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ASSESSMENT OF HAEMATO-BIOCHEMICAL AND THERAPEUTIC RESPONSES OF CHRONIC BRUCELLOSIS IN CROSSBRED DAIRY COWS IN BANGLADESH

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ABSTRACT

Background: Sero-molecular methods have been used for the study on the prevalence and economic impact of brucellosis in Bangladesh. The physio-pathological effects and therapeutic trials against brucellosis in animals or humans could not be traced in the available inland literature.

Objectives: This study was conducted to determine the sero-prevalence and haemato-biochemical and therapeutic responses in Brucella-infected dairy crossbred cows.

Materials and Methods: Sera samples of 552 cross-bred dairy cows of Military Dairy Farm, Jessore and smallholder dairy farms of Sirajgonj and Dhaka were initially screened for Brucella infection with Rapid kit test (RKT) and Rose Bengal test (RBT) and positive samples were tested further with ELISA and PCR for confirmatory diagnosis during the period from January 2018 to June 2019. Out of 11 all tests positive cows, of which four had history of abortion were selected for therapeutic trials with combined long acting oxytetracycline @ 25 mg/kg BW 16 doses at 72 hours intervals and streptomycin @ 25 mg / kg BW 10 doses at 24 hours interval injections. Blood samples of all the Brucella negative control and pre- and post-treatment stages of all the Brucella-infected cows were tested for haemato-biochemical changes and Brucella antibody responses by using ELISA and PCR.

Results: Of the 552 sera screened for Brucella infection, of which 18 (3.26%) cows were found positive with the RKT, RBPT and ELISA, whereas only 11 (1.99%) samples showed positive with PCR. The haemato-biochemical values between Brucella-negative and positive cows and antibiotic pre-treated and post-treated values of Brucella-infected cows were compared and discussed. The antibody titer decreased with antibiotic treatment and increased on with-drawl of the antibiotic at 180 days which indicates that antibiotics only effective against bacteremic form not intracellular stage that caused to relapse. However, of the four treated cows, one became pregnant on artificial insemination with normal reproductive cycle which needs to explore its status in further research.

Conclusions: The haemato-biochemical values in cows affected with sub-clinical brucellosis can determine the extent of harmful effects on the health of cows. Therapy with multiple antibiotics for long period, absence of effective vaccines and the most expensive quarantine and 'test and slaughter' methods, brucellosis remains as a challenge for its control and eradication in developing world. Human patients affected with brucellosis is treated with antibiotics with overall neglecting the animal reservoir of Brucella infection, therefore 'One Health' approach would be required to control this disease.

Keywords: Chronic brucellosis, Cross-bred cows, Rapid Kit test, Rose Bengal test, ELISA, PCR, Haemato-biochemicals, therapy trials, Antibodies response

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INTRODUCTION

Brucellosis, caused by intra-cellular Brucella species is one of the most important emerging zoonotic chronic diseases distributed worldwide since the discovery of *Brucella melitensis* by Bruce in 1887.[1] However, several countries traditionally considered to be endemic including western and northern Europe, Canada, Japan, Australia, New Zealand, Israel and most of the Latin America have achieved control.[2,3] Countries with highest incidence of human brucellosis per million population reported to be 1,604.4 cases in Syria,[2] followed by 391.0 cases in Mongolia, 268.8 in Iraq, 211.9 in Tajikistan, 149.5 in Saudia Arabia and 141.6 in Iran.[2,4-7] The highest numbers of 5514 brucellosis outbreaks mainly caused by *B. abortus* in cattle have been reported from Mexico, followed by 2138 in China, 1268 in Greece and 1142 in Brazil.[6] Outbreaks of brucellosis in cattle caused by *B. abortus* have also been reported from Canada,[8] *B. melitensis* in cattle from Spain,[9] *Brucella* spp. in humans from Brazil[10] and even from India[11,12] but so far not reported from Bangladesh.[13] This disease is specifically hinder animal productivity and human health in developing countries has led the WHO to classify it as one of the world's leading 'neglected zoonotic diseases.'[14,15] The *B. abortus* (cattle), *B. melitensis* (small ruminants) and *B. suis* (swine) are leading causes of animals and human brucellosis in developing world.[1,6] The cross-species transmission of certain *Brucella* spp. can occur and cattle may be affected with all these three species[16] with *B. abortus* is the predominant causal agent, less frequently by *B. melitensis* and occasionally by *B. suis*.[3] This is an endemic disease in developing world that can manifest not only as acute outbreak or chronic disease but also as sub-clinical or carrier infections persisting throughout the life with recurrences potentially during pregnancy associated with abortion in late pregnancy in cattle.[17] Although rarely fatal, brucellosis remains a major public health problem and a significant economic loss due to abortion with associated disorders in dairy cattle.[6,18] Brucella infection in pregnant cattle produces placentitis, followed by abortion in late pregnancy, temporary or permanent infertility and weak newborn calves and decreased milk production whereas it causes orchitis in male, and infertility and hygromas in both male and female cattle.[6,18] However, the disease remains asymptomatic in non-pregnant cattle and most infected animals abort only once in their life time and may become carrier and continue to shed the bacteria for long period especially at parturition.[6,18] The brucellosis has been reported to be associated with bovine infertility for the first time in 1967 from Bangladesh.[19] Recently the reviewed reports showed that the 3.7% sero-prevalence of brucellosis in cattle, 4.0% in buffaloes, 3.6% in goats and 7.3% in sheep,[20,21] and also 4.8% in pigs and 4.0% in dogs[21] in Bangladesh. The clinical occurrence of brucellosis has not yet been reported from Bangladesh and it might be associated with the reported 435 (0.70%) aborted cases.[22] However, the *B. abortus* biovar 3 has been isolated from dairy cattle in Bangladesh.[23] Due to false-positive classical serological tests,[24] both sero-molecular techniques and bacteriological culture for the isolation and identification of the causative bacteria are required.[25] In addition to reproductive organs and mammary gland, Brucella infection affects most of the vital organs including heart, liver, kidneys and muscles leading to impairment of their normal function through alteration of their biochemical constituents depending on the stage of infection and damage.[26] During acute bacteremia stage, it destroys the macrophages and lymphoid cells that may cause alteration of hemato-biochemical

