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The prevalence of *Brucella abortus* DNA in seropositive bovine sera in Bangladesh

Md. Siddiqur Rahman[1,3]*, Md Abu Sayed Sarker[1], A. K. M. Anisur Rahman[1], Roma Rani Sarker[1], Falk Melzer[2], Lisa D. Sprague[2] and Heinrich Neubauer[2,3]

[1]Department of Medicine, Faculty of Veterinary Science, Bangladesh Agricultural University, Mymensingh 2202, Bangladesh.
[2]Institute of Bacterial Infections and Zoonoses, Federal Research Institute for Animal Health, Friedrich-Loeffler-Institut, Naumburger Str. 96a, 07743 Jena, Germany.
[3]OIE Reference Laboratory for Brucellosis, Federal Research Institute for Animal Health, Friedrich-Loeffler-Institut, Naumburger Str. 96a, 07743 Jena, Germany.

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Prevalence of brucellosis has been widely investigated on the basis of serological test in livestock but the information on the prevalence of *Brucella* species is scarce in Bangladesh. The objective of this work was to determine the prevalence of *Brucella* species in cattle and buffaloes in Bangladesh. For these purpose, a total of 799 serum samples of cattle and buffaloes were collected from different districts of Bangladesh. Out of 799 serum samples, 45 serum samples reacted positively to the Rose Bengal test (RBT); among the RBT positive serum, 14 sera were found to contain *Brucella* DNA by genus specific IS711 screening using quantitative real time PCR (qRT-PCR); and all the 14 qRT-PCR positive samples were found to contain specifically *Brucella abortus* DNA. This report confirms that *B. abortus* is endemic in cattle and buffaloes in Bangladesh. A combination of SAT-iLEISA and PCR could be effective for future eradication programmes.

Key words: Brucellosis, cattle, buffalo, Bangladesh, serology, polymerase chain reaction (PCR).

INTRODUCTION

Brucellosis is considered to be the most widespread zoonosis throughout the world and is caused by different species of the genus *Brucella* (OIE, 2008). In animals, brucellosis mainly affects reproduction and fertility, with abortion, birth of weak offspring and reduced milk yield (Sewel and Blocklesby, 1990). In man, the clinical picture resembles many other febrile diseases, but sacroiliitis and hepato-splenomegaly are the most prominent symptoms. Severe complications are endocarditis and neurological disorders (Colmenero et al., 1996). Numerous serological tests, that is, Rose Bengal Test (RBT), serum agglutination test (SAT), complement fixation test (CFT) and ELISA are used for detecting *Brucella* antibodies in cattle and small ruminants at herd level. Presently, quantitative real time (qRT) PCR methods are used to corroborate serological diagnostics. *Brucella* DNA can

*Corresponding author. E-mail: prithul02@yahoo.co.uk. Tel: 88 01918181550. Fax: 88 091 61510.

Table 1. Oligonucleotide primers and probes in the real-time multiplex PCR assay for the detection of *Brucella* spp., *B. abortus*, *B. melitensis*.

Species	Forward primer[a]	Reverse primer[a]
Brucella spp.	GCTCGGTTGCCAATATCAATGC	GGGTAAAGCGTCGCCAGAAG
Brucella spp. TagManprobe[ab]	6FAMAAATCTTCCACCTTGCCCTTGCCATCABHQ1	
B. abortus	GCGGCTTTTCTATCACGGTATTC	CATGCGCTATGATCTGGTTACG
B. abortus Tag Manprobe[ab]	HEXCGCTCATGCTCGCCAGACTTCAATGBHQ1	
B. melitensis	AACAAGCGGCACCCCTAAAA	CATGCGCTATGATCTGGTTACG
*B. melitensis*TagMan probe[ab]	Texas RedCAGGAGTGTTTCGGCTCAGAATAATCCACABHQ2	

[a]Oligonucleotide sequence provided in 5´to 3´orientation. 5´Fluorophonre/3´quencher[b]: 6-FAM: 6-carboxyfluorescein; HEX: 6-hexachlorofluorescein; BHQ1: Black Hole Quencher 1; BHQ2: Black Hole Quencher 2.

readily be detected in serum of infected animals when blood culture fails, and species differentiation is done using serum and the IS711 species specific qRT-PCR is possible (Gwida et al., 2011).

