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Pathomorphological and Molecular Studies of Respiratory Mannheimiosis in Goats

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ABSTRACT

Background: *Mannheimia* is a Gram-negative, facultative anaerobe, non-spore forming and non-motile bacteria belongs to the family Pasteurellaceae. Caprine bacterial pneumonia is most often caused by *Mannheimia haemolytica* (previously known as *Pasteurella haemolytica*) and *Pasteurella multocida* which are more frequently associated with the outbreak of acute pneumonia and death of goats, particularly young animals. *Mannhemia hemolytica* is an opportunistic pathogen that particularly inhabits the nasopharynx and tonsils of goat and sheep. The present study was aimed to study *Mannheimiosis* in Guwahati, Assam in order to diagnose and control the disease among goats.

Methods: The present investigation was conducted during the year 2021-2022 in Guwahati, Assam. A total of 51 pneumonic lung samples were collected from nearby slaughter houses and during necropsy at Department of Pathology, C.V.Sc., A.A.U., Khanapara, Guwahati-22. All the samples were subjected to primary isolation in blood agar and MacConkey's agar and further confirmation was made using PCR.

Result: Out of total 51 pneumonic lung samples examined seven (13.72%) cases were found positive for *Mannheimia haemolytica* infection whereas 5 cases were found to be associated with other respiratory pathogens. Visible gross lesions observed during necropsy were broncho-interstitial pneumonia, suppurative pneumonia and fibrinous pneumonia with hemorrhagic tracheitis. Histopathological examination revealed presence of characteristic oat cells, fibrin clots, oedema, necrosis and polymorphonuclear cells infiltration in the lungs. Bacteriological culture for isolation and identification of *Mannheimia haemolytica* showed â haemolytic zone on MLA plates. Confirmatory diagnosis was made using PCR targeting *16s rRNA* gene with amplicon size 1500 bp and *Lkt gene* with amplicon size 206 bp. The phylogenetic analysis of *16s rRNA* gene from positive sample showed percent identity of above 97% with other strains of *Mannheimia haemolytica* present in NCBI website. The variability of lesions observed in the present study that contributed to the development of pneumonia rather than *Mannheimia haemolytica* alone and PCR could be used as a reliable technique for confirmatory diagnosis of respiratory mannheimiosis.

Key words: Goat, Isolation, Mannheimia, Pathology, PCR.

INTRODUCTION

Goat farming is one of the well-established livestock sectors in India, progressively gaining widespread popularity among landless, marginal farmers in the country. Goat has significant role in providing supplementary income and livelihood to millions of poor farmers and landless labourers of rural India who cannot afford to rear large farm animals like cows and buffaloes. Productivity of dairy goats largely depends on their good health and protection from diseases (Verma, 2013; Barde, 2016). Caprine bacterial pneumonia is most often caused by *Mannheimia haemolytica* (previously known as *Pasteurella haemolytica*) and *Pasteurella multocida* which are more frequently associated with the outbreak of acute pneumonia and death of goats, (Rawat *et al*., 2019) particularly young animals. *Mannhemia haemolytica* is an opportunistic pathogen that particularly inhabits the nasopharynx and tonsils of goat and sheep. They produce disease in immunocompromised animals due to several stress factors. *Mannheimia haemolytica* is a Gram-negative, facultative anaerobe, non-spore forming and non-motile bacteria belongs to the family Pasteurellaceae.

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Presumptive diagnosis of pneumonic pasteurellosis is made based on gross, histopathology, cultural and biochemical characteristics. However, molecular methods are required for confirmatory bacterial identification (Rawat *et al*., 2019, Dutta *et al*., 2020). Goat is an important food animal for sustaining the livelihood of rural families in Assam and goat farming is predominantly practiced across the state as a source of livelihood. In such situation outbreak of bacterial infection particularly pneumonia in goats causes severe economic losses. Looking at the importance of goat which often affected with respiratory illness, it becomes important to have an insight of bacterial infection with a special reference to *Mannheimia* spp., so that control and treatment measures could be practiced effectively.

MATERIALS AND METHODS

Sample collection and study period

The study was conducted for a period of one year from March, 2021 to February 2022. A total of 51 suspected lungs samples were collected from nearby slaughter houses and during necropsy at Department of Pathology, C.V.Sc., A.A.U., Khanapara, Guwahati-22.