constituents. These haemato-biochemical changes in blood can be used as indicators for the severity and stages of infection and consequently help in their diagnosis.[26] Brucella is the intra-cellular pathogen usually remains dormant inside the phagosome vacuoles of the affected macrophages which are inaccessible to common antibiotics and antibodies. Accordingly, the antibiotics are effective against active bacteremia stage but not against intracellular carrier stage. Therefore, treatments of brucellosis have been tried by different workers against intracellular stage but none was found successful.[27-29] Therefore, an appropriate antibiotic therapy for animals and humans beings is still disputed and would be too expensive in most of the animal species.[30] Antibiotic therapy has been successfully used in clinical human brucellosis[31] but it has not been widely used to treat sub-clinical (intracellular bacteria) and clinical brucellosis in animals mainly due to high cost of treatment and low efficacy rate of antibiotics. A combination of long-acting oxytetracycline (OTC) and streptomycin (ST) eliminated *Brucella abortus* in 67 to 71.4% of infected cows.[26,32,33] The combined treatment has eliminated *Brucella ovis* from 11 of 12 (91.6%) experimentally infected treated rams but treatment did not resolve clinical epididymitis in two rams affected before treatment.[32] The OTC and ST are capable of penetrating the bacterial cell wall, inhibiting protein synthesis and providing long lasting concentration in plasma and hence considered most effective treatment of brucellosis.[34] This paper describes the efficacy of combined OTC and ST therapy and changes of haemato-biochemical constituents of *Brucella* infected dairy cross-bred cows.

MATERIALS AND METHODS

This study was conducted on 552 Holstein-Friesian crossbred dairy cows, aged between 4 to 8 years, reared under smallholder farmers in the districts of Dhaka (Savar) and Sirajgonj (Shahjatpur) and an intensive management system of Military Dairy Farm in the district of Jessore for the period from January 2018 to June 2019. These cows were vaccinated against Foot and mouth disease (FMD), Haemorrhagic septicaemia (HS) and Anthrax as per recommendation of the vaccine manufacturers but none was vaccinated against brucellosis. Cows were bred by artificial insemination. All the selected cows were apparently healthy and have good appetite and health.

Approximately 10 ml of blood samples were collected from each of all the selected 552 dairy cows by using disposable sterile plastic syringe. The collected blood samples with a syringe were kept in a rack for 3 hours than transfer to the refrigerator and kept overnight at 4 ^{0}C then sera were poured into an Eppendorf tubes and centrifuge 1500 rpm for 15 minutes. After centrifugation, clear sera were obtained and sera were transferred to a sterilized labeled Eppendorf tubes by using sterilized Pasteur pipettes and stored at -20 °C until use for rapid kit test and Rose Bengal test. Animals positive in these two tests (Rapid test kit and Rose Bengal test) were subjected to ELISA and molecular test (PCR).

Rapid test kit (RTK)

The sera of cattle, were subjected to antigen Rapid test kit i.e. *Brucella* antibody test kit (Senspert® Brucella Ab Test Kit, Korea) to detect the antibodies of *B. abortus.* The kit was kept in temperature within the range of 2-30 °C. The kit was used within 10 minutes after opening

from the wrap. The test was performed as recommendation of the manufacturer. The test kit was removed from the pouch and was placed on the horizontal surface. The serum was obtained using a dropper as a pipette and one drop (10 µl) was dispensed into the specimen well. When the fluid was completely absorbed into the specimen well and then 2 drops (80 µl) of buffer was added. Result was read within 5 to 10 minutes. The purple band should appear on the control line regardless of the test result. The presence of another band on the test line determines the result as positive (Photo 1).

Rose Bengal Test (RBT)

The RBT with the sera of cattle was performed according to the method described.[35] Briefly, sufficient antigen (Instituto de Salud Tropical Universidad, Edificio, CIMA, Avda, Pio XII, 55 E-31008, Pampalona, Spain), test sera, and positive and negative control sera for a day's testing were removed from refrigeration and brought to room temperature. Equal volumes (30 µl) of serum were mixed and rotated on a glass plate for 4 minutes. Any sign of agglutination is considered as positive case of brucellosis in the tested serum and graded to be positive with clear agglutination and negative if there was no sign of agglutinations (Photo 2).

Enzyme-Linked Immunosorbent Assay (ELISA)

Level of antibody was detected by Antibody Test Kit (IDEXX Montpellier SAS, France) according to the protocol of the manufacturer and reading was performed by automated ELISA reader.

Procedure of ELISA test

All reagents were equilibrated at room temperature and the coated plate were removed from the foil sachet and inserted into the strip holder. According to the manufacturer instructions, four micro-wells were required for control (two positive controls and two negative controls). 190µl of dilution buffer N.2 was dispensed into each well. 10 µl of undiluted positive and negative control solution were pipetted into the respective control wells. 10 µl of undiluted samples were dispensed into remaining wells and gently mixed after tapping. Then the plate was incubated for one hour at room temperature. Then each micro well was washed with wash solution for three times. 100 µl of conjugate was added to each well and sealed the plate following incubation for 30 minutes at room temperature and then washed with the wash solution for three times. 100 µl TMB substrate was added to each well and kept for 20 minutes at RT away from direct light. Finally 100 µl of stop solution was added to each well and OD value was read at 450 nm within 5 minutes.

DNA extraction

DNA was extracted from serum of cows positive in serological test according to the instruction of Purelink DNA extraction mini kit (USA).

Primer design

Primer was designed by using primer 3 Plus online software. To design the primer, the option IS711 gene was searched and the species *B. abortus* was chosen. The nucleotide sequences of

IS711 gene was copied as FASTA format from NCBI. The sequence was pasted on the site of the software called "source sequence below". The target region of the source sequence was selected for picking primers. The size of amplicon (602 BP), forward and reverse primers were selected automatically by the software.[36]

PCR

PCR was performed for each sample to amplify *B. abortus* specific IS711 repetitive genetic element (602 bp). The forward and reverse primers used for this study were 5'-GCTTGCCTTGATCTTTTTGG-3' and 5'-AATGCAGACAGGCCCTAATG-3' respectively. Each 50 µl reaction mixture contained 2 µl of genomic DNA, 25 µl of PCR master mix (Promega Corporation, Madison, WI, USA), 2 µl of 10-20 pmol of the forward and reverse primers, and the final volume was adjusted to 50 µl with 19 µl of nuclease-free water. DNA amplification involved pre-heat at 95°C for 2 minutes, followed by 32 cycles consisting of denaturation at 95 °C for 30 s, annealing at 48 °C for 30s and extension at 72 °C for 45s. The final extension period was 5 minutes at 72 °C with overnight holding at 4 °C. The PCR products were analyzed by 1% agarose gel electrophoresis (Alpha Imager, Wiesbaden Germany), with ethidium bromide staining and a gel documentation system (Alpha Imager) was used for photography.

