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Histomorphological and molecular detection of pneumonic Pasteurellosis in bovines from slaughter house, Mizoram

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Abstract
Pneumonic Pasteurellosis, also known as respiratory mannheimiosis, is the most common infection having wide prevalence in ruminants. The disease is caused by *P. multocida*, Gram negative, non-motile, non-spore forming, facultative anaerobic, small rods or coccobacilli commensal in upper respiratory tract and gastrointestinal tract. During the study period, total 95 number of tissue samples were collected from slaughtered animals. Out of these, 30 swabs were collected in culture broth from suspected lungs samples. The tissue samples suspected for Pasteurellosis were collected in 10% NBF for fixation and then histopathological analysis was performed. Microscopic study revealed that the affected lungs were dominated by interstitial and alveolar oedema along with vascular thrombosis of capillaries and small blood vessels. And characteristic oat shape cells. The intestine and other visceral organs like liver, kidney and spleen showed moderate to severe haemorrhages. 7 samples were found positive for *Pasteurella sp*. expressing *hyaD-hyaC* gene with amplification at 1044 bp size.

Keywords: bovines, Histomorphological, Mizoram, molecular, Pasteurellosis, slaughter house

Introduction
Livestock plays an important role in economic status of a country. Cattle and buffaloes are being raised for milk, meat and leather and also as draught animals. The effective development of any livestock industry depends upon the prevention and control of diseases among the animals. Diseases in these animals cause heavy economic losses in milk, meat and wool industry. Bacterial and viral organisms and parasites play a significant role in diseases causing heavy morbidity and mortality as they are important etiological agents causing gastroenteritis in calves (Singh *et al.*, 2000) [1].

Respiratory tract infections are commonly observed in various species of domestic as well as in farm animals. However, among respiratory infections, Pneumonic Pasteurellosis, also known as respiratory mannheimiosis, is the most common infection having wide prevalence in ruminants. Pneumonic Pasteurellosis is an acute and often fatal epizootic disease of respiratory system of large ruminants (Blood *et al.*, 1983; Jubb *et al.*, 1985) [2, 3]. The disease is caused by *P. multocida*, Gram negative, non-motile, non-spore forming, facultative anaerobic, small rods or coccobacilli commensal in upper respiratory tract (URT) and gastrointestinal tract (Holt *et al.*, 1994; Lopez, 2001; Quinn *et al.*, 2002) [4, 5, 6]. It is most commonly observed in cattle and buffalo but also reported in sheep, horse and camel (Jubb *et al.*, 1985) [3]. Goats are considered relatively resistant (Wijewardana *et al.*, 1990) [7]. Prevalence of Pasteurellosis has been reported in all continents except Australia. The disease is characterized by an acute febrile course with severe fibrinous or fibrinopurulent bronchopneumonia, fibrinous pleurisy and septicaemia. Death may occur after few days of onset of clinical signs, those who survived acute infection may remain as chronically infected (Brogden *et al.*, 1998; Lopez, 2001) [8, 5]. Physical or physiological stress are important for occurrence of disease which include adverse environmental and climatic conditions such as extremely bad weather, overcrowding, transportation, poor management or previous infection with mycoplasma, respiratory viruses, or some other pathogenic organisms.

Some previous reports stated that *P. multocida* was associated with acute or chronic pneumonic lesions in human beings (Beyt *et al.* 1979; Klein & Cunha, 1997; Marinella, 2004) [9, 10, 11].

Also, the other species i.e. *P. dagmatis* was associated with fatal peritonitis and septicaemia in human beings (Ashley *et al.*, 2004) [12]. A slaughter house study helps in the assessment of the disease status of herds and also prevents distribution of the infected meat to human being (Mellau *et al.*, 2010) [13]. Abattoir data contain valuable informations about the incidence and epidemiology of animal diseases (Jobre *et al.*, 1996; Chhabra and Singla, 2009) [14, 15]. The present study was performed to assess the incidence of Pasteurellosis in the slaughterhouse inspection during slaughtering.

