Brucella abortus is prevalent in both humans and animals in Bangladesh

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Summary

To determine the role of different *Brucella* (*B.*) spp. in Bangladesh, 62 animal samples and 500 human sera were tested. Animal samples from cattle, goats and sheep (including milk, bull semen, vaginal swabs and placentas) were cultured for *Brucella* spp. Three test positive human sera and all animal samples were screened by *Brucella* genus specific real time PCR (rt PCR) and positive samples were then tested by IS711 rt PCR to detect *B. abortus* and *B. melitensis DNA*. Only *B. abortus DNA* was amplified from 13 human and six animal samples. This is the first report describing *B. abortus* as the aetiological agent of brucellosis in occupationally exposed humans in Bangladesh. Of note is failure to detect *B. melitensis* DNA, the species most often associated with human brucellosis worldwide. Further studies require to explore the availability of *Brucella melitensis* in Bangladesh.

Keywords: *Brucella abortus*, humans, animals, clinical samples, Bangladesh, real-time PCR

Impacts

- Only *Brucella abortus* was detected from humans and domestic ruminants
- Brucellosis may not be the principle reason for abortion in domestic ruminants
- *Brucella melitensis* was not detected from any of the human and animal sample.

Introduction

Brucellosis – caused by a range of *Brucella* spp. – is a widespread bacterial zoonosis which impacts both human health and animal production in endemic countries. It is a severely debilitating illness, characterized by fever, sweating, fatigue, weight loss, headache and arthralgia which can persist for weeks to months in humans (Ariza et al., 2007). Chronic or life-threatening sequelae can result. WHO considers brucellosis a neglected zoonosis. It is a serious occupational hazard for livestock farmers, dairy workers, butchers, hired animal caretakers, veterinarians, laboratory workers and for consumers of raw livestock products (Anon., 2005). The isolation of *B. abortus* in Bangladesh was first reported in 1981 (Pharo et al., 1981) from two seropositive cows. However, the procedures used to type these isolates as *B. abortus* were not fully described. Moreover, *Brucella* genus and *B. abortus DNA* have recently been amplified from seropositive humans (Rahman et al., 2012) and bovine sera (Rahman et al., 2014), respectively, using real time (rt) PCR. *Brucella abortus* DNA has also been detected from patients with prolonged fever who were serologically positive (Rahman et al., 2016). The *Brucella* spp. responsible for infections in occupationally exposed humans have not been defined. The seroprevalence of brucellosis in domestic ruminants ranges from 3.6 to 7.3% (Islam et al., 2013). In humans, 18.2% of dairy workers, 2.6% of livestock farmers, 21.6% of goat farmers, 2.5% of butchers and 5.3% of veterinary practitioners were found to be seropositive in Bangladesh (Rahman et al., 2012*;* Rahman et al., 1983; Rahman et al., 1988). It indicates this disease in endemic in Bangladesh but still the species of *Brucella* prevalent in both humans and different species of animals are not known completely.

Laboratory detection and identification of *Brucella* species is still based on culture and phenotypic characterization, respectively, performed in laboratories with level 3 biosafety facilities. The isolation of *Brucella* spp. helps in understanding the prevalent biovars of the dominants species and also the source of infection in outbreaks (Al Dahouk et al., 2007). PCR techniques have become popular for rapid detection of brucellae from clinical (blood or serum) samples (Zerv et al., 2001; Queipo-Ortuño et al., 2005). The IS711 based rt PCR is reported to be specific and highly sensitive

(Bounaadja et al., 2009). Most RT PCR assays developed to date are designed to detect brucellae at genus level, enabling early implementation of treatment. *Brucella* IS711 species specific multiplex rt PCRs for *B. abortus* and *B. melitensis* also exist (Probert et al., 2004).

A recent study estimated the cost of brucellosis on livestock production in neighbouring India to be USD 3.4 billion (Singh et al., 2015), and brucellosis is a disease of public health importance in both developed and developing countries (Dean et al., 2012). The global health burden caused by brucellosis has been recently estimated to be >250,000 Disability Adjusted Life Years (DALYs) (Kirk et al., 2015). The aim of the current study was to determine the *Brucella* species responsible for brucellosis in animals and humans in Bangladesh.