In the agro-based economy of Bangladesh, livestock contribute 2.73% of the total gross domestic product (GDP) and 75% of rural people are directly or indirectly involved in livestock rearing including 23.4 million cattle and 1.86 million buffaloes. Brucellosis was first identified serologically in cattle in 1967 (Mia and Aslam, 1967), and in buffalo in 1997 (Rahman et al., 1997). Besides, the serological prevalence of brucellosis has been reported in man and animals in Bangladesh (Nahar and Ahmed, 2009; Muhammad et al., 2010; Rahman et al., 2006; 2011; 2012). Pharo et al. (1981) for the first time in Bangladesh described the isolation of *Brucella abortus* from two cows both of which were MRT and RBT positive. In the same year, Rahman and Rahman (1981) claimed to isolate *Brucella* spp. from MRT positive milk in sub-clinical mastitic udder. Unfortunately, the detail procedure to validate the isolates as *Brucella* spp. is missing in these papers. Moreover, these isolates were not preserved in any laboratory in Bangladesh for further analysis. The culture of *Brucella* spp. requires BSL 3 facilities, highly skilled personnel and it has also high health risk to laboratory workers. However, real time PCR techniques are available to identify *Brucella* at species level which is more sensitive, specific, faster, safe and relatively cheaper than culture technique (Alton et al., 1988; Al Dahouk et al., 2007). Therefore, the aim of this study was to determine the species of *Brucella* in Bangladesh using sophisticated and sensitive technique, quantitative real time PCR.

MATERIALS AND METHODS

Blood samples from 99 adult buffaloes and 700 cattle were randomly collected between May and October 2011 for a preliminary study. RBT, SAT, CFT (all Pourquier, IDEXX, Montpellier, France) and the IDEXX Brucellosis Serum X2 Ab Test (IDEXX, Liebefeld-Bern, Switzerland) were performed according to the procedures described by the manufacturers. The RBT positive sera were re-tested with SAT, CFT, ELISA and qRT-PCR. For the qRT-PCR, DNA was isolated from 200 µL of seropositive serum using the High Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. *Brucella* IS711 targeting genus specific qRT-PCR was done according to the established and routine protocol (Tomaso et al., 2010) on a light cycler 2.0 instrument (Roche, Mannheim, Germany). Cycle threshold values (CT) ≤ 40 were interpreted as positive. Positive samples were then typed with the *Brucella* IS711species specific qRT-PCRs for *B. abortus* and *Brucella melitensis* according to Probert et al. (2004). CT values were calculated by the instrument's software MxPro3000P v 4.01. CT values ≤42 were interpreted as positive. The details primers list could be found in Table 1.

Statistical analysis

Descriptive statistics, 95% confidence interval of prevalence and Fisher Exact test to determine the level of significance between *B. abortus* detection level among RBT positive cattle and buffalo serum were performed in R 3.1.0 (The R foundation for Statistical Computing).

RESULTS

Out of total 700 cattle and 99 buffalo sera, 38 cattle and seven buffalo sera showed positive reaction to RBT with the overall prevalence of brucellosis 5.42% (95% Confidence Interval (CI): 3.87-7.38) in cattle and 7.07% (95% CI: 2.89-14.03) in buffalo (Table 2). Out of 38 RBT positive sera of cattle, 23.68% were *B. abortus* positive whereas out of 7 RBT positive buffalo sera, 71.43% were *B. abortus* positives. 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). Figure 1 shows the amplification plots of *B. abortus* specific real time PCR based on seropositive cattle and buffalo sera.

Out of 45 sera tested, six samples were three tests positive and can be considered as acute and active infection. Among 799 sera samples, 36 were positive only to RBT but negative to the other two tests (Table 3).

The relationship between serological tests and PCR is shown in Table 4. Out of nine *B. abortus* specific rt PCR positive cattle samples, 7 were positive only to RBT but negative to other two tests. On the other hand, out of five buffalo *B. abortus* specific rt PCR positive buffalo

Table 2. Prevalence of brucellosis and *B. abortus* infection in cattle and buffalo based on RBT and rt PCR.

Sera	Tested	Positive in RBT	Prevalence (95% CI)	*B. abortus* detected	Detection percentage (95% CI)	Fisher Exact Test Odd ratio (95% CI)
Cattle	700	38	5.42 (3.87-7.38)	9	23.68 (11.4-40.24)	1
Buffalo	99	7	7.07 (2.89-14.03)	5	71.43 (29.04-96.33)	7.61 (1.03-92.99)

*p-value=0.02.

