Molecular detection of virulent *Mannheimia haemolytica* and *Pasteurella multocida* in lung tissues of pneumonic sheep from semiarid tropics, Rajasthan, India

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**Abstract:** The present study was planned to detect the genetic elements of *Mannheimia haemolytica* and *Pasteurella multocida* in pneumonic sheep lungs. Pneumonia was diagnosed on the basis of gross pathological lesions. Lung tissues were collected at necropsy of sheep (n = 96) and subjected to isolation of total DNA. The *M. haemolytica*-specific PHSSA and Rpt2 genes, and the *P. multocida*-specific KMT1 and the Omp87 genes were amplified using polymerase chain reaction (PCR). A housekeeping gene targeting the sheep cellular mitochondrial 12S ribosomal DNA was used as an internal control. PCR reactions were optimized using the positive and negative controls. Gene-specific PCR products were subjected to nucleotide sequencing for confirmation. The pneumonic lungs showed congestion and hemorrhagic changes with consolidation, which was most evident in the whole of the apical lobes and parts of the diaphragmatic lobes. PCR amplification showed detection of PHSSA (327 bp) and Rpt2 (~1022 bp) genes specific to *M. haemolytica* in 52 (54.1%), and the KMT1 (457 bp) and Omp87 (2627 bp) genes specific to *P. multocida* in 16 (16.6%) lung samples. Sequence analysis confirmed the PCR products for specific genes. This study highlighted the culture-independent, rapid, and confirmatory diagnosis of ovine pneumatic pasteurellosis caused by *M. haemolytica* and/or *P. multocida*.

**Key words:** Bronchopneumonia, lung, *Mannheimia haemolytica*, *Pasteurella multocida*, sheep

1. **Introduction**

Ovine pneumonia has a significant economic impact in India, where a diverse ovine germplasm with 65.07 million sheep out of 1172.833 million sheep heads globally contributes to 5.54% of world sheep population (1,2). It is developed by a complex interaction between environmental factors, pathogens, and host body defense (3). *Mannheimia haemolytica* may act as a primary cause of ovine pneumatic pasteurellosis. However, *Pasteurella multocida* plays an important role in the development of bronchopneumonia in adult sheep and septicemia in nursing lambs (4). These bacteria are normal inhabitants of the respiratory system of healthy sheep. However, they may become opportunistic pathogens due to certain predisposition and stress factors leading to the development of clinical pneumonia. Different studies revealed occurrence of ovine pneumonia associated with *M. haemolytica*. However, reports on *P. multocida* related to ovine pneumatic pasteurellosis are scanty despite its proven role in the development of lamb respiratory diseases (5). In India, limited studies have been documented on ovine pneumonia associated with *M. haemolytica* (6). Pneumonic pathology due to these microorganisms has been recorded most often during the necropsy of dead sheep and is evident by congestion and consolidation of the lung tissue. Failure to diagnose ovine pneumonia during antemortem examination of the infected animals and gap in the accurate information related to the onset of lesions restrict our knowledge for its strategic control (7). Diagnosis of pasteurellosis has been conventionally based on the clinical symptoms of pneumonia, isolation and phenotyping, and capsular serotyping of the causative pathogens, which is time-consuming. However, the introduction of advanced genotypic methods that are more sensitive and specific helps to identify the bacteria and their genetic elements more accurately and rapidly (8,9). Therefore, the present study was designed to detect the specific genetic components of *M. haemolytica* and *P. multocida* directly from the lung tissues of dead pneumatic sheep.

2. **Materials and methods**

2.1. **Study area and overview of pneumonia**

The sampling area included the organized farms of ICAR-Central Sheep and Wool Research Institute, Avikanagar, Tonk, Rajasthan, India which comes under the agro-
climatic zone of semiarid eastern plain. It is located at 26.28°N, 75.38°E with an average elevation of 132 m. This region comes under the dry climate which has short southwest monsoon season from June to mid-September. Due to the semiarid climate, the pasture land is dry and dusty with scanty grass and fodder growth except the monsoon season. The animals graze in the pasture land during daytime by travelling through different pasture fields. The animals, in the evening, come back to the shed where they are provided with chopped fodder and formulated concentrates. There are different sheep breeds including Malpura, Avikalin, Patanwadi, Garole, and their crosses maintained at the farms.

