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Histopathology, Isolation and Molecular Detection of *Pasturella multocida* from Sheep in Gadag Region of Karnataka, India

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Bacterial pneumonia is the most common respiratory problem in small ruminants. The heart blood and lung tissue samples from two dead sheep suffering from pneumonia at the age of six month were subjected for histopathology, bacterial isolation & identification and antibiogram. Acute fibrinous bronchopneumonia was observed at necropsy and histopatholgy. The isolates were identified as *P. multocida* based on cultural and biochemical properties, and additionally confirmed by species-specific PCR amplification of *KMT1* (*Multocida* toxin hydrolase gene) gene with the

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amplicon of 460 bp. The antibiogram showed susceptibility to chloramphenicol, β-lactams, quinolones, fluoroquinolones and aminoglycosides. In this study, the occurrence of ovine pasteurellosis caused by *P. multocida* with acute fibrinous bronchopneumonia was recorded.

Keywords: Histopathology; KMT1gene; pasteurellosis; Pasteurella multocida.

1. INTRODUCTION

Pneumonia is the main cause of death of small ruminants with a significant economic loss due to the high mortality among affected animals [1]. *Pasteurella multocida* (*P. multocida*) is an opportunistic pathogen found mainly in the oral cavity, nasopharyngeal and upper respiratory tract of animals [2] that causes severe pneumonia in a wide range of animals [3] including sheep and goats of all ages [4]. Ovine pasteurellosis is characterized by an acute febrile course with severe fibrinous or fibrinopurulent bronchopneumonia, fibrinous pleurisy in adult sheep and septicaemia in suckling lambs that did not receive adequate colostrums [5]. Various virulence elements such as lipopolysaccharide capsule, adhesin, outer membrane proteins, and proteases produced by *P. multocida*, help them to invade the host respiratory system and establish the infection. Predisposing factors for pasteurellosis in animals are stress such as weaning, transport, production, reproduction or introduction of new animals from other farms and weather patterns of certain geographical area, and poor management practices [6]. In view of the above, the present study was carried out to know the histopathology and to diagnose the cause of death in two pneumonic sheep.

2. MATERIALS AND METHODS

2.1 Sample Collection

Two, six month old female, non descriptive sheep from Livestock Farm Complex were presented for necropsy to the Department of Pathology, Veterinary College, Gadag, KVAFSU, Bidar, Karnataka. Heart blood in sterile syringe, lung swabs and pieces of lung were samples collected aseptically.

2.2 Histopathology

The collected pieces of lung were preserved in 10% formal saline and processed by the standard histological technique [7]. Sections about 5µm thick were cut and stained with H & E (haematoxylin and eosin) for histopathological examination.

2.3 Bacterial Isolation and Biochemical Characterization

Samples were inoculated into BHIB (Brain heart infusion broth) (HiMedia, Mumbai) and incubated aerobically at 37 ℃ overnight. The incubated broth was streaked on BHIA (Brain heart infusion agar) and blood agar base (HiMedia, Mumbai) supplemented with 5% defibrinated sheep blood for isolation of organisms. The plates were examined for bacterial growth, colony morphology, color, odour, hemolysis pattern on blood agar and stained by Gram staining technique for morphological features. Isolated colonies were subjected to assays such as catalase, indole, oxidase, urease and streaked on Triple Sugar Iron (TSI) and MacConkey agar (HiMedia, Mumbai) for biochemical characterization [8].

2.4 Molecular Detection by Polymerase Chain Reaction

Genomic DNA was extracted from isolates using a DNA extraction kit (DNeasy Blood and Tissue Kit Qiagen, USA). Genomic DNA was subjected for PCR to identify *P. multocida* by species specific amplification of the *KTM1* gene (*Multocida* toxin hydrolase gene) using published primers (Forward primer 5`-ATCCGCTATTTACCCAGTGG-3` and Reverse primer 5`-GCTGTAAACGAACTCGCCAC-3`) with expected amplicon of 460 bp [9]. PCR was performed with an initial denaturation at 95 ℃ for 5 min, followed by 30 cycles consisting of denaturation at 95 ℃ for 1 min, annealing at 55 ℃ for 1 min, extension at 72 ℃ for 1 min, and final extension at 72 ℃ for 7 min. After PCR, the products were subjected to electrophoresis and examined in a gel documentation system.

