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SERO-MOLECULAR INVESTIGATION OF *COXIELLA BURNETII* INFECTION IN DOMESTIC RUMINANTS AND HUMANS AND ASSOCIATED RISK FACTORS BASED ON 'ONE HEALTH' PERSPECTIVES IN BANGLADESH

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Background: Q-fever is an important zoonotic disease caused by the bacterium *Coxiella burnetii* with wide host range of mammals, birds and arthropods worldwide. The prevalence of *C. burnetii* infection has been reported in domestic ruminants in Bangladesh with no attention on the 'One Health' approach for the epidemiological investigation associated with risk factors for prevention of the disease.

Objective: This study aimed to investigate the sero-molecular prevalence of *Coxiella burnetii* infection and associated risk factors in domestic ruminants and humans based on 'One Health' approach in Bangladesh

Materials and Methods: This study on *C. burnetii* infection was conducted in cattle, goats and humans of the four randomly selected districts (Kurigram, Sirajgonj, Pabna and Mymensingh) in Bangladesh during the period from 2018 to 2021. A total of 162 and 172 serum samples respectively from cattle and goats with the reproductive disorders, 159 serum samples from human patients with pyrexia of unknown origin lasting over a period of three weeks and who were in close contact with animal, 119 milk samples from cows with reproductive disorders and 6 aborted materials from goat were collected from these selected districts for the detection of *C. burnetii* antibody by ELISA and DNA by PCR assay. The research was performed with the collaboration of OIE reference laboratory for Q fever, Friedrich-Loeffler-Institute, Germany.

Results: The overall sero-prevalence of *C. burnetii* infection by ELISA was 3.01% in cattle, 7.6 % in goats and 0.63% in humans. The highest sero-positivity was recorded in cattle in Mymensingh (4.5%) in comparison to Pabna (2.8%), Kurigram (2.5%) and Sirajgonj (2.4%) districts. Risk factors associated with higher sero-positivity of *C. burnetii* infection was recorded in cattle with > 5 years (4.4%) than 3 to 5 years (2.1%), indigenous (4.2%) than cross-bred (2.6%), history of retention of placenta (5.6%) than abortion (2.3%) and natural service (5.9%) than artificial insemination (2.3%). The sero-positivity of *C. burnetii* infection in goat showed highest in Kurigram (10.0%), followed by Sirajgonj (9.5%) and Mymensingh (9.3%) with no positive reactors in Pabna district. Risk factors associated with sero-positivity of *C. burnetii* infection in goats showed higher in > 2 years (14.1%) than up to 2 years (2.1%) age group and higher with the history of abortion (10.0%) than retention of placenta (4.2%). Risk factors associated with positivity of *C. burnetii* infection detected in milk samples of cows by ELISA showed an overall 21.85% positivity associated with history of reproductive disorders, with highest positivity in anestrus (33.3%), followed by retention of placenta (24.4%), abortion (21.7%) and lowest with repeat breeding (8.3%). In addition, higher positivity was recorded in cross-bred (24.2%) than in indigenous (12.5%) cattle. In case of humans, only one human patient (0.63%) had positive for IgG phase-II ELISA. *C. burnetii* DNA was detected in two sero-positive milk samples but all of the intermediate positive milk samples by ELISA were negative by PCR assay and even none of the aborted material of goat was positive in PCR and cell culture.

Conclusions: This study recorded the prevalence and risk factors associated with the zoonotic *C. burnetii* infection in both the domestic ruminants and humans but still it is unrecognized and underestimated in both human and animal health and research in Bangladesh. The findings of this study support the further research on *C. burnetii* in both human and veterinary medicine under 'One Health' approach particularly targeting epidemiology on the agent, host and environment for the control and prevention of the disease in Bangladesh.

Keywords: C. burnetii, Ruminants, Humans, ELISA, PCR, Sera, Milk, Prevalence, Risk factors, 'One Health' approach

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INTRODUCTION

Coxiellosis, also known as Q fever is recognized zoonotic disease of 'One Health' concern, affecting most of the mammals including humans. It is caused by Gram-negative intracellular bacterium *Coxiella burnetii* which is endemic at varying prevalence on all the continents except New Zealand and Antarctica.[1,2] The disease is considered as endemic in more than 51 countries[3] but remains a largely neglected zoonosis.[4] In addition, this disease has been ranked as the most contagious and listed as one among the 13 global priority zoonosis.[5] The annual incidence of Q fever is varied in different countries with ranges from 0.28 to 2.40 cases per million persons in USA, England and Wales,[6] but has reported higher as 15-49 cases per million persons in Australia.[7] Moreover, several outbreaks of the disease in humans have been reported from different developed countries like the Netherlands,[8,9] Australia,[7,10] America[11] and Europe.[12] The disease is associated with significant impact on public health as well as socio-economic impact of livestock farmers.[5] Mammals including humans, birds and arthropods like ticks have been reported to be reservoir hosts of *C. burnetii* but domestic ruminants particularly cattle, sheep and goats are the major reservoir hosts. Coxiellosis is mostly asymptomatic in the hosts but occasionally causes late abortion, stillbirth, endometritis or infertility in ruminants. Infected animals shed *C. burnetii* into the environment in milk, colostrum, urine, vaginal discharges and especially in birth products.[1,2] The amniotic fluids and placenta contain higher number of organism during birthing e.g. 10^9 bacteria / g placenta.[13] The *C. burnetii* organism can survive for long periods in the environment and it is common for aerosols from infected herds to be carried by the wild and cause infection in humans. Inhalation of infectious aerosol or contaminated dusts containing air-borne bacterium the major route of acquiring the disease in humans, so that a single inhaled organism may produce clinical illness.[14] Oral route of transmission of this infection in humans may occur through consumption of contaminated raw milk and dairy products, skin or mucosal contact, tick bites, blood transfusion, sexual transmission and embryo transfer.[15,16] However, the *C. burnetii* is mostly occupationally acquired zoonotic disease transmitted from animals to humans with a significant public health problem throughout the world. The standard routine laboratory culture methods are usually not suggested to grow the *C. burnetii* because the isolation procedure requires biosafety level 3 (BSL3) facilities with appropriate personal protective equipment.[17,18] Accordingly, specific indirect diagnostic methods including molecular detection by PCR assays in clinical samples and serological methods like ELISA for the detection of specific antibodies against phase I and phase II antigens are usually used as a methods of choice for sero-epidemiological studies.[5,19] Very limited reports on *C. burnetii* infection mostly in ruminant animals have been published from Bangladesh,[20-22] probably be due to non-availability of diagnostic facilities to detect the sub-clinical and clinical infections. In rural Bangladesh, most of animal farmers share the same premises which may facilitates the transmission of Q fever and other emerging zoonotic diseases from animals to humans and vice versa. But the medical and veterinary medical practitioners and public health professionals have no any epidemiological data and clinical or sub-clinical status of the *C. burnetii* infection in animals and humans in Bangladesh. This paper describes the sero-molecular investigation of *C. burnetii* infection in ruminants and humans to identify the potential risk factors with 'One Health' perspective in Bangladesh.

