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ORIGINAL ARTICLE

Identification of Brucella spp. in Aborted Fetuses by Guineapig inoculation

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Abstract

Background: Brucellosis is a zoonotic disease which is endemic in Bangladesh. The prevalence of bovine brucellosis in subsistence management system in Bangladesh is low. However, the prevalence of bovine brucellosis at Central Cattle Breeding and Dairy Farm (CCBDF) is reported to be very high and Brucella abortus DNA has also been detected from cows' milk of this farm. The principal manifestation of bovine brucellosis is abortion in pregnant cows, which is common at CCBDF. The role of brucellosis in abortion at CCBDF has not been ascertained. Hence, this study was undertaken to confirm Brucella spp. as the etiology of abortion in cows at CCBDF.

Materials and Methods: Aborted fetal membranes and vaginal swabs from 3 cows, in which late abortion occurred, were collected aseptically from the CCBDF. The samples were initially stained with modified Zeihl-Neelsen staining method. The stain-positive samples were ground individually using a pestle and mortar, and a homogenized mixture was prepared by adding normal saline. Two milliliters of the homogenate from each sample were inoculated subcutaneously into a guinea pig. The sera of guinea pigs were collected after 3rd week of inoculation to perform rose Bengal test (RBT) and rapid antibody test (RAT).

Result: Samples from two cows showed positive staining result in which numerous pink-colored coccobacilli were seen. All the sera collected were tested positive for both RBT and RAT. It is evident from this study that two of three abortions at CCBDF were due to brucellosis.

Conclusion: Guineapig inoculation technique could be used as a good alternative of culture for confirming the diagnosis of brucellosis from contaminated clinical samples like placenta.

Key words: Abortion, Fetal membrane, Rose Bengal Test, Rapid Antibody Test Kit, Brucellosis

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Introduction

Brucellosis, a zoonotic disease, is endemic in many countries of the world including Bangladesh (Rahman *et al.*, 2017). It affects both, public health and animal production (Ariza *et al.*, 2007). Brucellosis is caused by a Gram-negative, facultative intracellular bacterium of the genus *Brucella* (*B.*) (Khurana *et al*., 2021).

In animals, brucellosis causes severe economic losses as result of stormy abortions in ruminants or reproductive failure, sterility and reduced milk production as well as lost trade in much of the developing world. In female cattle, it causes abortion, infertility, retained placenta, endometritis and to a smaller extent, orchitis, and infection of the accessory sex glands in males (Lopes *et al.*, 2010; McDermott *et al.*, 2013; Elkhansaa and Angara, 2014; Olsen and Palmer, 2014). Brucellosis is one of the most common zoonotic infections transmitted to human through consumptions of unpasteurized dairy product or through direct contact with infected animals or its body fluid after abortion. In man, the clinical picture resembles many other febrile diseases, but sacroiliitis and hepato-splenomegaly are the most prominent and cause debilitating condition if not promptly treated. It is an occupational hazard and farmers, dairy workers, animal caretakers, artificial inseminators, veterinary doctors, butchers and laboratory personals are at high risk (Rahman *et al.*, 2012).

There are several reports on seroprevalence, risk factors, molecular, epidemiological and review of brucellosis in human and animals in Bangladesh (Islam *et al.*, 2013; Rahman *et al.*, 2016; Ahasan *et al.*, 2017; Rahman *et al.*, 2017; Rahman *et al.*, 2019; Tithy et al., 2022).