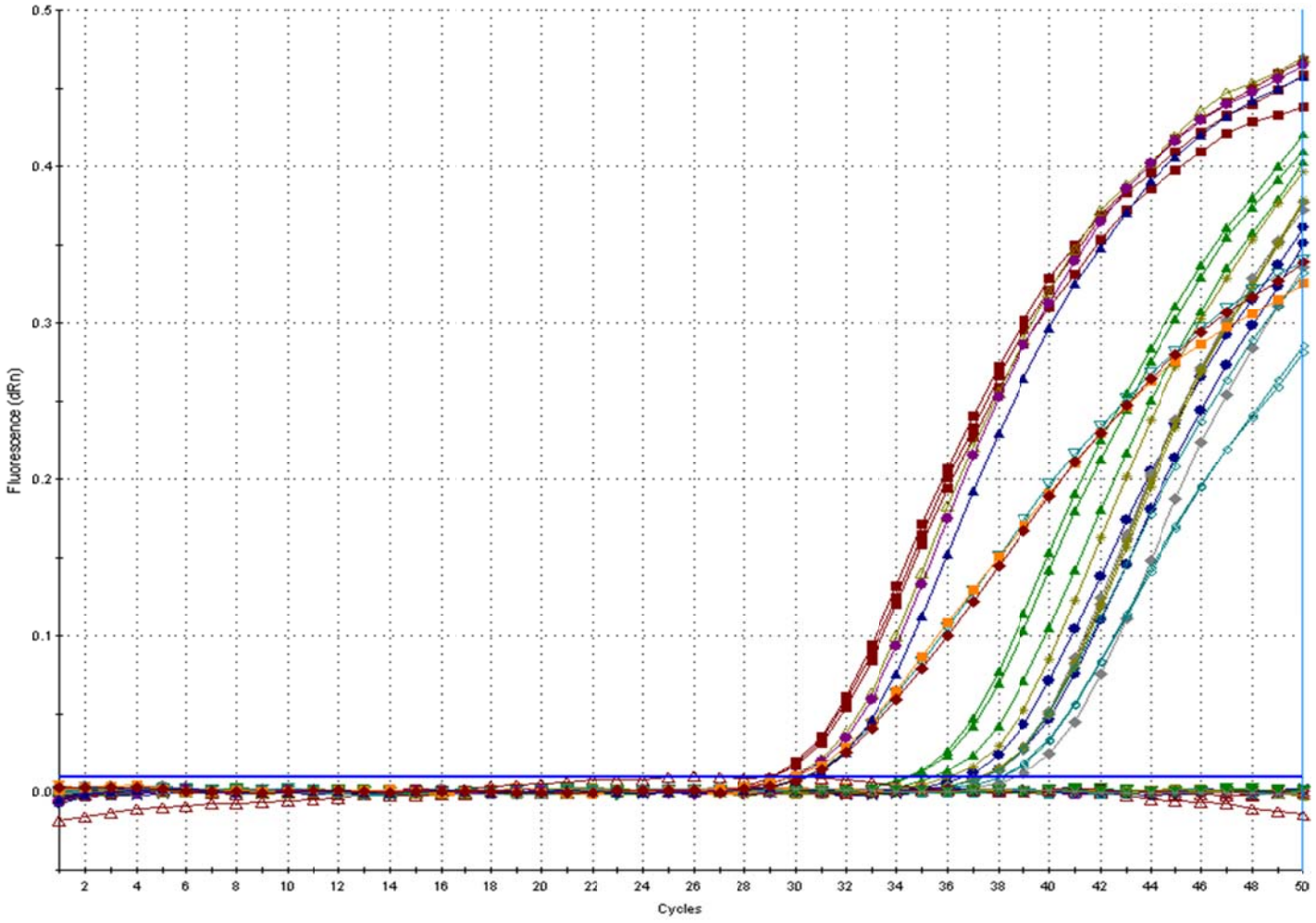

Figure 1. Amplification plots of *B. abortus* specific real time PCR with the DNA extracted from sera of cattle and buffalo in Bangladesh.

samples, only one was positive to RBT but negative to the other two tests. The genus specific screening by PCR detected *Brucella* DNA in 14 sera, the species specific IS711 PCR also detected *B. abortus* DNA from all the14 sera samples tested.

