TÜBİTAK

Turkish Journal of Veterinary and Animal Sciences

http://journals.tubitak.gov.tr/veterinary/

Research Article

Turk J Vet Anim Sci
(2018) 42: 556-561

doi:10.3906/vet-1802-73

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

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Received: 23.02.2018 • **Accepted/Published Online:** 04.10.2018 • **Final Version:** 10.12.2018

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 pneumonic 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 pneumonic 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 pneumonic 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 pneumonic 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-

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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 $MgCl_2$, and 1 U of Taq DNA polymerase (Sigma-Aldrich) in 1X PCR buffer.

Table. Primer sequence of different genes.

Gene	Primer sequence	Amplicon size (bp)	Annealing temp.	Reference
PHSSA	F-5`TTCACATCTTCATCCTC 3` R-5`TTTTCATCCTCTTCGTC 3`	327	48	10
Rpt2	F-5`TTCACATCTTCATCCTC 3` R-5`TTTTCATCCTCTTCGTC 3`	~1022	48	11
KMT1	F-5`TTCACATCTTCATCCTC 3` R-5`TTTTCATCCTCTTCGTC 3`	457	56	12
Omp87	F-5`ACCTCGGTTTTATGGCATTG 3` R-5`CTTATTAGAACGTCCCACCA 3`	2627	56	This study (from U60439.1)
12S rRNA	F-5`TAACCCTTGTMCCTTTTGSATRRK 3` R-5`AGACTAACTTTTAAAGATACAGTGGG 3`	270	48	13

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 suppurative 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

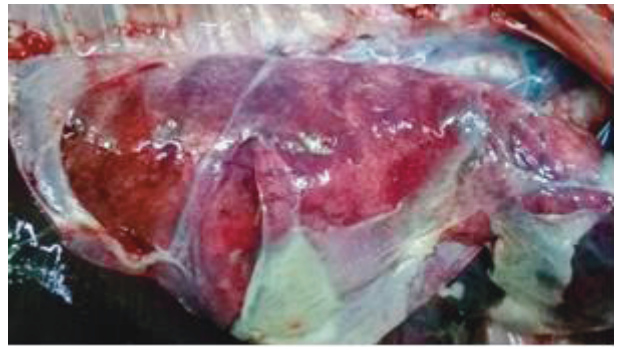

Figure 1. Congested, hemorrhagic, and consolidated diaphragmatic lobe of lung.

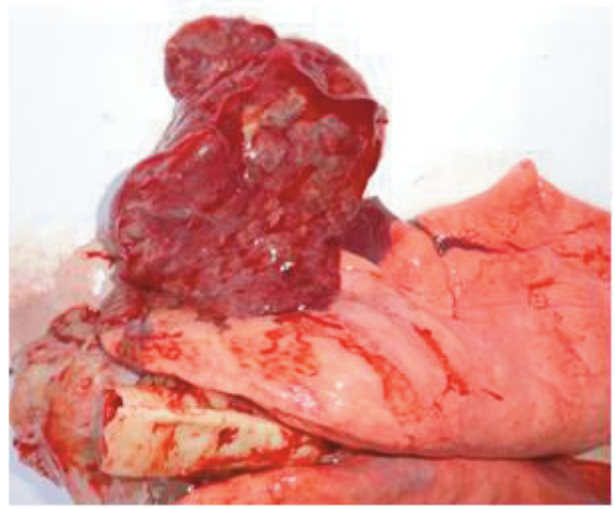

Figure 2. Hemorrhagic and consolidated apical lobe of lung.