Materials and Method

The present study was conducted during the period from March 2019 to February 2020. The Govt. slaughterhouse, Mizoram was regularly visited during the study period and the carcasses were examined for presence of any abnormal changes in the visceral organs. During the study period, total 95 number of tissue samples were collected slaughtered animals after from slaughtering. Out of these, 30 swabs were collected in culture broth from suspected lungs samples. The tissue samples suspected for Pasteurellosis were collected in 10% neutral buffered formalin (NBF) for fixation and then histopathological analysis was performed. After fixation the tissue were cut into 5mm thickness and washed in tape water for overnight. Then the tissues were processed through paraffin embedding technique. The sections were cut at 5µ in rotary microtome, then sections were stained with routine histological staining i.e. H & E. The stained slide was observed under light microscope and marked histopathological changes were recorded. The swabs from suspected lungs were also collected in broth culture for isolation of bacterial DNA which was used for molecular diagnosis (PCR). The primer used for PCR diagnosis is CAPA-FWD TGCCAAAATCGCAGTCAG, CAPA-REV TTGCCATCATTGTCAGTG (hyaD-hyaC gene of size 1,044bp) (Townsend *et al*., 2001) [16].

DNA Isolation

Swabs were collected from lungs suspected for Pasteurellosis, in broth culture and incubated at 35-37ºC overnight. Then 1 ml of inoculated broth culture was transferred to 1.5 ml Eppendorf tubes and centrifuged at 8000 rpm/10 minutes. The supernatant was discarded without disturbing the pellet and the pellet was vortexed vigorously by addition of 1 ml of normal saline solution (NSS). The mixture was centrifuged at 8000 rpm/5 minutes. The salt solution was discarded without disturbing the pellet followed by resuspension in 100 µl of nuclease free water (NFW) and the suspension was vortexed. The mixture was incubated on boiling water (100 ºC) for 10 mins followed by immediate transfer to -20 ºC for 15 mins. The lysate was centrifuged at 8000 rpm/5 mins. and the supernatant was transferred to clean Eppendorf tubes for storage at -80 ºC.

Polymerase chain reaction (PCR) protocol

The PCR reaction was carried out with 12.5 µl of master mixture which contained 1.25 µl of 10X PCR buffers (Thermo Scientific, USA), 0.1 µl of Dream Taq DNA polymerase (5U/µl), 0.5 µl of each primer (10 pmol/µl), 0.3 µl of 10 mM dNTPs, 1 µl of template DNA and rest volume included Nuclease free water. Amplification was performed using a C1000 thermal cycler (BioRad, USA) with 95°C, 5 minutes for initial denaturation, 95 °C, 1 minute for denaturation, 54 °C, 45 seconds for annealing, 72 °C, 1 minute for extension and 72 °C, 5 minutes for final extension (30 cycles). The PCR amplicons were checked by electrophoresis on 1.5% low melting agarose gel and purified by gel extraction by using Qiaex II gel extraction kit (Qiagen, Germany) following manufacturers protocol. Then the PCR result was recorded.

Results

Gross lesions

Grossly, the affected lungs were consolidated, haemorrhagic and the lesions were mostly observed in cranio-ventral parts (Fig. 1) but in severe cases infection were more extensive with involvement of diaphragmatic lobe. The consistency of affected lungs was firm and the cut surface usually revealed an irregular, variegated pattern of red, white and grey tissue due to haemorrhage and necrosis. In some cases, there was presence of pneumonic changes (Fig. 2) and in few pleurisy adhesion in lungs were observed. There was also presence of haemorrhages and congestion in visceral organs like liver, spleen and kidney. Haemorrhagic enteritis was also observed in few cases.

Fig 1: Lungs showing severe congestion and hemorrhages predominantly in apical lobe

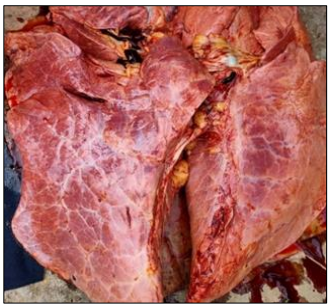

Fig 2: Lungs showing areas of consolidation and red hepatisation

Histopathology

Microscopic study revealed that the affected lungs were dominated by interstitial and alveolar oedema along with vascular thrombosis of capillaries and small blood vessels. The lumen of bronchiole showed presence of fibrinous exudates, cellular debris along with hyperplasia of bronchiolar epithelial cells. Interlobular septa were thickened and the alveoli were filled with inflammatory cells like neutrophils and mononuclear cells (Fig. 3). Characteristic oat shape cells were also observed (Fig. 4). The intestine showed haemorrhagic enteritis i.e. haemorrhages with infiltration of mononuclear cells (Fig. 5). The other visceral organs like liver (Fig. 6), kidney (Fig. 7) and spleen (Fig. 8) showed moderate to severe haemorrhages.

