemic in many developing countries of Asia, Africa and Latin America including Bangladesh. Since the first report in 1970, a lot of brucellosis seroprevalence reports are available in cattle, goats, sheep and humans in Bangladesh. Most of the previously reported prevalence studies were based on non-random samples, which may not give a true representation of the status of the disease in respective populations. Some authors also investigated the risk factors in cattle. The tests used for the diagnosis of brucellosis in domestic ruminants and humans are imperfect and their performance was not evaluated in Bangladesh. The true prevalence of brucellosis in domestic ruminants is not known and is essential for analyzing the impact of this disease in domestic ruminants in Bangladesh. Indeed, when diagnostic tests are used without evaluating their performance in a context usually generate unreliable results, which in turn may lead to wrong epidemiological inferences. In addition, information on risk factors of brucellosis in humans and animals is also scarce. Moreover, the different species of *Brucella* prevalent in animals is scarce and not known in humans in Bangladesh. The overall objective of this thesis was to investigate the epidemiology of brucellosis in humans and domestic ruminants in Bangladesh in terms of the evaluation of commonly used diagnostic tests, estimation of true prevalence, identification of risk factors and detection of *Brucella* species in order to provide information that will guide the selection of appropriate control strategies.

Study design and data analysis

Sampling

To collect random samples of animals a system of map digitization and selection of one geographical point from selected unions (Sub-Upazilla) using a hand held GPS machine was used. Blood (milk also where applicable) samples were then collected from livestock farmers and their animals within 0.5 km of the selected points. A convenient blood sample of butchers, dairy hands and veterinary practitioners were collected from Dhaka and Mymensingh districts. The sera of pyretic humans were collected from Mymensingh Medical College hospital randomly once in a week. Random milk samples were collected from Sirajgonj and Chittagong districts.

Systematic random milk and blood samples of cattle including breeding bulls (semen also) of central cattle breeding and dairy farm (CCBDF) were also collected. Milk and blood samples of gayals of a herd in regional Bangladesh Livestock Research Institute at Naikhonchari, Bandarban were also collected. Convenient samples of placenta and vaginal swabs were also collected from Mymensingh district.

Data collection and Analysis

Data on serology was generated by using Rose Bengal test (RBT), Slow Agglutination test (SAT) /Standard tube agglutination test (STAT) (animals/humans) and indirect enzyme linked immunosorbent assay (iELISA). Animal, their herd level data and human data on potential risk factors were collected using a pretested questionnaire. The data was stored in Microsoft Excels worksheets and transferred to respective software for analysis. To estimate true prevalence and evaluate three conditionally dependent serological tests, Bayesian latent class models were used. Random effect and Firth's logistic regression analyses were used to determine the risk factors of human brucellosis. The STATA, R and OpenBUGS softwares were used for data analyses. Staining, culture, genus and species-specific real time PCR assays were applied to isolate and to detect *Brucella* Spp./DNA in seropositive human sera and animal samples.

Main results

Only 0.29% (95% CI: 0.06-0.86) cattle were acutely infected whereas 0.49% (95% CI: 0.16- 8 1.1) were chronically infected with brucellosis in Mymensingh. On the other hand, in CCBDF 15.58% (95% CI: 11.89-19.89) cattle were acutely infected with brucellosis and only 3.2%(95%CI: 1.63-5.72) were chronically infected. The true prevalence of brucellosis among cattle in Mymensingh and CCBDF were 0.3% (95%CI: 0.03-0.7) and 20.5% (95% CI: 16.4-26.3) respectively. The performance of iELISA was best in both Mymensingh and CCBDF with the sensitivity of 90.5% and 91.3% and specificity of 99.3% and 99.2% respectively. The performance of RBT was better in Mymensingh than CCBDF with 81.0% and 76.1% sensitivity and 99.0% and 95.6% specificity respectively. Similar to RBT, the performance of SAT was also better in Mymensingh than CCBDF with 63.5% and 79.7% sensitivity and 98.6% and 95.3% specificity respectively.

Through this test validation study, a new cut-off of 5 IU/ml for iELISA was recommended both in low (as at Mymensingh) and high prevalence scenarios in cattle populations (as at CCBDF) for routine screening. It was recommended to do nothing for the control of bovine brucellosis under small-scale dairy and subsistence management systems in Bangladesh. However, vaccination should be applied in herds where the prevalence is very high as like CCBDF.

The true prevalence of brucellosis in goats and sheep were estimated as 1% (95% CI): 0.7–1.8) and 1.2% (95% CI: 0.6–2.2) respectively. The sensitivity of iELISA was 92.9% in goats and 92.0% in sheep with corresponding specificities of 96.5% and 99.5% respectively. The sensitivity and specificity estimates of RBT were 80.2% and 99.6% in goats and 82.8% and 98.3% in sheep. The sensitivity and specificity of SAT were 57.1% and 99.3% in goats and 72.0% and 98.6% in sheep.

The prevalence of brucellosis in occupationally exposed people (HROG) using three tests was observed to be 4.4% based on a parallel interpretation. The results of the multiple random effects logistic regression analysis with random intercept for district revealed that the odds of brucellosis

seropositivity among individuals who had been in contact with livestock for more than 26 years was about 14 times higher as compared to those who had less than 5 years of contact with livestock. In addition, when the contact was with goats, the odds of brucellosis seropositivity were about 60 times higher as compared to when contact was with cattle only. The seroprevalence of brucellosis among patients with pyrexia of unknown origin (PUO) was estimated to be 2.7% (95% CI: 1.2-5.2). The age, residence, type of patient, contact with animals, type of animal handled, arthralgia and backache were found to be significantly associated with a positive serological result in bivariable Firth's logistic regression. *Brucella abortus* was detected from seropositive pyretic patients.

Only *B. abortus* DNA was amplified from 19 seropositive human samples (both HROG and PUO) and six animal samples (3 cows milk, one goat milk, one gayal milk and one bull semen). No *Brucella* like organism was observed under microscope in stained smears. Similarly, no *Brucella* organism was isolated from any of the clinical samples.

Conclusion

The true exposure prevalence of brucellosis in cattle under small-scale dairy and subsistence/backyard management systems is very low (0.3%; 95% CI: 0.03-0.7). The active/acute infection is also very low (0.29%: 95% CI: 0.06-0.86) and similar to true exposure prevalence. The brucellosis in cattle under such management system is naturally controlled and further control program is not recommended considering the poor socioeconomic conditions.

The true exposure prevalence of brucellosis in CCBDF is very high (20.5%; 95% CI: 16.4-26.3). The acute infection in this farm is also very high (15.58%; 95% CI: 11.89-19.89). Immediate control measures by initiating calf hood (female calf) vaccination are recommended to protect a valuable herd which also provides frozen semen for artificial insemination all over the country.

The SAT and iELISA may simultaneously be applied to know the stage of brucellosis infection in domestic ruminants in high prevalence scenarios.

The true exposure prevalence of brucellosis in goats and sheep are also low and around 1%. Due to lower positive predictive value, these test results should be interpreted with caution to avoid misleading information.

Breeding bulls used for artificial insemination all over the country were found to be infected with brucellosis.

Brucellosis is not a serious problem for the general population in Bangladesh as drinking raw milk and milk products is unusual and not a risk factor. The apparent prevalence of brucellosis in high risk occupationally exposed people (4.4%; 95% CI: 2.8-6.6) and in pyretic patients (2.7%; 95% CI: 1.2-5.2) are also low.

The RBT may be applied as a screening test in humans having signs and symptoms of brucellosis along with the history of animal contact. In case of suspicion, genus or species specific rt PCR may be applied for confirmation.

Only *Brucella abortus* is dominant in humans and animals in Bangladesh. Regular screening of occupationally exposed people and pyretic patients with animal contact by serology and species specific rt PCR will indirectly help to know the species of *Brucella* prevalent in animals in Bangladesh.

Chapter 2

Preamble

This thesis is a compilation of two papers published in peer reviewed scientific journals like *Foodborne Pathogens and Disease* and *Preventive Veterinary Medicine*, three submitted or prepared for submission in the scientific journals (*PLoS One*, *Transboundary and Emerging Diseases*). The document consists of 13 chapters: Chapter 1 and chapter 2 consist of summary and preamble of the thesis.

Chapter 3 provides information on epidemiology of brucellosis in domestic ruminants and humans with especial emphasis on prevalence, risk factors, evaluation of serological tests in the absence of gold standard. It also briefly describes the context of this study.

Chapter 4 describes the objectives of this thesis.

Chapter 5 to chapter 9 isnclude 5 manuscripts published, submitted or prepared for submission.

Chapter 5 describes the efficacy of three conditionally dependent serological tests like Rose Bengal test (RBT), Slow Agglutination test (SAT) and indirect enzyme linked immunosorbent assay (iLEISA) for the diagnosis of bovine brucellosis in naturally infected cattle in Bangladesh; the true prevalence of bovine brucellosis and stage of infection are also reported. The performance of these three tests was also compared in low and high prevalence scenarios. Random blood samples of cattle from Mymensingh district and central cattle breeding and dairy farm were collected and used for this study.

In chapter 6, the Bayesian latent class model based evaluation of three conditionally dependent serological tests like RBT, SAT and iELISA for the simultaneous diagnosis and estimation of true revalence of brucellosis in small ruminants are described. Representative blood samples of goats and sheep populations in Bangladesh were collected and used in this study.

The dominant species of *Brucella* prevalent in humans and domestic ruminants Bangladesh is described in chapter 7. The chapter describes the prevalence of only *Brucella abortus* in humans and animals.

In chapter 8, the prevalence and risk factors of brucellosis in a high-risk group of people in Bangladesh is reported. The high risk group of people included in this study was livestock farmers, milkers, butchers and veterinary practitioners. The RBT, STAT and iELISA were used in every individual.

The chapter 9 describes the prevalence of brucellosis in pyretic people in Bangladesh and detection *Brucella* species from seropositive pyretic patients. Randomly collected blood samples from pyrexia of unknown origin patients were used for this study. The same tests were also used in pyrexic people.

The chapter 10 describes the general discussion. This chapter critically analyzes the results obtained from a series of studies and compared with relevant studies. The probable reasons of low prevalence of brucellosis in domestic ruminants were explained. The practical relevance of the generated knowledge form this study to design and implement future brucellosis control program was also discussed.

Chronologically, chapter 11, chapter 12 and chapter 13 consist of conclusions, recommendations and bibliography.

Chapter 3

General Introduction

3.1 Epidemiology of brucellosis in humans and animals

3.1.1 Brief history of brucellosis

Brucellosis is an ancient and one of the world's most widespread zoonotic diseases affecting both, public health and animal production (Ariza *et al.*, 2007) which is caused by a Gram-negative, facultative intracellular bacteria of the genus *Brucella* (*B.*). The paleo-pathological evidence form the partial skeleton of the late Pliocene *Australopithecus africanus* suggests that brucellosis occasionally affected our direct ancestors 2.3–2.5 million years ago (D'anastasio *et al*., 2011). The pathological, molecular (DNA analysis) and electron microscopy findings from the human skeletal remains (Rashidi *et al.*, 2001; Capasso, 2002; Mutolo *et al.*, 2012; D'Anastasio *et al*., 2011), remains of buried cheese (Capasso, 2002) also suggested the presence of brucellosis long time ago for example: 3000-1200 B.C. in Bahrain, Persian Gulf, 2100-1550 B.C. in Palestine and Jordan, 79 A.D. in Roman town Pompeii and Herculaneum. 1260-1020 A.D. in Butrint, Albania. However, the causative agent of brucellosis, "*Micrococcus melitensis*" (i.e. *Brucella melitensis*), was discovered in 1887 by British surgeon captain David Bruce, his wife Mary Elizabeth Steele and the Maltese microbiologist doctor Giuseppe Caruana-Scicluna from the liver of diseased soldiers in the Mediterranean island of Malta (Spink, 1956; Ruiz- Castañeda, 1986;Wyatt, 2009). After this discovery, the Maltese medical doctor Fioravanti Temistocle Archimede Laurenzo Giuseppe Sammut, (known as Temi Zammit) had revealed that the causative agent of Malta fever was transmitted from infected goats to humans through contaminated milk (Wyatt, 2005, 2011). After ten years of "*Micrococcus melitensis*" discovery, the Danish scientist Bernhard Bang identified "*Bacillus abortus*" (i.e. *Brucella abortus*) in bovine aborted fetuses (Bang, 1897). Another organism similar to "*Micrococcus melitensis"* was isolated from aborted pigs in United States, which was finally designated as *Brucella suis* (Traum, 1914). At that time, this disease was further studied by Alice Catherine Evans (an American microbiologist) and based on her findings pasteurization of milk was proposed as a preventive measure (Evans, 1918). Finally in 1920, Louis Meyer and Wilbur Shaw honored David Bruce by proposing a single new genus named *Brucella* to group these pathogenic bacteria (Meyer and Shaw, 1920).

3.1.2 Species, biovars and zoonotic potentials

Species identification and sub-typing of *Brucella* isolates are very important for epidemiologic surveillance (to know the species and/or biovar diversity) and investigation of outbreaks (to know the source of infection) in *Brucella*-endemic regions (Al Dahouk *et al.*, 2007; Marianelli *et al.*, 2007). There are several concepts (species concept) for the nomenclature for bacteria like "Taxospecies", "Nomenspecies", "Genomospecies". The "genomospecies" (based on DNA-DNA hybridization) is defined as a group of strains sharing approximately 70% or greater DNA-DNA relatedness with 5°C or less change in melting temperature (Wayne *et al.*, 1987). Based on this concept, the genus *Brucella* should

be mono-specific as DNA similarity is above 90% between the six classical species (Verger *et al.*, 1985). Hence, it was proposed and this proposal was supported by the Subcommittee on the Taxonomy of *Brucella* at that time with *B. melitensis* becoming the sole representative species and the other species being considered biovars of *B. melitensis* (Corbel, 1988). However, a monospecific genus concept (i.e. "genomospecies") did not get widespread support among the scientific community both from practical and scientific background (Moreno *et al*., 2002; Cohan, 2002; Gevers *et al.*, 2005). So, the classical *Brucella* species are "nomenspecies" with no true taxonomical standing. "Nomenspecies" is defined by the cluster of strains to which it is convenient to give a species name on basis other (e.g. host specificity) than taxonomical (Ravin, 1963).

Since 1920, in addition to *B. melitensis*, *B. abortus* and *B. suis*, at least 7 new species have been identified as belonging to the *Brucella* genus with several additional new species under consideration for inclusion (Oslen and Palmer, 2014) as shown in Table 3.1. Now, this genus consists of at least ten nomospecies having characteristic host preferences and zoonotic potential (Table 3.1). Of 10 recognized species of *Brucella*, infections with *B. abortus*, *B. melitensis*, *and B. suis* are the most pathogenic to humans, considered as bioweapons, and are listed as category B priority pathogens by the US Center for Disease Control (CDC). Because, the organism is highly infectious, can be readily aerosolized and outbreaks might be difficult to detect due to non-specific symptoms associated with infection (Doganay and Doganay, 2013). Out of seven biovars of *B. abortus*, biovar 1 is most frequently isolated from cattle in countries where biovar prevalence has been studied, such as the USA (Bricker *et al.*, 2003), Latin America (Lucero *et al.*, 2008), Brazil (Poester *et al*., 2002), India (Renukaradhya *et al*., 2002) and Pakistan (Ali *et al*., 2014). *Brucella melitensis* biovar 1 has been predominantly isolated from India (Sen and Sharma, 1977; Hemashettar *et al*., 1987), Libya (Refai, 2002), Iran (Zowghi *et al*., 2009) and Latin America (Lucero *et al.*, 2008). But *Brucella melitensis* biovar 3 is the most commonly isolated from China (Man *et al*., 2010), Egypt, Tunisia, Israel, Turkey and Jordan (Refai, 2002).

However, *B. melitensis* has never been reported from Brazil and Uruguay (Lilenbaum *et al.* , 2007; Garin, 2011) and surveys of 80% of the ovine and caprine populations in El Salvador and Costa Rica did not reveal antibodies against *Brucella*, suggesting the absence of *B. melitensis* in these countries (Moreno, 2002).

B. suis biovar 1 is most often found in South America and Asia. Both biovars 1 and 3 have been reported in the United States, Australia, and China (Cvetni´c *et al.*, 2009). Biovar 2 is the most common strain in Europe (Fretin *et al*., 2013; Szulowski *et al*., 2013). Biovars 1, 2, and 4 can be transmitted from swine to cattle, inducing transient seroconversion, which can confound *B. abortus* diagnostic assays (Musser *et al.*, 2013). Previously it was known that *B. suis* biovar 2 does not infect humans but it was isolated from wild boar hunters and are pathogenetically implicated (Garin-Bastuji *et al.*, 2006).

3.1.3 Ecology of Brucella spp.

Bacteria of the genus *Brucella* spp. are coccobacilli, Gram-negative, aerobic, non-spore-forming, non-motile and non-capsulated (Bargen *et al*., 2012). Although able to multiply in life-less media, *Brucella* organisms are better described as facultative extracellular intracellular parasites (Moreno and Moriyon, 2002). The brucellae are members of the α–proteobacteria (Moreno *et al*., 1990) and interestingly (being animal pathogens) have close relationships with soil organisms (e.g. *Ochrobactrum* spp.), with plant symbionts (e.g. *Rhizobium* spp.) and with phytopathogens (e.g. *Agrobacterium* spp.). The species *Ochrobactrum intermedium* is considered to be phylogenetically and taxonomically most closely related to *Brucella* (Scholz *et al*., 2008). All of these bacteria inhabit eukaryotic cells, and comparative genomic studies indicate that they have evolved from a common ancestor (Boussau *et al*., 2004).

The target organs and tissues of *Brucella* spp. are placenta, mammary glands, and epididymis in animal reservoir host (Adams, 2002; Xavier *et al*., 2009; Neta *et al*., 2010).

Table 3.1: The species, biovars/biotypes, host preferences and zoonotic potentials of *Brucella* species

Species	Biovars	Colony type	Host tropism	First reported, country	Zoonotic Potential
B. melitensis	1-3	Smooth	Goat, sheep, camels, cows	Bruce, 1887, Malta	High
B. abortus	1-6, 9	Smooth	Cattle, buffalo, camels, bison, elk, yaks	Bang, 1897, Denmark	High
B. suis	1-5	Smooth	Pigs (biotypes 1-3), wild boar and European hares (biotype 2), reindeer and caribou (biotype 4), wild rodents (biotype 5)	Traum, 1914, USA	High
B. neotomae	-	Smooth	Desert woodrat	Stoenner and Lackman, 1957, USA	Unknown
B. pinnipedialis	-	Smooth	Seal	Foster *et al.*, 2007, Scotland	Mild
B. ceti	-	Smooth	Dolphin, porpoise, whale	Foster *et al*., 2007, Scotland	Mild
B. microti	-	Smooth	Vole, fox, (soil)	Scholz *et al*., 2009, Czech Republic	Unknown
B. inopianata	-	Smooth	Unknown	Tiller *et al*., 2010, Australia	Mild
B. ovis	-	Rough	Sheep	McFarlane *et al.* 1952, New Zealand	No
B. canis	-	Rough	Dog	Carmichael and Kenney, 1968, USA	Mild
Future species					
Brucella papionis sp. nov.	-	Smooth	Baboon	Schlabritz-Loutsevitch *et al*., 2009, USA	Unknown
BO2	-	Smooth	Unknown	Hofer *et al*., 2012, Austria	Mild
Frog isolate (exceptionally motile)	-	Smooth	Bullfrogs	Eisenberg *et al*., 2012, Germany	Unknown

3.1.3.1 Transmission

Transmission within these hosts may occur 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 (Alexander *et al*., 1981; Godfroid *et al*., 2004). As the bacterial concentrations in fetal fluids or placenta after abortion can be as high as 10^9 to 10^{10} colony-forming units (CFUs)/g and minimum infectious doses are estimated in the 10^3 to 10^4 CFU range, abortion events can laterally transmit brucellosis to many cattle that have contact with birthing materials (Olsen and Tatum, 2010).

Moreover, transmission within the natural hosts can occur through milk or via semen or genital secretions during mating. Zoonotic transmission occurs most frequently via unpasteurized milk products in urban settings, while occupational exposure of farmers, veterinarians, or laboratory workers can result from direct contact with infected animals or tissues or fluids associated with abortion (Olsen and Palmer, 2014). Only rare cases of vertical and horizontal (Wyatt, 2010) transmission between humans have been reported (Ruben *et al*., 1991; Mantur *et al*., 1996; Çelebi *et al*., 2007; Meltzer *et al*., 2010) and humans are generally considered to be incidental, or dead-end hosts for *Brucella* species (Meltzer *et al*., 2010). The spillover of brucellae from wildlife to domestic ruminants is also possible (Mick *et al*., 2014).

3.1.3.2 Entry into the host and evading immune system

The most common portals of entry for *Brucella* in animals and humans are mucous membranes of the respiratory (aerosol) (Franz *et al*., 2001) and digestive tracts, and in the natural host, also the conjunctiva and membranes covering the sexual organs. Bacteria are eventually taken up by phagocytic cells (macrophages, dendritic cells, etc.) and reach the regional lymph nodes, leading to subsequent systemic dissemination (Ackermann *et al*., 1988; Salcedo *et al*., 2008). As *Brucella* cannot multiply outside their mammalian hosts, the most important aspect of *Brucella* ecology is their ability to establish an intracellular replicative niche and remain protected from the host immune responses (Bargen *et al*., 2012). Brucellae lack classic virulence factors like toxins, fimbriae and capsules which raises the possibility that they might have unique and subtle mechanisms to penetrate host cells, elude host defenses, alter intracellular trafficking to avoid degradation and killing in lysosomes and modulate the intracellular environment to allow long-term intracellular survival and replication (Delrue *et al*., 2004). The *Brucella* LPS O-polysaccharide appears to be a key molecule for cellular entry, to prevent complement-mediated bacterial lysis and to prevent apoptosis (i.e. programmed cell death) of the macrophages within which they reside allowing them to extend their longevity (de Bagüés *et al*., 2004; Lapaque *et al*., 2005).

Brucella has developed mechanisms to avoid innate immunity by minimizing stimulation of pattern recognition receptors (PRRs) of the host. The *Brucella* cell envelope has high hydrophobicity and its LPS has a non-canonical structure that elicits a reduced and delayed inflammatory response compared with other Gram-negative bacteria (Rittig *et al*., 2001) and has lower stimulatory activity on TLR4 receptors (Rittig *et al*., 2003). The O side chain on the LPS can form complexes with the major histocompatibility complex class II molecules that interfere with the ability of macrophages to present exogenous proteins. *Brucella* ornithine-containing lipids and lipoproteins in the outer membrane are poor activators of innate immunity.

The rough (vaccine) strains (i.e., strains with lipopolysaccharide lacking the O-side chain) are less virulent because of their inability to overcome the host defense system (Rittig *et al*., 2003). However,

under in vitro conditions, up to 90% of virulent *Brucella* and 99% of nonvirulent *Brucella* may be killed following intracellular entry (Porte *et al*., 1999).

3.1.3.3 Survival inside host cell

After entering into the host cell, smooth *Brucella* quickly traffic through the early endosomal compartment and depart the phagosome to form the modified phagosome (termed brucellosome). *Brucella* initially localize within acidified phagosomes (Rittig *et al.*, 2001), where they are exposed to free oxygen radicals generated by the respiratory burst of phagocytes. Brucellae have multiple mechanisms to detoxify free radicals. *Brucella* expresses 2 superoxide dismutases (SodA and SodC), which detoxify superoxide anions generated by the respiratory burst of phagocytes. *Brucellae* require acidification of the phagosomal compartment to a pH <4.5 before they display wild-type intracellular replication in initial stages of intracellular infection. Localization in an acidified environment induces expression of the VirB operon (virB 1–10), which controls expression of genes associated with a type IV secretion system. The VirB operon interacts with the endoplasmic reticulum to neutralize the pH of the phagosome (Anderson *et al*., 2008). The *Brucella*-induced modifications of the phagosome prevent fusion with the lysosome.

Virulent *Brucella* strains express a cyclic glucan synthase (cgs) that produces and secretes low molecular weight cyclic glucans. These molecules disrupt the lipid raft microdomain structures within intracellular membranes surrounding the bacteria. This modification of lipid raft distribution in phagosomal membranes inhibits phagosome maturation, prevents fusion with lysosomes (Arellano-Reynoso *et al*., 2005).

3.1.3.4 Survival outside host cell

Brucella may remain viable within the environment for a period of time. In general, the viability of *Brucella* spp. outside the mammalian host is enhanced by cool temperatures and moisture and decreased by high temperatures, dryness and direct exposure to sunlight. For example, *B. abortus* survives a couple of hours under direct sunlight but up to 185 days in the cold and shade. *Brucella abortus* also survives in aborted fetuses, manure and water for periods of up to 150 to 240 days (Saegerman *et al*., 2010).

3.1.3.5 *Brucella* is everywhere

Brucella can infect domestic and wild animals, rodents, sea mammals and even fresh water fish. If *Brucella* infected meat waste is thrown into river directly without proper treatment fresh water fish may also become infected with *Brucella*. As happened in case of a Nile river fish (*Clarias gariepinus*) in Egypt from which *B. melitensis* has been isolated (El-Tras *et al*., 2010). So, it is evident that *Brucella* knows no boundary as infecting humans, domestic and wild animals, fresh water fish and even marine mammals.

3.1.4 Prevalence, risk factors and impact in domestic ruminants

3.1.4.1 Bovine brucellosis

Brucellosis in cattle is caused almost exclusively by *B. abortus*. There are some areas where the co-existence of cattle and small ruminants facilitate cattle infection with *B. melitensis* (Samaha *et al*., 2008). Cattle can also become transiently infected by *B. suis* biovar 1 which prefer mammary gland as their preferred site (Olsen and Hennager, 2010). Brucellosis has been eradicated in many developed countries in Europe, Australia, Canada, and New Zealand (Minas, 2006). The European Union (EU) has granted brucellosis-free status to Sweden, Denmark, Finland, Germany, Cyprus, the UK (excluding Northern Ireland), Austria, Netherlands, Belgium, and Luxembourg (Pappas et al., 2006). Norway and Switzerland are also considered brucellosis-free countries. The areas at high risk of brucellosis infection are the countries of the Mediterranean Sea Basin (Portugal, Spain, South France, Italy, Greece, Turkey, North Africa), also countries of South and Central America, Asia, Africa, the Caribbean and Near East (Galínska and Zagórski, 2013). The prevalence of bovine brucellosis in some of the endemic countries is presented in Table 3.2.

Table 3.2: Reported 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, C-ELISA, FPA, CFT	12.4% (10.89–14.0)	2.10% (1.90–2.40)	de la Sota *et al*., 2005
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*., 2013
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; Megersa *et al*.,2011b; Megersa *et al*., 2011a; Megersa *et al*., 2012; Adugna *et al*., 2013
Libya	42	Sub-national			42.1% (20.3-66.5)	Ahmed *et al*., 2010
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*., 2006; Chimana *et al*., 2010; 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)	Sahin *et al.* , 2008
India	6813	Sub-national	iELISA		13.6% (12.8-14.4)	Kumar *et al*., 2005; Aulakh *et al*., 2008; Trangadia *et al*., 2010; Trangadia *et al*., 2012; Jagapur *et al*., 2013; Islam *et al*.., 2013a
Pakistan	3699	Sub-national	RBT, iELISA, cELISA, PAT		14.1% (12.9-15.2)	Nasir *et al*., 2004; Hamidullah *et al*., 2009; Abubakar *et al*., 2012; Shafee *et al*., 2011; Iqbal *et al*., 2013; Gul *et al*., 2014; Saleha *et al*., 2014

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: Slow Agglutination Test; 2-ME: 2-Mercaptoethanol Test; cELISA: Competitive ELISA; FPA: Fluorescence Polarization Assay; PAT: Plate Agglutination Test; NA: not available.

The range of reported prevalence varied from 2.0% to 42.1%. However, in countries where control measures have been undertaken, the prevalence was decreased gradually as in Brazil and Chile (Lopes *et al*., 2010).

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).

In some parts of Africa, hygromas and abscess in carpal joints are the major clinical signs in nomadic or semi-nomadic cattle herds infected with B. abortus biovar 3 (FAO, 2006; Bankole *et al*., 2010; Boukary *et al*., 2013). The brucellae localize in the supra-mammary lymph nodes and mammary glands of 80% of the infected animals and thus continue to secrete the pathogen in milk throughout their lives (Hamdy and Amin, 2002; FAO, 2006). Venereal transmission is not a major route of infection under natural conditions, but artificial insemination with contaminated semen is a potential source of infection (Radostits *et al*., 2010; Chiebao *et al*., 2013).

The reported animal level risk factors of bovine brucellosis include age, breed, history of abortion, etc. (Al-Majali *et al*., 2009; Ibrahim *et al*., 2010; Boukary *et al*., 2013; Chand and Chhabra, 2013; Patel *et al*., 2014). The herd level risk factors of bovine brucellosis identified are large herd size, mixed farming, agro-ecological zones, contact with wildlife, new entry in the herd, artificial insemination, etc. (Muma *et al*., 2007; Al-Majali *et al*., 2009; Ibrahim *et al*., 2010; Chiebao *et al*., 2013; Chand and Chhabra, 2013; Patel *et al*., 2014).

3.1.4.2 Brucellosis in small ruminants

Brucellosis in goats is caused mainly by *B. melitensis* but in countries where there is no *B. melitensis*, goats can get infected with *B. abortus* (Lilenbaum *et al*., 2007). As in cattle, brucellosis in goats is characterized by late abortion, stillbirths, decreased fertility and low milk production (Lilenbaum et al., 2007). Mammary gland is also commonly infected in sheep and goats. Mastitis is commonly observed feature of caprine brucellosis compared with bovine brucellosis. The affected mammary gland maybe characterized by multinodular firmness with watery, clotted milk (Cutler *et al*., 2005). Prolonged excretion of organisms in milk may occur in goats but less so in sheep (Poester *et al*., 2013).

Table 3.3: Reported prevalence of brucellosis in small ruminants in some endemic countries

Country	Sample size (flock/animal)	Level	Tests used	Flock Prev. (95% CI)	Animal Prev. (95% CI)	References
Mexico: Goat	83/1713	Sub-national	RBT, CFT	71.1% (60.1-80.5)	19.0% (17.2-20.9)	Montiel *et al*, 2013
Georgia: Sheep	3823	Sub-national	RBT		5.5% (4.8-6.3)	Mamisashvili *et al*., 2013
Georgia:Goat	1323	Sub-national	RBT		3.2% (2.3-4.3)	Mamisashvili *et al*., 2013
Kosovo: Sheep	3548	National	RBT		6.5% (5.7-7.4)	Jackson *et al*., 2004
Kosovo:Goat	511	National	RBT		7.8% (5.7-10.5)	Jackson *et al*., 2004
Egypt:Sheep	1604	National	RBT, iELISA		7.8% (6.6-9.3)	Samaha *et al*., 2008; Hegazy *et al*., 2011
Egypt: Goat	749	National	RBT, iELISA		6.7% (4.9-8.7)	Samaha *et al*., 2008; Hegazy *et al*., 2011
Libya: Goat	340	Sub-national	RBT		30.6% (25.7-35.8)	Ahmed *et al*., 2010
Libya: Sheep	188	Sub-national	RBT		23.9% (18.0-30.7)	Ahmed *et al*., 2010
Ethiopia: Goat	2005	Sub-national	RBT, CFT		1.6% (1.1-2.3)	Megersa *et al*., 2011b; Megersa *et al*., 2012
Niger: Sheep	1186	Sub-national	iELISA		2.5% (1.7-3.6)	Boukary *et al*., 2013
Niger: Goat	839	Sub-national			0.5% (0.1-1.2)	Boukary *et al*., 2013
Iran: Sheep	740	Sub-national	RBT		4.2% (2.9-5.9)	Akbarmehr and Ghiyamirad, 2011
Iran: Goat	160	Sub-national	RBT		5.0% (2.2-9.6)	Akbarmehr and Ghiyamirad, 2011
Jordan:Goat	69/1100	Sub-national	RBT, CFT	53.6% (41.2-65.7)	27.8% (25.1-30.5)	Al-Majali, 2005
Jordan:Sheep	66/1380	Sub-national	RBT, CFT	46.9% (34.6-59.6)	37.6% (35.0-40.2)	Al-Majali *et al*., 2007
Tajikistan: Sheep	6238	National	RBT		5.7% (5.2-6.3)	Jackson *et al*., 2007
Tajikistan:Goat	6767	National	RBT		5.5% (4.9-6.0)	Jackson *et al*., 2007
Kyrgyzstan: Sheep	2101	National	RBT, iELISA, FPA		12% (7%–23%)	Dürr *et al*., 2013
Kyrgyzstan:Goat	1310	National	RBT, iELISA, FPA		15% (7%–30%)	Dürr *et al*., 2013
Pakistan:Sheep	384	Sub-national	mRBT		7.0% (4.7-10.1)	Iqbal *et al*., 2013

Legend: CI: Confidence interval; Flock Prev.: Flock level prevalence; Animal Prev.: Animal level prevalence; RBT: Rose Bengal test; CFT: Complement Fixation Test; iELISA: Indirect ELISA; FPA: Fluorescence Polarization Assay; mRBT: modified RBT.

In sheep, brucellosis can be divided into classical brucellosis and ram epididymitis. Ram epididymitis is caused by non-zoonotic agent *B. ovis*, while classical brucellosis is caused by *B. melitensis* and remains a major public health threat equal to goat brucellosis (Acha and Szyfres, 2003). The reported prevalence of brucellosis in some of the endemic countries is summarized in Table 3.3. The highest prevalence of caprine and ovine brucellosis were reported from Libya and Jordan respectively.

Table 3.4: Reported prevalence/incidence of human brucellosis in some endemic countries

Country	Sample size	Type of sample	Test used	Prevalence/ Incidence	Reference
Brazil	180	General people	cELISA	13.3% (8.7-19.2)	Angel *et al*., 2012
Bosnia and Herzegovina	286	Unknown	RBT, STAT	20.6% (16.1-25.8)	Hamzi´c *et al*., 2005
Chad	860	Nomadic pastoralist	iELISA	3.3% (2.2-4.7)	Schelling *et al*., 2003
Egypt	4490			64-70 cases per 100 000 individuals	Jennings *et al*., 2007
Ethiopia	541	PUO patients, OEP	RBT, LFA, 2-ME	9.9% (7.6-12.8)	Kassahun *et al*., 2006; Regassa *et al*., 2009
Tanzania	199	OEP	RBT	5.5% (2.8-9.7)	Swai and Schoonman, 2009
Togo	683	OEP and general people	RBT, iELISA	1.0% (0.4-2.1)	Dean *et al*., 2013
Iran	39359			0-37.3 cases per 100 000 individuals	Mollalo *et al*., 2014
	1681	Referred patients, OEP	iELISA	4.1% (2.8-5.8)	Esmaeili *et al*., 2014; Nikokar *et al*., 2011
Kyrgyzstan	1777	Random sample	RB, iELISA, FPA	7.0% (4.-9.0)	Dürr *et al*., 2013
Turkey	2038	Farmers, veterinarian and general people	RBT, STAT	8.8% (7.6-10.1)	Cetinkaya *et al*., 2005; Otlu *et al*., 2008; Kutlu *et al*., 2014
Saudi Arabia	26613	Healthy and individuals with symptoms	STAT	4.2% (3.9-4.4)	Al-Sekait, 1999
India	1552	OEP, pyrexic patients	RBT, STAT, iELISA	8.4% (7.1-9.9)	Agasthya *et al*., 2007; Agasthya *et al*., 2012; Pathak *et al*. , 2014
Pakistan	852	OEP	RBT, SAT, iELISA	15.0% (12.7-17.6)	Hussain *et al*., 2008; Mukhtar and Kokab, 2008; Ali *et al*., 2013

Legend: OEP: Occupationally exposed people; RBT: Rose Bengal test; LFA: Lateral Flow Assay; STAT: Standard Tube Agglutination Test; SAT: Slow Agglutination Test; 2-ME: 2-Mercaptoethanol Test; cELISA: Competitive ELISA; FPA: Fluorescence Polarization Assay.

The herd level important risk factors for small ruminants brucellosis identified are large flock size, addition of new animals from unscreened sources, intensive system of management, history of abortion, grazing communal pasture, keeping sheep and goat together (Kabagambe *et al*., 2001; Lithg-Pereira *et al*., 2004; Solorio-Rivera *et al*., 2007; Al-Majali *et al*., 2007; Islam *et al*., 2010; Montiel *et al*., 2013; Teklue *et al*., 2013).

3.1.5 Prevalence, clinical symptoms and signs and risk factors in human

Brucellosis in humans almost always originates from an animal reservoir (Godfroid *et al*., 2013). The highest prevalence of human disease is currently found in areas of Africa, Asia, Latin America, and the Middle East. Despite being endemic in many developing countries, brucellosis remains under diagnosed and underreported (Godfroid *et al*., 2005). The range of reported incidence of human brucellosis cases per 100,000 people per year are 0.28-268.81 in North Africa and Middle East, 34.86 in Sub-Saharan Africa, 0.03-32.49 in Western Europe, 88.0 in Central Asia, 12.84-25.69 in Central and Southern Latin America and 0.02-0.09 in North America (Dean *et al*., 2012b). The highest incidence of human brucellosis is reported from Saudi Arabia, Iran, Palestinian Authority, Syria, Jordan and Oman (Pappas *et al*., 2006). The prevalence of human brucellosis in some endemic countries is given in Table 3.4. Human cases are useful indicator of the presence of disease in animal populations. It is also important to determine if the infection was acquired locally or elsewhere and if food products are implicated, to establish whether these were locally produced or imported (FAO, 2006).

3.1.5.1 Clinical symptoms and signs

The most common clinical symptoms are fever (78%), arthralgia (65%), myalgia (47%) and back pain (45%) (Dean *et al*., 2012a). As 78% patients with brucellosis suffers from fever, it is a diagnostic challenge in malaria-endemic areas. Hepatomegally and splenomegally are reported in 23% and 26% patients respectively (Dean *et al*., 2012a). Life-threatening focal complications are endocarditis and neurobrucellosis but the overall case fatality is less than1% (Godfroid *et al*., 2011; Olsen and Palmer, 2014). Severe complications of brucellosis infection are not rare, with 1 case of endocarditis and 4 neurological cases per 100 patients as reported by Dean *et al*. (2012a). It is also reported by Dean *et al*. (2012a) that one in 10 men suffers from epididymo-orchitis.

Poor diagnosis and treatment may result in complications like osteoarticular (sacroilitis, spondylitis, peripheral arthritis and osteomyelitis), dermal (erythematous papular lesions, purpura, dermal cysts), genitourinary (orchiepididymitis, glomerulonephritis and renal abscess), respiratory (pleural effusions and pneumonia), cardiovascular (endocarditis), and neurologic disorders (peripheral neuropahties, meningoencephalitis, transient ischemic attacks, psychiatric manifestations and cranial nerve compromise) resembling many other infectious and non-infectious diseases (Franco *et al*., 2007; Godfroid *et al*., 2011).

3.1.5.2 Risk factors

The most important species of *Brucella* responsible for human disease in order of their significance are *B. melitensis*, *B. abortus*, and *B. suis* (Acha and Szyfres, 2003). Transmission of brucellosis occurs from ingesting (foodborne), directly contacting (penetration through breaks in the epidermis), and inhaling the organism (occupationally exposed). The most common means of exposure is eating contaminated animal products in endemic areas (Olsen and Palmer, 2014). The less common means of infection include person to person (Meltzer *et al*., 2010; Wyatt, 2010), accidental infection with live vaccines (Ashford *et al*., 2004; Strausbaugh and Berkelman, 2003) and through blood donation and tissue transplantation (FAO, 2006).

Brucellosis is also one of the most common laboratory-acquired infections. Laboratory workers while handling specimens containing *Brucella* species may generate aerosol and may results in infection (Noviello *et al*., 2004). In countries where milk and dairy products are always pasteurized before consumption, brucellosis principally affect persons who are in close contact with animals and animal products (occupationally exposed) (Seleem *et al*., 2010).

The occupationally exposed vulnerable groups include veterinary doctors, butchers, veterinary technicians, insemination service employees, zoo technicians, farmers working on multi-herd farms, employees of meat and milk processing enterprises (Gali ´nska and Zagórski, 2013).

