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A sero-surveillance of *Brucella* spp. antibodies and individual risk factors of infection in cattle in Bangladesh

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Abstract

Brucellosis is a serious zoonosis, recognized worldwide. It primarily affects animals, which act as reservoirs for human infection as well as being of economic significance to the agri-food industry. Bangladesh has been reported as an endemic area for brucellosis. So a cross sectional study was conducted to determine the seroprevalence and potential risk factors of brucellosis in cattle in Dinajpur and Mymensingh districts of Bangladesh. A total of 182 cattle were examined by Rose Bengal Plate Test (RBPT) between September 2008 and October 2009. Then Positive, doubtful, and negative samples were further confirmed with slow agglutination test (SAT) and both indirect and competitive enzyme linked immunosorbent assay (iELISA and cELISA). A questionnaire was used to collect epidemiological information of the animals. The overall animal-level prevalence was 3.30%. Brucellosis seroprevalence was higher (4.76% by cELISA) in cattle above 48 months than those under 48 months. Female showed higher seroprevalence (10.67%) than male (6.25%). Higher seroprevalence was also found in cattle bred naturally (20.0%) than artificially (8.77%) and cattle that aborted or with previous abortion record (22.22%) showed higher seroprevalence than non-aborted (7.69%). The sensitivity of RBT and SAT was found 100% as compared to cELISA standard test, whereas specificity of RBT (95.35%) was higher than that of SAT (94.32%).

Key words : *Brucella* spp., Epidemiology, Serology, Cattle, Bangladesh

INTRODUCTION

Brucellosis is an emerging disease since the discovery of *Brucella melitensis* as the cause of Malta fever of fatal human case by David Bruce in 1887 in the island of Malta and so called "Malta Fever". In 1897, the isolation of *B. abortus* from aborted cattle by Bernard Bang and named "Bang's Disease" (Nicoletti, 1990). The disease also called as "Undulant Fever" and "Mediterranean fever" (WHO, 2006). It is an important zoonotic disease caused by small non-motile coccobacilli shaped facultative anaerobic gram-negative bacteria genus *Brucella* (Kakoma et al, 2003; Baek et al, 2003). It is a disease of economic and public health significance and had a worldwide distribution.

Brucellosis mainly affects reproduction and fertility, reduces the survival rate of newborns and reduce milk yield (Roth et al, 2003; Franco et al, 2007). It is essentially a disease of sexually matured animals. In human beings, the symptoms of disease are weakness, joint and muscle pain, headache, undulant fever, hepatomegaly, splenomegaly and night sweats. Sometimes it ischaracterized by influenza like clinical disease, which may be severe and may be followed by chronic intermittent relapses (Hugh-Jones, 2000). If untreated, recurrent

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fever (undulant fever) in humans can be developed and may persist for several months. Recently it has been reported that brucellosis can affect the central and peripheral nervous system of human (Al-Sous et al, 2004) and Domingo (2000) revealed that good relations exist between veterinary and health personnel in the field.

Brucellosis is a widespread zoonosis. *B. melitensis* mainly responsible for brucellosis in sheep and goats, but *B. melitensis* in cattle has emerged as an important problem in some southern European countries such as Israel, Kuwait, and Saudi Arabia. *B. melitensis* infection is particularly problematic because *B. abortus* vaccine do not protect effectively against *B. melitensis* infection; the *B. melitensis* Rev.1 vaccine has not been fully evaluated for use in cattle. Thus, bovine *B. melitensis* infection is emergingas an increasingly serious public health problem in some countries. A related problem has been noted in some South American countries, particularly Brazil and Colombia, where *B. suis* biovar 1 has become established in cattle (Garcia Carrillo, 1990). In some areas, cattle are now more important than pigs as a source of human infection.