MATERIALS AND METHODS

A total of 162 cattle, 172 goat and 159 human serum samples, 119 cattle milk samples and 6 caprine samples of aborted materials (5 placenta and 1 fetus) were collected from selected districts of Bangladesh (Table 1). Cattle and goats with the history of reproductive disorders (retained fetal membrane, abortion, anestrous, repeat breeding) and human patients with pyrexia of unknown origin (PUO) with body temperatures higher than 38°C and lasting over a period of three weeks were target populations for this study. Cattle and goats of Kurigram Sadar, Pabna Sadar, Shahjadpur and Mymensingh Sadar were included. Pyrexic humans with the history of close contact with animals who came for treatment at Mymensingh Medical College Hospital (MMCH) were studied. Systematic random samples (every third pyrexic patient) were sampled from MMCH were tested. If one animal in a herd/flock met inclusion criteria it was selected. When more than one animal had signs then one was selected using random selection.

Table 1. Collection of samples including species, number, types and location

SN	Upazilas and Districts	No. of serum samples			No. of cow milk samples	No. of aborted samples	
		Cattle	Goat	Human		Placenta	Fetus
1	Pabna Sadar	36	36	-	18	2	-
2	Kurigram Sadar	40	40	-	20	1	-
3	Mymensingh sadar	44	54	159	42	2	1
4	Shahjadpur, Sirajgonj	42	42	-	39	-	-

Collection of blood sample

Five to seven milliliters of blood were drawn from the jugular vein in case of cattle and goats or median cubital vein in case of humans using 10 ml sterile syringe and kept in slant position at room temperature for 12 hours. After clotting, the syringe with blood was kept overnight in refrigerator at 2-8°C. The blood was then centrifuged at 1500 ×g for 10 minutes. Then serum was separated using micropipette in sterile screw capped tube, labeled and stored at -20°C until further use.

Collection of milk sample

Ten ml milk samples were collected into sterile plastic tubes from each cattle and were stored at -20°C and sent from Bangladesh to Germany by World Courier Service (in dry ice) following the process recommended by the IATA (International Air Transport Association) for further tests.

Collection of tick sample

The ticks were hands picked from randomly selected 126 cattle, identified and sorted according to the area, species, sex and developmental stages. Later, ticks were gathered into groups of 1 to 7 ticks as to the area, species and gender for DNA extraction. The collected ticks were placed in aluminium foil and preserved at -20 °C. The collected ticks were identified

Morphologically with the aid of a photographic microscope using the tick identification keys.[23,24]

Collection of aborted material

The aborted materials were collected from goats (Photo 1 and 2) and preserved at -20°C until further use.

Data collection

A questionnaire was designed to collect information on individual animals, farms and household cattle from the animal owners. Animal-level data on age, breed, sex, reproductive disorder, pregnancy status and herd-level data on herd size, herd composition and location of the herd were considered. Questionnaire was also designed to collect information for human such as age, sex, occupation, exposure history with animals and consumption of livestock products etc.

Serology (ELISA)

Milk samples: Upon arrival, the milk samples were centrifuged, the fat fraction was removed and discarded. The non-fat fraction was frozen to be tested for antibodies at a later time. The samples were tested for antibodies against *C. burnetii* using the commercial CHEKIT® Q fever antibody ELISA Test Kit (IDEXX, Liebefeld-Bern, Switzerland). These ELISA plates are coated with *C. burnetii* inactivated phase 1 and phase 2 antigens. The optical densities (OD) of the samples were corrected by subtracting the OD of the negative control. The results were expressed as S/P values and estimated as the ratio between OD of the sample (S) and the OD of positive control (P) included in the test kit. According to the instructions from IDEXX S/P $\geq$ 40% was considered positive, S/P < 30% was considered negative, and results in the interval 30% $\leq$ S/P < 40% were considered to be intermediate. The remaining non-fat fraction of the milk samples was frozen and stored for possible later purposes like determination of *C. burnetii*.

Sera (cattle and goat): The sera (Photo 3) were tested by ELISA for *C burnetii* antibodies according to the procedure described.[25] All reagents were taken into 18-26°C before use. The reagents were mixed by shaking gently. All samples were tested in triplicate and the optical densities (OD) of the samples were averaged and corrected by subtracting the OD of the negative control. Serum based tests were performed using the commercial CHEKIT Q-Fever Antibody ELISA Test Kit (IDEXX, Liebefeld-Bern, Switzerland) based on *C. burnetii* inactivated phase 1 and phase 2 antigens.[26] The positive cut-off value (S/P ratio) of ELISA individual animal sera was $\geq$ 40%.