Brucellosis may also occur through international travel to endemic countries. Tourists or business travelers to endemic areas may acquire brucellosis by consumption of unpasteurized milk or dairy products. They may also import contaminated cheese or other dairy products into their countries and infect their families (Godfroid *et al*., 2005; FAO, 2006).

3.1.6. Control of human brucellosis

Every case of human brucellosis is directly or indirectly linked with infected animals or their products. So, the control of human brucellosis depends on minimizing/controlling disease burden in animals and reducing animal to human transmission (Zinsstag *et al*., 2007; Rubach *et al*., 2013). The control of animal brucellosis will be discussed in section 3.4.

The consumption of nonpasteurized dairy products from *Brucella*-infected animals is the most frequent route of human infection in general. So, pasteurization of milk will reduce *Brucella* transmission to humans (Rubach *et al*., 2013).

If pasteurization is not available, boiling or heating of milk at 80–85°C (176–185°F) for several minutes will also kill the *Brucella* (Corbel, 2006). The risk of infection among occupationally exposed group of people can be reduced through personal hygiene measures and adoption of safe working practices, including use of protective clothing, disinfection of protective clothing, and disinfection of potentially infected utensils and premises. To avoid aerosol transmission, eating and smoking must be forbidden in the abattoirs/heavily contaminated environments while handling animals and inhalation of dust or aerosols derived from dried excreta or tissues released at abortion, parturition or slaughter should be prevented by

the use of suitable respirators (Corbel, 2006; Islam *et al*., 2013b). However, these measures may be unfeasible in low and middle-income countries due resource limitations and nomadic animal husbandry practices (Corbel, 2006). In this situation, steps should be taken to reduce the impact of the disease by educating the population regarding the disease and its risk factors (Corbel, 2006; Islam *et al*., 2013b).

Ideally, occupationally exposed group of people should be kept under medical surveillance with periodic serological examinations. It is strongly recommended that new staffs provide a baseline blood sample before starting work. Those who develop clinical disease should be treated promptly. Young people less than 18 years of age and pregnant women should be excluded from high-risk occupations (Corbel, 2006).

3.2 Diagnosis in animals

In the history of microbiology, very few diseases have more diagnostic tests than brucellosis and the list of some indirect tests is given in Table 3.5. Diagnostic tests are applied for the following purposes: confirmatory diagnosis, screening or prevalence studies, certification, and, surveillance in order to avoid the reintroduction of brucellosis (in countries where brucellosis is eradicated) through importation of infected animals or animal products (Godfroid *et al*., 2010). The diagnostic methods include direct tests, involving isolation of organism or DNA detection by polymerase chain reaction (PCR)-based methods and indirect tests, which are applied either in vitro (mainly to milk or blood) or in vivo (allergic test). Isolation of *Brucella* spp. or detection of *Brucella* spp. DNA by PCR is the only method that allows certainty of diagnosis.

3.2.1 Direct diagnosis

3.2.1.1 Stained smears

Smears of placental cotyledon, vaginal discharge or fetal stomach contents may be stained using modified Ziehl-Neelsen (Stamp) method. The presence of large aggregates of intracellular, coccobacillus red organisms is presumptive evidence of brucellosis. It is still often used, even though this technique is not specific as other abortive agents such as *Chlamydophila abortus* or *Coxiella burnetii* are also stained red (Alton *et al*., 1988; FAO, 2006).

3.2.1.2 Culture

Bacterial culture plays an important role in confirming the presence of disease and it is essential for antimicrobial susceptibility, biotyping and molecular characterization which provides valuable epidemiological information to know the sources of infection in outbreak scenarios and the strain diversity in endemic regions (Kattar *et al*., 2008; Álvarez *et al*., 2011). However, two studies reported direct molecular typing of *Brucella* organisms without culture from clinical materials of both human and animal origin (Zhang *et al.*, 2013; Gopaul *et al*., 2014). The likelihood of obtaining a positive culture from material (other than samples collected from an abortion event) from a live infected animal is too low (15-

70%) for reliable diagnosis. Isolation of *Brucella* bacteria is time and resource-intensive; it requires level 3 biocontainment facilities and highly skilled technical personnel to handle samples and live bacteria for eventual identification and biotyping (Yu and Nielsen, 2010). Handling all live *Brucella* involves risk of laboratory infection and very strict biosafety rules must be observed. In order to avoid these disadvantages, methods based on PCR are becoming very useful and considerable progress has been made recently to improve their sensitivity, specificity, and technical ease and to lower costs. So, culture is not an appropriate technique for routine screening of brucellosis in animals and humans (Seleem *et al*., 2010; McGiven, 2013).

Important clinical samples include aborted fetuses (stomach, spleen, and lung), fetal membranes, vaginal secretions, colostrum, milk, sperm, and hygroma fluid. *Brucella* may also be isolated post-mortem from supra-mammary, internal iliac and retropharyngeal nodes, spleen, udder tissue, testes and gravid uterus. Care should be taken to minimize the fecal and environmental contamination of the material to give the greatest chance of successfully isolating *Brucella.* For the isolation of *Brucella* spp., the most commonly used medium is the Farrell medium (FM), which contains antibiotics able to inhibit the growth of other bacteria present in clinical samples.

However, due mainly to the nalidixic acid and bacitracin contained in its formulation, FM is inhibitory for *B. ovis* and also for some *B. melitensis* and *B. abortus* strains (Marin *et al*., 1996). A new selective medium (CITA) containing vancomycin, colistin, nystatin, nitrofurantoin, and amphotericin B was found to be more sensitive than FM (De Miguel *et al*., 2011). The cream and the sediment part of the milk obtained after centrifuge are spread on to the surface of at least three plates of solid selective medium. Placenta and other solid tissues need to ground manually or homogenize in a blender or stomacher with a small proportion of sterile water. Fetal stomach contents are collected, after opening the abdomen, by searing the surface of the stomach with a hot spatula and aspirating the liquid contents with a Pasteur pipette or syringe. As some *Brucella* species, like *B. abortus* biovars 1-4, need CO2 for growth the culture plates should be incubated at 35°C to 37°C in 5% to 10% CO2. *Brucella* colony may be visible after 2-3 days, but cultures are usually considered negative after 2-3 weeks of incubation (Alton *et al*., 1988).

3.2.1.3 Biotyping

Biotyping of *Brucella* spp. is performed using different tests, like agglutination tests with antibodies against rough (R antigen) or smooth LPS (against the A or M antigens); lysis by phages, dependence on CO2 for growth; production of H2S; growth in the presence of basal fuchsine or thionine; and the crystal violet or acriflavine tests (Alton *et al*., 1988).

These techniques must be carried out using standardized procedures by experienced personnel and usually performed only in reference laboratories.

3.2.2 Molecular methods

3.2.2.1 Identification

Several PCR based methods have been developed. The best-validated methods are based on the detection of specific sequences of *Brucella* spp., such as the 16S-23S genes, the IS711 insertion sequence or the BCSP31 gene encoding a 31-kDa protein (Ouahrani-Bettache *et al*., 1996; Baddour and Alkhalifa, 2008). These techniques were originally developed on bacterial isolates and are now also used to detect *Brucella* spp. DNA in clinical samples.

PCR-based assays are rapid and highly sensitive and specific, with some assays detecting down to 10 cells in less than two hours (Bounaadja *et al*., 2009).

3.2.3 Indirect diagnosis

There are two types *Brucella* colony known as 'smooth' and 'rough' based on the lipopolysaccharide (LPS) in their outer cell wall (Baldwin and Goenka, 2006). The 'smooth' phenotype has complete LPS consisting of lipid A, a core oligosaccharide and an O-side chain polysaccharide (S-LPS/OPS) while LPS of 'rough' strains lack the O-side chain (R-LPS). *B. ovis and B. canis* are both naturally 'rough' species and all the others are 'smooth' species (Cardoso *et al*., 2006). The S-LPS or *B. abortus* whole cells and R-LPS are usually used as antigen for the diagnosis of brucellosis caused by smooth and rough species respectively (Nielsen and Duncan, 1990; OIE, 2008). For example, the RBT uses *B. abortus* biotype 1 (Weybridge 99) whole cells as antigen, which will be able to detect antibody against *B. melitensis* also as both share common epitopes in OPS.

3.2.3.1 Tests used

Nielsen and Yu (2010) had reviewed serological diagnosis of brucellosis. The authors described the kinetics of immune response and discussed the principles, merits and demerits of the available diagnostic tests. As stated there, the serological tests detect different types of antibody produced in response to *Brucella* infection. For example, the IgM isotype antibody response usually appears 5 to 15 days post exposure.

The IgM antibody response is followed very shortly by production of IgG1 isotype of antibody and subsequently by IgG2 and IgA (Sutherland, 1984; Godfroid *et al*., 2002; Saegerman *et al*., 2004). As the IgM response commences early, theoretically it would be most suitable to measure this isotype as an indicator of exposure. There is, however, a number of other microorganisms containing antigens with epitopes similar to those of OPS and measurement of IgM antibody may result in a false positive reaction in serological tests. False positive reactivity would lead to specificity problems which would be of considerable consequence in an early control programme resulting in unnecessary slaughter; in the last stages of an eradication program and in free areas, resulting in expensive follow-ups. Production of IgG2 and IgA isotypes occurs later in infection and, as a result, measurement of these antibodies would

generally lower assay sensitivity. Based on these observations, the most useful antibody for serological testing for brucellosis is IgG1 (Nielsen and Yu, 2010).

Godfroid *et al*. (2010) had reviewed the diagnosis of brucellosis in livestock and wild life. It is stated there that the kinetics of production and disappearance of the principal immunoglobulin isotypes during infection, and the activity of these immunoglobulins in the different serological tests, will usually permit the distinction between acute and chronic infections.

Table 3.5: List of some available tests for the diagnosis of brucellosis

Tests	Agglutination tests	Primary Binding Assays
Slow	Slow Agglutination (SAT)	Radioimmunoassay
	SAT with added reducing agents such as 2-mercaptoethanol or dithiothreitol	Fluorescence immunoassay
	SAT with addition of rivanol to precipitate glycoproteins	Particle counting fluorescence immunoassay
	SAT with addition of ethylene diamine tetraacetic acid to reduce IgM binding (EDTA)	Indirect enzyme immunoassay
	SAT with antiglobulin added to enhance agglutination	Competitive enzyme immunoassay
	Milk ring test	Fluorescence polarization assay
Rapid	Rose Bengal	
	Modified Rose Bengal	
	Buffered Plate agglutination	
	Card	
	Heat Treatment of serum	
	Addition of 10% sodium chloride	
Tests	Precipitation Tests	Compliment Fixation Test
	Agar gel immunodiffusion	Warm
	Radial immunodiffusion	Cold
		Hemolysis in gel
		Indirect hemolysis
Tests	Allergic tests	
	Skin test	

The authors explained the following principles about the status of brucellosis based on serological test results:

• The concomitant presence of IgM and IgG suggests acute brucellosis, while chronic brucellosis is characterized by the presence of IgG alone.

• A positive response in an agglutination test, which detects mainly IgM, is not indicative of brucellosis if it is not confirmed by a positive IgG response by iELISA within one week.

Accordingly, in this research multiple testing strategy was used so that the stage of infection in domestic ruminants can be determined using three agglutinating (one rapid: RBT and two slow: MRT and SAT) and one primary binding assay (iELISA).

These four tests are further discussed in this section.

- Slow Agglutination Test with EDTA: In 1897, Smith and Wright (Weight, 1897) published the first description of this test for the serological diagnosis of brucellosis in man. The antigen used is *B. abortus* biotype 1 (Weybridge 99). The SAT detects IgM isotype of antibody efficiently. The SAT detects IgG less efficiently, especially IgG1, resulting in low assay specificity. Addition of EDTA has improved it specificity significantly (Alton *et al*., 1988). Reading is done on the basis of degree of agglutination and expressed in international units (IU). Any serum with an antibody titer greater than or equal to 30 IU/ml, as prescribed by the EU (Shey-Njila *et al*., 2005), is considered positive. Due to specificity problem, the OIE has recommended the discontinuation of this test as a diagnostic tool for bovine brucellosis (OIE, 2009). However, it is a standardized and extremely robust test that has shown good results and has proven efficacious in several countries now declared officially free of brucellosis (Emmerzaal *et al*., 2002). This test is simple, cheap and if used simultaneously with other test IgG detecting test like iELISA will help to determine the stage of infection in animals based on the above-mentioned principle (Godfroid *et al*., 2010).
- Milk Ring test: The test consists of mixing colored *Brucella* whole-cell antigen with fresh bulk/tank milk. In the presence of anti-*Brucella* antibodies, antigen-antibody complexes form and migrate to the cream layer, forming a purple ring on the top of the column of milk. In the absence of antigen-antibody complexes, the cream remains colorless. This test is not considered sensitive and may fail to detect a small number of infected animals within a large herd.

  However, this lack of sensitivity is compensated by the fact that the test can be repeated, usually monthly, due to its very low cost and gives a good reflection of serum antibody.

  False positive reactions may also occur due to abnormal milk such as mastitic milk, colostrum and late lactation cycle milk. This test is prescribed by the OIE for use only with cow milk.

  Bulk milk can be screened to detect the presence of infected animals within the herd, which can then be identified by blood testing. This method of screening is extremely effective and is usually the method of choice in dairy herds (OIE, 2009).
- Rose Bengal Test: *B. abortus* biotype 1 (Weybridge 99) whole cells stained with Rose Bengal and suspended in a buffer which when mixed with the appropriate volume of serum results in a final pH of 3.65±0.05. After thorough mixing of the serum and antigen, agglutination must be visible be within 4 minutes for positive result. The low PH used reduces non-specific reactions because it prevents some agglutination by IgM and encourages agglutination by IgG1. The RBT is a rapid

agglutination tests lasting 4 minutes done on a glass plate with the help of an acidic-buffered antigen (pH 3.65 ±0.05). These tests have been introduced in many countries as the standard screening test followed by confirmatory testing because it is very simple, the consumables are cheap, there is a low equipment requirement and the assay is standardized (Nielsen and Ewalt, 2010). False negative reactions may occur, due to pro-zoning with sera containing very high levels of antibody. In addition, false positive serological reactions (FPSR) may occur due to some cross-reacting antibodies and antibodies resulting from *B. abortus* S19 vaccination (OIE, 2009).

- Indirect ELISA: Most iELISAs use purified *B. abortus* biotype 1 (Weybridge 99) S-LPS as antigen. The iELISA is highly sensitive but lacks the capability to fully resolve the FPSR problem and the problem of differentiating between antibodies resulting from cross-infection and S19 vaccination (OIE, 2009). Competitive ELISA and fluorescence polarization assay have been reported to circumvent the FPSR problems due to cross-reacting antibodies and S19 vaccination (Nielsen and Ewalt, 2010). However these tests are not in use in Bangladesh for the diagnosis of brucellosis.

3.3 Evaluation of diagnostic test and estimation of true prevalence in the absence of gold standard

Using diagnostic tests without evaluating their performance in a context may generate unreliable results, which may also lead to wrong epidemiological inferences (Godfroid *et al*., 2013). The performance of a diagnostic test is typically described by two quantities, the sensitivity (Se) and the specificity (Sp), each describing the capacity of the test to reflect the unknown "true" disease status (Speybroeck *et al*., 2013). The Se is the probability of a positive test given the disease is present and Sp is the probability of a negative test result given the disease is absent. An important fact, which is often overlooked, is that the Se and SP are population specific parameter, as opposed to some intrinsic constant, as it depends upon the specific biological characteristics of the study population (Greiner and Gardner, 2000). The evaluation of the performance of a diagnostic test is straightforward when a gold standard test (Se=1 and Sp=1) is available for verifying disease status or from an experiment where a proportion of the subjects are artificially infected (diseased) and a proportion of the subjects are known to be disease-free as shown in equations 3.3.1, 3.3.2 and 3.3.3 from Table 3.6.

Table 3.6: Evaluation of a diagnostic test by a gold standard test

	Gold Standard Test		Total
Test under evaluation	Positive	Negative	
Positive	*TP*	*FP*	*TP + FP*
Negative	*FN*	*TN*	*FN + TN*
	TP + FN	*FP + TN*	*n = TP + FP + FN + TN*

Legend: *n* indicates the number of subjects; *TP*, *FP*, *FN*, and *TN* indicate true positive, false positive, false negative and true negative respectively; *TP + FN* indicates diseased and *FP + TN* indicate disease-free; TP =True positive: truly infected individual with a positive test result; TN =True negative: truly non-infected individual with a negative test result; FP =False positive: truly non-infected individual with a positive test result; FN =False negative: truly infected individual with a negative test result

The sensitivity, specificity of the test under evaluation and the true prevalence of the disease can be calculated from the following equations based on the results of Table 3.6:

$$Se = \frac{TP}{TP + FN} \qquad 3.3.1$$

$$Sp = \frac{TN}{FP + TN} \qquad 3.3.2$$

$$True\ prevalence = \frac{TP + FN}{TP + FP + FN + TN} \qquad 3.3.3$$

However, gold standard tests are rarely available and most diagnostic tests are imperfect (Se <1 and/ or Sp <1). For example, although culture is considered as gold standard for the diagnosis of brucellosis, its sensitivity varies from (30 -70% in chronic cases and 80-90% in acute cases) in humans (Franco *et al*., 2007; Espinosa *et al*., 2009).

Apparent prevalence (test positive / sample size) can be converted to true prevalence based on the results of a screening test using the Rogan-Gladen estimator as shown in the equation 3.3.4 (Rogan and Gladen, 1978) when the Se and Sp of that test are known.

$$True\ prevalence = \frac{Apparent\ prevalence + Sp - 1}{Se + Sp - 1} \qquad (3.3.4)$$

In this equation, the only variable is apparent prevalence, the Se and Sp are fixed parameters which is not realistic as many factors like the presence of cross-reacting pathogens (Saegerman *et al*., 2004) and low infection pressure, may influence the test parameter values (Speybroeck *et al*., 2012). Even if, the Se and Sp are considered as variables, as it should be, it is not possible to estimate three unknown quantities (true prevalence, Se and Sp) from a single equation like 3.3.4. Under certain conditions, using several diagnostic tests may partially solve this problem, but most often external information on the diagnostic test characteristics will be needed (Berkvens *et al*., 2006).

It is also fully explained that the Rogan-Gladen estimator can produce estimates of prevalence, which exceed one or are negative (Hilden, 1979; Speybroeck *et al*., 2013).

Several other approaches have been developed for evaluation of tests in the absence of gold standard including both frequentist (maximum likelihood estimation based) and Bayesian latent class models (Lewis and Torgerson, 2012). The class of models where the disease status of the individual is unknown are known as latent class models as the disease status is latent: existing but not presently evident or realized (Toft *et al*., 2005). Among the frequentist approach, the most widely used latent class model of test accuracy based on maximum likelihood estimation was proposed by Hui and Walter (1980). Hui and Walter model depends on three assumptions:

- the tested individuals are divided into two or more populations with different disease prevalences;
- the tests have the same properties in all populations;
- the tests are conditionally independent given the true (but latent) disease state.

In fact, these assumptions are not always true ((Dendukuri and Joseph, 2001; Branscum *et al*., 2005; Toft *et al*., 2005; Berkvens *et al*., 2006).

Bayesian latent class model for the simultaneous estimation of true prevalence and evaluation of diagnostic test characteristics are preferred over the frequentist approach as it assumes Se and Sp as random population parameters (not fixed) and can combine observed field data (likelihood) with any external (a prior) information on Se, Sp and apparent prevalence within a single model (Dunson, 2001; Speybroeck *et al*., 2013). The external information may be obtained from similar previous studies or even the beliefs of investigators (i.e. expert opinions). The choice of prior information thus plays a central role when interpreting diagnostic test results and every effort should be made to verify that the prior probability distributions assigned to the different variables are not in conflict with the data (Berkvens *et al*., 2006). The deviance information criterion (DIC) (Spiegelhalter *et al*., 2002) and Bayesian p value (Bayes-p) (Gelman *et al*., 2013) are two measures of discordance which can be used to verify whether the prior information is in conflict with the data/test results. A systematic review summarizing data using appropriate meta-analysis is preferred to obtain informative priors on diagnostic test performance (EFSA, 2009). A beta or uniform distribution of the priors is usually used in Bayesian latent class models. The sensitivity analysis of prior information should always be performed to assess its potential influence on estimates (Rahman *et al*., 2013).

In Bayesian latent class model, rather than using a single imperfect test, multiple imperfect tests (either in series or parallel) are combined to estimate the true prevalence of the disease and diagnostic test accuracy parameters (Joseph *et al.*, 1995; Black and Craig, 2002). The overall misclassification errors are reduced in multiple testing strategy (Sanogo *et al*., 2014).

An important consideration in the evaluation of multiple diagnostic tests is whether or not the tests can be assumed conditionally independent of each other given the true disease status. It has been demonstrated that the assumption of conditional independence may lead to biased estimates for test characteristics if in fact the tests are conditionally dependent (Vacek, 1985; Gardner *et al*., 2000). For example, in the context of brucellosis serology, the IgG iELISA detects only IgG, whereas RBT detects IgG mainly (IgM and IgA also) and SAT detects IgM mainly (IgG and IgA also) antibodies. So, they can be considered to be partially conditionally dependent on each other given the true disease status (Gardner *et al*., 2000; Nielsen and Yu, 2010). Therefore, the estimation procedures should be adjusted for the dependencies among the tests (Georgiadis *et al*., 2003; Branscum *et al*., 2005). In this scenario, Bayesian latent class model should incorporate the covariance factor expressing the extent of the dependence among positive and negative results, to estimate the Se and Sp of the tests under evaluation and by taking into account the testing strategy also (Gardner *et al*., 2000).

3.4 Control and eradication of brucellosis in domestic ruminants

Before developing a national control strategy, the impact of brucellosis on the livestock economy and human health, adequacy of national veterinary service organization to carry out the strategy, collaboration between public health and veterinary services and the costs of the different control or eradication strategies must be evaluated as part of this strategy (Blasco and Molina-Flores, 2011). Some of the developed countries have successfully eradicated animal brucellosis by combined vaccination and test-and-slaughter programs (Pappas *et al.*, 2006), along with effective disease surveillance and animal movement control (Godfroid *et al*., 2013).

3.4.1 Eradication

In order to eradicate brucellosis, the combined test and slaughter program is usually implemented initially by compulsory vaccination, then vaccination is gradually restricted and eventually prohibited during removal of seropositive animals or herd depopulation (when the herd or flock prevalence is low: for example <2%). More than a decade is usually needed to complete the brucellosis eradication program by a "test-and-slaughter" policy and key for success is a sufficient financial compensation scheme for farmers for their culled livestock (Corbel, 2006; Godfroid *et al*., 2013). Other than expense, a good record keeping, infrastructure, cooperation between all related stakeholders and epidemiologic surveillance are also essential for successful eradication program.

The vaccination primarily prevents clinical effects of the disease (i.e., abortions or infected calves) that lead to transmission (Olsen and Stoffregen, 2005). The most widely used live attenuated vaccines are *B.*

melitensis Rev1 (Rev1) for sheep and goats and *B. abortus* S19 (S19) for cattle. Both induce good protection but can induce abortion if administered during pregnancy. In the United States, routine brucellosis vaccination is administered via intramuscular or subcutaneous injection and limited to prepubescent heifers (4–12 months) (Olsen and Tatum, 2010). Both S19 and Rev1 interfere serological diagnostic testing. However, conjunctival vaccination with reduced doses before the age of 4 months avoids the serological interference as well as the abortions and udder infections (Godfroid *et al*., 2011). For example, the original dose of S19 vaccine is ($2.5–12x10^{10}$ CFUs) for calfhood vaccination, but the United States and some other countries switched to a reduced dosage ($3–10x10^{9}$ CFUs) in the 1980s in an effort to reduce the number of calfhood vaccinates having retained antibody titers (Olsen and Tatum, 2010). Since 1996, the United States and some Latin American countries are using *B. abortus* strain RB51 vaccine instead of S19. The RB51 is a rough strain and hence does not induce antibody responses that are detected by conventional brucellosis serologic tests. Moreover, cattle that were calfhood vaccinated with S19 did not seroconvert as adults when boostered with calfhood dosages of RB51. Under experimental conditions, cattle vaccinated with 1 to $3.4x10^{10}$ CFUs of RB51 have reduced incidence of abortion or *Brucella* infection at necropsy when compared with non-vaccinated cattle (Poester *et al*., 2006). As with S19, adult vaccination with RB51 has been used to protect cattle against brucellosis. Under experimental conditions, parenteral vaccination of pregnant cattle with 1 to 3 x 109 CFUs of RB51 is safe and efficacious for the subsequent pregnancy (Olsen, 2000). Similar to S19, RB51 can induce abortions under field conditions (Van Metre *et al*., 1999). The data suggests that calfhood vaccinated cattle can be safely booster vaccinated with RB51 ($1–3\ x10^{9}$ CFUs) as pregnant adults (Leal-Hernandez *et al*., 2005).

However, some authors have questioned the efficacy and safety of RB51 with regard to bovine brucellosis (Moriyón *et al*., 2004; Mainar-Jaime *et al*., 2008; Bagnat and Manett, 2011) and it is not effective against *B. melitensis* or *B. ovis* infections in sheep (Moriyón *et al*., 2004). In addition, human infections due to RB51 have also been described (Villarroel *et al*., 2000); this strain is resistant to rifampin, a widely used antibiotic in the treatment of human brucellosis (Ariza *et al*., 2007).

S19 strain of *B. abortus* has also been found to be associated with human infection (Strausbaugh and Berkelman, 2003; Wallach *et al*., 2008). In countries having a high prevalence of brucellosis and/or limited regulatory programs, S19 may be the vaccine of choice because it may be slightly more protective and many countries can produce commercial S19 vaccines. However, in countries, where prevalence of brucellosis is low and serologic surveillance is high, the RB51 vaccine is preferred because of its lack of interference with serologic surveillance and comparable efficacy in protecting against brucellosis (Olsen and Tatum, 2010).

The only available vaccine against *B. melitensis* infection is Rev1 has been proved to be effective for prevention of brucellosis in sheep and goats (Blasco, 1997). The individual doses of $1–2x10^{9}$CFUs subcutaneously induces long-lasting serologic response, which makes an eradication program based on combined test and slaughter impractical. But, that problem may be solved by vaccinating via conjunctival instillation (using same dose) where the immune response is similar to that induced by the classic

subcutaneous method, but the serologic responses evoked by the vaccine are significantly reduced, making this program fully compatible with the application of an eradication program based on vaccination combined with test and slaughter (Blasco and Molina-Flores, 2011). Rev1 in both routes may induce abortion in pregnant animals (Blasco, 1997). Another alternative may be vaccinating only sexually immature female animals to minimize stimulation of postvaccinal antibodies, which may confuse the interpretation of diagnostic tests, and also to prevent possible abortions induced by the vaccines (Corbel, 2006).

A potential public health risk with the Rev1 vaccine is that this strain also can infect humans (Ollé-Goig and Canela-Soler, 1987; Blasco and Diaz, 1993) and is resistant to streptomycin, an antibiotic that in combination with doxycycline constitutes the most effective treatment of human brucellosis (Ariza *et al*., 2007).

3.4.2 Control

Eradication of brucellosis by test-and-slaughter is unfeasible in developing countries because of limited resources to compensate farmers whose animals are slaughtered during such screening programs (Godfroid *et al*., 2011). However, a mass vaccination strategy (avoiding pregnant animals in mid-gestation) may be applied to control brucellosis in developing countries.

The herd/flock level control of animal brucellosis may be achieved using some general principles: reducing the exposure to *Brucella* spp. and increasing the resistance to infection of animals in the populations

1. The reduction of exposure to *Brucella* spp.

Farm sanitation and biosecurity: Aborted fetuses, placentae and contaminated litter should be disposed by incineration or deep burial mixing with lime at sites away from water courses. Any area in which an abortion or infected parturition has occurred should be washed down with an approved disinfectant. Dung should be cleaned daily and stored in a secluded area until rendered safe by natural decay (this will probably require about one year) or else burnt or soaked in disinfectant before disposal. Premises that have held *Brucella*-infected animals should not be re-stocked until at least four weeks have elapsed between cleaning and disinfection. Rodent control measures should be enforced and insect infestation kept to a minimum by the use of fly screens, light traps and insecticides. The use of maternity pens to isolate animals during and post-parturition is essential as these animals shed the most *Brucella*. Isolation of post-parturient animals reduces the spread of infection to the rest of the herd or flock (Corbel, 2006).

Control of animal movement: The control of animal movements between herds, and especially from farms or regions with a high prevalence of disease is a basic principle of animal disease control and is a necessary and highly effective measure. The control of animal movement within a country is sometimes impossible without regulatory/legislative support. The permanent, individual identification of animals is also very important to identify the inter state/division/district and cross-border movements and market

chain interactions of livestock within the country and the region is also necessary. Unauthorized sale or movement of animals from an infected area to other areas should be forbidden. Replacement stock should be procured from brucellosis free herds/flocks. The application of pre-movement testing will reduce the risk of spread of brucellosis between herds/holdings and provides additional assurance for the purchaser in this regard. Isolation of purchased replacements for at least 30 days and a serological test prior to entering the herd/flock is necessary. In case of porous borders with neighboring countries, a regional control strategy should be developed to prevent illegal trafficking of livestock. All imported livestock should be monitored in quarantine stations before entered into the country (Corbel, 2006; Loth *et al*., 2011; Islam *et al*., 2013b; Mondal and Yamage, 2014).

2. Increasing the resistance to infection of animals in populations:

Vaccination: The most successful method for prevention and control of brucellosis in animals is through vaccination. While the ideal vaccine does not exist, the attenuated strains of *B. melitensis* strain Rev.1 for sheep and goats and *B. abortus* strain 19 have been used widely as described earlier.

3.5 Diagnosis in human

The diagnosis of human brucellosis is usually based on the isolation of *Brucella* spp. from blood, tissue specimens, body fluids and bone marrow, the serological tests for the detection of anti-*Brucella* spp. antibodies and the molecular methods for the detection of *Brucella* spp. DNA (Sakran *et al*., 2006). In countries where brucellosis is enzootic (i.e. present in animal reservoirs), human confirmed cases are based on clinical symptoms associated with positive serology without attempts to isolate *Brucella* spp. (CDC, 1997).

3.5.1 Isolation of brucellae from blood and tissue

The confirmatory diagnosis of brucellosis necessitates the isolation of the pathogen from blood, bone marrow or other tissues and body fluids. The isolation of *Brucella* depends on the stage of disease (acute versus chronic), antibiotic pre-treatment, the existence of an appropriate clinical specimen and the culturing methods used. Isolation is much higher during the first two weeks of symptomatic disease and in blood cultures taken during the pyrexial phase (Memish *et al*., 2000). However, isolation of *Brucella* spp. has the risk of laboratory-acquired infections and is time consuming, and the sensitivity of culture method is often low (30 -70% in chronic cases and 80-90% in acute cases), depending on the culture medium, *Brucella* species, disease stage and quantity of circulating bacteria (Franco *et al*., 2007; Espinosa *et al*., 2009).

Brucella selective media like such as Farrell's medium (FM) is most commonly used for the isolation (Farrell, 1974). Due to the presence of nalidixic acid and bacitracin in FM, growth of *B. suis* and several *B. melitensis* and *B. abortus* strains can be significantly inhibited.

So, a new selective medium containing vancomycin, col-istin, nystatin, nitrofurantoin and amphotericin B has been developed for veterinary samples (De Miguel *et al*., 2011). However, the new selective media

has not been evaluated for the diagnosis of human brucellosis. Automated continuously monitored blood culture systems such as Bactec (BD Diagnostics, Sparks, MD, USA) and BacTAlert (bioMerieux, Durham, NC, USA) give higher yields than the conventional culture method and reduces the time to detection significantly (Mantur and Mangalgi, 2004; Raj *et al*., 2014).

Bone marrow cultures have proven to be more sensitive (15-20% more than peripheral blood culture) than blood cultures for the detection of *Brucella* spp. at any stage of disease, and the mean time to detection is significantly reduced (Gotuzzo *et al*., 1986; Mantur *et al*., 2008). This method has also proven its usefulness in patients treated with antibiotics. As bone marrow aspiration and biopsy is painful, the procedure should be restricted to seronegative patients in whom there is a strong clinical suspicion of brucellosis (Gotuzzo *et al*., 1986).

3.5.2 Identification and Typing

The colonies of *Brucella* spp. are usually recovered from clinical specimens on FM or other media, within 24–48 h of aerobic incubation or under 5–10% CO2 incubation at 37°C. The colonies of smooth *Brucella* strains are raised, convex, circular, translucent, and 0.5 mm to 1 mm in diameter. Colony morphology, as well as virulence, antigenic properties and phage sensitivity of the bacteria, is subject to changes after subcultivation or prolonged culture (more than four days) (Cardoso *et al.*, 2006). Thus, smooth brucellae dissociate to rough forms, which grow in less convex and more opaque colonies with a dull, dry, yellowish-white granular appearance. The identification of *Brucella* species and biovars is based on CO2 requirement, H2S production, urease activity, agglutination with monospecific sera (A and M), selective inhibition of growth on media containing dyes such as thionin or basic fuchsin, and phage typing (Alton *et al*., 1988). But, these procedures are cumbersome, time consuming, hazardous and have a high risk of laboratory-acquired infection. A good alternative to the standard microbiology methods is the semi-automated metabolic biotyping system (MicronautTM), based on a selection of 93 different substrates (Al Dahouk *et al*., 2010).

This new method reduces time and also minimizes the risk of laboratory-acquired infections. Using this technology, *Brucella* can be identified and differentiated up to the species and biovar level within 48 hours. Ferreira *et al*. (2010) developed a powerful tool for bacterial identification termed as matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS). In comparison with conventional phenotypic techniques or molecular methods, it is rapid, precise and cost effective. MALDI-TOF MS is able to identify *Brucella* spp. directly, from both culture plates and blood culture bottles.

3.5.3 Serological diagnosis

Serological testing is fast, non-hazardous and more sensitive than culture and therefore preferred in routine clinical practice. However, serological tests can only indirectly prove *Brucella* infections by high or rising titers of specific antibodies. Agglutination titers ≥1: 160 or a fourfold rise of titers in follow-up sera are considered to be indicative of active infection. Diagnostic titers, however, can be detected months or even years after acute infection despite therapeutic success and negative blood cultures (Ariza *et al*., 1992). In addition, a high proportion of the population in endemic regions may have persistent antibody titers due to ongoing exposure to *Brucella.* Validation of serological tests and setting cut-off in the context of its use are crucial for the diagnosis of human brucellosis (Kiel and Khan, 1987; Memish *et al*., 2002).

Just detection of antibody without clinical signs and symptoms or a history of potential exposure does not indicate brucellosis (Ariza *et al*., 1992; Al Dahouk and Nöckler, 2011). Serological tests may be negative, especially early in the course of the disease, and laboratory testing should be repeated after one to two weeks in clinically suspicious cases. Sequential serological testing also allows the monitoring of treatment response. In the first week of infection, immunoglobulin (Ig) M isotype antibodies predominate, followed by a shift to IgG in the second week (Al Dahouk *et al*., 2002). Titers of both subtypes rise continuously and reach a peak within four weeks. Successful antibiotic therapy is usually indicated by a rapid decline in antibody titers, whereas persisting high IgG titers reflects treatment failure (Casanova *et al*., 2009; Bosilkovski *et al*., 2010). Relapse is often characterized by a second peak of anti-*Brucella* IgG and IgA, but not IgM, immunoglobulins.

The diagnostic antigen of classic serological tests is usually made from whole-cell extracts containing large amounts of smooth lipopolysaccharides (S-LPS). As the humoral immune response during natural infection is mainly mediated by antibodies directed against S-LPS, these assays reliably detect agglutinating and/or non-agglutinating antibodies. However, because of cross-reactivity with various other clinically relevant bacteria, the specificity of LPS based assays can be low. The immune-dominant epitope of the *Brucella* O-polysaccharide (OPS) resembles the corresponding epitopes of *Yersinia enterocolitica* O:9, *Salmonella urbana* group N, *Vibrio cholerae*, *Francisella tularensis*, *Escherichia coli* O157 and *Stenotrophomonas maltophilia* (Al Dahouk *et al*., 2002). In contrast, the S-LPS antigen is not shared by *B. canis* and *B. ovis,* thus explaining why canine brucellosis cannot be diagnosed by standard serological methods based on smooth *Brucella* antigens (Lucero *et al.*, 2005). These strains exist in a rough colony form and do not share cross-reacting antigens with the other *Brucella* spp. (Araj and Kaufmann, 1989). The list of some diagnostic tests available for the diagnosis of brucellosis is presented in Table 3.5 (Nielsen and Yu, 2010).

In this study, the RBT, STAT and iELISA were used simultaneously for the diagnosis of human brucellosis. The principle and procedures are similar as described in previous section. Although STAT was introduced over a century ago, in 1897, it is widely used in human brucellosis diagnosis (Alton *et*

al., 1988). The test is performed in tubes by reacting a known standardized volume and concentration of whole *Brucella cell* suspension with a standardized volume of doubling serum dilutions, usually ranging from 1:20 to 1:1280. The suspension mixture is incubated in a water bath at 37°C for 24 h, and agglutination at the bottom of the tubes is examined visually. Most authors considered a STAT titer of 1:160 as diagnostic in conjunction with a compatible clinical presentation (Konstantinidis *et al*., 2007; Gómez *et al*., 2008). But, in regions where brucellosis is endemic, a large proportion of the population may have persistent *Brucella*-specific antibody titers. In this scenario some authors recommend to use a STAT titers of 1:320 or higher to make the test more specific (Kiel and Khan, 1987; Memish *et al*., 2002; Mantur *et al*., 2007).

WHO and OIE provide guidelines for STAT and RBT standardization, but not for the iELISAs. In general a 'new' cut-off should be determined under local conditions to avoid false positives.

3.5.4 Molecular method

Polymerase chain reaction (PCR) assays can be applied to detect *Brucella* DNA in pure cultures and in clinical specimens, i.e. serum, whole blood and urine samples, various tissues, cerebrospinal, synovial or pleural fluid, and pus (Colmenero *et al*., 2010; Debeaumont *et al*., 2005; Queipo-Ortuño *et al*., 2006, 2008a). The PCR is more sensitive than blood cultures and more specific than serological tests (Al Dahouk *et al*., 2013). The analytical sensitivity can be further increased by using real-time PCR assays, which can detect as few as five bacteria per reaction (Navarro *et al*., 2006; Al Dahouk *et al*., 2007). Moreover, real-time PCR enables high-throughput screening of clinical samples and delivers results within a few hours.

3.5.4.1 Standard PCR

For the diagnosis of human brucellosis, a PCR assay with one pair of primers was developed, which amplifies the target genomic sequence of *Brucella* species. Primer pairs include the primers for sequences encoding 16S rRNA (Romero *et al*., 1995; Nimri, 2003), outer membrane protein (omp2a, omp2b) (Leal-Klevezas *et al*., 1995; Sifuentes-Rincan *et al*., 1997; Bardenstein *et al*., 2002), 31-kDa immunogenic *Brucella abortus* protein (BCSP 31) (Matar *et al*., 1996; Queipo-Ortuño *et al*., 1997), 16S-23S ribosomal DNA interspace region (Fox *et al.*, 1998) and insertion sequence (IS711) (Cloeckaert *et al*., 2000; Elfaki *et al*., 2005). In fact, blood samples are often used for the diagnosis of human brucellosis by the standard PCR (Navarro *et al*., 1999). Several factors were reported to affect PCR results in a blood specimen such as the high concentrations of leukocytes DNA and heme compounds (Morata *et al*., 1998). Additionally, human genomic DNA affects the sensitivity of peripheral-blood PCR assay for the detection of *Brucella* DNA (Navarro *et al*., 2002). Zerva *et al*. (2001) reported that serum samples should be used preferentially over whole blood for diagnosis of human brucellosis by PCR, but Mitka *et al*. (2007) revealed that buffy coat and whole blood were the optimal specimens.

Genus specific PCR assays are generally adequate for the molecular diagnosis of human brucellosis (Al Dahouk and Nöckler, 2011). The BCSP31 gene, coding for a 31-kDa immunogenic outer membrane protein conserved among all *Brucella* spp., is the most common molecular target in clinical applications (Baily *et al*., 1992). Such a genus-specific PCR can help to avoid false-negative results in patients infected with unusual species and biovars.

