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Brucella abortus Killed Vaccine: The Achievement of 52 Years (1967-2019) in Bangladesh

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COLUMN ARTICLE

Brucellosis is a zoonotic disease with economic impacts attributable to human, livestock and wildlife disease. In animals, brucellosis mainly affects reproduction and fertility, with abortion and reduced milk yield. In man, the clinical picture resembles many other febrile diseases, but sacroiliitis and hepato-splenomegaly are the most prominent [1]. Brucellosis was first investigated in 1967 [2] in Bangladesh (former East Pakistan). The overall animal-level sero prevalence of bovine brucellosis in Bangladesh varied from 2.4% - 18.4% [3,4]. Variable prevalence of bovine brucellosis in the subsistence and commercial management systems in Bangladesh were also reported which ranged from 0.6% and 20.4% [5-7]. Brucellosis in humans and animals in Bangladesh were reported to be caused by *Brucella abortus* [8-10]. Pharo., *et al.* [11] for the first time in Bangladesh described the isolation of *Brucella abortus* from two cows both of which were MRT and RBT positive. In the same year, Rahman and Rahman [12] claimed to isolate *Brucella* spp. from MRT positive milk in sub-clinical mastitic udder. Unfortunately, the detail procedure to validate the isolates as *Brucella* spp. is missing in these papers. Moreover, these isolates were not preserved in any laboratory in Bangladesh for further analysis and some author reported *Brucella abortus* DNA in humans and animals sera in Bangladesh [4,8].

Only one study recently described the isolation of *Brucella abortus* biovar 3 from placenta of an aborted cow [13]. More studies on the isolation and characterization of *Brucella* species from aborted cows is required to understand the species and biovar diversity. The knowledge of available *Brucella* species and their biovars are essential in disease control decisions. As a zoonosis, the control of animal brucellosis will lead to the control of human brucellosis [10,14-17]. To control bovine brucellosis two types of vaccines are available. In bovines, the most successful vaccine (S19) is only used in calves, as adult vaccination results in orchitis in male, prolonged infection, and possible abortion complications in pregnant female cattle [18]. S19 vaccine also creates problem in serological diagnosis of brucellosis. Another vaccine produced from *Brucella abortus* strain RB51 (SRB51), does not interfere serological diagnosis of brucellosis but may cause abortion when administered in pregnant animal [19]. On the other hand the heat killed vaccine produces less immunity but there is no adverse effect. Vaccination against brucellosis in Bangladesh has not yet been initiated. Brucellosis is endemic in Bangladesh [10] and the prevalence varies greatly in different management systems. In low prevalence subsistence management systems, vaccination is not recommended due to very low level of prevalence [7]. However, in intensive management system the prevalence of

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bovine brucellosis is reported to be higher and vaccination may be initiated. Before importing live vaccine which have some adverse effects locally prepared vaccine can be tested for its immune response. Therefore, the objective of this communication was to isolate *Brucella* spp. from placentas of aborted dairy cows and to evaluate the immune response in the guinea pig with the killed vaccine prepared from the local isolate.

The study area was the Government owned Central Cattle Breeding and Dairy Farm (CCBDF) in Savar, located in the Dhaka district of Bangladesh. The aborted foetal membranes were collected from Central Cattle Breeding and Dairy Farm (CCBDF). Foetal membranes were collected with the permission of the authorities from late aborted cases (between the 5^{th} and 8^{th} months of gestation) [20]. A total of 4 foetal membranes were collected aseptically in a sterile plastic bag. The samples were frozen for 24 hours before transfer. Next day, it was transferred from Savar to Mymensingh maintaining frozen condition within ice box by the use of gel ice pack. The frozen samples were thawed in room temperature. After washing with normal saline the placentas were cut into small pieces to expose the cutting surface. The cut surface was pressed over a clean glass slide to create impression smear of the foetal membrane. Then the smear was dried and fixed over a flame. The dried smear was stained by modified Ziehl-Neelsen staining method [21]. *Brucella* selective media was prepared as per the instruction of manufacturer (HIMEDIA, M348-500G) and the stain positive aborted fetal membranes inoculums were prepared by washing the membranes properly and cut into small pieces (about 2 gm) and grinded with PBS by using mortar and pestle. Ten ml of liquid was collected by using sterile syringe. Prepared inoculum was separately streaked into the petridishes after 24 hours of incubation at 37°C. Inoculated plates were placed in incubator in inverted position at 37°C for 3 - 5 days under the supply of 5% CO_2. The petridishes were examined every 24 hours after passing the initial 48 hours of incubation. Then the resultant colonies were sub-cultured maintaining the same procedure. One drop of PBS was placed in a sterile glass slide and thoroughly mixed with a loopful of *Brucella* colony. Then the slide was placed under the phase contrast microscope and the slide was examined for the motility of the *Brucella* organism at 40×10 X object.

