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Immunopathological and Molecular Approaches for the Diagnosis of Salmonellosis in Natural Cases of Bovine Abortion

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Rapid and precise diagnosis in natural field cases of bovine abortion warrants use of most sensitive and reliable diagnostic methods. In the present study, histopathological, immuno-histochemical and PCR technique(s) have been applied for diagnosis of abortion cases due to Salmonellosis. Histopathologically, multifocal lymphoid granulomas were observed in liver of aborted fetuse(s) which showed positive immuno-reactivity to the anti-*Salmonella* polyclonal antibody. PCR for diagnosis of salmonellosis in the aborted bovine fetuses found two positive samples out of the total 113 samples tested. Primers S18/ S19 pair amplified a 159 bp region of the sequence encoding an ompC gene of *Salmonella* species.

Key words: Abortion, Immuno-histochemistry, PCR, Salmonellosis.

The *Salmonella* spp. though typically enteric pathogens in calves have also been well recognized as a cause of abortion in pregnant cows (Rings, 1985). Most *Salmonella* serovars are potential pathogens and their isolation is quite exhaustive. The rapid detection of members of this genus is particularly useful from zoonotic point of view. Since the early 1990's, PCR has been increasingly used as a diagnostic tool for etiologic diagnosis of abortion in cows either as a complement or as a replacement of time consuming traditional diagnostic methods such as bacterial isolation (Anderson, 2007). Immunohistochemistry (IHC) is a fast, specific and highly sensitive method for antigen detection and can also be used on retrospective samples for diagnosis. In the present study, diagnosis of salmonellosis in field cases of bovine abortion was done on the basis of histopathological, immuno-histochemical and PCR technique(s).

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MATERIALS AND METHODS

Pathological studies

Tissue sample from aborted fetuse(s) (27) and placental cotyledon(s) (16) were collected in 10% neutral buffered formalin for fixation of samples for histopathology and immuno-histochemistry. Fixed tissue samples were given overnight washing under tap water. Then dehydration of samples was done through ascending grades of alcohol followed by clearing with acetone and benzene. Tissues were later embedded in paraffin wax (Leica Microsystem, Paraplast tissue embedding medium, 56°C) for further processing and 4-5µ thin sections were cut. The paraffin sections were later stained with routine hematoxylin and eosin technique (Luna, 1968).

For immuno-histochemical studies 4-5µ thick paraffin embedded tissue sections were cut and mounted on Superfrost Plus, positively charged microscopic slides (Fisher Scientific, USA). The slides were then kept on hot plate to melt the paraffin at 60°C for 30 minutes and stored

till further use. The dewaxing and rehydration was performed by dipping tissue sections in EZ-AR™ Common Solution (BioGenex Laboratories Inc., San Ramon, California, USA), and heated at 70° C for 10 minutes in EZ-Retriever ™ System (BioGenex Laboratories Inc., San Ramon, California, USA). Then heat induced epitope retrieval (HIER) was performed by using EZ-AR™ 3 Solution by heating at 95° C for 10 minutes in EZ-Retriever ™ System. Immuno-histochemical staining was performed by using advanced SS™ One-Step Polymer–HRP IHC Detection System (BioGenex Laboratories Inc., San Ramon, California, USA) as per manufacturer's instructions. A polyclonal antibody against *Salmonella* (IVRI, Izatnagar) was used in a dilution of 1:500. As a negative control, section(s) were incubated with PBS instead of the primary antibody. The antigen-antibody-peroxidase reaction was visualized by using freshly prepared 3, 3-diaminobenzidine (DAB) solution (50 µl of DAB chromogen with 1 ml of DAB buffer provided by the manufacturer and adding 5 µl of hydrogen peroxide).