3.5.4.2 Real Time PCR

Real-time PCR for the rapid detection and differentiation of *Brucella* species in clinical samples has recently been developed, targeting 16S-23S internal transcribed spacer region (ITS) and the genes coding omp25 and omp31 (Kattar *et al*., 2007), BCSP 31 (Colmenero *et al*., 2005; Debeaumont *et al*., 2005; Queipo-Ortuño *et al*., 2008b), and IS711 (Queipo-Ortuño *et al*., 2005; Zhang *et al*., 2013). The major advantages of real-time PCR are that it can be performed in a very short time, does not require electrophoretic analysis, and avoids contamination. The samples that can be tested by real-time PCR include *Brucella* isolates (Redkar *et al*., 2001), serum (Queipo-Ortuño *et al*., 2005), blood, and paraffin-embedded tissues (Kattar *et al*., 2007; Zhang *et al*., 2013). A study analyzed the sensitivity and specificity of the 3 established real-time PCR methods using primers and TaqMan probes targeting the IS711, BCSP31 and per genes, and it also compared their efficiencies for the detection of the *Brucella* genus. The results showed that the IS711-based real-time PCR was the most sensitive, specific and efficient to detect *Brucella* spp (Bounaadja *et al*., 2009).

However, a primary molecular diagnosis must always be confirmed using a second gene target (Scholz *et al.*, 2007). For confirmation and distinction from closely related microorganisms, 16S rRNA gene sequencing can be used (Gee *et al*., 2004). Using real time PCR, species and even biovars level differentiation of the isolates is possible (Huber *et al*., 2009).

Subtyping at the strain level is useful for differentiating re-exposure from relapse (Al Dahouk *et al*., 2005). A multiple locus VNTR (variable number of tandem repeats) analysis assay based on 16 markers (MLVA-16) has proven its usefulness for diagnostic purposes in human brucellosis (Al Dahouk *et al*., 2007).

A relapse or primary treatment failure can be confirmed by assessing identical MLVA-16 genotypes of *Brucella* strains isolated from the same patient before and after first-line therapy, and, as a consequence, antibiotic therapy should be prolonged. Re-infection is usually characterized by divergent genotypes of *Brucella* isolates, and standard therapy can be repeated without substantial loss of efficacy (Al Dahouk *et al*., 2013). Quantitative real-time PCR using human sera maybe applied not only for initial diagnosis but also for differentiating active from past *Brucella* infections (Queipo-Ortuño *et al*., 2008b).

Detection of *Brucella* at species level using real time PCR assays is also possible from clinical samples of both human and animal origin (Gwida *et al*., 2011; Ali *et al*., 2014). Recently direct molecular

typing of *Brucella* organisms from clinical material (ct value <26) was also reported using MLVA-16 (Gopaul *et al*., 2014).

3.6 Treatment

The prerequisite for an effective therapy of brucellosis are that treatment should start on time, should consists of combination of drugs along with at least one drug having good penetration into macrophages and can act in the acidic intracellular environment cells and should be prolonged (Mantur *et al*., 2007). In 1986, the World Health Organization issued guidelines for the treatment of human brucellosis. The guidelines discuss two regimens, both using doxycycline (100 mg twice daily) for a period of six weeks, in combination with either streptomycin (15 mg/Kg body weight intramuscularly) for two to three weeks or rifampin (600-1200mg/day) for six weeks. A recent meta-analysis of the efficacy of various combinations of drugs against brucellosis proved superiority of doxycycline and streptomycin over doxycycline and rifampicin with less relapses (4.2%) and less side effects (18.6%) (del Pozo and Solera, 2012).

Childhood brucellosis can be successfully treated with a combination of two drugs: doxycycline 4 mg/Kg/day and rifampicin 10 mg/Kg/day orally for six weeks (Mantur *et al*., 2004). Rifampicin with or without Trimethoprim–sulfamethoxazole (960 mg twice daily for 6 weeks) has proved safe to treat brucellosis during pregnancy (Ozbay and Inanmis, 2005; Pappas *et al*., 2006).

Relapses occur about 10% cases and are often milder in severity than initial disease and can be treated with a repeated course of the usual antibiotic regimens (Roushan *et al*., 2006).

3.7 Scenario of brucellosis in human and animals in Bangladesh

3.7.1 Human population

According to the Population and Housing Census 2011, there are about 150 million people and 32 million households in Bangladesh. The male and female ration is 100.3. The population density and growth rate are 1015/km^{2}and 1.37% respectively (BBS, 2011). About 71% of the total population is rural and 85% of the rural households own animals. The exact number of skilled veterinary practitioners, milkers, butchers, artificial inseminators and unskilled veterinary practitioners are not known in Bangladesh. About 5000 staffs (including officers and supporting staffs) are working in the DLS to deliver veterinary services.

3.7.2 Livestock population, its impact on national economy and an overview of the context of the study

3.7.2.1 Livestock population

Livestock in Bangladesh include cattle, buffalo, goats, sheep, chickens and ducks. In the fiscal year of 20122013, there were about 23.4, 1.5, 25.2, 3.1 and 296.3 (Total 349 million) million heads of cattle,

buffalo, goats, sheep and poultry respectively (DLS, 2014). Bangladesh has one of the highest cattle densities: 145 large ruminants/km^2 compared with 90 for India, 30 for Ethiopia, and 20 for Brazil. The average weight of local cattle ranges from 125 to 150 kg for cows and from 200 to 250 kg for bulls that falls 25-35% short of the average weight of all-purpose cattle in India. Milk yields are extremely low: 200-250 liter during a 10-month lactation period in contrast to 800 liter for Pakistan, 500 liter for India, and 700 liter for all Asia. Despite highest cattle densities in Bangladesh, the current production of milk, meat and eggs are inadequate to meet the current requirement and the deficits are 85.9%, 77.4% and 73.1% respectively (Anon., 2007; Teufel *et al*., 2010).

3.7.2.2 Breeds of livestock

The common cattle breed are indigenous and their crosses with Holstein Friesian and Sahiwal (Teufel *et al*., 2010). The exact proportion of crossbred cattle population in Bangladesh is not known but it is assumed that around 15% of the cattle population in the country are crossbred (Jabbar *et al*., 2010). However, this figure differs in certain areas, for example, in milk sheds the proportion of crossbred cows may be more than 80% where targeted milk marketing and input supply systems are operational (Jabbar *et al*., 2005; Uddin *et al*., 2010). There are two common breeds of goats: Black Bengal and Jamunapari (Anon., 2013). The Black Bengal is the outstanding breed for meat and skin production, and is characterized by high prolificacy and disease resistance. It is an important asset for the resource-poor farmers, providing them with animal protein and improved economic security. The sheep of Bangladesh are also indigenous, are kept for mutton and coarse wool, although mutton is not preferred by people (Teufel *et al*., 2010).

3.7.2.3 Contribution of livestock in national economy

The cattle and buffalo in Bangladesh provide power for cropping (traction for ploughing), transport, threshing and oil seed crushing, manure as a source of fuel and fertilizer (increase crop production), a ready source of cash and meat and milk for human consumption. The 30% of the total tillage is still covered by draught animals (DLS, 2014). However, with mechanization and improved rural transport network, need for tillage and transportation has been declining and will decline further over time (Jabbar *et al*., 2010). In Bangladesh, around 8% of total protein (44% of animal protein) for human consumption comes from livestock. Hides and skin of cattle, buffaloes, goats and sheep are valuable export items, ranked third in earnings after ready-made garments and shrimp. About 4.31% of the total export earning comes from leather and leather goods (DLS, 2014). In the year of 2012-2013, the Govt. earned about US$ 534.0 million by exporting livestock products and byproducts (4133.52 crore BD Taka). Another report indicates that livestock plays an important role in the national economy of Bangladesh with a direct contribution of around 3% percent to the agricultural GDP and providing 15% of total employment (Anon., 2007).

Livestock are often one of the main assets of rural households. Although, livestock ownership is often a symbol of wealth, but for many cases, the poor households typically move up the 'livestock ladder'from poultry to goats or sheep, to cattle/buffalo (Deshingkar *et al*., 2008). Livestock (mainly poultry and small ruminants) are an important asset for women because it is often easier for many women in developing countries to acquire livestock assets, whether through inheritance, markets or collective action processes, than it is for them to purchase land or other physical assets or to control other financial assets (Rubin *et al*., 2010; Kristjanson *et al*., 2014). For the poor people, livestock serve as 'piggy banks', to save and store money and manage risk (Herrero *et al*., 2013). As 70% (20% directly and 50% indirectly) of the population rely on livestock to some extent for their livelihood, the poverty reduction potential of the livestock sub-sector is very high (Anon., 2007, 2013; DLS, 2014).

3.7.2.4 Veterinary services and disease surveillance

Inadequate veterinary services are one of the major obstacles for livestock development in Bangladesh. The ratio of Veterinary Surgeons to farm animals and birds was estimated at 1: 1.7 million (Anon., 2007). Department of Livestock Services (DLS) under the Ministry of Fisheries and Livestock, offers rudimentary public goods services and some private goods services. At present, the veterinary services of DLS are dominated by private goods services like treatment of sick animals and birds, production of vaccines and frozen semen for artificial insemination, fodder production, etc. The national livestock development policy recommended DLS to engage increasingly in delivery of public goods services like enforcement of laws and regulations, quality control of feeds, drugs, vaccines, semen and breeding materials, extension services, disease investigation and surveillance, veterinary public health, conservation and development of native breeds, policy formulation and strategy development (Anon., 2007).

The veterinary services are provided through Upazilla (sub-district) Livestock Development Centers (ULDC). The ULDCs also serve as passive disease surveillance centers for livestock in Bangladesh as the limited manpower and fund restrict the ability of the veterinary authorities for active surveillance and the passive surveillance below Upazilla level (i.e., unions and villages). The Upazillas are the lowest administrative level up to which all extension and animal health services of DLS are available. In the meantime, the USAID funded cell phone-web based SMS-(Short Message Service) gateway system for active surveillance of HPAI H5N1 was successful and got popularity at national and international level (Yamage and Ahmed, 2011). It may also be of value for the surveillance of other important animal diseases provided the availability of fund for the sustenance. Recently, a web-based software, namely Livestock Disease Information System (LDIS), has been developed with the support from FAO ECTAD for reporting livestock diseases in Bangladesh, which will enable tracking field cases of all diseases on a daily basis at least at Upazilla level (Mondal and Yamage, 2014). The most common clinical diseases of cattle include parasitic diseases, mastitis and FMD and of small ruminants include PPR in addition to parasitic diseases.

The Veterinary public health section exists in DLS but it is neither equipped nor does it have the funds to deal with disease surveillance and reporting, control of zoonotic diseases, food safety and other public health issues. It does not have a supporting legal framework to implement its mandate (Anon., 2007).

The private sector and NGOs are gradually participating in livestock development activities.

3.7.2.5 Food habit of people and close contact with animals

The possibility of foodborne zoonosis like brucellosis in Bangladesh is very high as the food safety laws are conventional, inadequate and ineffective (Rahman *et al.,* 2014). However, due to the habit of proper boiling and cooking before consumption, the prevalence of infectious foodborne illness may be low.

Being unable to make separate houses for livestock, some poor farmers pose themselves to zoonotic disease risk through sharing same premises with their livestock (mostly small ruminants) (Halder and Barua, 2003).

3.7.2.6 Livestock slaughter for meat

Round the year in Bangladesh, livestock are slaughtered for meat. It is estimated that around 3.5 million cattle are slaughtered annually in the country of which 40% are imported through cross-border illegal trade (Anon., 2002, 2007; Rweyemamu *et al.,* 2008; Ghosh, 2014; Mondal and Yamage, 2014; Khatun *et al.,* 2014). Approximately 15 million goats are also slaughtered annually mostly of local origin. Of the total slaughter of cattle and goats, around 40 percent is performed during Eid-ul-Azha (a muslim festival) (Anon., 2007).

3.7.2.7 Cross-border cattle movement

India has an enormous cattle population (approximating 200 million) used for dairy production and draught purposes; the slaughter of cattle is forbidden (for religious cause) in most Indian provinces and their meat is not consumed (Anon., 2002; Ghosh, 2014). The Indian policy does not allow the export of live animals from India. Despite the restriction, cattle export continues from India to Bangladesh (Ahmed and Khan, 2011). Religious practice in India and Nepal and insufficient beef production in Bangladesh also facilitate market price driven movement of animals (Loth *et al.,* 2011). Bangladesh is almost surrounded by India and shares a poorly patrolled 2,400-mile border through which cattle informally moves into Bangladesh from various parts of northern and northeastern borders of India. It is estimated that up to 25,000 Indian cattle illegally enter into Bangladesh from West Bengal every single day (Ghosh, 2014). Another report indicates that from West Bengal's border district of Murshidabad more than 0.35 million animals are entering into Bangladesh every year (Anon., 2002). Survey results from 407 respondents including 11 Bangladesh land port areas, also support above statements regarding cattle smuggling from India. Clinical diseases like FMD and black

quarter and clinical signs like diarrhea were also reported by the respondents in imported animals. About 98% of the imported cattle come from India and the remaining 2% from Nepal. Almost 89% of the imported bovines are cattle and the rest are buffaloes. Out of 11 land port areas only in Meherpur, young cattle are found to be reared for fattening and in the remaining areas animals are distributed to larger cities for slaughter (Khatun *et al*., 2014). Uncontrolled movement of animal is a potential risk factor for most of the transboundary animal diseases like FMD, PPR, brucellosis, etc. (Rweyemamu *et al*., 2008; Loth *et al*., 2011; Mondal and Yamage, 2014).

The Scientific evidence also supports this fact. For example, analysis of sequence data showed very close relationship of FMD isolates from Bangladesh with FMD serotype O viruses from Nepal and from India (Loth *et al*., 2011). The possibility of introduction of other transboundary diseases like brucellosis in Bangladesh from border countries cannot be ruled out. However, Bangladesh government has approved a project to establish and run 24 quarantine stations at air, land and sea ports (DLS, 2014). If quarantine is applied properly in different ports of entry it will no doubt help to prevent introduction of trasnboundary diseases. However, it will fail to do so in case of informal/illegal import, as usually occurring between India and Bangladesh, where trader and suppliers will not cooperate, which is essential for effective quarantine (Bhuyan and Hasan, 2014).

3.7.2.8 Livestock identification system

The animals and even their herds/flocks are not identified in Bangladesh. It is also an important constraint for research, extension and thereby the livestock development. Animal identification system is also necessary for disease surveillance and control programs. As all people of Bangladesh are not yet identified (without national ID), probably it will take some more time for the Govt. to pay attention to this need.

3.7.3 Animal husbandry practice

3.7.3.1 Production system

Mixed crop-livestock production system is predominant in Bangladesh. In this system, majority of rural population keep cattle, goats, native chickens and ducks together for multiple uses (Tiller *et al*., 2010; Saadullah, 2012). Poor and landless livestock farmers either rear their own or take care of others' livestock as a paid service. Households having single species of livestock are common (Shamsuddin *et al*., 2010). Domestic ruminants live on scavenging roadside and riverbank grasses either freely or under tethering system. Rice gruel and rice straw are provided usually at night.

The small-scale dairy farms consisting of 1-4 cattle are also found all over the country (Uddin *et al*., 2010). Medium (herd size 5-10) and large (herd size >10) scale dairy farms are also found in milk rich pockets like Chittagong, Satkhira, Sirajgoanj, Munshiganj, etc. (Jabbar *et al*., 2005; Shamsuddin *et al*., 2010).

The small-scale dairy system mainly practices zero grazing (cut-and-carry system) with occasional semi-zero and tethering systems (Saadullah, 2001). The medium and large-scale dairy system practices only zero grazing all over Bangladesh with the exception in Pabna-Sirajgonj districts. There the cattle graze freely and remain in the pasture ("Bathan: common grazing land") for about six months (December to May).

3.7.3.2 Feeds and feeding

Crop residues (rice straw), tree leaves, native roadside grasses, household waste, tree fodder are the principal forms of roughage for livestock (Saadullah *et al*., 2012). The rice straw constitutes 80% of the total dry roughage for ruminants. The concentrate feed are cereal grain, rice bran, wheat bran, oil cake, pulse bran, salt, vitamin-mineral premix and molasses but their supply to animals are low, irregular and restricted mostly to milking cows. Poor farmers do not provide concentrates to their animals and depend on natural grass.

3.7.3.3 Breeding practice

With regard to breeding, sporadic and indiscriminate crossbreeding has been practiced with insignificant proportion in livestock species of Bangladesh like buffalo, goat and sheep. As a result, some quasi-indigenous animals have been generated as well but their impact on the total production system is negligible except in the case of cattle. Cattle crossbreeding through AI program has been initiated since 1969 where both tropical and temperate dairy breeds have mainly been used but without following any consistent breeding policy. No doubt, it gradually narrows the genetic diversity of our indigenous genetic resources. Artificial insemination is gradually getting more popularity for breeding cows. In commercial dairy farms more than 80% cows are bred by AI (combining regular and irregular use) (Hossain *et al*., 2005; Jabbar *et al*., 2010). The national coverage of AI is around 36%. The DLS is expanding its coverage by increasing semen production and also by establishing more AI sub-centre/points all over Bangladesh. For example, in 2006, there were 23 district AI centers (including CCBDF), 423 subcenters and 554 AI points. The DLS is increasing the number of AI subcenters/points to 3212. The production of semen in 2007-2008 was 2.31 million doses and in 2012-2013 it has been increased to 3.43 million doses (Anon, 2014; DLS, 2014).

Studies reported that crossbreeding in Black Bengal goat is more intense in the urban and peri-urban areas of North Western region of Bangladesh utilizing several Indian goat breeds like Jamunapari, Sirohi and Beetal. The male mediated introgression is a great threat for Black Bengal goat germplasm and their conservation efforts. The situation is not much worse in case of species buffalo and sheep where crossbreeding practices is negligible. It is notable to mention that yet there is no national body to oversee and control any germ-plasm introduction in this country (Bhuiyan and Bhuiyan, 2014).

Table 3.7a: Reported seroprevalence of brucellosis in cattle in Bangladesh

Year	Area (Serology)	Sample size (positive)	Tests used	Prevalence (95% CI)	References
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)	Islam *et al.*, 2013c; Belal and Ansari, 2013; Dey *et al*., 2013
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*., 2012b
2011	Bagherhatt, Bogra, Gaibandha, Mymensingh and Sirajgong	188 (4)	RBT, iELISA	2.1% (0.6-5.4)	Rahman *et al*., 2011b
2010	Dinajpur, Mymensingh	182 (6)	RBT, iELISA, cELISA	3.3% (1.2-7.0)	Ahasan and Song, 2010
09	Mymensingh	200 (9); 200 (10): 400 (19)	RBT	4.8% (2.9-7.3)	Nahar and Ahmed, 2009; Rahman *et al*., 2009
2006	Mymensingh, Sherpur	300 (7)	TAT	2.3% (0.9-4.7)	Sikder *et al*., 2012
2005	Mymensingh	120 (4)	RBT, PAT, TAT	3.3% (0.9-8.3)	Amin *et al*., 2005
2004	Mymensingh	250 (5)	RBT, PAT, TAT	2.0% (0.7-4.6)	Amin *et al*., 2004
1992	Chittagonj, Comilla, Jessore, Manikgonj	350 (17)	RBT, PAT, TAT	4.9% (2.9-7.7)	Ahmed *et al*., 1992
	Sub-total	2865 (137)		4.8% (4.1-5.7)	
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.

3.7.3.4 Disease control strategy

The only disease control effort of DLS is initiation of vaccination against 16 different types of diseases (like FMD, anthrax, hemorrhegic septicemia, black quarter for cattle, PPR for small ruminants and Newcastle disease, infectious bursal disease, chicken pox, duck cholera, duck plague for poultry) for domestic animals and poultry. The quantity of vaccines produced and delivered by the DLS are inadequate. The government subsidy in vaccine production in present form is a possible obstacle for the private investors. The regulatory authority to check the quality of domestically produced or imported vaccines does not exist (Anon., 2007). Vaccination is done in a haphazard manner without any strategic plan for controlling the targeted diseases. Approximately 112 million doses of vaccine were produced in 2013-2014 for the 349 million livestock and poultry (Anon, 2014).

Vaccination against brucellosis in domestic ruminants has never been initiated in Bangladesh.

Table 3.7b: Reported prevalence of brucellosis in cattle in Bangladesh based on milk ring test

Year	Area (Milk based)	Tested (Positive)	Tests used	Prevalence (95% CI)	References
2012	Chittagong	500 (25)	MRT (Individual milk)	5.0% (3.3-7.3)	Sikder *et al*., 2012
1983	Dhaka, Tangail, Mymensingh	1992 (80)	MRT (Individual milk)	4.2% (3.2-4.9)	Rahman *et al*., 1983
1981	Sirajgonj, Mymensingh, Dhaka	234 (23), 527 (40): 761 (63)	MRT (Indivudal and Bulk/herd milk)	8.3% (6.4-10.75)	Pharo *et al*., 1981; Rahman and Rahman, 1981
1978	Dhaka, Mymensingh, Tangail	490 (42)	MRT (Bulk milk)	8.6% (6.2-11.4)	Rahman *et al*., 1978
	Overall	3743 (210)		5.6% (4.8-6.3)	

Legend: MRT: Milk Ring Test.

3.7.4 Reported prevalence and risk factors of brucellosis in humans and animals

The reported seroprevalence of brucellosis in cattle in Bangladesh is summarized in Tables 3.7a and 3.7b. The seroprevalence seems to be static over the years except in 1970 and 2013. It is not clear what happened in these years with regard to brucellosis. The report of Islam *et al*. (2013c) used RBT reagent procured from South Korea and found 23 (15.3%) seropositive out of 150 cattle. In the same area and in the same year Dey *et al.* (2013) also tested 190 cattle sera but found only 2 (1.1%) positive cases, however, using different test. So, such a significant difference of seroprevalence in cattle of the same area is unusual. The quality of antigen may influence the test result. In addition, the RBT antigen could deteriorate when repeatedly cycled between refrigerator and room temperature during use (MacMillan, 1990). Similarly, the problem with the test may also be the reason for higher prevalence of bovine brucellosis in 1970. The overall seroprevalence of cattle brucellosis is 5.3% (4.8-6.2). The overall prevalence of brucellosis in cows based on MRT is 5.6% (4.8-6.3).

Similarly, the reported seroprevalence of brucellosis in small ruminants is summarized in Table 3.8. The overall seroprevalence of brucellosis in goats and sheep respectively are 2.9% (95% CI: 2.1-4.1) and 5.4% (95% CI: 3.9-7.1). In small ruminants also, the temporal variation of seroprevalence was not noticed. Among the three species of domestic ruminants, the overall seroprevalence of brucellosis is higher in sheep than goats and cattle.

Table 3.8: Reported seroprevalence of brucellosis in goats and sheep in Bangladesh

Year	Area (goats)	Sample size	Tests used	Prevalence (95% CI)	References
2014	Mymensingh, Netrakona	113 (7)	RBT	6.2% (2.5-12.3)	Akhter *et al*., 2014
2012	Bagerhat, Bogra, Gaibandha, Mymensingh, Sirajgonj, Nilphamari	154 (4), 230 (5): 384 (9)	RBT, iELISA, cELISA, FPA	2.3% (1.1-4.4)	Rahman *et al*., 2012c,b
2011	Bagherhatt, Bogra, Gaibandha, Mymensingh and Sirajgong	127 (4); 120 (3): 247 (7)	RBT, iELISA	2.8% (1.1-5.8)	Rahman *et al*., 2011b,a
2010	Dhaka, Mymensingh, Rajshahi	208 (8)	RBT, SAT	3.8% (1.7-7.4)	Islam *et al*., 2010
2007	Dhaka, Mymensingh	300 (6)	RBT, PAT, TAT, MET	2.0% (0.7-4.3)	Uddin *et al*., 2007b
1988	Mymensingh, Tangail, Manikgonj	350 (51)	PAT, TAT	14.5% (11.0-18.7)	Rahman *et al*., 1988
	Overall	1252 (37)		2.9% (2.1-4.1)	
Year	Area (sheep)	Sample size (positive)	Tests used	Prevalence (95% CI)	References
2014	Mymensingh, Netrakona	102 (6); 101 (6): 203 (12)	RBT, iELISA	5.9% (3.1-10.1)	Ahsan *et al*., 2014; Akhter *et al*., 2014
2012	Bagerhat, Bogra, Gaibandha, Mymensingh, Sirajgonj	206 (14); 170 (12); 80 (1): 456 (27)	RBT, iELISA, cELISA, FPA	5.9% (3.9-8.9)	Rahman *et al*., 2011a, 2012b,d
2011	Bagherhatt, Bogra, Gaibandha, Mymensingh and Sirajgong	130 (4)	RBT, iELISA	3.1% (0.8-7.7)	Rahman *et al*., 2011b
2007	Dhaka, Mymensingh	60 (2)	RBT, TAT, PAT	3.3% (0.4-11.2)	Uddin *et al*., 2007a
	Overall	839 (45)		5.4% (3.9-7.1)	

Legend: RBT: Rose Bengal test; SAT: Slow Agglutination Test; MET: 2-Mercaptoethanol Test; cELISA: Competitive ELISA; iELISA: Indirect ELISA; FPA: Fluorescence Polarization Assay; PAT: Plate Agglutination Test; TAT: Tube Agglutination Test.

The reported prevalence of human brucellosis especially in HROG is summarized in Table 3.9. Only four studies so far reported brucellosis seroprevalence in humans. The sample size varied from 50-210. Temporal variation was noted in seroprevalence of human brucellosis over the years. The

seroprevalence of brucellosis up to 1988 and after 2009 differed significantly in humans. The reason is not clear as the seroprevalence in animals are static over the years. The type human sample, tests used and their interpretation may the reasons for such difference.

Commercial production system and abortion were reported to be significantly associated bovine brucellosis seroprevalence (Islam *et al*., 2013b). Pregnancy status in goats and age in sheep were also found to be significantly associated with brucellosis (Islam *et al*., 2013b).

Table 3.9: Reported seroprevalence of human brucellosis in Bangladesh

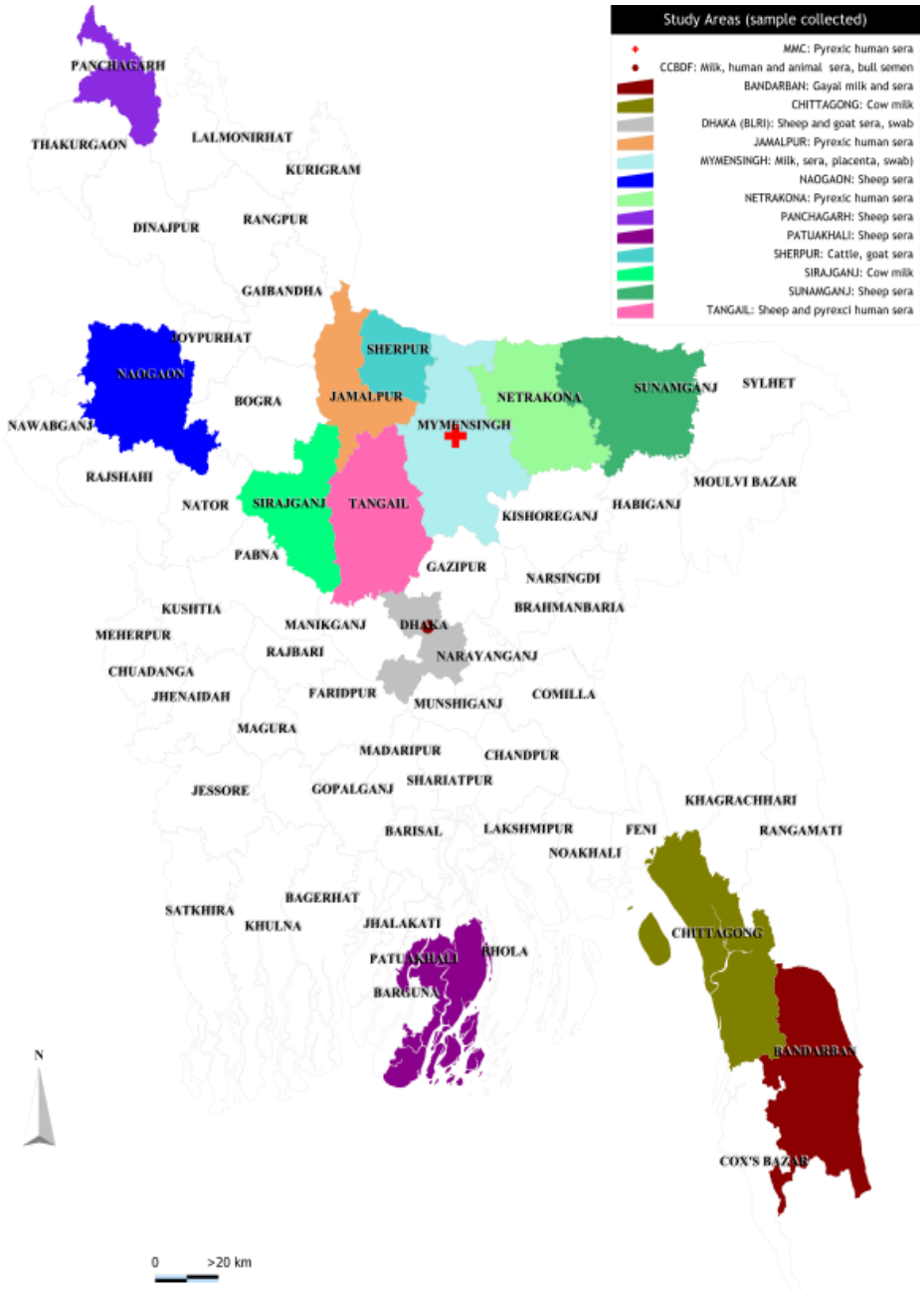
Year	Area	Sample size (Positive)	Sample type	Tests used	Prevalence (95% CI)	Reference
1983	Dhaka, Mymensingh, Tangail	190 (21)	Milker, livestock and crop farmer	RBT, STAT	11.1% (6.9-16.4)	Rahman *et al*., 1983
1988	Tangail, Mymensingh	116 (25)	Goat farmer	TAT	21.6% (14.5-30.1)	Rahman *et al*., 1988
2009	Mymensingh	50 (3)	Livestock farmer	STAT	6.0% (1.3-16.5)	Nahar and Ahmed, 2009
2010	Mymensingh	210 (7)	Livestock farmer, butcher, milker	RBT, STAT	3.3% (1.4-6.7)	Muhammad *et al*., 2010
	Overall	566 (56)			9.9% (7.6-12.7)	

Legend: RBT: Rose Bengal Test; STAT: Standard Tube Agglutination Test; TAT: Tube Agglutination Test.

It is evident from the above review that brucellosis is also endemic in Bangladesh. Several studies on seroprevalence brucellosis in animals and humans and its risk factors in cattle, sheep and goats are available as shown above. Most of the previous studies used non-random sampling and the size of the samples were also very small in some cases like 50, 60 and 80 (Uddin *et al*., 2007b; Nahar and Ahmed, 2009; Rahman *et al*., 2011a). 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 diagnosis of brucellosis in domestic ruminants and humans are imperfect and their performance was not evaluated in Bangladesh context. The true prevalence of brucellosis in domestic ruminants is not known and is essential for analyzing the impact of this disease in domestic ruminants in Bangladesh. Indeed, when diagnostic tests are used without evaluating their performance in a context usually generate unreliable results, which in turn may lead to wrong epidemiological inferences (Godfroid *et al*., 2013). The prevalence of brucellosis in pyrexic patients and risk factors of human brucellosis were not studied. Moreover, the information on different species of *Brucella* prevalent in animals is scarce and not known in humans in Bangladesh (Rahman *et al*., 2014). The epidemiological understanding of brucellosis in domestic ruminants and humans in

Bangladesh is incomplete and sometimes misleading for the decision makers to initiate a control strategy. The figure 3.7.1 shows the study areas and type of samples collected for this study.

Figure 3.7.1 Map of Bangladesh showing study areas and type of samples



Chapter 4

Objectives

The overall objective of this thesis was to investigate the epidemiology of brucellosis in humans and domestic ruminants in Bangladesh in terms of the evaluation of commonly used diagnostic tests, estimation of true prevalence, identification of risk factors and identification of *Brucella* species in order to provide information for appropriate control strategies.

The specific objectives and underlying research questions are:

1. To evaluate the performance of Rose Bengal test (RBT), Slow agglutination test (SAT) and indirect enzyme linked immunosorbent assay (iELISA) for the diagnosis of bovine brucellosis and to estimate the true prevalence of bovine brucellosis (Chapter 5). The research questions addressed under objective 1 are:
    - How do the RBT, SAT and iELISA perform for the diagnosis of bovine brucellosis in Bangladesh?
    - Does the true prevalence of bovine brucellosis vary greatly from the reported apparent prevalences?
    - What is the proportion of cattle infected with acute and chronic brucellosis?
2. To evaluate the performance of RBT, SAT and iELISA for the diagnosis of brucellosis in small ruminants and estimation of true prevalence of small ruminants brucellosis (Chapter 6). The research questions are the same as those in objective1 but for small ruminants.
3. To identify the species of *Brucella* prevalent in human and animals in Bangladesh (Chapter 7). The underlying research question is:
    - Which species of *Brucella* is prevalent in humans and animals in Bangladesh?
4. To study the prevalence and risk factors of brucellosis in high-risk group of people in Bangladesh (Chapter 8). The research questions are:
    - Are the livestock keepers, butchers, dairy hands and veterinarians at high risk to be infected with brucellosis?
    - What are the factors associated with brucellosis in occupationally exposed group of people?
5. To estimate the prevalence of brucellosis in pyretic patients in Bangladesh (Chapter 9). The research question is:
    - Do the pyretic people also suffer from brucellosis?

Chapter 5

Bovine brucellosis

Preamble

There are about 23.4 million heads of cattle in Bangladesh. The study on bovine brucellosis was started in 1970. Since then, several studies focused on the seroprevalence of bovine brucellosis in Bangladesh. However, most of them were based on non-representative and non-random samples. A prevalence study based on non-representative and non-random sample does not reflect true status of that disease in a population. The true prevalence of bovine brucellosis, the proportion of cattle infected with acute and chronic brucellosis and also the performance of commonly used tests are unknown in Bangladesh. This chapter addresses these issues based on Bayesian latent class analysis of three serological test applied on randomly collected cattle blood samples from one district and one government dairy farm.

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Bayesian estimation of true exposure prevalence and characteristics of three serological tests for bovine brucellosis diagnosis in Bangladesh

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Abstract

A Bayesian latent class analysis framework was used to estimate the true prevalence of bovine brucellosis and to evaluate the performance of three conditionally dependent serological diagnostic tests: indirect Enzyme Linked Immunosorbent Assay (iELISA), Rose Bengal Test (RBT) and Slow Agglutination Test (SAT) in Bangladesh. One thousand three hundred and sixty randomly selected cattle sera were tested in parallel from Mymensingh district and one Government (Govt.) owned dairy farm of Bangladesh. Only 0.29% (95% Credible Interval (CI): 0.06-0.86) cattle were acutely infected whereas 0.49% (95% CI: 0.16-1.1) were chronically infected with brucellosis in Mymensingh. On the other hand, in Govt. farm 15.58% (95% CI: 11.89-19.89) cattle were acutely infected with brucellosis and only 3.2% (95% CI: 1.63-5.72) were chronically infected. The estimated true prevalence of brucellosis among cattle of Mymensingh and Govt. dairy farm were 0.3% (95% CI: 0.03-0.7) and 20.5% (95% CI: 16.4-26.3) respectively. The performance of iELISA was best in both Mymensingh and Govt. farm with the sensitivity of 90.5% and 91.3% and specificity of 99.3% and 99.2% respectively. The performance of RBT was better in Mymensingh than Govt. farm with 81.0% and 76.1% sensitivity and 99.0% and 95.6% specificity respectively. Similar to RBT, the performance of SAT was also better in Mymensingh than Govt. farm with 63.5% and 79.7% sensitivity and 98.6% and

95.3% specificity respectively. Through this test validation study, we recommend iELISA with a new cut-off of 5 IU/ml both in chronically (as at Mymensingh) and acutely infected cattle population (as at the Government Farm) for routine screening. We recommend to do nothing for the control of bovine brucellosis under the small-scale dairy and subsistence management system in Bangladesh. However, vaccination should be applied in herds where the prevalence is very high such as at Govt. farm.

Introduction

Bovine brucellosis, an economically important reproductive disease of livestock, is one of the most widespread zoonoses and remains a major public health problem in many developing countries [1]. It is predominantly caused by *Brucella abortus* and induces infertility and delayed heat, leads to loss of calves and interrupts lactation thereby leading to reduction in milk yield.

Brucellosis is endemic in Sub-Saharan Africa, the Middle East, South America, and South East Asia [2]. In Bangladesh, it was reported by [3] that 37% of adult cows were infertile and that brucellosis could play an important role in causing infertility in cows.

The total annual economic loss due to bovine brucellosis in indigenous cows in Bangladesh was estimated at €720,000 and the loss per 1,000 cross-bred cows at €12,000 [4]. The range of reported animal level seroprevalence in cattle of Bangladesh was 0.0%-18.4% [5–15]. Previous serological studies of brucellosis in Bangladesh used the Rose Bengal Test (RBT), Plate Agglutination Test or Standard Tube Agglutination Test (SAT) either alone or in series. As none of these tests is considered to be a gold standard, seroprevalence reported using these tests were apparent prevalence. Moreover, the performance of these tests has not been evaluated in naturally infected cattle of Bangladesh. Using diagnostic tests without evaluating their performance may generate unreliable results which may also lead to wrong epidemiological inferences. The IgG iELISA detects only IgG, whereas RBT detects mainly IgG (also IgM and IgA) and SAT detects mainly IgM (plus also IgG and IgA) antibodies. So, they can be considered to be partially conditionally dependent on each other given the true disease status. The *Brucella* spp. induced humoral IgG immune responses persist after the peak of the response (3-4 weeks post-infection) and remain detectable over long periods of time (up to several years); in contrast, the IgM response is rapidly induced 2-3 weeks after exposure and may disappear after a few months. Considering this fact it can be concluded that, the simultaneous presence of IgM and IgG indicates acute brucellosis, while the presence of IgG alone is an indication of chronic brucellosis. Moreover, a positive response in an agglutination test like SAT, which detects mainly IgM, is not indicative of brucellosis if it is not confirmed by a positive IgG response by iELISA within one week [16]. So, the selection of three tests in this study will also help us to know the stage of infection of brucellosis (acute vs chronic) in cattle.

