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Full Length Research Paper

Milk ring, rose bengal tests and conventional PCR based detection of *Brucella abortus* infected dairy cattle in Bangladesh

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The objective of this study was to detect dairy cattle infected with *Brucella abortus* from Jamalpur, Rangpur districts and Central Cattle Breeding and Dairy Farm (CCBDF), Savar, Dhaka in Bangladesh. Both milk and serum samples of 510 dairy cattle were initially screened by milk ring test (MRT) and Rose Bengal Test (RBT). Twelve samples positive in both MRT and RBT were further confirmed by conventional polymerase chain reaction (PCR). The overall prevalence of brucellosis based on MRT and RBT was 2.7 and 2.4%, respectively. The prevalence of brucellosis was found to be significantly ($p<0.001$) higher in CCBDF than Jamalpur district. Out of 7 MRT and RBT positive samples, 42.9% samples of CCBDF were PCR positive and none of the five samples of Jamalpur and Rangpur districts was PCR positive. These results strongly suggest that the use of MRT, RBT and PCR technique could lead to more reliable diagnosis of brucellosis from dairy cow in Bangladesh.

Key words: Seroprevalence, molecular detection, brucellosis, dairy cattle, Bangladesh.

INTRODUCTION

Brucellosis is an ancient and one of the world's most widespread zoonotic diseases affecting both, public health and livestock production (Ariza et al., 2007), which is caused by Gram-negative, facultative intracellular bacteria of the genus *Brucella.* Bovine brucellosis is caused almost exclusively by *Brucella abortus,* which is associated with abortion during the last trimester of gestation and production of weak newborn calves, and

infertility in cows and bulls (Xavier et al., 2009). Bovine brucellosis may also be responsible for retention of placenta and metritis and results in 25% reduction in milk production in infected cows (Acha and Szyfres, 2003; Anonymous, 2006). The overall seroprevalence of bovine brucellosis reported in Bangladesh is 5.3% (4.8 to 6.2) (Ahmed et al., 1992; Amin et al., 2004, 2005; Nahar and Ahmed, 2009; Ahasan and Song, 2010; Rahman et al., 2012; Sikder et al., 2012; Belal and Ansari, 2013; Dey et al., 2013; Islam et al., 2013) and overall prevalence of brucellosis in cows based on milk ring test (MRT) is 5.6% (4.8 to 6.3) (Pharo et al., 1981; Rahman and Rahman, 1981; Rahman et al., 1983; Sikder et al., 2012). *B. abortus* DNA has also been detected from bovine milk and serum samples using real time polymerase chain reaction (PCR) assays (Rahman et al., 2014, 2017) in Bangladesh. However, facilities to perform real time PCR are not widely available in Bangladesh. On the other hand, conventional PCR facility is available in most of the research and educational institutions. The organism is shed in the milk of infected cows. Zoonotic transmission occurs most frequently via unpasteurized milk products in urban settings, while occupational exposure of farmers, veterinarians, or laboratory workers can result from direct contact with infected animals or tissues or fluids associated with abortion (Olsen and Palmer, 2014). In Bangladesh, among the high-risk occupationally exposed people, seroprevalence of brucellosis in humans was reported to be the highest in dairy workers (Rahman et al., 2012). Long time direct contact with infected cows may be responsible for such a higher level of brucellosis among dairy workers as consumption of raw milk is unusual in Bangladesh. So, identification of cows, which shed *B. abortus* in milk, is valuable in culling decision. This study describes the Rose Bengal, MRT and conventional PCR based detection of *B. abortus* infected dairy cattle in Bangladesh.

MATERIALS AND METHODS

Both blood and milk samples were collected randomly from 510 dairy cattle, originating from Jamalpur, Rangpur districts and Central Cattle Breeding and Dairy Farm, Savar, Dhaka. The study was conducted between August and October, 2013.

Milk Ring Test (MRT)

Milk ring test (MRT) on individual milk sample was conducted according to Alton et al. (1988). In brief, antigen (Ring Test reagent, Institut Pourquier, Montpellier, France) was kept at room temperature (18 to 23°C) for 1 h before starting the test. After proper mixing, 1.0 ml of milk sample and 50 µl of MRT of antigen were added in each tube. The milk and MRT reagent was mixed with vortex mixture and incubated for 1 h at +37°C and then between +2 and +8°C for 18 to 20 h. The result was read as positive if the ring of cream is equally or more colored than the underlying milk and as negative if the ring of cream is less colored than the underlying milk.