One drop of *Brucella abortus* specific serum was taken in a sterile glass slide and a loopful of bacterial colony were thoroughly mixed with the *Brucella abortus* specific serum and the slide was examined up to 4 minutes for agglutination [21]. BD oxidase reagent were used for the identification of Gram negative criteria of *Brucella* spp. Oxidase reagent contain 0.5 ml of a 1% aqueous solution of N, N, N', N'-tetra methyl-p-phenylenediaminedihydrochloride. The test is based on the production of an enzyme called indophenol oxidase which oxidizes the dye present in the reagent which results in a color change of yellow to dark-purple compound [22]. A filter paper was soaked with the reagent and loopful of bacterial colony was streaked onto the soaked area of the filter paper. Catalase reagent contain a 3% solution of hydrogen peroxide. The enzyme catalase decomposes hydrogen peroxide into water and oxygen. Organisms that possess catalase demonstrate the positive reaction by the rapid appearance of gas bubbles. Two drops of catalase reagent were placed in glass slide and a loopful colony was added. Then the mixture was examined immediately for the rapid production of gas bubbles.

Brucella selective broth were also prepared as per the instruction of the manufacturer (HIMEDIA, M348-500G). Some of the colony from *Brucella* selective agar media were transferred in the *Brucella* selective broth with a sterile metallic inoculation loop and incubated at 37°C for 3 - 5 days under the supply of 5% CO_2. The capped bottles containing broth were examined every 24 hours after 48 hours of incubation. Fifty milligram of colonies from the *Brucella* selective culture media and 2 ml of cultured broth were used for the extraction of DNA. DNA was extracted by using Wizard Genomic DNA Purification Kit (Promega, USA) according to manufacturer's instruction. Primer for PCR was designed by using primer3Plus online software. To design the primer, the option IS711 gene was searched and the species *Brucella 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 [23]. The conditions of PCR and analysis of PCR products were performed as per the method of Yeasmin [24].

Ten test tubes were taken containing 9 ml PBS. One ml of the cultured broth was transferred to the 1st test tube and then mixed thoroughly by the use of sterile stirrer. After that 1 ml solution was taken and transferred respectively to next test tubes to make tenfold serial dilution. One ml inoculum was taken by sterile micropipette from the 10^{-6} dilution and spread to the *Brucella* selective agar media thoroughly by using a sterile spreader. Similarly, three plates were prepared from 10^{-8} dilution. Then six plates were inverted and incubated at 37°C for 3 - 5 days under the supply of 5% CO_2 and regularly examined after 48 hours at 24 hours interval. Colonies that grew on the surface of the medium generally were small in size, transparent or honey color with smooth round surface was carefully counted and average was done from the three plates of the same dilution. Between 30 and 300 colonies were counted on a single plate [25]. Each colony represented a "colony forming unit" (CFU). Centrifugation of 200 ml of the cultured broth were done three times at 10,000 rpm for 10 mins and the supernatant were discarded. Then bacterial pellet were washed every time after centrifugation with PBS. The bacterial pellet was mixed with required amount of PBS to obtain 40×10^9 cfu organisms in 2 ml dose for guinea pig inoculation [21]. The bacterial pellet was mixed thoroughly with PBS by the use of vortex machine every time after centrifugation [21]. Organisms were killed by heating the suspension at 80°C for 90 minutes in hot water bath for producing heat killed vaccine [21].