Four cows which were positive at serological and molecular test subjected to blood collection for haematological, biochemical and ELISA test. Blood was collected to same way for serological test, 10 ml of blood was collected from jugular vein using disposable sterile syringe (12.0 ml), about 5 ml of blood immediately transferred to vacutainer tube and 5 ml vial to EDTA vial (Becton Dickson) and labeled the vacutainer tubes and maintained at 4 °C in refrigerator until they were processed. In laboratory, sera were separated by centrifugation at 1500 rpm for 15 min and stored in 1.5 ml Eppendorf tubes at -20 until tested.

Therapeutic evaluation

Cattle found positive in both serological and molecular tests were selected for therapeutic trial. Seven dairy cows, divided into two groups, control (Brucella negative) group (n = 3) and treated (Brucella positive) group (n = 4). The Brucella positive four Holstein-Friesian cross-cows had history of abortion and stillbirth (Photo 3 & 4) were selected for this treatment trial. Each of the four Brucella positive cows were treated with long acting oxytetracycline (Renamycin LA,® Renata Ltd., Bangladesh) @ 25 mg / kg BW at 72 hours interval for 16 IM injections and Streptomycin (Streptomycin sulfate,® Barcelona, Spain) @ 20 mg / kg BW IM at 24 fours interval daily for 10 days.

Antibody titer against *B. abortus* in treated cows was measured at day 0 (pre-treatment) and at day 30, 90 and 180 (post-treatment) using ELISA. The effectiveness of the treatment was evaluated on the basis of antibody titer and antigen detection with PCR.

Haematological studies

The haematological values especially Haemoglobin (Hb), Packed cell volume (PCV), Erythrocyte sedimentation rate (ESR), Total erythrocytes count (TEC), Total leukocytes count (TLC) and Differential leukocytes count (DLC) were carried out within 6 hours of blood

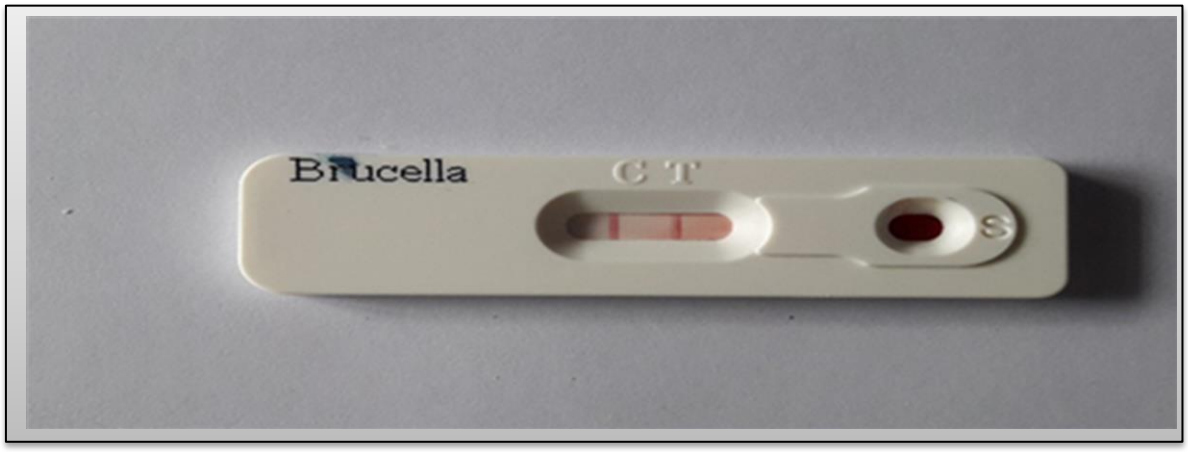


Photo 1.Rapid test kit results showing a purple control band and a Brucella positive band

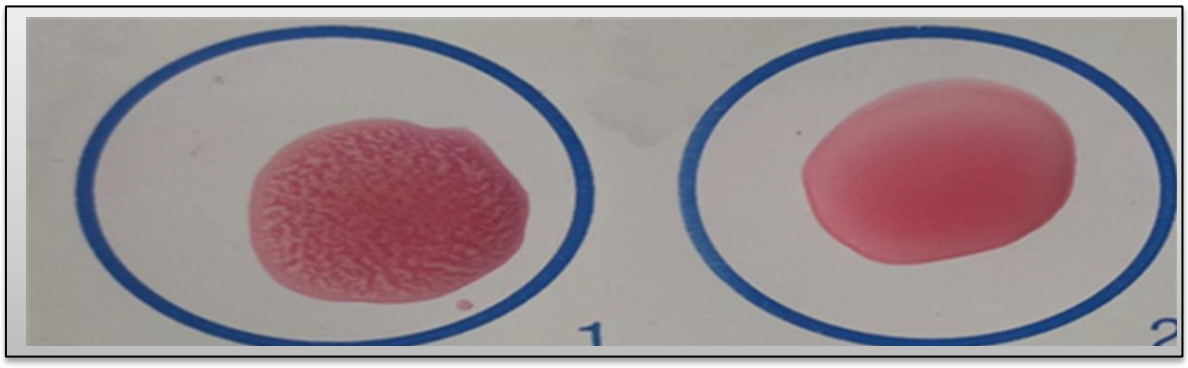


Photo 2. Rose Bengal test results showing a Brucella positive clear agglutinations (1) with a no sign of agglutination in negative sample (2)

Photo 3. A Holstein-Friesian crossbred cow with sero-molecularly positive to Brucella infection delivered a stillborn calf at her late pregnancy

Photo 4. A stillborn calf delivered by a sero-molecularly positive Holstein-Friesian cross-bred cow at her late pregnancy

collection by using ABX Penta-80 Automated Hematology Analyzers (Horiba Co. Ltd., Japan) at the Diagnostic Section, Sodesh Hospital, Mymensingh as described.[37]

Biochemical studies

The standard panel of liver biochemical test (Serum alanine aminotransferase (ALT), Aspartate aminotransferage (AST), Creatine kinase (CK), Glucose and Total serum protein) were determined by using the reagent kits (Merck Millipore Corporation, Darmstadt, Germany) in Microlab 300- Semi Auto Analyzer (ELI Tech Group., Puteaux, France) at the Diagnostic Section, Sodesh Hospital, Mymensingh, Bangladesh as decribed.[38]

Statistical analysis

The paired sample t-test was used for statistical analyses by using SPSS program version 22 computer program to find the significant different between Brucella negative and Brucella positive, pre-and post-treatment groups of haemato-biochemical parameters and antibody level detected by ELISA. P value < 0.05 was assumed for statistical significance.

