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PRODUCTION OF ANTISERA AGAINST *BRUCELLA ABORTUS* IN RABBITS AND ITS USE IN IMMUNOHISTOCHEMISTRY TO DETECT *BRUCELLA ABORTUS* ANTIGEN IN SPLEEN OF ABORTED BOVINE FETUS IN BANGLADESH

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ABSTRACT

Background: Brucellosis is one of the most important emerging zoonotic chronic diseases distributed worldwide since the discovery of *Brucella melitensis* by Bruce in 1887. This disease is specifically hindering animal productivity and human health in developing countries, which has led the WHO to classify it as one of the world's most important neglected zoonotic diseases. The isolation of *Brucella* from host tissues, milk, vaginal exudates, etc. continues to be the "gold standard", followed by bacteriological characterization. However, it is time-consuming and has low sensitivity because *Brucella* is a fastidious microorganism that can easily be overgrown by other contaminating bacteria. To overcome these adversities, visualization of antigen-antibody interaction assays has been tried for safe and timely diagnosis ahead of conventional isolation. Immunocytochemistry and immunohistochemistry techniques have been used to detect *Brucella abortus* antigen and it is mainly based upon staining with species-specific monoclonal or polyclonal antibodies.

Objective: The main objective of this study is to produce polyclonal antisera against *Brucella abortus* and its use in immunocytochemistry for the detection of *Brucella* isolates in clinical samples.

Materials and Methods: A total of 10 rabbits were selected for 2 groups consisting of 7 tests and 3 controls. From 7 test samples pooled polyclonal antisera against *Brucella abortus* was prepared in the same way pooled sera was prepared from control. *Brucella abortus* strain RB 51 was administered subcutaneously at 21-day intervals on rabbits three times. Blood samples were collected at 21-day intervals and antisera were prepared by centrifugation of the sera at 20000 rpm for 15 minutes and preserved in a deep freezer in the laboratory. Sections of the spleen from aborted fetuses clinically suspected of brucellosis were processed and immunohistochemistry (ICH) was performed.

Results: The sample was immune stained with a developed polyclonal *Brucella* antibody. It gave a brown color positive reaction in the macrophage of the spleen. The immune-stained slides were visualized using a photographic microscope. The results of this study showed that the immunohistochemical technique was sufficiently sensitive for detecting *B. abortus* antigens in formalin-fixed tissues of artificially inoculated *B. abortus* RB 51 strain in rabbits.

Conclusions: Further studies are necessary to detect the sensitivity of this test for the diagnosis of brucellosis abortion cases in ruminant animals and to find the possibility of using this immunochemical technique as a complementary tool to serology and bacteriology for the diagnosis of brucellosis.

Keywords: *Brucella abortus*, rabbit, antisera, immunohistochemistry, spleen of bovine fetus

