Bangl. J. Vet. Med. (2016). 14 (2): 221–226 ISSN: 1729-7893 (Print), 2308-0922 (Online)

SEROPREVALENCE AND MOLECULAR DIAGNOSIS OF *BRUCELLA ABORTUS* AND *BRUCELLA MELITENSIS* IN BANGLADESH

M. A. S. Sarker[1], R. R. Sarker[1], M. M. Begum[2], N. M. Shafy[1], M. T. Islam[1], M. A. Ehsan[1], P. K. Bhattacharjee[1], M. F. Rahman[3], F. Melzer[4], H. Neubauer[4] and M. S. Rahman[1*]

[1]Department of Medicine, Bangladesh Agricultural University, Mymensingh; [2]Youth Training Center, Munshiganj, Bangladesh; [3]Commandant Armed Forces Medical College, Dhaka Cantonment; [4]Federal Research Institute for Animal Health, Jena, Germany

ABSTRACT

The importance of brucellosis is primarily due to its public health significance and economic loss for the animal industry. The present study was performed to determine the seroprevalence of brucellosis in man and animals. A total of 1,452 samples (cattle 913, buffaloes 99, sheep 40, goats 50 and persons 350) were collected from Central Cattle Breeding and Dairy Farms, Savar, Dhaka and 5 districts (Mymensingh, Jamalpur, Gaibandha, Rangpur and Bagerhat) of Bangladesh. All samples were screened by RBT and the RBT positive sera were re–tested with SAT CFT, ELISA and qRT-PCR. Out of 913 cattle and 99 buffalo sera, 48 cattle and 7 buffalo sera showed positive reaction to RBT i.e. prevalence of brucellosis is 5.3% in cattle and 7.1% in buffaloes. The 350 human sera and all sheep and goats samples were negative to RBT. Among the RBT positive sera, 12 sera were found to contain *Brucella* DNA by genus specific IS711 quantitative real time PCR (qRT-PCR); and all PCR positive samples were found to contain *Brucella abortus* DNA. This report confirms that *B. abortus* is endemic in cattle and buffaloes in Bangladesh.

Key words: Brucellosis, molecular diagnosis, Bangladesh

INTRODUCTION

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. Brucellosis is an important disease of animals caused by small non-motile coccobacilli shaped Gram-negative bacteria. Brucellosis can affect domestic animals (like cattle, buffaloes, sheep, goats, pigs) and humans. The genus *Brucella* has 12 species on the basis of host specificity. Important zoonotic *Brucella* species are *B. abortus*, *B. suis* and *B. melitensis*. Brucellosis in cattle is caused almost exclusively by *B. abortus* which causes abortion and infertility in dairy cows and in small ruminants (sheep and goats), brucellosis in small ruminants is caused by *B. melitensis*. 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 with the mammary gland as their preferred site (Olsen and Hennager, 2010). Domestic animals get infection from ingestion of contaminated feed and water, inhalation of aerosolized bacteria, sexual intercourse and direct contact with contaminated materials (Radostits *et al*., 2000). 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. 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). Human infections can result from direct contact with infected animals and brucellae can be transmitted to consumers with contaminated raw milk and milk products (Radostits *et al*., 2000).

*Corresponding e-mail address: prithul02@yahoo.co.uk
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Polymerase chain reaction (PCR) assays can be applied to detect *Brucella* DNA from 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; 2008). The PCR is more sensitive than blood culture and more specific than serological tests (Al Dahouk *et al*., 2013). Culture for brucellosis diagnosis requires BSL 3 facilities and skilled personnel. In Bangladesh, no laboratory exists with BSL3 facilities for isolation of brucellae. Therefore, it is more feasible to detect *Brucella* DNA from clinical samples in Bangladesh. 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. The aim of this study was to determine the seroprevalence of brucellosis in animals and humans and to detect *Brucella* DNA at the species level using real-time PCR.