Gross and histopathology

For histopathological examination, samples were collected in 10% formal saline solution and processed, sectioned and stained following standard procedure adopted by Luna (1968). Duplicate sections were stained with Brown and Brenn stain for demonstration of bacterial organism in the tissue following standard protocol (Culling, 1974).

Bacteriological studies

Sample processing and microbiological examination

Infected lung tissue aseptically collected in a sterile zip bag, a loopful colony from that tissue was streaked in brain heart infusion (BHI) broth near a flammable lamp. For microbiological isolation, goat respiratory tract tissues were also aseptically obtained and immediately placed in 0.9 percent sterile normal saline. BHI broth having lung and respiratory tract tissues was then incubated for 24 hours at 37°C. A loop full colony was inoculated using the streak plate method on blood agar, MacConkey's agar and BHI agar and then incubated at 37°C (24-48 hours). Following incubation, the colonial morphology of isolated colonies was examined and identified. The colonies were then stained using the Gram's staining method and the Methylene blue staining method for demonstration of bipolar characteristic of the organism. The stained colonies were examined under the 100X lens of a light microscope. Small colonies that were gram negative with rods or coccobacilli and bipolar appearance suggestive of *Mannheimia haemolytica* were picked up and subculture onto MacConkey agar plates for purification and preliminary identification of the isolates. Suspected colonies of *Mannheimia haemolytica* isolates were preserved for further study in 80% glycerol stock in Nutrient Agar broth at -20°C. In order to conduct a biochemical test, single isolated colony was subcultured on nutrient agar using the methods outlined by Barrow and Feltham (1993).

Biochemical characterization for *Mannheimia haemolytica* isolates

Various biochemical test such as catalase test, oxidase test, distinctive odour test, urease activity test, lactose test was performed following standard protocol (Steel, 1962; Susmitha, 2019; Mohamed *et al*., 2018).

PCR confirmation of *Mannheimia haemolytica*

In culturally and biochemically positive isolates *of Mannheimia haemolytica*, were confirmed by PCR amplification.

Extraction of bacterial DNA

The template DNA extracted from suspected colonies were subjected to PCR targeting *16s rRNA* and *Lkt* gene using the specific primers (Tan *et al*., 2016) (Tabatabaei and Abdollahi, 2018). The isolated colonies of the culture were centrifuged at 5000 rpm for five minutes. The supernatant was discarded. The pellet was washed with 100 µl nuclease free water, resuspended and vortexed followed by centrifugation at 5000 rpm for 5 minutes. The supernatant was discarded, 50 to 70 µl nuclease free water was added in the pellet and votexing was done. It was transferred to dry bath at 100°C for 30 minutes. Then the sample was kept at -20°C for 10 minutes. Again, centrifugation was done at 12000 rpm for 10 minutes. The resulted supernatant collected in fresh tube was DNA. Details of oligonucleotide primer sequences for 16s rRNA gene are depicted in Table 1. The forward and reverse primers used in this study were commercially procured. The extracted DNA samples were quantified using NanoDrop UV spectrophotometer and stored at -20°C until use.

PCR amplification

The polymerase chain reaction (PCR) was performed in a thermocycler (Applied Biosystems). PCR amplification was carried out using the specific primers to detect the different genes. 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. For PCR amplification of 16sRNA gene, 4 µl of DNA template, 1 µl of each primer and 12.5 µl of 2X Green Taq Master mix and 6.5 µl of Nuclease free water added to prepare 25 µl of reaction mixture. For PCR amplification of *Lkt* gene, 5 µl of DNA template, 1 µl of each primer, 12.5 µl of 2X Green Taq Master mix and 5.5 µl of Nuclease free water added to prepare 25 µl of reaction mixture.

The PCR conditions for 16s rRNA included initial denaturation at 94°C for 3 min followed by 40 cycles consisting of denaturation at 94 °C for 30 secs, annealing at 55°C for 30 secs, extension at 72°C for 1 min and the final extension at 72°C for 10 mins. PCR conditions for Lkt gene included initial denaturation at 94°C for 40 secs followed by 35 cycles consisting of denaturation at 94°C for 40 secs,

annealing at 50°C for 40 secs, extension at 72°C for 40 secs and the final extension at 72°C for 6 min. The amplified products were confirmed by agarose gel electrophoresis, using 1.5% agarose containing ethidium bromide in 1X tris-Acetic acid- EDTA (TAE) buffer (40 mM Tris-HCl, 1 mM EDTA and 0.1 per cent glacial acetic acid with pH8). Electrophoresis was carried out at 80-100 V for 1-2 hrs. The gel was visualized under UV light in Gel Doc System (BioRad, USA) and images were captured by Imagelab software.