Ovine pneumonia was the most prevalent disease condition leading to high mortality of sheep at farms. It affected animals irrespective of their age and breed. The affected animals showed coughing, sneezing, and respiratory distress. The animals appeared dull, depressed, and anorectic, and with respiratory grunts in the advanced stage of the disease. The clinical course of the disease was acute and short leading to sudden death of the lambs. The animals that survived the acute course of the disease became chronically ill with poor performance.

2.2. Collection and processing of tissue samples
Lung tissue samples were aseptically collected at the time of postmortem examination of dead pneumonic sheep (n = 96) from December 2014 to March 2016. All the samples were brought immediately to the laboratory for further investigation. About 0.5 g of each lung tissue sample was triturated individually in a sterile mortar and pestle. A homogenous tissue suspension was made with 1 mL of autoclaved distilled water. The tissue suspension was collected in 1.5-mL autoclaved Eppendorf plastic tubes (Hamburg, Germany) for isolation of DNA.

2.3. Isolation of DNA from lung tissues
Each of the tissue homogenates (200 µL) was used to isolate the total DNA using DNeasy Blood and Tissue Kit (Qiagen, Germantown, MD, USA) as per manufacturer’s instructions. Simultaneously, the genomic DNA was also isolated from Escherichia coli ATCC 25922 and M. haemolytica and P. multocida strains (maintained at the laboratory) and used as negative and positive controls, respectively, for PCR reactions. The extracted DNA samples were quantified using NanoDrop UV spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and stored at −20 °C until use.

2.4. PCR amplification
The polymerase chain reaction (PCR) was performed in peqSTAR 96 Universal Gradient thermocycler (Peqlab Biotechnology, Erlangen, Germany). PCR amplification was carried out using the specific primers (Sigma-Aldrich, St. Louis, MO, USA) to detect the different genes (Table). The reaction mixtures and amplification conditions were optimized for all the genes. Both the positive and negative control DNA samples were used for PCR reactions. The presence of M. haemolytica and P. multocida was confirmed by species-specific amplification for PHSSA and KMT1 gene, respectively. The PHSSA-positive tissue DNA samples were subjected to further identification by amplification of the methyltransferase (Rpt2) gene. Moreover, the Omp87 gene coding for outer membrane protein-87 was identified as specific to P. multocida. A housekeeping gene targeting the sheep cellular mitochondrial 12S ribosomal DNA was amplified as an internal control along with the bacterial genes. For PCR amplification, 50 ng of DNA was added to 20-µL reaction mixture containing 200 µM of dNTPs, 0.2 µM of each primer, 1.875 mM of MgCl₂, and 1 U of Taq DNA polymerase (Sigma-Aldrich) in 1X PCR buffer.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>Amplicon size (bp)</th>
<th>Annealing temp.</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHSSA</td>
<td>F-5’TTCACATCTTCATCCCTC 3’ R-5’TTTTCATCCTCTTCGTC 3’</td>
<td>327</td>
<td>48</td>
<td>10</td>
</tr>
<tr>
<td>Rpt2</td>
<td>F-5’TTCACATCTTCATCCCTC 3’ R-5’TTTTCATCCTCTTCGTC 3’</td>
<td>~1022</td>
<td>48</td>
<td>11</td>
</tr>
<tr>
<td>KMT1</td>
<td>F-5’TTCACATCTTCATCCCTC 3’ R-5’TTTTCATCCTCTTCGTC 3’</td>
<td>457</td>
<td>56</td>
<td>12</td>
</tr>
<tr>
<td>Omp87</td>
<td>F-5’ACCTCGGTTTTATGGCATTG 3’ R-5’CTTATTAGAACGTCCCACCA 3’</td>
<td>2627</td>
<td>56</td>
<td>This study (from U60439.1)</td>
</tr>
<tr>
<td>12S rRNA</td>
<td>F-5’TACCCCTTGMCCCTTTTGSATRRK 3’ R-5’AGACTAACCTTTAAGATACGTTGG 3’</td>
<td>270</td>
<td>48</td>
<td>13</td>
</tr>
</tbody>
</table>
The PCR conditions for PHSSA, Rpt2, and sheep-specific 12S ribosomal DNA included initial denaturation at 95 °C for 3 min followed by 35 cycles consisting of denaturation at 95 °C for 1 min, annealing at 48 °C for 1 min, extension at 72 °C for 30 s, and the final extension at 72 °C for 5 min. PCR conditions for KMT1 and Omp87 gene included initial denaturation at 95 °C for 3 min followed by 35 cycles consisting of denaturation at 95 °C for 45 s, annealing at 56 °C for 45 s, extension at 72 °C for 1 min for KMT1 and 2 min for Omp87 and the final extension at 72 °C for 5 min. The amplified PCR products (5 µL) were separated in agarose gel (1.5% w/v) stained with ethidium bromide (0.5 µg/mL) by running in horizontal submarine electrophoresis unit using 1X (TAE) as running buffer and examined under the gel documentation system (UVP , Upland, CA, USA).