MATERIALS AND METHODS

Venous blood samples were randomly and aseptically collected from cattle, buffaloes, sheep, goats and humans. A total of 1,452 blood samples was collected i.e. 913 from cattle, 99 from buffaloes, 40 from sheep, 50 from goats and 350 from human of Mymensingh Medical College Hospital, Central Cattle Breeding and Dairy Farm, Savar Dhaka; Mymensingh, Jamalpur, Gaibandha, Rangpur and Bagherhat districts of Bangladesh (Table 1). Clinical, epidemiological, managemental and reproductive information was recorded using questionnaires. Data on age, sex, geographical area, status of pregnancy, disease history, hygroma, reproductive disorder such as abnormal abdominal uterine discharge, abortion, retention of placenta, and reproductive diseases were recorded. The RBT was used as a screening test to identify infected animals and humans. iELISA (Svanova Biotech AB, Uppsala Sweden), RBT, SAT, CFT were performed according to the procedures described by OIE (2009) and the manufacturers. RBT positive sera were re-tested with SAT, CFT, ELISA and qRT-PCR.

DNA extraction and real time PCR

DNA was isolated from 200 µL of RBT positive sera using the High Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. The resulting DNA concentration was determined photometrically using a Nano Drop ND-1000 UV-Vis spectrophotometer (Nano-Drop Technologies, Wilmington, DE, USA). Samples tested RBT positive were investigated with *Brucella IS711* genus specific RT PCR. Reactions were conducted in plastic capillaries using the light cycler 2.0 instrument (Roche, Mannheim, Germany) according to the method of Tomaso *et al*. (2010). Briefly, each reaction contained the primer pair IS711_S-5'-TTGTCGATGCTATCGGCCTAC-3'/IS711_R-5'-GGCAATGAAGGCC CTTAAGT-3' at a concentration of 500nM and the probes IS711_FL 5'-GAAGCTTGCGGACAGTCACCATA AT-Fluo-3'/IS711_LC-5'-Red640-GCCGGGTGTTGGCTTTATTCG-Pho-3' at a concentration of 200nM. The final 20µL reaction mixture included 4µL LC FastStart DNA Master Plus Master mix (Roche) supplemented with 1 µL primers and 0.4µL probes and 2µL sample. Cycling parameters were as follows: a ten minute activation step at 95°C followed by 45 cycles of 95°C for 10 sec, 55°C for 10 sec, and 72°C for 10sec (annealing and extension). Melting curve parameters were: 0 sec at 95°C, 30 sec at 45°C and 0 sec at 95°C followed by a 30 sec cooling step at 40°C. Data acquisition and evaluation was calculated by the instrument's software. Ct values below 40 were interpreted as positive. Subsequently the samples were examined with the *Brucella IS711* species specific RT PCRs for *B. abortus* and *B. melitensis* according to the method of Probert *et al*. (2004). Briefly, for the detection of *B. abortus* each reaction contained the primers BabortF 5'-GCGGCTTTTCTATCACGGTA TTC-3', BabortR 5'-CATGCGCTATGATCTGGTTACG-3' at a final concentration of 300 nM each and the genus specific probe 5'-6FAM-CGCTCATGCTCGCCAGACTTCAATG-BHQ1-3' at 100nM. The final 25 µL reaction mixture included 12.5 µL of TaqMan Universal Master Mix (Applied Biosystems) supplemented with 0.75 µM primers, and 0.25 µM probes and 2 µL of sample DNA. Cycling parameters were as follows: an initial incubation at 50°C for 2 min, followed by an initial denaturation step at 95°C for 10 min, 50 cycles of 95°C for

25 s and 57°C for 60 s (annealing and extension). The identical conditions also applied to the reactions for detecting *B. melitensis* using the primer pair BmelitF 5'-AACAAGCGGCACCCCTAAAA-3', BmelitR 5'-CATGCGCTATGATCTGGTTACG-3' and the genus specific probe 5'-6FAM-CAGGAGTGTTTCGGCTCA GAATAATCCACABHQ1-3'. The cycle threshold value (Ct) was calculated by the instrument's software MxPro3000P v 4.01. Ct values below 40 were interpreted as positive.