Human sera: Human sera (Photo 4) for detection of IgG antibodies to *C. burnetii* phase I and phase II antigen, a commercial ELISA was used (Serion ELISA classic, Virion/Serion, Wurzburg, Germany). IgG phase I and II were processed on a fully automated 4 plate ELISA processing system (DSX). Different dilution protocols were used according to the manufacturer‘s instructions, using a 1:500 dilution for the IgG phase I and phase II assay. Data

were analyzed according to the Virion/Serion protocol, reporting IgG phase I qualitatively and IgG phase II quantitatively. IgG phase I was positive whenever the measured absorbance was more than 10% above the extinction of the cutoff control. Ambiguous results were added to negative results. IgG phase I extinctions were expressed in optical density (OD) values. IgG phase II extinctions were expressed in iu/ml titer using a logistic-log-model calculation. Samples with titer values of <20 iu/ml were considered negative, values of 20-30 iu/ml were scored as borderline. Those that had values of > 30 iu/ml were considered as positives.

DNA extraction

Sera: DNA from seropositive samples of *C. burnetii* were extracted according to the method described[27] using the High Pure PCR Template Preparation Kit™ (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. DNA quantification was performed with a Taq Man based real-time PCR assay targeting the transposase element IS1111 as described.[28] The real-time PCR assay was performed with the primers and the conditions (Table 2 and 3). Samples were considered positive with a cycle threshold (Ct) < 40.[29]

Table 2. Primer and probe for IS 1111 Real-time PCR

Oligo	Name	Sequence (5′ to 3
Primer	Cox-R	CCCCGAATCTCATTGATCAGC
	Cox-F	GTCTTAAGGTGGGCTGCGTG
Probe	Cox-TM	6FAM-AGCGAACCATTGGTA-TCGGACGTTXTATGG-PH

Table 3. PCR conditions for Real-time PCR

Cycler: Strata gene M 3000	Temp.	Time
Initial denaturation	50 ^{0}C	02 min
	95 ^{0}C	10 min
Denaturation, Cycles 50	95 ^{0}C	15 secs
Anneal/Elong. Cycles 50	60 ^{0}C	30 secs

Milk: A volume of 1 ml milk was centrifuged at 8000 g for 60 minutes, the cream and milk layers were removed and the pellet was washed and resuspended twice in 50 µl distilled water.[30] Then DNA extraction was applied by using QIAamp kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions for milk samples.

Aborted material: Total genomic DNA was extracted from fetus and placenta using QIAamp DNA Mini Kit (Qiagen, Germany) according to the manufacturer's instructions.

Real-time PCR

Sera, aborted material, milk and tick

DNA quantification was performed with a Taq Man based real-time PCR assay targeting the transposase element IS1111 as described.[28] The real-time PCR assay was performed with the primer, probe and the conditions (Table 2 & 3). Samples were considered positive with a cycle threshold (Ct) < 40.[29]

Real-time PCR was performed in a total volume of 25 µl containing: 2.5 µl of 10× PCR buffer, 2.0 µl of 50 mM MgCl2, 2.0 µl of 10 mM dNTP, 2.0 µl of 10 p mol forward and reverse primer, 0.75 µl of 10 pmol FAM / TAMRA dual labeled probe (FAM-TCATCAAGGCAC-

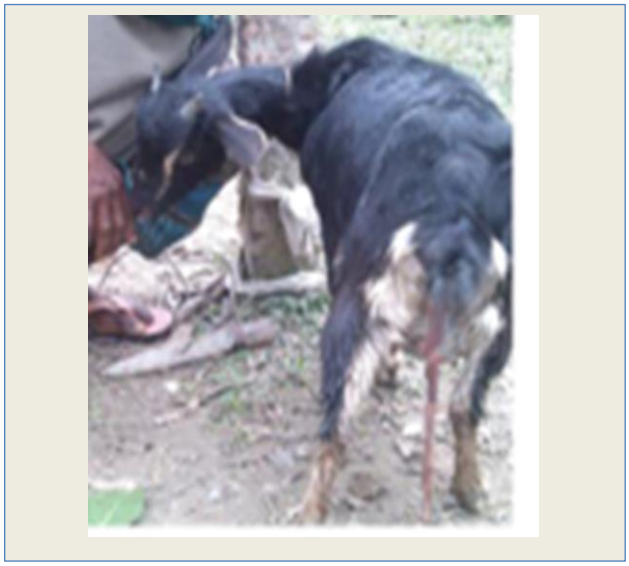

Photo 1. A doe with retained placenta used for sampling in Pabna district

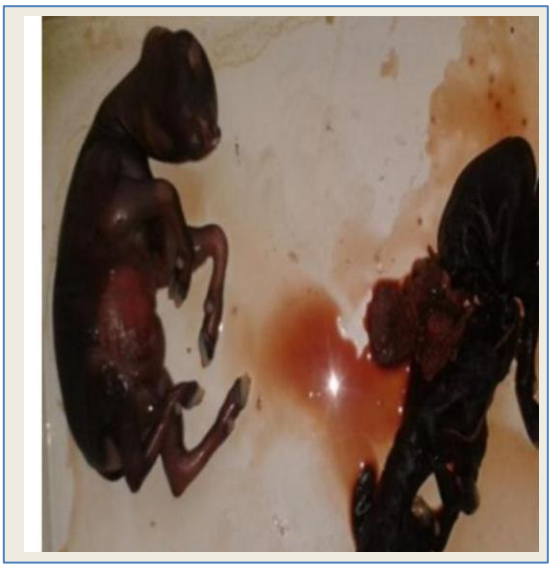

Photo 2. Aborted fetuses of goat used for sampling in Mymensingh district

Photo 3. Collected serum samples from animals

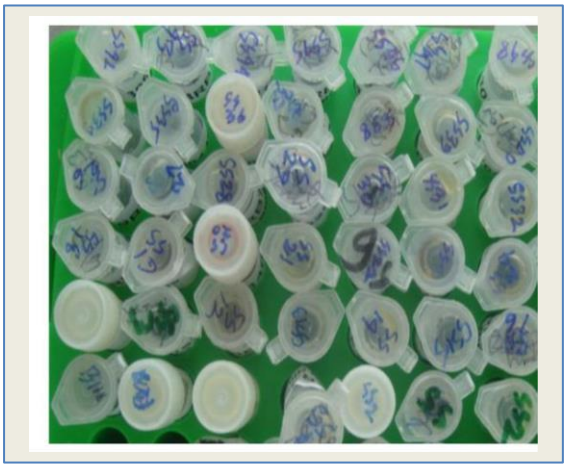

Photo 4. Collected serum samples from humans

CAATGGTGGCCA-TAMRA) (in deviation to the published minor groove binder probe),[31] 8.5 µl sterile water, 0.25 µl of 5 U/ µl thermostable polymerase DNA (Invitrogen, Germany), and 5 µl of extracted DNA. Cycling conditions in a 7500 fast real-time PCR system (Applied Biosystems, USA) were as follows: 94℃ for 2 minutes and 45 cycles of 94℃ for 15 seconds and 60 ℃ for 30 seconds. The reagents for real-time PCR were from Invitrogen, Germany; primers and probes from Genecam, Germany. As positive control *C. burnetii* DNA (Genecam, Germany) and negative control (water) were used.