The factors influencing the epidemiology of brucellosis in cattle in any geographical region can be classified into factors associated with the transmission of the disease among herds and the factors influencing the maintenance and spread of infection within herds (Crawford et al, 1990). The density of animal populations, the herd size, the type and breed of animal (dairy or beef), the type of husbandry system and other environmental factors are thought to be important determinants of the infection dynamics (Salman and Meyer, 1984).

Worldwide, Brucellosis remains a major source of disease in humans and domesticated animals. The true incidence of human brucellosis is clearly notknown. However, most cases of brucellosis in human are occupational and occur in the farmers, lab technicians, veterinarians, people working in meat processing industry, sheep herders etc (Radostits, 2000; Al-Ani et al, 2004) although other factors such as methods of food preparation, heat treatment of dairy products, and direct contact with animals also influence risk to the population.

Rose bengal test (RBT), serum agglutination test, tube agglutination test, mercaptoethanol test and/or ELISA are generally used for the serological detection of *Brucella* infections in livestock. The serological assay allows the detection of *Brucella* specific antibodies in a whole blood sample collected at a farm or in the field from an animal directly after the sample is collected.

In Bangladesh, the current status of brucellosis is not clearly known. There are a lot of undiagnosed cases of abortion, stillbirth and retained placenta which is thought to be brucellosis. This brucellosis in animals plays an important constraint to the development of livestock in Bangladesh and may have a considerable impact on both human and animal health as well as socioeconomic effects.

Recent serosurveys (Rahman et al, 2008; Rahman et al, 2006) for brucellosis in Bangladesh indicated a low level of seropositivity for brucellosis. However, seropositivity often indicates the presence of a larger problem rather than the absolute number of serologic reactors detected and also, because those animals were tested with *B. abortus* antigen, seropositivity to *B. melitensis* may not have been detected. In Bangladesh, brucellosis was first detected in cattle in 1967 (Mia and Islam, 1967) but there are no study conducted with ELISA. Therefore, the study was carried out with the following objectives: (1) Determination of brucellosis seroprevalence by RBT and Slow Agglutination Test (SAT) in Mymensingh and Dinajpur Districts of Bangladesh, (2) Application of ELISA to confirm the seroprevalence of brucellosis, and (3) Epidemiological study of brucellosis in Dinajpur and Mymensingh Districts.

MATERIALS AND METHODS

Experimental design

The study was conducted for 14 months from September 2008 to October 2009 in the Department of Medicine, Faculty of Veterinary Science, Bangladesh Agricultural University, Mymensingh. Venous blood samples were randomly and aseptically obtained from sexually matured cattle of both sexes. A total number of 182 blood samples were collected from cattle in

Mymensingh and Dinajpur District of Bangladesh. In Dinajpur District, 50 cattle sera were collected from Dinajpur Sadar Veterinary Hospital, Fakir para and Hossain pur village and in Mymensingh District, 132 sera were collected from Bangladesh Agricultural University Veterinary Clinic (44 samples), Pagla bazaar (68 samples), and Boyra (20 samples) (Table 1). During the sampling, a questionnaire regarding species, age, sex, location, pregnancy status, abortion record, types of insemination, reproductive problem was completed. All of the blood samples were processed and tested by RBT as a screening test. Then later, SAT, indirect enzyme linked immunosorbent assay (i-ELISA), and competitive enzyme linked immunosorbent assay (c-ELISA) were used for confirmatory diagnosis.

Serological tests

The serological tests for diagnosis of brucellosis in cattle include RBT, SAT, and both indirect and competitive ELISA.

For RBT, antigen preparation and testingwas done according to the procedure of the manufacturer [Veterinary Laboratory Agency (VLA), United Kingdom]. All sera and antigen were brought into room temperature for 45 minutes before use. The control sera, test sera, and *B. melitensis* Rose Bengal antigen prepared by a heat killed stained suspension of *B. abortus* S99 cells suspensed in 0.5% phenol saline.