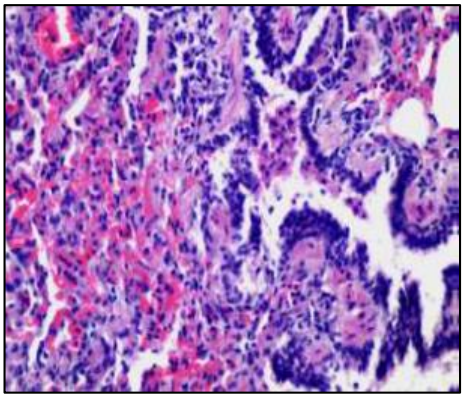

Fig 3: Lungs showing hyperplasia of bronchiolar epithelium with congestion and hemorrhages and bronchiolar lumen containing fibrinous exudates with cellular debris (H & E, 400x)

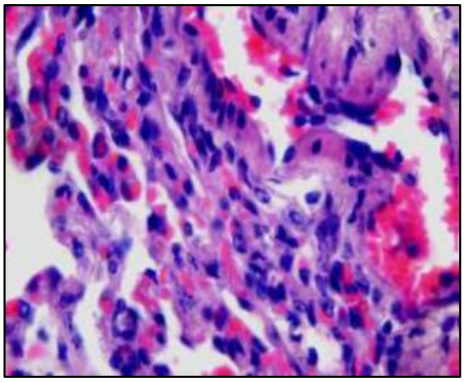

Fig 4: Lungs showing bronchopneumonia with presence of Oat shaped cell (H & E, 1000x)

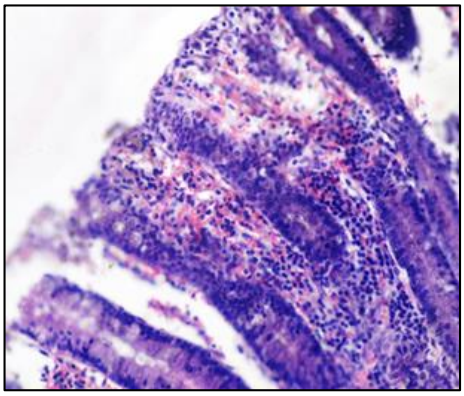

Fig 5: Intestine showing enteritis with hemorrhages and infiltration of mononuclear cells (H & E, 400x)

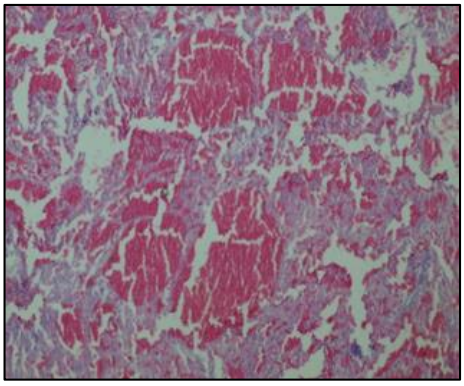

Fig 6: Liver showing severe congestion and hemorrhages (H & E, 200x)

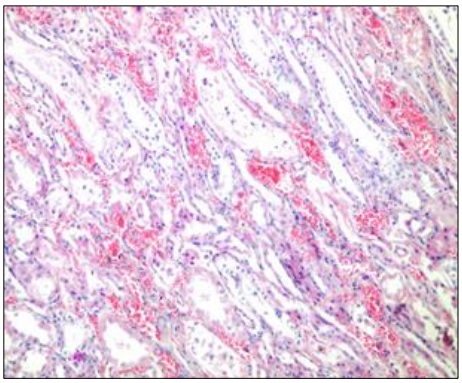

Fig 7: Kidney showing congestion, hemorrhages in intertubular capillaries (H & E, 200x)

Molecular Detection

Out of 30 swabs collected in culture broth from suspected lungs samples, 7 samples were found positive for *Pasteurella sp*. expressing *hyaD-hyaC* gene with amplification at 1044 bp size (Fig. 8).