The guinea pigs were collected from ICDDRB (International Center for Diarrheal Disease Research, Bangladesh), Dhaka. The animals were quarantined for 10 days. The selected guinea pigs were screened for brucellosis by Rose Bengal Test (RBT) and Rapid Antibody Kit Test and four negative guinea pigs were selected for inoculation. Sufficient nutritional feed and clean water were supplied and the guinea pigs were kept in a hygienic condition. Two ml of inoculum (4×10^{10} cfu) of heat killed bacterial suspension was injected to each of four guinea pigs subcutaneously [21]. The guinea pigs were observed for nine weeks. Blood was collected from the guinea pigs at 1st, 2nd, 4th, 6th and 9th week after inoculation of heat inactivated *Brucella* organism. The guinea pigs were restrained properly, the injection site was disinfected with 70% alcohol and 1.5 ml of blood was collected from each of the guinea pig directly from the heart. The collected blood was kept undisturbed in syringe in a slightly inclined position on a tray for 1 hour to facilitate clotting and separation of serum. The separated serum was taken in a tube and then centrifuged at 2500 rpm for 10 minutes and the sera were transferred to the sterile and labeled eppendorf tube. The sera samples were stored at -20°C for future use.

The RBT was performed to determine the reciprocal antibody titer based on the procedure described by Baek., *et al* [26]. Briefly 30 µl of antigen was placed on a fine plastic plate circled approximately 1.5 cm in diameter and two fold dilutions of 30 µl of tested serum were done (1:5, 1:10, 1:20, 1:40, 1:80 respectively up to the dilution where agglutination stop) with the use of PBS and were put beside each of the antigen respectively up to the disappearing of the agglutination. The antigen and serum was mixed on the plate with a stirrer and rotated for 4 mins. Indirect ELISA was performed as per the method of Yeasmin [24]. Briefly, microplates are coated with *Brucella* Lipopolysaccharide (LPS). Samples to be tested are incubated in the wells. Upon incubation of the test samples in the coated wells, *Brucella* specific antibodies form immune complexes with *Brucella* LPS. After the washing away unbound material, an anti-ruminant antibody enzyme conjugate is added which binds to any immune-complex *Brucella* LPS antibody. Unbound conjugate is washed away and enzyme substrate (TMB) is added. In the presence of the enzyme, the substrate is oxidized and develops a blue compound becoming yellow after blocking. Subsequent color development is directly related to the amount of antibody to *Brucella* present in the test sample. The result is obtained by comparing the sample optical density with the positive control mean optical density. OD values were read at 450 nm within 5 minutes.

Numerous pink colored *Brucella* like organism with blue background were observed under microscope. Small, translucent, honey colored, round, and convex colonies

were seen in the media with smooth margins (Figure 1). Brownian movement was found under phase contrast microscope at 40×10X object. Agglutination was found within one minute of mixing of bacterial colony with *Brucella abortus* specific serum (Figure 2). The bacterial isolate was oxidase positive as indicated by the change of color (violet) immediately (Figure 3). The bacterial isolate was catalase positive as indicated by bubble formation immediately in the slide (Figure 4). Extracted DNA from the colonies and broth (represents foetal membrane samples) gave positive result in PCR (Figure 5). Turbidity developed within 4 days into the broth which was the indicator of growth of *Brucella* spp. into the selective broth. The graph showing that the reciprocal antibody titer was 0 at the 0^{th} of inoculation of heat killed *Brucella abortus* vaccine and start to significantly ($p < 0.01$) rise from the 2^{nd} week and reach a peak level 4^{th} week and then start to significantly ($p < 0.01$) decline upto 9^{th} week and at 9^{th} week antibody level was similar to the 0^{th} week of inoculation (Figure 6). The graph showing that the OD value of the serum of guinea pig after inoculation of heat killed *Brucella abortus* vaccine was 0.0945 at 0^{th} week and 0.1025 at 1^{st} week which was near about the negative control OD 0.106. After that, the OD value started to rise significantly ($p < 0.01$) from the 2^{nd} week (OD 0.2287) and reached to a peak level at 4^{th} week (OD 0.2842) and then started to decline significantly ($p < 0.01$) from 6^{th} week (OD 0.1832) to 9^{th} week (OD 0.1015). The OD value of 9^{th} week post vaccination was also near about the negative control OD 0.106 (Figure 7).

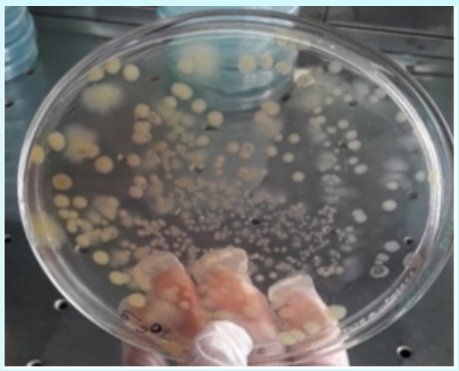

Figure 1: Small, translucent, round colonies grew in the Brucella selective media.