RESULTS

The results on the sero-prevalence of brucellosis in dairy cows are presented in the Table 1. Out of the 552 tested samples, 18 (3.26%) were found positive in RTK, RBT and ELISA, whereas molecular test (Photo 5) showed 11 (1.99%) tested cows positive (Table 1). Serological result was available from 552 animals as the animals shown negative reaction with kit test, RBT and ELISA were considered as negative to brucellosis. Again, an animal was considered as positive if it became positive in all four tests (RKT and RBT, ELISA and PCR). Here, among the 552 samples 11 (1.99%) cows were shown positive reaction with all four tests. Out of 11 animals, 4 cows had history of abortion and stillbirth which was selected for therapeutic trials. Table 1 shows that the sub-clinical bovine brucellosis is distributed in all the three districts in both the smallholder and intensive military dairy farm in Bangladesh.

Table 1. Comparative evaluation of different serological and molecular methods for the detection of sero-prevalence of brucellosis in dairy cows

SN Districts	No. of cows tested	Rapid kit test No.+ ve	RBPT kit No. +ve	ELISA kit test No. +ve	PCR test No.+ve	True prevalence No. (%)
1. Jessore Military Dairy Farm	**133**	**4**	**4**	**4**	**2**	**2 (1.50)**
2. SHDF, Shahjadpur, Sirajgonj	254	9	9	9	5	5
3. SHDF, Savar, Dhaka	165	5	5	5	4	4
Sub-total	**419**	**14**	**14**	**14**	**9**	**9 (2.14)**
Total / Overall	552	18	18	18	11	11 (1.99)

SHDF = Smallholder Dairv Farms

Effects on haemato-biochemical constituents

Dairy cattle found Brucella positive with the help of sero-molecular tests were selected for the therapeutic trials. Treatment efficacies were evaluated on the basis of haemato-biochemical changes and antibodies levels at pre- and post-treatment values (Table 2 & 3).

Brucella-infected cows (pre-treatment) showed significantly ($p < 0.05$) lower values of TEC (6.47 ± 0.45; 7.69 ± 0.68), Hb (8.75 ± 0.87; 10.93 ± 1.56), TLC (3.90 ± 0.21; 7.85 ± 0.76) and neutrophils count (26.80 ± 0.89; 28.96 ± 0.67) in comparison to non-infected control cows (Table 2), whereas PCV (37.87 ± 1.0 ; 39.33 ± 1.83), ESR (00.6.0 ± 0.1; 00.75 ± 0.13), eosinophils (3.02 ± 0.21; 9.93 ± 0.21) and basophils (2.15 ± 0.42; 0.92 ± 0.11) values were higher in Brucella-infected than non-infected dairy cows (Table 2). Comparison of haematological values between pre-and post-treatment reveals that the TEC (6.47± 0.45; 6.99 ± 0.52), Hb (8.75 ± 0.87 ; 10.38 ± 1.02), PCV (39.33 ± 1.83; 40.83 ± 1.17) and neutrophils (26.80 ± 0.89 ; 27.20 ± 1.02) increased significantly ($p < 0.05$) at post-treatment, whereas eosinophils (4.93 ± 0.21; 3.93 ± 0.28) and basophils (2.15 ± 0.42; 1.50 ± 0.25) count decreased significantly ($p < 0.05$) at post treatment but the TLC (3.90 ± 0.21; 4.24 ± 0.28) and ESR (0.75 ± 0.13; 0.70 ± 0.08) values did not differ significantly ($p > 0.05$) between pre-and post-treatment values (Table 2).

Table 2. Comparison of haematological changes between chronic brucellosis affected and treated cows

SN Parameters	Brucella negative control cows Gr. A (n = 3)	Brucella positive cows		Gr. A and Gr. B		Gr. B and Gr. C	
		Pre-treatment Gr. B (n = 4)	Post-treatment Gr. C (n = 4)	t value	p value	t value	p value
1. TEC (10^6/µl)	07.69 ± 0.68	06.47 ± 0.45	06.99 ± 0.52	1.84	0.207	-5.1	0.0156*
2. Hb (g/dl)	10.93 ± 1.56	08.75 ± 0.87	10.38 ± 1.02	3.80	0.063	-3.65	0.036*
3. PCV (%)	37.87 ± 1.00	39.33 ± 1.83	40.83 ± 1.17	-1.12	0.378	-4.08	0.027*
4. ESR (mm/1st hr)	00.60 ± 0.10	00.75 ± 0.13	00.70 ± 0.08	-0.92	0.456	1.00	0.391
5. TLC (10^3/µl)	07.85 ± 0.76	03.90 ± 0.21	04.24 ± 0.28	13.08	0.006*	-2.76	0.070
a. Lymphocytes %	67.02 ± 1.76	66.12 ± 1.10	66.26 ± 0.96	2.43	0.136	-0.849	0.46
b. Neutrophils %	28.96 ± 0.67	26.80 ± 0 .89	27.20 ± 1.02	7.40	0.180	-3.7	0.034*
c. Eosinophils %	03.02 ± 0.21	04.93 ± 0.21	03.93 ± 0.28	-8.50	0.014*	-3.15	0.051
d. Basophils %	00.92 ± 0.11	02.15 ± 0.42	01.50 ± 0.25	-7.20	0.019*	3.50	0.039*

*Significant at ($p < 0.05$)

Hb = Haemoglobin TEC = Total Erythrocyte Counts PCV = Packed Cell Volume

TLC = Total Leukocyte Counts ESR = Erythrocyte Sedimentation Rate

The significantly ($p < 0.05$) higher values of serum glucose (77.8 ± 3.37; 72.35 ± 3.55), creatinine (1.82 ± 0.19; 0.96 ± 0.20), total serum protein (6.70 ± 0.37; 5.71 ± 0.37), ALT (23.01 ± 1.27; 31.60 ± 0.71) and AST (95.84 ± 4.36; 75.98 ± 1.24) were recorded in Brucella-infected than non-infected control dairy cows (Table 3). It appears that the glucose (77.8 ± 3.37; 71.50 ± 5.73) and AST (95.84 ± 4.36; 91.33 ± 4.45) values decreased significantly ($p < 0.05$) in antibiotic post-treated animals than pre-treatment respective values (Table 3).