RESULTS AND DISCUSSION

Gross and histopathology

Most common gross lesions recorded in pneumonic lungs was consolidation followed by areas of emphysema, congestion and hemorrhage, edema and pleural adhesion. The right cranial lobe was found to be the most affected during the present study. This might be due to presence of an additional apical bronchus which enters the right cranial lobe at the level of third rib and through which infection enters. Consolidation was observed at the adjacent surface of the nearby lobes close to each other like right cranial and medial lobe (Fig 1). This might be due to spread of infection by endobronchial way or spread by direct contact to the adjacent lobe (Ozyildiz *et al*., 2013). In a few cases, rib impression over lungs was also observed. Hemorrhagic tracheitis was recorded along with frothy exudate in the tracheal lumen. Swollen and sometimes hemorrhagic lymph node was evident. These findings were in accordance with the reports of (Dar *et al*., 2012, Ozyildiz *et al*., 2013 and Dutta *et al*., 2020). Classification of pneumonia is most debatable topic and due to similar changes found in different cases there may be clash in classification. In present study, classification of pneumonia associated with respiratory mannheimiosis was based on morphological changes like distribution, color, appearance, texture and exudation of the diseased lungs which was in accordance with the reports of Mohamed and Abdelsalam (2008); Tijjani *et al*. (2012) and Ozyıldız *et al*. (2013).

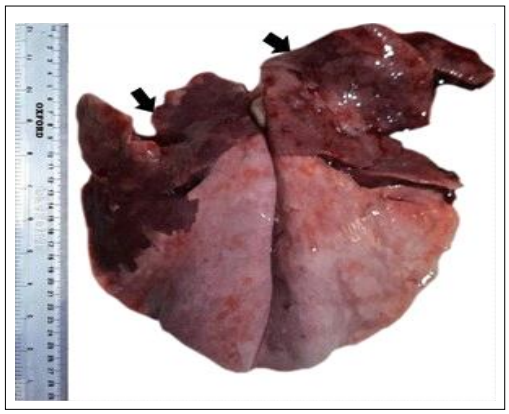


Fig 1: Lung showing antero-ventral consolidated areas indicating bronchopneumonia.

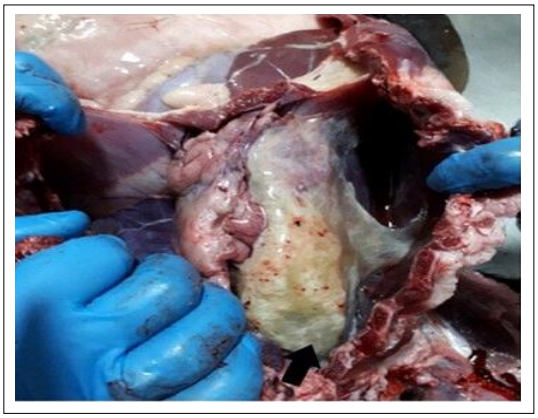


Fig 2: Deposition of thick layer of fibrin mass in the lungs leading to adhesion with the thoracic wall.

Various types of pneumonia associated with respiratory mannheimiosis recorded in the present study were bronchopneumonia, interstitial pneumonia, haemorrhagic pneumonia, suppurative pneumonia and fibrinous pneumonia which was intermixed with fibrinous bronchopneumonia, fibrinous, suppurative and fibrinopurulant pneumonia. Bronchopneumonia was recorded more because more branching bronchi occur at cranioventral part and the lumens are narrow so bacteria stick to lumen of the bronchi and gravitates the infection (Sastry and Rao, 1968). Most common gross alterations recorded was cranio-ventral consolidation or hepatization of the lung. The consolidated areas were dark red moist and meaty (Fig 1). Consolidation on right apical lobe, right middle lobe, left apical lobe and a small extent of left caudal lobe were noted. Lung also showed areas of emphysema and necrosis. Proteases released from the necrotic macrophages and leukocyte cause degeneration of elastin present which results emphysema in the lungs (Dar *et al*., 2012). Cut section of lungs revealed frothy fluid in some bronchial and bronchiolar lumen. Similar findings were reported by Dag *et al.* (2018) and Dutta *et al.* (2020). Multifocal, patchy to diffuse areas of haemorrhage was observed throughout all the lobes of lungs. In few cases, lobes of the lungs were covered with stringy net like material.