2.5 Antibiogram

The antimicrobial sensitivity test was performed using Kirby Bauer disc diffusion method [10] on Muller Hinton agar (Himedia, India) based on standards of the Clinical and Laboratory Standards Institute [11]. The isolates were tested for their susceptibility to the following antibiotics, aminoglycosides (amikacin 30 mcg, gentamicin

10 mcg), β-lactams (cefotaxime 30 mcg, penicillin G 1 unit) quinolones (norfloxacin 10 mcg), fluoroquinolones (ciprofloxacin 5 mcg, enrofloxacin 10 mcg, ofloxacin 5 mcg), lincosamide (clindamycin 2 mcg), amoxicillin-clavulanic acid (10 mcg), amoxicillin-clavulanic acid (20/10 mcg), chloramphenicol (30 mcg), tetracycline (30 mcg)and vancomycin (30 mcg) (Himedia, India). *Escherichia coli* ATCC 25922 was used as a quality control strain. The zone of inhibition was interpreted based on the CLSI guidelines.

3. RESULTS AND DISCUSSION

3.1 Clinical Signs and Autopsy Lesions

Before death, the animals showed clinical signs such as dullness, inappetence, pyrexia with mucopurulent nasal discharge with dyspnoea. At necropsy, the conjunctival mucosa was congested, and cyanotic with petechiae. A thick cyanotic line was present on the gums in the lower jaw. Severe lung congestion, a glistening surface with subpleural and parenchymal haemorrhages and fibrinous exudates on the pleural surface with adhesions to the thoracic wall (Fig. 1) and multiple areas of discoloration were seen. The liver was congested and enlarged with pale areas. The heart vessels were congested by diffuse petechial to ecchymotic haemorrhages on the epicardium (Fig. 2). The spleen and kidney showed congestion. In the small intestine segmental congestion, hemorrhage on the mucosa and serosa with ballooning was observed. The observed changes were similar to the observations of Marru et al. [1], Dar et al. [12], Singh et al. [13] and Mekibib et al. [14].