Table 3. Comparison of biochemical changes between chronic brucellosis affected and treated cows

SN	Parameters	Brucella negative control cows Gr. A (n = 3)	Brucella positive cows		Gr. A and Gr. B		Gr. B and Gr. C	
			Pre-treatment Gr. B (n = 4)	Post-treatment Gr. C (n = 4)	t value	p value	t value	p value
1.	Glucose (mg/dl)	72.35 ± 3.55	77.8 ± 3.37	71.50 ± 5.73	-1.39	0.297	4.22	0.024*
2.	SCK (mg/dl)	00.96 ± 0.20	01.82 ± 0.19	01.79 ± 0.15	-7.71	0.016*	5.73	0.011*
3.	TSP (g/dl)	05.71 ± 0.37	06.70 ± 0.37	06.32 ± 0.37	-37.57	0.001*	3.16	0.051
4.	ALT (unit/L)	31.60 ± 0.71	23.01 ± 1.27	23.33 ± 1.14	39.46	0.001*	-4.35	0.022*
5.	AST (unit/L)	75.98 ± 1.24	95.84 ± 4.36	91.33 ± 4.45	-6.10	0.026*	1.45	0.244*

*Significant at ($p < 0.05$) SCK = Serum creatinine TSP = Total serum protein
ALT=Alanine transaminase AST=aspartate aminotransferase

Therapeutic efficacy

Out of four Brucella-infected dairy cows treated with antibiotics (oxytetracycline and streptomycin) injections for 48 days showed only one (25.0%) animal negative to Brucella-infection by using sero-molecular methods. These four treated animals received dual antibiotics therapy showed significantly ($p < 0.05$) increased level of Hb (10.38 ± 1.02) and PCV (40.83 ± 1.17) in comparison to pre-treatment Hb (8.75 ± 0.87) and PCV (39.33) values (Table 3). The biochemical constituents especially glucose and AST values decreased in comparison to pre-treatment values (Table 3).

The graph shows that the mean OD value (antibody titer) of the serum of cows affected with *Brucella* was 2.28 at 0 day of therapy and 1.39, 0.98, 1.17 at day 30 and 90 and 180 days respectively (**Fig. 1**). The OD value started to decline significantly at day 30 ($p < 0.004$) than from day 0 and decrease up to 90 days ($p < 0.0001$) then started to rise at day 180 insignificantly ($p < 0.210$). After complete the therapy (180 days) change of OD value (antibody titer) is statistically significant from the start of the therapy ($p < 0.003$).

Side-effects and cost of treatment

All the antibiotic treated dairy cows showed anorexia and swelling, painful sensation at the site of repeated LA-OTC injections, particularly in the thigh muscles but these lesions disappeared within 48 hours of each injection. However, this combined long-term antibiotic therapy caused only one animal negative from Brucella-infection by using sero-molecular tests. The local side effects of local swelling and pain at the injection sites could be minimized with a pain killer (Keto-A Vet® Acme Ltd., Bangladesh @ 3mg / kg BW IM) and antihistaminic (Astavet® Acme Ltd., Bangladesh @ 15 ml / animal IM) injections. The cost of treatment of the Brucella-infected cows was estimated as BDT 22,500/- per cow. Only one antibiotic treated cow was found negative to Brucella-infection after antibiotic therapy. This recovered cow showed regular oestrus cycle and artificial insemination was done by frozen semen (**Photo 6**). The pregnancy of the inseminated cow was confirmed by both rectal examination and ultrasonography (Photo 7) at day 60 post-insemination.

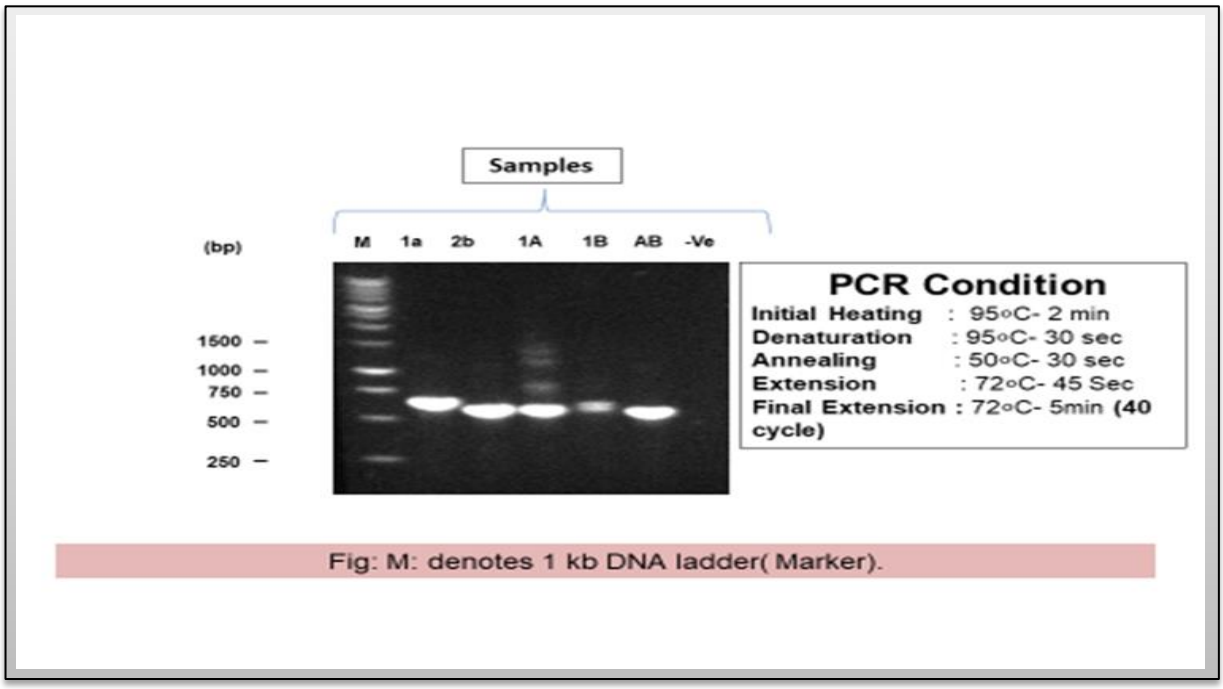


Photo 5. *Brucella abortus* species specific Polymerase chain reaction (PCR) products of samples collected from serologically positive cross-bred dairy cows

