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Prevalence and Diagnostic Test Comparison of Brucellosis in Cattle in Pabna and Mymensingh Districts of Bangladesh

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Abstract. Present study was undertaken to determine the seroprevalence of brucellosis in cattle of Pabna and Mymensingh districts in Bangladesh. A total of 260 cattle sera samples were collected from Pabna and Mymensingh districts. The epidemiological data were collected by structured questionnaire. RBT and SAT were used as screening tests and further confirmed by I-ELISA. The seroprevalence of *Brucella* in cattle was estimated to be 4.23%, 3.07% and 2.31% by RBT, SAT and I-ELISA, respectively. The comparison of the serological tests result revealed the highest prevalence in RBT than SAT and I-ELISA. The prevalence of *Brucella* was 2.5% in Pabna and 2.14% in Mymensingh. It was observed that, a higher prevalence of *Brucella* was found in female (2.67%) than in male (1.82%), natural breeding (2.67%) than artificial breeding (1.81%), in aged animals (3.33%) than young (1.25%). But these differences were not statistically significant. There exists significant difference between prevalence of *Brucella* in cattle with history of abortion than without history of abortion (P value=0.013).

Keywords: brucellosis, cattle, diagnosis, epidemiology, Bangladesh

Introduction

Brucellosis is a zoonotic disease caused by different species of the genus *Brucella* that are pathogenic for a wide variety of animals and humans. In animals, brucellosis mainly affects reproduction and fertility, reduces the survival of newborns and milk yield. Mortality in adult animals is insignificant (Sewell and Brocklesby, 1990). According to the Food and Agricultural Organization (FAO), the World Health Organization (WHO) and the World Organization of Animal Health (OIE), brucellosis is considered to be the most widespread zoonosis worldwide (Mustafa and Nicoletti, 1993).

Although it has been eradicated in many developed countries in Europe, Australia, Canada, Israel, Japan and New Zealand (Geering *et al*., 1995) in some other areas it has emerged as a major zoonotic disease in sheep and goats. The importance of brucellosis was primarily due to its public health significance and economic loss to the animal industry (WHO, 1971). Bangladesh has been reported as an endemic country for brucellosis because of a considerable number of human and animal populations are exposed to the infection each year.

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Brucellosis in humans is caused by exposure to livestock and livestock products. Infection can result from direct contact with infected animals and can also be transmitted to consumers through raw milk and milk products. Brucellosis spreads between animals in a herd and the disease is a systemic infection that can involve many organs and tissues. Once the acute period of the disease is over, symptoms of brucellosis are mostly not pathognomonic, however, the organism can be located in the supramammary lymph nodes and mammary glands of 80% of infected animals. Thus they continue to secrete *Brucella* in their body fluids (Redkar *et al*., 2001).

Under the name Malta fever, the disease (now called brucellosis) first came to the attention of British medical officers in the 1850s in Malta during the Crimean war. The causal relationship between the organism and disease was first established in 1887 by Dr. David Bruce (Wilkinson and Lise, 1993). Brucellosis is endemic worldwide including Bangladesh (Das *et al*., 2008) but often a neglected disease.

It causes a great economic loss to the livestock industries through abortion, infertility, birth of weak and dead offspring, increased calving interval and reduction of milk yield (Rahman *et al.*, 2006).

In Bangladesh it was first reported in cattle in 1967 (Mia and Islam, 1967), and in humans in 1983 (Rahman *et al*., 1983). Islam *et al*. (1983) estimated the annual economic loss in Bangladesh due to bovine brucellosis in indigenous cows as 720,000 EUR (total) and 12000 EUR per 1000 cross-bred cows and a total of 276000000 EUR in cross-bred cows. Recently brucellosis has been reported in cattle, buffalo, sheep, goat and pig in different regions of Bangladesh (Rahman *et al*., 2011a, 2011b; Rahman *et al*., 2010; Nahar and Ahmed, 2009; Uddin *et al*., 2007a, 2007b; Rahman *et al*., 2006; Amin *et al*., 2004). The diagnosis of brucellosis is confirmed by isolation of *Brucella* spp. by bacteriological culture or by the detection of an immune response by serological test to its antigens (Orduña *et al*., 2000). But the diagnosis of brucellosis based exclusively on *Brucella* isolation presents several drawbacks. The slow growth of *Brucella* may delay diagnosis for more than 7 days and also, the sensitivity is often low, ranging from 50 to 90% depending on disease stage, *Brucella* spp., culture medium, quantity of bacteria and culture technique employed (Gotuzzo *et al*., 1986). Hence, the serological tests are important for diagnosis of brucellosis.

