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Original Article

Seroprevalence of brucellosis in patients with prolonged fever in Bangladesh

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Abstract

Introduction: This study describes the seroprevalence of human brucellosis among pyretic patients and detection of *Brucella abortus* DNA from seropositive pyretic patients using real-time polymerase chain reaction (rtPCR) for the first time in Bangladesh.

Methodology: Blood samples were collected from 300 pyretic patients from October 2007 to May 2008 and subjected to three serological tests: Rose-Bengal plate test (RBT), standard tube agglutination test (STAT), and indirect enzyme-linked immunosorbent assay (iELISA). Risk factors were identified by multivariate Firth's logistic regression analysis. *Brucella* genus (BCSP31) and species-specific (IS711) rtPCR were applied to six human sera samples.

Results: The seroprevalence of brucellosis among pyretic patients was estimated to be 2.0% (95% confidence interval [CI]: 0.74–4.30). The odds of brucellosis seropositivity were 8.9 (95% CI: 1.26–63.0) times higher in pyretic patients who handled goats than those who handled only cattle, whereas the odds of brucellosis seropositivity were 9.7 (95% CI: 1.28–73.68) times higher in pyretic patients who had backache compared to those without backache. *B. abortus* DNA was amplified from all six human sera that tested positive by RBT, STAT, and iELISA. As the agreement between the tests was very strong, RBT is recommended as a screening test for the diagnosis of human brucellosis in Bangladesh because it is easier to use, cheaper, and faster.

Conclusions: Brucellosis among pyretic patients is common, and *B. abortus* is responsible for brucellosis in such patients. Pyretic patients who handle goats and those with backaches should be screened for brucellosis.

Key words: Brucellosis; risk factors; pyrexia; real-time PCR; *Brucella abortus*; Bangladesh.

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Introduction

Human brucellosis is a zoonotic bacterial infection caused by a Gram-negative facultative intracellular bacteria of the genus *Brucella.* The most pathogenic and invasive species for humans is *Brucella melitensis*, followed in descending order by *Brucella suis*, *Brucella abortus*, and *Brucella canis* [1]. The transmission to humans mostly results from the consumption of fresh milk and dairy products prepared from unpasteurized milk such as soft cheeses, yogurts, and ice creams. However, direct contact with infected animals is an important transmission route, especially among abattoir workers, herdsmen, veterinarians, butchers, and also through the inhalation of infected aerosolized particles by personnel in microbiologic laboratories [2].

Human brucellosis poses major economic and public health challenges in affected countries, especially in the Mediterranean countries of Europe, northern and eastern Africa, Near East countries, India, Central Asia, Mexico, and Central and South America. However, there are only a few studies where the seroprevalence of brucellosis among patients with prolonged fever has been estimated. For example, Baba *et al*. [3] estimated the seroprevalence in northeastern Nigeria to be 5.2%, whereas Tolosa *et al.* [4] obtained a slightly lower seroprevalence of 3.6% in southeastern Ethiopia. The study by Kadri *et al*. [5] yielded a seroprevalence of 0.8% among patients with prolonged fever in Kashmir-India, and a prevalence rate of 1.0% (1/100) among hospitalized patients with prolonged fever was reported by Aniyappanavar *et al*. [6]. The

wide variability in estimated seroprevalence reported might be due to differences in the sampling design schemes used, the number of samples, exposure to *Brucella* spp., the number of diagnostic tests used, and the manner in which the tests were interpreted.

The status of brucellosis among humans in Bangladesh is not well documented. There is no official report about the prevalence or incidence of this disease in humans in Bangladesh. Several study findings revealed that 4.4%–12.8% of people in high-risk occupational groups were serologically brucellosis positive in some selected areas of Bangladesh [7-10].

Moreover, brucellosis is known to be a pyretic disease, and the prevalence of brucellosis in pyretic patients of Bangladesh is not yet known. The infection in humans is not clearly defined; it is mainly characterized by fever yielding body temperatures of up to 38.3°C [11]. Other symptoms include backache, arthralgia, headache, chills, night sweats, weakness, and weight loss [12]. Malaria, typhoid fever, tuberculosis, and rheumatic fever are endemic in Bangladesh [13-16]. Since pyrexia is a characteristic of the aforementioned diseases, including brucellosis, clinical examinations should always be accompanied by laboratory tests. The Rose-Bengal test (RBT), standard tube agglutination test (STAT), and indirect enzyme-linked immunosorbent assay (iELISA), either alone or in combination, were used in previous studies. None of these tests is perfect. However, if multiple imperfect tests are used in parallel on each sample, the agreement between two test pairs can be calculated, and their serial interpretation increases specificity and thereby the positive predictive value, which is very important in cases of human patients [17].