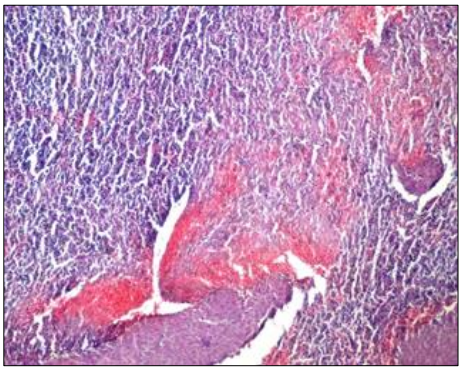

Fig 8: Spleen showing severe hemorrhages (H & E, 100x)

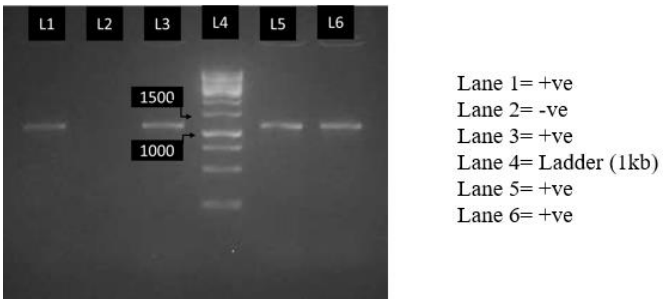

Fig 9: 1.2% Agarose gel electrophoresis stained with Ethidium bromide showing 1044 bp hyaD-hyaC gene fragment of *Pasteurella sp.* in swab of lungs

Discussion

Pneumonic Pasteurellosis is an acute and often fatal epizootic disease of respiratory system of large ruminants (Blood *et al.*, 1983; Jubb *et al.*, 1985) [2, 3]. The disease is caused by *P. multocida*, Gram negative, non-motile, non-spore forming, facultative anaerobic, small rods or coccobacilli commensal in upper respiratory tract (URT) and gastrointestinal tract (Holt *et al.*, 1994; Lopez, 2001; Quinn *et al.*, 2002) [4, 5, 6]. It is most commonly observed in cattle and buffalo but also reported in sheep, horse and camel (Jubb *et al.*, 1985) [3]. Grossly, the lungs of *Pasteurella* infected cattle were consolidated, haemorrhagic and the lesions were mostly observed in cranio-ventral parts but in severe cases infection were more

extensive with involvement of diaphragmatic lobe. There was also presence of haemorrhages and congestion in visceral organs like liver, spleen and kidney. Same type of lesions was previously observed (Doaust, 1989; Holt *et al*., 1994; Brennan *et al*., 1997) [17, 4, 18]. Microscopically, the affected lungs were dominated by interstitial and alveolar oedema along with vascular thrombosis of capillaries and small blood vessels. The lumen of bronchiole showed presence of fibrinous exudates, cellular debris along with hyperplasia of bronchiolar epithelial cells. Congestion and haemorrhages were also observed in the tissue section. Interlobular septa was thickened and the alveoli were filled with inflammatory cells like neutrophils and mononuclear cells. Characteristic oat shape cells were also observed. The intestine from these cattle's showed haemorrhagic enteritis. Haemorrhages were also observed in liver, kidney and spleen. Same type of microscopic changes were reported by De Alwis, 1992; Andrews, 2004; Patrick, 2015 [19, 20, 21]. Out of 30 lungs swab collected from suspected cases 7 were positive for *Pasteurella* organisms which was confirmed by conventional PCR method. PCR detection was done by targeting the gene *hyaD-hyaC* of size 1044 bp (Townsend *et al*., 2001) [16].

Conclusion

Pneumonic Pasteurellosis is an acute and often fatal disease of respiratory system of large ruminants mainly cattle and buffalo. A slaughter house study helps in the assessment of the disease status of herds and also prevents distribution of the infected meat to human being. Abattoir data contain valuable informations about the incidence and epidemiology of animal diseases. During this study period, total 95 tissue samples and 30 swabs were collected. Out of 30 swabs 7 swabs showed positive in molecular detection. Pasteurellosis in slaughtered animals were confirmed based on gross, microscopic and molecular tests.

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