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Indirect enzyme linked immunosorbent assay for the diagnosis of brucellosis in cattle

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Abstract

Brucellosis is a major zoonosis caused by Gram negative facultative intracellular bacterial organisms of the genus *Brucella* that are pathogenic for a wide variety of animals and human beings. Because of its economic impact on animal health and the risk to the human population,most countries have a brucellosis control program. Brucellosis is also an economically important andprevalent disease in Bangladesh. The accurate and prompt diagnosis is very important in controlling and eradicating of the disease in animals. The present study was undertaken to determine the seroprevalence of brucellosis in cattle in Mymensingh and Patuakhali district of Bangladesh. A total of 120 serum samples were collected from the two districts along with a questionnaire related to the epidemiology of the disease. The sampleswere screened by using slow agglutination test and conformed by indirect enzyme linked immunosorbent assay. The overall seroprevalence of brucellosis in cattle was 5% and it was observed that, a higher prevalence of *Brucella* was found in female than male, through natural breeding than artificial insemination (AI) and animal above 4 years old are highly susceptible than younger ones. Higher prevalence was found in aborted animals in comparison with non aborted animal. Finally, the study revealed that the female animal has more susceptible to brucellosis and healthy semen should be used for AI.

Key words : *Brucella* spp., I-ELISA, Epidemiology, Cattle, Bangladesh

INTRODUCTION

Brucellosis is an important zoonosis and serological surveillance is essential to its control (Erdenebaatar et al., 2004). Brucellosis is caused by different species of the genus *Brucella* that are pathogenic for a wide variety of animals and human beings (Mathur, 1971). In animals, the brucellosis mainly affects reproduction and fertility, reduces the survival of newborns, and reduce milk yield. Mortality of adult animals is insignificant (Sewell et al, 1990). In humanbeings, the symptoms of disease are weakness, joint and muscle pain, headache, undulant fever, hepatomegaly, splenomegaly and night sweats (Hugh-Jones, 2000).

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According to the Food and Agriculture Organization (FAO), the World Health Organization (WHO) and the world Organization of Animal Health (OIE), brucellosis is considered the most widespread zoonosis worldwide (Mustafa and Nicoletti, 1995).

The first description of an outbreak of undulant fever caused by *B. abortus* involved college students who drank raw cow milk in the dormitory (Hugh-Jones, 2000). Brucellosis has been an emerging disease since the discovery of *B. melitensis* as the cause of Malta fever in the spleen of a fatal human case on the island of Malta in 1886 and isolated by David Bruce one year later in 1887 and *B. abortus* isolated from the aborted cattle by Bernard Bang, in 1897 (Nielsen and Duncan, 1990; Hugh-Jones, 2000).

Historically, in the Indian subcontinent the credit of

first investigation of contagious abortion in livestock associated with brucellosis, goes to the Imperial Veterinary Research Institute (now Indian Imperial Veterinary Research Institute), Muketswar, in northern India (Anonymyous M,1918). In Bangladesh, brucellosis was first identified in cattle in 1967 (Mia and Islam, 1967), in buffalo in 1997 (Rahman et al, 1997).

The importance of brucellosis is not known precisely, but it can have a considerable impact on human and animal health, as well as on socioeconomic impacts, especially in which rural income relies largely on livestock breeding and dairy products (Islam et al, 1983). Human brucellosis is caused by exposure to livestock and livestock products. Infections can result from direct contact with infected animals and can be transmitted to consumers through raw milk and milk products. Most cases occur in people employed in meat processing industry while sources include the domestic cattle, pig, sheep, goat and unpasteurized dairy products (Radostits, 2000).

Prevalence of brucellosis has been reported in cattle from different parts of the world. The higher prevalence of brucellosis in cows of better managed farms and estimated of human brucellosis as 12.8% in herders and agricultural workers and 21.6% in goat farmers (Rahman et al, 1983). The seroprevalence of brucellosis in cattle was 2.4~18.4% whiles the herd-level seroprevalence in cattle was 62.5% in Bangladesh (Rahman et al, 2006) The seroprevalence of brucellosis was 4.5% in cattle and 6% in human (Azimun, 2007).