Rose Bengal Test (RBT)

RBT (Rose Bengal, Institut Pourquier, Montpellier, France) was performed following the procedure described by Alton et al. (1988). The detail description of the test procedure can be found in Rahman et al. (2013).

DNA extraction and PCR

DNA was extracted from milk samples by using Wizard Genomic DNA Purification Kit (Promega, USA) according to manufacturer's instruction and then PCR was performed. Briefly, at first, the required number of PCR tubes were labeled and kept on ice. Then 23 µl of reaction mixture was dispensed into each of the PCR tubes and 2 µl of DNA template from each sample was added to the respective tube and mixed well with the help of the micropipette. The tubes were placed in a 24 wells thermo cycler (Eppendorf, Germany). Then the temperature of the thermo cycler was set according to the following thermal profile. Initial denaturation at 95ºC for 10 minutes, denaturation at 94ºC for 15 seconds, annealing at 54ºC for 1 minute, extension at 72ºC for 1 minute and final extension at 72ºC and 40 cycles for 10 minutes. After completion of PCR, the products were separated by electrophoresis in a 1X TAE 1% agarose gel stained by ethidium bromide. The band was then visualized with a medium wavelength UV light (Figure 1). Primer sequence of alkB genes used in this study was 5'-GCGGCTTTTCTACACGGTATTC-3' (F) and 5'-CATGCGCTATGATCTGGTTACG-3' (R) as per Terzi et al. (2010).

RESULTS AND DISCUSSION

The overall prevalence of brucellosis based on MRT and RBT were 2.7 and 2.4% respectively. The prevalence of brucellosis was significantly ($p<0.01$) higher in CCBDF than Jamalpur district (Table 1). Out of seven MRT and RBT positive milk samples of CCBDF, 42.9% were PCR positive, but all of the five samples that originated from Jamalpur and Rangpur districts were PCR negative (Table 2).

Brucellosis has been recognized as an important zoonotic disease as it hampers both animal production and human health. None of the diagnostic tests available in Bangladesh are perfect, so screening results need to be verified by confirmatory test. In this study, milk and serum samples were screened by MRT and RBT and conventional PCR was used as confirmatory test. The MRT is prescribed by OIE for screening of dairy milk samples. It is very easy to perform, cheap and it gives a good reflection of serum antibody (Nielsen, 2002; OIE, 2009). The RBT is also used as the standard screening test followed by confirmatory testing due to its simplicity, cheap consumables, low equipment requirement and standardized assay (Nielsen and Ewalt, 2010). Individual cow milk was screened by both MRT and RBT in parallel to increase the sensitivity of detecting *Brucella* shedding cows. It was possible to test 12 samples by conventional PCR, which were positive in both tests. Only 42.9% of CCBDF samples were PCR positive and none of the five samples that originated from Jamalpur and Dinajpur