Statistical analysis

The questionnaire based data was processed by Microsoft Excel and MSTATC, the results were statistically analyzed for interpretation by using Chi-square tests (χ^2). Significance was determined at 1 to 5% level where applicable.

RESULTS AND DISCUSSION

Out of 913 cattle and 99 buffalo sera, 48 cattle and 7 buffalo sera showed positive reaction in RBT resulting in a prevalence of 5.3% (95% Confidence Interval (CI): 3.87-7.38) in cattle and 7.1% (95% CI: 2.89-14.03) in buffaloes (Table 2).

Table 1. Prevalence of brucellosis in cattle and buffaloes, sheep & goats, and humans.

Serum	Tested	RBT n (%)	SAT n (%)	CFT n (%)	iELISA n (%)	BCSP n (%)	IS711 n (%)	*B. abortus* n (%)
Cattle	913	48 (5.3)	5 (0.5)	26 (2.8)	1 (0.1)	17 (1.9)	2 (0.2)	8 (0.9)
Buffaloes	99	7 (7.1)	4 (4.0)	5 (5.1)	4 (4.0)	6 (6.1)	4 (4.0)	4 (4.0)
Sheep & goats	90	-	-	-	-	-	-	-
Humans	350	-	-	-	-	-	-	-

Out of 48 RBT positive sera of cattle 7 were *B. abortus* positive whereas out of 7 RBT positive buffalo sera 4 were *B. abortus* positive. The difference in detection level of *B. abortus* from cattle and buffalo sera was statistically significant (p=0.02). The odds of getting *B. abortus* DNA from RBT positive buffalo sera was 7.61 times higher than the same from cattle sera (Table 2).

Table 2. Serological test results

RBT	SAT	CFT	iELISA	Number
1+	+	-	-	2
1+	+	+	-	3
2+	+	+	+	4
3+	+	-	+	6
3+	+	+	-	7
1+	-	+	-	24
2+	+	+	+	6
2+	-	+	-	3
Sub Total				55
Suspicious	-		-	957
Total				1,012

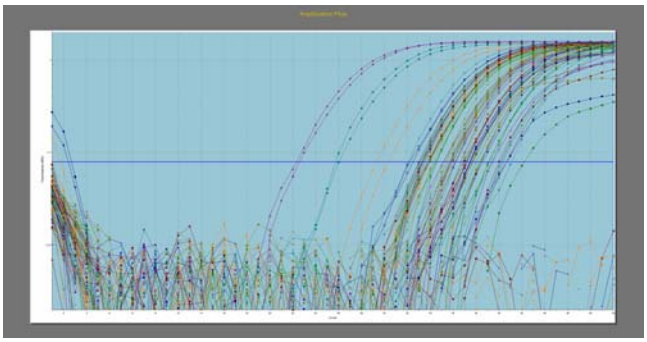

Figure 1. Amplification plot of *B. abortus* specific real-time PCR with DNA extracted from sera of cattle and buffaloes in Bangladesh

Figure 1 shows the amplification plot of *B. abortus* specific real time PCRs of seropositive cattle and buffalo sera. Out of 55 sera tested, 6 samples were seropositive in three additional tests used and infection can be considered as acute and active. Among 1,012 sera samples, 55 were positive only in RBT and 957 samples were suspicious negative in RBT (Table 2).

The relationship of serological tests and PCR is shown in Table 3 & 4. Seven cattle samples contained 1 *Brucella abortus* DNA, 48 sera were positive only in RBT (but negative in other two tests). On the other hand, 4 buffalo sera contained *Brucella abortus* DNA and 7 sera were positive in RBT (but negative in other two tests). The genus specific screening by PCR detected *Brucella* DNA in 4 sera, the species specific IS711 PCR also detected *B. abortus* DNA from the same 4 sera samples.