In Bangladesh approximately 80 percent of people live in villages, and rural income is largely dependent on livestock; the people are in close contact with livestock on a daily basis. 6.5% of national income and 3.5% GDP come from livestock there are a lot of undiagnosed cases of abortion, stillbirth and retained placenta which is thought to be brucellosis and play an important constraint for the development of livestock in Bangladesh (Rahman et al, 2006).

Serological testing using the RBT, SAT, TAT, mercaptoethanol test and ELISA are general used for the detection of *Brucella* infection in livestock. Recently a simple and rapid field test for the "penside" diagnosis of brucellosis in livestock was developed (Abdoel et al, 2008). ELISA has been evaluated for many years for their diagnosis performance to detect serum antibody to brucellosis in domestic animals. It has gained popularity over recent years as an alternative to other serological tests. ELISA for diagnosis of brucellosis has several advantages when compared with other tests. Firstly, it has 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 o disease. Fourthly, ELISA results provide an epidemiological tool for investigation the infective status of flocks (Rahman, 2003). To the best of knowledge, an ELISA for the diagnosis of brucellosis has not been practiced yet in Bangladesh.

In previous studies, reports of brucellosis in Bangladesh both in animal and human were made using only *B. abortus* antigen but there were no report of *B. abortus* specific prevalence in cattle in Bangladesh by ELISA. Therefore, the present study was carried out to study the ELISA for the diagnosis of brucellosis in Mymensingh and Patuakhali districts of Bangladesh with the following objectives. (1) Detection of brucellosis in cattle by SAT, and (2) application of I-ELISA for the diagnoses of brucellosis in above area.

MATERIALS AND METHODS

Sources of samples

The study was conducted for 9 months from April 2009 to November 2009 in the Department of Medicine, Faculty of Veterinary Science, Bangladesh Agricultural University, Mymensingh. A total of 120 blood samples were collected from cattle in Mymensingh, and Patuakhali: 50 samples were collected from Mymensingh and 70 samples were collected from Patuakhali as summarized in Table 1. The study recorded clinical, epidemiological,

and reproductive information: age, gender, breed, area, client's complaint, pregnancy and breeding status, number of animals in herds, disease history, reproductive problems such as abnormal uterine discharge, abortion, repeat breeding in cows and reproductive diseases in bulls were recorded by completing a questionnaire when the samples were collected.

All blood samples were processed for sera preparation and were tested with slow agglutination test (screening test) and indirect enzyme linked immunosorbent assay (I-ELISA) for confirmatory diagnosis.

Blood and sera samples collection

At first the owner and the attendant controlled the animal and then the site of blood collection at jugular furrow was soaked with tincture of iodine or alcohol. About 4~7ml of blood was collected from jugular vein of each cattle with sterile disposable syringe and needle and was kept undisturbed on a tray for at least 30 minute

Table 1. Collection of sera from cattle in various locations of Mymemsingh and Patuakhali district

District	Area/Location	No. of cattle
Mymensingh	BAU Veterinary clinic	21
	Digarkanda	19
	Sesh morr	10
Patuakhali	Thana veterinary hospital (Bauphal)	25
	Kasubpur,	23
	Bazemahal	22
	Kuakata (Kalapara).	20
Total		120

at room temperature in a slightly inclined position to facilitate clotting and separation of serum. After this period, the clotted blood samples with sera are transferred to refrigerator at 4°C and kept overnight. Then the clotted blood samples were centrifuged at 3,000rpm for 10min for clarification. After centrifugation, clear sera were poured into sterilized and labeled microcentrifuge tubes and stored at −20°C until used.