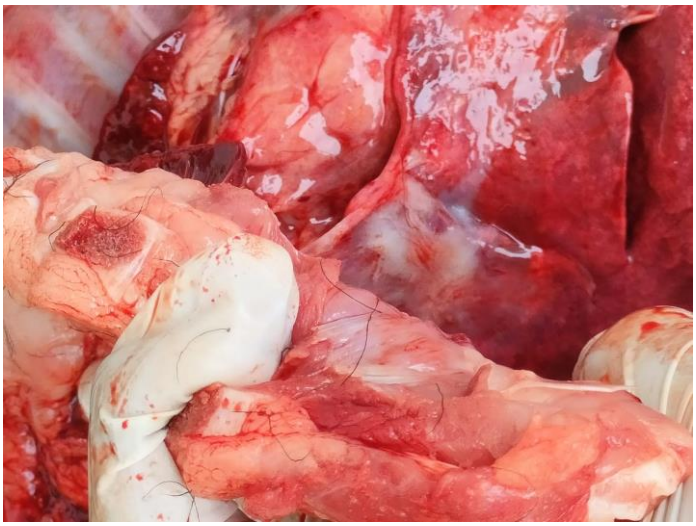

Fig. 1. Lung showing fibrinous adhesion

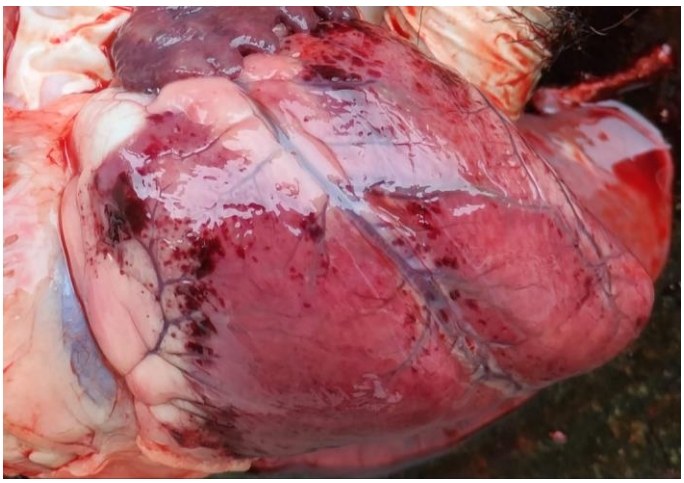

Fig. 2. Heart showing diffuse pin point and ecchymotic haemorrhages on the epicardium

3.2 Histopathology

Histopathology revealed thickening of the visceral pleura, congested vessel, and infiltration of the neutrophils with extensive subpleural necrosis were present (Fig. 3). In the intraalveolar space there was alveolar necrosis, haemorrhage, and brown black haemosiderin pigments, eosinophilic edema fluid with neutrophils and mononuclear cells. Widespread necro-haemorrhagic lesions with lung edema, fibrin (Fig. 4) and desquamation of the bronchiolar epithelia with peribronchial and intra luminal inflammatory exudate were observed. In the liver, diffuse vacuolar changes in the hepatocytes with an narrowing of sinusoidal space and spleen showed congestion, with increased haemosiderin content. The above histopathological findings were also reported by Marru et al. [1], Dar et al. [12], Singh et al. [13] and Mekibib et al. [14]. Gross and histopathological lesions in the present study indicate underlying vascular damage due to septicemia and accompanied inflammation.

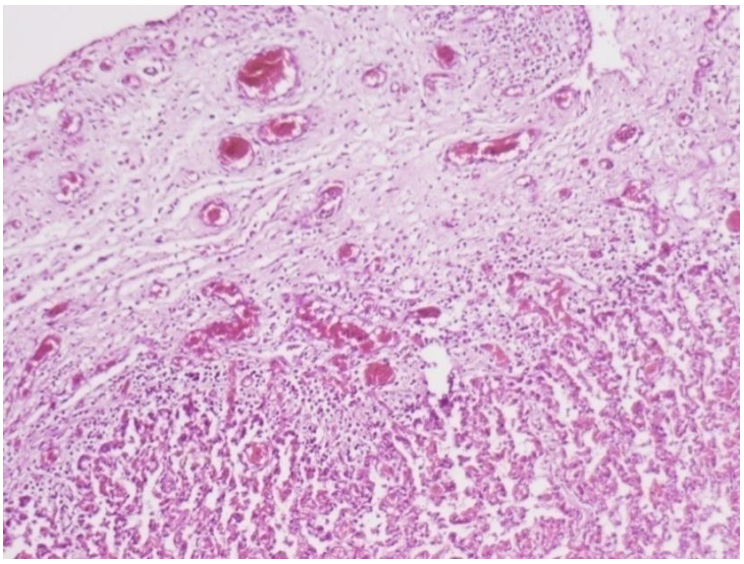

Fig. 3. Showing thickening of the visceral pleura, congested vessels and extensive subpleural necrotic changes

