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SHORT COMMUNICATION

Pathological Investigations of Organ Affinity of *Brucella* Species and their Cross Species Transmission

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ABSTRACT

The present study was conducted to determine the localization of *Brucella* species within different aborted tissues. Another objective was to determine its cross species transmission of *Brucella* in three main districts of Punjab, Pakistan with significant bovine population. The results revealed the presence of *Brucella abortus* (23.3%) only and none of bovine fetus was positive for *Brucella melitensis* (0%) while lung was the most prevalent targeted tissue for *Brucella* as compare to liver and stomach respectively in both cattle and buffaloes fetuses. The results also indicated increased *Brucella* incidence in cattle fetuses (30 & 13.3%) than buffalo fetuses (16.6 & 6.66%) (P=0.034) by both AMOS PCR and IHC respectively. We also found significantly more positive cases by AMOS PCR than IHC (P=0.024). These novel findings about best specimen selection for isolation of *Brucella* and absence of *Brucella melitensis* in bovines of Punjab Pakistan can be helpful in designing brucellosis control programs.

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INTRODUCTION

Bovine brucellosis is caused by three *Brucella* species including *B. abortus*, *B. melitensis* and *B. suis* (Aznar *et al*., 2014; Gul *et al*., 2015). Unfortunately, most of the *Brucella* work in bovines is focused on determining the status of *B. abortus* in Pakistan and there has been no endeavor to determine the status of other *Brucella* species particularly *B. melitensis* in bovines with a view to determine cross species transmission of this organism. *B. suis* is not that important in Pakistan as we do not have the breeding of specific host (pigs). On the other hand, *B. melitensis* is very important since because of large scale mixed farming of large and small ruminants in Pakistan. This may enhance the transmission possibility of *Brucella* across the species. Moreover, *B. melitensis* is of zoonotic importance and causes occupational exposure of more than 500,000 humans annually (Figueiredo *et al*., 2015) therefore it is mandatory to screen our bovines for the presence of *B. melitensis* as they may be possible spreaders of disease to humans. This will be a step towards the control of human brucellosis though consumption of contaminated milk from infected bovines. Another novel aspect of this study was to determine which organ has more affinity for *Brucella* as this information can be useful for pathologists, microbiologist and other stakeholders to determine the ideal tissue from which to isolate or detect *Brucella* and to gain into the tissue survival and pathogenesis of *Brucella*.

The present study also compared two diagnostic techniques including AMOS PCR and immunohistochemistry (IHC) for antigenic detection of *Brucella*. As the diagnosis of brucellosis is mainly through bacteriological isolation and serological techniques but long incubation periods, sample handling, false-positive results due to cross-reactions with other bacteria (Wareth *et al*., 2014) and requirement of BSL-3 hinders the confirmatory diagnosis of this disease. Therefore, reliable techniques for the differentiation and detection of *B. abortus* and *B. melitensis* are needed to know the exact status of both organisms in our animals. The results of this multi-purpose comparative study can provide the basic information required for brucellosis eradication programs in Pakistan.

MATERIALS AND METHODS

Samples of lung, liver and stomach were collected from 60 aborted fetuses of cattle and buffaloes (30 each) at private and government livestock farms located in and around Lahore district. The aborted fetuses were included on the bases of abortion in last month and from dams with no history of vaccination against brucellosis. The samples were preserved in 10% neutral buffered formalin for further processing by AMOS PCR and IHC for analysis of *B. abortus* and *B. melitensis*.

The DNA was extracted from lungs, liver and stomach of aborted bovine fetuses using QIAamp™ DNA mini kit (Qiagen Inc., Valencia, CA) and was purified by using genomic DNA purification kit Catalog # K0512. DNA quantification was done by using Nanodrop. The IS*711*genetic element was targeted for amplification in both *B. abortus* and *B. mellitensis* using primer sequences as follows:

IS*711* for *B. abortus* at 498bp.
5-GACGAACGGAATTTTTCCAATCCC-3
5-TGCCGATCACTTAAGGGCCTTCAT-3
IS*711* for *B. melitensis* at 731bp
5-AAATCGCGTCCTTGCTGGTCTGA
5-TGCCGATCACTTAAGGGCCTTCAT-3

Amplification of DNA was carried out by denaturation at 94ºC for five minutes, primers annealing at 60ºC for one minute, initial extension at 72ºC for one minute and the final extension at 72ºC for 10 minutes. The amplicons were run on 1.2% agarose gel along with DNA markers and imaging was performed using gel documentation system (Bricker and Halling, 1995).