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INTRODUCTION

Brucellosis is a re-emerging bacterial disease caused by species of the Brucella genus and affects a wide range of domesticated as well as wildlife species.[1] It is caused by gram-negative, aerobic, non-spore-forming, and facultative intracellular coccobacilli or short rods. It has a significant economic impact on the public health and the livestock sector. Brucellosis is enlisted as the second leading zoonotic infection followed by rabies by the Office International des Epizooties and classified as risk group III in the laboratory biosafety manual of the World Health Organization.[2] It is one of the common zoonotic infections transmitted to humans through the consumption of unpasteurized dairy products or direct contact with infected animals or their body fluids after abortion. Twelve species are currently recognized in domestic, wildlife animals, and marine mammals.[3] The species of Brucella are genetically very similar although each has different host preferences. Most of the species cause abortion in their specific host with other signs and symptoms. Among them importantly five species are reported to be zoonotic: *B. abortus, B. melitensis, B. suis, B. canis, and B. ceti*.[4] Brucellosis in domestic animals has been known as bovine contagious abortion, enzootic abortion, epizootic abortion, contagious abortion, infectious abortion, ram epididymitis, and Bang's disease.[5] It is endemic in the Middle East, Africa, South America, and Asia. Brucellosis is a widespread neglected zoonotic disease all over the world with more than 500,000 human cases reported annually.[6] In humans, the clinical picture resembles many other febrile diseases, but sacroiliitis and hepato-splenomegaly are the most prominent and cause debilitating conditions if not promptly treated. Brucellosis is also considered an occupational hazard that mainly affects slaughterhouse workers, butchers, livestock producers, shepherds, farmers, veterinarians, artificial inseminators, and laboratory personnel.[7] Bovine brucellosis is predominantly a disease of sexually mature animals.[8] It has been reported as the cause of abortion in many countries including Bangladesh and is mainly caused by *Brucella abortus*.[9] In female cattle, brucellosis appears as different clinical signs, including late abortions, stillbirth, weak calves, placentitis, and infertility whereas in males epididymitis and orchitis.[10] Brucellosis is a notifiable disease in many countries, in Bangladesh it was first serologically investigated in 1967 in the cow.[11] It is endemic in Bangladesh with several reports of seroprevalence in man and animals but there is no proper guideline for vaccination and control measures against this disease performed in Bangladesh.[7,12-14] There are many reports on seroprevalence, and a few reports on the characterization of *Brucella* of large ruminants at the species level are available in Bangladesh.[14] Since *B. abortus* is prevalent both in humans and animals in Bangladesh.[7] The prevalence of brucellosis in the subsistence management system is low (0.6%).[15] Bovine brucellosis is endemic in both humans and animals in Bangladesh.[8] Previous studies estimate the overall seroprevalence of brucellosis in cattle to be 2.4-18.4%, while the herd-level seroprevalence in cattle was estimated as 62.5% in Bangladesh.[15,16] The diagnosis of brucellosis continues to be challenging in developing countries like Bangladesh. Rose Bengal Plate Test (RBPT) is the most used conventional screening test for brucellosis in animals.[17] RBPT relies on the unique antigenic properties of lipopolysaccharides (LPS) that are present within the cell membrane of *Brucella* spp.; however, the LPS antigen is also present in several other gram-negative bacteria, including Vibrio and *Yersinia enterocolitica*, which may cross-react on the brucellosis diagnostic assays.[18] The isolation of *Brucella* from host tissues,

milk, vaginal exudates, etc. continues to be the "gold standard", followed by bacteriological characterization. However, it is time-consuming and has low sensitivity because *Brucella* is a fastidious microorganism that can easily be overgrown by other contaminating bacteria. Moreover, the technique is less sensitive in chronic infections. More significantly, the World Health Organization (WHO) classified the genus *Brucella* as a risk group III pathogen[19] which requires to be handled with utmost care and the isolation must be performed by a person with sound technical skill. To overcome these adversities, visualization of antigen-antibody interaction assays has been tried for safe and timely diagnosis ahead of conventional isolation.[20] Immunocytochemistry and immunohistochemistry techniques have been used to detect *Brucella abortus* antigen and it is mainly based on staining with species-specific monoclonal antibodies.[20] In addition, immunohistochemical detection of *B. abortus* and *B. melitensis* antigens in cases of naturally occurring abortions in cattle and sheep, respectively elsewhere.[21,22] This paper describes the production of polyclonal antisera against *Brucella abortus* in rabbits and the use of polyclonal antisera for immunohistochemistry to detect *B. abortus* antigens in suspected brucellosis aborted fetus spleen.

MATERIALS AND METHODS

This study was carried out at the "Livestock and Human Brucellosis Laboratory" under the Department of Medicine and Department of Pathology, Bangladesh Agricultural University, Mymensingh- 2202.

Sample collection

The rabbit was collected from different areas of the Mymensingh district. A total of 10 rabbits were selected for 2 groups consisting of 7 used for antisera production and 3 served as controls.

Antigen used for antibody production

Brucella abortus RB 51 strain was collected from Spain. It was administered subcutaneously @1 ml/rabbit at 21-day intervals three times.

Sample collection and preservation

Blood samples (2 ml) were collected from the rabbit ear vein on days 0, 21, 42, and 63 before inoculation of antigen. The rabbits were restrained properly, the injection site was disinfected with 70% alcohol, and 1.5 ml of blood was collected from each of the rabbits from ear veins.

Antisera preparation

The collected blood was kept undisturbed in the 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 2000 rpm for 15 minutes and the sera were transferred to the sterile and labeled Eppendorf tube. From 7 test samples pooled polyclonal antisera against *Brucella abortus* was prepared in the same way pooled sera was prepared from control.[23] The sera samples were preserved in a deep freezer in the laboratory.