Among people with prolonged fever, risk factors that have been shown to be significantly associated with *Brucella melitensis* include gender, age, and occupation [4-5,18].

In Bangladesh, there is no published report on the isolation of *Brucella* species from man or animals, but Rahman *et al*. [10] reported the presence of *Brucella* DNA at genus level from seropositive human sera. Laboratory detection and species identification is still based on culture and phenotypic characterization, respectively, which are time consuming and resource intensive. Moreover, the risk of laboratory-acquired infections during handling of infectious samples or isolates is very high [19]. Polymerase chain reaction (PCR) techniques are gradually becoming popular for rapid detection of *Brucellae* from clinical samples such as blood or serum [20-22]. The IS711-based real-time PCR is reported to be specific and highly sensitive [23]. Most rtPCR assays so far developed are designed to detect *Brucellae* at genus level to enable early onset of treatment. *Brucella* IS711 species-specific multiplex real-time PCRs for *B. abortus* and *B. melitensis* also exist for investigation of cultures [24].

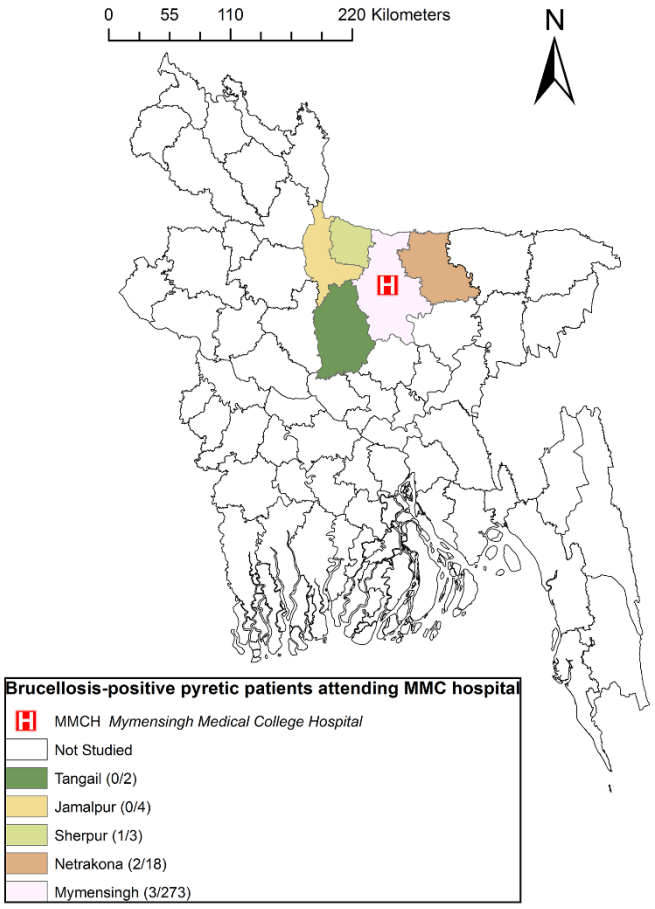
The objectives of this study were to determine the seroprevalence of brucellosis among patients with prolonged fever and to detect species of *Brucella* prevalent among pyretic patients using real-time PCR.

Methodology

Study population and study area

Patients with prolonged fever were defined as those with body temperatures higher than 38°C on several occasions and lasting over a period of three weeks. Patients were recruited from Mymensingh Medical College (MMC) hospital. The geographical position of MMC hospital and place of residence of patients are shown in Figure 1. MMC is the only medical college in the region. Therefore, patients from the surrounding districts have to visit MMC hospital to receive specialized treatment.

Figure 1. Study areas showing the origin of patients with prolonged fever from Mymensingh and surrounding districts.