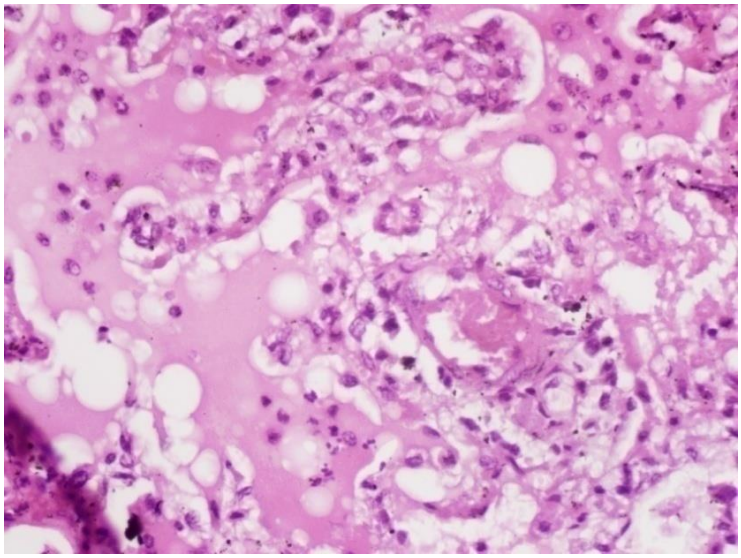

Fig. 4. Showing lung edema intra-alveolar neutrophilic and cell debris, and eosinophilic fibrin deposits

3.3 Identification of Organisms

The *P. multocida* could be identified by phenotypic characteristics such as cultural or biochemical features [5,6]. Colonies on BHIA were small, smooth, white-creamy to grey in color, mucoid, and on blood agar, the colonies were non- hemolytic with the same characteristics as seen on BHIA. In grams staining, neat heart blood smears and colonies, revealed Gram negative coccobacilli with bipolar appearance suggestive of *P. multocida.* The isolates on MacConkey agar showed no growth after 48 hours of aerobic incubation. In the biochemical profile, the isolates were catalase, indole, oxidase positive, urease negative and on TSI slants yellow butt, yellow slant without H_2S production were observed. Based on cultural and biochemical characteristics the isolates were identified as *P. multocida*. In agreement with our findings, many studies have been reported on the isolation and identification of *P. multocida* based on the cultural, biochemical characteristics [13,15-22].

PCR assays can be used to identify *P. multocida* isolates from different hosts, regardless of geographic location [23]. The PCR products on electrophoresis revealed the amplicon of 460 bp (Fig. 5) which was specific for *KTM1* gene of *P. multocida* organism. Many researchers [9,21,22,24] confirmed *P. multocida* by species-specific amplification of *KTM1* gene by the PCR assay which was comparable with our results.

3.4 Antibiogram

The isolates showed complete susceptibility to chloramphenicol (30 mcg), cefotaxime (30 mcg), ciprofloxacin (5 mcg), enrofloxacin (10 mcg), gentamicin (10 mcg), norfloxacin (10 mcg), penicillin G (1 unit) and intermediate susceptibility to amikacin (30 mcg) (Fig. 6). Contrary to our findings, studies [1,22] showed complete resistance to penicillin G, gentamicin and recorded intermediate sensitivity to gentamicin, cefotaxime, ciprofloxacin, enrofloxacin and norfloxacin [13,20]. However, the result of intermediate susceptibility to amikacin was in accordance with the findings of Singh et al. [13] and Rawat et al. [20]. In the present study, antibiotics amoxicillin-clavulanic acid (10 mcg), amoxicillin-clavulanic acid (20/10 mcg), vancomycin (30 mcg), ofloxacin (5 mcg), clindamycin (2 mcg) and tetracycline (30 mcg) showed complete resistance. However, Singh et al. [13] andRawat et al. [20] have recorded complete susceptibility to amoxicillin-clavulanic acid and tetracycline. Reduced susceptibility to tetracyclins and amoxicillin could be a consequence outcome of frequent use of these antibiotics on farms and in field conditions.