Standardization of developed antisera for immunohistochemistry *for detection of B. abortus*

The spleen from the aborted fetus of cattle was clinically suspected of brucellosis as there was a

history of retention of the placenta. This sample was confirmed by immunohistochemistry.

Immunohistochemistry

Immunohistochemistry (IHC) was performed on clinical samples. It includes tissue staining and immunostaining.[20]

Protocol for tissue processing for paraffin sectioning

Spleen from aborted fetus was collected from Mymensingh, and was processed for paraffin sectioning for immunohistochemical studies as given below:

- Block preparation with paraffin.
- Sectioning (4-5 μm thickness) with a microtome.
- Picking of sections on grease-free glass slides.
- Drying of sections by incubating them in an incubator at 60 °C for one hour.

Table 1.

S/N	Reagents	Duration	S/N	Reagents	Duration
1.	10% neutral buffered formalin	12-24 hours	08.	Acetone II	15 minutes
2.	70% alcohol	1 hour	09.	Benzene I	15 minutes
3.	80% alcohol	1 hour	10.	Benzene II	15 minutes
4.	90% alcohol	1 hour	11.	Paraffin Wax I	2 hours

Immunohistochemical studies on tissue

1. Five-micron sections were cut and mounted on super frost positively charged slides (Biogenex).

2. Sections were deparaffinized (Xylene) and rehydrated to water (descending grades of alcohol).

3. After fixation, heat-induced antigen retrieval was done in citrate buffer-based antigen unmasking solution (H-3300, Vector Laboratories USA) and heating in the microwave at 98 °C for 10 minutes.

4. Slides were then left for 30 min in hot antigen retrieval solution and washed in 0.1 M phosphate-buffered saline (at pH 7.4).

5. The endogenous peroxidase activity was blocked by immersing the sections in 3% (v/v) H_2O_2 in methanol for 20 min followed by washing in 0.1 M phosphate-buffered saline (at pH 7.4).

6. To prevent nonspecific binding of antibodies sections were blocked with normal horse serum (Vector's Laboratories, USA).

7. The sections were incubated with primary antibodies (*Brucella abortus*, RB 51 strain) at 4 °C for overnight in the humid incubation chamber. After washing in 0.1 M phosphate-buffered saline (at pH 7.4), the sections were incubated with a universal secondary antibody (Vector Laboratories, USA).

8. The chromogen used was 3, 3'-diaminobenzidine tetrahydrochloride (DAB) (Vector Laboratories, USA) with Gill's III hematoxylin counterstaining.

9. The sections were washed in running tap water, dehydrated, cleared, and mounted with DPX.

Microscopic examination and image analysis

The immune-stained slides were imaged using a microscope with a photographic unit. The images evaluated for the presence of *Brucella* which was visualized by the presence of brown-colored DAB reaction.[20]

RESULT

Pooled polyclonal antisera were prepared by standard protocol. Then these polyclonal antisera were used for immunohistochemistry to detect *Brucella* antigen in tissue. The spleen from the aborted fetus showed positive signals that were brown color staining in the cytoplasm of the macrophage.

Immunohistochemical (IHC) staining of spleen sections demonstrated numerous labeled brown color staining in the cytoplasm of macrophage (Fig. 1).

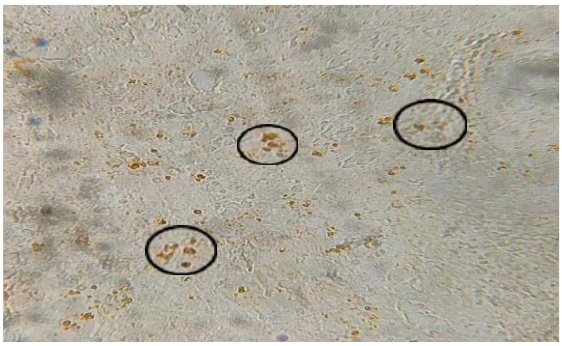

Figure 1. Immunocytochemistry on the spleen of aborted fetus sample (100 ×)