SAT

SAT was carried out with EDTA as described by Garin et al (1985). The SAT (synbiotics, concentrated suspention of *B. abortus*, weybridge, stain 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 (1 tab /100ml distilled water) One milliliter of the antigen SAW was diluted with 19ml of SAT buffer solution. A positive control serum was included in every test.

Briefly, the slow agglutination test was performed in "U" bottom 96 well microplates. At first for each test serum, a raw of 3 wells of the 96 well microplates was selected to make 2-fold (?) dilution of the sera. 168µl of SAW buffer was pipette in first well and 100µl in the 2nd well and 3rd well respectively of the microplate. Then 32µl 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 100µl was taken from this well and was placed in the second well (1/12.5). 100µl from the 2nd well was transferred into the 3rd well and finally 100µl of liquid in excess was discarded from 3rd well. Note that all wells contained 100µl.

Then in each well 100µl of standardized SAW antigen was added. This gives the serial serum dilution of 1/1.25, 1/25, 1/50. The plate was then incubated at 37°C for 24 hours (+/−4 hrs) for reading.

Interpretation

After 24hrs, agglutination reaction was observed by using a magnifying mirror against illumination source. Reading was taken on the basis of this protocol and the standardization we performed (75% agglutination of the OIEISS), A table (Appendix-111) provide by Veterinary Agrochemical Research Center, Groeseleaberg 99, 1180 Brussels, Belgium were used for the interpretation of the result.

Indirect enzyme linked immunosorbent assay (i-ELISA)

The assay was performed according to the protocol provided by the manufacturer (Svanova Biotech AB, art. No. 10-2700-10, SE-751 83 Uppsala, Sweden).

Preparation of PBS-tween buffer for I-ELISA

According to the procedure, 20x concentrate PBS-tween solution (PBST) was diluted into 1/20 in distilled water (DW). 500ml per plate was prepared by adding 25ml PBST solution to 475ml DW and was mixed thoroughly.

Preparation of anti-bovine IgG conjugate for i-ELISA

According to the procedure, lipholized HRP Conjugate was reconstrituted with 11.5ml PBS-tween buffer. Buffer was added carefully to the bottle. Then the solution was leftfor one minute and mixed thoroughly. According to the recommendation, the solution was prepared immediately before use.

Test procedure

All reagents supplied by the manufacturer company were equilibrated to room temperature 18 to 25°C (64 to 77°F) before use. 100μl of sample dilution buffer was added to each well that would be used for serum samples and serum controls. After that, 4μl of positive control serum (Reagent A) and 4μl of negative control serum (Reagent B) was added to selected wells coated with *B. abortus* antigen. For confirmation purposes it was run the control sera in duplicates. 4μl of serum sample was also added to a selected well coated with *B. abortus* antigen. The samples were also run in duplicates for confirmation. The plate was shacked thoroughly, sealed, and incubated at 37°C (98.6°F) for 1 hour. The plates were washed 3 times with PBS-tween buffer then 100μl of HRP conjugate was added to each well and incubated at 37°C (98.6°F) for 1 hour. The plate was washed once more. Then 100μl Substrate Solution was added to each well and incubated for 10 minutes at room temperature. The reaction was stopped by adding 50μl 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 450nm in a microplate photometer. The OD was measured within 15 minuafter the addition of stop solution to prevent fluctuation in OD values.

Calculations: Calculations of results were done as described below.

Calculation of percent positivity values (PP)

All OD values for the test samples as wellas the negative control (Neg C) are related to the OD value of the positive control as follows:

$$PP = \frac{\text{Test sample or Neg C (OD)}}{\text{Positive control (OD)}} \times 100$$

Interpretations

The assay was calibrated with the OIE ELISA standard sera and standardized with the EU directives 64/432/EEC.

Data processing and statistical analysis

The data obtained by questionnaire was statistically analyzed by using Microsoft excel and MStatc.