More than 80% of the population of this area lives in villages, and crop-based livestock farming is their main source of income. Drinking non-pasteurized milk and eating milk products is very unusual for these villagers. Milk is usually consumed after boiling, albeit milkers occasionally drink raw milk during milking. Cheese, yogurt, and butter are usually consumed only by the wealthy city population. Blood samples from pyretic patients were collected randomly once a week. Every day, around 100 patients visit the outpatient facilities of MMC hospital. Ambulant and hospitalized patients meeting the inclusion criteria were recruited on the same date. Blood samples were collected from a total of 300 patients starting October 2007 until May 2008.

Ethical considerations

The study protocol was peer reviewed and cleared for ethics by the ethical review committee of MMC. Informed verbal and written consent was also taken from all individuals prior to blood sample collection.

Questionnaire data collection

Information was collected through personal face-to-face interviews. Questionnaires recorded information on age, sex, education, occupation, residency, type of patient (out and in), consumption of unpasteurized milk, contact with livestock (yes or no), animals handled, duration of contact in years, type of pyrexia, and presence of arthralgia, sweating, and backache (yes or no).

Collection and handling of blood samples

The collection and handling of blood samples was described previously by Rahman *et al*. [10].

Serological tests

All blood samples were tested in parallel by indirect IgG ELISA, RBT, and STAT. The detailed procedures for all three tests were described previously by Rahman *et al.* [10]. The estimated sensitivity and specificity of the iELISA, RBT, and STAT were 69.6% and 99.4%, 79.2% and 99.2%, and 80.6% and 97.9%, respectively (unpublished data).

DNA extraction from human serum

DNA was extracted from six human sera positive by all three serological tests applied. DNA was extracted using the DNeasy Spin Column Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol.

BSCP31 genus-specific and B. abortus- and B. melitensis-specific IS711 real-time PCR

The IS711/BCSP31 real-time PCRs originally described as a multiplex PCR assay [24] were performed as single assays to detect *Brucella* spp. DNA and/or to distinguish between *B. melitensis* and *B. abortus* DNA, respectively. No further modification of the protocols was done. The species-specific assays were applied when a genus-specific assay had detected *Brucella* DNA in a sample. The primers and probes were obtained from TIB MOLBIOL (Berlin, Germany). Amplification reaction mixtures were prepared in volumes of 25 µL containing 12.5 µL TaqMan Universal Master Mix (Applied Biosystems, New Jersey, USA), 0.75 µL of each of the two specific primers (0.3 µM) and 0.5 µL TaqMan probe (0.2 µM), 5 µL of template, and 6.25 µL of nuclease-free water. The real-time PCR reaction was performed in duplicate in optical 96-well microtiter plates (qPCR 96-well plates, Micro Amp, Applied Biosystems, Foster City, USA) using an Mx3000P thermocycler system (Stratagene, La Jolla, California) with the following run conditions: 2 minutes at 50°C, 10 minutes at 95°C, followed by 50 cycles of 95°C for 15 seconds and 57°C for 1 minute. Cycle threshold values below 40 cycles were considered positive. The instrument set the threshold automatically. The samples scored positive by the instrument were additionally confirmed by visual inspection of the graphical plots showing cycle numbers versus fluorescence values.

Statistical analyses

To determine the potential risk factors and clinical symptoms associated with brucellosis seropositivity in patients with prolonged fever, individuals were considered positive if they had at least one of the clinical symptoms and tested positive in all of the three serological tests and also in real-time PCR.

Firth's logistic regression analysis was preferred in place of the traditional exact logistic regression analysis to overcome the computational limitations and convergence issues caused by the sparseness (separation) of the data. Initially, a univariate analysis was performed using Firth's logistic regression model [25]. The model used as response the brucellosis status of the individuals and each risk factor or indicator variable as the independent variable. Variables with a p value ≤ 0.10 in the univariate analysis were further analyzed in a multivariate Firth's logistic regression model. A manual forward stepwise model building approach was used with Akaike's information criterion (AIC) as the calibrating parameter to select the final

model. The model with the lowest AIC value was considered as the best univariate model in this approach. The remaining variables are then added, each in turn, to form two variable models. Similarly, the best two-variable model was selected based on the AIC. This was repeated until the addition of one more variable failed to improve the model fit. The model with the smallest AIC was considered to be the most appropriate model. Firth's logistic regression analyses were performed using STATA version 12.1 software (Stata Corp, College Station, USA).