The prevalence of bovine bucellosis at subsistence management system in Bangladesh was reported to be below 1% (Rahman *et al*., 2019). However, the true prevalence of bovine brucellosis at Central Cattle Breeding and Dairy Farm (CCBDF), Savar was reported to be 20.5% (Rahman *et al.*, 2019). *Brucella spp.* was also detected form milk of dairy cows by PCR of this herd (Rahman *et al*., 2017). Hence it is expected that a high proportion of abortion at CCBDF might be due to *Brucella spp*. To confirm the etiology of abortion, the *Brucella spp.* should be isolated from aborted fetal membranes, vaginal swabs or fetal contents. The isolation of *Brucella spp.* is a difficult task, and it requires level three biosafety cabinet. Moreover, in the presence of competing microflora *Brucella spp.* does not grow (Dahouk *et al.*, 2002). Aborted fetal membranes might be contaminated with other pathogens. In this case, animal inoculation technique is a better alternative for detecting *Brucella spp*. from clinical specimens (Alton *et al.*, 1988). It can also be used for production of vaccine (Yeasmin et al., 2019). Hence, the objective of this study was to detect *Brucella spp*. from aborted fetal membranes using guineapig inoculation technique.

Materials and methods

Ethics Statement

The study protocol of ethical statement was peer reviewed and approved by the Ethical Review Committee of appropriate authority. Animal research was approved by the Faculty of Veterinary Science, Bangladesh Agricultural University.

Sample collection

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 fetal membranes were collected from CCBDF. Fetal membranes were collected from late aborted cases (between the 5th and 8th months of gestation) (Megid *et al.*, 2010). A total of 3 fetal membranes were collected aseptically in a sterile plastic bag. The sample was frozen for 24 hours until it was completely solid. Next day, it was transferred from Savar to Mymensingh maintaining frozen condition. The frozen sample was thawed in room temperature. After thawing the placenta was washed with normal saline and cut into pieces to expose the cutting surface.

Staining

A clean glass slide was taken for impression smearing. The cut surface was pressed over the slide to create the impression of a smear of the organ (fetal membrane, cotyledon). Then the smear was dried and fixed over a flame. The dried smear was stained by modified Ziehl-Neelsen staining method (Alton *et al.*,

1988). Briefly, the impression smears were stained with working carbol fuchsin solution for 10 min, and then decolorized by 3% acetic acid solution for 1 min and counterstained with 1% malachite green solution for 20 sec. After washing in tap water, dried and observed under microscope using 100x objective (oil immersion) to find pale red *Brucella* organism in blue background.

Five grams of fetal membranes were taken and washed very well. The sample was minced and grinded with normal saline using pestle and mortar. Six ml of liquid was collected by using a sterile syringe. The inoculums were prepared for both stain positive and stain negative samples. Three guineapigs were selected for each sample (total 3 samples) and 2 ml of inoculum was injected subcutaneously. Those guineapigs were collected for ICDDRB. The guineapig was observed for six weeks. Blood was collected after 3 weeks and Rose Bengal test, Rapid Antigen Kit test was done for the detection of the antibody of *Brucella* organism.

Rose Bengal Test

The Rose Bengal test was done according to the description of (Alton *et al.*, 1988). Equal volumes (30 µl) of serum and antigen (concentrated suspension of *B. abortus* biotype 1 [Weybridge 99]; Instituto de Salud Tropical Universidad, Edificio, CIMA, Avda, Pio XII, 55 E-31008, Pampalona, Spain) were mixed and rotated on a glass plate for 4 min.

Rapid Antibody Kit test

The serum of guinea pigs was subjected to antigen Rapid Brucella Antibody test Kit (Senspert® Brucella Ab Test Kit, Korea) to detect the antibodies of *B. abortus*. The kit was used according to the instruction of the manufacturer. The kit is equipped with two indicators: one for control (C) and another for displaying the test (T) result. The presence of one red band in the control area indicates a negative result, whereas the presence of two bands in both the control and test sections indicates a positive result. If there is only one band in the test section, it indicates an invalid result.

Result and Discussion

Staining result

Numerous pink colored *Brucella* like organism with blue background was observed under microscope. Among three samples, two (66.7%) were stain positive (Fig. 1).