RESULTS

A total of 120 sera samples were collected from cattle in Patuakhali and Mymensingh. The sera were tested by

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

Sera tested	Positive No. (%) of reactor by		
	SAT	I- ELISA	All tests
120	9 (7.5%)	6 (5.00%)	6 (5.00%)

Table 3. Age related seroprevalance of brucellosis based on SAT and I-ELISA in cattle

Age (years) of animals	Sera tested	No. (%) of positive reactors by	
		SAT	I- ELISA
1-2	11	1 (9.09)	0 (0.00)
2-4	86	5 (5.81)	4 (4.46)
Above 4	23	3 (13.04)	2 (8.69)

SAT and I-ELISA and the results have shown in Tables 2~8. The majority serum samples were collected from 2~4 years old cattle (76 out of 120) from female (90 out 120), from cattle bred by natural breeding (85 out of 120), and from cattle with no previous abortion (113 out of 113).

The seroprevalence of brucellosis in cattle was 5.00% (n=6) among 120 cattle by I-ELISA (Table 2).

Age related seroprevalance of brucellosis based on SAT, I-ELISA was shown in Table 3. One positive reactor was found within the age of 1 to 2 years and the prevalence was 9.09% by SAT and 0 (0.00)% by I-ELISA. Among 86, 2~4 years old cattle, the prevalence was 5.81% by SAT and 4.46% by I-ELISA, Among 23 over 4 yearsold cattle, the prevalence was 13.04%.by SAT and 8.69% I-ELISA.

Sex related seroprevalance of brucellosis based on SAT and I-ELISA in cattle shown in Table 4. The higher prevalence was found in female than male and female showed a prevalence of 8.08% by SAT and (5.05%) by I-ELISA. The male showed a prevalence of 4.76% by SAT and 4.76% by I-ELISA.

Breeding related seroprevalance of brucellosis based on SAT and I-ELISA in cattle shown in Table 5. More positive cases werefound in cattle bred by natural breeding than artificial insemination. The prevalence was 7.05% by SAT and 5.88% by I-ELISA in the case of natural breeding. The prevalence was 8.57% by SAT and 2.85% I-ELISA in the case of artificial insemination.

Abortion related seroprevalance of brucellosis based on SAT and I-ELISA in cattle shown in Table 6. Out of 120 cattle, only 7 had the history of Abortion. The prevalence in aborted cattle was 28.57% by SAT or I-ELISA, while the prevalence in non aborted cattle was 6.19% and 3.53% by SAT and I-ELISA, respectively.

Area related seroprevalance of brucellosis based on SAT and I-ELISA was shown in Table 7. In Patuakhali, the prevalence was 5.88% or 9.43% by SAT and 5.88% or 5.66% by I-ELISA for male or female, respectively. In Mymensingh, the prevalance was detected only in female, which was 6.52% by SAT and 4.35% by I-ELISA.

Pregnancy related seroprevalance of brucellosis based on SAT, I-ELISA shown in Table 8. A higher prevalence (16.12% by SAT and 9.69% by I-ELISA) was found in pregnant cattle than non pregnant cattle.

Table 4. Sex related seroprevalance of brucellosis based on SAT and I- ELISA in cattle

Sex	Sera tested	No. (%) of positive reactors by	
		SAT	I-ELISA
Male	21	1 (4.76)	1 (4.76)
Female	99	8 (8.08)	5 (5.05)

Table 5. Breeding related seroprevalance of brucellosis based on SAT and I-ELISA in cattle

Types of breeding	Sera tested	No. (%) of positive reactors by	
		SAT	I-ELISA
Natural breeding	85	6 (7.05)	5 (5.88)
Artificial insemination	35	3 (8.57)	1 (2.85)

Table 6. Abortion related seroprevalance of brucellosis based on SAT and I-ELISA in cattle

Types of condition	Sera tested	No. (%) of positive reactors by	
		SAT	I-ELISA
History of abortion	7	2 (28.57)	2 (28.57)
No previous abortion.	113	7 (6.19)	4 (3.53)