The performance of a diagnostic test is traditionally evaluated by comparison to a perfect or reference test, i.e. a gold standard test, a diagnostic test with 100% sensitivity and specificity [17]. Isolation and identification of *Brucella* spp. is considered as the "gold standard" for brucellosis diagnosis [18],

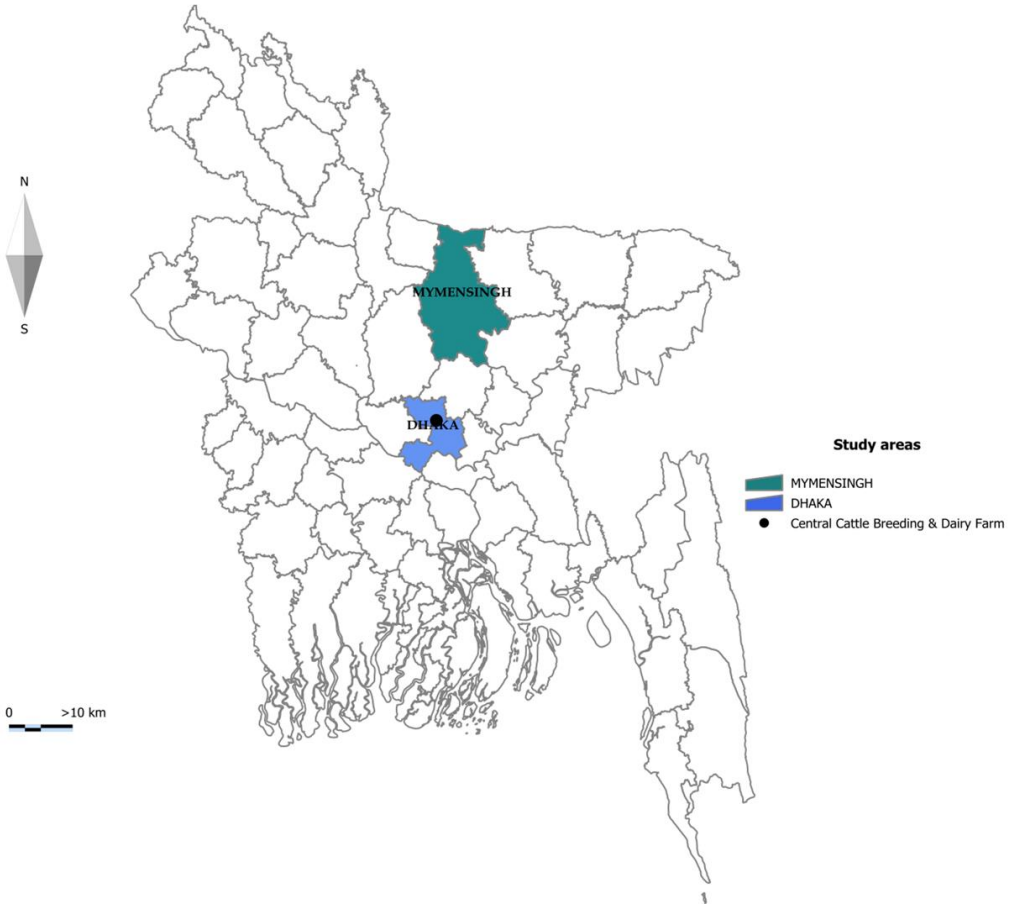
which is difficult to perform in developing countries due to the lack of trained personnel and the requirement of sophisticated laboratory facilities with high level safety containment. Moreover, the sensitivity of the culture method is low [19, 20]. However, there are alternative ways of investigating accuracy of diagnostic tests even in the absence of a gold standard test. Among them, latent class analysis has been increasingly gaining acceptance in veterinary medicine [21, 22]. In this situation, rather than using a single imperfect test, multiple imperfect tests are combined to estimate the true prevalence of antibodies to bovine brucellosis and diagnostic test accuracy parameters [17, 23]. An important consideration in the evaluation of multiple diagnostic tests is whether or not the tests can be assumed conditionally independent of each other given the true disease status. It has been demonstrated that the assumption of conditional independence may lead to biased estimates for test characteristics if in fact the tests are conditionally dependent [24]. As iELISA, RBT and SAT are partially dependent on each other, any estimation procedure should therefore adjust for the dependencies among the tests. Different theoretical frameworks have been developed over the years [21, 25]. Specific publications where authors considered test dependence in a multiple testing strategy for the diagnosis of brucellosis are [26, 27]. At the other end of the spectrum a system allowing estimation without explicit external information exists as well: this approach assumes conditional test independence and constant values for sensitivity and specificity across different populations [28]. In this study, the true exposure prevalence to bovine brucellosis in a population of naturally infected cattle in Mymensingh district and the largest Government (Govt.) dairy farm of Bangladesh was estimated. In addition, the performance of three conditionally dependent serological tests namely indirect ELISA (iELISA), Rose Bengal Test (RBT) and Slow Agglutination Test (SAT) was evaluated using a Bayesian analysis.

Materials and Methods

Study area and animal husbandry practice

The study area included Mymensingh district (MD) and the Government owned Central Cattle Breeding and Dairy Farm (CCBDF) in Savar, located in the Dhaka district of Bangladesh (Figure 5.1). These areas are located between latitudes 23°31’ and 25°12’N and longitudes 90°01’ and 90°47’E. The areas were chosen because of the location of Bangladesh Agricultural University (BAU) which manages the brucellosis diagnostic laboratory and because they have the highest livestock population density (>600 km^{-2}) in Bangladesh. On average the study area has an elevation of 10m above sea level. CCBDF is the largest farm in Bangladesh with major objectives to produce crossbred heifers and bulls for distribution to farmers, to collect semen from tested bulls to support the national artificial insemination (AI) program and to supply milk to Dhaka city. During the last 25 years, this farm maintains on average a herd of about 2500 cattle. Holstein Friesian and Sahiwal breeds are mainly used for semen production. The system of animal management is intensive and only AI is used for reproduction.

Figure 5.1. Map of Bangladesh showing the study areas



The cattle management system in MD is small-scale dairy with traditional crop-based subsistence management systems. The small-scale dairy system mainly practices zero grazing ("cut-and-carry system") with occasional semi-zero and tethering systems. Occasionally when there is no crop in the field, animals of separate owners graze together in villages. Common ration supplements are rice polish, wheat bran and oil cake, but their supply to animals is low, irregular and restricted mostly to milking cows. The common breeds are indigenous and their crosses with Holstein Friesian and Sahiwal.

The study was carried out between September 2007 and August 2008. Vaccination against brucellosis has not been initiated in any livestock species of Bangladesh.

Sampling design

A cross-sectional study was carried out to investigate the seroprevalence of bovine brucellosis in the Mymensingh district of Bangladesh. Since there is no livestock databank in Bangladesh, the first step of the sampling process was the digitisation of the map of Mymensingh district using ArcView Version 3.2 (Environmental Systems Research Institute, Inc. Redlands, California). Out of a total of the 146 unions (sub Upa-Zilla) of MD, 28 were randomly selected. One geographical coordinate was randomly selected from each selected union and located by a hand held GPS reader. Livestock farmers within 0.5 km radius of the selected point were informed about the survey [29]. To encourage livestock farmers to participate, free anthelmintic and vitamin-mineral premix were supplied to their animals when sampling took place and all animals of a selected herd were sampled. Blood samples were collected at CCBDF, including all breeding bulls and systematic random samples of cows (every 10th cow). In addition, a questionnaire designed to collect animal and herd level data was administered during blood sampling of each herd.

Ethics statement

The study protocol was peer reviewed and cleared for ethics by the Faculty of Veterinary Science of Bangladesh Agricultural University (ref. 01/2007/EB/FVS). In this research, farm owned animals were used just once for jugular venepuncture following established techniques. Minimal restraining of the animals was needed during jugular venepuncture and it was mainly done by using a halter. This level of intervention has no impact on the well-being of the animal and is also routinely performed by the veterinarians for the purpose of disease diagnosis, treatment and research. It was not an experimental research on animals and thereby approval by the ethical committee was not needed. Verbal consent of farm owners were obtained prior to the collection of blood samples from their animals. The study took place on the territory of Mymensingh District of Bangladesh and within the confines of the Central Cattle Breeding and Dairy Farm in Dhaka District of Bangladesh. The study did not involve endangered or protected species.

Processing of blood samples

About 5-7 ml of blood was collected from each animal by jugular venipuncture with disposable needles and Venoject tubes, labelled and transported to the laboratory on ice (after clotting) within 12 hours of collection. Blood samples were kept in the refrigerator (2-8°C) in the laboratory and one day later sera were separated by centrifuging at 6000g for 10 minutes. Each serum was labelled to identify the animal and stored at -20°C. Each serum was divided into two tubes each containing about 1-1.5 ml of serum. One aliquot was used for testing and the other was preserved in a serum bank.

Serological tests

All blood samples were tested in parallel using iELISA, SAT and RBT in the Medicine Department laboratory of BAU, Mymensingh, Bangladesh.

iELISA was performed according to [30] using *B. abortus* biotype 1 (Weybridge 99) S-LPS (*Brucella* smooth lipopolysaccharide) as antigen. A detailed description of the method can be found in [31]. The accepted cut-off value for a positive result is 2 IU/ml of test serum [30]. The effect of using a different cut-off value for iELISA (5 IU/ml) was evaluated in one of the statistical models (Model 2, see below). RBT was performed as described by [18]. The detailed procedure was described in a previous paper by [31]. The result was considered positive when agglutination was noticeable after 4 minutes.

SAT was carried out with ethylenediaminetetraacetic acid (EDTA) as described by [32]. The antigen used was *B. abortus* biotype 1 (Weybridge 99) (Synbiotics Europe, France). One hundred and sixty eight micro litre of SAW buffer in the first well and 100 μl in the second and the third wells were added in 96-well microtitre plate. Thirty two micro litre of serum was added in the first well (Dilution 1/6.25). After well mixing of diluent and serum, 100 μl from first well was transferred to the second well (1/12.5). In the same way 100 μl was transferred from second to the third well (dilution 1/25) and 100 μl discarded from the third well. Then in each well, 100 μl of standardised SAW antigen was added giving the serial serum dilutions of 1/12.5; 1/25 and 1/50. The plates were agitated and incubated at 37°C for 20-24h. Reading was done on the basis of degree of agglutination and expressed in international units (IU). Any serum with an antibody titre greater than or equal to 30 IU/ml, as prescribed by the EU [33], was considered positive.

Statistical analysis

A Bayesian analysis framework was used in WinBUGS 1.4 [34] and R 3.1.1 (R Foundation and Statistical Computing, 2014) to estimate the prevalence and the sensitivity and specificity of the three tests. Stata/MP 13.1 [35] was used to compute the ordered logistic regression.

As fully explained in [21], converting the apparent prevalence (laboratory prevalence, seroprevalence) into the so-called true prevalence always requires one to solve a system of overparametrized equations (one test yields one independent equation with three unknown variables, two tests yield three independent equations with seven unknowns variables, ...). This invariably requires the input of

external (prior, independent) information, either in the form of prior estimates of test sensitivity or test specificity, or in the form of some hypothesis, such as conditional independence of tests or constancy of test characteristics across different populations. Several solutions have been proposed, going from the so-called Hui-Walter model [28], based on two conditionally independent diagnostic tests applied in two populations with sensitivity and specificity constant over the two populations, to the fully parametrized models proposed by (e.g.) [36], [25], [37] and [21].

This paper examines two modelling approaches:

The Hui-Walter model [28], using the three test combinations (iELISA/RBT, iELISA/SAT and RBT/SAT) in the two localities (MD and CCBDF). Prevalence and test characteristic estimation is done probabilistically by means of the WinBUGS model shown in S1 Listing 1, which gives the example of the iELISA/RBT combination. The posterior mean and the 95% credibility interval are reported. The general equation is:

$$p_i(t_j) = pr_i \prod_j \left(1^{1-t_j} - (-1)^{t_j} se_j\right) + (1 - pr_i) \prod_j \left(1^{t_j} - (-1)^{1-t_j} sp_j\right)$$

with

$$i \in \{1,2\}\ (\text{locality}); t \in \{0,1\}\ (\text{-ve, +ve}); j \in \{1,2\}\ (\text{test})$$
$$pr = \text{prevalence}; se = \text{sensitivity}; sp = \text{specificity}$$

The prior (external) information for this scheme is strictly deterministic (sensitivity/specificity of second test independent of result of first test and sensitivity/specificity identical in two localities). It reduces the number of parameters to be estimated to six (two prevalences, two sensitivities and two specificities) and it has six independent equations. Because of reasons of symmetry ({p, Se, Sp} and {1-p, 1-Sp, 1-Se} are solutions, see [21] for full details), two of the parameters have to be constrained to the domain [0.5,1].

2. The full model assuming conditional dependence has the implicit characteristic of being over-parametrized (it has seven independent equations and requires fifteen variables to be estimated for each of the two locations). It thus requires explicit external (prior) information for the test characteristics (sensitivity and specificity). An approach, proposed by (*e.g.*) [36] makes use of covariances to model the conditional dependence. The WinBUGS code for the case of MD is shown in S2 Listing 2. The posterior mean and 95% credibility intervals are reported. The general equation is:

$$p(t_I) = pr \cdot \left(\sum_{J|\#J\neq 1} \gamma_J \cdot \prod_i \zeta_J(i) \right) + (1-pr) \cdot \left(\sum_{J|\#J\neq 1} \gamma'_J \cdot \prod_i \xi_J(i) \right)$$

with

$$I = \{1,2,3\}; \forall i \in I \;\; t_i \in \{0,1\} \text{ (-ve, +ve)}; J \in \mathcal{P}(I)$$
$$\gamma_J = cov_{se\ldots}; \gamma'_J = cov_{sp\ldots}; \gamma_\varnothing = \gamma'_\varnothing = 1$$
$$\zeta(i) = \begin{cases} -(-1)^{t_i} & \forall i \in J \\ t_i \cdot se_i + (1-t_i)\cdot(1-se_i) & \forall i \notin J \end{cases}$$
$$\xi(i) = \begin{cases} -(-1)^{1-t_i} & \forall i \in J \\ (1-t_i)\cdot sp_i + t_i \cdot (1-sp_i) & \forall i \notin J \end{cases}$$
$$pr = \text{prevalence};\ se = \text{sensitivity};\ sp = \text{specificity}$$

Prior information was obtained from a meta-analysis (see further) with the extra prior condition that the most likely value for the iELISA specificity was to exceed 99%, hence a very narrow prior beta distribution was used. Two cut-off values were tested: 2 IU/ml and 5 IU/ml.

Model selection proceeded according to the method described in [21], making use of *DIC*, *pD* and *Bayes-p*. All models were run with a burn-in period of 50,000 iterations and estimates were based on a further 50,000 iterations using three chains. The external (prior) information was generated by means of a meta-analysis, carried out in Stata 13.1 [35], using the results published in the following references: (iELISA) [38], [39], [40], [41], [42], [43]; (RBT) [38], [41], [43], [44], [45], [46]; (SAT) [38], [43], [47], [48]. The choice of priors was driven by the aim to get as diverse as possible a range of field conditions, even if that meant that the actual status of the animals was not always clear. Note that this will result in higher uncertainty in the priors –*i.e.* wider distributions–, giving more weight to the data. The exception was the iELISA specificity, as explained above, where a narrow prior distribution was used.

Results

Descriptive statistics

Mymensingh district: A total of 1,020 cattle were subjected to the three serological tests in MD. About 86.3% and 70.1% of the cattle in MD were indigenous and female respectively. The mean age was 3.72±0.09 years (mean±standard error) and ranged from 0.03 to 15 years. The average body weight of cattle was 72.2±1.82 with a median of 75 Kg. About 73.8% of the herds consisted of one to three cattle. Only one herd had more than 10 cattle in MD. The herd size ranged from 1 to 11 with a median of 2 animals.

Government dairy farm: In the CCBDF herd, 340 sera samples (including 89 from breeding bulls) were tested by the three serological tests. The average age of cattle was 5.3 ±0.21 years and ranged from 0.03 to 17 years. About 87.6% and 64.1% of the GF cattle were cross-bred and female respectively. The average body weight of cattle was 256.83±10.43 with a median of 200 Kg.

Serological results

Table 5.1 shows the numbers of animals that tested positive in the three tests in function of the iELISA cut-off value. The following summary is valid for an iELISA cut-off value of 5 IU/ml. Only 6.1% (22/362) herds from MD were serologically positive in at least one of the three tests (one animal and 2 animals positive per herd respectively in 19 and 3 herds). Only 0.29% (3/1020) (95% Credible Interval (CI): 0.06-0.86) cattle were acutely infected whereas 0.49% (95% CI: 0.16-1.1) were chronically infected in Mymensingh. About 2.2% (95% CI: 1.4-3.2) cattle were positive in at least one serological test in MD. The apparent prevalence were 0.4% (95% CI: 0.1-1.0) based on iELISA, 0.9% (0.4-1.7) based on RBT and 1.3% (95% CI: 0.7-2.2) based on SAT. About 22.6% (95% CI: 18.3-27.5) cattle were positive in at least one serological test in Govt. dairy farm. In CCBDF, about (53/340) 15.58% (95% CI: 11.89-19.89) cattle were acutely infected with brucellosis and only (11/340) 3.2% (95% CI: 1.63-5.72) cattle were chronically infected. The apparent prevalence were 17.6% (95% CI: 13.7-22.1) based on iELISA, 18.2% (14.3-22.8) based on RBT and 19.7% (15.6-24.3) based on SAT respectively.

Table 5.1. Cross-classified test results for brucellosis in cattle in Mymensingh district and Government Dairy Farm of Bangladesh. Results are based on iELISA (cut-off = 2 IU/ml and cut-off = 5 IU/ml), RBT and SAT

iELISA	RBT	SAT	Mymensingh		Government Farm	
			2 IU/ml	5 IU/ml	2 IU/ml	5 IU/ml
1	1	1	2	1	51	47
1	1	0	6	1	3	2
1	0	1	6	2	7	6
1	0	0	137	5	53	11
0	1	1	0	1	8	12
0	1	0	1	6	0	1
0	0	1	5	9	0	12
0	0	0	863	995	217	259
		Total	1020	1020	340	340

Legend: iELISA =indirect Enzyme-Linked Immuno Sorbent Assay; RBT =Rose Bengal Test; SAT =Slow Agglutination Test; 1/0 =positive/negative test result.

Meta-analysis

Table 5.2 summarises the results of the meta-analysis and the corresponding parameters for the respective prior beta distributions.

Table 5.2. Summary values of the meta-analysis estimation of test characteristics and corresponding beta distribution parameters

	Sensitivity		Specificity		Sensitivity		Specificity	
Test	Mean	95% CI	Mean	95% CI	shape1	shape2	shape1	shape2
iELISA	93.9	86.9–97.2	99.8	99.1–99.9	85.00	6.4867	750.00	2.6544
RBT	91.0	70.6–97.7	99.6	84.3–99.9	18.80	2.7583	22.60	1.0837
SAT	82.6	27.8–98.3	99.7	97.4–99.9	3.45	1.5142	190.00	1.6483

Legend: iELISA =indirect Enzyme-Linked Immuno Sorbent Assay; RBT =Rose Bengal Test; SAT =Slow Agglutination Test; shape1, shape2 =first and second shape parameter of the respective beta distribution.

Hui-Walter Model

The estimates obtained from the application of the Hui-Walter model (iELISA cut-off at 2 IU/ml and 5 IU/ml) for the three possible combinations of two tests are shown in Table 5.3. The increase in cut-off (as per definition) results in an increase of the iELISA specificity and a drop in the estimated prevalence. When iELISA is in the combination (HW_{ER} and $HW_{ES)}$ there is also an increase in *pD* and a drop in *DIC*, indicating a better fit (Table 5.4, *e.g.* in the case of HW_{ER} the *pD* increases from 4.89 to 5.39 and the *DIC* decreases from 39.15 to 36.37, whereby this decrease is due almost entirely for MD, namely 18.32 to 15.76, the value for CCBDF remaining virtually constant around 20.5).

Table 5.3. Estimated values for true prevalence and test characteristics (and their 95% credibility intervals) using the Hui-Walter Model for Mymensingh district and the Government Farm, when setting the iELISA cut-off at respectively 2 IU/ml (a) and 5 IU/ml (b) (a) iELISA cut-off = 2 IU/ml

(a) iELISA cut-off= 2IU/ml								
Model	PrevM	PrevG	iELISA		RBT		SAT	
			Se	Sp	Se	Sp	Se	Sp
HW_{ER}	0.014 (0.006-0.025)	0.267 (0.197-0.339)	0.872 (0.782-0.943)	0.861 (0.837-0.883)	0.681 (0.532-0.866)	0.998 (0.994-0.999)		
HW_{ES}	0.013 (0.005-0.023)	0.269 (0.199-0.342)	0.867 (0.778-0.938)	0.860 (0.837-0.882)			0.729 (0.569-0.915)	0.998 (0.994-0.999)
HW_{RS}	0.003 (0.001-0.008)	0.195 (0.153-0.240)			0.907 (0.809-0.984)	0.993 (0.987-0.997)	0.959 (0.886-0.998)	0.989 (0.981-0.994)
(b) iELISA cut-off= 5IU/ml								
Model	PrevM	PrevG	iELISA		RBT		SAT	
HW_{ER}	0.005 (0.001-0.012)	0.235 (0.187-0.288)	0.799 (0.681-0.901)	0.993 (0.987-0.998)	0.748 (0.629-0.854)	0.993 (0.986-0.998)		
HW_{ES}	0.006 (0.002-0.014)	0.234 (0.186-0.285)	0.808 (0.693-0.909)	0.994 (0.988-0.999)			0.807 (0.694-0.902)	0.990 (0.983-0.996)
HW_{RS}	0.003 (0.001-0.008)	0.195 (0.153-0.240)			0.907 (0.809-0.984)	0.993 (0.987-0.997)	0.959 (0.886-0.998)	0.989 (0.981-0.994)

Legend: iELISA =indirect Enzyme-Linked Immuno Sorbent Assay; RBT =Rose Bengal Test; SAT =Slow Agglutination Test; PrevM =true prevalence MD; PrevG =true prevalence CCBDF herd; Se =sensitivity; Sp =specificity; HW = Hui-Walter; E = iELISA; R = RBT; S = SAT.

Table 5.4. Values of DIC and pD for Hui-Walter models at two iELISA cut-off values

Model	iELISA with cut-off=2 IU/ml				iELISA with cut-off=5 IU/ml			
	DIC_M	DIC_G	DIC_{tot}	p_D	DIC_M	DIC_G	DIC_{tot}	*pD*
ER	18.3	20.9	32.2	4.89	15.8	20.6	36.4	5.39
ES	19.7	21.2	40.9	5.51	16.4	20.4	36.8	5.44
RS	16.1	18.1	34.2	4.84	16.1	18.1	34.2	4.84

Legend: iELISA = indirect Enzyme-Linked Immuno Sorbent Assay; DIC_M = Deviance Information Criterion at MD; DIC_G = Deviance Information Criterion at CCBDF; DIC_{tot} = total Deviance Information Criterion; p_D = effective number of parameters estimated; ER = combination indirect Enzyme-Linked ImmunoSorbent Assay and Rose Bengal Test; ES = combination between indirect Enzyme-Linked Immuno-Sorbent Assay and Slow Agglutination Test; RS = combination between Rose Bengal Test and Slow Agglutination Test.

Conditional dependence model

The values of the various statistics (*DIC*, *pD* and *Bayes-p*) using two different cut-off values for iELISA (2 IU/ml and 5 IU/ml) are summarised in Table 5.5. The estimates of the prevalence and test characteristics for Mymensingh and Government Farm using an iELISA cut-off of 5 IU/ml are shown in Table 5.6.

Table 5.5. Cut-off selection for iELISA at Government Farm and Mymensingh

		Unconstrained				Constrained	
Locality	Cut-off	*DIC*	*pD*	*Bayes-p*	*DIC*	*pD*	*Bayes-p*
Govt. Farm	2	37.98	4.84	0.6394	40.14	4.35	0.7704
	5	37.82	5.29	0.5123	37.85	4.96	0.5088
Mymensingh	2	35.87	4.58	0.6320	56.19	4.04	0.9969
	5	32.47	4.56	0.5341	31.20	4.50	0.4429

Legend*:* p_D = effective number of parameters estimated; *DIC* = Deviance Information Criterion; *Bayes-p*=Bayesian p-value

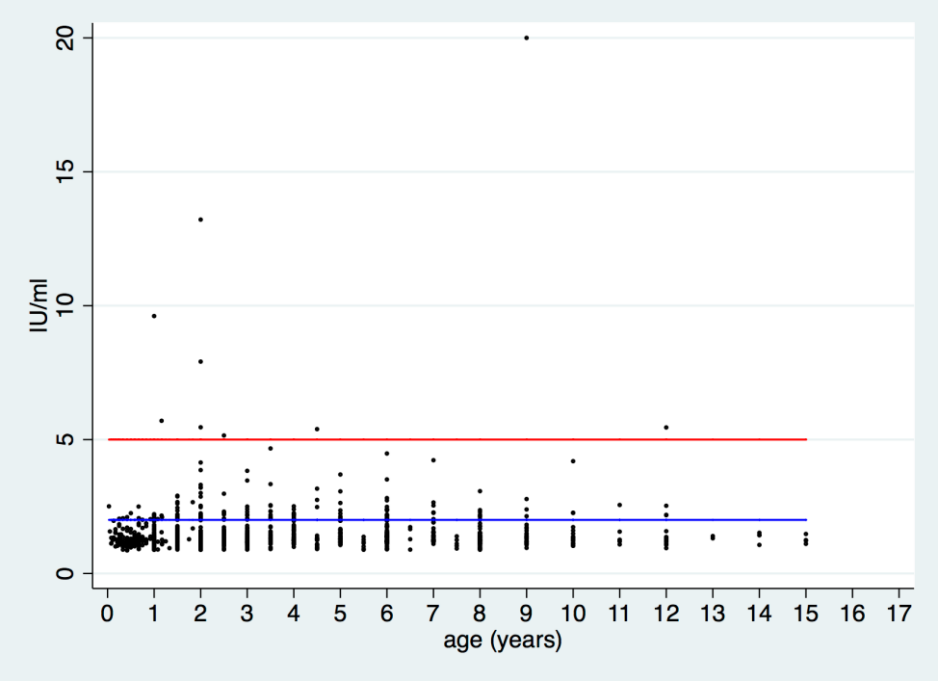
Table 5.6. Variable estimates (prevalence and test characteristics at Government Farm and Mymensingh) when putting iELISA cut-off at 5 IU/ml

Locality	Prevalence	iELISA		RBT		SAT	
		Se	Sp	Se	Sp	Se	Sp
Govt. Farm	0.205	0.913	0.992	0.761	0.956	0.797	0.953
Mymensingh	0.003	0.905	0.993	0.810	0.990	0.635	0.986

Legend: iELISA= indirect Enzyme-Linked Immuno Sorbent Assay; RBT =Rose Bengal Test; SAT =Slow Agglutination Test; Se=Sensitivity; Sp=Specificity

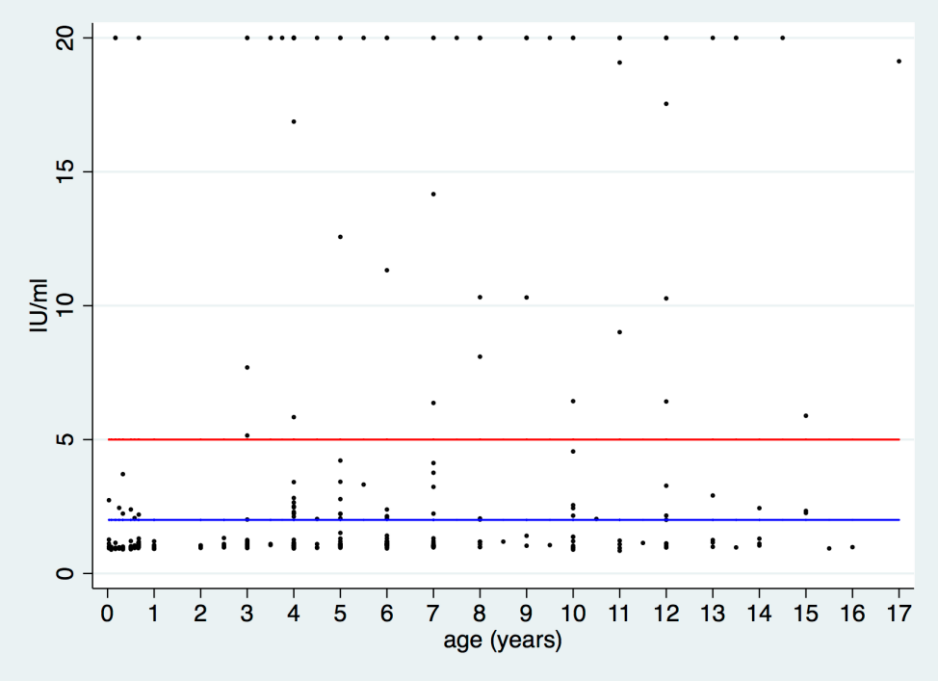
The distribution of the iELISA values (with a ceiling of 20 IU/ml) in function of age of the animals at MD and CCBDF are shown respectively in Figure 5.2 and Figure 5.3. The predictions of the ordered logistic regression of the serological classification (0 = negative [ELISA < 2 IU/ml]; 1 is false positive [2 ~ ELISA < 5 IU/ml]; 2 is true positive [5 IU/ml < ELISA]) in function of age at the two localities are presented in Figure 5.4 and Figure 5.5.

Figure 5.2. iELISA IU/ml values in function of age of the animal at Mymensingh



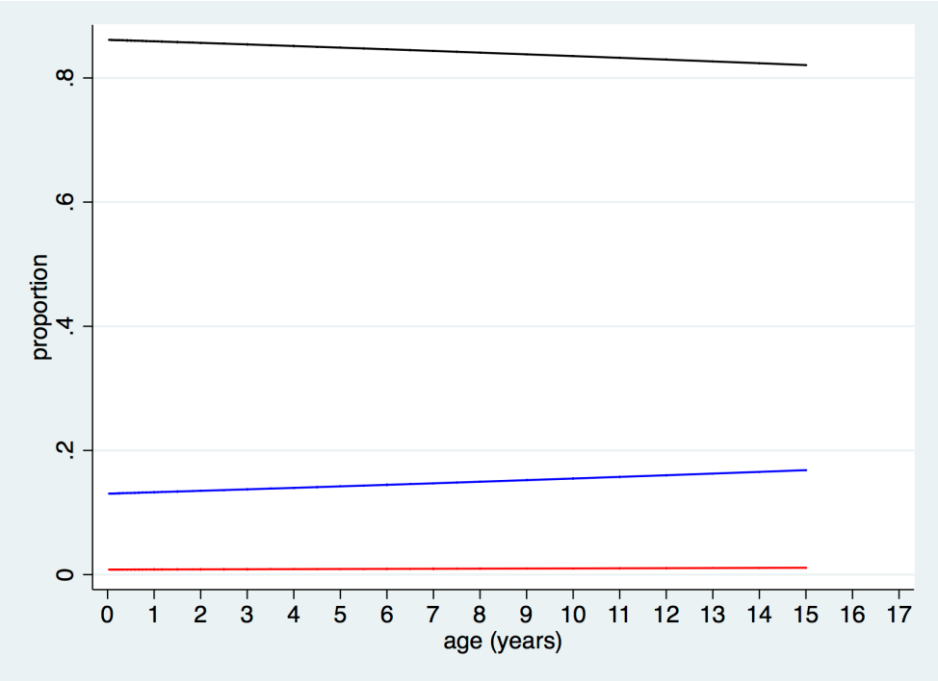
Legend: horizontal blue line and red line indicate the cut-off of 2 IU/ml and 5 IU/ml, respectively.

Figure 5.3. iELISA IU/ml values in function of age of the animal at Government Farm



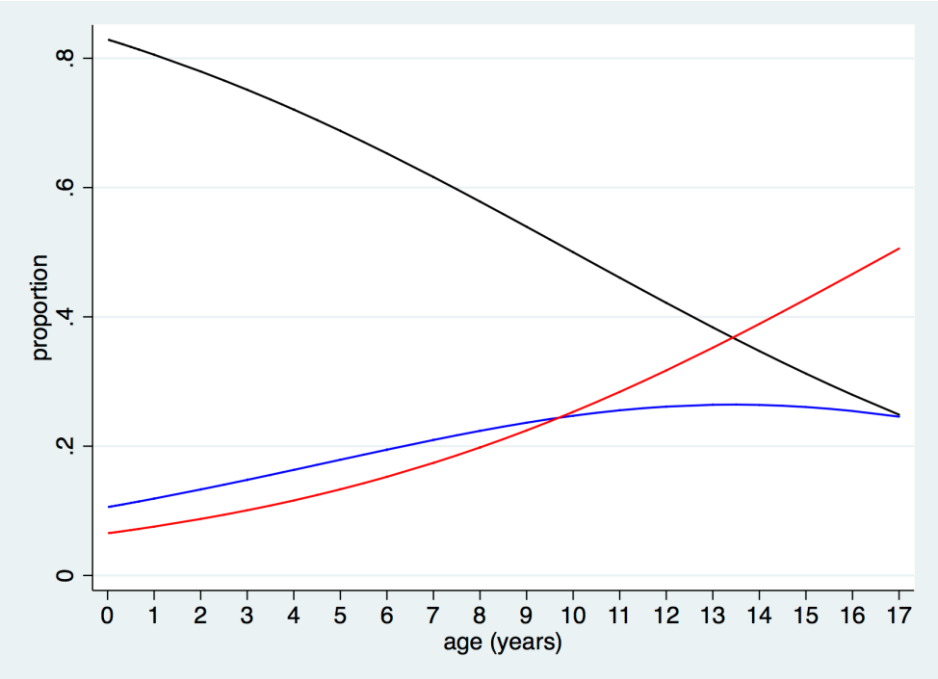
Legend: horizontal blue line and red line indicate the cut-off of 2 IU/ml and 5 IU/ml, respectively.

Figure 5.4. iELISA serological class in function of age at Mymensingh: predictions from the ordered logistic regression



Legend: Black line, proportion animals with ELISA < 2 IU/ml (Negative); Blue line, proportion animals with 2 ≤ ELISA < 5 IU/ml (False positive); Red line, proportion animals with ELISA ≥ 5 IU/ml (True positive).

Figure 5.5. iELISA serological class in function of age at Government Farm: predictions from the ordered logistic regression



Legend: Black line, proportion animals with ELISA < 2 IU/ml (Negative); Blue line, proportion animals with 2 ≤ ELISA < 5 IU/ml (False positive); Red line, proportion animals with ELISA ≥ 5 IU/ml (True positive).

Discussion

This study estimated the true prevalence of brucellosis applying parallel multiple tests on blood samples from cattle in Bangladesh. The true seropositive status of a disease is an essential piece of information for decision makers prior to establishing prevention and control measures. Through this study, prevalence of brucellosis in cattle of MD and CCBDF of Bangladesh were updated to 0.3% (95% CI: 0.03-0.7) and 20.5% (95% CI: 16.4-26.3) respectively through a Bayesian analysis framework. The estimated true prevalence of brucellosis in MD is much lower than the previous reports 0.0%-18.4% [5–15] of apparent prevalences from Bangladesh. The smaller size and non-randomness of the samples, types of test used, differences in management system prevailing in different parts of Bangladesh may be responsible for the great variation in apparent prevalences in earlier studies. In Bangladesh, indigenous cattle are reared in subsistence/backyard management system whereas in commercial management system mostly cross-bred cattle are maintained. The prevalence of brucellosis is reported to be significantly higher in commercial production system [15]. This is also supported by our data that about 86.3% cattle of MD are indigenous. However, the prevalence in the largest CCBDF exceeds the upper limit of the previous prevalence reports. Farmers are aware of the disease and the cows having signs suggestive of brucellosis such as anoestrous, repeat breeding syndrome, retained placenta and abortions are usually sold to butchers. Moreover around 3.5 million cattle are slaughtered annually in the country and about 40% of them are performed during the festival of Eid-ul-Azha [49]. During this mass slaughter, the animals infected with brucellosis may be removed from the population, partially explaining the very low prevalence in the subsistence management system such as in MD. CCBDF does not represent the cattle population of Bangladesh even those of the privately managed larger farms. The disease management system, especially with respect to reproduction, is better in private farms than that at CCBDF. The prevalence structure obtained in MD will represent brucellosis status in cattle of other districts especially where small-scale dairy and subsistence/backyard management system prevails. Higher brucellosis prevalence was observed at CCBDF than in MD. Several reasons may be responsible for this observation, including larger herd size, irregularly/not testing cattle, high proportion of cross-breed cattle, new introduction of animals in the herd from local and international market without proper testing and sole use of artificial insemination (AI) practice at CCBDF. CCBDF also maintains breeder bulls for the production and dissemination of frozen semen throughout the country for AI. Breeder bulls of CCBDF were also found to be brucellosis positive and after reporting removed from semen production (Unpublished data of the same authors).Although the study of brucellosis started before liberation from Pakistan [5], no progress has been made regarding control of this disease at national level in Bangladesh. Estimation of the true prevalence along with its true credible interval may help decision makers to quantify the impact of this disease in bovines of Bangladesh. The average herd level apparent prevalence of brucellosis observed in the MD of Bangladesh was around 2.33% (Unpublished data of the same authors). It indicates that with a subsistence management system

brucellosis may be naturally controlled without any directed control measures. With such a low prevalence of brucellosis, both at herd and animal level, test and slaughter policies of control may be implemented [50,51]. But considering the poor socioeconomic status of Bangladesh, we recommend to do nothing for further control of bovine brucellosis under small-scale dairy and subsistence management system. But, vaccination should be initiated to reduce the prevalence of brucellosis in herds where the prevalence is very high as in the largest govt. farm. Information on the performance of diagnostic tests is needed by clinicians, decision-makers in the context of clinical diagnoses or quantitative risk assessments as well as for prevalence estimation or risk-factor studies [52]. This study is the first to validate three serological tests for the diagnosis of bovine brucellosis in Bangladesh. Latent class evaluation of diagnostic tests is increasingly gaining acceptance in veterinary medicine with few studies noted in the literature for brucellosis diagnosis [46, 53]. According to the results of our study, the iELISA yielded the highest sensitivity and specificity in both MD and CCBDF. This was the case when we increased the cut-off to 5 IU/ml. Both validation by the Hui-Walter model and the full conditional dependence model yielded a better fit when using a cut-off of 5 IU/ml than when using 2 IU/ml as cut-off (expressed in terms of DIC and pD, Table 4 and Table 5). Based on these results, we recommend only iELISA with a cut-off value of 5 IU/ml for routine screening of bovine brucellosis in Bangladesh context. The iELISA test kits provided by different companies have different cut-offs and thereby it is difficult to compare the results of those test kits. To avoid false positives an appropriate cut-off should be determined under local conditions. The stage and duration of disease affect sensitivity of a diagnostic test and they are thought to be different in low and high-prevalence population [52]. As the prevalence of acute brucellosis is higher at CCBDF, the sensitivity of SAT (which detect mainly IgM produced in acute stage of infection) at CCBDF (79.7%) is also higher than that in MD (63.5%). Similarly, since the prevalence of chronic infection is relatively higher in MD, the sensitivity of IgG detecting (indicate chronic infection) tests gradually increases (SAT-RBT-iELISA: 63.5-81.0-90.5%). The decreased specificity of RBT and SAT at CCBDF might be due to presence of some false positive reactions, as we did not change their reading or cut-off. It is supported by our data also. In Table 1, twelve cases positive in both RBT and SAT, one case positive in RBT only and two cases positive in SAT only may be false positive reactions. Similarly, the relatively lower specificity of SAT in MD may also be due to the presence of false positive reactions (in Table 1 there are nine cases positive only in SAT). The iELISA titre declined significantly with the age of the cattle, which is a normal phenomenon of humoral immune response. The IgG response reaches peak after 3-4 weeks post-infection and stays detectable over long periods of time [16]. So, with the age of cattle the IgG titers should decline normally unless re-exposure to *Brucella* occurs.