The immunohistochemical analysis was performed using *INVITROGEN* Histostain®-*Plus* 3rd Gen IHC detection kit Cat.No.85-9673 according to manufacturer's instructions (Xavier *et al*., 2009). The tissue samples were fixed for 24 hrs in 10% neutral buffered formalin solution, dehydrated and embedded in paraffin wax. Paraffin-embedded tissue sections were sectioned (4 µm), mounted on positively charged Superfrost Plus glass slides and stained with hematoxylin and eosin (H & E).

Primary antibodies were raised in three healthy adult rabbits. Their serum was checked by ELISA for antibodies against *Brucella.* The inoculum was prepared with *Brucella* vaccine, injected subcutaneously @ 0.2 mL per rabbit and bled periodically from day zero to 7, 15, 30, 45 to check the antibody titer in their serum against *Brucella*. Slides were treated with the primary antibody (100 µL) for 60 minutes in a moist chamber. After washing with phosphate-buffered saline (PBS), the slides were incubated with biotinylated secondary antibody (100 µL) for 10 min at room temperature and rinsed twice in PBS. The slides were incubated with streptavidin–peroxidase conjugate (100µL) for 10 min at room temperature and rinse again with PBS. The reaction was developed with a 0.026% diaminobenzidine solution and counter staining was done with hematoxylin. Tissue sections positive for *B. abortus* and *B. melitensis* by culture were used as tissue controls while bovine tissues from *Brucella*-free cattle were used as negative controls. The normal rabbit serum replaced primer antibody in negative control. The statistical analysis was made by the chi square test using SPSS version 22.

RESULTS AND DISCUSSION

Of the 60 aborted bovine tissue samples, AMOS PCR detected 14 samples (23.3%) and IHC detected six (10%) samples to be positive for *B. abortus* while none of the sample was positive for *B. melitensis* (Fig. 1). The absence of *B. melitensis* in bovines is noteworthy as this species is considered to cause human mortality as compared to other *Brucella* species (Osler and Palmer, 2014). This may be due to host specificity of *Brucella* species.

Tissue burden of *Brucella* in lung, liver and stomach was also observed by IHC (Fig. 2, 3 & 4). Interestingly we detected more *Brucella* antigens in lung tissue as compare to liver and stomach respectively (P=0.032). These findings may be due to more susceptibility of respiratory epithelium as a predilection site for *Brucella* as compare to gastrointestinal tract. The low amount of *Brucella* antigen in liver of infected fetuses may be due to the presence of mononuclear phagocytes in liver that are mainly involved in removing *Brucella*. This variable *Brucella* load may also depend upon the time post infection and affinity of receptors in each organ for entry of organism (Castaneda-Roldan *et al*., 2004). It may also be possible that the number of *Brucella* organisms in liver and stomach was less than the threshold of immunohistochemical detection. Therefore, we need to culture the tissue to determine tissue burden/concentration of *Brucella*.

The ratio of *B. abortus* positive samples was more in cattle fetuses (30 & 13.3%) than buffalo fetuses (16.6 & 6.66%) (P=0.034) by both AMOS PCR and IHC respectively. This was again good news for stakeholders as there are more buffalo milk consumers in Pakistan than those consuming cow milk. This increased incidence in buffaloes could be due to resistance of buffaloes to brucellosis in comparison to cattle (Borriello *et al*., 2006).

In order to replace the laborious culture techniques for routine analysis we compared two antigenic techniques for direct detection of *Brucella* and found more positive cases by AMOS PCR than IHC (P=0.024). This indicates more sensitivity of AMOS-PCR as compare to IHC. Moreover, AMOS PCR detects four *Brucella* species simultaneously and is a single step procedure as compare to IHC. The cost comparison of two showed that IHC was more expensive than AMOS-PCR. Therefore, based upon our results we recommend use of AMOS-PCR for quick, accurate and cost effective diagnosis of brucellosis.