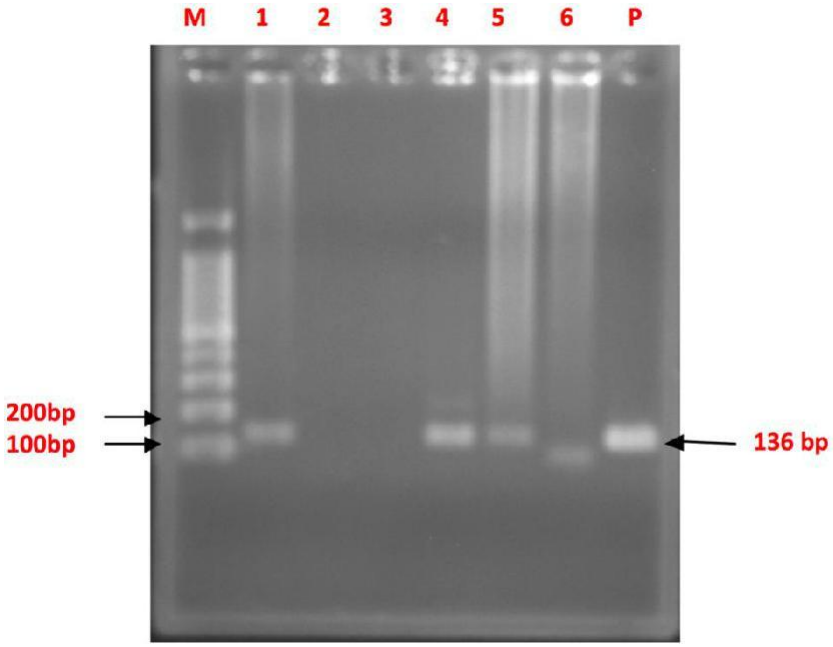

Figure 1. PCR for detection of *B. abortus* from MRT positive milk samples. M, DNA marker (100 bp ladder); 1 to 6 samples; P, positive control. PCR amplicon is analysed in 2% agarose and visualized by Transiiluminator UV Solo, Germany.

Table 1. Breed and district wise prevalence of brucellosis based on MRT and RBT in dairy cattle.

Breed	Positive		Prevalence (%)	
	MRT	RBT	MRT	RBT
Holstein Friesian (n=410)	12	10	2.92	2.43
Sahiwal (n=100)	2	2	2.0	2.0
Fisher's Exact Test P-value	-	-	1	1
District				
Dhaka, CCBDF (n=71)	9	7	12.7	9.9
Rangpur (n=238)	3	3	1.3	1.3
Jamalpur (n=201)	2	2	0.99	0.99
Fisher's Exact Test P-value			<0.001	<0.001
Overall	14	12	2.7	2.4

Table 2. Comparative analysis between MRT, RBT and PCR results.

MRT and RBT positive	Tested	PCR positive	Prevalence (%)
CCBDF	7	3	42.9
Jamalpur and Rangpur districts	5	0	0

districts was positive in PCR. Even being positive in both screening tests, the samples originating from Jamalpur and Rangpur district were negative in conventional PCR, which may be due to low seroprevalence (1.1 to 2.1%) of brucellosis in these areas (Dey et al., 2013). Even if the sensitivity and specificity of a test is very high due to low

prevalence, the positive predictive value of a test may be very low (Rahman, 2015). It is also possible that some milk samples may contain bacteria below the detection limit and failed to be found as positive (O'Leary et al., 2006). Moreover, it is not possible to detect *Brucella* DNA by PCR in majority of the MRT positive samples from cows in their chronic phase of the disease (Terzi et al., 2010). Conversely, the true prevalence and acute infection of brucellosis in CCBDF were reported to be 20.5 and 15.6%, respectively (Rahman, 2015). As a result, more *Brucella* organisms will be shed in milk in this farm increasing the likelihood of detection in PCR. As vaccination against brucellosis in animals was never introduced in Bangladesh, the prevalence indicates natural infection. Identification and culling of acutely infected animals from the population will help to reduce the transmission of the pathogen in animal populations and thereby the zoonotic transmission to humans.

PCR amplification targeting the genus and species-specific genes *alkB* was performed to confirm the presence of *Brucella* DNA in milk samples. Detection of an amplicon of 136 bp confirmed the presence of *B. abortus* DNA. Similar finding was reported by Terzi et al. (2010).

Currently, veterinary diagnostic laboratories utilize MRT for diagnosis of bovine milk samples, which indirectly identifies *Brucella* spp. in the host (Chimana et al., 2010). Just MRT positivity does not indicate acute infection. To declare acute infection evidence of *Brucella* organism or detection of *Brucella* DNA in animal samples is essential (Bricker, 2002; Hamdy and Amin, 2002; Gupta et al., 2006).

The main limitation was that it does not represent diary rich areas like Sirajganj, Chittagong, Satkhira and Munshiganj in Bangladesh. The status of acutely infected dairy cattle of these regions will reveal the importance of this disease in both human and animal health in Bangladesh.

Acute infection of brucellosis in dairy cattle can be determined by MRT, RBT and conventional PCR techniques. This finding will help to cull dairy cattle acutely infected with brucellosis having serious public health hazard.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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