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Supporting Information - Listing 1: WinBUGS code for the Hui-Walter model

```
list(r1=c(8,143,1,868), n1=1020, r2=c(54,60,8,218), n2 = 340)
# model
Model{
r1[1:4] ~ dmulti(p1[1:4], n1)
p1[1] <- prev[1]*se[1]*se[2] + (1-prev[1])*(1-sp[1])*(1-sp[2])
p1[2] <- prev[1]*se[1]*(1-se[2]) + (1-prev[1])*(1-sp[1])*sp[2]
p1[3] <- prev[1]*(1-se[1])*se[2] + (1-prev[1])*sp[1]*(1-sp[2])
p1[4] <- prev[1]*(1-se[1])*(1-se[2]) + (1-prev[1])*sp[1]*sp[2]
r2[1:4] ~ dmulti(p2[1:4], n2)
p2[1] <- prev[2]*se[1]*se[2] + (1-prev[2])*(1-sp[1])*(1-sp[2])
p2[2] <- prev[2]*se[1]*(1-se[2]) + (1-prev[2])*(1-sp[1])*sp[2]
p2[3] <- prev[2]*(1-se[1])*se[2] + (1-prev[2])*sp[1]*(1-sp[2])
p2[4] <- prev[2]*(1-se[1])*(1-se[2]) + (1-prev[2])*sp[1]*sp[2]
prev[1] ~ dbeta(1,1)
prev[2] ~ dbeta(1,1)
se[1] ~ dbeta(1,1)
sp[1] ~ dbeta(1,1)
se[2] ~ dunif(0.5,1)
sp[2] ~ dunif(0.5,1)
r3[1:4] ~ dmulti(p1[1:4], n1)
for (i in 1:4) {
  d1[i] <- r1[i]*log(max(r1[i],1)/(p1[i]*n1))
  d2[i] <- r3[i]*log(max(r3[i],1)/(p1[i]*n1))
}
G01 <- 2*sum(d1[])
Gt1 <- 2*sum(d2[])
bayesp[1] <- step(G01 - Gt1)
r4[1:4] ~ dmulti(p2[1:4], n2)
for (i in 1:4) {
  d3[i] <- r2[i]*log(max(r2[i],1)/(p2[i]*n2))
  d4[i] <- r4[i]*log(max(r4[i],1)/(p2[i]*n2))
}
G02 <- 2*sum(d3[])
Gt2 <- 2*sum(d4[])
bayesp[2] <- step(G02 - Gt2)
bayesp[3] <- step(G01 + G02 - Gt1 - Gt2)
}
```

Supporting Information - Listing 2: WinBUGS code for the full model. Conditional dependence is modelled by means of covariances

```
list(r = c(1,1,2,5,1,6,9,995), n = 1020)
# model
model {
r[1:8] ~ dmulti(p[1:8], n)
prob_se[1] <-  se[1] * se[2] * se[3] + se[1] * a[3] + se[2] * a[2] + se[3] * a[1] + a[4]
prob_se[2] <-  se[1] * se[2] * (1 - se[3]) - se[1] * a[3] - se[2] * a[2] + (1 - se[3]) * a[1] - a[4]
prob_se[3] <-  se[1] * (1 - se[2]) * se[3] - se[1] * a[3] + (1 - se[2]) * a[2] - se[3] * a[1] - a[4]
prob_se[4] <-  se[1] * (1 - se[2]) * (1 - se[3]) + se[1] * a[3] - (1 - se[2]) * a[2] - (1 - se[3]) * a[1] + a[4]
prob_se[5] <- (1 - se[1]) * se[2] * se[3] + (1 - se[1]) * a[3] - se[2] * a[2] - se[3] * a[1] - a[4]
prob_se[6] <- (1 - se[1]) * se[2] * (1 - se[3]) - (1 - se[1]) * a[3] + se[2] * a[2] - (1 - se[3]) * a[1] + a[4]
prob_se[7] <- (1 - se[1]) * (1 - se[2]) * se[3] - (1 - se[1]) * a[3] - (1 - se[2]) * a[2] + se[3] * a[1] + a[4]
prob_se[8] <- (1 - se[1]) * (1 - se[2]) * (1 - se[3]) + (1 - se[1]) * a[3] + (1 - se[2]) * a[2] + (1 - se[3]) * a[1] - a[4]
prob_sp[8] <-  sp[1] * sp[2] * sp[3] + sp[1] * b[3] + sp[2] * b[2] + sp[3] * b[1] + b[4]
prob_sp[7] <-  sp[1] * sp[2] * (1 - sp[3]) - sp[1] * b[3] - sp[2] * b[2] + (1 - sp[3]) * b[1] - b[4]
prob_sp[6] <-  sp[1] * (1 - sp[2]) * sp[3] - sp[1] * b[3] + (1 - sp[2]) * b[2] - sp[3] * b[1] - b[4]
prob_sp[5] <-  sp[1] * (1 - sp[2]) * (1 - sp[3]) + sp[1] * b[3] - (1 - sp[2]) * b[2] - (1 - sp[3]) * b[1] + b[4]
prob_sp[4] <- (1 - sp[1]) * sp[2] * sp[3] + (1 - sp[1]) * b[3] - sp[2] * b[2] - sp[3] * b[1] - b[4]
prob_sp[3] <- (1 - sp[1]) * sp[2] * (1 - sp[3]) - (1 - sp[1]) * b[3] + sp[2] * b[2] - (1 - sp[3]) * b[1] + b[4]
prob_sp[2] <- (1 - sp[1]) * (1 - sp[2]) * sp[3] - (1 - sp[1]) * b[3] - (1 - sp[2]) * b[2] + sp[3] * b[1] + b[4]
prob_sp[1] <- (1 - sp[1]) * (1 - sp[2]) * (1 - sp[3]) + (1 - sp[1]) * b[3] + (1 - sp[2]) * b[2] + (1 - sp[3]) * b[1] -
b[4]
for (i in 1:8) {
  p[i] <- pr * prob_se[i] + (1 - pr) * prob_sp[i]
}
pr ~ dunif(0,1)
se[1] ~ dbeta(85,6.4867)
se[2] ~ dbeta(18.8,2.7583)
se[3] ~ dbeta(3.45, 1.5142)
sp[1] ~ dbeta(750,2.6544)
sp[2] ~ dbeta(22.6,1.0837)
sp[3] ~ dunif(0,1)
for (i in 1:4) {
  a[i] ~ dunif(-1, 1)
  b[i] ~ dunif(-1, 1)
}
r2[1:8] ~ dmulti(p[1:8],n)
for (i in 1:8) {
```

```
  ## p > 0
  constraint1[i] <- step(p[i])
  O1[i] ~ dbern(constraint1[i])
  O1[i] <- 1
  ## p < 1
  constraint2[i] <- step(p[i] - 1)
  O2[i] ~ dbern(constraint2[i])
  O2[i] <- 0
  ## prob_se > 0
  constraint3[i] <- step(prob_se[i])
  O3[i] ~ dbern(constraint3[i])
  O3[i] <- 1
  ## prob_se < 1
  constraint4[i] <- step(prob_se[i] - 1)
  O4[i] ~ dbern(constraint4[i])
  O4[i] <- 0
  ## prob_sp > 0
  constraint5[i] <- step(prob_sp[i])
  O5[i] ~ dbern(constraint5[i])
  O5[i] <- 1
  ## prob_sp < 1
  constraint6[i] <- step(prob_sp[i] - 1)
  O6[i] ~ dbern(constraint6[i])
  O6[i] <- 0
  d[i] <- r[i]*log(max(r[i],1)/(p[i]*n))
  d2[i] <- r2[i]*log(max(r2[i],1)/(p[i]*n))
}
bayesp <- step(sum(d[]) - sum(d2[]))
}
# Initial values of variable nodes for three chains
list(pr = 0.5,
    se = c(0.93, 0.77, 0.22),
    sp = c(0.95, 0.99, 0.7),
    a  = c(0.0189, -0.0196, -0.0594, 0.001134),
    b  = c(0.0045, 0.01, 0.002, -0.0004),
    r2 = c(44,2,5,2,15,1,3,268))
list(pr = 0.5,
    se = c(0.965 , 0.835 , 0.6),
    sp = c(0.975 , 0.995 , 0.85),
    a  = c(0.011725, 0.0035, 0.0165, -0.000595),
```

```
    b  = c(0.002375, 0.00875, 0.00175, -0.0007875),
    r2 = c(44,2,5,2,15,1,3,268))
list(pr = 0.5,
    se = c(0.999, 0.9, 0.983),
    sp = c(0.999, 0.999, 0.999),
    a  = c(0.0004, 0.000483, 0.0068, -0.0001864),
    b  = c(0.000499, 0.000499, 0.000499, -0.000248502),
    r2 = c(44,2,5,2,15,1,3,268))
```

Chapter 6

Brucellosis in small ruminants

Preamble

There are almost 28.3 million heads of small ruminants in Bangladesh of which 89.0% are goats. Small ruminants are reared mostly by poor farmers and sometimes share same premises with animals exposing them to the risk of zoonosis. Similar to bovine brucellosis, some studies reported apparent prevalence of brucellosis in small ruminants in Bangladesh. The true prevalence of brucellosis in these two species, the performance of serological tests for the diagnosis of brucellosis in small ruminants were not studied. The information about true prevalence and characteristics of commonly used diagnostic test will help to know impact of this disease in goats and sheep populations. This chapter describes the true status of brucellosis along with the sensitivity and specificity of three serological tests for the diagnosis of brucellosis in goats and sheep using Bayesian latent class analysis.

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Bayesian estimation of true prevalence, sensitivity and specificity of indirect ELISA, Rose Bengal Test and Slow Agglutination Test for the diagnosis of brucellosis in sheep and goats in Bangladesh

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Abstract

The true prevalence of brucellosis and diagnostic test characteristics of three conditionally dependent serological tests were estimated using the Bayesian approach in goats and sheep populations of Bangladesh. Serum samples from a random selection of 636 goats and 1044 sheep were tested in parallel by indirect ELISA (iELISA), Rose Bengal Test (RBT) and Slow Agglutination Test (SAT). The true prevalence of brucellosis in goats and sheep were estimated as 1% (95% credibility interval (CI): 0.7–1.8) and 1.2% (95% CI: 0.6–2.2) respectively. The sensitivity of iELISA was 92.9% in goats and 92.0% in sheep with corresponding specificities of 96.5% and 99.5% respectively. The sensitivity and specificity estimates of RBT were 80.2% and 99.6% in goats and 82.8% and 98.3% in sheep. The sensitivity and specificity of SAT were 57.1% and 99.3% in goats and 72.0% and 98.6% in sheep. In this study, three conditionally dependent serological tests for the diagnosis of small ruminant brucellosis in Bangladesh were validated. Considerable conditional dependence between IELISA and RBT and between RBT and SAT was observed among sheep. The influence of the priors on the model fit and estimated parameter values were checked using sensitivity analysis. In multiple test validation, conditional dependence should not be ignored when the tests are in fact conditionally dependent.

1. Introduction

Brucella melitensis, primarily responsible for brucellosis in sheep and goats is by far, the most important zoonotic agent among Brucella spp. (Anonymous, 1986; Solorio-Rivera et al., 2007). Brucellosis in sheep and goats is rarely caused by *Brucella abortus* and *Brucella suis* (EC, 2001). *Brucella ovis* causes epididymitis in rams but rarely causes abortion in ewes (Van Tonder et al., 1994) and does not cause disease in humans. In the majority of industrialized countries, bovine brucellosis has been eradicated or controlled. However, small ruminant brucellosis remains a problem in some of these countries as well as in all developing countries. Basically, brucellosis is almost always present where small ruminants are kept (Godfroid et al., 2005; Franco et al., 2007).

There are about 36.5 million goats and 1.69 million sheep representing more than 57% of the total livestock of Bangladesh. About 85% of rural households own animals and 75% of the population rely on livestock to some extent for their livelihood (Anonymous, 2005; BBS, 2004). More than 98% of goats are owned by the small, marginal and landless farmers in the villages. Their small body size and easy management especially by feeding on road side grasses, tree leaves and kitchen vegetable wastes i.e. investing practically nothing, attracts poor women and children to small ruminant rearing (Amin, 2006). A good proportion of humans in Bangladesh have very close contacts with small ruminants and direct contact with animals is the principal route of brucellosis transmission. The epidemiological understanding of small ruminant brucellosis is in a very preliminary stage in Bangladesh. The estimated seroprevalence of brucellosis in Bangladesh based on previous studies ranges from 0.7% to 14.6% in goats (Mustafa, 1984; Rahman et al., 1988, 2011a,b) and 0 to 4.8% in sheep (Mustafa, 1984; Amin, 2003; Uddin, 2006; Rahman et al., 2011a,b).

The serological tests used in previous studies were the Rose Bengal Test (RBT), Standard Tube Agglutination Test, ELISA or Plate Agglutination Test. None of the aforementioned tests are perfect. So, the prevalence reported using these tests are not true prevalence due to misclassification of some of the tested animals. Moreover, the performance of these tests has not been validated in naturally infected small ruminants of Bangladesh. Tests are normally validated by comparing with the gold standard or perfect test. However, the gold standard for the diagnosis of brucellosis is isolation and identification of the organism (Alton et al., 1988; OIE, 2008) which is not easy to perform in a developing and resource-limited country like Bangladesh. In the absence of a gold standard, simultaneous estimation of true prevalence and diagnostic test characteristics can be performed successfully when applying multiple diagnostic tests to every individual subject, using a Bayesian approach which combines test results and external information (Berkvens et al., 2006; Adel et al., 2010; Praud et al., 2012).

An important consideration in the evaluation of multiple diagnostic tests is whether or not the tests can be assumed conditionally independent of each other given the true disease status. It has been demonstrated that the assumption of conditional independence may lead to biased estimates for test

characteristics if in fact the tests are conditionally dependent (Vacek, 1985; Gardner et al., 2000). Since iELISA, RBT and SAT are based on the same biological process (Nielsen, 2002) i.e. detection of anti-*Brucella*-smooth-lipopolysaccharide (SLPS) antibodies, they can be considered to be conditionally dependent (Gardner et al., 2000). Therefore, the estimation procedures should be adjusted for the dependencies among the tests (Dendukuri and Joseph, 2001; Branscum et al., 2005). Few reports have been noted where authors considered test dependence in a multiple testing strategy for the diagnosis of porcine and bovine brucellosis (Ferris et al., 1995; Mainar-Jaime et al., 2005; Praud et al., 2012) but none was noted for the diagnosis of small ruminants brucellosis.

The aim of this study was to estimate the true prevalence of brucellosis in small ruminants of Bangladesh and to evaluate the performance of three conditionally dependent serological tests namely indirect ELISA, RBT and SAT using a Bayesian modeling approach.

2. Materials and methods

2.1. Study and sampling design

Livestock herds in Bangladesh are not identified regionally or centrally in the form of a data bank. To obtain random samples in this context a map digitization and herd selection procedure was followed in the Mymensingh district of Bangladesh. Out of a total of the 146 unions (sub Upa-Zilla) of Mymensingh district (consisting of several Upa-Zillas), 28 were randomly selected. Usually one geographical coordinate was randomly selected from each selected union and located by a hand held GPS reader. Livestock farmers within 0.5 km radius of the selected point were informed about the survey. All animals of the selected herds were sampled. Since there were very few sheep in Mymensingh district, blood samples were also collected from all other divisions of Bangladesh except in Khulna through the nationwide network of the Bangladesh Livestock Research Institute (BLRI) using the same sampling design scheme. The study area is shown in Figure 6.1. The study was conducted initially between September 2007 and August 2008 and then between January 2010 and May 2010 additional sheep samples were collected. In addition, a pretested questionnaire designed to collect animal and herd level data during blood sampling was administered.

2.2. Processing of blood samples

About 4 ml of blood was collected from each animal by jugular venipuncture with disposable needles and venoject tubes, labeled and transported to the laboratory on ice (after clotting) within 12 h of collection. Blood samples were kept in the refrigerator (2–8 °C) in the laboratory and one day later sera were separated by centrifuging at 6000 x g for 10 min. Each serum was labeled to identify the animal and stored at -20 °C. Blood samples collected from other districts were processed in respective districts and sera stored at -20 °C in regional BLRI field stations and conveniently transferred to the medicine department laboratory of Bangladesh Agricultural University (BAU). Each serum was

divided into two tubes each containing about 1 ml of serum. One aliquot was used for testing and the other was preserved in a serum bank.

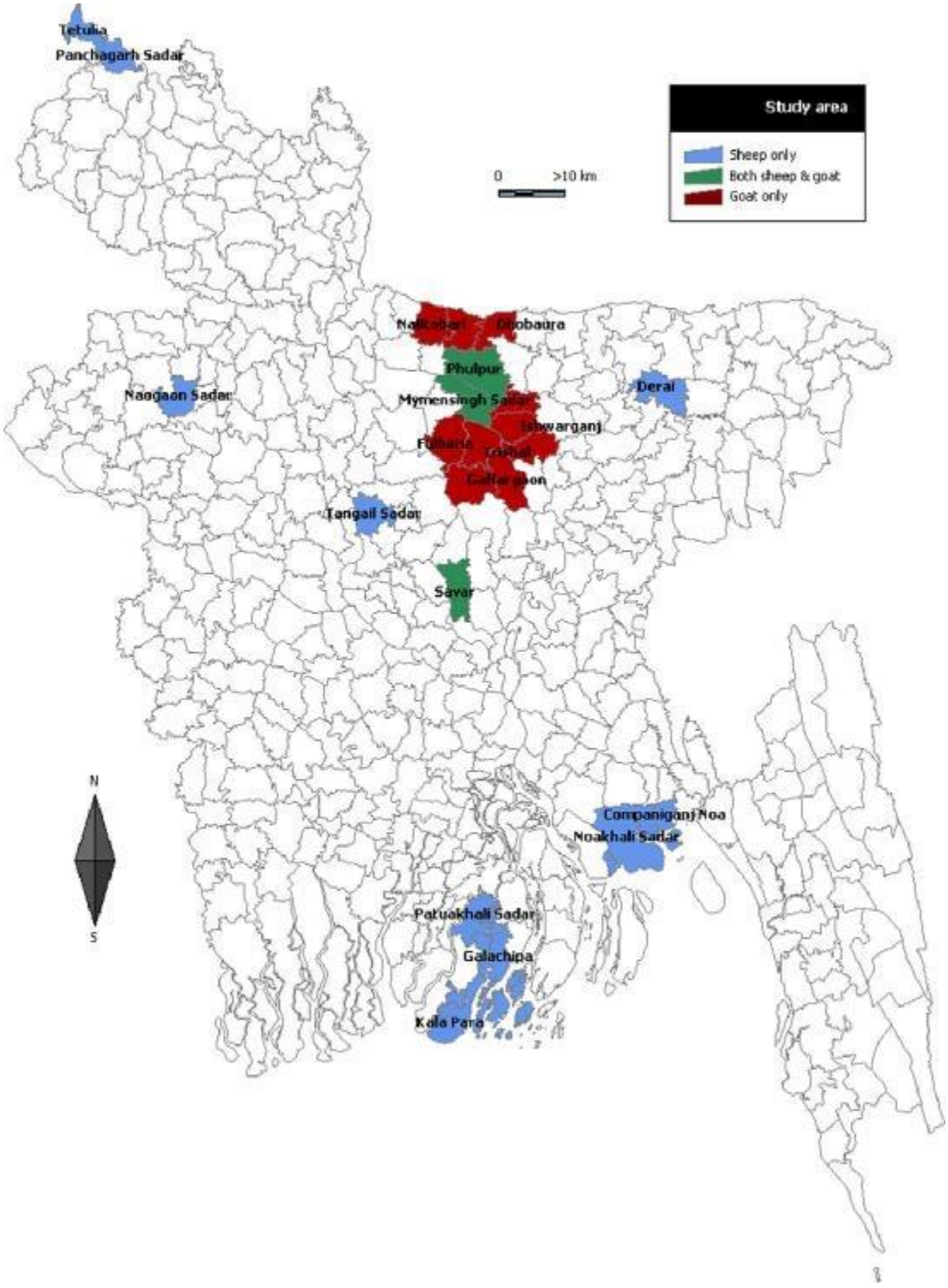
2.3. Serological tests

All blood samples were tested in parallel by iELISA, RBT and SAT in the medicine department laboratory of BAU, Mymensingh, Bangladesh.

iELISA was performed according to Limet et al. (1988) using *B. abortus* biotype 1 (Weybridge 99) as antigen. The detail procedure was described in a previous paper by Rahman et al. (2012). The cut-off value for a positive result was defined at 2 U/ml of test serum for goats (Godfroid et al., 2002) and 6 U/ml of test serum for sheep (Pers. Comm. David Fretin).

RBT was performed as described by Alton et al. (1988). Briefly, sufficient antigen, test sera, positive and negative control sera for a day's testing were removed from refrigeration and brought to room temperature (22 ± 4 ◦C). Equal volumes (30 µl) of serum and antigen (concentrated suspension of *B. abortus* biotype 1 (Weybridge 99); Institut Pourquier, France) were mixed and rotated on a glass plate for 4 min. The result was considered positive when agglutination was noticeable after this delay.

Figure 6.1. Map of Bangladesh showing the study areas



SAT was carried out with ethylenediaminetetraacetic acid (EDTA) as described by Garin et al. (1985). The antigen used was *B. abortus* biotype 1 (Weybridge 99) (Synbiotics Europe, France). One hundred and sixty eight microliter of SAW buffer in the first well and 100 µl in the second and the third wells were added in 96-well microtiter plate. Thirty two microliter of serum was added in the first well (dilution 1/6.25). After proper mixing of diluent and serum, 100 µl from the first well was transferred to the second well (1/12.5). In the same way 100 µl was transferred from the second to the third well (dilution 1/25) and 100 µl discarded from the third well. Then in each well 100 µl of standardized SAW antigen was added giving the serial serum dilutions of 1/12.5, 1/25 and 1/50. The plates were agitated and incubated at 37 ◦C for 20–24 h. Reading was done on the basis of degree of agglutination and expressed in international units (IU). Any serum with an antibody titer greater than or equal to 30 IU/ml, as prescribed by the EU (Shey-Njila et al., 2005), was considered positive.

2.4. Statistical analysis

2.4.1. Model building

A Bayesian latent class analysis was implemented in WinBUGS 1.4 (Spiegelhalter et al., 2003) and R 2.14.2 (R Foundation and Statistical Computing 2012) to estimate the prevalence, sensitivity and specificity of the three tests, using models developed by Branscum et al. (2005), Berkvens et al. (2006), Nérette et al. (2008) and Haley et al. (2011) separately for sheep and goats. In a three test scenario, 7 parameters need to be estimated by the multinomial model under the assumption of conditional independence namely; the prevalence, and the sensitivities and specificities of the three tests. However, under the assumption of conditional dependence, 8 additional parameters need to be estimated namely the conditional covariance between each pair of tests among infected and non-infected subjects. This model is in fact non-identifiable since the data only allows for seven parameters to be estimated. As none of the three tests is considered a gold standard test and the tests are not conditionally independent, constraints have to be imposed on a subset of the parameters in order to make the models identifiable (Branscum et al., 2005). To evaluate the goodness of fit of the models, the posterior predictive p-value, Deviance Information Criterion (DIC) (Spiegelhalter et al., 2002) and the number of effectively estimated parameters (pD) (Berkvens et al., 2006) were used as calibrating parameters. Briefly, the DIC ensures that a parsimonious model is selected. It is calculated as DIC = pD + D with D the mean posterior deviance and pD the number of parameters effectively estimated by the model. Models with a smaller DIC should be preferred to models with larger DIC. The posterior predictive p-value is a posterior predictive check that detects lack-of-fit of the model to the data. It is based on the difference between the deviance of the observations and the deviance of observations generated randomly from the currently fitted model and for models that provide adequate fit to the data, the value should be around 0.50. A posterior predictive p-value of 0.5 is the value that would be obtained if the distribution of the deviances based on the observed and simulated data sets overlapped perfectly (Kelly and Smith, 2011). The apparent prevalence for sheep in 2007/2008 was 3.7% [18/482]

(95% credibility intervals (CI): 2.2–5.8) and that in 2010 was 2.5% [14/562] (95% CI: 1.4–4.1). Using the "prtesti" command in Stata 12.1, we observed that the difference between the two proportions was not statistically significant (p-value = 0.3015) therefore data for the two phases were combined.

2.4.2. Modeling conditional dependence

Using the model that assumes conditional independence among the three tests given the true disease status of individuals as the baseline model, conditional dependence between each pair of tests was estimated using different parameterizations of the model that assumed conditional dependence between tests (Branscum et al., 2005; Berkvens et al., 2006; Nérette et al., 2008). Jones et al. (2010) proposed that in the construction of conditional dependence models, mainly simple extensions of the conditional independence model should be considered. Essentially, in the first set of simple parameterizations, the conditional dependence between iELISA and RBT, between iELISA and SAT and between RBT and SAT were each added in turn to the conditional independence model. In addition, three models were constructed with conditional dependence between the pairs: iELISA–RBT and iELISA–SAT, iELISA–RBT and RBT–SAT and between iELISA–SAT and RBT–SAT respectively (Nérette et al., 2008). Finally a model with conditional dependence among all the three tests was considered (all pairs inclusive) separately among infected and non-infected individuals and also among infected and non-infected animals combined. The models for both goats and sheep along with their corresponding parameters are presented in Table 6.3.

Letting π to be the true prevalence, T1, T2 and T3 to represent the test outcomes for iELISA, RBT and SAT respectively, with positive test outcomes denoted by 1 (or +), negative test outcomes by 0 (or −), and sensitivities and specificities by Se and Sp respectively, the expected cell probabilities (p) based on these three tests under the assumption of conditional dependence are given as follows:

$$p(1\,1\,1) = P(T_1^+, T_2^+, T_3^+) = \pi(\mathrm{Se}_1\mathrm{Se}_2\mathrm{Se}_3 + \mathrm{Se}_1 a_{23} + \mathrm{Se}_2 a_{13} + \mathrm{Se}_3 a_{12}) +$$
$$(1-\pi)((1-\mathrm{Sp}_1)(1-\mathrm{Sp}_2)(1-\mathrm{Sp}_3) + (1-\mathrm{Sp}_1)b_{23} + (1-\mathrm{Sp}_2)b_{13} + (1-\mathrm{Sp}_3)b_{12})$$
$$p(1\,1\,0) = P(T_1^+, T_2^+, T_3^-) = \pi(\mathrm{Se}_1\mathrm{Se}_2(1-\mathrm{Se}_3) - \mathrm{Se}_1 a_{23} - \mathrm{Se}_2 a_{13} + (1-\mathrm{Se}_3)a_{12}) +$$
$$(1-\pi)((1-\mathrm{Sp}_1)(1-\mathrm{Sp}_2)\mathrm{Sp}_3 - (1-\mathrm{Sp}_1)b_{23} - (1-\mathrm{Sp}_2)b_{13} + \mathrm{Sp}_3 b_{12})$$
$$p(1\,0\,1) = P(T_1^+, T_2^-, T_3^+) = \pi(\mathrm{Se}_1(1-\mathrm{Se}_2)\mathrm{Se}_3 - \mathrm{Se}_1 a_{23} - (1-\mathrm{Se}_2)a_{13} - \mathrm{Se}_3 a_{12}) +$$
$$(1-\pi)((1-\mathrm{Sp}_1)(1-\mathrm{Sp}_2)\mathrm{Sp}_3 - (1-\mathrm{Sp}_1)b_{23} - (1-\mathrm{Sp}_2)b_{13} + \mathrm{Sp}_3 b_{12})$$
$$p(1\,0\,0) = P(T_1^+, T_2^-, T_3^-) = \pi(\mathrm{Se}_1(1-\mathrm{Se}_2)(1-\mathrm{Se}_3) + \mathrm{Se}_1 a_{23} - (1-\mathrm{Se}_2)a_{13} - (1-\mathrm{Se}_3)a_{12})$$
$$+(1-\pi)((1-\mathrm{Sp}_1)\mathrm{Sp}_2\mathrm{Sp}_3 - (1-\mathrm{Sp}_1)b_{23} - \mathrm{Sp}_2 b_{13} - \mathrm{Sp}_3 b_{12})$$
$$p(0\,1\,1) = P(T_1^-, T_2^+, T_3^+) = \pi((1-\mathrm{Se}_1)\mathrm{Se}_2\mathrm{Se}_3 + (1-\mathrm{Se}_1)a_{23} - \mathrm{Se}_2 a_{13} - \mathrm{Se}_3 a_{12}) +$$
$$(1-\pi)(\mathrm{Sp}_1(1-\mathrm{Sp}_2)(1-\mathrm{Sp}_3) + \mathrm{Sp}_1 b_{23} - (1-\mathrm{Sp}_2)b_{13} - (1-\mathrm{Sp}_3)b_{12})$$
$$p(0\,1\,0) = P(T_1^-, T_2^+, T_3^-) = \pi((1-\mathrm{Se}_1)\mathrm{Se}_3(1-\mathrm{Se}_3) - (1-\mathrm{Se}_1)a_{23} - \mathrm{Se}_2 a_{13} - (1-\mathrm{Se}_3)a_{12})$$
$$+(1-\pi)(\mathrm{Sp}_1(1-\mathrm{Sp}_2)\mathrm{Sp}_3 - \mathrm{Sp}_1 b_{23} + (1-\mathrm{Sp}_2)b_{13} - \mathrm{Sp}_3 b_{12})$$
$$p(0\,0\,1) = P(T_1^-, T_2^-, T_3^-) = \pi((1-\mathrm{Se}_1)(1-\mathrm{Se}_3)\mathrm{Se}_3 - (1-\mathrm{Se}_1)a_{23} - (1-\mathrm{Se}_2) * a_{13} + \mathrm{Se}_3 * a_{12})$$
$$+(1-\pi)(\mathrm{Sp}_1\mathrm{Sp}_2(1-\mathrm{Sp}_3) + \mathrm{Sp}_1 b_{23} + \mathrm{Sp}_2 * b_{13} + (1-\mathrm{Sp}_3) * b_{12})$$
$$p(0\,0\,0) = P(T_1^-, T_2^-, T_3^-) = \pi((1-\mathrm{Se}_1)(1-\mathrm{Se}_3)(1-\mathrm{Se}_3) + (1-\mathrm{Se}_1)a_{23} + (1-\mathrm{Se}_2)a_{13} + \mathrm{Se}_3 * a_{12})$$
$$+(1-\mathrm{Se}_3)a_{12}) + (1-\pi)(\mathrm{Sp}_1\mathrm{Sp}_2\mathrm{Sp}_3 + \mathrm{Sp}_1 b_{23} + \mathrm{Sp}_2 * b_{13} + (\mathrm{Sp}_3) * b_{12})$$

Representing the conditional covariance between pairs of tests among infected animals by "a" and among the non-infected population by "b" (Table 6.5), and median posterior estimates were obtained along with their 95% CI. In addition, conditional correlations were computed as described in Georgiadis et al. (2003), Haley et al. (2011) and Branscum et al. (2005). According to Georgiadis et al. (2003), when the conditional correlations are low (≤0.2), the estimates of the conditional dependence and independence models are similar whereas when the correlations are high (>0.2) the conditional dependence model should be considered. All models were compared using the DIC and posterior predictive p-values. To be considered significantly different, the reduction in DIC between any two models should be more than 3 units (Spiegelhalter et al., 2002; Kostoulas et al., 2006; Nérette et al., 2008). In situations where the difference in DIC was smaller than 3 units, the models were assumed to be similar and selection was based on parsimony (the smaller the number of effective parameters estimated (pD) the better) (Spiegelhalter et al., 2002).

2.5. Prior distributions for parameters

Based on a review of the literature, limited information was available regarding the true prevalence and test sensitivities and specificities for brucellosis among small ruminants in Bangladesh. Therefore, prior information from other similar studies was used. A very important source of prior information was the EFSA report of 2006 (EFSA-Q-2006) in which a thorough meta-analytic approach was used to estimate priors of Se and Sp for RBT, iELISA and SAT in sheep and goats. Based on several studies obtained from the literature, a meta-analysis (Random effect) was performed using "metandi" in Stata 12.1 (Harbord and Whiting, 2009). To perform metandi, a minimum of four studies is required. However, for SAT, only two studies were available therefore, the meta-analysis was performed for RBT and iELISA. In addition, mentandi requires that the number of true positives, true negatives, false positives and false negatives be known for each study. This was not available for the meta-analytic study based on the EFSA report so the priors were combined: the lowest limit was used as the lower bound and the higher value as the upper bound in uniform distributions. The same set of priors for the sensitivity and specificity were used both for sheep and goats data. The prior interval estimates used in uniform distributions for the Se and Sp were (0.870, 0.986) and (0.962, 1.00) for iELISA (0.670, 0.934) and (0.915, 1.00) for RBT and (0.301, 0.967) and (0.977, 1) for SAT respectively. The priors used for the prevalence of brucellosis in goats and sheep in Bangladesh were based on local prevalence reports 0.7–14.6% in goats (Mustafa, 1984; Rahman et al., 1988, 2011a,b) and 0–4.8% in sheep (Mustafa, 1984; Amin, 2003; Uddin, 2006; Rahman et al., 2011a,b).

The prior sources for sensitivities and specificities of the three serological tests used for the Bayesian analysis in this study are summarized in Table 6.1. Prior information on the 8 covariance parameters

(4 for infected and 4 for the non-infected individuals) were not available so initial values were generated in R 2.14.2 based on the range of possible values of the sensitivities and specificities listed in Table 6.1 (see Appendix B).

Table 6.1. Sources of priors used for estimation of diagnostic test characteristics for brucellosis in goats and sheep in Bangladesh

Reference	Species	iELISA		RBT		SAT	
		SE	SP	SE	SP	SE	SP
Blasco et al. (1994)	SG	100	100	91.8-92.5	100		
Baum et al. (1995)	SG					90.3-96.7	97.7-1
Abu-Harfeil and Abu-Shehada (1998)	S	66.5-78.7		34.4-47.8			
Burriel et al. (2004)	SG	88.1-96.7	94.7-99.2				
Nielsen et al. (2004)	SG	82.1-96.6	96.4-98.4	64.7-85.3	99.0-99.9		
Nielsen et al. (2005)	G	94.5-97.5	99.3-99.9				
Minas et al. (2005)	S	92.7-96.3	100	67.0-74.1	99.3-1		
EFSA-Q-2006	SG	94.5-95.8	99.1-99.3	91.6-93.4	99.8-1		
Minas et al. (2008)	SG	97.6-98.8	99.8-1	74.0-77.7	99.5-99.9		
Ramirez-Pfeiffer et al. (2008)	SG			76.5-85.2	61.9-74.4		
Gupta et al. (2010)	G					30.1-79.2	50.6-90.4

Legend: SG: sheep and goat; G: goat only; S: sheep only; iELISA: indirect ELISA; RBT: Rose Bengal Test; SAT: Slow Agglutination Test; SE: Sensitivity: SP: Specificity.

2.6. Model diagnostics

All models were run using three chains, a burn-in period of 50,000 iterations and another 100,000 iterations to obtain the posterior estimates. Trace plots were used to explore how fast the chain explores the posterior distribution (Ntzoufras, 2011). A more formal test for convergence, the Brooks, Gelman and Rubin convergence statistic was used to assess model convergence (Gelman and Rubin, 1992). The WinBUGS codes used are presented in Appendices A and B.

2.7. *Sensitivity analyses of selected models*

The influence of the prior information on the estimates of the diagnostic test characteristics were verified using sensitivity analysis (Branscum et al., 2005; Kostoulas et al., 2006; Praud et al., 2012). This was done by using standard uniform priors and slight perturbations (in steps of 10% or 15%) of the prior intervals (Haley et al., 2011). The following sets of priors were considered:

- Uniform prior (UP) for prevalence (Pr) and informative priors (IP) for sensitivities (Se) and Specificities (Sp)
- UP for Pr and for Se and IP for Sp
- UP for Pr and for Sp and IP for Se
- IP for Pr and UP for Se and Sp
- IP for Pr and for Se and UP for Sp
- IP for Pr and for Sp and UP Se
- Perturbations of the prior interval

For each set of alternative prior distributions considered for the model parameters, the model was run with the same number of chains and similar diagnostics were performed.

3. Results

3.1. Data exploration

The study was conducted initially between September 2007 and August 2008 for both sheep and goats and later between January 2010 and May 2010 for sheep. The mean age for goats was 1.6 ± 0.06 (mean ±se) years ranging from 0.17 to 8 years whereas the mean body weight was 10.0 ± 0.19 (mean ± se) kg ranging from 2 to 30 kg. About 95% of goats were of the Black Bengal breed and the rest were of Jamuanpari breed of origin. Sixty-six percent of the sampled goats were female. The median herd size of goats was 2 ranging from 1 to 18. The mean age of sheep was 2.1 ± 0.0.04 (mean ± se) years ranging from 0.08 to 8 years whereas the mean body weight of was 14.6 ± 0.15 (mean ± se) kg ranging from 2 to 40 kg. All sheep were of the indigenous type and 77% of them were female. The median herd size of sheep was 5 and ranged from 1 to 75.

The cross classified test results of the three serological tests on the 636 sera of goats and 1044 sera of sheep are shown in Table 6.2. Two (0.3%) out of a total of 636 goats were positive for all three tests and 94% (598/636) were test negative. Similarly 8 (0.8%) out of the total of 1044 sheep were positive for all three tests and 96.5% (1007/1044) were negative for all three tests (Table 6.2).

Table 6.2. Cross-classified test results for brucellosis in goats and sheep of Bangladesh based on iELISA, RBT, and SAT

iELISA	RBT	SAT	Goat	Sheep
1	1	1	2	8
1	1	0	1	3
1	0	1	0	0
1	0	0	29	5
0	1	1	0	9
0	1	0	2	6
0	0	1	4	6
0	0	0	598	1007
	Total		**636**	**1044**

Legend: iELISA: indirect ELISA; RBT: Rose Bengal Test; SAT: Slow Agglutination Test; 1: Positive; 0: Negative.

3.2. Model selection and posterior estimates

The priors used in the Bayesian analyses were the same for the models for both goats and sheep. For the data for goats, the DIC for the conditional independence model was 26.09. None of the models with conditional dependence terms led to a significant reduction (of greater than 3) in DIC (Table 6.3). In addition, all the median estimates of the conditional correlations were close to 0.2. The conditional independence model was therefore selected as a plausible model for the data for goats. The median estimates of the true prevalence of caprine brucellosis, sensitivity, and specificity of the three tests are summarized in Table 6.4. The true prevalence of caprine brucellosis in Bangladesh was updated to 1% with 95% CI of 0.7–1.8. The highest sensitivity (92.8% and 95% CI 87.3–98.3) with corresponding lowest specificity (96.5% and 95% CI 96.2–97.3) was estimated for iELISA among goats. The specificity of both RBT and SAT were greater than 99.2% and the sensitivity of RBT was higher (80.2%) than that of SAT (57.3%) among goats. For the data for sheep, all models that included the conditional covariance between RBT and SAT yielded significantly lower DICs (33.47–35.13) compared to the conditional independence model (52.3).

Table 6.3. Comparison of model diagnostic parameters for conditional independence and different conditional dependence models used to estimate true prevalence of brucellosis in small ruminants and sensitivity and specificity of three diagnostic tests

Models	Goat			Sheep		
	Post.	pD	DIC	post	pD	DIC
Conditional independence	**0.55**	**2.30**	**26.09**	1.00	4.54	52.3
Conditional Dependence (CD) between iELISA and RBT	0.62	3.03	26.43	1.00	4.82	53.46
CD between iELISA and SAT	0.63	3.10	27.30	1.00	5.03	52.87
CD between RBT and SAT	0.61	3.33	27.60	0.49	5.19	34.10
CD between iELISA and RBT and between iELISA and SAT	0.69	3.24	27.75	0.99	5.33	54.97
CD between iELISA & SAT and between RBT & SAT	0.66	3.48	28.68	0.53	5.14	35.15
CD between iELISA and RBT and between RBT and SAT	0.64	3.35	27.74	0.48	**5.10**	**33.50**
CD among all tests for infected animals	0.57	3.02	25.65	0.99	4.44	46.88
CD among all tests for non-infected animals	0.71	3.47	29.37	0.64	5.50	37.61
CD among all tests	0.69	3.54	28.90	0.52	4.98	34.53

Legend: iELISA: indirect ELISA; RBT: Rose Bengal Test; SAT:Slow Agglutination Test; Bold Models were used to estimate prevalence and test characteristics for goat and sheep respectively; $_{pD}$: the number of parameters effectively estimated by the model; Post.: Post predictive *p*-value; DIC: Deviance Information Criterion.

Table 6.4. Median posterior estimates of prevalence, sensitivity and specificity of iELISA, RBT and SAT for the diagnosis of brucellosis in goats in Bangladesh

Test	Variable	Median	95% Credibility interval
	Prevalence	1.0	0.7, 1.8
iELISA	Se	92.9	87.3, 98.3
	Sp	96.5	96.2, 97.3
Rose Bengal	Se	80.2	67.7, 92.7
	Sp	99.6	98.9, 99.9
Slow Agglutination	Se	57.1	31.7, 91.4
	Sp	99.3	98.4, 99.8

Table 6.5. Bayesian median posterior estimates of prevalence, conditional correlations, sensitivity and specificity of iELISA, RBT and SAT for the diagnosis of brucellosis in sheep in Bangladesh.

Test	Variable	Median	95% Credibility Interval
	Prevalence	1.2	0.6, 2.2
iELISA	Se	92.0	87.2, 98.2
	Sp	99.5	98.7, 99.9
Rose Bengal	Se	82.8	68.1, 92.9
	Sp	98.3	97.4, 99.0
Slow Agglutination	Se	72.0	43.6, 94.5
	Sp	98.6	97.8, 99.2
Dependence coefficient			
Between iELISA and RBT among infected sheep	ρ_{a12}	0.18	0.0, 0.46
Between RBT and SAT among infected sheep	ρ_{a23}	0.53	0.32, 0.72
Between iELISA and RBT among non- infected sheep	ρ_{b12}	0.29	-0.11, 0.82
Between RBT and SAT among non-infected sheep	ρ_{b23}	0.40	-0.13, 0.87

Legend: ρaij stands for the conditional correlation between test i and test j among infected subjects and ρbij stands for the conditional correlation between test i and test j among non-infected subjects.