Serological test like the rose Bengal test (RBT), slow agglutination test (SAT), mercaptoethanol test, enzyme linked immunosorbent assay (ELISA) and complement fixation test (Islam *et al*., 1983) are generally used for the detection of *Brucella* infection in animals. Enzyme linked immunosorbent assay (ELISA) has been evaluated for many years for the detection of serum antibody to *Brucella* in domestic animals. It has gained popularity over recent years as an alternative to other serological tests because it has several advantages compared with other tests such as, (a) it is direct method of identification of specific antibody, (b) it is more sensitive test than the slow aggluti-nation test, (c) the antibody enzyme conjugate employed has light chain reactivity and thus able to detect all classes of antibody. Despite having these advantages, there has been limited use of ELISA for the diagnosis of brucellosis in Bangladesh. Therefore, the present study was designed to diagnose brucellosis by adopting I-ELISA as well as RBT and SAT to detect antibodies to *Brucella* organism and to identify the risk factor and distribution of brucellosis in cattle in Pabna and Mymensingh districts of Bangladesh for prevention and control.

Materials and Methods

A total of 260 blood sera samples were collected from cattle of Pabna and Mymensingh districts of Bangladesh. Among cattle sera samples, 120 were collected from Bhangoora, Shordarpara, Kashipur and Gojatola of Pabna and 140 samples were collected from BAU Veterinary Clinic area, Sasmore, Sutiakhali and Digharkanda of Mymensingh during the period from May to December, 2011 (Table 1). Sampling was carried out as multistage sampling with the farm being selected first in the study area and then cattle randomly selected within each farm. The sampling frame within each farm was the list of all cattle on farm record. The sampling unit consisted of the animals selected from the list of all cattle within each farm using computer generated random numbers. The questionnaire based data on age, sex, breeding strategy, pregnancy status, area, history of abortion in cows were recorded.

Table 1. Collection of serum samples from cattle in Pabna and Mymensingh districts

Area/location	No. of cattle samples
Pabna district	120
Mymensingh district	140
Total	260

Blood and sera samples collection. Animals were restrained with the help of the owner. Then the site of blood collection at the jugular furrow was soaked with iodine or alcohol. About 5-7 mL of blood was collected from the jugular vein of each cattle using a sterile disposable syringe and needle and was kept undisturbed on a tray for at least 1 h at room temperature in a slightly inclined position to facilitate clotting and separation of serum. After this period, the clotted blood samples with sera were transferred to a refrigerator and were kept overnight at 4 °C. Then the blood samples with sera were centrifuged at 3000 rpm for 10 min. Later on, the sera were aliquated into sterilised labeled Eppendorf tube and stored at -20 °C until used.

Serological study. The serological test for the diagnosis of brucellosis in cattle was performed by rose Bengal test (RBT), slow agglutination test (SAT) for screening and indirect enzyme linked immunosorbent assay (I-ELISA) for confirmatory diagnosis.

Rose Bengal plate test. Rose Bengal test (RBT) was performed according to the procedure as described by OIE (2004) which is being routinely used and described previously by Uddin *et al*. (2007a, 2007b) using *B. abortus* antigen (obtained from Dae Sung Microbiological Lab, South Korea). The serum samples and *B. abortus* antigen were kept 1 h at room temperature before starting the test. Thirty µL of each serum to be tested was placed on a glass plate circled approximately 2 cm in diameter. Then the vial of antigens was shacked gently and 30 µL of antigen was put beside each of the sera. The antigens and the serum were mixed on the plate with a stirrer and spread over the entire area enclosed by the circle. Then the plate was placed on a mechanical rotator as 80-100 rpm for 4 min and the reading was noted immediately. Any agglutination or precipitation was considered as positive, whereas, no reaction (negative) indicated the absence of *Brucella* antigen in the sera.