SAT was carried out with EDTA as described by Garin et al (1985). The SAW (Synbiotics, concentrated suspension of *B. abortus*, Weybridge, strain 99) buffer was prepared by adding 0.93g EDTA (5mM, Triplex®) to 500ml PBS which was prepared by adding 5 tablets of PBS (DULBECCO-A Oxoid) to 500ml distilled water (with 1 tablet/100ml distilled water). One ml of SAW antigen was diluted with 19ml SAW buffer. A positive control serum was included in every test.

Both indirect ELISA and competitive ELISA were performed according to the protocol provided by the manufacturer (Svanova Biotech AB, art. No. 10-2700-10, SE-751 83 Uppsala, Sweden). PBS- Tween buffer for ELISA was prepared through 20x concentrate PBS-Tween solution (PBST) was diluted into 1/20 with distilled water (DW). In case of iELISA, anti-Bovine IgG conjugatewas prepared by lipholized HRP conjugate was reconstrituted with 11.5ml PBS-Tween buffer. Buffer was added carefully to the bottle. Then the solution was leaved for one minute and mixed thoroughly. All of the reagents were prepared immediately before use according to the recommendation. Finally, the assay was calibrated against the DIE ELISA Standard sera and standardized against EU directives 64/432/EEC. Following this, the test sample results should be interpretated as follow) (Table 2).

For cELISA, freeze dried mAb was reconstituted with 6ml Sample Dilution Buffer. Buffer was added carefully to the bottle and was prepared just immediately before use and mixed gently.

Data processing and statistical analysis

The questionnaire based data was processed by Microsoft excel and MStatc. The results were analyzed by Chisquare tests (x^2). Significance was determined at 1% and 5% level.

Table 1. Area/location and number of cattle sera collection

Area / Location	No. of cattle
Mymensingh district	
BAU Veterinary clinic	44
Pagla bazaar	68
Boyra	20
Dinajpur district	
Sadar Vet. hospital	26
Fakirpara	10
Hossain pur	14
Total	182

Table 2. Interpretation of test sample

Sample material	Sample incubation		Interpretation
	1 hr/+37°C PP	ON /+4°C PP	
Individual serum	<40 >40	<50 >50	Negative positive
Pool of serum	<25 >25	<30 >30	Negative positive
Individual or tank milk	<10 >10	<15 >15	Negative positive

RESULTS

Serological results

Two different districts (Dinajpur and Mymensingh) were considered for serum collection and tested by RBT, SAT, iELISA, and cELISA.The results has shown in Table 3 ~ 10. The majority of the serum samples were collected from 2 ~ 4 years of age (114 out of 182), from female (150 out of 182), from cows bred by artificial insemination (114 out of 91),and from cows with no previous abortion record (130 out of 91).

The overall seroprevalence of brucellosis in cattle has shown in Table 3 was 3.30% (6 cattle) among 182 cattle, respectively.

Comparison of the serological tests results

Seroprealence of brucellosis was compared among different tests including RBT, SAT, iELISA and cELISA and the results have been shown in Table 4.

Table 3. Overall seroprevalence of brucellosis in 182 cattle

No. (%) of positive cases			
RBT	SAT	ELISA	All tests
18 (9.89)	16 (8.79)	6 (3.30)	6 (3.30)

Table 4. Comparison of seropositivity of brucellosis in 182 cattle

No. (%) of reactors by							
RBT		SAT		iELISA		cELISA	
Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
18 (9.89)	164 (90.11)	16 (8.79)	166 (91.21)	2 (1.10)	180 (98.90)	6 (3.30)	176 (96.70)

Table 5 . Sensitivity and specificity of RBT and SAT by comparing with cELISA (standard test) for detection *Brucella* antibodies in cattle

	Test	C-ELISA		Subtotal	Sensitivity (%)	Specificity (%)
		Positive	Negative			
RBT	Positive	6	12	18	100	95.35
	Negative	0	164	164		
	Total	6	176	182		
SAT	Positive	6	10	16	100	94.32
	Negative	0	166	166		
	Total	6	176	182		