The percentage of agreement and coefficient of agreement between two test pairs were calculated according to Langenbucher *et al*. [26]. Calculations of the different parameters were carried out in R version 3.1.0 (The R Foundation for Statistical Computing, 2014) using a two-by-two contingency table.

Results

Descriptive statistics

The overall estimated seroprevalence of human brucellosis was 2.0% following a serial interpretation of the three tests. The distribution of brucellosis seropositivity among the patients with prolonged fever is presented in Tables 1a and 1b. The mean age of the individuals was 24.4 years and ranged from 2 to 80 years, with males representing 66% of the study population. All six of the seropositive patients with prolonged fever had clinical symptoms and recovered after therapy with streptomycin (1 g intramuscular injection daily) for 15 days and doxycycline (100 mg orally every 12 hours) for 45 days (data not shown).

The seroprevalence was found to be highest for those older than 40 years of age (12.5%). None of the patients with prolonged fever who had college- to university-level education (0/37) were found to be serologically positive for brucellosis. All six of the seropositive patients had none to secondary-level education (6/263). The seroprevalence of brucellosis was higher in males (2.5%) compared to females

Table 1a. Univariate analysis of potential risk factors and clinical symptoms for brucellosis among 300 people with prolonged fever in Bangladesh.

Factors	Tested	Positive (%)	Exact binomial 95% CI	P value*
Age group (years)				
2–20	149	0	0.0–2.4	-
21–40	111	1 (0.90)	0.02–4.9	0.39
41–80	40	5 (12.5)	4.2–26.8	0.01
Education				
College to university	37	0 (0)	0.0–9.5	-
None to secondary	263	6 (2.3)	0.84–4.9	0.66
Sex				
Female	101	1 (0.9)	0.03–5.4	-
Male	199	5 (2.5)	0.82–5.8	0.49
Residence				
Urban	106	0 (0)	0–3.4	-
Rural	194	6 (3.1)	1.1–6.6	0.18
Type of patient				
Outpatient	262	3 (1.1)	0.24–3.3	-
Inpatient	38	3(7.9)	1.7–21.4	0.01
Occupation				
Business	31	0 (0)	0.0–11.2	-
Crop farming	37	0 (0)	0.0–9.5	0.93
Day labor	7	0 (0)	0.0–40.9	0.48
Housewife	28	1 (3.6)	0.09–18.3	0.46
Livestock farming	78	5 (6.4)	2.1–14.3	0.30
Not applicable (age under 5 years)	20	0	0.0–16.8	0.83
Service	12	0	0.0–26.5	0.64
Study	87	0	0.0–4.2	0.61
Contact with animals				
No	219	1 (0.46)	0.01–2.5	-
Yes	81	5 (6.2)	2.0–13.8	0.01

*P values obtained from Firth's logistic regression analysis; CI: confidence interval.

(0.9%). None of the pyretic patients from urban areas (106) were serologically positive for brucellosis. All six of the pyretic patients serologically positive for brucellosis originated from rural areas (6/194). The estimated seroprevalence of brucellosis was higher among inpatients (7.9%) than outpatients (1.1%). Livestock farmers (5/78) and housewives (1/28) were serologically positive among different occupations of the patients. The other occupational groups included study (87), crop farming (37), business (31), minor (20), service (12), and day labor (7). The estimated seroprevalence of brucellosis was found to be higher among those who had contact with livestock (6.2%) as compared to those who had no contact with livestock (0.46%). Among 81 pyretic patients who had known contact with animals, 57, 4, and 20 handled cattle only, both cattle and goats, and goats only, respectively. However, 25% (5/20) of those handled goats were serologically positive for brucellosis. Only one seropositive pyretic patient had no known contacts with animals. There was no positive case among those who drank unpasteurized milk products. The rising and falling type of pyrexia was relatively higher (12.5%) than the irregular and continuous type of pyrexia. Most of the patients (91%) in this study originated from the Mymensingh district. There were no positive cases in the Tangail and Jamalpur districts.