DISCUSSION

Brucellosis is an important zoonosis and serological surveillance is essential to its control, although many countries have eradicated *B. abortus* from cattle (Erd-

Table 7. Locations related seroprevalance of brucellosis based on SAT and I- ELISA in cattle

Location	Sex	Sera tested	No. (%) of positive reactors by	
			SAT	I-ELIS
Patuakhali	Male	17	1 (5.88)	1 (5.88)
	Female	53	5 (9.43)	3 (5.66)
Mymensingh	Male	4	0 (0.00)	0 (0.00)
	Female	46	3 (6.52)	2 (4.34)

Table 8. Pregnant related seroprevalance of brucellosis based on SAT and I- ELISA in cattle

Criteria of animal	Sera tested	No. (%) of positive reactors by	
		SAT	I- ELISA
Non pregnant	68	3 (4.41)	2 (2.94)
Pregnant	31	5 (16.12)	3 (9.69)

enebaatar et al, 2004). Economic losses can be heavy due to abortion and infertility and herd should be monitored for the presence of infection. Despite eradication programs, including vaccination, testing and slaughter out, the disease has remained prevalent in many areas in the world. The brucellosis is still endemic in Africa, South America, Near and Middle East and isolated or sporadic outbreaks are reported from Europe (Timoney et al, 1988). Each year half of a million cases of brucellosis are reported worldwide but according to WHO, these numbers greatly underestimate the true prevalence. The importance of brucellosis is not known precisely, but it can have a considerable impact on human and animal health, as well as on socioeconomic impacts, especially in which rural income relies largely on livestock breeding and dairy products (Islam et al, 1983).

A total of 120 cattle serum samples were collected to study serodiagnoses of brucellosis in cattle. The overall seroprevalence of brucellosis in cattle was 5.00% (n= 120) which is higher than the overall seroprevalence of brucellosis, 2% (n=250) reported by(Amin et al, 2004) and 2.33% (n=300) reported by (Amin, 2003). But this finding is in agreement with the study conducted by 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%.

A higher prevalence (13.04%) was found in cattle with increasing of age of the animals. A similar finding was recorded by Aulakh et al (2008). It may be considered that the higher prevalence of brucellosis among older cattle might be due to maturity with the advance age. However, the older animals supposed to be more infected, because of more contact with infectious agents and sometimes from malnutrition during pregnancy.But there was no significant association between age group and the prevalence when sera sample tested with SAT and I-ELISA (Sergeant ES, 1994), also found that there was no apparent association between age serological status, or age and the prevalence. But (Ghani et al, 1998) stated that several epidemiological factors, such as age, sex, breed, location, herd size and living condition influence the seroprevalence of brucellosis.

The prevalence of brucellosis in cattle was found to be higher in female than male by both SAT and ELISA which is similar to the findings recorded by Sharma et al (2003). The prevalence of brucellosis was found to be higher in pregnant cows (16.61%) than non-pregnant cows (4.41%) by SAT. Similar results were reported by Amin et al (2004) and they reported 3.45% and 1.23% in pregnant and non-pregnant cows, respectively. This finding also correlates with the reports by Amin et al (2003) and Plommet et al (1971). The prevalence of brucellosis in cattle bred by AI (8.57%) by SAT was found to be higher than cattle bred by natural breeding (7.05%) by SAT. It is similar reported by Sarumathi (2003).

In this study, the highest prevalence of brucellosis in cattle was found in Patuakhali district especially in female 9.43% by SAT and 5.66% by I-ELISA than Mymensingh 6.52% by SAT and 4.34% by I-ELISA.

In conclusion, it is observed that a higher prevalence of *Brucella* was found in female than malenatural breeding than artificial one aged animal between 2~4 years old than young below 2 years old pregnant animals than non-pregnant animals and aborted animals than non aborted animals. The result shows that female animal has higher susceptibility to infection of brucellosis and healthy semen should be used for AI. Further studies on isolation, identification and typing of *Brucella* are recommended.

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