Table 6 . Age-related seroprevalence of brucellosis in cattle

Age (Months) of animals	Sera tested	No. (%) of positive reactors by				Level of significance (x^2-test)
		RBT	SAT	iELISA	cELISA	
13 - 24	26	4 (15.38)	4 (15.38)	0 (0.00)	0 (0.00)	NS*
25 - 48	114	8 (7.02)	6 (5.26)	0 (0.00)	4 (3.51)	
Above 48	42	6 (14.29)	6 (14.29)	2 (4.76)	2 (4.76)	

*NS=Not significant

Table 7 . Sex related seroprevalence of in cattle

Sex	No. of sera	No. (%) of positive reactors by			
		RBT	SAT	iELISA	cELISA
Male	32	2 (6.25)	2 (6.25)	0 (0.00)	2 (6.25)
Female	150	16 (10.67)	14 (9.33)	2 (1.33)	4 (2.67)

Table 8 . Area related seroprevalence of brucellosis in cattle

Location (District)	Sera tested	Sex	No. of sera	No. (%) of positive reactors by			
				RBT	SAT	iELISA	cELISA
Dinajpur	50	Male	10	2 (20.0)	2 (20.0)	0 (0.00)	2 (20.0)
		Female	40	2 (5.00)	2 (5.00)	0 (0.00)	0 (0.00)
Mymensingh	132	Male	22	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
		Female	110	14 (12.73)	12 (10.91)	2 (1.85)	4 (3.70)

Table 9 . Breeding related seroprevalence of brucellosis in cattle

Types of breeding	Sera tested	No. (%) of positive reactors by			
		RBT	SAT	iELISA	cELISA
Natural breeding	30	6 (20.00)	6 (20.00)	2 (6.67)	4 (13.33)
Artificial insemination	114	10 (8.77)	8 (7.02)	0 (0.00)	0 (0.00)

Table 10 . Prevalence of brucellosis in non-aborted and aborted /previously aborted cattle

Record condition of animals	Sera tested	No. (%) of positive reactors by				Level of significance (x^2-test)
		RBT	SAT	iELISA	cELISA	
No previous abortion	130	10 (7.69)	10 (7.69)	0 (0.00)	2 (1.54)	
Previous abortion	18	4 (22.22)	2 (11.11)	2 (11.11)	2 (11.11)	**

**: Significant at 1% level of probability ($P < 0.01$)

DISCUSSION

Worldwide, brucellosis remains a major bacterial source of disease in humans and domesticated animals. The objectives of the study were to implement of ELISA, SAT and to improve the understanding the epidemiology of *Brucella* in cattle and toprovide information for disease control in livestock and human being. Seropositivity was considered to be due to natural infection because vaccination has never been practiced in Bangladesh.

Bangladesh has been reported as an endemic area for brucellosis because of a considerable number of human and animal populations wereexposed to the infection each year (Rahman et al. 1978). Definitive diagnosis of infectious disease 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). Accurate presumptive diagnosis can be achieved from serological techniques used in combination with clinical observations and case histories. Although classical serological techniques suffer from several drawbacks, poor performance, and lack of standardization, RBT has been used as a screening test of *Brucella* infection (MacMillan, 1990) and more sensitive than the CFT when testing culture positive animal (Blasco et al, 1994). ELISA techniques have the potential to solve many of these problems using classical serological techniques (Wright et al, 1993).

Widely accepted,agglutination tests (SAT and to lesser extent RBT) are not recommended for the diagnosis of chronic brucellosis since these tests mainly detect IgM. The amount of IgMfound in sera will decline with time and become undetectable in agglutination tests in most chronic cases (OIE, 2000). However, in experimental conditions, agglutination tests are able todetect infections as early as two weeks postinfection and thus remain excellent tools to use in order to detect early infections (Godfroid and Kasbohrer, 2002).