Molecular studies

Total of 103 samples comprising of stomach contents (46), placental cotyledons (16), pooled tissue samples (27) viz. lungs, heart, liver, kidneys, spleen, brain of aborted fetuses and the vaginal mucus/uterine discharges (14) of aborted animals were collected and stored at -20^{0}C till further use. DNA extraction from frozen tissues samples was performed using a commercial kit (HiPura mammalian genomic miniprep purification spin kit Himedia) following the manufacturer's instructions with slight modifications. Placenta (2-4 cotyledons) and tissue fragments of approximately 2 g weight from kidney, lung, liver, spleen and heart were pooled and triturated in sterile pastle and mortar with sterilized sand along with 8 ml phosphate-buffered saline and centrifuged at 4000 rpm for 15 minutes. The supernatant (200µl) was collected and DNA extraction was carried out using HiPura mammalian genomic miniprep purification spin kit (Himedia) as per protocol. Stomach contents of aborted fetuses and vaginal discharges of recently aborted animals stored at -20^{0}C were thawed and centrifuged at 4000 rpm for 15 minutes and 200µl of the supernatant was used for DNA extraction using HiPura blood genomic DNA miniprep purification spin kit (Himedia) as per manufacturer's guidelines.

Polymerase Chain Reaction (PCR) for Salmonellosis

The PCR amplification of DNA using primers specific for *Salmonella* genus was standardized by varying the concentration of the reaction mix and cycling conditions. All the reactions were performed in a Master cycle Gradient thermocycler (Hybaid, Touch Down) with a preheated lid. Primers S18-ACCGCTAACGCTCGCCTGTAT and S19-AGAGGTGGACGGGTTGCTGCCGTT used in the present study were based on the DNA sequence of the ompC of *Salmonella* species described previously by Kwang *et al.*, (1996). The PCR amplifications were carried out in a volume of 50µl and reaction mixture for amplification of target gene comprised of PCR Master Mix (2X) - 25 µl, Forward Primer (10 pmol/µl)- 1.25 µl, Reverse Primer (10 pmol/µl)-1.25 µl, Template DNA-8 µl, Distilled water-14.5 µl. The reaction was performed as per method of Amavisit *et al.*, (2001). The amplified product was analyzed on 1.5% agarose gel (50 min at 70 V) by electrophoresis and visualized by using gel documentation system (Bio rad, USA)

RESULTS AND DISCUSSION

In the present study, abortion due to salmonellosis was reported in 2 cows which aborted at 7 month of gestation. Grossly, liver was enlarged and showed single or multifocal pale area of necrosis (Fig. 1).There was marked spleenomegaly and hemorrhagic areas were evident. Histopathologically, multifocal early lymphoid granulomas (Figs. 2-3) were observed in liver of aborted fetuses. These findings were similar to the findings of Skoric *et al.*,(2007), who found Gram-negative bacteria that cause granuloma in liver include *Salmonella* sp., *Brucella* sp., *Francisella* sp., *Pseudomonas* sp., *Yersinia* sp., and *Actinobacillus* sp.. Immunohistochemical staining revealed positive immuno-reactivity (Figs 4-5) to the anti-*Salmonella* polyclonal antibody and confirmed the histopathological findings. Immuno-histochemistry allows detecting the distribution of the antigen and its attestation with microscopic lesions (Haines and clark, 1991). Moreover, immuno-histochemical method can be used as a complementary tool in the suspected

Fig. 1. Enlarged pale liver

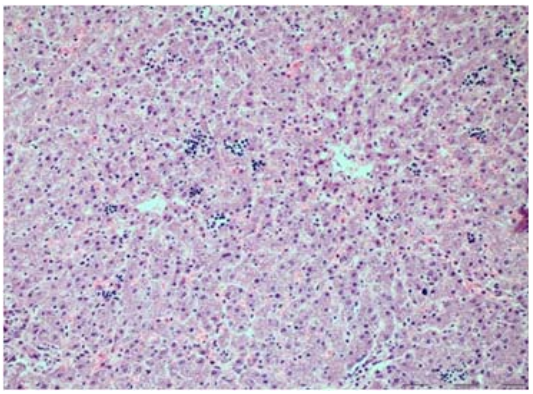


Fig. 2. Liver- Multifocal early typhoid granuloma seen as lymphoid aggregates (H &E, 20X)