Slow agglutination test (SAT). SAT was carried out with EDTA as described by Garin *et al*. (1985). The SAW (synbiotics, concentrated suspension of *B. abortus*, Weybridge, stain 99) antigen was diluted (1 mL antigen with 19 mL SAT buffer solution). The SAT buffer was prepared by adding 0.93 g EDTA (5 mM, Triplex®) to 500 mL PBS, where PBS was prepared by adding 5 tablets of PBS (Dulbecco-A, Oxoid, UK) to 500 mL distilled water. Briefly, the slow agglutination test was performed in flat bottom 96 well micro plates. At first for each test serum, a row of 3 wells of the 96 well micro plates was selected to make double dilution of the sera. 168 µL of SAW buffer was pipetted in first well and 100 µL in the 2nd well and 3rd well, respectively, of the micro plate. Then 32 µL of serum was added in 1st well (dilution 1/6.25) after well mixing of the serum and PBS EDTA in the 1st well and 100 µL was taken from this well and was placed in the second well (1/12.5). 100 µL from the 2nd well was transferred into the 3rd well and finally 100 µL of liquid in excess was discarded from 3rd well. Note that, all wells contained 100 µL. Then in each well 100 µL of standardized SAW antigen was added. This gives the serial serum dilution of 1/12.5, 1/25, 1/50. The plate was then incubated at 37 °C for 24 h (+/-4 h) for reading. After 24 h, the agglutination reaction was observed by using a magnifying mirror against illumination source. Notably, for every group of samples tested, a positive control serum was included. Reading was taken on the basis of this protocol and the standardization was performed (75% agglutination of the OIEISS). The results were interpreted according to instruction of Veterinary Agrochemical Research Centre (Groeseleaberg 99, 1180 Brussels, Belgium).

Indirect enzyme linked immunosorbent assay (I-ELISA). All the samples found to be positive in RBT were further confirmed using I-ELISA. The assay was performed according to the protocol provided by the manufacturer's instructions (Svanova Biotech AB, art No.10-2700-10, SE-751 83 Uppsala, Sweden).

All reagents supplied by the manufacturer company were equilibrated to room temperature 18 to 25 °C (64 to 77 °F) before use. An amount of 100 µL of sample dilution buffer was added to each well that would be used for serum samples and serum controls. After that 4 µL of positive control serum (reagent A) and 4 µL of negative control serum (reagent B) was added, respectively, to selected wells coated with *B. abortus* antigen. For confirmation purposes it was run the control sera in duplicates. The plate was shaken thoroughly and sealed then incubated at 37 °C (98.6 °F) for l h. The plate was rinsed 3 times with PBS-Tween buffer and filled up the wells at each rinse, emptied the plate and tapped thoroughly to remove all remains of the fluid. Then 100 µL of HRP conjugate was added to each well and incubated at 37 °C (98.6 °F) for 1 h. The plate was rinsed again according to the previous way. Then 100 µL substrate solution was added to each well and incubated for 10 min at room temperature 18 to 25 °C (64 to 77 °F). Begin timing after the first well was filled. The reaction was stopped by adding 50 µL of stop solution to each well and mixed thoroughly. The stop solution was added in the same order as the substrate solution was added. The optical density (OD) of the controls and samples was measured at 450 nm in a micro plate photometer. The OD was measured within 15 min after the addition of stop solution to prevent fluctuation in OD values. Any change in colour observed by naked eye indicated positive reaction.

Statistical analysis. The questionnaire-based data was processed in Microsoft Excel and analysed in SPSS. The z-test for proportions was used to compare the results between the serum tests. The z-test for proportions was done to find out the significant differences in the prevalence of *Brucella* based on the result of RBT in terms of age, sex, history of abortion, breeding strategies and study area.

Results and Discussion

A total of 260 sera samples from cattle were collected from Pabna and Mymensingh, 120 were collected from cattle of Pabna district and 140 from cattle of Mymensingh district (Table 1). The sera were tested by rose Bengal test (RBT), slow agglutination test (SAT) and indirect enzyme linked immunosorbent assay (I-ELISA) and the results are shown in Table 2 and 3. The overall prevalence of *Brucella* was found to be 4.23%, 3.07% and 2.31% by RBT, SAT and I-ELISA, respectively. It was shown that out of 260 cattle sera examined by RBT, 11 cattle sera showed positive reaction to RBT with a prevalence of 4.23%; 8 were positive to SAT with a prevalence of 3.07% and 6 to indirect ELISA with the prevalence of 2.31% (Table 2). The comparison of the serological test result revealed the highest prevalence in RBT than SAT and I-ELISA. But the prevalence of *Brucella* determined by RBT, SAT and I-ELISA did not differ significantly at 5% level of significance (P value=0.456).