Factors associated with brucellosis seropositivity among people with prolonged fever based on univariate analysis

The results of the univariate Firth's logistic regression analysis are shown in Tables 1a and 1b. It was revealed that the age, type of patient, contact with animals, type of animal handled, arthralgia, and backache were significantly associated with a positive serological result ($p < 0.05$).

Table 1b. Univariate analysis of potential risk factors and clinical symptoms for brucellosis among 300 people with prolonged fever in Bangladesh.

Factor	Tested	Positive (%)	Exact binomial 95% CI	P value*
Animal handled				
Cattle	57	0 (0)	0–6.3	-
Cattle and goat	4	0 (0)	0.0–60.2	0.22
Not known	218	1 (0.46)	0.01–2.5	0.51
Goat	21	5 (23.8)	8.2–47.2	0.01
Drinking of raw milk				
No	296	6 (2.0)	0.75–4.4	0.29
Yes	4	0 (0.0)	0.0–60.2	-
Nature of fever				
Irregular	281	4 (1.4)	0.38–3.6	-
Continuous	11	1 (9.1)	0.2–41.3	0.03
Rising & falling	8	1 (12.5)	0.3–52.7	0.01
Sweating				
No	71	4(5.6)	1.6–13.8	0.02
Yes	229	2 (0.9)	0.1–3.1	-
Arthralgia				
No	282	3 (1.1)	0.2–3.1	-
Yes	18	3 (16.7)	3.6–41.4	< 0.001
Backache				
No	288	3 (1.0)	0.21–3.01	
Yes	12	3 (25.0)	5.4–57.2	< 0.001
Headache				
No	43	4 (9.3)	2.6–22.1	0.002
Yes	257	2 (0.8)	0.09–2.8	-
District				
Jamalpur	4	0 (0)	0.0–60.2	-
Mymensingh	273	3 (1.1)	0.2–3.2	0.18
Netrokona	18	2 (11.1)	1.4–34.7	0.85
Sherpur	3	1 (33.3)	0.8–90.6	0.35
Tangail	2	0 (0)	0.0-84.2	0.79

*P values obtained from Firth's logistic regression analysis; CI: confidence interval.

Factors associated with brucellosis seropositivity among people with prolonged fever on multivariate analysis

Type of animal handled was found to be significantly associated with brucellosis seropositivity among patients with prolonged fever ($p < 0.03$). Backache was found to be a significant clinical symptom ($p < 0.03$) for brucellosis seropositivity among patients with prolonged fever (Table 2).

Real-time PCR results

From the six sera positive in the three serological tests, *B. abortus* DNA was amplified (Table 3). No *B. melitensis* DNA could be amplified from any of the six human sera.

Agreement between test pairs

The percentage agreement, kappa value, and corresponding 95% confidence interval are shown in Table 4. More than 99.3% agreement was observed between RBT-iELISA, RBT-STAT, and iELISA-RBT. The kappa values ranged from 0.85–0.93, indicating very strong agreement between tests.

Discussion

The seroprevalence of brucellosis among patients with prolonged fever is described for the first time in Bangladesh and was estimated to be 2.0%. A lower seroprevalence of 0.8% was reported from Kashmir-India [5], whereas a slightly higher prevalence of 5.2% was observed in northeastern Nigeria among patients with pyrexia of unknown origin [3]. The seroprevalence of 2.0% for our study is an indication that the majority of the patients with prolonged fever were not infected with brucellosis. It is known that only about 30% of cases of fever are due to infections [27]. Malaria, typhoid, tuberculosis, and rheumatic fever are common pyretic diseases of humans in Bangladesh and are routinely referred by physicians for laboratory testing. Brucellosis as a cause of pyrexia was neglected by medical professionals in Bangladesh; a simple RBT facility is not even available in most laboratories. In this study, it was observed that about 2.0% of the pyretic patients suffered from brucellosis. However, this study may not represent the total pyretic patients in Bangladesh, as not all pyretic people visit hospitals for health services. So, there might have been some bias in

Table 2. Final model of risk factors and clinical symptoms associated with human brucellosis seropositivity among 300 people with prolonged fever in Bangladesh.