Table 1: List of primer sequences used for detection of 16s rRNA and *Lkt* gene of *Mannheimia haemolytica* isolates.

Primer	Sequence (5′-3′)	Target	Product size	Reference
27F	AGAGTTTGATCCTGGCTCAG	16s rRNA	1500 bp	Tan *et al*., 2016.
1392R	GGTTACCTTGTTACGACTT			
F	GCAGGAGGTGATTATTAAAGTGG	Lkt	206 bp	Tabatabaei and Abdollahi (2018)
R	CAGCAGTTATTGTCATACCTGAAC			

Excess straw-colored serous fluid was present in the pleural and peritoneal cavities. Deposition of thick layer of fibrin on the lungs leads to adhesion of lungs to the thoracic wall (Fig 2). Marbling appearance of lungs was prominent due to thickening and widening of interlobular septa as a result of accumulation of fibrin. Similar findings were observed by Brogd Dutta *et al*., (2020). Suppurative pneumonia was characterized by presence of multiple suppurative foci on lung surface. There was cranio bronchial and cranio lateral consolidation with suppuration on the left and right lungs. Presence of white frothy fluid in tracheal lumen and cut surface of the lungs. Trachea revealed hemorrhagic mucosa and frothy exudate in lumen and bronchi. The tracheal, mediastinal and bronchial lymph nodes were frequently congested, oedematous and haemorrhagic.

Microscopically, bronchi and bronchioles were filled with cellular debris, mucus, fibrin and large number of polymorphonuclear cells along with bronchiolar hyperplasia and desquamation of bronchiolar epithelium. Necrosis and sloughing of bronchiolar epithelium recorded in the present study might be attributed to release of proinflammatory cytokines (TNF α, IL1 and IL8), adhesion molecule and histamine by alveolar macrophages and neutrophils. Leucocytes also contribute to injury and necrosis of bronchiolar epithelium by releasing enzymes and free radicals (Zachary and McGavin, 2012). Multinucleated syncytial cells and spindle-shaped oat cell were present in the alveolar lumen. Oat cells are basophilic spindle shaped cells that were originated from neutrophils and macrophages. Oat cell originate from blood monocyte which transform into oat shape when developing in the necrotic and hypoxic environment created by *Mannheimia haemolytica* (Herceg *et al*., 1982). These cells were commonly described in pneumonias induced by *Mannheimia haemolytica,* however, they are not pathognomonic because they can also be seen in other pathological conditions of the lungs. Oat cells are known to be due to leukotoxins produced by the bacteria (Dag *et al*., 2018). In some sections, widespread neutrophilic infiltration and coagulative necrosis were present in and around the bronchus. Peribronchiolar lymphoid hyperplasia and thrombus formation were also evident.

Microscopically fibrinous pneumonia was characterized by thickened pleura and presence of intra alveolar fibrin in the form of “fibrin balls” within the alveolar spaces (Fig 3). Apart from this, there was interalveolar fibrin accumulation. In few cases within the vicinity of the interalveolar fibrin accumulation, an interstitial lymphocytic infiltration was present. Leukotoxin secreted from *Mannheimia hemolytica* in the lungs cause increase in procoagulant activity and decreases fibrinolytic activities of leukocytes leading to deposition of fibrin in alveoli. Vasculitis with fibrin thrombi was also recorded. Bronchiolar lumen were filled s with fibrin-rich exudate. Alveolar oedema, thickened pleura, thrombus formation (Fig 4) was also evident. These observations lend to support the findings of Dutta *et al.* (2020). Diffuse hemorrhage in alveoli and interalveolar septae with erythrocytes and inflammatory cells was observed. Hemorrhage and leukocytic infiltration were also observed in the bronchial lumen. The wall of the bronchus showed inflammatory changes. In case of suppurative pneumonia multiple suppurative foci scattered throughout

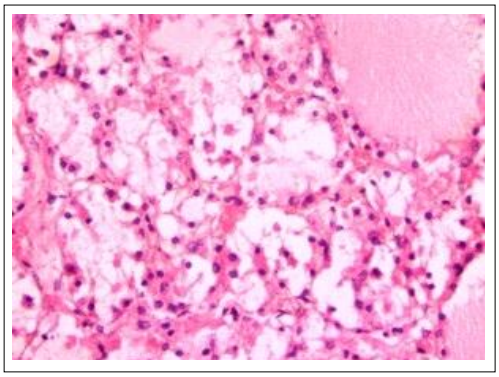


Fig 3: Lungs showing fibrin balls, oedema and oat cells in the alveolar lumen H and E×400.