Table 3. Relationship of serological tests and PCR, Cattle sera (n=913)

Area	RBT positive	RBT intensity	SAT		CFT		iELISA		BCSP		IS711		*B. abortus*	
			+	-	+	-	+	-	+	-	+	-	+	-
Mymensingh	5	1+	1	4	4	1	0	5	4	1	0	5	0	5
Mymensingh	2	2+	0	2	0	2	0	2	2	0	0	2	0	2
Savar	11	1+	0	11	0	11	0	11	0	11	0	11	0	11
Kurigram	27	1+	2	25	18	9	0	27	17	10	0	27	6	21
Kurigram	2	2+	1	1	2	0	1	1	1	1	1	1	0	2
Kurigram	1	3+	1	0	1	0	0	1	1	0	1	0	1	0

Table 4. Relationship of serological tests and PCR, buffalo sera (n=99)

Area	RBT positive	RBT intensity	SAT		CFT		iELISA		BCSP		IS711		*B. abortus*	
			+	-	+	-	+	-	+	-	+	-	+	-
Mymensingh	1	1+	1	0	1	0	0	1	1	0	0	1	0	1
Mymensingh	1	2+	1	0	1	0	1	0	1	0	1	0	1	0
Bagerhat	2	1+	0	2	1	1	0	2	1	1	0	2	0	2
Bagerhat	2	2+	2	0	2	0	2	0	2	0	2	0	2	0
Bagerhat	1	3+	1	0	0	1	1	0	1	0	1	0	1	0

Out of 48 RBT positive sera of cattle, 7 (14.6%) were *B. abortus* DNA positive whereas out of 7 RBT positive buffalo sera, 4 (57.1%) contained *B. abortus* DNA. The difference in detection level of *B. abortus* from cattle and buffalo sera was statistically significant (p=0.02). The odds of getting *B. abortus* DNA from RBT positive buffalo sera was 7.61 times higher than from cattle sera (Table 2).

As shown above seroprevalence of brucellosis in individual cattle and buffalo by RBT is 5.3% and 7.1%, respectively (Table1) which is in line with published data showing a range between 2.4 to 18.4% at individual animal level and 62.5% at herd level. Serological prevalence in buffaloes was reported to be 2.87% (Amin *et al*., 2005; Rahman *et al*., 1997). In this study 10.9% (6/55) RBT positive bovines were found acutely infected by brucellosis. These acutely infected animals were positive in all tests. IgM and IgG are produced in early and later stage of the infection/disease, respectively. For this reason if a sample is positive in SAT and ELISA, it is considered as an acute infection. On the other hand, if a sample is positive only in the IgG ELISA, The infection is considered to be chronic. When a sample is positive only in agglutination tests like SAT, brucellosis has to be confirmed by an IgG detecting test like IgG ELISA (Godfroid *et al*., 2010; Seleem *et al*., 2010). However, this requires repeated sampling from the same animal which was not possible and not purpose of this study also. From the 7 cattle sera from which *B. abortus* DNA was detected, 2 were negative in SAT and 6 were negative in iELISA but all samples were positive in RBT. The infection in these animals might have been in the early stage which was detected by RBT only. In humans, *Brucella* DNA can be detected a long time after clinical cure reported by Navarro *et al*. (2006). This also indicates that the presence of only *Brucella* DNA does not mean acute infections. This may be similar in animals as seen in this study. The key shortcoming of PCR based techniques is that biovars can not be identified. Culture from milk and sera will be part of future investigations. It can be concluded that a combination of real time PCR with RBT, SAT and iELISA should be applied for detection brucellosis in cattle and buffaloes from Bangladesh in a future eradication program. The knowledge on prevalent *Brucella* species in humans and animals will help to initiate appropriate control measures against brucellosis.

ACKNOWLEDGEMENTS

The authors are grateful to National Reference Laboratory for brucellosis, Friedrich-Loeffler-Institute, Jena, Germany for laboratory and financial support.

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