Out of 260 cattle, 110 were male and 150 were female. The prevalence of *Brucella* in female was 4.67%, 3.33% and 2.67% in RBT, SAT and I-ELISA, respectively, and in male 3.64%, 2.73% and 1.82% in RBT, SAT and

Table 2. Overall seroprevalence of brucellosis in cattle based on RBT, SAT and I-ELISA

Total no. of samples collected and tested	No. of positive reactors			Percentage of positive reactors			Level of significance
	by RBT	by SAT	by I-ELISA	by RBT	by SAT	by I-ELISA	
260	11	8	6	4.23%	3.07%	2.31%	NS

NS = not significant at 5% level of significance (P value=0.456).

Table 3. Demographic factors related seroprevalence of brucellosis in cattle based on RBT, SAT and I-ELISA

No. of sera samples collected and tested		No. and % of positive reactors						#Level of significance
		by RBT	95% CI	by SAT	95% CI	by I-ELISA	95% CI	
Age								
< 2 years	60	2(3.33%)	1.21-7.87%	1(1.67%)	1.57-4.91%	1(1.67%)	1.57-4.91%	NS
≥ 2-4 years	80	3(3.75%)	0.41-7.91%	2(2.5%)	0.92-5.92%	1(1.25%)	1.18-3.68%	
> 4 years.	120	6 (5.0%)	1.1-8.9%	5(4.17%)	0.59-7.75%	4(3.33%)	0.12-6.54%	
Sex								
Male	110	4(3.64%)	0.14-7.14%	3(2.73%)	0.32-5.78%	2(1.82%)	0.68-4.32%	NS
Female	150	7(4.67%)	1.29-8.05%	5(3.33%)	0.46-6.2%	4(2.67%)	0.09-5.25%	
History of abortion								
Yes	20	3(15%)	0.65-30.65%	2(10%)	3.15-23.15%	2(10%)	3.15-23.15%	*
No	240	8(3.33%)	1.06-5.6%	6(2.25%)	0.37-4.13%	4(1.67%)	0.05-3.29%	
Breeding								
Breed by AI (Cross breed)	110	4(3.64%)	0.14-7.14%	3(2.73%)	0.32-5.78%	2(1.81%)	0.68-4.31%	NS
Natural breeding (Indigenous)	150	7(4.67%)	1.29-8.05%	5(3.33%)	0.48-6.2%	4(2.67%)	0.1-5.25%	
Location/Area								
Pabna	120	6(5%)	1.1-8.9%	4(3.33%)	0.12-6.54%	3(2.5%)	0.29-5.29%	NS
Mymensingh	140	5(3.57%)	0.5-6.64%	4(2.86%)	0.2-5.62%	3(2.14%)	0.26-4.54%	

#Level of significance determined based on the results of RBT; NS = not significant; * = significant at 5% level of significance (P value=0.013); 95% CI = 95% confidence interval.

ELISA, respectively. The prevalence of *Brucella* was higher in female than male (Table 3) which was not statistically significant (P value=0.683).

In the present study, higher prevalence of 5.0%, 4.17% and 3.33% by RBT, SAT and I-ELISA, respectively was reported in cattle of more than 4 years old in comparison to 3.75%, 2.5% and 1.25% in age group of ≥ 2-4 years and 3.33%, 1.67% and 1.67% in the age group of < 2 years by RBT, SAT and I-ELISA, respectively (Table 3). The difference among different age groups of cattle was statistically insignificant (P value=0.884).

A higher prevalence of *Brucella* was found in cattle with history of abortion that was 15%, 10% and 10% than without history of abortion that was 3.33%, 2.25% and 1.67% by RBT, SAT and I-ELISA, respectively (Table 3). There exists a statistically significant difference between prevalence of *Brucella* in cattle with history of abortion than without history of abortion (P value= 0.013).