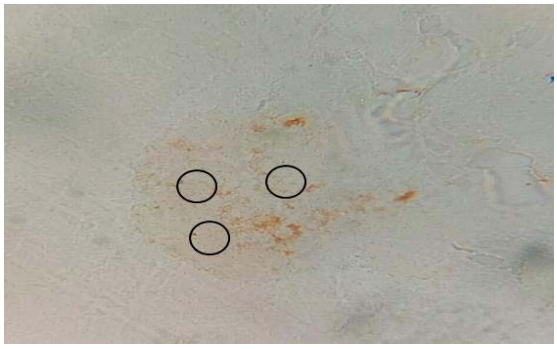

Figure 2. Immunocytochemistry on aborted fetal membrane lymph node sample (100 ×)

The lymph node samples were subjected to immunocytochemistry yielded positive results with DAB chromogen. The immunohistochemistry gave a brown color positive reaction indicating the presence of *Brucella* spp. in the lymph node sample (Fig. 2). Thus, immunohistochemical examination of paraffin wax-embedded tissues for *B. abortus* antigens was found to be both sensitive and specific.

DISCUSSION

Brucellosis is still a neglected zoonotic disease.[24] However, for the diagnosis of brucellosis some direct tests such as isolation, polymerase chain reaction (PCR), culture, immunohistochemistry (IHC), etc., and some indirect tests such as Rose Bengal test (RBT), standard tube agglutination test (SAT), complement fixation test (CFT), enzyme-linked immunosorbent assay (ELISA), etc. are used.[25] However, bacterial culture is the gold standard of

brucellosis diagnostic methods but bacterial culture is time-consuming and labor-intensive, and accordingly, serological tests based on agglutination are utilized as a conventional method of diagnosis. However, this paper has been presented at the conference[26] and then submitted to this journal for publication which has been processed as per journal style and peer-reviewed.

Polyclonal antibodies are relatively inexpensive and can be prepared in large quantities in different animals like rabbits, goats, and horses. Rabbits are more commonly used than other animals. The appropriate size of the animal, easy handling, and antigenic precipitation properties of the antibodies are some of the important advantages of rabbits. The positive control used in the kit is prepared by rabbit immunization against *Brucella abortus*. However, the goat has been reported to be a better and more appropriate choice, in terms of both cost and quantity, when a high concentration of serum is required.[23]

Brucellosis is a zoonotic disease, and the affected animal needs to be disposed of. So, for further disposal of affected animals, direct tests are being used worldwide. The immunohistochemical technique has been reported to be sufficiently sensitive for detecting B. abortus antigens in formalin-fixed lung tissues from naturally aborted bovine fetuses.[21] Another study on the comparison of diagnostic tests revealed PCR and IHC provide a reliable test for the diagnosis of bovine brucellosis in aborted fetal tissues and placental cotyledons whereas serology is most important for the detection of Brucella-positive animals in a herd.[27] In this study, we only used immunohistochemistry in artificially *B. abortus*-infected rabbit tissues. So far, with the detection of *Brucella* organisms at the tissue level by immunohistochemistry, an attempt was made to develop pooled polyclonal antibodies in rabbits with standard protocol. The staining process had a limitation in that the positive control slide was not used for comparison. However, in one instance, the positive immunohistochemical staining was indicated by the presence of brown color signals within the cytoplasm of macrophages.

Immunohistochemistry (IHC) is an affordable and straightforward procedure that can be conducted with minimal resources. It serves as a potent technique for investigating the localization and presence/absence of a target at both tissue and cellular levels. It allows for the storage and retrieval of paraffin-embedded and frozen tissue samples as needed, and stained tissue sections can be preserved for future reference.

CONCLUSIONS

Commercially available antibodies against *Brucella* organisms used in immune-histochemistry (IHC), were not available when this small research was conducted. So, we developed pooled polyclonal antiserum in rabbits using *Brucella abortus* organisms from Spain. We successfully developed pooled polyclonal antiserum which detected *Brucella* organisms in macrophages by immunohistochemistry (IHC). Further studies are necessary to apply this test in bovine species associated with abortion to find out that this immunohistochemical technique could be a complementary tool to serology and bacteriology for the diagnosis of brucellosis.

ETHICAL APPROVAL

All animal-related procedures and methods were carried out by the Animal Welfare and Experimentation Ethical Committee of the Bangladesh Agricultural University, Mymensingh. (Ethical approval number- AWEEC/BAU/2023(55)

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