3.3. Sensitivity analyses results

The results of the sensitivity analyses of the models for goats and sheep are shown in Tables 6.6 and 6.7 respectively.

The conditional independence model for goats and conditional dependence model for sheep were used for the sensitivity analyses. The model diagnostic parameters indicated that the different set of priors yielded reasonable fit to the data. The true prevalence of caprine as well as ovine brucellosis and specificities of all three tests obtained from the different models of sensitivity analyses were similar to those of the selected models since their 95% credibility intervals overlapped. Whereas the estimated specificities were the same as those of the selected models regardless of the set of priors used, the sensitivities were observed to vary and yielded wider confidence intervals. However, since the 95% credibility intervals overlapped, the observed differences were not statistically important (Tables 6.6 and 6.7). For example, the true median prevalence of goats and sheep were 1.0% (95% CI: 0.7–1.8%) and 1.2% (95% CI: 0.6–2.2%) respectively and the ranges of the median prevalence obtained in sensitivity analyses respectively for goat and sheep ranged from 0.6–5% to 0.5–4.3% respectively. Decreasing the lower limits of all the prior intervals by 10% led to only slight and statistically unimportant changes in the estimated parameter values and their 95% Cr Intervals in the models for both goats and sheep.

Table 6.6. Median posterior estimates of prevalence, sensitivity and specificity of iELISA, RBT and SAT based on a sensitivity analysis of the conditional independence model used to estimate true prevalence of caprine brucellosis and diagnostic test characteristics

Models & Tests	Post.	pD	DIC	Prevalence (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)
UP for Prev and IP for Se and Sp	0.55	2.89	25.92	0.6 (0.2, 1.6)		
ELISA					93.0 (87.3, 98.3)	96.5 (96.2, 97.3)
RBT					81.2 (67.8, 92.8)	99.6 (98.8, 99.9)
SAT					60.1 (32.1, 92.9)	99.3 (98.4, 99.8)
UP for Prev and Sp and IP for Se	0.40	3.65	25.32	0.6 (0.2, 1.5)		
ELISA					93.0 (87.3, 98.3)	95.3 (93.5, 96.8)
RBT					81.7 (67.9, 92.9)	99.6 (98.8, 99.9)
SAT					62.1 (32.3, 93.5)	99.3 (98.4, 99.8)
IP for Prev and UP for Se and Sp	0.57	3.42	27.97	1.1 (0.7, 3.3)		
ELISA					69.6 (23.9, 98.5)	95.5 (93.6, 97.2)
RBT					62.5 (15.3, 98.1)	99.7 (98.9, 100)
SAT					46.0 (10.2, 90.8)	99.3 (98.5, 99.9)
IP for Prev and Se and UP for Sp	0.39	3.06	25.57	0.9 (0.7, 1.7)		
ELISA					92.8 (87.3, 98.3)	95.4 (93.5, 96.9)
RBT					80.9 (67.7, 92.8)	99.6 (98.9, 99.9)
SAT					59.0 (31.9, 92.1)	99.3 (98.4, 99.8)
IP for Prev and Sp and UP for Se	0.70	2.07	28.03	1.3 (0.7, 5.0)		
ELISA					71.1 (26.1, 98.5)	96.6 (96.2, 98.0)
RBT					51.1 (10.2, 97.0)	99.7 (98.9, 100)
SAT					37.5 (6.9, 87.1)	99.3 (98.5, 99.9)
Perturbation example: 10% decrease of lower limits of Se and Sp	0.39	3.13	25.69	0.9 (0.7, 1.8)		
ELISA					88.0 (77.6, 98.1)	95.4 (93.5, 96.8)
RBT					85.3 (77.4, 93.0)	99.6 (98.9, 99.9)
SAT					56.6 (23.4, 91.9)	99.3 (98.4,99.8)

Legend: UP: uniform prior; IP: informative prior; Prev: prevalence; Se: sensitivities; Sp: specificities; CI: Credibility interval; iELISA: indirect ELISA; RBT: Rose Bengal Test; SAT: Slow Agglutination Test; Post.: Post. pred. *p*-value; p_D: the number of parameters effectively estimated by the model; DIC: Deviance Information Criterion.

Table 6.7. Median posterior estimates of prevalence, sensitivity and specificity of iELISA, RBT and SAT based on a sensitivity analysis of a conditional dependence model used to estimate true prevalence of ovine brucellosis and diagnostic test characteristics

Models & Tests	Post.	pD	DIC	Prevalence (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)
UP for Prev and IP for Se and Sp	0.48	5.09	33.49	1.2 (0.6, 2.2)		
ELISA					92.1 (87.2, 98.2)	99.5 (98.7, 99.9)
RBT					82.8 (68.1, 92.9)	98.3 (97.4, 99.0)
SAT					72.2 (43.6, 94.5)	98.6 (97.8, 99.2)
UP for Prev and Sp and IP for Se	0.49	5.18	33.70	1.2 (0.5, 2.2)		
ELISA					92.1 (87.2, 98.2)	99.5 (98.7, 99.9)
RBT					82.7 (68.1, 92.9)	98.3 (97.3, 99.0)
SAT					72.0 (43.6, 94.5)	98.5 (97.7, 99.2)
IP for Prev and UP for Se and Sp	0.49	4.43	31.84	2.1 (0.8, 4.3)		
ELISA					52.7 (25.1, 94.3)	99.5 (98.7, 100)
RBT					75.0 (37.0, 99.0)	98.8 (97.6, 99.9)
SAT					66.4 (35.2, 96.6)	99.1 (98.0, 99.9)
IP for Prev and Se and UP for Sp	0.49	5.21	33.77	1.2 (0.5, 2.2)		
ELISA					92.1 (87.2, 98.2)	99.5 (98.7, 99.9)
RBT					82.8 (68.1, 92.9)	98.3 (97.3, 99.0)
SAT					72.0 (43.7, 94.5)	98.5 (97.7, 99.2)
IP for Prev and Sp and UP for Se	0.49	4.41	31.79	2.1 (0.8, 4.3)		
ELISA					52.7 (25.0, 94.3)	99.5 (98.7, 100)
RBT					75.2 (37.2, 98.9)	98.8 (97.7, 99.9)
SAT					65.5 (35.3, 96.7)	99.1 (99.1, 99.9)
Perturbation example: 10% decrease of lower limits of Se and Sp	0.50	5.11	33.45	1.3 (0.6, 2.5)		
ELISA					85.5 (77.4, 97.7)	99.5 (98.7, 100)
RBT					80.1 (59.6, 92.8)	98.3 (97.4, 99.1)
SAT					70.5 (41.4, 94.3)	98.6 (97.7, 99.3)

Legend: UP: uniform prior; IP: informative prior; Prev: prevalence; Se: sensitivities; Sp: specificities; CI: Credibility interval; iELISA: indirect ELISA; RBT: Rose Bengal Test; SAT: Slow Agglutination Test; Post.: Post. pred. *p*-value; p_D: the number of parameters effectively estimated by the model; DIC: Deviance Information Criterion.

4. Discussion

In this study, the true prevalence and diagnostic test characteristics for brucellosis in goats and sheep were determined using a Bayesian analysis framework. More than 90% of the goats in the country were of the Black Bengal breed. The study area had the highest density of small ruminants (>300 km2) in Bangladesh (Anonymous, 2005) and about 95% of the goats sampled were of the Black Bengal breed. The sheep sample covered almost all the divisions except Khulna division of Bangladesh. The breed of sampled sheep was indigenous which is predominant all over Bangladesh (Bhuiyan, 2006). However, a study based on micro-satellite markers by Khan et al. (2009) described Garole sheep of Satkhira district (within Khulna division) as an independent sheep breed in Bangladesh. So, the prevalence estimated in this study is based on a representative sample of goats and sheep and would therefore be applicable to the goats and sheep (except Khulna division) populations of Bangladesh. About 1% of goats and 1.2% of sheep of Bangladesh were found to be serologically positive for brucellosis. The prevalence of brucellosis in goats and sheep are within the range of previously reported apparent prevalence. However, through this study we obtained the true prevalence along with their true probability interval (credibility interval contains the true parameter with 95% certainty (Mustafa, 1984; Rahman et al., 1988; Enøe et al., 2000; Amin, 2003; Uddin, 2006; Rahman et al., 2011a,b). The relatively higher seroprevalence in sheep may be due to the relatively larger herd sizes of sheep compared to goats in Bangladesh. Larger herd sizes have been reported to be significantly associated with brucellosis seropositivity among livestock (Mikolon et al., 1998; Kabagambe et al., 2001; Solorio-Rivera et al., 2007).

In Bangladesh, among livestock farmers about 49% rear small ruminants either alone or with large ruminants and about 53% farmers who share same premises with animals are goat owners (Rahman et al., 2012). As small ruminants come in very close contact with humans, brucellosis in goats and sheep should be controlled with the highest priority in order to control this zoonosis in humans. In Bangladesh, goats are a very valuable asset especially for the poor people. They mature sexually quite early, at 6–8 months of age, and breed around the year. They kid twice a year and meat and skin obtained from the Black Bengal are of excellent quality and fetch high prices, even in the local market. Sheep of Bangladesh are also as prolific as goats. Small ruminants with clinical signs suggestive of brucellosis (abortion, retained fetal membrane, anestrous, etc.) are usually sold and eventually slaughtered by butchers. Moreover, around 15 million goats are slaughtered annually and of them about 40% are performed during the annual festival of Eid-ul-Azha (Anonymous, 2007). It has been shown that the longer infected animals are in contact with the rest of the herd, the greater the number of seropositive animals (Radostits et al., 2000). Large scale slaughtering of small ruminants for meat consumption may reduce the number of infected animals in the population. These factors may be responsible for low prevalence in goats and sheep of Bangladesh. In such an intermediate (1–5%) prevalence scenario of small ruminants brucellosis in Bangladesh, eradication can be achieved mainly

by test and slaughter policy. However, pre-requisites for undertaking eradication programs such as: good organization of farmers and veterinary services, the implementation of strict movement control measures, an efficient identification system of the animals, no chance of sharing common grazing places and availability of financial resources are not yet at hand. The complete understanding of the disease including the species and biovars of *Brucella* involved in small ruminants should also be known for planning control programs (Anonymous, 2006; Minas, 2006).

In this study, the performance of iELISA and RBT were relatively better than that of SAT in goats and sheep. The specificity estimates of SAT and RBT were very similar. The sensitivities of iELISA and RBT were similar in both sheep and goats. However, the specificity of iELISA (95.5%) was slightly lower in goats compared to sheep whereas that of RBT was slightly lower in sheep compared to goats. The increased specificity of iELISA in sheep was due to the higher cut-off values than that of goats. The sensitivity and specificity of iELISA estimated were in accordance with results from other studies (Abuharfeil and Abo-Shehada, 1998; Burriel et al., 2004). The sensitivity of RBT in goats and sheep were 80.2% and 82.8% respectively even though the specificity of RBT in both goats and sheep was more than 98%. The estimated sensitivity and specificity of RBT were coherent with findings from previous studies (Nielsen et al., 2004; Ramirez-Pfeiffer et al., 2008). The sensitivity and specificity of SAT in goats and sheep were 57.1%, 99.3% and 72.0%, 98.6% respectively. The sensitivity and specificity of SAT were also in accordance with results from other studies (Baum et al., 1995; Gupta et al., 2010). The iELISA was the most sensitive and specific test explaining the fact that acutely infected animals were less common in the population. The serological response observed in this study includes both *B. abortus* and *B. melitensis* infections but excludes *B. ovis* as its antibody does not react with antigens prepared by SLPS. The proportion of goats and sheep infected with *B. abortus* and *B. melitensis* in Bangladesh is not yet known. But *B. abortus* was detected from goat milk using real time PCR assay (unpublished data). Among the three tests none was sensitive and specific enough to be used alone for the diagnosis of caprine brucellosis in Bangladesh. In the model for goats, the hypothesis of conditional dependence among the three tests was not important. This might have been due to small and sometimes zero cell frequencies observed for goats. In sheep, considerable conditional dependence between iELISA and RBT and between RBT and SAT among infected as well as non-infected sheep were observed. The iELISA is a quantitative test which detects only IgG, SAT quantifies both IgM and IgG (but mainly IgM) and RBT qualitatively detects both IgM and IgG (Christopher et al., 2010; Godfroid et al., 2010; Dìaz et al., 2011). The conditional correlation between RBT and SAT for sheep may be explained by the similarity of the type of antibody detected. The weaker conditional correlation between iELISA and RBT among infected as well as non-infected sheep may be explained by the fact that RBT also partially detects IgG. The sensitivity analysis of the conditional independence model for goats and a conditional dependence model for sheep revealed that the results can be considered to be robust. Slight differences in prevalence and sensitivities were

observed but the differences were not statistically important as the credibility intervals of the estimates overlapped with those of the prevalence and sensitivities of the serological tests in the chosen models for goats and sheep (Tables 6.4–6.7).

5. Conclusion

This study is the first to evaluate the accuracy of brucellosis diagnostic tests among sheep and goats in Bangladesh considering conditional dependence between the diagnostic tests. An intermediate level of true prevalence of brucellosis among goats and sheep respectively was estimated. Such low prevalence will allow test and slaughter policy to control this zoonosis in small ruminants. There was considerable conditional dependence between iELISA and RBT and between RBT and SAT implying that a combination of the three serological tests may be a plausible choice unless other tests with very high sensitivity and specificity are validated. In multiple test validation, conditional dependence should not be ignored when the tests are in fact conditionally dependent.

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Appendix A.

Appendix A presents the WinBUGS code used to estimate true prevalence and test characteristics for iELISA, SAT, and RBT among goats using a model in which the three tests are assumed to be conditionally independent.

The conditional independence model had the smallest DIC value among all models including condition dependence between any of the two tests. In the code presented below, the sensitivity and specificity of iELISA are represented by: se [1] and sp [1] respectively, of RBT by se [2] and sp [2] respectively, and of SAT by se [3] and sp [3] respectively.
For this model, initial values were automatically generated in WinBUGS.

```
#Model
model
{
r[1:8] ~ dmulti(p[1:8], n)
## p[1] = p(111); p[8] = p(000); pr = true prevalence
p[1] <- pr*(se[1]*se[2])*se[3] + (1-pr)*((1-sp[1])*(1-sp[2]))*(1-sp[3])
p[2] <- pr*(se[1]*se[2])*(1-se[3]) + (1-pr)*((1-sp[1])*(1-sp[2]))*(sp[3])
p[3] <- pr*(se[1]*(1-se[2]))*se[3] + (1-pr)*((1-sp[1])*sp[2])*(1-sp[3])
p[4] <- pr*(se[1]*(1-se[2]))*(1-se[3]) + (1-pr)*((1-sp[1])*sp[2])*(sp[3])
p[5] <- pr*((1-se[1])*se[2])*se[3] + (1-pr)*(sp[1]*(1-sp[2]))*(1-sp[3])
p[6] <- pr*((1-se[1])*se[2])*(1-se[3]) + (1-pr)*(sp[1]*(1-sp[2]))*sp[3]
p[7] <- pr*((1-se[1])*(1-se[2]))*se[3] + (1-pr)*(sp[1]*sp[2])*(1-sp[3])
p[8] <- pr*((1-se[1])*(1-se[2]))*(1-se[3]) + (1-pr)*(sp[1]*sp[2])*sp[3]
pr ~ dunif(0.007,0.146)
se[1] ~ dunif(0.870,0.986)
se[2] ~ dunif(0.670,0.934)
se[3] ~ dunif(0.301,0.967)
sp[1] ~ dunif(0.962,1.00)
sp[2] ~ dunif(0.915,1.00)
sp[3] ~dunif(0.977,1)
r2[1:8] ~ dmulti(p[1:8],n)

for ( i in 1:8)
{
d[i] <-(pow(r[i]-p[i]*n,2)/(p[i]*n))
d2[i] <- (pow(r2[i]-p[i]*n,2)/(p[i]*n))
I[i] <- step(p[i] -1)*step(-p[i])
}
bayesp <- step(sum(d[]) - sum(d2[]))
}
#data
list(r=c(2,1,0,29,0,2,4,598), n=636)
```

Appendix B:

Appendix B presents the WinBUGS code used to estimate true prevalence and test characteristics for iELISA, SAT and RBT among sheep using model in which the three tests were considered to be conditionally dependent given true disease status.

The model with conditional dependence between the Se and Sp of iELISA and RBT (a12 and b12) and between the Se and Sp of RBT and SAT (a23 and b23) had the smallest DIC value among all models including condition dependence between any of the two tests. In the code presented below, the sensitivity and specificity of iELISA are represented by: se [1] and sp [1] respectively, of RBT by se [2] and sp [2] respectively, and of SAT by se [3] and sp [3] respectively.
For this model, initial values were calculated using the R program and added at the end of Appendix B.

```
#model
model
{
r[1:8] ~ dmulti(p[1:8], n)
## p[1] = p(111); p[8] = p(000); pr = true prevalence

p[1] <- pr*(se[1]*se[2]*se[3]+se[1]*a23+se[3]*a12) + (1-pr)*((1-sp[1])*(1-sp[2])*(1-sp[3])+(1-
sp[1])*b23+(1-sp[3])*b12)

p[2] <- pr*(se[1]*se[2]*(1-se[3])-se[1]*a23+(1-se[3])*a12) + (1-pr)*((1-sp[1])*(1-sp[2])*sp[3]-(1-
sp[1])*b23+sp[3]*b12)

p[3] <- pr*(se[1]*(1-se[2])*se[3]-se[1]*a23-se[3]*a12) + (1-pr)*((1-sp[1])*sp[2]*(1-sp[3])-(1-
sp[1])*b23-(1-sp[3])*b12)

p[4] <- pr*(se[1]*(1-se[2])*(1-se[3])+se[1]*a23-(1-se[3])*a12) + (1-pr)*((1-sp[1])*sp[2]*sp[3]+(1-
sp[1])*b23-sp[3]*b12)

p[5] <- pr*((1-se[1])*se[2]*se[3]+(1-se[1])*a23-se[3]*a12) + (1-pr)*(sp[1]*(1-sp[2])*(1-
sp[3])+sp[1]*b23-(1-sp[3])*b12)

p[6] <- pr*((1-se[1])*se[2]*(1-se[3])-(1-se[1])*a23-(1-se[3])*a12) + (1-pr)*(sp[1]*(1-sp[2])*sp[3]-
sp[1]*b23-sp[3]*b12)

p[7] <- pr*((1-se[1])*(1-se[2])*se[3]-(1-se[1])*a23+se[3]*a12) + (1-pr)*(sp[1]*sp[2]*(1-sp[3])-
sp[1]*b23+(1-sp[3])*b12)

p[8] <- pr*((1-se[1])*(1-se[2])*(1-se[3])+(1-se[1])*a23+(1-se[3])*a12) + (1-
pr)*(sp[1]*sp[2]*sp[3]+sp[1]*b23+sp[3]*b12)
pr ~ dunif(0,0.048)
se[1] ~ dunif(0.870,0.986)
se[2] ~ dunif(0.670,0.934)
se[3] ~ dunif(0.301,0.967)
sp[1] ~ dunif(0.962,1.00)
sp[2] ~ dunif(0.915,1.00)
sp[3] ~dunif(0.977,1)
```

```
ll1 <- max(-(1-se[1])*(1-se[2]), -se[1]*se[2])
ul1 <- min(se[1]*(1-se[2]),(1-se[1])*se[2])
a12 ~ dunif(ll1,ul1)
ll3 <- max(-(1-se[2])*(1-se[3]), -se[2]*se[3])
ul3 <- min(se[2]*(1-se[3]),(1-se[2])*se[3])
a23 ~ dunif(ll3,ul3)
ll4 <- max(-(1-sp[1])*(1-sp[2]), -sp[1]*sp[2])
ul4 <- min(sp[1]*(1-sp[2]),(1-sp[1])*sp[2])
b12 ~ dunif(ll4,ul4)
ll6 <- max(-(1-sp[2])*(1-sp[3]), -sp[2]*sp[3])
ul6 <- min(sp[2]*(1-sp[3]),(1-sp[2])*sp[3])
b23 ~ dunif(ll6,ul6)

###Correlation between tests
rhoDplus12 <- a12/(sqrt(se[1]*(1-se[1]))*sqrt(se[2]*(1-se[2])))
rhoDplus23 <- a23/(sqrt(se[2]*(1-se[2]))*sqrt(se[3]*(1-se[3])))

rhoDmin12 <- b12/(sqrt(sp[1]*(1-sp[1]))*sqrt(sp[2]*(1-sp[2])))
rhoDmin23 <- b23/(sqrt(sp[2]*(1-sp[2]))*sqrt(sp[3]*(1-sp[3])))

r2[1:8] ~ dmulti(p[1:8],n)
for ( i in 1:8)
{
d[i] <-(pow(r[i]-p[i]*n,2)/(p[i]*n))
d2[i] <- (pow(r2[i]-p[i]*n,2)/(p[i]*n))
I[i] <- step(p[i] -1)*step(-p[i])
}
bayesp <- step(sum(d[]) - sum(d2[]))
}


#####Data
list(r=c(8,3,0,5,9,6,6,1007), n=1044)

####Initial values
list(pr=0.025, se=c( 0.895 , 0.765 , 0.45 ), sp=c( 0.964 , 0.977 , 0.987 ), a12= 0.027825 ,a13= -0.00525
,a23= -0.01175 ,b12=0.010672 ,b13= 0.006032 ,b23= 0.00620100000000001,
r2=c(8,3,0,5,9,6,6,1007))

list(pr=0.015, se=c( 0.917 , 0.85 , 0.68 ), sp=c( 0.985 , 0.985 , 0.995 ), a12= 0.02905 ,a13= 0.01494
,a23= 0.027 ,b12= 0.00727500000000001 ,b13= 0.002425 ,b23= 0.002425 , r2=c(8,3,0,5,9,6,6,1007))

list(pr=0.005, se=c( 0.958 , 0.896 , 0.867 ), sp=c( 0.998 , 0.998 , 0.998 ), a12= 0.016632
,a13=0.015414 ,a23=0.038168 ,b12=
0.000996000000000001,b13=0.000996000000000001,b23=0.000996000000000001 ,
r2=c(8,3,0,5,9,6,6,1007))
```

Chapter 7

Brucella abortus is dominant in Bangladesh

Preamble

Brucellosis is endemic in Bangladesh. However, the information on different species of *Brucella* prevalent in animals is scarce and not available in humans in Bangladesh. It is a zoonosis and almost every human case is directly or indirectly linked to animals or their products. So, the species of *Brucella* responsible for human infection will also reflect the prevalent species in animal populations. In Bangladesh, no laboratory exist with BSL-3 facilities for routine isolation of *Brucella* species from human and animal samples. The knowledge on prevalent *Brucella* species in humans and animals will help to initiate appropriate control measures against brucellosis. This chapter describes the detection of *Brucella* species both from humans and animals in Bangladesh in collaboration with Belgian and German laboratories.

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Brucella abortus is the dominant species in both, man and animals in Bangladesh

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Brief short title: *Brucella abortus* in humans and animals in Bangladesh

Summary

The aims of this study were to isolate *Brucella* spp. from samples of small ruminants, gayal and cattle and to identify the prevalent *Brucella* species in human and animal samples using species-specific real-time PCR (rt PCR) assays. Twenty-three placentas and 17 vaginal swabs from cattle, goats and sheep were initially investigated by Stamp staining to visualize *Brucella*-like organisms. A total of 62 animal samples including Milk Ring Test (MRT) positive bulk milk, bull semen, vaginal swabs and placentas were cultured in Farrell medium (FM) for isolation of *Brucella* organisms. The samples were initially screened by *Brucella* genus specific BCSP31 rt PCR. The *Brucella* genus positive samples were then tested by IS711 rt PCR to detect *Brucella abortus* and *Brucella melitensis* DNA. No *Brucella* organism was seen in stained smears and in FM. Only *B. abortus* DNA was amplified from six animal samples. *Brucella abortus* was found to be the only prevalent species in domestic and semi-wild ruminants in Bangladesh. Interestingly, no *B. melitensis* DNA was detected, which is the species most often associated with human disease. In 13 seropositive human patient sera only *B. abortus* DNA was found corroborating our conclusions and findings. No *Brucella* DNA was amplified from any of the placenta and vaginal swabs indicating that brucellae may not be a major cause of abortion in domestic ruminants in Bangladesh. This is the first report describing species of *Brucella* prevalent in both humans and animals in Bangladesh.

Keywords: *Brucella abortus*, humans, animals, clinical samples

Introduction

Brucellosis is a widespread bacterial zoonosis hampering both human health and animal production. In humans brucellosis is a severely debilitating and disabling illness, with fever, sweating, fatigue, weight loss, headache, and joint pain persisting for weeks to months (Ariza et al., 2007). Brucellosis may become chronic or provoke life-threatening sequelae. The World Health Organization (WHO) has considered brucellosis as a neglected zoonosis. Brucellosis is a serious occupational hazard for livestock farmers, milkers, butchers, hired animal caretakers, veterinarians, and for consumers of raw livestock products in general (Anon., 2005). In animals, abortion, infertility and chronic wasting of affected animals have a considerable negative impact on the family income of peasants in developing countries. Brucellosis is caused by Gram-negative, facultative intracellular bacteria of the genus *Brucella.* This genus consists of at least ten nomospecies having characteristic host range and pathogenicity (Atluri et al., 2011). Four out of the ten known *Brucella* species infect humans commonly and the most pathogenic and invasive species for humans is *Brucella melitensis* (host: sheep and goats), followed in descending order by *Brucella suis* (pigs), *Brucella abortus* (cattle and buffaloes) and *Brucella canis* (canids) (Acha and Szyfres, 2003). In Bangladesh the first report on the isolation of *B. abortus* was made by Pharo et al. (1981) from two cows which were found to be positive using Milk Ring Test (MRT) and Rose Bengal Test (RBT). However, the authors failed to describe the procedure of typing their isolates as *B. abortus*. The true and apparent seroprevalence in domestic ruminants were found to vary from 0.3 to 1.2% and 3.6 to 7.3%, respectively, indicating that brucellosis is endemic in Bangladesh but with a low prevalence (Islam et al., 2013a; Rahman et al., 2013). Indeed, *Brucella* spp and *B. abortus* DNA was amplified from human and bovine sera, respectively, using real time PCR (rt PCR) (Rahman et al., 2012; Rahman et al., 2014a). No data on the prevalent *Brucella* species in humans or gayals, sheep and goats exist although sero-prevalence is reported also for these farm animals.

No recent *Brucella* isolates are available for Bangladesh to support serological or PCR generated data. Isolation also enables the identification of biovars of the prevalent species and thus the tracing back of the source of an outbreak (Al Dahouk et al., 2007). Pure culture is also needed for molecular typing e.g. MLVA to obtain adequate amounts of DNA. Cultivation however is time consuming and is associated with a high risk for laboratory-acquired infections (Yu and Nielsen, 2010).

The aims of this study were therefore to isolate *Brucella* spp. from samples of small ruminants, gayal and cattle, or at least to identify the prevalent *Brucella* species in these samples using species specific rt PCR assays.

Materials and Methods

Ethical statement

The study protocol was peer reviewed and cleared for ethics by the Ethical Review Committee of Mymensingh Medical College. Informed written consent was taken from all individuals prior to the collection of blood.

Milk and semen samples of farm animals were collected during routine milking and semen collection in the farm. Placenta and vaginal swabs from farm animals were collected after abortion where minimal restraining of the animals was needed. This level of intervention has no impact on the well-being of the animal and is also routinely performed by the veterinarians for the purpose of treatment and research.

The faculty of veterinary science of Bangladesh Agricultural University has approved the animal part of this research. Verbal consent of farm owners was obtained prior to the collection of milk, semen, placenta and vaginal swabs from their animals.

Animal samples

Randomly collected milk ring test (MRT) positive bulk milk samples (cattle, goat and gayal [*Bos frontalis*]), convenience samples of placentas, vaginal swabs from different animals (cattle, goat and sheep) and semen samples of bulls (cattle) were used to isolate *Brucella* spp. and to detect *Brucella* genus and species specific DNA (Table 7.1).

Staining

The impression smears of vaginal swabs and placentas were stained by the Stamp method as described by Alton et al. (1988). In brief, the impression smears were dried by flame and stained with working carbol fuchsin solution for 10 min, then decolorized by 3% acetic acid solution for 1 min and counterstained with 1% malachite green solution for 20 seconds. After washing in tap water, they were dried and assessed by microscopy using 100X objective (oil immersion). *Brucella* organisms are pale red in a blue background.

Bacteriology

For the isolation of brucellae, samples were cultured in Farrell's medium using the method described by Alton et al. (1988). Briefly, milk samples were centrifuged at 6,000 g for 15 min and cream and sediment were spread to half of the plate. Swab and semen (150 µl) samples were treated the same way. Placentas were cut into small pieces of about 5 g and 4-5 ml normal saline was added. A homogenate was prepared in a stomacher. About 150 µl of the homogenate was spread as described above. Plates were incubated at 37°C in 7.5% CO_2 and observed after 48 hours of incubation as

brucellae are slow-growing organisms and colonies are only visible after 48 hours of incubation. A sample was considered culture-negative if no growth occurred within 7 days.

DNA extraction from human serum and animal samples

DNA was extracted from a total of 62 animal samples (Table 7.2). DNA was extracted using the DNeasy spin column kit (QIAGEN) according to the manufacturer's protocol. Moreover, DNA originating from 13 human sera which were positive in *Brucella* genus specific rt PCR and were described in a previous paper by Rahman et al. (2012), were also included in this study to identify the disease causing *Brucella* species.

BCSP31 genus specific and *Brucella abortus* and ***Brucella melitensis* specific IS711 real-time PCR**

The BCSP31/IS711 rt PCRs originally described as a multiplex PCR assay (Probert et al., 2004) were performed as single assays to detect *Brucella* spp. *DNA* and/or to distinguish between *B. melitensis* and *B. abortus DNA*, respectively. No further modification of the protocols was done. The species specific assays were applied when a genus specific assay had detected *Brucella DNA* in a sample. Primers and probes were obtained from TIB MOLBIOL (Berlin, Germany). Amplification reaction mixtures were prepared in volumes of 25 µl containing 12.5 µl TaqManTM Universal Master Mix (Applied Biosystems, New Jersey, USA), 0.75 µl of each of the two specific primers (0.3 µM) and 0.5 µl TaqMan probe (0.2µM), 5 µl of template, and 6.25 µl nuclease-free water. The rt PCR reaction was performed in duplicate in optical 96-well microtiter plates (qPCR 96-well plates, Micro AmpTM, Applied Biosystems) using a Mx3000P thermocycler system (Stratagene, La Jolla, California) with the following run conditions, 2 min at 50°C, 10 min at 95°C, followed by 50 cycles of 95°C for 15s and 57°C for 1 min. Cycle threshold values below 40 cycles were considered positive. The threshold was set automatically by the instrument. The samples scored positive by the instrument were additionally confirmed by visual inspection of the graphical plots showing cycle numbers versus fluorescence values.

Table 7.1. Animal samples used to detect *Brucella* species using culture and real time PCR

Sample	Origin	Tested	Serology	Animal Purchase	Abortion	Retained placenta	Repeatbreeding
Cow milk **	Chittagong	5	ND	3 yes; 2 No	4 Yes; I no	2 yes; 3 N0	3 Yes; 2 No
	Mymensingh	3	ND	No	No	No	1 Yes; 2 No
	Sirajganj	5	ND	2 Yes; 3 No	2 Yes; 3 No	2 yes; 3 N0	3 Yes; 2 No
	Dhaka, CCBDF	1	At least one of the three tests	Yes	Yes	Yes	Yes
Goat milk**	Rajshahi Goat Farm	1	At least one of the three tests	Yes	Yes	Yes	No
	Savar Goat Farm	1	At least one of the three tests*	Yes	Yes	Yes	No
Gayal milk**	Bandarban	1	RBT, SAT and iELISA positive	No	Yes	Yes	Yes
Bull semen	Mymensingh	1	RBT positive	NA	NA	NA	NA
	Savar, CCBDF	4	ND	NA	NA	NA	NA
Placenta, cattle	Mymensingh	5	ND	No	Yes	Yes	Yes
Placenta, goat	Mymensingh	10	ND	Yes	Yes	Yes	No
Placenta, sheep	Mymensingh	6	ND	Yes	Yes	Yes	No
	Savar, Dhaka	2	At least one of the three tests	Yes	Yes	Yes	No
Vaginal swab (cattle)	Mymensingh	4	ND	No	Yes	Yes	No
Vaginal swab (goat)	Mymensingh	10	ND	Yes	Yes	Yes	No
Vaginal swab (sheep)	Mymensingh	3	ND	Yes	Yes	Yes	No

Legend: * Three tests indicate RBT, SAT and iELISA; **All milk samples (herd/flock) were Milk Ring Test (MRT) positive, ND: Not done; NA: Not applicable; RBT: Rose Bengal Test; SAT: Slow Agglutination Test.

Table 7.2. Summary of real time PCR results

		Positive		
Sample	Number	*Brucella* genus rt PCR	*Brucella abortus* rt PCR	*Brucella melitensis* rt PCR
Milk cow	14	3	3	Negative
Milk goat	2	1	1	Negative
Milk gayal	1	1	1	Negative
Placenta cattle	5	Negative	ND	ND
Placenta goat	10	Negative	ND	ND
Placenta sheep	8	Negative	ND	ND
Vaginal swab cattle	4	Negative	ND	ND
Vaginal swab goat	10	Negative	ND	ND
Vaginal swab sheep	3	Negative	ND	ND
Bull semen	5	1	1	Negative
Human serum	13	13	13	Negative
Total	75	19	19	Negative

Legend: rt PCR: Real Time PCR; ND: Not done.

Results

Brucella-like bacteria were not found in any of the stained smears. No growth of *Brucella* spp. was noted in any of the clinical samples. Six out of 62 animal samples investigated were positive in the genus specific BCSP31 rt PCR assay (Table 2). Five milk (three cattle, one goat and one gayal) and one semen sample were positive in the *B. abortus* specific rt PCR. *Brucella abortus* DNA was amplified from 13 human serum samples. Data on demographic characteristics, occupation and animal contacts of 13 humans are given in Table 3. All patients (all of male sex) were positive in three serological tests, had one or more clinical symptoms and signs suggestive of brucellosis and had been treated (data not shown). No *B. melitensis* DNA could be amplified from either human or animal samples.

Table 7.3. Characteristics of 13 patients diagnosed positive with a *Brucella abortus* real-time PCR

ID	Age	Education	Contact with animal	Duration of contact	Occupation
HS 69	65	Secondary	Cattle	35 years	Milkers
HS 70	53	Secondary	Cattle	9 years	Milkers
HS 72	40	Secondary	Cattle	20 years	Milkers
HS 75	45	Primary	Cattle	30 years	Livestock farmer
HS 76	40	Primary	Cattle	20 years	Milkers
HS 77	60	Primary	Cattle	40 years	Milkers
HS 78	42	Primary	Cattle, goat	10 years	Livestock farmer
HS 80	40	Primary	Goat	25 years	Livestock farmer
HS 83	45	Primary	Cattle	22 years	Milkers
HS 85	45	Primary	Cattle, goat	20 years	Butcher
HS 86	28	Secondary	Cattle, goat	14 years	Livestock farmer
HS 87	12	Secondary	Goat	2 years	Livestock farmer
HS 88	35	None	Cattle	14 years	Milkers

Discussion

We describe the successful amplification of *B. abortus* DNA from human serum, dairy milk (cattle, goat, gayal) and bull (cattlel) semen from Bangladesh. Animals and their products are the almost exclusive source of human infection. Consequently, the presence of *B. abortus* DNA in human samples is also proof of presence of *B. abortus* within the (dairy) animal populations of Bangladesh. The presence of *B. abortus* DNA in milk and semen also supports this hypothesis. Moreover, the detection of *B. abortus* DNA in bovine sera from other parts of the country added independent evidence in this regard (Rahman et al., 2014). At present, it can be supposed that *B. abortus* is the main disease causing *Brucella* species in patients and infected ruminants (Figure 7.1).

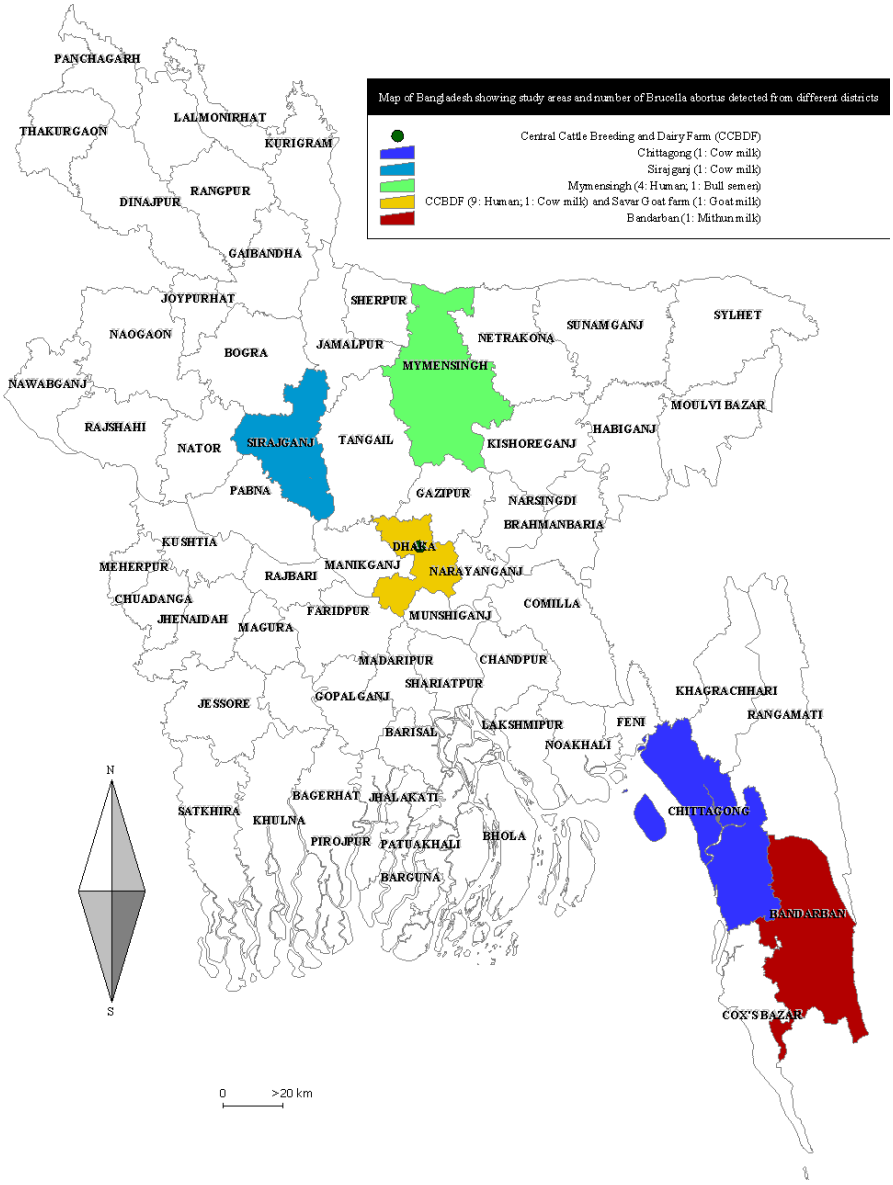
Brucella abortus DNA was detected from milk samples of gayal in the Bandarban hill district of Bangladesh. The gayal (*Bos frontalis*), synonymous with mithan or mithun, is a semi-domestic ruminant found in the hill regions of northeast India, Myanmar, Bhutan, Bangladesh, China and Malaysia (Simoons and Simoons, 1968; Mason, 1988). The results of phylogenetic analyses indicate that the gaur (*Bos gaurus*) is the wild ancestor of the gayal (Dorji et al., 2010; Tanaka et al., 2011). The source of infection in gayals is not clear. However, Bandarban shares a common boundary with

Mizoram State of India and Chin State of Myanmar. Although no reports on isolation of *Brucella* species from gayal in India exist, *Brucella* seroprevalence in gayal has been reported, with the highest in the Mizoram gayal (Rajkhowa et al., 2005). Moreover, gayals from Tripura and Mizoram cross into Bangladesh due to their contiguous habitat in north-eastern India and Bangladesh (Choudhury et al., 2002).