Materials and Methods

Ethical statement

The study protocol was peer reviewed and approved by the Ethical Review Committee of Mymensingh Medical College. Informed written consent was taken from all individuals prior to the collection of blood. Animal research was approved by the Faculty of Veterinary Science of Bangladesh Agricultural University.

Human samples

Collection of human sera has been previously described (Rahman et al., 2012). Briefly, a survey of livestock farmers, milkmen, butchers, and veterinary practitioners in the Mymensingh, Sherpur, and Dhaka districts of Bangladesh was conducted between September 2007 and August 2008. In addition, workers at two government-owned farms in Dhaka District were sampled. A total of 500 individuals were sampled. In this survey the prevalence of *Brucella* antibodies (based on parallel interpretation of three serological tests – Rose Bengal Test [RBT], Standard Tube Agglutination Test [STAT] and an Indirect ELISA) was estimated to be 4.4% (Rahman et al., 2012). Twenty-two human sera were positive in at least one serological test. Out of 22, 13 were positive in three serological tests and in a *Brucella* genus specific RT PCR (Rahman et al., 2012) targeting the BCSP31 gene encoding a 31-kDa antigen conserved among *Brucella* spp (Navarro et al., 2004) and included

in this study. The BCSP31-PCR assay was carried out using standard procedure (Bounaadja et al., 2009; Baily et al., 1992), and IS711-PCR was done using the procedure described by Halling et al. (1993).

Animal samples

Randomly collected milk ring test (MRT) positive bulk milk samples (cattle, goat and gayal [*Bos frontalis*]), convenience samples of placentas, vaginal swabs from different animals (cattle, goats and sheep) post-abortion (within 0-3 days) and semen samples from bulls (with orchitis) were collected and used to isolate and detect *Brucella* genus and species specific DNA.

Staining

Impression smears of vaginal swabs and placentas were stained by the Stamp method (Alton et al., 1988). In brief, the impression smears are dried by flame and stained with working carbol fuch-sin solution for 10 min. Then decolorized by 3% acetic acid solution for 1 min and counterstained with 1% malachite green solution for 20 seconds. After washing in tap water, dried and observed under microscope using 100X objective (oil immersion), *Brucella* organisms appear pale red in a blue background.

Bacteriology

For the isolation of *Brucella* organisms, clinical samples were cultured in Farrell's medium us-ing standard methods (Alton et al., 1988). Briefly, milk samples were centrifuged at 6000 g for 15 min; cream and sediment were spread on half of the plate and streaked on the other half. Swab and semen (150 microliter) samples were also spread and streaked in the same way.

The cotyledons of the placentas were cut into small pieces (about 5 g). Approximately 4–5 ml of normal saline was added and a homogenate was prepared in a stomacher or blender. About 150 microliter of the homogenate was spread and streaked as for milk, swab and semen samples. Plates were incubated at 37°C in 7.5% CO_2 and first inspected after 48 hours. A sample was considered culture negative if no growth occurred within 7 days. DNA was extracted from samples using the DNeasy spin column kit (QIAGEN) according to the manufacturer's protocol.

IS711 genus specific and *Brucella abortus* and *Brucella melitensis* specific real-time PCR

The IS711 rt PCRs originally described as a multiplex PCR assay (Probert et al., 2004) were performed as single assays to detect *Brucella* spp. *DNA* and to distinguish between *B. melitensis* and *B. abortus DNA*, respectively, without further modification. The species-specific assays were applied when *Brucella DNA* had been detected in a sample in the genus-specific assay. The same primers and probes were obtained from TIB MOLBIOL (Berlin, Germany). Amplification reaction mixtures were prepared in volumes of 25 µl containing 12.5 µl TaqManTM 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 (0.2µM), 5 µl of template, and 6.25 µl nuclease-free water. The rt PCR reaction was performed in duplicate in optical 96-well microtiter plates (qPCR 96-well plates, Micro AmpTM, Applied Biosystems) using a Mx3000P thermocycler system (Stratagene, La Jolla, California) with the following run conditions, 2 min at 50°C, 10 min at 95°C, followed by 50 cycles of 95°C for 15s and 57°C for 1 min. Cycle threshold values below 40 were considered positive. The threshold was set automatically by the instrument. In addition, the samples scored positive by the instrument were confirmed by visual inspection of plots of cycle numbers versus fluorescence values.