2.5. Nucleotide sequencing

The amplified PCR products of all the targeted bacterial genes were subjected to nucleotide sequencing for confirmation. For nucleotide sequencing, the samples were amplified using Pfu polymerase. The amplified PCR products (50 µL) were resolved in 1.5% agarose gel and stained with ethidium bromide, electrophoresed, and illuminated under a UV transilluminator. UV-visualized gene-specific PCR bands in the gel were excised with the help of a clean B.P. blade and kept in 1.5-mL Eppendorf tubes. PCR products in the excised gel were purified by using a gel purification kit (Qiagen) as per manufacturer’s instructions. The PCR products were eluted in 40 µL of elution buffer, quantified using NanoDrop UV spectrophotometer (Thermo Fisher Scientific) and the DNA concentration was equalized at ~100 ng/µL and stored at −20 °C until use for bidirectional Sanger’s sequencing (SciGenom Labs, Kochi, India).

3. Results

The postmortem examination showed congestion and petechial and ecchymotic type of hemorrhages in the lung tissues of dead sheep (Figures 1 and 2). Patchy to diffuse, reddish-brown to greyish-red areas of consolidation and hepatization of lungs was predominantly recorded on gross pathological examination. Consolidation was recorded in the whole of the apical lobes and parts of the diaphragmatic lobes. The pathological changes were most commonly recorded in apical, cardiac, and diaphragmatic lobes of the lungs. The incised surfaces of the lung tissue showed straw-yellow colored mucus and release of frothy fluid from the bronchi and bronchioles. Furthermore, the chronic and complicated cases were evident with supplicative lesions and small abscesses in the lung tissue. Also, there was hard texture of the lung tissue that was found adhered to the diaphragm and thoracic wall. The pleural membrane was thickened. The tracheal rings were hemorrhagic with mucoid froth in the tracheal lumen. Besides the lungs and trachea, the petechial hemorrhagic changes were also seen on the other organs such as heart, spleen, and kidneys. Moreover, the pneumonic animals also showed hepatic and intestinal congestion.

PCR reactions revealed amplification of PHSSA (327 bp) and Rpt2 (~1022 bp) genes (Figure 4) specific to M. haemolytica in 52 (54.1%) of the lung tissue samples. The KMT1 (457 bp) (Figure 5) and Omp87 (2627 bp) (Figure 6) genes specific to the P. multocida were detected in 16 (16.6%) of the tissue samples. The genetic elements of both M. haemolytica and P. multocida were recorded together in 10 (10.4%) lung tissues. However, 42 (43.75%) and 6 (6.25%) of the lung tissues showed an exclusive presence of M. haemolytica and P. multocida, respectively. A sheep-specific 12S ribosomal DNA product of 270 bp was amplified from all the lung tissue samples.

Variability in the nucleotide sequences of Rpt2 (methyltransferase) gene was recorded which indicated the colonization of lungs with diverse strains or serotypes.
Furthermore, the variability due to single nucleotide substitution in the nucleotide sequences of Omp87 gene was also recorded. The nucleotide sequences have been submitted to NCBI GenBank database with their respective accession numbers (MF776876.1, MF776877.1, MF776878.1, MF776879.1, MF776880.1, MF776881.1, and MF776882.1).

4. Discussion

Ovine pneumonic pasteurellosis caused by M. haemolytica and/or P. multocida is a fatal disease of sheep which requires immediate attention. Specific and rapid diagnosis of pneumonia is essential for control of this disease at the farm level. In acute cases, the clinical course of pneumonic pasteurellosis is short and many times it goes unnoticed due to the high virulence of the causative agents and severity of the disease. Postmortem examination explores different types of pathological changes developed in the respiratory system of the affected animals for the presumptive diagnosis of ovine pneumonia. The severity of the pathological lesions in the lungs and trachea may vary depending on the virulence of invading bacterial strains and immunity of the host and the prevailing environmental conditions of a particular geographical area.