Photo 6. The Brucella-infected Holstein-Friesian cross cow became pregnant after antibiotic therapy

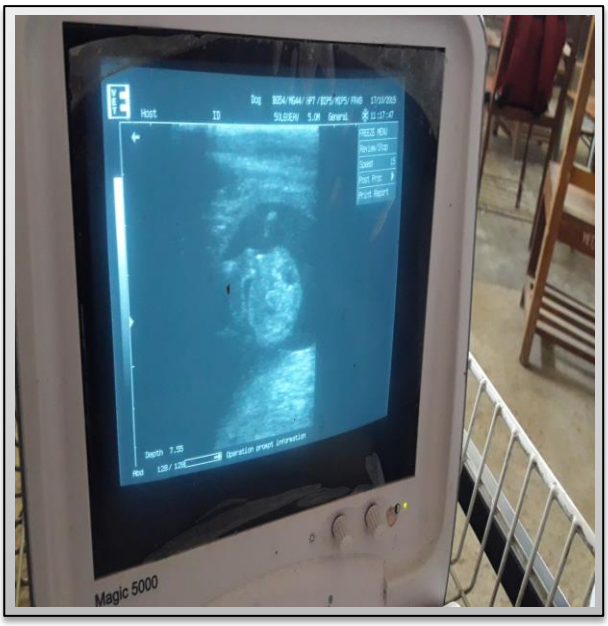
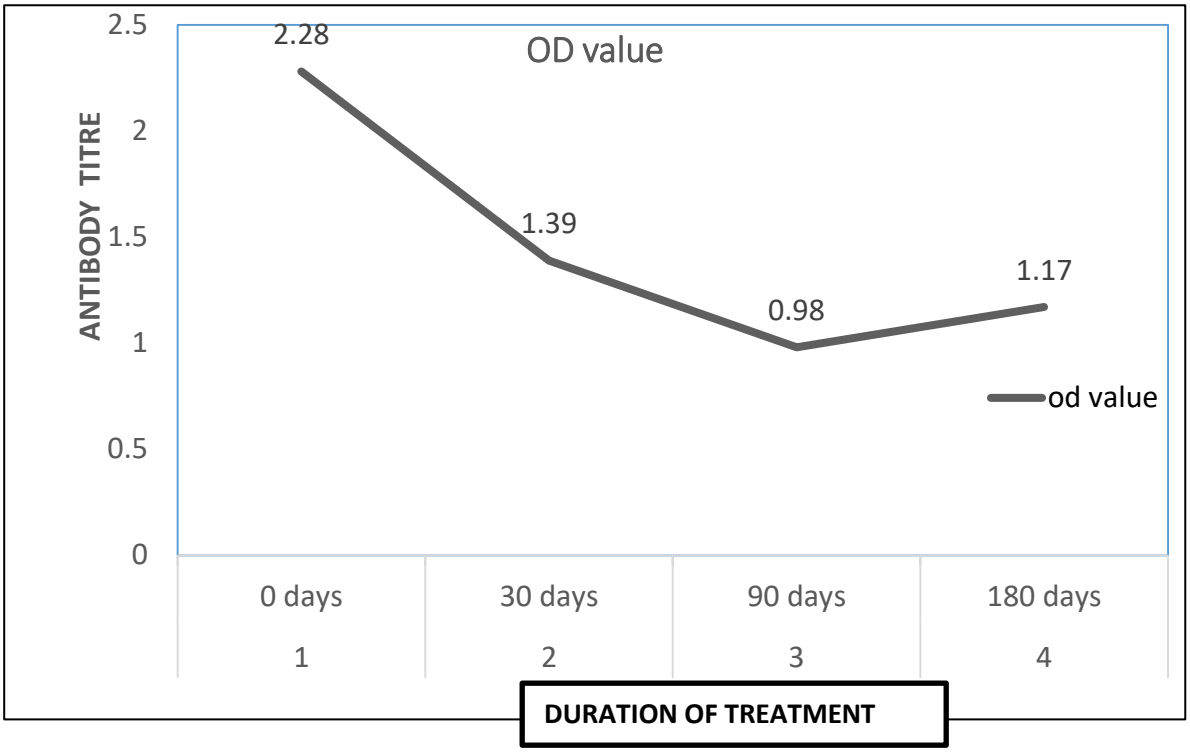


Photo 7. Ultrasonography detection of pregnancy in Brucella-treated Holstein-Friesian cross cow



The last antibiotic was injected on 48 days

Fig. 1. Changes of antibody titer (OD value) of antibiotic treated *Brucella* affected cows.

DISCUSSION

Brucellosis is one of the most economically important chronic zoonotic diseases affecting different species of food and companion animals including human worldwide.[29,39] This disease has been controlled and eradicated in some developed countries in their cattle herd but it is still widespread endemic disease in most of the developing countries including Bangladesh.[20,21] Bovine brucellosis has become a serious problem in Indian dairy cattle due to band of slaughtering cattle because of religion ground, social system and animal husband practices.[40] It has been estimated to be BDT 60 million economic losses due to brucellosis in 1983[41] and more recently it has been estimated as BDT 48.4364 million (US $ 0.61million) annually only from Mymensingh district in Bangladesh.[42] A large numbers of sero-prevalence studies on brucellosis in animals and humans have confirmed widespread prevalence of brucellosis in Bangladesh.[20,21] Endemic brucellosis in animals and humans in Bangladesh due to huge human population, poor sanitary conditions, inadequate management practices, unrestricted cattle importation through boarders, minimum attention on sero-survey and monitoring and control of chronic and zoonotic brucellosis. Lack of government policies resulted increased of brucellosis associated with abortion and stillbirth in dairy cattle. Analysis of the 50 years research findings on the prevalence of clinical diseases in cattle based on hospital records revealed that the clinical cases of brucellosis have not yet been reported from Bangladesh.[22] The occurrence of

clinical brucellosis might be only associated with the abortion and stillbirth at late gestation and such aborted cases are not usually reported at the veterinary hospitals and usually go undiagnosed. However, treatment of these aborted cases are not usually practiced due to absence of clinical signs, moreover the attention of treatment of the sub-clinical cases are not given due to intracellular pathogen, treatment failure and relapse rates are high, depend on the drug combinations and high cost of treatment.[40] Cattle are usually kept in close association with small ruminants and infection can also be caused by *B. melitensis*. Bovine brucellosis, being zoonotic remains a serious obstacle in public health, social and economic progress, food security and food safety in developing countries, where appropriate preventive and control measures are not in place.[40] Expansion of animal industries and urbanization and lack of hygienic measures in animal husbandry and in food handling partly account for brucellosis remains a public health hazard.