DISCUSSION

We found seroprevalences of 5.42 and 7.07% in cattle and buffalo by RBT, respectively (Table 2). The seroprevalence of brucellosis in cattle in Bangladesh is reported to lie between 2.4 - 18.4% at animal level and at 62.5% at herd level. Serological prevalence in buffaloes was reported to be 2.87% (Rahmanet al., 1997; Amin et al., 2005).

About 13.3% (6/45) RBT positive bovines were found to be acutely infected with brucellosis. These animals were positive to both IgG (iELISA) and IgM (SAT) detecting tests. The IgM and IgG are produced respectively in early and later stage of the disease. So, if a sample is positive in SAT and ELISA, it is considered as an active and acute infection. Whereas, if a sample is positive only to

Table 3. Summary of three serological test results.

RBT	SAT	iELISA	Number	Remarks
1+	+	+	0	
1+	+	-	3	Probably false positive (if RBT detected IgG)/Acute infection (if RBT detected IgM)
1+	-	-	33	Probably false positive
2+	+	+	4	Acute infection*
2+	+	-	0	
2+	-	-	3	Probably false positive
3+	+	+	2	Acute infection*
3+	+	-	0	
3+	-	-	0	
Sub-total			45	Tested by genus and species specific rt PCR
Suspicious	ND	Negative	93	Probably false positive
Suspicious	ND	ND	15	Probably false positive
Negative	ND	Negative	50	
Negative	ND	ND	596	
Total			799	

ND: Not done, only two sera were tested by CFT and found positive. They were positive in at least 2+ in RBT and also in iLEISa and SAT.

Table 4. Relationship of serological tests and PCR.

Sample	Area	RBT	SAT	iELISA	BCSP genus specific rt PCR	IS711 genus specific rt PCR	*B. abortus* specific IS711 rtpcr	Number
Cattle serum	Kurigram	1+	Negative	Negative	Positive	Not done	Positive	7
Cattle serum	Kurigram	2+	Positive	Positive	Positive	Positive	Positive	1
Cattle serum	Kurigram	3+	Positive	Positive	Negative	Positive	Positive	1
Buffalo serum	Mymensingh	1+	Negative	Negative	Positive	Not done	Positive	1
Buffalo serum	Mymensingh	2+	Positive	Positive	Positive	Positive	Positive	1
Buffalo serum	Bagerhat	2+	Positive	Positive	Positive	Positive	Positive	2
Buffalo serum	Bagerhat	3+	Positive	Positive	Positive	Positive	Positive	1
Total								14

IgG ELISA, it is considered as chronic infection. A sample positive to only agglutination test like SAT cannot be considered as brucellosis unless confirmed by an IgG detecting test like IgG ELISA within one week (Godfroid et al., 2010; Seleem et al., 2010). However, it requires repeated sampling from the same animal which was not possible and also not the purpose of this study. From all cattle and buffalo sera investigated, only two cattle sera from Kurigram could be analysed by CFT due to the low quality of the sera. These two sera were also positive in the ELISA.

Out of 9 cattle sera from where *B. abortus* DNA were detected 7 had negative test results both in SAT and iELSA. The biological explanation of this phenomenon is not clear. However, these animals were positive to RBT (1+). The infection in these animals may be in the very early stage which was detected by the qualitative test (RBT) but not by the quantitative tests like SAT and iLEISA for the presence of antibody below cut-off level. Similarly, for buffalo sera only one sample was positive to RBT but negative to SAT and iELISA. In humans, presence of *Brucella* DNA after a long time after clinical cure was also reported by Navarro et al. (2006). This indicates that the presence of only *Brucella* DNA does not indicate acute infection. Similar phenomenon may also occur in animals as we have notice in this study. Contrarily, serological cross reactivity with other abortion causing agents could explain the high number of RBT 'positives'which is regularly reported for females older than four years (Chantal and Thomas, 1976). However, the low number of animals investigated in this study does not allow statistical proof of these assumptions.

The major shortcoming of PCR based techniques is that the biovar cannot be determined. Cultivation from sera often fails and was thus not attempted in our preliminary study but has to be part of future investigations. It can be concluded that a combination of real-time PCR with SAT and iELISA should be applied to

detect brucellosis in cattle and buffalo from Bangladesh in a future eradication program. This paper for the first time detected the presence of *B. abortus* using real time PCR technique in the cattle and buffalo populations in Bangladesh.

Conflict of Interest

The author(s) have not declared any conflict of interests.

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