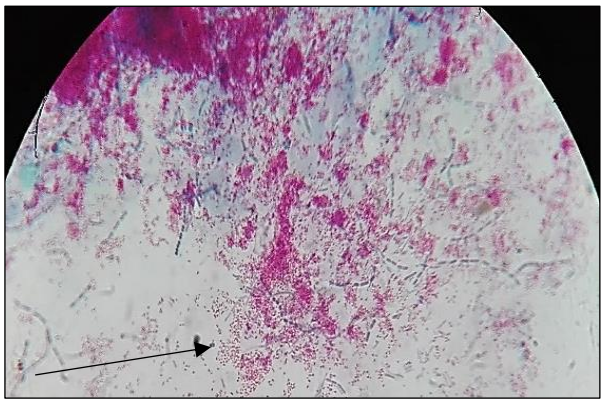


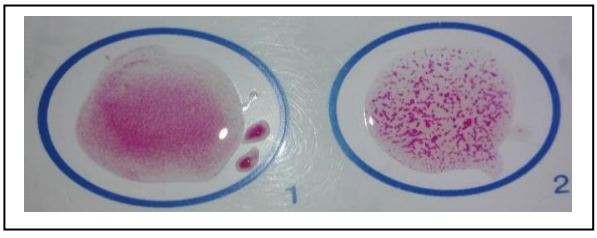
Figure 1: (arrowhead) indicates numerous pink colored *Brucella* like organism in Modified Zeihl-Neelsen staining (100x).

Rose Bengal Test result

All sera samples of inoculated guinea pigs showed positive result. In other word, it can be said that out of three fetal membrane samples two showed positive result. Agglutination was observed within 1 minute (Fig. 2).

Rapid Antibody Kit test result

All the RBT positive samples were also tested positive in Rapid Antibody test. The two red marks indicates the brucella positive sample.



The study was conducted to confirm *Brucella* spp. as an etiology of abortion in dairy cows at CCBDF. For a confirmatory diagnosis of brucellosis isolation of *Brucella* bacteria is the best method which is also considered as a "gold standard" test (Alton *et al*., 1988). However, culture requires level 3 biosafety cabinet and skilled personnel to handle samples and live bacteria for eventual identification and biotyping (Yu and Nielsen, 2010). The likelihood of obtaining

a positive culture from aborted fetal material is high but *Brucella spp.* does not grow readily in the presence of competing microflora (Dahouk *et al.*, 2002).

Figure 2: Agglutination in Rose Bengal Test

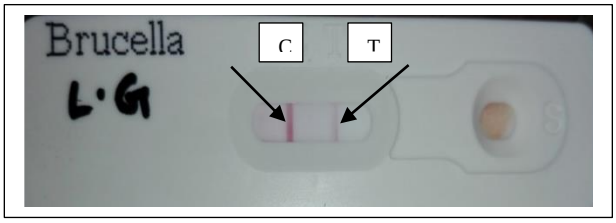


Figure 3: Two red marks indicate a positive result (C indicates control band and T indicates Test positive band)

The clinical sample like aborted fetal membranes might contain lot of contaminants and from such sample guineapig inoculation method should be the best choice for the isolation of *Brucella* (Alton *et al.*, 1988). In the case of guineapig inoculation fetal membranes must be initially screened by Modifies Ziehl Neelsen technique. The presence of pink color coccobacilli in stained smear indicates probable presence of *Brucella* organism. The stain positive samples should be inoculated in guineapig. In this study, *Brucella* organism multiplied and induced humoral immune response when two stain positive fetal membranes were inoculated in guineapigs (García-Carrillo C, 1990, Yeasmin *et al*., 2019). The humoral immune response was detected by RBT and RAT. The isolation through guinea pig inoculation was not reported yet from Bangladesh. This study for the first time in Bangladesh reports the successful detection of *Brucella spp.* from aborted fetal membranes using guineapig inoculation technique.

Two of the three tested fetal membranes were positive for *Brucella spp.* indicating that a high proportion of abortion at CCBDF might be due to brucellosis which is expected as the prevalence of brucellosis is very high (Rahman *et al*., 2019). The isolation of *Brucella spp.* from infected guineapig tissues can be used for further research like culture, vaccine production.

Conclusion:

Two out of three abortions were caused by *Brucella* spp. Guineapig inoculation method could be an alternative of isolation at least at genus level.

Conflict of interest

The author does not have any conflict of interest.

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