The pathological lesions of pneumonic lung tissues in the present study were similar to those in the previous studies on pasteurellosis (14,15). Akloul and Menoueri (16) also recorded consolidation in the whole of the apical lobe of the pneumonic lung with bacterial infections, which supports the findings of the present study. Presence of petechial hemorrhages on different organs viz. epicardium, spleen, and kidneys indicated septicaemia. Watson and Davis (4) also reported septicemia of neonatal lambs due to P. multocida infection.
At necropsy, pneumonia is usually diagnosed on the basis of gross lesions in the lungs of the dead animals. This trend of disease diagnosis by practicing veterinarians is adapted in the sheep flocks of rural areas. Hence, an early diagnosis of infectious diseases like pneumonia is very important for record keeping and National Disease Reporting System. However, due to the laborious and time-consuming methodology of Pasteurella sp. and Mannheimia sp. isolation and the lack of confirmatory diagnostic techniques, a specific disease recording could not be established in the field. Moreover, the pathology of pneumonia cannot be known exactly due to a diverse interaction of different causative agents with the respiratory system (17). Furthermore, the respiratory tract is more vulnerable to injurious factors because of various noxious agents, agro-climatic changes, and an increase in the incidence of viral respiratory diseases (18). Therefore, due to the multiple etiological occurrences of ovine pneumonia, it is important to identify the type of specific pathogen using molecular detection techniques. Though pneumonia in small ruminants is primarily caused by viral agents such as Parainfluenza 3 virus and, Respiratory syncytial virus, or Mycoplasma ovipneumoniae (19) and is predisposed by an extreme of environmental insults, M. haemolytica, a most frequently isolated bacterial pathogen, is considered to be the main cause of the disease (20). Among bacterial causes, P. multocida, compared to M. haemolytica, develops less severe respiratory pneumonic pasteurellosis in sheep; it contributes to the incidence of disease in field conditions (5). However, M. haemolytica was the most common organism associated with ovine pneumonic lung tissues in the present study similar to the earlier reports (11,14).

The PHSSA resembles the virulence markers of M. haemolytica (21). Due to the presence of serotype 1-specific antigen (ssa1) gene, the commensal M. haemolytica strains may become pathogenic to their host in stress-prevailing environments. Moreover, a genetic correlation between ssa1 and leukotoxin (lkt) indicates that the PHSSA could have a significant pathobiological effect in the progression of pneumonic pasteurellosis (22,23). Therefore, the PHSSA represents a species-specific and virulence-associated gene of M. haemolytica. Furthermore, the species-specific Rpt2 locus of M. haemolytica may possibly modulate the type III restriction-modification system which acts as a barrier to the introduction of foreign DNA (24). Thus, the PHSSA and Rpt2 genes are the important virulence markers responsible for the pathogenic potential of M. haemolytica to develop ovine pneumonia.

Similarly, the presence of P. multocida infection was specifically identified by amplification of KMT1 gene (9). The Omp87 (previously oma 87) gene encoding for an immunodominant protein of 87 kDa on the surface of P. multocida serotypes was also detected (25). The Omp87 protein is also known as an important adhesin for P. multocida (26). The outer membrane proteins (OMPs) of these gram-negative bacteria play an important role in the progression of ovine pneumatic pasteurellosis. They are involved in the process of nutrient uptake by the bacteria, transport of molecules in and out of the bacterial cell, colonization and invasion of the host, evasion of the host immune response, and injury to the host tissue and thus aid the development of productive infection (27).

Variability in the nucleotide sequences of Rpt2 gene of M. haemolytica and the Omp87 gene of P. multocida indicated the acquisition of multiple bacterial strains in the flock possibly from different sources during the interaction of animals. The cross-sharing of bacterial strains by the animals provides a broad opportunity to pathogens for their survivability and evolution.

The present study concluded that ovine pneumonia was associated with mortality of sheep. Acute bronchopneumonia was predominantly recorded on pathological examination of dead sheep. Virulent M. haemolytica and P. multocida were detected in lung tissues of sheep affected by bronchopneumonia. Molecular detection of genetic components of M. haemolytica and P. multocida directly from lung tissues indicated the bacterial-culture-independent, rapid, and confirmatory diagnosis of pneumonia pasteurellosis and/or mannheimiosis in sheep.

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References