Limited research reports are available on therapeutic efficacy trials and effects on haemato-biochemical constituents in Brucella-affected animals.[34] This might be due to occurrence of mostly sub-clinical disease and occasionally associated with abortion, stillbirth and infertility in animals. This study recorded lower values of TEC (6.47 ± 0.45), TLC (3.90 ± 0.321), Hb (8.75 ± 0.87) and neutrophils (26.80 ± 0.89) in Brucella-infected cows in comparison to non-infected healthy cows. These findings support the lower values of Hb, TEC, TLC and neutrophils counts reported in Brucella-infected cows in India.[34] But the comparatively higher values of PCV (39.33 ± 1.83), eosinophils (4.93 ± 0.21) and basophils (2.15 ± 0.42) counts recorded in this study contradict the lower values of eoinophils reported.[34] However, within reference value, decreased or even increased values of Hb in Brucella-affected cattle have been reported.[43]
A significant decreased of TEC, Hb and PCV values while significantly increased value of TLC has been reported in Brucella-affected cattle.[44] The Hb, PCV, TEC, TLC, lymphocytes and basophil values reported within the range of reference values in Brucella-infected cattle.[43] However, the marked reduction of TEC in Brucella-infected cows could be attributed to the reduction of RBC as a result of inadequate production of erythropoietin hormone.

The higher values of serum glucose, serum creatinine, total serum protein (TSP) and AST were recorded in Brucella-infected cows than non-infected cows in this study. Lower value was recorded only with ALT in Brucella-affected cows in comparison to non-infected cows. These findings could be compared with the higher values of glucose, TSP and creatinine values and lower values of AST and ALT than the reference values (Kushwaha et al., 2014). However, significantly ($p < 0.05$) increased values of AST and ALT and non-significantly variation of creatinine in Brucella-infected cattle.[45]

The effect of the antibiotic treatment on the murine antibody response to Brucella antigens have been reported with infection and treatment methods.[24,46] They have reported that early antibiotic therapy affects the antibody response to cytoplasmic proteins of Brucella in mice.

Infections with intracellular bacterial pathogens are difficult to treat due to the inability of conventional antimicrobial agents to penetrate, accumulate or be retained in the mammalian cells.[47] Some pathogenic bacteria are facultative intracellular e.g. *Mycobacterium* spp., *Listeria monocytogenes*, *Salmonella* spp. and *Brucella* spp. going through an intracellular phase during infectious cycle without being strictly dependent on the cellular medium, while others are

obligate intracellular pathogens (*Chlamydia* spp. and *Rickettsia* spp.) which do not survive in the extra-cellular medium of the host.[47]

Brucella organisms enter into macrophages through interaction between bacterial lipopolysaccharide (LPS) and membrane of the microphages to form a phagosome that is a membrane bound protein (vacuole). The bacteria resist to respiratory burst metabolites of the phagosome and acidic pH, secrete cyclic beta-1,2 glucans proteins and inserts in the outer side of phagosome membrane thus inhibiting fusion with lysosomes with phagosome. Lack of phagosome-lysosomal fusion (usually kill and digest the pathogens) provides a safe haven for bacteria replication within phagosome.[39] After release from macrophages, the bacteria disseminate within host body through lymphatic system and blood circulation (bacteremia) and localize in the predilection sites of placenta and fetus of pregnant cattle associated with abortion and stillbirth of calves. Abortion of dairy cattle is commonly defined as a loss of fetus between the age of 42 and 260 days, and pregnancy lost before 42 days are usually referred to as early embryonic death, whereas a calf that is born dead between 260 days and full term is defined a stillbirth.[48] The life span of mammalian macrophages is more than 90 days[49] and at the end of life span, routine programed death of the affected macrophages and bacteria may be released out and cause bacteremia.

The ELISA antibody OD values in Brucella positive cows treated with oxytetracycline and streptomycin at pre-treatment (0 days) was 2.28 and antibodies response to the administration of antibiotics decreased the OD to 1.39 at 30 days and 0.98 at 90 days and then increased the antibody ELISA OD to 1.17 at 180 days due to ceased of antibiotic injections on 48 days. This indicates that the intracellular Brucella might be sensitive to used antibiotics against the continuous release of Brucella organism from the program death of the macrophages. It also might be the reason to increase antibodies level when stop the antibiotic injections to the affected animals. Therefore, the antibiotic therapy against brucellosis may not inactivate the bacteria hiding in the macrophages but may be effective against bacteremia stage. Moreover, the chemotherapy over a long period of time is tiring, uneconomical, stressful and a public health concern due to the antibiotic residues in milk.[50]

Currently, there is great challenge in the development of effective antibacterial agents against intracellular pathogens especially Mycobacterium, Salmonella, Brucella, Listeria, Shigella and methicillin-resistant *Staphylococcus aureus* (MRSA) that inhibit inside the phagocytic macrophages. Within these intracellular safe havens the bacteria reproduce and form a repository, often causing chronic infections. Infected patients become life-long carriers of the pathogens and chronically suffer from the infection or die from invasive forms of the pathogens. Most of the antibiotics like aminoglycosides, glycopeptides and macrolides do not effectively accumulate within infected macrophages and the therapeutic values are limited in these intracellular bacteria.[51] The cationic amphiphilic polyproline helices (CAPHs) is a unique class of cell penetrating peptides that have intrinsic antimicrobial activity have been shown to effectively target intracellular bacteria as broad spectrum antibiotics.[52]

Brucella organisms are facultative intracellular pathogens localize within the mononuclear phagocytic cells of the lymph nodes, liver, spleen and bone marrow. A variety of antimicrobial drugs have activity against Brucella but the results of *in vitro* susceptibility tests do not always

correlate with clinical efficacy. This might be due to intra-cellular location of the organism which provides some protection against host defenses and antimicrobial drug effects. Several reports have been published on the therapeutic trials against Brucella infections in animals and humans but none was reported entirely successful.[29,53] The Bruceella pathogen is protected from antibiotics as well as antibodies since it remains active within phagosome vacuoles inside macrophages (phagocytic cells) of the reticulo-endothelium system. Therefore, the antibiotics those have permeability into the phagocytic cells where this bacterium is protected and multiplied in the infected hosts are required for treatment of brucellosis.