Variable	Odds ratio	P value	95% confidence interval
Type of animal handled			
Cattle	1		
Cattle and goat	1.58	0.81	0.04–70.51
Not known	0.09	0.15	0.04–2.37
Goat	8.92	0.03	1.26–63.0
Clinical symptoms			
Backache			
No	1		
Yes	9.71	0.03	1.28–73.68

Table 3. *Brucella* genus and *Brucella* species-specific real-time polymerase chain reaction among seropositive patients.

PCR type	Tested	Positive	CT values	Range	
			Mean ± SE	Minimum	Maximum
BCSP31 *Brucella* genus	6	6	36.5 ± 0.36	34.9	38.2
IS711 *Brucella genus*	6	6	34.2 ± 0.29	32.8	35.6
IS711 *Brucella abortus*	6	6	33.5 ± 0.83	31.04	36.0
IS711 *Brucella melitensis*	6	0	Not done	None	None

Table 4. Agreement between two diagnostic tests.

Test combination	Percent agreement	Kappa	95% confidence interval	Remarks
RBT-iELISA	99.7	0.92	0.81–1.03	Almost perfect agreement
RBT-STAT	99.7	0.93	0.82–1.04	Almost perfect agreement
iELISA-STAT	99.3	0.85	0.74–0.97	Almost perfect agreement

RBT: Rose-Bengal plate test; iELISA: indirect enzyme-linked immunosorbent assay; STAT: standard tube agglutination test.

the selection of pyretic patients, which is also a limitation of this study.

Therefore, besides recommending that patients with prolonged fever be tested for tuberculosis, typhoid, malaria, and rheumatic fever, clinicians should also consider brucellosis for routine testing.

Even though our results show that gender was not significantly associated with human brucellosis seropositivity, other studies have shown otherwise [5,12,18]. Brucellosis is an occupational disease and therefore mostly affects livestock farmers, dairy workers, butchers, veterinarians, and laboratory personnel. These occupations are male dominated in Bangladesh, making males more commonly affected than females.

All six of the brucellosis-infected pyretic people were of rural origin. More than 80% of the people lived in rural areas and were involved with livestock production and thereby exposed to brucellosis-positive animals.

Significantly higher seropositivity was estimated for pyretic patients who handled goats compared to those who handled only cattle. Rahman *et al.* [10] also observed a relatively higher seroprevalence of brucellosis in people who handled only goats than in those who handled only cattle and in those who handled both cattle and goats, respectively. The same authors also reported that about 14.2% livestock farmers shared the same premises with their animals and 52.7% of them kept goats in their houses. This close contact to animals could be the reason for high prevalence among goat handlers.

Backache was a significant clinical symptom for brucellosis seropositivity among the patients with prolonged fever. Similar observations were also made by other authors [12,28-29].

Based on its easy handling and low costs, the RBT is recommended as a screening test for the diagnosis of human brucellosis in Bangladesh. A more specific test, such as serum-based genus or species-specific real-time PCR can be used for confirmation [10,20] to avoid unjustified costs, drug toxicity, and masking of other potentially dangerous diseases such as tuberculosis, which are also endemic in Bangladesh. At the time of this investigation, the real-time PCR assay had to be performed in Germany, but now the facilities to perform this test are available in Bangladesh. The percentage agreement between the two tests pairs and corresponding kappa values indicate similar performance of the tests.

Detection of *Brucella* DNA was reported even for serum samples that were taken a long time after clinical signs of disease had ceased in these patients [30-31]. Our six ELISA-, STAT-, and RBT-positive patients presented with clinical symptoms suggestive of brucellosis, and indeed they recovered after typical brucellosis treatment had been administered. Amplification was successful, as we had expected. Thus, we could demonstrate that confirmatory diagnosis by species-specific real-time PCR is adequate for a well-timed onset of a combination treatment necessary for brucellosis [12].

The isolation of *Brucella* from seropositive patients was not performed due to lack of facilities, which was a limitation of this study.

The small sample size of 300 patients lead to sparseness (the distribution of the individuals within the different categories of the risk factors was not even and the frequencies were sometimes very low) of the data. This limitation can be resolved by future studies involving a larger number of patients.

Conclusions

Brucellosis among pyretic patients is common, and *Brucella abortus* is responsible for brucellosis in such patients. Pyretic patients who handle goats and those with backache should be screened for brucellosis.

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