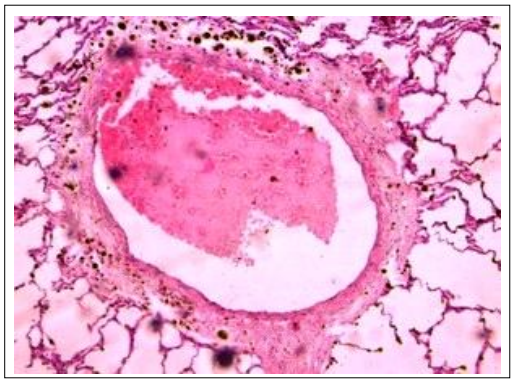


Fig 4: Lungs showing thrombus formation, H and E×400.

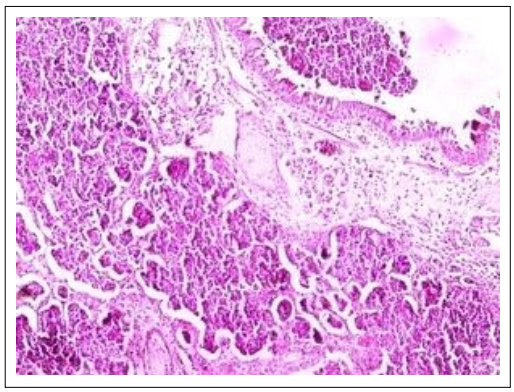


Fig 5: Lungs showing suppurative pneumonia, H and E×100.

the lung parenchyma (Fig 5). Heavy infiltration of neutrophils could be seen in bronchial and alveolar lumen. In a few cases, central necrotic mass admixed with bacterial colonies surrounded by thick connective tissue capsule with infiltration of inflammatory cells could be seen. Vascular congestion and inflammatory exudate were seen in the alveoli. Intravascular thrombosis was also evident. In trachea, necrosis and sloughing of tracheal epithelium was evident. There was polymorphonuclear cell infiltration in tracheal submucosa associated with submucosal congestion. The mediastinal lymph nodes revealed haemorrhage with mild to moderate depletion of lymphocytes inwhite pulp. Brown and Brenn stained tissue sections revealed presence of large number of gram-negative bacteria (red) in the trachea and lungs (Fig 6).

Bacteriological and biochemical studies

Out of total 51 lung samples subjected to primary bacterial isolation 7 isolates were confirmed as *Mannheimia haemolytica* based on typical cultural and biochemical characteristics. Other isolates associated with *Mannheimia haemolytica* were identified as *E. coli, Staphylococcus spp* and *Klebsiella spp*. Isolation and identification considered as golden standard for detection of bacteria from *Pasteurellaceae* family (*Mannheimia haemolytica*), this was in accordance with (Oruc, 2006 and Tijjani *et al*., 2012 and Dag *et al*., 2018). On blood agar plates, the isolated colonies of *Mannheimia haemolytica* formed â haemolytic zone with translucent colonies (Fig 7). On MacConkey's Agar plates pin point red colonies were formed (Fig 8). These findings were in accordance with (Quinn *et al*., 2011; Barde, 2016; Susmitha, 2019 and Laishevtsev, 2020). Impression smears stained with Gram's stain and Methylene blue revealed Gram-negative rods or coccobacilli with bipolar characteristic.

In different biochemical tests *Mannheimia spp* showed positive in catalase, oxidase and lactose test, negative in distinctive odour from colonies and urease activity. These findings were in accordance with (Quinn *et al*., 2011 and Mohamed *et al*., 2018).

Molecular studies

PCR was used as the diagnostic tool for the detection of *16s rRNA* and *Lkt* gene of *Mannheimia haemolytica* with amplicon size of 1500 bp and 206 bp respectively (Fig 9, 10). All the 7 positive isolates were screened by PCR and the samples were found positive for *16s rRNA* and *Lkt* gene.