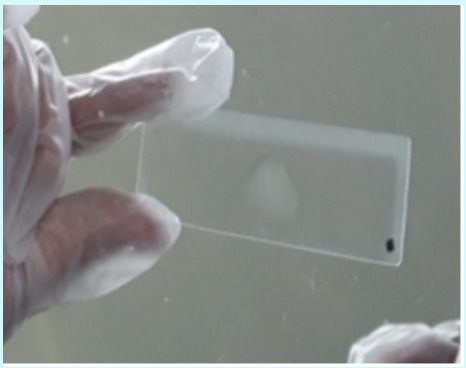

Figure 2: Agglutination in the Brucella abortus specific serum agglutination test.

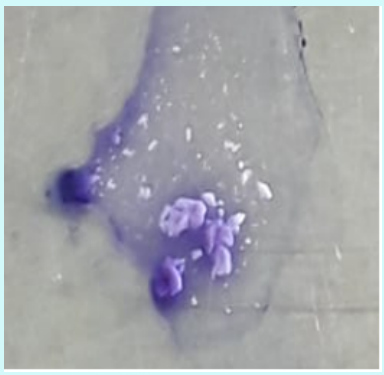

Figure 3: Color change of the colonies indicates oxidase positive reaction.

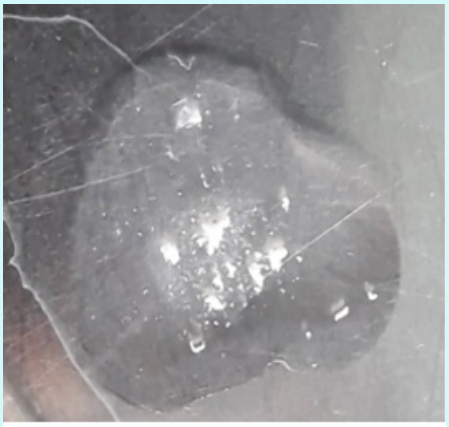

Figure 4: Bubble formation indicates catalase positive reaction.

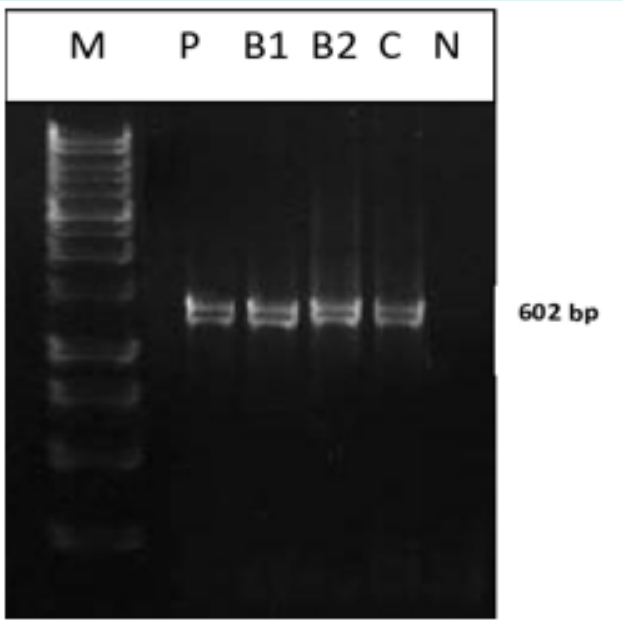

Figure 5: Gel electrophoresis image of amplicons produced from: IS711 PCR assay (602 bp) from colonies of culture media and broth. Lane M for molecular marker, lane P for positive control, lane B1-B2 for broth, lane C for colonies and lane N for negative control.

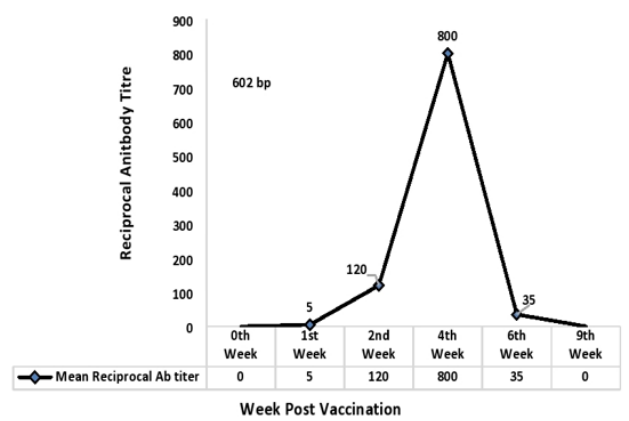

Figure 6: A graph showing the reciprocal antibody titer of guinea pig serum by RBT after inoculation of heat killed vaccine of Brucella abortus.