Sera and tick sample values falling below cycle threshold (Ct) of 38 were considered positive. A milk sample was considered positive when the cycle threshold (Ct) value was ≤36, negative when the Ct was ≥40 and doubtful when the Ct was between >36 and <40.[32]

Cell culture

Isolation of *C. burnetii* was performed using Vero cells (ATCC^{R} CCL-81^{TM}). The Vero cell-line was kept at 37±1℃ with 5% CO_2 in MEM (Eagle's Minimum Essential Medium Sigma Aldrich, USA) supplemented by 10% foetal bovine serum (FBS, Sigma Aldrich, Poland). Antibiotic and anti-mycotic solutions (contains 10.000 units/ml penicillin G, 10 mg/ml streptomycin sulfate, 25 µg/ml amphotericin B) (Sigma Aldrich, USA) were added at the beginning of the culture to a final concentration of 1%. Tissue samples were cut into small pieces and homogenized in 500-1000 µl of sterile PBS and incubated at 37±1℃ for 1 hour. Aliquots of 200 µl of sample were transferred to two 25 cm^2 flasks with a confluent monolayer of Vero cells. The medium was changed weekly. Formation of vacuoles was checked regularly and tested for growth of *C. burnetii* at two-week intervals, by real-time PCR. The used culture method is an own validated method.

Analysis of data

Data were entered into MS excel 2007. Cleaning and processing of data were performed then transferred to R statistical software to estimate prevalence and its 95% confidence interval. Chi-square test was conducted to evaluate the univariable association between explanatory variables and Q fever status in different species.

RESULTS

The serological results obtained by ELISA illustrated that the sero-positivity for *C. burnetii* were recorded in 3.01% and 7.6% in cattle and goat samples, respectively (Table 4 and Photo 5). The highest prevalence in cattle (4.5%) was recorded in Mymensingh district while the lowest (2.4) was recorded in Sirajgonj districts. Breed-wise distribution revealed that there was a higher prevalence in indigenous (4.2%) than the cross-bred (2.6%) cattle (Table 5). With respect to various reproductive problems, the number of positive reactors was recorded and it was found that cattle with the history of retention of placenta (5.6%) showed a significantly higher prevalence of *C. burnetii* than abortion (2.3%). No positive reactors were found in anestrus and repeat breeding (Table 5). A higher sero-prevalence was recorded in Kurigram (10.0%) than Sirajgonj (9.5%) and Mymensingh (9.3%) but no positive reactors were found in Pabna district (Table 6). In this study, sero-prevalence was significantly higher in the more than 2 year's age (14.1%) group than in the up to 2 years age (2.1%) group (Table 6).

Testing of 119 milk samples by ELISA revealed that the prevalence of Q fever in cattle with a history of reproductive disorder was 21.85% (Table 7). Breed-wise distribution of Q fever revealed that there was a higher prevalence in cross-bred (24.2%) than in the indigenous (12.5%) cattle (Table 8). Cows with a history of artificial insemination (23.5%) showed a significantly higher prevalence than cows with natural (11.8%) breeding (Table 8).

Table 4. Sero-prevalence of *Coxiella burnetii* infection in cattle and goats

SN	Species	No. of sera tested	ELISA + ve	Prevalence (%)	95% Confidence interval	PCR
1	Cattle	162	05	3.01	1.1-7.4	- ve
2	Goat	172	13	7.60	4.3-12.8	- ve

Table 5. Contingency tables and Chi-square tests conducted to evaluate the association between explanatory variables and Q fever sero-prevalence in cows with reproductive disorders in different districts in Bangladesh

SN	Variable	Category	No. of samples tested	Prevalence (%)	95% CI	p-value
1	District	Pabna	36	2.8	0.1-16.2	0.93
		Kurigram	40	2.5	01-14.7	
		Mymensingh	44	4.5	0.8-16.7	
		Sirajganj	42	2.4	0.1-14.1	
2	Age (years)	3-5	94	2.1	0.4-8.2	0.71
		> 5	68	4.4	1.1-13.1	
3	Breed	Indigenous	48	4.2	0.7-15.4	0.98
		Cross-bred	114	2.6	0.7-8.1	
4	Breeding	Natural service	34	5.9	1.0-21.1	0.62
		AI	128	2.3	0.6-7.2	
5	History of reproductive disorders	Repeat breeding	10	0	0-34.5	0.62
		Abortion	86	2.3	0.4-8.9	
		Anestrus	12	0	0-31.1	
		Retained placenta	54	5.6	1.4-16.3	

In this study only one human patient tested a positive result in the IgG phase-II ELISA. The prevalence recorded for acute Q fever was 0.63% out of 159 tested but none was positive for IgG phase-I ELISA (Table 9).

C. burnetii DNA was detected in 2 sero-positive milk samples (Figure 7) when a specific quantitative real-time PCR assay, LSI VETMAX PCR (LSI, Life Technologies Lyon, France), targeting the IS 1111 element was used. However, all of the intermediate milk samples by ELISA were negative by PCR assay.

This study revealed *C. burnetii* DNA in only one tick (0.79%) out of 126 tick samples. None of the sero-positive sera samples of cattle, goat and human was positive in PCR. Moreover, none of our aborted material was positive in real-time PCR and cell culture examination.