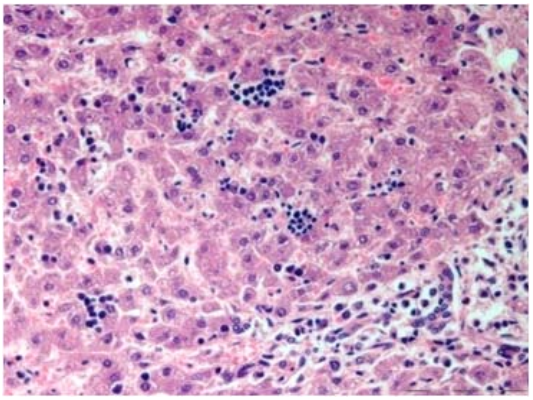


Fig. 2. Liver- Multifocal Lymphoid granuloma (H &E, 40X)

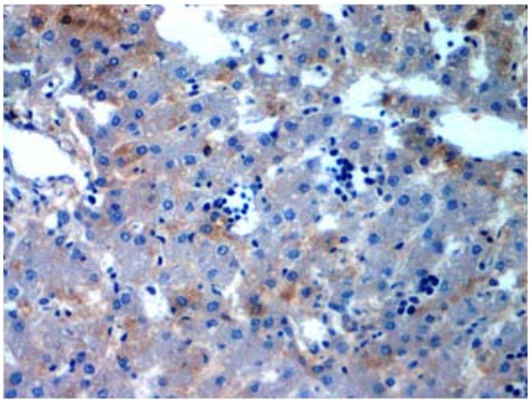


Fig. 4. Liver- immunoreactivity to the anti-*Salmonella* polyclonal antibody (40X)

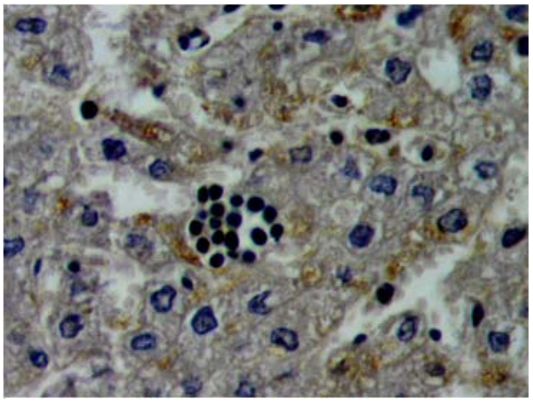
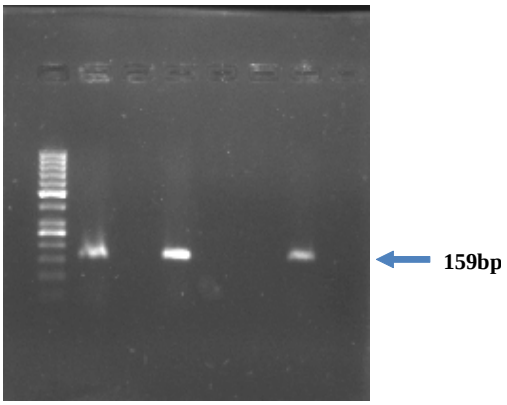


Fig. 5. Liver- immunoreactivity to the anti-*Salmonella* polyclonal antibody (100X)



Lane M : Molecular weight marker (50bps)
Lane P &N : Positive and Negative controls
Lane 1-5 : Samples (1 and 3 tested positive for *Salmonella* at 159bp).

Fig. 6. *Salmonella* genus species PCR from aborted material

aborted cases; when isolation is not possible or material submitted is fixed in formalin The PCR protocols employed in this study efficiently amplified the sequences from the positive control samples and without any amplification of negative control(s). Primers S18/ S19 pair amplified a 159 bp region of the sequence encoding an ompC of *Salmonella* species. Two of the 113 samples (one from stomach contents and one from pooled tissue) produced 159 bp amplicon (Figure 6). In the present study, PCR amplify the omp C gene that encodes an outer membrane protein which is one of the major structural proteins of *Salmonella*. Similar findings were reported by Da Silva *et al*., (2009) who amplified the *Salmonella* spp. DNA from aborted fetal tissue in bovine. PCR based assays have been proved to be an important alternative rapid technique that overcome problems and disadvantages of currently used traditional methods. The specificity and high sensitivity of PCR provide a valuable tool for the diagnosis.

Thus, immuno-histochemical and PCR technique(s) were found sensitive and useful for diagnosis of natural field cases of bovine abortion caused by *Salmonella* spp. and have a good supplement to the gross and histopathological alteration(s) as a result of the disease.

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