Results

Brucella-like bacteria were not seen in any of the stained smears. No growth of *Brucella* spp. was noted in any of the clinical samples, but six (9.8%) of 62 animal samples investigated were positive in the genus specific BCSP31 rt PCR assay (Table 1). Among animals, only five milk (three cattle, one goat and one gayal) and one semen sample were positive in the *B. abortus* specific rt PCR.

Brucella abortus DNA was amplified from all of the 13 human serum samples tested. The clinical characteristics of these 13 patients and treatment received are shown in Table 2. Among brucellosis infected patients 76.9%, 61.5%, 46.2% and 53.8% had fever, arthralgia, backache and sweating respectively.

No *B. melitensis* DNA could be amplified from human or animal samples.

Discussion

We have described the successful amplification of *B. abortus* DNA from human serum, milk (cattle, goat, gayal) and bull semen from Bangladesh. Animals and their products are the sole source of human infection. Consequently, the presence of *B. abortus* DNA in human samples together with their history of contact demonstrates that dairy animal populations in Bangladesh are a source of brucellosis in humans, and that *B. abortus* is the most common (and potentially only) aetiological agent of human brucellosis in Bangladesh. The presence of *B. abortus* DNA in milk and semen supports this conclusion and moreover, the recent detection of *B. abortus* DNA from bovine sera in Bangladesh (Rahman et al., 2014) provides further evidence of the causal role of *B. abortus*. Detection of *B. abortus* DNA from five districts of Bangladesh (Bandarban, Chittagong, Dhaka, Mymensingh and Sirajganj) suggests that infection of dairy animals is widespread. However, due to small size and non-randomness of the sample our result does not represent whole Bangladesh.

Brucella abortus DNA was detected from milk samples of gayal in the hilly district of Bandarban, Bangladesh. The gayal (*Bos frontalis* syn.: mithan or mithun) is a semi-domestic ruminant found in the hill regions of northeast India, Myanmar, Bhutan, Bangladesh, China and Malaysia (Simoons and Simoons 1968*;* Mason, 1988). The source of infection of these animals is not clear. Bandarban district shares a common border with Mizoram state, India and Chin state, Myanmar. Although no report of the isolation of *Brucella* spp. from gayal in India exists, high *Brucella* seroprevalence in gayal in Mizoram has been reported (Rajkhowa et al., 2005). Moreover, it is reported that gayal populations from Tripura and Mizoram in India and from Bangladesh mingle due to the porous nature of this border (Choudhury, 2002). Another possible source of *Brucella* in this gayal population is crossbreeding with cattle. Holstein Friesian bull semen originating from one of the government farms included in the current study – and found to be *Brucella*-infected – has been used for this crossbreeding purpose (Huque et al., 2011). *B. abortus* DNA was also detected from one goat milk sample. Although *B. melitensis* is the most common agent of caprine brucellosis, *B. abor-*

tus infection of goats has been reported – especially in countries where *B. melitensis* is absent, for example Brazil (Lilenbaum et al., 2007). Including the current study, *B. melitensis* has not been reported from Bangladesh. The failure to detect *B. melitensis* DNA may also be due to very small number of goat samples we tested.