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

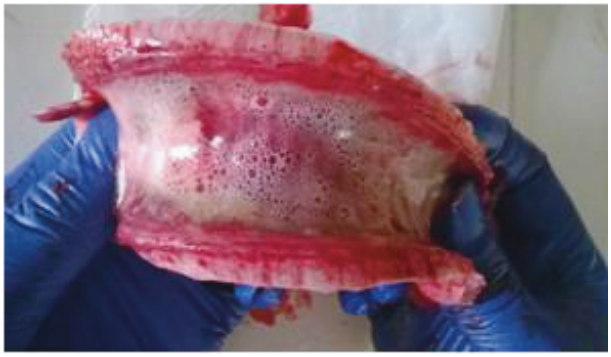

Figure 3. Congested and hemorrhagic trachea filled with mucus and froth.

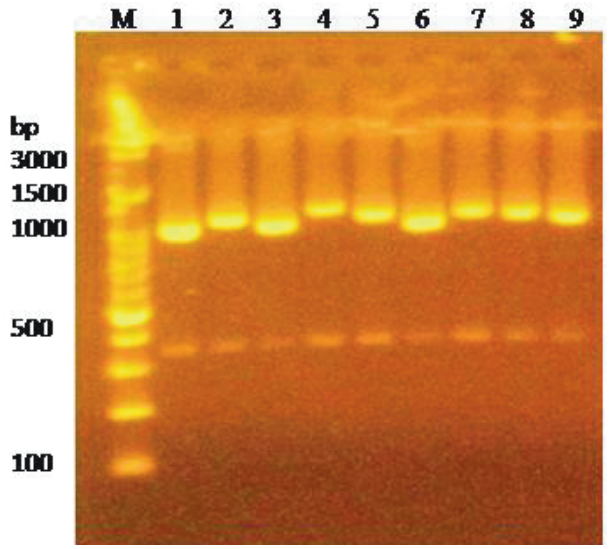

Figure 4. Amplification of *Rpt2* (~1022 bp) and *PHSSA* gene (327 bp) of *M. haemolytica.* Lanes 1–9 denote test-positive. Lane M: ladder.

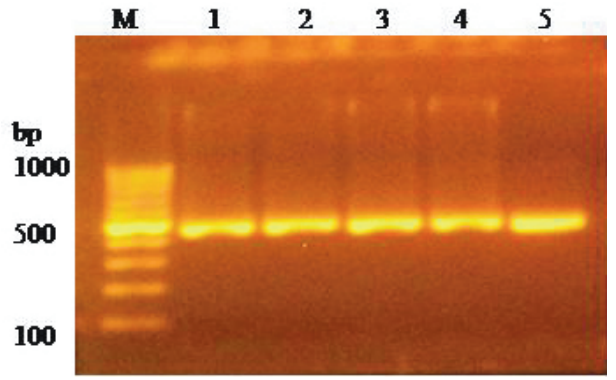

Figure 5. Amplification of *KMT1* gene (457 bp) specific to *P. multocida.* Lanes 1–5 denote test-positive. Lane M: 100 bp DNA ladder.

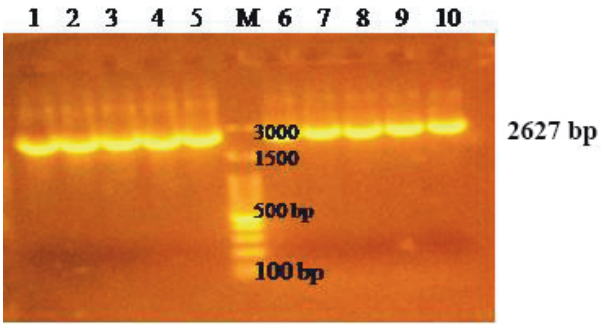

Figure 6. Amplification of full coding region of *Omp87* gene (2627 bp) of *P. multocida.* Lanes 1–5 and 6–10 denote test-positive. Lane M: ladder.

of *M. haemolytica*. 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 septicemia. 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 pneumonic 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 pneumonic pasteurellosis and/or mannheimiosis in sheep.

Acknowledgment

The authors are thankful to Dr. Dhirendra Singh, head of the Animal Health Division and the director of ICAR-Central Sheep and Wool Research Institute in Rajasthan for providing the necessary facilities to conduct the research.

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