Table 6. Contingency tables and Chi-square tests conducted to evaluate the association between explanatory variables and Q fever sero-prevalence in does (she goats) with reproductive disorders in different districts in Bangladesh

SN	Variable	Category	No. of samples tested	Prevalence (%)	95% CI	p-value
1	District	Kurigram	40	10	3.3-24.6	0.29
		Mymensingh	54	9.3	3.5-21.1	
		Pabna	36	0	0-12.0	
		Sirajganj	42	9.5	3.1-23.5	
2	Age (years)	Up to 2	94	2.1	0.4-8.2	0.007
		> 2	78	14.1	7,6-24.34	
3	History of reproductive disorders	Abortion	100	10	5.2-18.0	0.26
		Retained placenta	72	4.2	1.1-12.5	

Table 7. Prevalence of *Coxiella burnetii* infection in cattle, goat and tick

SN	Samples	Host species	No. tested	ELISA + ve	INTD	Prevalence (%)	95% Confidence interval	PCR	Culture
1	Milk	Cows	119	26	7	21.85	15.9-30.5	2	-
2	Aborted materials	Goat	06	-	-	-	-	-	-

INTD = Intermediate - = Negative

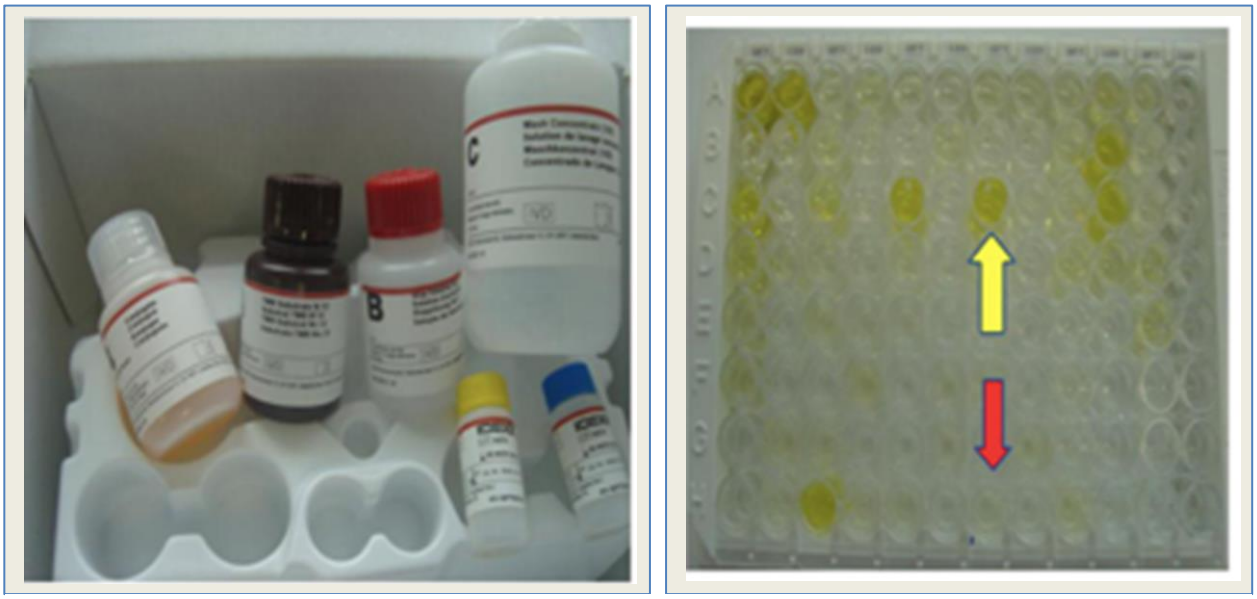

Photo 5. ELISA test kit component (left photo) and its reaction-yellow arrow indicating positive reaction of *Coxiella burnetii* infection and red arrow indication negative reaction

Table 8. Contingency tables and chi-square tests conducted to evaluate the association between explanatory variables and *Coxiella burnetii* status in milk samples in cattle with reproductive disorders in different districts in Bangladesh.

SN	Variable	Category	No. of samples tested	Prevalence (%)	95% Confidence interval	p-value
1.	Districts	Pabna	18	0	00.0-21.9	0.08
		Kurigram	20	30.0	12.8-54.3	
		Mymensingh	42	21.4	10.8-37.2	
		Sirajgonj	39	28.2	15.5-45.1	
2.	Age (years)	3-5	72	22.2	13.6-33.8	1.00
		> 5	45	21.3	11.2-36.1	
3.	Breed	Indigenous	24	12.5	03.3-33.4	0.33
		Cross	95	24.2	16.3-34.3	
4.	Breeding	Natural service	17	11.8	02.1-37.7	
		AI	102	23.5	15.9-33.2	
5.	History of reproductive disorders	Repeat breeding	12	08.3	0.43-40.2	0.59
		Abortion	60	21.7	12.5-34.5	
		Anestrus	06	33.3	05.9-75.9	
		Retained placenta	41	24.4	12.9-40.6	

Table 9. Sero-molecular prevalence of *Coxiella burnetii* infection in humans

SN	Species	No. of samples tested	ELISA		Prevalence, % (95% Confidence interval)		PCR
			Ph2 IgG	Ph1 IgG	Acute	Chronic	
1.	Human	159	1	Negative	0.63 (0.03-3.9)	Negative	Negative