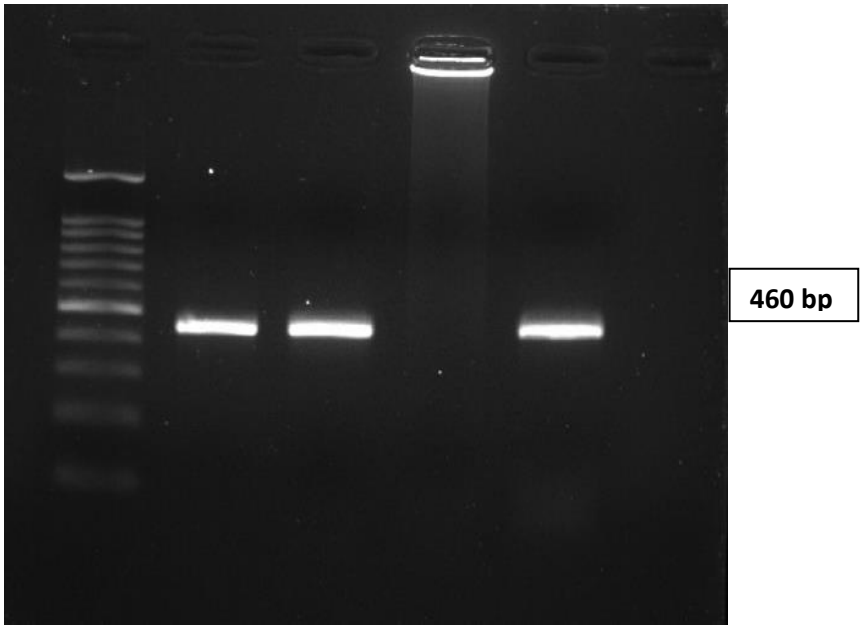

Fig. 5. PCR amplification of *KMT1* gene of *P. multocida*

(M: 100 bp Marker, Lane 1: Sample 1, Lane 2: Sample 2, Lane 3: Negative control, Lane 4: Positive control, Lane 5: Non template Control)

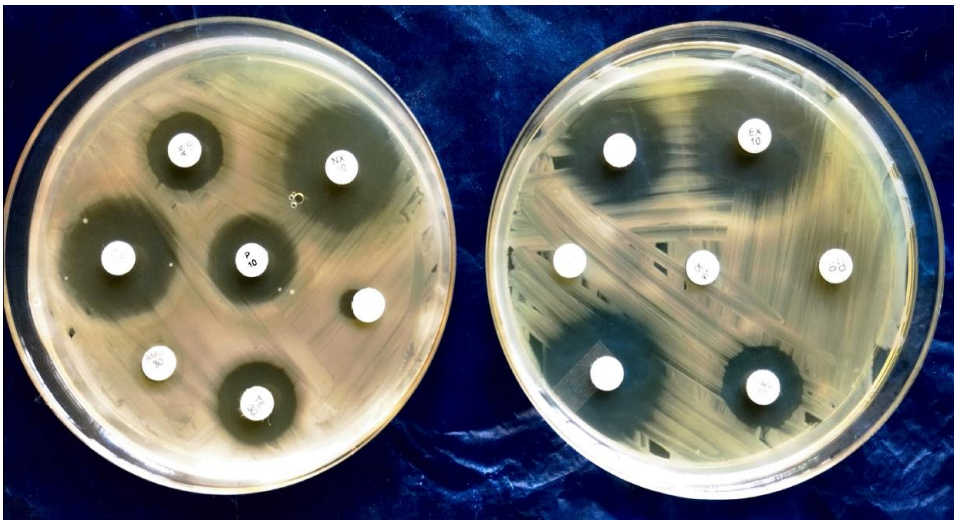

Fig. 6. Antibiogram of *Pasturella multocida*isolates

4. CONCLUSION

Acute fibrinous bronchopneumonia was the prominent post mortem finding recorded in two dead sheep with suspected pasteurellosis. *P. multocida* was isolated, identified from pulmonary tissues and heart blood by cultural, biochemical tests and confirmed by PCR assay. The antibiogram showed sensitivity to chloramphenicol, β-lactams, quinolones, fluoroquinolones and aminoglycosides.

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COMPETING INTERESTS

Authors have declared that they have no known competing financial interests or non-financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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