The therapy of brucellosis remains an unsolved problem both in medical and veterinary medical mainly due to relapse of treated cases and failure of mono-therapy. Recently, the treatment of brucellosis involves the use of two or more antibiotics to avoid relapses and to prevent prolonged use of these drugs. The selection of successful antimicrobial treatment of brucellosis is the use of antibiotics that penetrate into macrophages and thus active against the pathogen, active in the acidic environment of the macrophages infected with Brucella species, use of combination of antibiotics and evaluation of duration of treatment.[54]

When Brucella organisms could no longer be recovered from udder secretions or from any of the selected tissue specimens collected from treated animals, the respective cows were considered to be cured (successful treatment). Continued or resumed shedding of Brucella organisms in udder secretions or the isolation of Brucella organisms from any of the tissue specimens obtained are considered as conclusive evidence of treatment failure.[40] Use of broad-spectrum antibiotics such as aureomycin, terramycin, tetracyclines and streptomycin (ST), singly or in combination has resulted in the reduction of abortions in infected cows. However, cost of therapy, presence of antibiotic residue in milk and failure to cure udder infections in many cases led to general conclusion that such treatment may not be suitable for the control of bovine brucellosis, especially in countries where cow slaughter is not prohibited.

Oxytetracycline and streptomycin are capable of penetrating the bacterial cell wall, inhibiting protein synthesis and providing long lasting concentrations in the plasma and hence considered most effective in the treatment of brucellosis.[55] Combination of streptomycin and oxytetracycline has demonstrated synergistic effect in vitro.[56] Combination of oxytetyracycline and streptomycin injections reported better results than the drugs used alone.[34] However, they did not cure infection completely but reduced the antibody levels in treated cows recorded in this study are in conformity of the earlier reports.[34,40] The increased of antibody level at the 180 days of post-treatment in this study indicates that the combination of oxytetracycline and streptomycin did not eliminate the intra-cellular Brucella bacteria from treated animals. Therefore, shedding of Brucella organisms from treated animals should be monitored for longer duration, not only by PCR but also by bacteriological isolation and identification. However, the relapse of infection or abortion in subsequently pregnancy should also be monitored to validate the results of the treatment trials.[57,58]

Considering these facts, long acting oxytetracycline and streptomycin were selected for therapeutic trials in sero-molecular Brucella positive dairy cows. This antibiotic trial results showed that only one (25%) cow was found negative after the end of therapy with sero-molecular tests and this cow returned to normal breeding cycle and finally conceived with AI.

But other three (75%) treated cows remained positive with sero-molecular tests and even the antibodies titer increased on ceased of antibiotic therapy at the 180 days of post-treatment. These findings support the earlier Indian report[40] in which they have provided two therapeutic schedules A (Streptomycin + Isoniazide + Rifampicin + LA OCT) and B (Streptomycin + Rifampicin + Enrofloxacin) showed reduction in titers of anti-Brucella antibodies in both the treatment schedules. Of the 27 cows treated with two therapeutic schedules, 12 (44.44%) became pregnant, of which 10 (83.33%) had normal calving. They have concluded that the naturally infected cows the two schedules of treatment though costly proved effective for therapeutic management of Brucella infection.

The significantly increases of the liver enzyme (AST) in Brucella infected cows in comparison with the values of healthy cows indicates damage of the liver, which supports the earlier reports.[25,33,59] However, this study recorded lower level of ALT which contradicts the earlier reports. The insignificantly higher level of CK in Brucella-infected cows (1.82 ± 0.19) in comparison with healthy cows (0.96 ± 0.20) is in conformity with earlier reports.[33] The insignificantly increased total serum protein concentration in Brucella-infected cows (6.70 ± 0.37) in comparison to healthy cows (5.71 ± 0.37) was recorded. Chronic or sub-acute bacterial infections can cause increases in globulin fractions, particularly the γ-globulins resulting from production of different immunoglobulins by plasma cells in response to chronic antigenic stimulation.[33]

CONCLUSIONS

Bangladesh is an endemic country for brucellosis and therefore periodic use of sero-prevalence studies on susceptible animals would help for early diagnosis of Brucella infection that would be required for the control and eradication program of brucellosis. Haemato-biochemical studies would help to identify the effects of brucellosis on animal health and especially estimation of liver enzymes can determine the extent of hepatic damage. However, the haemato-biochemical alterations recorded in this study cannot be considered as indicator of Brucella infection because these alterations are also evidenced in other bacterial infections. Therefore, the haemato-biochemical alterations and sero-molecular tests should be carefully interpreted for final decision. The efficacy of combined long acting oxytetracycline and streptomycin against sub-clinical Brucella infection showed some encouraging result in crossbred dairy cows but the antibodies titer increased on with-drawl of drugs at 180 days of post-treatment that indicates relapsed. Commonly used anti-bacterial showed poor cellular uptake and often have limited access to intracellular targets resulting in low anti-bacterial activity against intracellular pathogens. It may be concluded that the cell-penetrating peptides (CPPs) showed great potential as delivery vehicles for anti-bacterial agents and may contribute to the generation of new therapeutic tools to treat infectious diseases cause by intracellular pathogens. Therefore, the interdisciplinary 'One Health' nature of the effects that brucellosis has indicate for collaboration of veterinary medical, medical, public health and other related experts for the control and eradication of brucellosis both in animals and humans.

ETHICAL APPROVAL

All animal-related procedures and methods were carried out in accordance with the Animal Ethical Committee of the University.

CONFLICT OF INTEREST

No conflict of interest to declare

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