Conclusions: The results of this study showed the presence of only *B. abortus* in bovine fetuses without any evidence of *B. melitensis* and the incidence of brucellosis was more in cattle fetuses than in buffalo fetuses. This study for the first time reported the increased affinity of fetal lung tissue for *Brucella* localization than liver and stomach. This information may help to propose the selection of best specimen/tissue for detection of *Brucella* species as well as the route of vaccine to target the most affected organs. Moreover, AMOS PCR detected more cases than IHC; hence, it can be suggested as a better diagnostic tool for the detection of brucellosis.

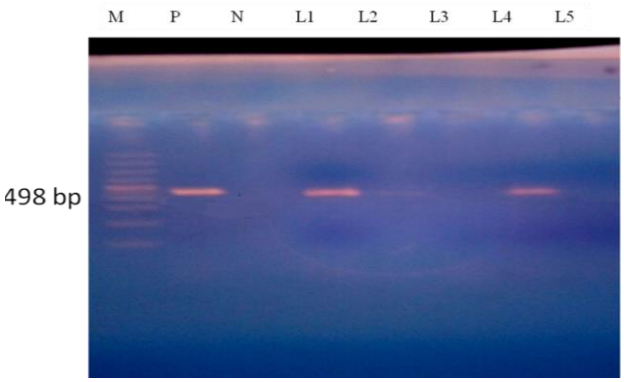

Fig. 1: Agarose gel electrophoresis of *Brucella abortus IS711* genomic region (498 bp).

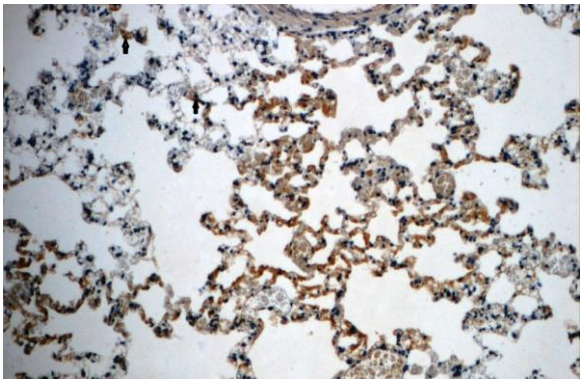

Fig. 2: Immunolabelled *Brucella abortus* in alveolar sacs of aborted cattle fetus at 100X stained with DAB chromogen and counter stain with hematoxylin.

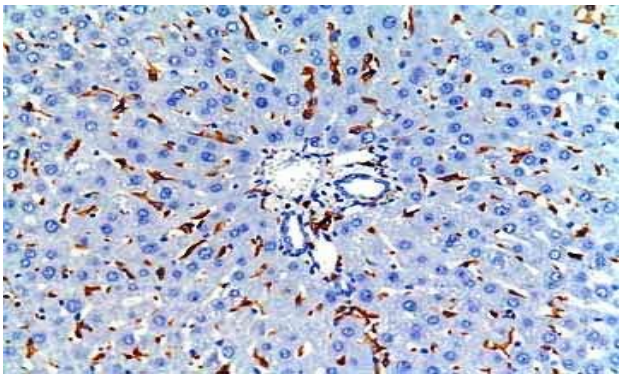

Fig. 3: Immunolabelled *Brucella abortus* in liver of aborted buffalo fetus at 100X stained with DAB chromogen and counter stain with hematoxylin.

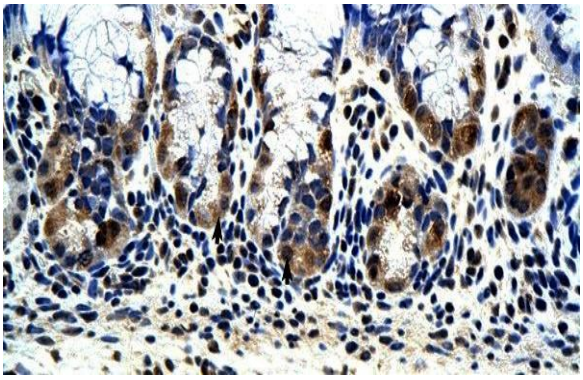

Fig. 4: Immunolabelled *Brucella abortus* in stomach of aborted buffalo fetus at 400X stained with DAB chromogen and counter stain with hematoxylin,

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Authors contribution: RA, IK and AA designed the study. RA, MNA, GM and SFR conducted the experiments. RA, HEA and FM drafted the manuscript. HN, SN and MZS analyzed the data. All authors critically reviewed the manuscript for intellectual content and gave final approval for final version.

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