Amplification of *Lkt* and *16s rRNA* gene of *Mannheimia haemolytica* by PCR in the present study was evident with other workers who found that *Lkt* and *16s rRNA* based PCR assay was confirmatory diagnosis for detection of *Mannheimia haemolytica*. As Leukotoxin (*Lkt*) secreted by the bacteria and caused the disease and *16s rRNA* gave confirmation of the bacteria thus *Lkt* and *16s rRNA* had a great importance in the field of diagnosis (Mohamed and Abdelsalam 2008, Tan *et al*., 2016; Tabatabaei and Abdollahi 2018).

Molecular characterization

Among the 7 PCR positive samples, two samples of *Mannheimia haemolytica were* sequenced and used for phylogenetic analysis. A phylogenetic tree was constructed using the neighbor-joining method (Fig 11). The sequences

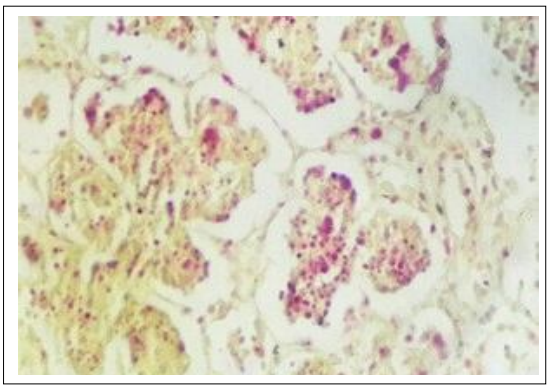


Fig 6: Gram negative bacteria (red stained) in lungs, Brown and Brenn × 400.

Fig 7: *Mannheimia haemolytica* showing translucent colonies forming â-haemolytic zone on blood agar.

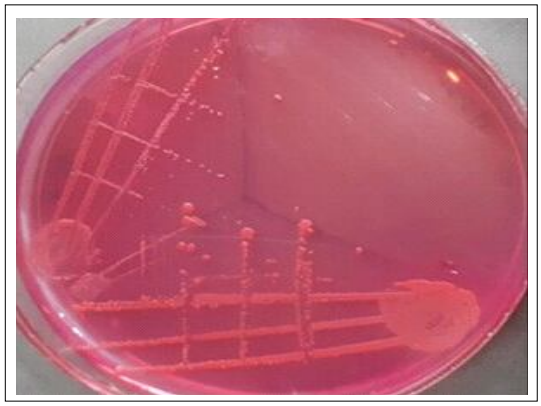
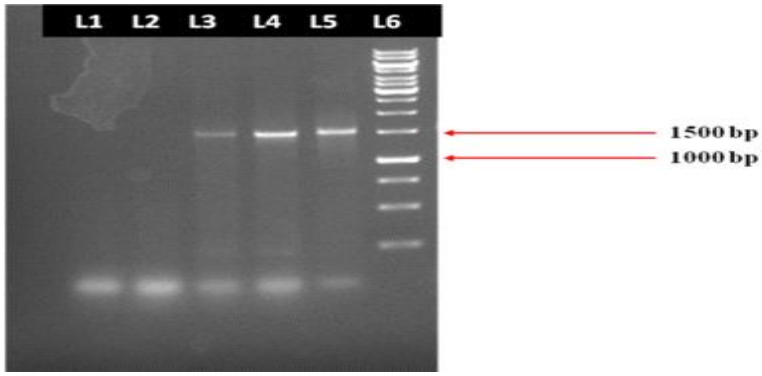
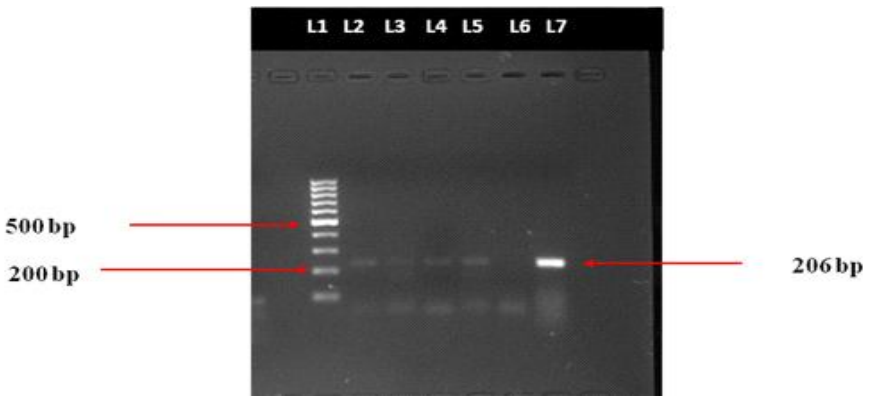


Fig 8: *Mannheimia haemolytica* showing pin point red colonies on MLA.