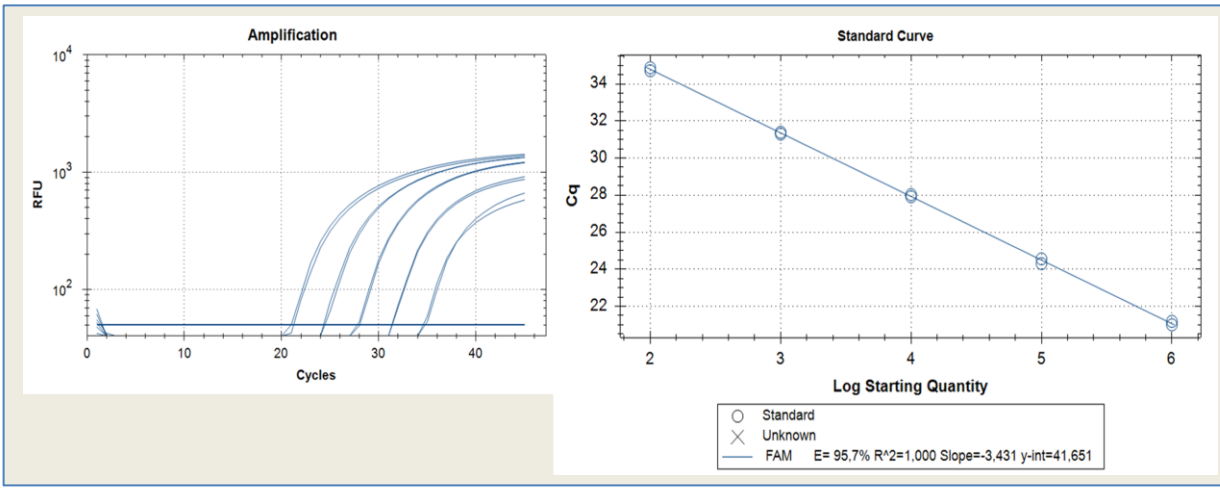

Photo 6. Real-time PCR amplification plots for DNA extracted from milk samples of Bangladesh and standard curve. The Ct results for the positive milk sample are amplified

DISCUSSION

Q fever is an infectious disease may have a significant impact on animal welfare, human health and economics.[33,34] It was first recognized clinically in 1935 in Brisbane, Queensland, Australia by Derrick during an outbreak of a febrile illness of unknown origin among abattoir workers.[35] The 'Q' stands for 'query' and was applied at the time when the cause was unknown by Derrick.[35] Burnet and Freeman, as well as Davis and Cox demonstrated that the etiological agent was filterable and displayed properties of both viruses and rickettsiae.[36,37] Then Cox succeeded in propagating the infectious agent in embryonated eggs.[38] The etiological agent of Q fever was first named *Rickettsia burnetii*. In 1938, Philip proposed the creation of a new genus called *Coxiella* and the renaming of the etiological agent as *Coxiella burnetii*, a name which honors both Cox and Burnet, who had identified the Q fever agent as a new Rickettsial species.[39] Recent phylogenetic investigations, based mainly on 16S rRNA sequence analysis, have shown that the *Coxiella* genus belongs to the gamma subdivision of Proteobacteria, characterizing it closer to Legionella and Francisella than to Rickettsia.[39,40]

C. burnetii is an obligate intracellular pleomorphic Gram-negative bacterium (0.2-0.4 × 0.4-1.0 μm) possessing a membrane similar to a Gram-negative bacterium stained by the Gimenez method. The usual host cell of *C. burnetii* is the macrophage, which is unable to kill the bacterium. This organism has two distinct antigenic forms, phase I and phase II variants based on the variation of the surface lipopolysaccharide (LPS). The phase I variants are highly infectious forms found in naturally infected hosts with a complete LPS on their surface whereas, phase II variants are less infectious and isolated during serial passage in an immunological incompetent host like cell cultures and embryonated eggs that have an incomplete LPS due to a spontaneous genetic deletion of 25,992 bp.[41,42] The anti-phase II antibodies (IgG & IgM) have been reported at high levels in acute Q fever whereas anti-phase I antibodies (IgG & IgA) have been reported at high levels only during chronic infection.[41] These antigenic variations are important for serological diagnosis and pathogenesis.[43]

Inhalation of contaminated dust and aerosols is the main route of transmission of *C. burnetii* in the hosts, which shows tropism for the reproductive tissue and the mammary gland and accordingly, the infectious agent is primarily shed during parturition and via the milk.[34] Inhalation of <10 bacteria is adequate to establish infection in the hosts.[44,45] C. burnetii develops spore-like forms, which are highly resistant to adverse environmental changes and disinfectants like desiccation and sunlight, so it can survive for months and even years in the environment.[46] The organism was found to be more heat resistant than *Mycobacterium tuberculosis* and recommended vat pasteurization was raised from 61.7 to 63 ^{0}C (holding time 30 minute) to ensure destruction of the organism. The current high temperature short time (HTST) pasteurization temperature of 72 ^{0}C for 15 second is also effective. This organism is primarily affect the domestic ruminants particularly sheep, goat and cattle but may also infect other livestock, pets, wild mammals, birds, reptiles and humans. Domestic ruminants are considered the main reservoir for the pathogens which is especially concentrated in placental tissues, replicates in trophoblasts and reproductive fluids. The infected animals contaminate the environment by shedding *C. burnetii* in milk, feces, urine, saliva[47] and very importantly in vaginal secretions, placenta, amniotic fluids and other products of conception.[47-49] Evidence

suggests a prominent role of airborne dispersal, *C. burnetii* travels up to 18 km on gale force winds, highest infection risk occurs within 5 km of sources in rural areas, whereas urban outbreaks generally occur over small distances.[50] Inhalation of contaminated droplets released by infected animals is the most common route of infection in both animals and humans.[51]

Arthropod vectors particularly Ixodid and Argasid ticks are considered to be the natural primary reservoir of *C. burnetii* where it replicates and excreted in saliva and feces. Tick feces are extremely rich in bacteria and may reach a concentration of 10^9 organisms per gram feces.[52] *Dermacentor* spp.[52] and *Ixodes ricinus*[53] have been reported to be infected, whereas none of the ticks analyzed were positive when tested by PCR despite the area being endemic for Q fever.[54]