Brucella DNA was detected in human serum samples even though these had been collected a long time after clinical symptoms had resolved in patients (Navarro et al., 2006). All 13 patients, which were seropositive in the RBT, SAT and iELISA, presented with clinical symptoms consistent with brucellosis and indeed all had recovered after a 'typical' brucellosis treatment had been administered. Confirmatory diagnosis by species-specific rt PCR is sufficient for timely initiation of brucellosis treatment. Probert et al. (2004) developed a multiplex rt PCR to detect *B. abortus* and *B. melitensis* from culture growth. Modifying this technique, we were able to show that IS711 species-specific rt PCR is capable of amplifying *Brucella DNA* from human sera and animal samples at species level. Estimating the positive predictive value of combinations of serological tests – using rt PCR as the gold standard – was not an objective of this study. Where feasible, use of rt PCR to confirm brucellosis in patients with clinical symptoms consistent with this disease and suspected dairy animal contacts is warranted.

Abortion is the most common clinical sign of brucellosis in female domestic ruminants and usually aborted fetuses, fetal membranes and fluids contain high bacterial loads contaminating the environment and causing a high risk of infection to other animals (Saegerman et al., 2009). In our study, none of the 40 fetal membranes and vaginal swabs originating from cattle, sheep and goats contained *Brucella* DNA. Although the sample size is small, it indicates that *Brucella* may not be a major cause of abortion in domestic ruminants in Bangladesh.

Brucella DNA was detected from some of the MRT positive, culture negative milk samples. The possible reason for unsuccessful recovery of isolates may be that the samples had been stored for 2–3 years after collection prior to shipment to Belgium (for isolation and molecular detection). Indeed, isolation is most likely during the acute phase of infections caused by *B. melitensis* or *B.*

suis and less successful in *B. abortus* infections. Brucellae are rarely isolated from samples with a competing microflora (Al Dahouk et al., 2002). The presence of competing organisms (observed during culture) may be another potential reason for isolation failure (Das et al., 2010).

This study further confirms that *B. abortus* is a cause of human brucellosis in Bangladesh and that infection is likely endemic in livestock. Contaminated milk represents a potential source of infection. Testing representative number of goat fetal membranes from different parts of the country will help understanding the presence of *B. melitensis* in Bangladesh.

The strength of this study is that for the first time it described the presence of *Brucella abortus* in occupationally exposed humans, goats and gayals in Bangladesh. This study has also some limitations including small size and non-randomness of the samples. Blood culture to isolate *Brucella* from seropositive humans was not possible due to lack of facility. The results of this study also do not represent whole of Bangladesh.

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Table 1. Summary of results of testing of human and animal samples for *Brucella* in Bangladesh using real time PCR

Sample type	Number	Positive		
		Brucella genus Real time PCR (%)	*Brucella abortus* rt PCR (%)	*Brucella melitensis* rt PCR
Milk, cow	14	3 (21.4)	3 (21.4)	0
Milk, goat	2	1 (50.0)	1 (50.0)	0
Milk, gayal	1	1 (100.0)	1 (100.0)	0
Placenta, cattle	5	0	–	–
Placenta, goat	10	0	–	–
Placenta, sheep	8	0	–	–
Vaginal swab cattle	4	0	–	–
Vaginal swab goat	10	0	–	–
Vaginal swab sheep	3	0	–	–
Bull semen	5	1 (20.0)	1 (20.0)	0
Human serum	13	13 (100.0)	13 (100.0)	–
Total	75	19 (25.3)	19 (25.3)	0

rt PCR, Real Time PCR

–, not tested

Table 2. Characteristics of 13 patients diagnosed positive with a *Brucella abortus* real-time PCR in Bangladesh.

Patient	Fever	Arthralgia	Backache	Sweating
1	Yes	Yes	No	Yes
2	No	Yes	Yes	No
3	Yes	No	Yes	Yes
4	Yes	Yes	No	Yes
5	No	Yes	No	No
6	Yes	Yes	Yes	No
7	Yes	No	No	No
8	Yes	No	No	Yes
9	Yes	Yes	No	No
10	No	Yes	Yes	No
11	Yes	Yes	No	Yes
12	Yes	No	Yes	Yes
13	Yes	No	Yes	Yes
%	76.9	61.5	46.2	53.8

All patients were treated by Doxycycline 100 mg twice daily plus rifampicin 600 mg daily for 6 weeks