In this study, the prevalence of *Brucella* in indigenous cattle was 4.67%, 3.33% and 2.67% in RBT, SAT and I-ELISA, respectively, while cross-bred cattle had a prevalence of 3.64%, 2.73% and 1.81% in RBT, SAT and I-ELISA, respectively (Table 3). This difference was not statistically significant (P value=0.683).

In this study, the highest prevalence of *Brucella* in cattle was found in Pabna district especially in female 5%, 3.33% and 2.5% compared to the prevalence of 3.57%, 2.86%, 2.14% in Mymensingh district as detected by RBT, SAT and ELISA, respectively. The difference in prevalence of *Brucella* in cattle between the two areas was not statistically significant (P value=0.568).

The overall seroprevalence of *Brucella* in cattle was 2.3% which is similar with the reports of Amin *et al*. (2004) and Rahman *et al*. (2006) who reported that the prevalence was 2.33%, 2% and 2.4%, respectively. The prevalence is lower than the finding of Nahar and Ahmed (2009) who reported 4.5% prevalence. This variation in the prevalence may be due to variation in the age, breed, sex, pregnancy status of the animal, study area, hygienic condition, breeding techniques, herd size, reproductive diseases and diagnostic tests applied (Kebede *et al*., 2008).

The comparison of the serological test result revealed a higher prevalence in RBT when compared to SAT and I-ELISA. The RBT showed more positive reaction to *Brucella* as compared to SAT and I-ELISA. Higher sensitivity and specificity of RBT was also reported by Muktaderul *et al*. (2011) and Muma *et al*. (2007), while Chakraborty *et al*. (2000) reported lower sensitivity but higher specificity of RBT.

The prevalence of *Brucella* was higher in female than male. This finding was similar to the findings recorded by Sharma *et al.* (2003). This may be due to presence of allantoic factors including erythritol, possibly steroid hormones and other substances in the female reproductive tract, especially in the gravid uterus which stimulate the growth of most of the *Brucellae* (Radolf, 1994). In the present study, higher prevalence was reported in cattle of more than 4 years old than in age group of ≥ 2-4 years and in the age group of < 2 years. This finding is coincided with the finding of Kazi *et al*. (2005). In contrast to the findings of the present study, Rahman *et al*. (2011a) reported the prevalence of brucellosis in the cows aged 2.5-4 years as 2·59%, while, in the cows over four years of age as 4·35%. Similarly, Amin *et al*. (2004) reported 2.3% and 4% prevalence in the < 4 and > 4 years age group, respectively. Age wise prevalence has also been studied by Abubakar *et al*. (2010) who showed that the incidence of brucellosis increased with age, and the incidence is high in sexually mature animals. The older animals are more susceptible to brucellosis due to more contact with infectious agents. Aged females suffering from malnutrition during pregnancy are more likely to be infected. Sergeant (1994) also found that there was no apparent association between age and serological status, or age and the prevalence. Ghani *et al*. (1998) and Uddin *et al*. (2007a, 2007b) stated that several factors such as age, sex, breed, location, herd size and living condition influence the seroprevalence of *Brucella.* It appears that the higher prevalence of brucellosis among older cows might be related to maturity with the advancing age. Thereby, the organism may have propagated to remain either as latent infection or it may cause clinical manifestation of the disease (Amin *et al*., 2005).

A higher prevalence of *Brucella* was found in cattle with history of abortion than without history of abortion. The present finding is in agreement with Rahman *et al*. (2006) who reported brucellosis to be higher in cattle with a history of abortion (15%) as compared to those with a history of returns to service (1·45%). In this study, the prevalence of *Brucella* in indigenous cattle was slightly more than cross-bred cattle. The difference

between the two groups was not statistically significant. Akbarmeher and Ghiyamirad (2011) reported that there exists differences in the prevalence of brucellosis in different breeds, but not statistically significant. In this study, the highest prevalence of brucellosis in cattle was found in Pabna district especially in female compared to the prevalence of *Brucella* in Mymensingh district.

Definitive diagnosis of brucellosis can be accomplished only through the direct demonstration and identification of the causative agent(s) by culture and isolation procedures (Orduña *et al*., 2000). But culture requires level 3 biological safety cabinet as the chance of laboratory personnel to be infected is high. Due to the lacking of laboratory facilities causative agent could not be isolated by culture. Further study is required for definitive diagnosis of brucellosis by highly sophisticated techniques like culture, PCR etc.

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