Human infection results from inhalation of contaminated dust aerosols and droplets but can also be transmitted by consumption of contaminated raw milk and raw milk products, direct contact between human to human, animal to human, tick feces (10^9 *C. burnetii* / g) to human, semen of infected animals, contact with placenta (10^9 *C. burnetii* / g) of animals and parturient women, blood transfusion and cases of sexual transmission have been reported.[15,55-57] *C. burnetii* is extremely infectious for humans, 1-10 viable organisms suffice to induce an infection via the aerogenic route.[57,58] However, Q fever in humans is mostly an occupational hazard for people who may be exposed to aerosolized *C. burnetii* infection from animal sources. Humans working in slaughter houses, veterinarians, veterinary technicians, livestock farmers, dairy workers, transporting of infected animals and laboratory workers handling potentially infected veterinary samples are at high risk occupations.[7,58] Animal contact with manure and birth products and even living or working close to dairy farms transmitted in USA and Australia whereas outbreak of Q fever has reported on living within one kilometer of an animal farm with infected animals in the Netherlands. The *C. burnetii* has spread via airborne dispersal from infective sheep and goat not cattle and travels up to 18 km on gale force winds.[50] These results suggest that occupational and environmental factors are pivotal in Q fever transmission.[6] Hence, 'One Health' approaches need to be based on human, animal and environmental domains for *C. burnetii* infection.

The reproductive disorders specially abortion, stillbirth, premature delivery and delivery of weak offspring are mainly associated with Q fever in domestic ruminants but mainly expressed in small ruminants whereas it is usually asymptomatic in cattle and camel which may develop infertility, metritis and mastitis.[59,60] *C. burnetii* has tropism for the reproductive organs, mainly the placenta and the most effective immune response against infection is through macrophages and natural killer cells but during pregnancy result in immunosuppression which favor multiplication of the bacterium in the tissues.[61] The bacteria may colonize the placenta resulting in vasculitis, diffuse inflammatory processes, necrosis, calcification in the intercotyledonary region and the possibility of abortion[59,62] birth of weak offspring and perinatal mortality.[48]

The occurrence of Q fever in goats have been reported to be associated with outbreaks in humans due to their extensive raising and close contact with humans.[63]

Currently, the *C. burnetii* infection is endemic at varying prevalence on all continents except New Zealand and Antarctica.[1,16,64] Recently, outbreaks of Q fever in goats in Australia[7] and Netherlands[8,65] associated with human outbreaks have been reported. Human infection is usually acquired from ruminants and is characterized by asymptomatic, acute or chronic

(acute cases become chronic 5-15%) disease with endocarditis (10%), pneumonia, hepatitis or more rarely hematological, neurological or cardiovascular involvement can occur.[7,57]

The Coxiella can be propagated in embryonated eggs and cell culture in a variety of culture media which is considered as the gold standard for diagnosis of *C. burnetii* infection but it is technically difficult, time consuming and highly hazardous zoonotic pathogen which is not routinely available in most microbiology laboratories because biosafety level III laboratory is required for its isolation.[4] A confirmed case of Q fever requires either definitive laboratory evidence (detection of *C. burnetii* by nucleic acid testing or by culture, sero-conversion or a significant increase in antibody level to phase II antigen in paired sera tested in parallel in the absence of recent Q fever vaccination, or a clinically compatible syndrome accompanied by detection of IgM specific for Phase II *C. burnetii* in the absence of recent Q fever vaccination.[7,66,67]

Detection of *C. burnetii* DNA in various samples by a range of PCR assays is progressively becoming available and is considered useful especially in the acute clinical stages of the illness when sero-conversion is not sufficient to be detected by serological methods.[43,68] However, ELISA and PCR assays have been recommended to gain a sensitive diagnosis in patients presenting with fever of unknown origin and enable appropriate management of the patients.[23,28,43,69]

This study recorded an overall sero-prevalence of 3.01% (1.1-7.4%) *C. burnetii* infection in cattle, 7.7% (4.3-12.8%) in goats and only 0.63% (0.03-3.9%) in humans by using ELISA, whereas cow milk samples showed highest positivity rate of 21.85% (15.9-30.5%) with this test. The sero-positivity of IgG phase I and Phase II antibodies of *C. burnetii* infection in humans found negative and 0.63% (95% CI: 0.03-3.9%), respectively.

The overall 3.01% sero-prevalence of *C. burnetii* infection in cattle recorded in this study supports the earlier inland finding of 3.57% in cattle[22] but both the higher sero-prevalence rate of 6.1%[21] and lower rate of 0.65%[20] have been reported from Bangladesh. However, sero-prevalence of *C. burnetii* infection in cattle varies widely from countries to countries like 31.37% in Iran,[70] 6.2% Northern Ireland,[71] 8.5% in Bulgaria,[72] 15% in China,[73] 16.0% in Netherlands,[74] 30.4% in Cameroon,[75] 24.5% in India[5] and 10.4% in Pakistan.[75]

This study recorded an overall 7.7% sero-prevalence of *C. burnetii* infection in goat population in Bangladesh which is in conformity with the earlier inland report of 7.6% sero-prevalence of this infection in goats.[21] However, comparatively lower sero-prevalence rates of 3.33%[22] and 0.76%[20] of *C. burnetii* infection has been reported from Bangladesh. Mostly higher sero-prevalence percentage rates of *C. burnetii* infection in goats have been reported elsewhere including 31.97% in Iran,[70] 13-23% in Africa,[77] 20-46% in Kenya,[43] 0.8-60.6% in China,[71] 7.8% in Netherlands,[78] 13.7% in Bulgaria,[72] 15.0% in Australia,[7] 15.0% in Pakistan.[79]

The outbreaks of Q fever in humans have been reported to be reported to be linked to an intensive goat and sheep dairy farms in the Netherlands[80] and Australia.7 It appears that the goats play a significant role in the transmission of *C. burnetti* infection in human outbreaks. In Bangladesh, comparatively higher sero-prevalence rate of *C. burnetii* infection in goats (7.7%) in comparison to cattle (3.01%) which might be possible goat as a source of major infection in humans.