Another possible way of introducing brucellosis in the gayal herd may be crossbreeding with cattle. Holstein-Friesian bull semen, which originated from Central Cattle Breeding and Dairy Farm (CCBDF) was used for crossbreeding purposes (Huque et al., 2001). As breeding bulls in CCBDF were found to be infected with brucellosis (unpublished observations), semen used for crossbreeding gayals could also have been the source of infection.

Brucella abortus DNA was also detected from one goat milk sample. Although *B. melitensis* is the most common agent of caprine brucellosis, infection in goats due to *B. abortus* may also occur and is a public health hazard especially in countries where *B. melitensis* is non endemic e.g. Brazil (Lilenbaum et al., 2007). *Brucella melitensis* has also never been reported from Uruguay, Ecuador, El Salvador and Costa Rica (Moreno, 2002; Garin, 2011; Jorge Ron-Roman pers. comm.). We do believe that *B. melitensis* is yet not endemic in Bangladesh and *B. abortus* is spread due to cohabitation of different small ruminants and infected cattle on the same premise or pasture. The distribution of *Brucella* species and their biotypes may vary within a country and even within states of a country. In Indian cattle, *B. abortus* is most frequently isolated (22/78). *Brucella melitensis* is rarely isolated from cattle and buffalo (3/46) (Polding, 1942; Sen and Sharma, 1975; Mathur, 1985; Hemashettar et al., 1987). Among cattle, *B. abortus* biotype 1 (21/39) is reported to be the predominant biotype in most parts of India (Sen and Sharma 1975). About 84.2% (32/38) and 15.8% (6/38) sheep isolates and 78.0% (39/50) and 22.0% (11/50) goat isolates are *B. melitensis* and *B. abortus,* respectively (Mathur, 1985). *Brucella melitensis* is the dominant (191/191; 53/53) cause of human brucellosis in India (Mathur, 1985; Mantur et al., 2006). Sporadic isolation of *B. abortus* from human cases was also reported (Sen and Sharma, 1975; Mathur, 1985; Mantur et al., 1994; Pathak et al., 2014).

Figure 7.1. Map of Bangladesh showing the study areas and number of *Brucella abortus* infected/detected from different districts



In neighbouring Myanmar, the reported seroprevalence of brucellosis in dairy cattle is 4.01% (25/623) but no report on isolation of *Brucella* species from humans and animals was found in the available literature (Tun et al., 2008). *Brucella abortus* infection seems to be a crossborder problem and cooperation effort is needed to control this disease in the region.

Detection of *Brucella* DNA was reported even from serum samples that were taken a long time after clinical signs of disease had ceased in these patients (Navaro et al., 2006). The thirteen individuals positive for the three serological tests presented clinical signs and symptoms suggestive of brucellosis and indeed all recovered after standard brucellosis treatment had been administered. We could demonstrate that confirmatory diagnosis by species specific rt PCR is adequate for a well-timed onset of treatment or switch of medication. Probert et al. (2004) developed a multiplex rt PCR to detect *B. abortus* and *B. melitensis* from culture growth. Modifying this technique, we were able to show that IS711 species-specific rt PCR is capable of amplifying *Brucella* DNA from human sera and animal samples at species level. .

None of the stained smears showed *Brucella* like organisms and culture results were negative. Abortion is the most common clinical sign of brucellosis in female domestic ruminants and usually aborted foetuses, foetal membranes and fluids contain high bacterial loads contaminating the environment and thereby resulting in a high risk of infection to other animals (Saegerman et al., 2010). In our study, none of the 40 foetal membranes and vaginal swabs originating from cattle, sheep and goats contained *Brucella* DNA. Although the sample size may be small, it can be supposed that *Brucella* is not a major cause of abortion in domestic ruminants in Bangladesh. The status of other infectious agents causing abortion in domestic ruminants in Bangladesh is not well documented. Rahman (2014) reported that 15.95% (15/94) dairy cattle herds in Bangladesh are Q fever positive in bulk milk ELISA, a disease reported to be responsible for abortion in domestic ruminants (Angelakis and Raoult, 2010). Although toxoplasmosis is endemic in domestic ruminants and humans in Bangladesh, its association with abortion has not been elucidated yet (Samad et al., 1993; Samad et al., 1997; Ashrafunnessa et al., 1998; Shahiduzzaman et al., 2011; Rahman et al., 2014b). The status of other infectious causes of abortion like *Neospora*, *Leptospira*, *Listeria*, *Chlamydia*, bovine viral diarrhoea virus, infectious bovine rhinotracheitis virus, etc. (Holler, 2012) in Bangladesh is not known at all.

Brucella DNA was detected from MRT positive milk samples but they proved culture negative. The possible reason for unsuccessful recovery of isolates may be that the samples were old (in storage for 2-3 years after collection and having been thawed and refrozen several times). Indeed, isolation is most likely during the acute phase of infections caused by *B. melitensis* or *B. suis* and less successful in *B. abortus* infections. (Corbel, 1997; Al Dahouk et al., 2002). The presence of competing organisms (possibly with presence of antibiotic resistance) may be another potential reason of isolation failure when the samples were cultured in the presence of *Brucella* selective supplements (Al Dahouk et al.,

2002). Other authors from Bangladesh also failed to isolate *Brucella* from vaginal swabs and foetal membranes (Das et al., 2013; Dey et al., 2013; Islam et al., 2013b).

Brucella-like organisms were not seen in stained smear and *Brucella* was not isolated from any of the clinical samples. *Brucella abortus* was found to be the only prevalent species among humans and animals in Bangladesh. Interestingly, no *B. melitensis* DNA was detected which is the species most often associated with human disease.

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Chapter 8

Brucellosis in occupationally exposed people

Preamble

Brucellosis is a zoonosis and considered as an occupational hazard of livestock farmers, dairy workers, veterinarians, slaughterhouse workers, and laboratory personnel. In Bangladesh, there are almost 19 million livestock farmers (12.7% of total population); the exact number of diary workers, veterinarians, and slaughterhouse workers are not known. Very little is known about the prevalence and risk factors of brucellosis in this group of people in Bangladesh. This chapter describes the prevalence and risk factors of brucellosis in occupationally exposed peoples in Bangladesh based on a random sample of livestock farmers and convenient sample of diary workers, veterinarians and butchers.

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Seroprevalence and Risk Factors for Brucellosis in a High-Risk Group of Individuals in Bangladesh

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Abstract

Brucellosis is an occupational hazard of livestock farmers, dairy workers, veterinarians, slaughterhouse workers, and laboratory personnel, all of whom are considered to belong to the high-risk occupational group (HROG). A study was undertaken to determine the seroprevalence of brucellosis, identify risk factors associated with brucellosis seropositivity, and detect *Brucella* at genus level using real-time polymerase chain reaction (PCR) among people in the HROG in the Dhaka division of Bangladesh. A sample of 500 individuals from the HROG was collected from three districts of Dhaka division of Bangladesh. A multiple random effects logistic regression model was used to identify potential risk factors. Two types of real-time PCR methods were applied to detect *Brucella* genus–specific DNA using serum from seropositive patients. The prevalence of brucellosis based on the three tests was observed to be 4.4% based on a parallel interpretation. The results of the multiple random effects logistic regression analysis with random intercept for district revealed that the odds of brucellosis seropositivity among individuals who had been in contact with livestock for more than 26 years was about 14 times higher as compared to those who had less than 5 years of contact with livestock. In addition, when the contact was with goats, the odds of brucellosis seropositivity were about 60 times higher as compared to when contact was with cattle only. Noticeable variation in brucellosis seropositivity among humans within the three districts was noted. All of the 13 individuals who tested positive for the serological tests were also positive in two types of real-time PCR using the same serum samples. Livestock farmers of brucellosis positive herds had a significantly higher probability to be seropositive for brucellosis. The study emphasized that contact with livestock,

especially goats, is a significant risk factor for the transmission of brucellosis among individuals in the HROG.

Introduction

Brucellosis is an occupational hazard of livestock farmers, dairy workers, veterinarians, slaughterhouse workers, and laboratory personnel, all of whom are considered to belong to the high-risk occupational group (HROG). It is caused by bacteria of the genus *Brucella*, which manifests in different variants in different animal species. For example, *Brucella abortus* is mostly associated with cattle and *B. melitensis* with sheep, goats, and humans (Pappas *et al.*, 2005). Infection can be acquired through ingestion of unpasteurized dairy products such as soft cheeses, yogurts, and ice creams. However, direct contact with infected animals and contact with vaginal discharge, urine, feces, or blood of infected animals (especially among abattoir workers, herdsmen, veterinarians, butchers, and personnel in microbiologic laboratories) is an important transmission route. Also, *Brucella* can be transmitted through skin lesions and the mucous membrane of conjunctiva, and by inhalation of infected aerosolized particles (Wise, 1980; Young, 1983; Pappas, 2005).

Human brucellosis remains the commonest zoonotic disease worldwide, with more than 500,000 new cases reported annually (Pappas *et al.*, 2006). It is associated with a chronic debilitating infection with substantial residual disabilities. The onset of the disease may be sudden, over a period of a few days, gradual, over a period of weeks to months, or associated with non-specific symptoms that include undulating fever, fatigue, malaise, headache, backache, and arthralgia (Mantur *et al.*, 2007).

Human brucellosis poses major economic and public health challenges in affected countries especially in the Mediterranean countries of Europe, northern and eastern Africa, Near East countries, India, Central Asia, Mexico, and Central and South America (Pappas *et al*., 2006). A limited number of studies have estimated the seroprevalence of human brucellosis in Bangladesh. These studies revealed that the prevalence of human brucellosis is 6–12.8% (Rahman *et al*., 1983, 1988; Muhammad *et al*., 2010). The variations in the seroprevalence reported may be due to differences in the number of samples (which ranged from 100 to 210 people in the HROG) and number of diagnostic tests used and the manner in which they were interpreted. None of these studies rigorously investigated risk factors associated with human brucellosis seropositivity despite substantial evidence that various factors such as occupational status, consumption of unpasteurized dairy products, type of animal handled, religious background, and whether or not assisted parturition (or assisted calf birth) is practiced influence the likelihood of brucellosis seropositivity (Abo-Shehadan *et a*l., 1996; Al-Shamahy *et al*., 2000; Swai and Schoonman, 2009; Sofian *et al*., 2008; John *et al.*, 2010).

The diagnosis of human brucellosis in Bangladesh has predominantly been based on serological tests namely the Rose Bengal Plate Test (RBT), Standard Tube Agglutination Test (STAT), and the Indirect Enzyme-Linked Immuno-sorbent Assay (iELISA), which are not gold standard tests (Rahman *et al*., 1983, 1988; Muhammad *et al*., 2010). These tests may not be able to differentiate between an active and a nonactive infection (Nimri, 2003). Isolation of *Brucella* spp. is the gold standard test for brucellosis. However, this is a slow process that sometimes requires Level 3 biocontainment facilities and highly skilled technical personnel, leading to high costs (Navarro *et al*., 2004). Handling of live *Brucella* species is also associated with possible infection to laboratory personnel if biosafety rules are not strictly monitored (Yu and Nielson, 2010). Due to the speed, safety, and high sensitivity and specificity of the polymerase chain reaction (PCR), all of the positive samples based on three serological tests were subjected to PCR.

The aim of this study was to determine the seroprevalence of brucellosis, identify risk factors associated with human brucellosis seropositivity, and detect *Brucella* at the genus level using real-time PCR. The results of this study may be used to inform the development and implementation of control measures bent on sensitizing the population at risk, regulating management practices at abattoirs and farms, and abating the incidence of human brucellosis in Bangladesh.

Methods

Ethical clearance

The study protocol was peer reviewed and cleared for ethics by the Ethical Review Committee of Mymensingh Medical College. Verbal and written consents were also taken from all individuals prior to blood sample collection.

Study population and survey area

The study was carried out between September 2007 and August 2008 among livestock farmers, milkers, butchers, and veterinary practitioners in the Mymensingh, Sherpur, and Dhaka districts of Bangladesh.

In Bangladesh, about 85% of rural households own animals, and 75% of the population rely on livestock to some extent for their livelihood (www.fao.org). Livestock farmers considered for this survey were the owners or hired animal caretakers of 571 herds of Mymensingh and Sherpur districts and also workers in two government-owned farms in Dhaka District from where blood samples were taken for determining the seroprevalence of brucellosis in domestic ruminants.

Veterinary professionals at risk for brucellosis in these districts include approximately 100 individuals (approximately 25 veterinarians and animal production specialists, including their assistants) in the Department of Livestock Services (DLS) of the Bangladesh Government. Some veterinarians (actual number not known) work in Non-Governmental Organizations (NGOs) having livestock development

programs. The exact number of butchers and slaughterhouse workers, as well as the actual number of milkers in these areas are not known. Estimates of butchers and milkers in these areas are 200 and 300, respectively.

Convenience samples from the population of milkers, butchers, and veterinary practitioners were obtained from Mymensingh and Dhaka districts. Milkers were selected from the Central Cattle Breeding and Dairy Farm (CCBDF) in Savar and commercial dairy farms, and those who collect and sell milk from small holder dairy farms (vendors) were also counted in this group. Butchers were selected from different locations of Mymensingh district, where a great proportion of the people are involved in this profession. Veterinary practitioners included were veterinary surgeons, veterinary field assistants, and veterinary students of Mymensingh and Dhaka districts of Bangladesh.

Questionnaire data collection

Information was collected through personal face-to-face interviews. Questionnaires recorded the following information for each subject: age, sex, address with mobile telephone number where available, level of education, occupation, type of animal handled and duration of contact in years, and previous history and presence of symptoms (pyrexia, sweating, arthralgia, backache, and headache) suggestive of brucellosis (Mantur *et al*., 2006). The full questionnaire is available upon request from the corresponding author.

Collection and handling of blood samples

About 4 mL of blood was collected with disposable needles and Venoject tubes, labeled, and transported to the laboratory on ice (after clotting) within 12 h of collection. Blood samples were kept in the refrigerator (2–8°C) in the laboratory, and 1 day later sera were separated by centrifuging at 6000 g for 10 min. Each serum was labeled to identify the individual and stored at - 20°C. Each serum was divided into two tubes, each containing about 1 mL of serum. One aliquot was used for testing, and the other was preserved in a serum bank. Among the total of 500 individuals considered to be in the HROG, 386 were livestock farmers. The serological status of these farmers was compared with that of the herd they managed.

Serological tests

All blood samples were tested in parallel using the RBT, STAT, and iELISA in the Medicine Department laboratory of Bangladesh Agricultural University (BAU), Mymensingh, Bangladesh. The tests are briefly described next.

Rose Bengal Test (RBT). RBT was performed according to standard procedure (Alton et al., 1988). Briefly, sufficient antigen, test sera, and positive and negative control sera for a day's testing were removed from refrigeration and brought to room temperature (22 – 4°C). Equal volumes (30 lL) of serum and antigen (concentrated suspension of *B. abortus* biotype 1 [Weybridge 99]; Institut

Pourquier, Montpellier, France) were mixed and rotated on a glass plate for 4 min. The result was considered positive when agglutination was noticeable after this delay.

Standard Tube Agglutination Test (STAT). STAT was carried out on doubling dilution of serum from 1:20 to 320 according to standard procedure (Alton *et al*., 1988). *Brucella abortus* antigen (Cypress Diagnostics, Langdorp, Belgium) was used according to the instruction of the manufacturer. The test tubes were incubated at 37°C for 24 h. Positive reactions were determined by observing agglutination in 1:160 or more dilution of test serum.

Indirect Enzyme-Linked Immunosorbent Assay (iELISA). iELISA was performed according to Limet *et al.* (1988) using *B. abortus* biotype 1 (Weybridge 99) as antigen. For the standard curve, six dilutions (1/270 to 1/8640) of the positive reference serum (no. 1121) were prepared. Fifty microliter of serum dilutions (1:50 in buffer consisting of 0.1M glycine, 0.17M sodium chloride, 50mM EDTA, 0.1% (volume) Tween 80, and distilled water, pH 9.2) were added to the wells in duplicate. The plates were incubated for 1 h at room temperature. Binding antibodies were detected using a Protein G-horseradish peroxidase (G-HRP) conjugate as described by Saegerman *et al.* (2004). Citrate-phosphate buffer containing 0.4% O-phenylenediamene and 2 mM H2O2 was used to visualize the peroxidase activity. Reading of optical densities (OD) was done at 492 nm and 620 nm using VMax® Micro-plate Reader. The results (OD_{492} –OD_{620}) were expressed as antibody units in comparison with a reference serum. The conversion of ODs into units (U/mL) was done using six dilutions of the reference serum to establish a standard curve. The cut-off value for a positive result was defined at 20 U/mL of test serum.

Real-time PCR

Real-time PCR was used to detect *Brucella* spp., mainly B. abortus and B. melitensis. DNA was isolated from 13 sera that tested positive on all three serological tests. About 200 ll of serum was used for extraction of DNA from sera using DNeasy spin columns (Qiagen Inc., Valencia, CA) according to the manufacturer's recommendations. The most frequently described PCR target for the diagnosis of human brucellosis is the bcsp31 gene encoding a 31-kDa antigen conserved among *Brucella* spp. (Navarro *et al*., 2004). The BCSP31-PCR assay was carried out using standard procedure (Baily *et al*., 1992; Bounaadja *et al*., 2009), and IS711-PCR was done using the procedure described by Halling *et al.* (1993). The cut off for the positivity is 40. Above this threshold, the sample is considered negative and below the threshold it is considered positive. The real-time PCR assay was performed at the National Reference Centre for Brucellosis, Veterinary and Agrochemical Research Centre (CODA-CERVA) in Belgium.

Statistical analysis

To determine the potential risk factors associated with human brucellosis sero-positivity, individuals were considered positive if they tested positive in at least one serological test along with the presence of any of the clinical symptoms suggestive of brucellosis as mentioned in questionnaire's data collection section.

Firstly, a univariate analysis was performed using a random effects logistic regression model. The model uses, as response, the brucellosis status of the individuals and each risk factor or indicator variable in turn as the independent variable. Occupational status was forced into the model as it is of primary interest. The possible effects of variations in brucellosis seropositivity among districts were accounted for by incorporating district as a random effect in the model (Van-Leeuwen *et al.*, 2010).

Variables with a p-value ≤ of 0.10 in the univariate analysis were further analyzed in a multivariable random effects logistic regression model. A manual forward stepwise model building approach was employed with the Akaike's Information Criterion (AIC) as the calibrating parameter to select the final model. In this approach, the best univariate model is selected as the model with the lowest AIC value. The remaining variables are then added each in turn to form three variable models. The best three-variable model is selected based on the AIC. This is repeated until the addition of one more variable fails to improve the model fit; in other words if the AIC does not change or starts to increase. The model with the smallest AIC is considered to be the most appropriate model. The effects of confounding were investigated by observing the change in the estimated coefficients of the variables that remain in the final model once a non-selected variable is included. When the inclusion of a non-significant variable led to a change of more than 25% of any parameter estimate, that variable was considered to be a confounder and was included in the model. All two-way interaction terms of the variables remaining in the final model were assessed for significance based on the AIC values, i.e., comparing the AIC values of the model with the desired interaction term and the corresponding model with no interaction terms (Dohoo *et al*., 2003).

The intra-class correlation coefficient (ICC), which is a measure of the degree of clustering of individuals belonging to the same district, was computed. In random effects logistic regression models, the individual level variance δ^2 on the logit scale is usually assumed to be fixed to ${\pi^2}/{3}$ (Snijders and Bosker, 1999). The variability attributed to differences among districts is given by:

$$ICC_{District} = {\delta^2}_{INT:DISTRICT} \Big/ ({\delta^2}_{INT:DISTRICT} + {\pi^2}/{3})$$

If the ICC is zero, it implies that there is no variability in brucellosis seropositivity among districts but rather a higher variability among humans within districts.

The models were built using the xtmelogit () function in STATA, version 11, software (StataCorp LP, College Station, TX). Model selection was done using Laplacian approximation, whereas parameter estimates from the final model were obtained using Adaptive Gaussian Quadrature (Twisck, 2003).

The robustness of the final model was assessed by increasing the number of quadrature (integration) points and monitoring changes in parameter estimates (Franken *et al*., 2009).

Results

Descriptive statistics

There were a total of 500 individuals from the Mymensingh, Sherpur, and Dhaka districts of Bangladesh. The prevalence of brucellosis based on the three tests was observed to be 4.4% following a parallel interpretation of the three tests. The prevalence of brucellosis for each category of each of the factors considered is presented in Table 8.1.

The prevalence was found to be highest (28.3%) among individuals who indicated symptoms linked to brucellosis. The prevalence of brucellosis was also found to be higher among milkers as compared to livestock farmers, butchers and veterinary practitioners. The prevalence of brucellosis appeared to be higher among individuals who handled only goats; the prevalence was found to be higher with increased duration of contact with animals. The prevalence of brucellosis among males was higher (5.6%) compared to that of females (0.8%). Finally, among those who consumed raw milk, the prevalence was higher (11.4%) as compared to those who did not consume raw milk (3.9%). Out of 571 herds, 386 people of 337 (59.0%) herds agreed to provide blood samples.

Factors associated with brucellosis seropositivity in humans based on a univariate analysis

The results of the univariate random effects logistic regression analysis with occupation forced into the model and a random intercept for district revealed that, type of animal handled, and duration of contact with animals were highly significantly associated with human brucellosis seropositivity ($p < 0.05$; Table 8.1). On the other hand, gender was not significant at the 5% level, but since its p-value was ≤ 0.10, it was considered as a potential risk factor and was thus included in the multivariable random effects model.

Multiple random effect logistic regression model

Out of the potential risk factors initially considered in the multiple random effects logistic regression model, four were included in the final model. None of the two-way interaction terms were statistically significant ($p > 0.05$). Gender appeared to be a confounding variable and was therefore included in the model. Increasing the number of quadrature points had no influence on the estimated fixed effects and the variance component parameters indicating that the model is robust. The estimated odds ratios (ORs) and their 95% confidence intervals (CIs) are presented in Table 8.2.

Table 8.1. Potential risk factors associated with household level seroprevalence of Brucellosis based on a univariate random effects model

Factor	*Tested*	*Positive*	*Prevalence*	*95% CI*
Age group (years)				
14-20	44	1	2.3	(0.06-12.0)
21-40	231	7	3.0	(1.2-6.1)
41-80	225	14	6.2	(3.4-10.2)
District				
Dhaka	63	12	19.0	(10.2-30.9)
Mymensingh	410	10	2.4	(1.2, 4.4)
Sherpur	27	0	0.0	(0, 12.8) [a]
Education				
None to secondary	468	22	4.7	(3.0, 7.0)
College to university	32	0	0.0	(0, 10.9) [a]
Sex [b]				
Female	125	1	0.8	(0.02, 4.4)
Male	375	21	5.6	(3.5, 8.4)
Occupation				
Livestock farmer	386	10	2.6	(1.2, 4.7)
Milker	55	10	18.2	(9.1, 30.1)
Butcher	40	1	2.5	(0.06, 13.2)
Veterinary practitioner	19	1	5.3	(0.1, 26.0)
Duration of contact with animals (years) [c]				
0.08-5	169	1	0.59	(0.01, 3.3)
6-15	166	3	1.8	(0.4, 5.2)
16-25	91	6	6.6	(2.5, 13.8)
≥ 26	76	14	16.2	(8.7, 26.6)
Type of animal handled [c]				
Cattle only	343	12	3.5	(1.8, 6.0)
Cattle and goat	86	4	4.7	(1.3, 11.5)
Goat	71	6	8.5	(3.2, 17.5)
Drinking raw milk				
No	465	18	3.9	(2.3, 6.0)
Yes	35	4	11.4	(3.2, 26.7)
Symptoms [c]				
No	440	5	1.1	(0.4, 2.7)
Yes	60	17	28.3	(17.5, 41.4)

Legend: [a]Exact binomial confidence interval; [b]Significant at 10% but not at 5% so was considered as a potential risk factor or indicator variable and therefore included in the multivariable random effects logistic regression model; [c]Highly significant ($p < 0.001$); CI, confidence interval.

Table 8.2. Final model of risk factors associated with human Brucelosis seropositivity among 500 people at high risk for Brucellosis within the Mymensingh, Sherpur, and Dhaka districts of Bangladesh

Risk factors	*OR*	P-*value*	*95% CI*
Occupational status			
Butcher	1	—	—
Livestock farmer	2.8	0.384	(0.28, 26.94)
Milker	16.9	0.053	(0.99, 293.85)
Veterinary practitioner	3.7	0.468	(0.11, 122.59)
Animal handled			
Cattle only	1	—	—
Cattle and goat	9.5	0.053	(0.97, 98.83)
Goat	59.8	< 0.001	(6.40, 559.93)
Duration of contact with animals (years)			
0.08–5	1	—	—
6–15	2.6	0.427	(0.24, 28.43)
16–25	9.9	0.047	(1.03, 95.30)
≥ 26	14.2	0.019	(1.56, 129.6)
Sex			
Women	1	—	—
Men	6.2	0.120	(0.62, 60.98)
Variance components	Estimate	SE	
District	1.22	0.81	(0.34, 4.46)

Legend: OR, odds ratio; SE, standard error; CI, confidence interval.

The variance component of the model with no covariates yielded an ICC of 0.28. This implies that 28% of the variance in the log odds of brucellosis seropositivity is attributed to differences among districts. After incorporating the significant risk factors, the ICC for districts remained almost the same at 0.27.

The between-district variability of 27% suggests that there is a weak variability in human brucellosis cases among districts in Bangladesh but a high between-human variability within districts. From the final model (Table 8.2), it can be seen that for those people who owned or handled mainly goats, the odds of brucellosis seropositivity were significantly higher than those of people who handled only cattle (OR = 59.8, $p < 0.001$).

Also though, not statistically significant, relative to those who owned or handled only cattle, those who handled cattle and goats were 9.5 times more likely to be brucellosis seropositive. The odds of human brucellosis seropositivity increased significantly with an increase in the duration of contact with animals.

In fact, for individuals who had been working with livestock for more than 26 years, the odds of brucellosis seropositivity were significantly higher compared to those who had been working for less than 26 years (OR = 14.2, p = 0.02).

Results of the real-time PCR

The findings from the real-time PCR for the seropositive cases are shown in Table 8.3. All of the 13 positive human cases based on the three tests were positive in both PCR. The mean Ct values of BCSP31 and IS711 real-time PCR test were 37.03 and 34.40, respectively, indicating a positive reaction in both situations.

The relationship between brucellosis-positive animal herds and occurrence of human infection is shown in Table 8.4. Livestock farmers of brucellosis-positive herds had significantly higher odds to be infected (OR = 10.2; 95% CI: 2.8–37.1).

Table 8.3. Real time polymerase chain reaction (PCR) confirmation of seropositive patients

PCR type	*Tested*	*Positive*	*CT values*		
			Mean ±SE	*Min*	*Maximum*
BCSP31	13	13	37.03±0.46	33.2	39.4
IS711	13	13	34.40±0.44	31.0	36.0

Legend: Ct, cycle threshold; SE, standard error.

Table 8.4. Relationship between Brucellosis seropositivity status of livestock herds (involving cattle, sheep, and goats) and human Brucellosis seropositivity

Herd status	*Livestock farmers*			
	Tested	*Positive*	*Prevalence*	*95% Confidence Interval*
Herds negative	309	3	0.1	(0.2, 2.8)
Herds positive	77	7	9.1	(3.7, 17.8)

Legend: CI, Confidence Interval.

Discussion

The present study represents the first report on the risk factors for brucellosis among individuals in high-risk occupations in Bangladesh. The results of this study suggest that the presence of brucellosis-related symptoms, type of animals owned or handled, and duration of contact with animals are highly significantly associated with brucellosis seropositivity in humans in the Mymensingh, Sherpur, and Dhaka districts of Bangladesh. In addition, there is considerable variability in brucellosis seropositivity among humans due to heterogenous distribution of different HROG people in different districts. In Mymensingh District, all type of HROG people were sampled; in Dhaka the sample was predominantly composed of milkers; and in Sherpur, all people sampled were livestock farmers. It was observed from this study that milkers have relatively higher brucellosis seroprevalence than livestock farmers, butchers, and veterinary practitioners. These factors may explain the variability of brucellosis seropositivity among individuals within district.

The prevalence of brucellosis in the HROG based on parallel interpretation of the three tests was observed to be 4.4%. This seroprevalence is comparable to those of other reports from this area (Muhammad *et al*., 2010; Thakur and Thapliyal, 2002). Brucellosis in humans in Bangladesh is ignored, misdiagnosed, and thought to have very low sporadic incidence. The findings of this study reveal that brucellosis among people in the HROG is not uncommon. In this study, about 28.5-fold increased odds of infection was found in HROG individuals having clinical symptoms suggestive of brucellosis. So, medical doctors should use these findings as a diagnostic clue in HROG individuals for brucellosis (Araj and Azzam, 1996).

The duration of contact with animals was found to be strongly associated with human brucellosis seropositivity. This finding is consistent with results from other studies (Rahman et al., 1983; Abo-Shehada *et al*., 1996). This could be due to long-term cumulative exposure by individuals to brucellosis-infected livestock or to a contaminated environment (which increases the chance of getting infected).

It was observed in this study that about 14.2% (55/386) livestock farmers shared same premises with animals, and the majority (29/55) of livestock species kept are goats. The relatively low socioeconomic status of the farmers makes it impossible to build separate animal houses for protection from predators, especially in the case of small ruminants. Among those who keep goats inside their houses, the seroprevalence of brucellosis was 6.9%. This finding of very intimate contact with goats may explain the relatively higher seroprevalence of brucellosis in HROG individuals having contact with goats. Similar observations were also made by other authors (Rah-man *et al*., 1988; Omer *et al*., 2002).

Brucellosis is an occupational disease in livestock farmers, dairy workers, butchers, veterinarians, and laboratory personnel. For this reason, occupational status was forced into the final model. The odds of

brucellosis seropositivity appeared to be high for milkers (OR = 16.9), which was consistent with findings from other studies (Rahman *et al*., 1983; Omer *et al*., 2002). Among dairy farm workers, undulant fever seems to be almost (but not entirely) limited to those who handle and milk the cows. The higher seroprevalence in milkers confirms the impression that intimate contact with animals is more important than consumption of infected milk (McDevitt, 1971).

Even though gender was an important confounding variable in this study, its non-significance as a risk factor for brucellosis seropositivity in this study may be explained by the very low proportion of brucellosis seropositive cases among females (one out of 25). Males were apparently about six times more likely to be brucellosis seropositive as compared to females. This is because the occupations described in this study are male dominated in Bangladesh. Several other studies have indicated gender as significant risk factors for brucellosis (Wassif *et al*., 1992; Shehata *et al*., 2001; Mantur *et al*., 2004; Meky *et al*., 2007).

In other studies, the consumption of raw milk has been shown to be the most significant risk factor for the transmission of brucellosis among humans (Godfroid *et al.*, 2011). However, in this study, consumption of unpasteurized dairy products was not a significant risk factor. This is probably due to the fact that our study subjects are limited to those in the HROG, most of whom are not the main consumers of the finished dairy products. To investigate the role of consumption of unpasteurized dairy products, a study should be performed that covers the entire population and not only those people in the HROG.

A total of 13 individuals from the HROG were positive in all three serological tests. From the results of both real-time PCR methods, *Brucella* genus–specific DNAs were detected in all of those 13 seropositive cases. This indicates that, among the test positive cases, there were no false positives. The detection of *Brucella* genus specific–DNA using real-time PCR from human sera is in agreement with findings from other studies (Zerva *et al*., 2001; Debeaumont *et al*., 2005; Queipo-Ortuno *et al.*, 2005). Detection of *Brucella* genus–specific DNA using real-time PCR is a rapid, highly sensitive, specific, and not hazardous test for laboratory personnel, which can be used as a better alternative to culture. At least at the regional level, a laboratory can be established with the facilities for performing serum-based real-time PCR. This will assist in the confirmation of the disease in the HROGs having signs of brucellosis. It can be added here that all of the 13 seropositive patients were treated with a combination of doxycycline and rifampicin, which successfully cured them, except for one relapse case (data not shown).

Working in a brucellosis-positive herd would normally increase the probability of getting infected with brucellosis. In this study, this risk was quantified as 10 times more likely for the livestock farmers having at least one seropositive animal in their herds.

This is one of the first studies that rigorously investigated and quantified risk factors for brucellosis seropositivity in Bangladesh using a random effects logistic regression model. The advantage of such a modeling approach is that it accounted for clustering of individuals within districts. However, the limitation is that samples of milkers, butchers, and veterinary practitioners are convenience samples generated by the use of nonprobabilistic sampling methods, which has the effect of limiting the generalization of the results to the entire, at-risk Bangladesh population. Given the unavailability of a sampling frame, randomness of the sample from these groups of individuals is almost impossible. Moreover, such a study is based on the contentment of patients, and it is difficult to evince this constraint.

Evidence from this study on risk factors for brucellosis seropositivity in humans can be strengthened by increasing the number of samples and ensuring a more representative sample including milkers, butchers, and veterinary practitioners. The large odds ratios with wide CIs obtained in our study should be cautiously interpreted, given that the distribution of the individuals within the different categories of the risk factors was not even and the frequencies were sometimes very low.

In conclusion, our study revealed that the duration of contact with animals and the type of animal handled appeared to be the most significant risk factors for human brucellosis seropositivity in the Mymensingh, Sherpur, and Dhaka districts of Bangladesh. These two factors can be easily altered by educating individuals at HROG on the potential risks of extensive contact with livestock. The non-existence of a vaccine against brucellosis in humans or the difficulty of accessing a safe and efficacious vaccine implies that controlling this zoonotic disease in animals will directly lead to prevention in humans (especially with respect to biosecurity). The significant risk factors identified in this study can be regarded as proxies for many other management factors that were not included in the questionnaire. Intervention studies will therefore be needed to confirm the role of these factors on human brucellosis seropositivity.

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Disclosure Statement

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Chapter 9

Brucellosis in pyretic patient

Preamble

Pyrexia is an important clinical symptom of human brucellosis. It is also caused by other diseases like tuberculosis, malaria, typhoid that are endemic in Bangladesh. However, there is no report on the prevalence of brucellosis in patients with pyrexia of unknown origin (PUO) in Bangladesh. Moreover, the species of *Brucella* responsible for brucellosis in pyretic patients is also unknown in Bangladesh. This chapter describes the prevalence of brucellosis in randomly collected PUO patients and detection of *Brucella* species from seropositive pyretic patients.

Manuscript in preparation.

Seroprevalence of brucellosis among people with pyrexia of unknown origin in Bangladesh

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Abstract

This study describes the seroprevalence of human brucellosis among patients with pyrexia of unknown origin (PUO) and detection of *Brucella* (*B.*) *abortus* DNA from seropositive PUO patients using real-time (rt) PCR for the first time in Bangladesh. Blood samples were collected from a total of 300 patients with PUO over a period of eight months starting from October 2007 until May 2008 and subjected to three serological tests; the Rose Bengal plate test (RBT), Standard Tube Agglutination Test (STAT) and the indirect enzyme-linked immunosorbent assay (iELISA). A univariate analysis was performed using Firth's logistic regression model. *Brucella* genus (BCSP31) and species specific (IS711) real time PCR (rt PCR) were applied on six human sera samples. The seroprevalence of brucellosis among patients with PUO was estimated to be 2.7% (95% CI: 1.2-5.2). The age, residence, type of patient, contact with animals, type of animal handled, arthralgia and backache were found to be significantly associated with brucellosis seropositivity. *Brucella abortus* DNA was amplified from all six human sera which tested positive in RBT, STAT and iELISA. Based on the performance, simplicity and cost, the RBT is recommended as a screening test for the diagnosis of human brucellosis in Bangladesh.

Keywords: Brucellosis, seroprevalence, risk factors, pyrexia, real-time PCR, Bangladesh

1. Introduction

Human brucellosis is a zoonotic bacterial infection caused by a Gram-negative facultative intracellular bacteria of the genus *Brucella.* The most pathogenic and invasive species for humans is *Brucella* (*B.) melitensis*, followed in descending order by *Brucella suis*, *Brucella abortus* and *Brucella canis* (Acha and Szyfre, 2003). The transmission to humans mostly results from the consumption of fresh milk and dairy products prepared from unpasteurized milk such as soft cheeses, yoghurts and ice creams. However, direct contact with infected animals is an important transmission route especially among abattoir workers, herdsmen, veterinarians, butchers and also through the inhalation of infected aerosolized particles in personnel in microbiologic laboratories (Pappas et al., 2005).

Human brucellosis poses major economic and public health challenges in affected countries especially in the Mediterranean countries of Europe, northern and eastern Africa, Near Eastern countries, India, Central Asia, Mexico and Central and South America. However, there are only a few studies where the seroprevalence of brucellosis among patients with PUO has been estimated. For example, Baba et al. (2001) estimated the seroprevalence in northeastern Nigeria to be 5.2% whereas Tolosa et al. (2007) obtained a slightly lower seroprevalence of 3.6% in south eastern Ethiopia. The study by Kadri et al. (2000) yielded a seroprevalence of 0.8% among patients with PUO in Kashmir-India and 1.0% (1/100) among hospitalized patients with prolonged fever was reported by Aniyappanavar et al. (2013). The wide variability in estimated seroprevalence reported may be due to differences in the sampling design schemes used, the number of samples, exposure to *Brucella* spp., the number of diagnostic tests used and the manner in which tests were interpreted.

The status of brucellosis among humans in Bangladesh is not well documented. There is no official report about the prevalence or incidence of this disease in humans in Bangladesh. Several study findings revealed that 4.4-12.8% of people in high-risk occupational groups were brucellosis seropositive in some selected areas of Bangladesh (Rahman et al., 1983; Rahman et al. 1988; Muhammad et al., 2010; Rahman et al., 2012).

Moreover, brucellosis is known to be a pyrexic disease and the prevalence of brucellosis in pyrexic patients of Bangladesh is not yet known. The infection in humans is not clearly defined; it is mainly characterized by PUO yielding body temperatures of up to 38.3 °C (Petersdorf, 1992). Other symptoms include: backache, arthralgia, headache, chills, night sweats, weakness and weight loss (Mantur et al., 2007). Malaria, typhoid fever, tuberculosis and rheumatic fever are endemic in Bangladesh (Ahmed et al., 2005; Ram et al., 2007; Haque et al., 2009; Zaman et al., 2011). Since pyrexia is a characteristic of the aforementioned diseases including brucellosis, clinical examinations should always be accompanied by laboratory tests. The Rose Bengal (RBT), Standard Tube Agglutination (STAT) and indirect ELISA (iELISA) either alone or in combination were used for previous studies. None of these tests is perfect. However, if multiple imperfect tests are used in parallel on each sample, the agreement between two test pairs can be calculated.

Among people with PUO, risk factors that have been shown to be significantly associated with *Brucella melitensis* include: gender, age, and occupation (Kadri et al., 2000; Tolosa et al. 2007; Al-Fadhi et al., 2008).

In Bangladesh, there is no published report on the isolation of *Brucella* species from man or animals, but Rahman et al. (2012) reported the presence of *Brucella DNA* at genus level from seropositive human sera. Laboratory detection and species identification is still based on culture and phenotypic characterization respectively, which are time consuming and resource-intensive. Moreover, the risk of laboratory acquired infections during handling of infectious samples or isolates is very high (Yu and Nielsen, 2010). The PCR techniques are gradually becoming popular for rapid detection of brucellae from clinical samples like blood or serum (Zerva *et al.,* 2001; Queipo-Ortuño *et al.,* 2005; Debeaumont *et al.,* 2005). The IS711 based rt PCR is reported to be specific and highly sensitive (Bounaadja *et al.,* 2009). Most rt PCR assays so far developed are designed to detect brucellae at genus level to enable early onset of treatment. *Brucella* IS711 species specific multiplex rt PCRs for *B. abortus* and *B. melitensis* also exist for investigation of cultures (Probert *et al.,* 2004).

The objectives of this study were to determine the seroprevalence of brucellosis among patients with PUO and to detect species of *Brucella* prevalent among PUO patients using real-time PCR.