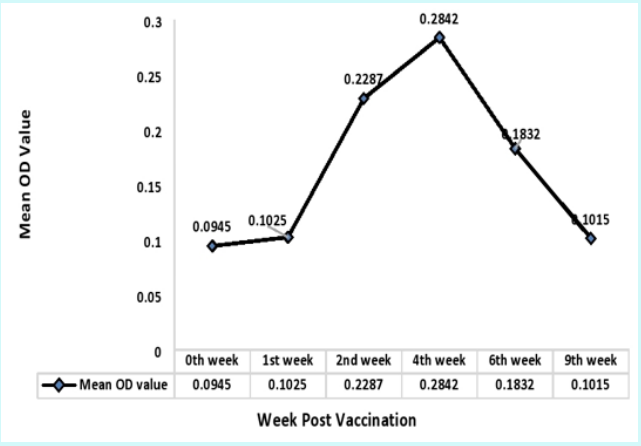

Figure 7: A graph showing the OD value of ELISA of guinea pig serum after inoculation of heat killed vaccine of Brucella abortus.

The study was conducted to isolate *Brucella abortus* as an etiology of abortion in dairy cows at CCBDF maintaining the standard cultural method for *Brucella* spp. For a confirmatory diagnosis of brucellosis, isolation of *Brucella* bacteria is the best method which is also considered as a "gold standard" test [21]. The likelihood of obtaining a positive culture from aborted foetal material is high but *Brucella abortus* does not grow readily in presence of competing microflora [27] which can be solved by using *Brucella* selective media with the addition of selective supplements [21]. On the selective media the culture positive samples produced smooth, small, translucent, dewdrop like round and convex colonies which were characteristics of the genus *Brucella* [28]. *Brucella* is a coccobacillus and non-spore forming bacterium [29]. To identify this character most popular method of staining is modified Ziehl Neelsen staining method. According to Baily., *et al.* (1992), *Brucella* is a non-motile organism and which is identified observing Brownian movement in the phase contrast microscope [30]. In this study, colonies grown on *Brucella* agar were examined by Modified Ziehl Neelsen staining and revealed that the organism was *Brucella* spp. since there were aggregates of red color coccobacilli in the microscopic field. Brownian movement was observed under phase contrast microscope at 40×10 X object. Culture positive isolates were tested positive in

catalase and oxidase test [31]. According to Alton., *et al.* [21], *Brucella abortus* colony shows agglutination when mixed with the *Brucella abortus* specific serum. The isolate of this study also showed agglutination with *Brucella abortus* specific serum. Moreover, the resultant colonies were tested by PCR for further confirmation. *Brucella abortus* specific IS711 primer was used and tested samples were positive for *Brucella abortus* DNA. For the detection of *Brucella* spp. both conventional and real time-PCR can be used [32,33]. Conventional PCR assay is a sensitive, specific and relatively inexpensive method for detecting *Brucella* DNA. However, real time PCR is also sensitive, specific, faster but more expensive than conventional PCR [33]. The protective immunity from the heat killed isolate of *Brucella abortus* can be measured by reciprocal antibody titer and ELISA [26,34]. The antibody level in case of ELISA started to rise significantly ($p < 0.01$) from the 2nd week (OD value 0.2287) and reach a peak level at 4th week (OD value 0.2842) and then started to decline significantly ($p < 0.01$) up to 9th week (OD value 0.1015). Similarly in case reciprocal antibody titer, started to significantly ($p < 0.01$) rise from the 2nd week (1:120) and reach to a peak level at 4th week (1:800) and then started to significantly ($p < 0.01$) decline up to 9th week (1:35). There is no significant variation of the OD values in ELISA and reciprocal antibody titer by RBT within the 0thand 9th week of post vaccination and also with the negative control. It is reported that the heat killed vaccine prepared from *Brucella abortus* smooth strain 544 with adjuvant (water-in-oil emulsion) shows 230-fold more protective immunity in guinea-pigs than the same without the adjuvant [35]. As, in this study there was a significant rise in the antibody level, so use of adjuvant with this heat killed vaccine may produce satisfactory rise of antibody level and may also give a protection against brucellosis for longer duration.

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