ELISA has been evaluated for many years for their diagnostic performance to detect serum antibody to brucellosis in domestic animals. ELISA for diagnosis of brucellosis has several advantages when compared with

other tests. Firstly, it is a direct method of identification of specific antibody and therefore, it is not prone to false positive reactions. Secondly, it is more sensitive than other the agglutination test and thus has the potential to detect infected animals. Thirdly, the antibody enzyme conjugate employed has light chain reactivity and thus is able to detect all classes of antibody. A combine determination of all classes of antibody allows accurate serological diagnosis at any stages of disease. Fourthly, ELISA results provide an epidemiological tool for investigating the infective status of flocks (Rahman, 2003). To the best of our knowledge, an ELISA for the diagnosis of brucellosis has not been practiced yet in Bangladesh.

The present investigation revealed that the overall seroprevalence of brucellosis in cattle was 3.30% which is higher than the overall seroprevalence of brucellosis, 2% reported by Amin et al (2004) and 2.33% reported by Amin (2003). But this finding is in agreement with Rahman et al (2006) who reported animal-level seroprevalence of brucellosis in cattle is 2.4~18.4% while the herd-level seroprevalence in cattle is 62.5%.

ELISA (indirect and competitive) testing of all the RBT and SAT positive and suspicious sera ensured a very high sensitivity and specificity. Sensitivity of RBT and SAT, in both cases was found 100% in cattle with considering cELISA as a standard test while specificity was found to be of 95.35% and 94.32% in cattle, respectively (Table 5). Thus, RBT was found more specific than SAT and equally sensitive in cattle. The results of the further testing of the suspicious and positive RBT and SAT samples gave some guidance about the likely costs and benefits of using ELISAs on positive animalsin the late stage of an eradication program. The results suggest that iELISA provided better estimates of the actual prevalence of the infection although RBT could be used as a screening test for brucellosis due to its low cost and easy execution.

In the case of age-related seroprevalence in cattle, among the three age groups, though highest prevalence was found 15.38% of 13 to 24 months age group by RBT, but whereas by both iELISA and cELISA was 0%. But in age group between 25 to 48 months and above 48 months seroprevalence was found 3.51% and 4.76% by cELISA respectively which agreed the findings by Aulakh et al (2008); brucellosis increases with age.

The prevalence of brucellosis in cattle was found to be higher in female (10.67%, 9.33%, 1.33%, and 2.67%) than male (6.25%, 6.25%, 0% and 6.25%) when determined by RBT, SAT, iELISA and cELISA tests, respectively (Table 7). This finding was similar to the findings recorded by Sharma et al (2003).

In this study, the highest prevalence of brucellosis was shown in Dinajpur district and especially in male with a highest prevalence 20% by RBT, SAT and cELISA tests than in Mymensingh district in female with 12.73% prevalence by RBT. Siriwardane (1987) showed the prevalence of brucellosis in Sri Lanka was varied from 1.2 to 20% in various areas. Prahlad et al (1997) reported that the prevalence of brucellosis in goats was highest in Rajasthan (29.7%) followed by Uttrar Pradesh (29.0%) and Punjab (15.8%).

The significantly ($P<0.01$) higher prevalence of brucellosis (22.22% by RBT and 11.11% by SAT, iELISA and cELISA) was found in aborted or previously aborted animals than that of non aborted animals (7.69% for sheep and 2.56% for goats). Similar relationship with seropositivity was found by Kubuafor et al (2000) and England et al (2000).

The prevalence of brucellosis in cattle bred by natural breeding (20% by RBT and SAT and 13.33% by cELISA) was found to be higher than cattle bred by AI (8.77% by RBT) but higher prevalence of brucellosis in cattle bred by AI was reported by Sarumathi et al (2003). The study stated that the higher prevalence in cattle bred by natural breeding may be due to presence of infectious bulls used for natural breeding.

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