This study recorded only 0.63% sero-prevalence of *C. burnetii* infection in humans for the first time in Bangladesh by using ELISA which appears very low rate in comparison to the rates reported from different countries in the world. As examples, 10.0% sero-positivity of *C. burnetii* infection in humans in China,[71] 1-32% in Africa,[77] 35.8% in Kenya,[43] 12.3-32% in Turkey,[81,82] 15.3% in Spain,[83] 5.2% in Australia,[84] 11.0% in Denmark,[85] 3.1% in USA,[86] 19.80-32.86% in Iran,[70] and 18.6% in India.[87]

These differences on the sero-prevalence rates of *C. burnetii* infection in humans of different countries could be due to varieties in ecologic, social, cultural, behavioral and economic conditions and also levels of animal infections, which affect the exposures of people in each of the country of the world (Mobarez et al. 2027).[70]

The ELISA positive samples of cattle (3.01%/5), goats (7.6%/13) and humans (0.63%/1) showed negative results with PCR, which might be attributed to the enduring immunological response of these animals and human to *C. burnetii* that has been correlated with the elimination of the pathogen.[5,88] On the other hand, the PCR positivity that shows negative result with ELISA can be attributed to the early acute phase of the infection, whereas positivity in both PCR as well as ELISA emphasizes an active circulation of the pathogen with the population.[88,89]

Of the 119 cow milk samples tested for *C. burnetii* infection, of which 21.85% (n=33) found positive with ELISA and only two ELISA positive milk samples showed positive reaction by real-time PCR. These findings are in conformity with the earlier finding of 15.6% positivity of *C. burnetii* infection in cow milk samples in Bangladesh.[22] Recent surveys conducted in many countries have revealed that the presence of *C. burnetii* in raw milk can vary over a wide range from 0 to 95%[90] with some particular examples of 15.0% in Lebanon,[91] 18.8% in the Netherlands (Van Engelen et al. 2014),[92] 27.0% in Italy[93] and 25.0% in Egypt.[94] The retail market samples of milk and milk products have reported to be infected with *C. burnetii* as 27.08% raw milk, 6.25% yogurt, 4.35% cheese, 4.16% dough and 0% ice cream in Iran.[95]

Shedding of *C. burnetii* in milk of domestic ruminants is intermittent and it can last for several months in goats and cattle,[13,96] whereas sheep shedding of *C. burnetii* occurs for a short period usually 1 to 8 days after the abortion.[97] Therefore, shedding of *C. burnetii* in milk by cow milk is the most important route of spreading this bacterium in the environment and transmission through consumption of raw milk and products prepared with raw milk. Similarly, *C. burnetii* infection have been detected in sheep and goat milk elsewhere[98] but it seems that no such research works have yet been conducted in Bangladesh. Moreover goat milk is usually consumed in non-pasteurized form in developing countries including Bangladesh and accordingly, serious attention must be taken to detect and presence of *C. burnetii* in goat milk.

This study detected *C. burnetii* DNA in only one (0.79%) tick out of 126 tick samples collected from selected animals. An early attempt has failed to detect *C. burnetii* DNA in 24 ticks collected from selected ruminants in Bangladesh.[21] However, the detection of *C. burnetii* DNA in tick samples for the first time in Bangladesh which is very low rate in comparison to high rate reported from different countries. As examples, 2.5% and 20.0% of ticks contained *C. burnetii* DNA collected from cattle and dogs in Kenya,[99] 25.0% collected from domestic animals in Ethiopia,[100] 31.0%, 4.8% in Europe[34] and 7.7% of ticks collected from sheep and

goats in Pakistan[79] and 2.4% of ticks collected from domestic and wild animals with highest sampled from wild deer (5.09%) in Slovenia and approximately the pathogen is present in more than 40 tick species.[101] The occasional reports of an unexpectedly high prevalence of *C. burnetii* DNA in ticks may reflect their role as a vector for the transmission of Q fever.[102] However, it is evident that the ticks are not primary source of infection of domestic animals and humans, which are infected by inhalation of contaminated aerosols or dust containing C. burnetii shed by infected animals.

Some of the variables obtained from the questionnaire and the risk analysis based on the result of the ELISA and PCR assay of sera and milk samples tested to detect infection of *C. burnetii* in cattle, goats and humans. Serum based detection of *C. burnetii* infection showed higher rate of infection at older age than young ages of both cattle (3-5 years 2.1% & >5 years 4.4%) and goats (up to 2 years 2.1% & > 2 years 14.1%) with ELISA. These findings are in conformity with the earlier reports published from different countries in different species of domestic ruminants.[103-105] Adult animals are more likely to form antibodies due to a higher chance of getting in contact with the pathogen during their lifetime.[103-105] Therefore, age is identified as one of the risk factor of *C. burnetii* infection in domestic ruminants based on the ELISA results at animal level. Farm practices especially improper aborted material disposal and not separating the animals from the rest of the herd during parturition are important risks for the occurrence of *C. burnetii* infection in the bovine populations in India.[106] Contact with other herds and purchased animals reported as risk factors for sero-positivity to *C. burnetii* infection in cattle in Algeria.[107] However, higher sero-positivity of 16% and 22% in populations living close contact with animals and those living in rural establishment has been reported respectively in Egypt.[108] Proximity to farm animals and contact with infected animals or their birth products have been identified as the most important risk factors for human disease[64]

CONCLUSION

This study and previous reports suggest that *C. burnetii* infection is prevalent in both humans and animal population in Bangladesh. These findings urge for further research to elucidate the epidemiology to assess the correlations and effects of the *C. burnetii* infection in animal and humans considering the local host, nature of the agent, environmental conditions and animal husbandry practices in Bangladesh. In addition, intensive livestock farming especially goat farming is a growing industry in the world including Bangladesh. The *C. burnetii* infection risk management complexities that need to address the triad of agent-host-environmental aspects which is the essential requirement of a 'One Health' approach. A close collaboration between medical and veterinary medical authorities particularly 'One Health' approaches will be required on all the zoonotic diseases at both the national and regional levels to develop an integrated program for zoonotic disease surveillance and prevention simultaneously both in livestock and humans in Bangladesh.

ETHICAL STANDARD

This research work was carried out with the ethical approval of the ethical authorities of the Mymensingh Medical College (No. MMC/IRB/2021/348) and Bangladesh Agricultural University, Mymensingh (No. AWEEC/BAU/2021(19), Bangladesh.

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