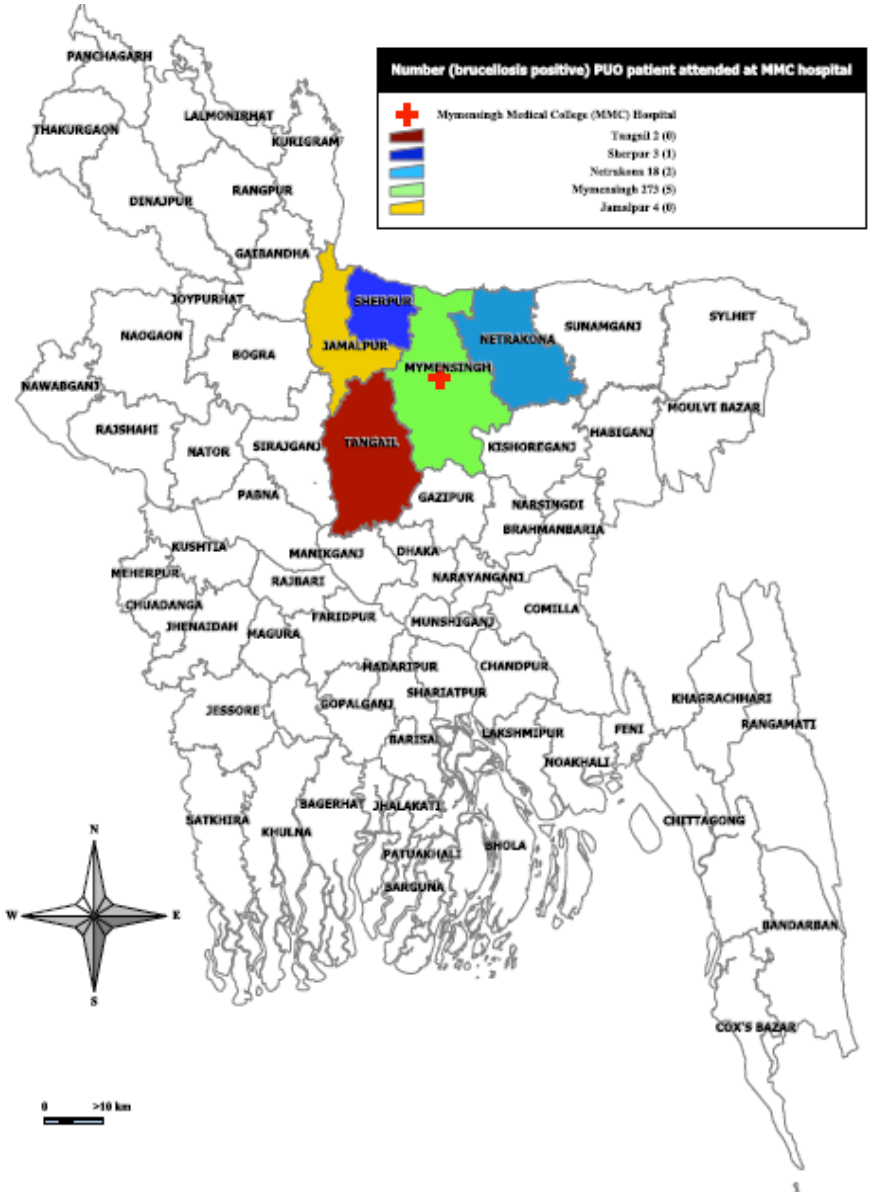
2. Materials and Methods

2.1. Study population and study area

Patients with PUO were defined as those with body temperatures higher than 38°C on several occasions and lasting over a period of three weeks. Patients were recruited from Mymensingh Medical College (MMC) hospital. The geographical position of MMC hospital, place of residence of patients and origin of animal samples are shown in Figure 9.1. MMC is the only medical college in the region. Therefore, patients from the surrounding districts have to visit MMC hospital to receive specialized treatment.

More than 80% of the population of this area lives in villages and crop-based livestock farming is their main source of income. Drinking of non-pasteurized milk and eating milk products is very unusual for these villagers. Milk is usually consumed after boiling, albeit milkers occasionally drink raw milk during milking. Cheese, yogurt and butter are usually consumed only from the wealthy city population. Blood samples from PUO patients were collected randomly once a week. Every day around 100 patients visit the outpatient facilities of MMC hospital. Ambulant and hospitalized patients meeting the inclusion criteria were recruited on the same date. Blood samples were collected from a total of 300 patients starting October 2007 until May 2008.

Figure 9.1. Study areas showing the origin of PUO patients from Mymensingh and surrounding districts



2.2. Ethical considerations

The study protocol was peer reviewed and cleared for ethics by the Ethical Review Committee of Mymensingh Medical College. Informed verbal and written consents were also taken from all individuals prior to blood sample collection.

2.3. Questionnaire data collection

Information was collected through personal face-to-face interviews. Questionnaires recorded information on age, sex, education, occupation, residency, type of patient (out and in), consumption of unpasteurized milk, contact with livestock (yes or no), animals handled, duration of contact in years, type of pyrexia, presence of arthralgia, sweating and backache (yes or no).

2.4. Collection and handling of blood samples

The collection and handling of blood samples was described in a previous paper by Rahman et al. (2012).

2.4.1. Serological tests

All blood samples were tested in parallel by indirect IgG ELISA, RBT and STAT. The detailed procedures for all three tests were described in a previous paper by Rahman et al. (2012). The estimated sensitivity and specificity of the iELISA, RBT and STAT were 78.6% and 99.6%, 88.4% and 99.4% and 81.7% and 99.6% respectively (Unpublished data).

2.4.2. *DNA extraction from human serum*

DNA was extracted from six human sera being positive in all three serological tests applied . DNA was extracted using the DNeasy spin column kit (QIAGEN) according to the manufacturer's protocol.

2.4.3. BSCP31 genus specific and Brucella abortus and Brucella melitensis specific IS711 real-time PCR

The IS711/BCSP31 rt PCRs originally described as a multiplex PCR assay (Probert *et al.,* 2004) were performed as single assays to detect *Brucella* spp. *DNA* and/or to distinguish between *B. melitensis* and *B. abortus DNA,* respectively. No further modification of the protocols was done. The species specific assays were applied when a genus specific assay had detected *BrucellaDNA* in a sample. The primers and probes were obtained from TIB MOLBIOL (Berlin, Germany). Amplification reaction mixtures were prepared in volumes of 25 µl containing 12.5 µl TaqManTM Universal Master Mix (Applied Biosystems, New Jersey, USA), 0.75 µl of each of the two specific primers (0.3 µM) and 0.5 µl TaqMan probe (0.2µM), 5 µl of template, and 6.25 µl nuclease-free water. The rt PCR reaction was performed in duplicate in optical 96-well microtiter plates (qPCR 96-well plates, Micro AmpTM, Applied Biosystems) using a Mx3000P thermocycler system (Stratagene, La Jolla, California) with the

following run conditions, 2 min at 50°C, 10 min at 95°C, followed by 50 cycles of 95°C for 15s and 57°C for 1 min. Cycle threshold values below 40 cycles were considered positive. The threshold was set automatically by the instrument. The samples scored positive by the instrument were additionally confirmed by visual inspection of the graphical plots showing cycle numbers versus fluorescence values.

2.5. Statistical analyses

To determine the potential risk factors and clinical symptoms associated with brucellosis sero-positivity in people with pyrexia of unknown origin, individuals were considered positive if they tested positive in at least one of the three serological tests used.

A univariate analysis was performed using a Firth's logistic regression model. The model used as response, the brucellosis status of the individuals and each risk factor or indicator variable in turn as the independent variable.

The percent agreement and coefficient of agreement between two test pairs were calculated according to Langenbucher et al. (1996). Calculations of the different parameters were carried out in R 3.1.0 (The R Foundation for Statistical Computing, 2014) using a 'two-by-two' contingency table.

3. Results

3.1. Descriptive statistics

The distribution of brucellosis seropositivity among the 300 PUO patients is presented in Table 9.1a and 9.1b. The overall seroprevalence of human brucellosis was 2.7% following a parallel interpretation of the three tests. The mean age of the individuals was 24.4 years and ranged from 2 to 80 years with 66% of the study population being males. Clinical symptoms of the 8 brucellosis seropositive PUO patients disappeared after therapy with streptomycin (1g i.m. daily) for 15 days and doxycycline (100mg p.o. every 12 hours) for 45 days.

3.2. Factors associated with brucellosis seropositivity among people with PUO based on a univariate analysis

The results of the univariate Firth's logistic regression analysis revealed that, age, residence, type of patient, contact with animals, type of animal handled, arthralgia and backache were significantly associated with a positive serological result ($p < 0.05$) (Table 9.1a and 9.1b).

Table 9.1a. Univariate analysis of potential risk factors and clinical symptoms for brucellosis among 300 people with pyrexia of unknown origin in Bangladesh

Factor	Tested	Positive (%)	Exact Binomial 95% CI	p-value*
Age Group (Years)				0.003
2-20	149	1 (0.7)	0.0-3.7	
21-40	111	2 (1.8)	0.2-6.4	
41-80	40	5 (12.5)	4.2-26.8	
Education				0.517
College to University	37	0 (0)	0.0, 9.5	
None to secondary	263	8 (3.0)	1.3, 5.9	
Sex				0.374
Female	101	2 (2.0)	0.2-7.0	
Male	199	6 (3.0)	1.1-6.4	
Residence				0.020
Urban	106	0 (0)	0, 3.4	
Rural	194	8 (4.3)	1.9, 8.3	
Type of patient				0.048
Out patient	262	5 (1.9)	0.6, 4.4	
Indoor	38	3(7.9)	1.7, 21.4	
Occupation				0.885
Business	31	0 (0)	0.0, 11.2	
Crop farmer	37	0 (0)	0.0, 9.5	
Day labour	7	0 (0)	0.0, 40.9	
Housewife	28	2 (7.1)	0.9, 23.5	
Livestock farmer	78	5 (6.4)	2.1, 14.3	
Not applicable (Age below 5 years)	20	0(0)	0.0, 16.8	
Service	12	0 (0)	0.0, 26.5	
Study	87	1 (1.1)	0.03, 6.2	
Contact with animals				0.023
No	219	3 (1.4)	0.3-4.0	
Yes	81	5 (6.2)	2.0-13.8	

Legend: *p values obtained from Firth's logistic regression analysis; CI, confidence interval.

Table 9.1b. Univariate analysis of potential risk factors and clinical symptoms for brucellosis among 300 people with pyrexia of unknown origin in Bangladesh

Factor	Tested	Positive (%)	Exact Binomial 95% CI	p-value*
Animal handled				0.001
Cattle	57	0 (0)	0, 6.3	
Cattle and goat	4	0 (0)	0.0-60.2	
Not known	219	3 (1.4)	0.3-3.9	
Goat	20	5 (25.0)	8.6, 49.1	
Drinking of raw milk				1
No	296	8 (2.70.03)	1.2-5.3	
Yes	4	0 (0.0)	0.0-60.2	
Nature of fever				0.247
Irregular	281	6 (2.1)	(0.8, 4.6)	
Continuous	11	1 (9.1)	(0.2, 41.3)	
Rising & falling	8	1 (12.5)	(0.3, 52.7)	
Sweating				0.001
No	71	6(8.5)	3.2, 17.5	
Yes	229	2 (0.9)	0.1, 3.1	
Arthralgia				<0.001
No	282	4 (1.4)	0.4, 3.6	
Yes	18	4 (22.2)	6.4, 47.6	
Backache				<0.001
No	287	4 (1.4)	0.4, 3.6	
Yes	13	4 (30.8)	9.1, 61.4	
Headache				0.001
No	43	5 (11.6)	3.9, 25.1	
Yes	257	3 (1.2)	0.2, 3.4	
District				0.486
Jamalpur	4	0 (0)	0.0-60.2	
Mymensingh	273	5 (1.8)	0.6-4.2	
Netrokona	18	2 (11.1)	1.4-34.7	
Sherpur	3	1 (33.3)	0.8-90.6	
Tangail	2	0 (0)	0.0-84.2	

Legend: **p* values obtained from Firth's logistic regression analysis; CI, confidence interval.

3.3. Real time PCR result

From those six sera, which were positive in the three serological tests, *Brucella abortus* DNA was amplified (Table 9.2). No *B. melitensis DNA* could be amplified from any of the six human serums.

Table 9.2. Brucella genus and *Brucella* species specific real time PCR among seropositive patients

PCR type	Tested	Positive	CT values	Range	
			Mean ±SE	Min	Maximum
BCSP31 *Brucella* genus	6	6	36.5±0.36	34.9	38.2
IS711 *Brucella genus*	6	6	34.2±0.29	32.8	35.6
IS711 *Brucella abortus*	6	6	33.5 ±0.83	31.04	36.0
IS711 *Brucella melitensis*	6	0	-	-	-

3.4 Agreement between test pairs

The percent agreement, kappa value and corresponding 95% confidence interval are shown in Table 9.3. More than 99.3% agreement was observed between RBT-iELISA, RBT-STAT and iELISA-RBT. The kappa value ranged from 0.85-0.93 indicates very strong agreement between tests.

Table 9.3. Agreement between two diagnostic tests

Test combination	Percent agreement	Kappa	95% Confidence Interval	Remarks
RBT-iELISA	99.7	0.92	0.81, 1.03	Almost perfect agreement
RBT-STAT	99.7	0.93	0.82, 1.04	Almost perfect agreement
iELISA-STAT	99.3	0.85	0.74, 0.97	Almost perfect agreement

4. Discussion

The seroprevalence of brucellosis among patients with pyrexia of unknown origin is described for the first time in Bangladesh and was estimated to be 2.7% (95% CI: 1.2-5.2). A lower seroprevalence of 0.8% was reported from Kashmir-India (Kadri et al., 2000) whereas a slightly higher prevalence of 5.2% was observed in north eastern Nigeria among patients with pyrexia of unknown origin (Baba et

al., 2001). The seroprevalence of 2.7% for our study is an indication that the majority of the patients with pyrexia of unknown origin were not infected with brucellosis. It is known that only about 30% of cases of PUO are due to infections (Williams and Bellamy, 2008). Malaria, typhoid, tuberculosis and rheumatic fever are common pyrexic diseases of humans in Bangladesh and are routinely referred by physicians for laboratory testing. Brucellosis as a cause of PUO was neglected by medical professionals in Bangladesh. Through this study it was observed that about 2.7% of the PUO patients suffer from brucellosis. However, this study may not represent the total PUO patients in Bangladesh as not all PUO people visit hospitals for health services. So, there might have some bias in the selection of PUO patients, which is also a limitation of this study.

Therefore, besides recommending that PUO be tested for tuberculosis, typhoid, malaria and rheumatic fever, clinicians should also consider brucellosis for routine testing.

Sero-prevalence of brucellosis in PUO patients increased with age which tallies with findings from other studies (Abo-Shehada *et al.*, 1996; Tolosa *et al.*, 2007). A possible reason for this that with age exposure of adults to livestock or livestock related activities increases.

Even though our results show that gender was not statistically significantly associated with human brucellosis seropositivity, other studies have shown otherwise (Kadri et al., 2000; Mantur et al., 2007; Al-Fadhi et al., 2008).

Brucellosis is an occupational disease and therefore mostly affects livestock farmers, dairy workers, butchers, veterinarians and laboratory personnel. These occupations are male dominated in Bangladesh making them more commonly affected than females.

All of the 8 brucellosis infected pyretic people were of rural origin. More than 80% of the people live in rural areas and involved with livestock production and thereby exposed to brucellosis positive animals.

Hospitalized pyretic patients had significantly higher seroprevalence than outpatients. Hospitalized patients showed acute course of infection resulting in immediate hospitalization. On the other hand, outpatients were chronically infected and more often presented with intermittent fever.

Livestock farmers, housewives and students were found to be brucellosis positive among different occupational groups. Housewives may get contact with *Brucella* contaminated meat and milk during preparations for cooking. Some housewives also assist in livestock production by herding, feeding, watering and milking in rural areas. Students living in villages also help their parents in livestock production by herding, feeding and watering during their free time. The brucellosis seropositivity among patients with pyrexia who were in contact with animals was significantly higher than for those who had no contact with animals. This is an indication that for those patients with chronic pyrexia and with a history of animal contact, brucellosis testing should be suggested.

Significantly higher seropositivity was estimated for pyrexic patients who handled goats compared to those who handled only cattle. Rahman et al. (2012) also observed a relatively higher seroprevalence of brucellosis in people who handled only goats than those who handled only cattle and those who

handled both cattle and goats, respectively. The same authors also reported that about 14.2% livestock farmers shared the same premises with their animals 52.7% kept goats in their houses. This close contact to animals could be responsible for the high prevalence among goat handlers.

Arthralgia and backache were significant clinical symptoms for brucellosis among the PUO patients. Similar observations were also made by other authors (Alsubaie *et al.,* 2005; Dokuzoğuz *et al.,* 2005; Mantur *et al.,* 2007).

Based on its easy handling and low costs, the RBT is recommended as a screening test for the diagnosis of human brucellosis in Bangladesh. A more specific test like serum based genus or species specific rt PCR can be used for confirmation (Zerva *et al.,* 2001; Rahman *et al.,* 2012) to avoid unjustified costs, drug toxicity and masking of other potentially dangerous diseases like tuberculosis which are also endemic in Bangladesh. At the time of this investigation rt PCR assay had to be performed in Germany, but now the facilities to perform this test are available in Bangladesh. The percent agreement between the two tests pairs and corresponding Kappa values indicate similar performance of the tests.

Detection of *Brucella* DNA was reported even for serum samples that were taken a long time after clinical signs of disease had ceased in these patients (Navarro et al., 2006; Vrioni et al., 2008). Our six ELISA, STAT and RBT positive patients presented with clinical symptoms and signs suggestive for brucellosis and indeed they recovered after 'typical' brucelosis treatment had been administered. Amplification was successful as we had expected. Thus, we could demonstrate that confirmatory diagnosis by species specific rt PCR is adequate for a well-timed onset of a combination treatment necessary for brucellosis (Mantur et al., 2007).

The small sample size of 300 patients leads to sparseness (the distribution of the individuals within the different categories of the risk factors was not even and the frequencies were sometimes very low) of the data. This limitation can be resolved by future studies involving a larger number of patients.

Conclusion

Brucellosis among PUO patients is not uncommon. *Brucella abortus* was found to be the only species prevalent in pyretic patients in Bangladesh. Based on easy handling and low costs, the RBT is recommended as the screening test and rt PCR as the confirmatory test whenever necessary for the diagnosis of human brucellosis in Bangladesh.

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Chapter 10

General Discussion

This chapter critically analyzes the knowledge generated from the chapter 5 to chapter 9 and discusses their practical relevance to design and implement future brucellosis control program whenever necessary. Brucellosis is an ancient and one of the world's most widespread zoonotic diseases affecting both, public health and animal production (Ariza et al., 2007). Since the first report on seroprevalence of brucellosis (Rahman and Mia, 1970), the brucellosis study in Bangladesh focused only on seroprevalence and few reports on risk factors mainly in domestic ruminants (Tables 3.7-3.9). As the previous prevalence reports were mostly based on non-representative samples, it helped little to know about the true status of the disease in animal populations. The other important aspect like estimation of true prevalence, evaluation of serological tests, risk factors of human brucellosis and identification of species responsible for brucellosis in humans and animals are not yet addressed in Bangladesh. The overall objective of this thesis was to investigate the epidemiology of brucellosis in humans and domestic ruminants in Bangladesh in terms of the evaluation of commonly used diagnostic tests, estimation of true prevalence, identification of risk factors and detection of *Brucella* species in order to provide information for appropriate control strategies.

Bangladesh has 64 districts (sub-division) and crop-livestock mixed farming system dominates all over the country as mentioned earlier. Out of 64 districts, Mymensingh was chosen to have a better understanding of the epidemiology of brucellosis in humans and domestic ruminants. Mymensingh is one of the highest livestock dense (>600/Km^2) areas in Bangladesh. The studies presented in this thesis were mostly Mymensingh district based. So, the findings of the studies will also represent other districts especially where small-scale dairy and subsistence/backyard management system prevails.

Small ruminant serum samples were also collected from other districts as they are not numerous in Mymensingh. Milk samples were also collected from Sirajgonj and Chittagong: two important milk pockets in Bangladesh. The Central Cattle Breeding and Dairy Farm (CCBDF) is the largest dairy farm and the sole government supplier of frozen semen for artificial insemination. The status of brucellosis of this herd including breeding bulls and animal caretakers were also investigated. A gayal herd under BLRI regional station at Naikhongchari, Bandarban, Bangladesh experienced sporadic abortion and thereby also studied for brucellosis.

So, the brucellosis status in domestic ruminants (cattle, goats and sheep), pyretic and occupationally exposed people and in a semi-domestic animal like gayal were revealed and sometimes updated. The species of *Brucella* responsible for brucellosis in humans and animals was also described.

10.1 The estimated true prevalence of brucellosis in domestic ruminants in Bangladesh

The estimated true median prevalence of brucellosis in goats and sheep were 1.0% (95% CI: 0.7–1.8%) and 1.2% (95% CI: 0.6–2.2%) respectively. The estimated true prevalence of brucellosis among cattle of Mymensingh and Govt. dairy farm were 0.3% (95% CI: 0.03-0.7) and 20.5% (95% CI: 16.4-26.3) respectively. The prevalence estimated in this study is based on a representative sample of goats and sheep and would therefore be applicable to the goats and sheep populations of Bangladesh. Similarly, the prevalence structure obtained in Mymensingh district will represent brucellosis status in cattle of other districts especially where small-scale dairy and subsistence/backyard management system prevails. It is very likely that there might have some reasons for this level of prevalence without any intervention. The overall apparent prevalence of brucellosis reported by other authors in Bangladesh are 5.3% (4.8-6.2), 2.9% (2.1-4.1) and 5.4% (3.9-7.1) for cattle, goats and sheep respectively as shown in Table 3.7 and 3.8. The apparent and true prevalence might vary from each other although both relate to exposure status not active infection. All of the previously reported seroprevalences were based on non-representative laboratory results. However, in a multi-testing environment along with the estimation of true exposure prevalence, the acute or active infection can also be revealed which may help as a decision support tool in terms of test and slaughter policy. For example, 0.3% (2/636), 0.8% (8/1044) sheep and goats respectively were found to be acutely infected (positive in both SAT and iELISA) with brucellosis (Rahman *et al*., 2013). The estimated true prevalence and acute infection (15.6% i.e. 53/340) of bovine brucellosis were very high in Govt. dairy farm. Brucellosis infected animals shed organism not only through abortion but also through vaginal discharge, feces, urine and milk (D'anastasio *et al*., 2011). So, culling of acutely infected animals from the population will help to reduce the transmission of the disease in animal populations.

The CCBDF is the herd where highly valuable breeding bulls, pure exotic breeds like Holstein Friesian cattle are also maintained. The central AI laboratory is also located within this herd which produces frozen semen for the whole country. It seems that the prevalence has been increasing steadily at CCBDF. In 2007 only one collecting/breeding bull was found RBT positive (Islam *et al*., 2007) (only bulls were studied) and in this study (done in 2008) one breeding bull and two pre-collecting bulls were found to be acutely infected in addition to a lot of dairy cattle. Such a high level of exposure prevalence and acute infection in this herd is also a serious threat for the other livestock in Bangladesh. After reporting all of these acutely infected cattle including the breeding bulls were culled. In such a high level of prevalence, vaccination should be initiated immediately to reduce the burden of this disease.

Prevalence structure may also vary in different regions within a country. Calculating mean prevalence at national level is a frequent error of decision makers, as those figures may not reflect local conditions (Blasco and Molina-Flores, 2011). Similarly, the control strategy will also vary based on the prevalence status in different regions/district. For example, it is recommended to do nothing for further control of brucellosis in Mymensingh and similar districts because of very low prevalence. Such a low prevalence of this zoonosis may be the result of a balance between host and pathogen due to their

coexistence for millennia without significant intervention measures to control the disease (Moreno, 2014). However, sufficient information to decide whether to initiate control strategy for brucellosis in dairy rich areas like Sirajgonj, Chittagong, Satkhira, etc., is unavailable. The possible reasons for such a low level of prevalence of domestic ruminants may be due to the:

- large scale slaughter of cattle and small ruminants throughout the year: as already mentioned earlier that about 15 million goats and 3.5 million cattle are slaughtered annually in Bangladesh. Due to this mass slaughter, brucellosis infected carcass may be removed from the cattle populations. This phenomenon resembles test and slaughter method of disease control, the only difference is that it is done without brucellosis testing. In such a scenario, the average life span of animals will be short in general which was also reflected in this study: the mean (mean ±se) age for goats, sheep in Bangladesh and cattle of Mymensingh district were 1.6 ±0.06 years, 2.1 ±0.04 and 3.72 ±0.09 years respectively. The age at puberty in cattle varies from 2-3 years (Mukasa, 1989), whereas the average age of puberty in goats and sheep are 6.5 months (Hassan et al., 2007) and 8.2 months (Hassan and Talukder, 2011) respectively in Bangladesh. The most important clinical manifestation of brucellosis in domestic ruminants is abortion, through which the infection spreads from one animal to other, which is less likely in case of shorter life span of animals. The shorter life span of animals, the lower the risk of *Brucella* transmission as this disease is most common in sexually mature animals (Reviriego *et al*., 2000; Solorio-Rivera *et al*., 2007; Islam *et al*., 2010; Lopes *et al*., 2010). Moreover, young animals tend to be more resistant to infection and they frequently clear infection before puberty (Walker, 1999; Godfroid *et al*., 2004).
- the other important factor for low prevalence of brucellosis in livestock is probably infrequent abortion in animals due to Brucellosis in Bangladesh: abortion is the main clinical sign of brucellosis in sexually mature female domestic ruminants (Lilenbaum *et al*., 2007; Xavier *et al*., 2009). Vaginal discharges, fetal membranes are the most important sources of transmission of this disease. Based on the results of this study and also from others, it seems that *Brucella* is not the most important cause of infectious abortion in ruminants in Bangladesh. *Brucella abortus* or *Brucella melitensis* were not detected from any of the aborted fetal membranes, vaginal swabs studied in this study. Similar observations were also made by others (Das *et al*., 2008; Dey *et al*., 2013; Islam *et al*., 2013c). As a result, the chance of transmission of brucellosis is less likely in the animal populations and thereby the low prevalence in animals.
- the third probable cause of low prevalence of brucellosis in small ruminants may be that only *Brucella abortus* is prevalent in this country. The preferred hosts of *B. abortus* are cattle and buffalo. However, *B. abortus* may also infect goats and sheep but due to non-preferred host the intensity of infection in sheep and goats may be low (Godfroid *et al*., 2013). The higher apparent and true prevalence in sheep may be due to their prolonged lives (less slaughter).

10.2 The performance of serological tests for the diagnosis of brucellosis in domestic ruminants

The Sensitivity, specificity, positive predictive value and negative predictive value of RBT, SAT and iELISA in cattle of two locations, sheep and goats are summarized in Table 10.1.

Table 10.1. Performance of RBT, SAT and iELISA for the diagnosis of brucellosis in cattle, goats and sheep in Bangladesh

Test	Cattle_Mym.		Cattle_Govt.		Goats		Sheep	
	Se (PPV)	Sp (NPV)	Se (PPV)	Sp (NPV)	Se (PPV)	Sp (NPV)	Se (PPV)	Sp (NPV)
RBT	81.0% (19.9%)	99.0% (99.4%)	76.1% (81.6%)	95.6% (93.9%)	80.2% (66.9%)	99.6% (99.8%)	82.8% (37.2%)	98.3% (99.8%)
SAT	90.5% (5.8%)	95.6% (99.9%)	79.7% (81.4%)	95.3% (94.7%)	57.1% (45.2%)	99.3% (99.6%)	72.0% (38.4%)	98.6% (99.7%)
iELISA	90.5% (28.0%)	99.3% (99.9%)	91.3% (96.7%)	99.2% (97.8%)	92.9% (21.1%)	96.5% (99.9%)	92.0% (69.0%)	99.5% (99.9%)

Legend: Mym: Mymensingh, Govt.: Government farm, Se: Sensitivity, Sp: Specificity, PPV:Indicates the probability that an animal with positive test truly has the disease, NPV: Indicates the probability that an animal with a negative test is truly free from disease.

The PPV and NPV of the tests were calculated using formula 10.2.1 and 10.2.2 based on the Bayes' theorem:

$$Positive\ Predictive\ Value\ (PPV) = \frac{Se*prevalence\ (Pr)}{Se*Pr + (1-Sp)*(1-Pr)} \qquad (10.2.1)$$

$$Negative\ Predictive\ Value\ (NPV) = \frac{Sp*(1-Pr)}{(1-Se)*Pr+Sp*(1-Pr)} \qquad (10.2.2)$$

The performance of iELISA is relatively better in in cattle of Mymensingh and government farm and in sheep in terms of sensitivity and specificity. Similarly, the performance of RBT is better in goats. The iELISA cut-offs were different in three species of animals (5 IU/ml for cattle, 2 IU/ml for goats and 6 IU/ml for sheep). Using a higher cut-off will increase the specificity of iELISA in goats. It is evident from Table 10.1 that even though the RBT, SAT and iELISA are highly specific the PPV are very low. It indicates that majority of the seropositive cattle in Mymensingh are not positive at all. For, example, the PPV of RBT in cattle of Mymensingh is 19.9% which means that 80.1% seropositive animals are actually not infected.

On the other hand, when using the same test but in high prevalence scenario as in CCBDF, the PPV of RBT becomes 81.6%. In a low prevalence scenario, the serological test (having a very low PPV) result based recommendation of initiating control program may be speculative and misleading as noted from different authors (Rahman *et al.*, 2011b; Islam *et al*., 2013b) in Bangladesh.

For this reason, after validating three serological tests, it was recommended to do nothing (even not testing as it gives very high false positive serological reactions (FPSR)) for the control of brucellosis in Mymensingh and to initiate immediate vaccination in govt. dairy farm. However, if testing is required in any case, simultaneous use of SAT and iELISA in an animal and their parallel interpretation will increase the sensitivity and NPV and their serial interpretation will further increase the specificity and PPV. The NPV of all the three tests are very high in all of the three species of livestock in low prevalence area but relatively lower in high prevalence area. In essence, the information about PPV and NPV are essential for the decision makers and clinicians but their estimation is dependent on three parameters: the prevalence of the disease in the population (both PPV and NPV), the sensitivity (NPV) and the specificity (PPV) of the tests used.

10.3 The species of *Brucella* dominant in humans and animals in Bangladesh

Brucella abortus is the only species detected from humans, cattle, goats and gayal. Species of *Brucella* infecting humans reflects the species prevalent in animals also in that area. Detection of the same species from animals also provides further evidence to support preceding hypothesis. Detection of *B. abortus* from 19 humans by PCR (7 milkmen, 7 livestock farmers, 2 house wives, one student, one butcher and one veterinary practitioner) originating from Mymensingh, Dhaka, Netrakona and Sherpur districts in Bangladesh. *Brucella abortus* was detected from six animals by PCR (3 dairy cow, one dairy goat, one dairy gayal and one bull semen). The detection of *Brucella abortus* DNA from cattle and buffalo sera from other districts in Bangladesh added further evidence in this regard (Rahman *et al*., 2014). However, isolation from the *Brucella abortus* DNA positive milk samples was not successful in Farrell's medium. The probable reasons may be that the samples were old, repeatedly frozen and thawed for several times and the presence of competing microflora... Indeed, samples potentially containing *Brucella* spp. should be cultured within two hours or, if this is not possible, cooled at 2-8°C. It can also be stored at -20°C by adding several drops of sterile saline to keep the tissue moist. Isolation is most likely during the acute phase of infections caused by *B. melitensis* or *B. suis* and less successful in *B. abortus* infections. Brucellae are rarely isolated from samples with a competing microflora (Corbel, 1997; Al Dahouk *et al*., 2002).

The presence of only *B. abortus* in a country may have some extra advantages over the presence of several species *Brucella* in animals and humans for future control and eradication program in Bangladesh. The presence of *B. abortus* in goats is probably a spill over infection and control of *B. abortus* infection in cattle (preferred host) will suffice to control it in spill over host (Godfroid *et al*., 2011).

Another important advantage is that it is less pathogenic to humans in comparison to *Brucella melitensis.* Isolation, identification and typing of *Brucella* at biovar is very important to understand the prevalent biovars of *Brucella abortus* in Bangladesh and to trace back the source of infection in an outbreak. These findings are important for planning and execution of disease control program also.

However, if the facility to work with *Brucella* spp. is absent in a country like Bangladesh, the molecular detection of *Brucella* species from clinical samples is also of paramount importance. Indeed, isolation from samples in Bangladesh will add further knowledge of biovar diversity (if any) and sources of infection through molecular characterization.

10.4 The prevalence and risk factors of human brucellosis

The overall prevalence of brucellosis in high risk group and pyretic people in Bangladesh were 4.4% and 2.7% respectively based on parallel interpretation of three tests. The important risk factors identified are contact with goats and duration of contact with animals. *B. abortus* was also detected from goat milk and goats are sometime kept inside human house especially by poor farmers posing them to the risk of infection (Rahman *et al*., 1988, 2012a). The previously reported higher prevalence in humans seems to be for higher FPSR as observed in livestock in Bangladesh in low prevalence area. The highest prevalence of brucellosis among HROG was found in milkmen (18.2%) but it was not statistically significant. Most of the seropositive milkmen worked in CCBDF where the prevalence of cattle brucellosis is very high.

So, relatively higher prevalence of brucellosis in milkmen may not indicate milking as a risky profession in Bangladesh. Consumption of raw milk is very rare and was not significantly associated with human brucellosis. So, in Bangladesh the main source of human brucellosis is occupational and the impact of brucellosis in general people is relatively lower (Seleem *et al*., 2010). Livestock farmers, butchers, milkmen, veterinary practitioners and AI worker are at risk. Previous reports from Bangladesh also support this fact (Rahman *et al*., 1983, 1988). Awareness building about the risk factors of brucellosis among the people at risk through education may also help to reduce the level of exposure and thereby the disease (Marcotty *et al*., 2009).

The prevalence of human brucellosis is dependent on the burden of the disease in animal populations and also the prevailing species of *Brucella* in animals of that area. For example, when *B. abortus* is a major problem in cattle, seroprevalence in humans are estimated to be in the range of 1.0–5.0% (Swai and Schoonman, 2009) but if *B. melitensis* is endemic as in the middle East, higher prevalence have to be expected (Pappas *et al*., 2006). The prevalence of human brucellosis observed in this study also support the above statement. The sole (probably) presence of *B. abortus* which is less pathogenic to humans and also the lower level of prevalence in animals are responsible for the lower prevalence in humans. The presence of brucellosis in pyretic patients and HROG people has some diagnostic value for the physicians.

In addition to clinical signs and symptoms, the history of animal contact should help clinicians to refer brucellosis testing in HROG and pyretic people. The RBT is recommended for routine screening. Along with RBT, serum based rt PCR of the RBT positive patients may also be used for confirmation of the serological finding in doubtful cases.

In high prevalence endemic countries, clinical signs and symptoms suggestive of brucellosis along with a positive serological test result suffice for the diagnosis of human brucellosis (CDC, 1997). But in low prevalence endemic areas, as there is high probability of FPSR, the serum based genus or specific specific rt PCR will help to confirm the serological test result. In this scenario of low prevalence, the confirmatory diagnosis is essential otherwise it may lead to useless treatment in individuals for 45 days. Other than unjustified costs it may lead to drug toxicity and mask other potentially dangerous disease like tuberculosis which is endemic in Bangladesh. In Bangladesh, the rt PCR based testing of disease is gradually becoming available in different laboratories.

Chapter 11

Conclusions

This thesis described the true prevalence of brucellosis in domestic ruminants, the prevalence and risk factors of brucellosis in humans and also the species of *Brucella* prevalent in animals and humans in Bangladesh. The results were generated based on the collection and analysis of field data using appropriate study design and statistical and molecular methods. The following conclusions can be drawn from the thesis:

- The true exposure prevalence of brucellosis in cattle under small-scale dairy and subsistence/backyard management system is very low (0.3%; 95% CI: 0.03-0.7). The active/acute infection is also very low (0.29%; 95% CI: 0.06-0.86) and similar to true exposure prevalence. The brucellosis in cattle under such management system is naturally controlled and further control program is not recommended considering the poor socio-economic condition. The main reason behind such low level of prevalence of brucellosis under small-scale dairy and subsistence management system is probably mass slaughter of livestock for meat all the year round. Another probable reason may be infrequent abortion due to *Brucellosis*.
- The true exposure prevalence of brucellosis in government dairy farm (CCBDF) is very high (20.5%; 95% CI: 16.4-26.3). The acute infection in this farm is also very high (15.58%; 95% CI: 11.89-19.89). Immediate control measure by initiating calfhood (female calf) vaccination is warranted to protect a valuable herd, which also provides frozen semen for AI all over Bangladesh.
- The SAT and iELISA simultaneously may be applied to know the stage of brucellosis infection in domestic ruminants both in high and low prevalence scenario. The iELISA with a new cut-off of 5 IU/ml may be used both in chronically (as at Mymensingh) and acutely infected cattle population (as at the Government Farm) for routine screening.
- The infection in breeding bulls with brucellosis is a serious hazard for the AI industry.
- The true exposure prevalence of brucellosis in goats and sheep are also low and around 1%. Due to the lower positive predictive value, these test results should be interpreted with caution to avoid misleading information.
- Brucellosis is not a serious problem for the general people in Bangladesh as drinking raw milk is unusual and is not a risk factor. The apparent prevalence of brucellosis in a high risk occupationally exposed people (4.4%; 95% CI: 2.8-6.6) and in pyretic patients (2.7%; 95% CI: 1.2-5.2) are also low.
- The RBT may be applied as a screening test in humans having signs and symptoms of brucellosis along with the history of animal contact. In case of suspicion, genus or species specific rt PCR may be applied for confirmation.

- Only *Brucella abortus* is dominant in humans and animals in Bangladesh. Regular screening of HROG and pyretic patients with animal contact by serology and species specific rt PCR will indirectly help to know further about the species diversity of *Brucella* in animals in Bangladesh.

Chapter 12

Recommendations

While conducting this research, the importance of animal and or herd/flock identification system was badly felt to draw random sample from population. Due to the absence of livestock data bank, an alternative way of map digitization and selection of livestock farms randomly using hand held GPS machine was used. In this study, only one district was studied to understand the epidemiology of brucellosis in cattle. The prevalence of a disease vary in different areas due to the variation of animal husbandry practices. The milk rich dairy pockets in Bangladesh were not studied extensively where the prevalence of this disease may vary from Mymensingh. The isolation of *Brucella abortus* was not possible from old specimens but DNA of *B. abortus* was detected from human sera and animal samples. Based on the above facts, the following recommendations are made:

- The Department of Livestock Services should initiate animal identification system, which is essential for planning research and extension work and also for disease surveillance. The notification of abortion should make mandatory and all abortion should be tested. However, the legislative support is essential for successful implementation of the above need.
- The epidemiology of brucellosis in milk rich dairy pockets should be investigated using appropriate study design and using both IgM (e.g. SAT) and IgG (e.g. iELISA) detecting tests in parallel. Research program for the isolation and identification of *Brucella* species from humans and animals should be conducted to know the species and biovar diversities in Bangladesh. Milk ring test positive samples should be preferred over abortion specimens and initially guinea pig inoculation technique should be preferred over culture for successful recovery of *Brucella* organism. Isolation of *Brucella* species from pigs will further help to know whether *Brucella suis* is circulating in Bangladesh as recently 6.7% seroprevalence has been reported in pigs.
- All infected cattle including breeding bulls should be culled from CCBDF. Immediately, all female calves should be vaccinated to protect this valuable herd.
- Breeding bull should be tested regularly (once a year) for brucellosis. Before introducing for semen production, brucellosis status in a bull must be checked rigorously.
- Collaboration between Department of Livestock Services and Public Health Department in terms of research and extension for the further control of this disease is warranted (“one health” approach). The activities of the One Health Hub, Bangladesh should be enhanced and brucellosis should be included in the priority list. At least one reference laboratory should be established for the diagnosis of brucellosis in humans and animals in Bangladesh. People should be educated about personal hygiene and risk factors of brucellosis by poster, leaflets and other mass media.

- Regional cooperation among bordering countries with regard to movement of animals is also essential to control this communicable disease. Because, Bangladesh share a common boundary of 2400 miles with India, which is known as porous, and 40% of the total cattle slaughtered annually in Bangladesh illegally come from India.

Chapter 13

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