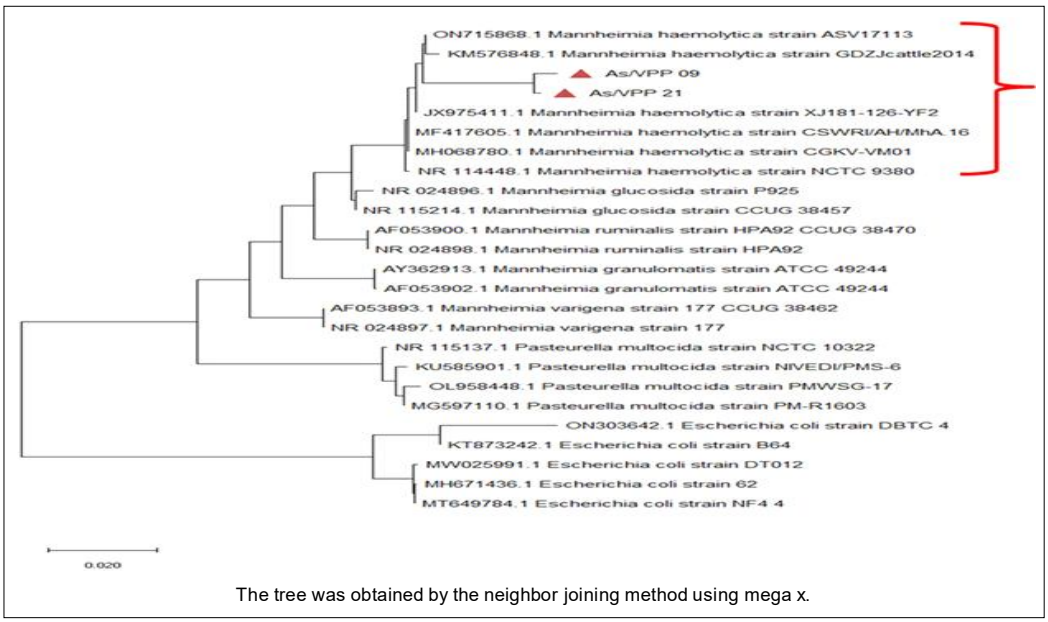
Lane 1 = No template control, Lane 2, 3, 4, 5 = Samples, Lane 6 = 1 kb ladder.

Fig 9: PCR amplification of *16s rRNA*gene (1500 bp) of *Mannheimia haemolytica* isolates.



Lane 1 = 100 bp ladder, Lane 2, 3, 4, 5 = Samples,
Lane 6 = No template control, Lane 7 = Positive control.

Fig 10: PCR amplification of *Lkt* gene (206 bp) of *Mannheimia haemolytica* isolates.



The tree was obtained by the neighbor joining method using mega x.

Fig 11: Phylogenetic tree based on the partial length *16s rRNA* gene sequences of *Mannheimia haemolytica* and other related bacterial species.

Percent Identity Matrix - created by Clustal2.1

1: NR_114448.1	100.00	99.78	98.80	99.86	99.86	99.86	96.35	97.25
2: JX975411.1	99.78	100.00	99.78	99.93	99.93	99.93	97.32	97.80
3: KM576848.1	98.80	99.78	100.00	99.08	100.00	99.08	97.40	97.25
4: MH068780.1	99.86	99.93	99.08	100.00	100.00	100.00	96.77	97.39
5: ON715868.1	99.86	99.93	100.00	100.00	100.00	100.00	97.89	97.88
6: MF417605.1	99.86	99.93	99.08	100.00	100.00	100.00	96.77	97.39
7: 09	96.35	97.32	97.40	96.77	97.89	96.77	100.00	99.19
8: 21	97.25	97.80	97.25	97.39	97.88	97.39	99.19	100.00

Fig 12: Per cent identity matrix.

of 16s rRNA gene of 2 isolates reported in the present study were compared with 6 references isolate reported in NCBI. From the phylogenetic tree it was evident that the two *Mannheimia haemolytica* isolates from this study clustered along with other *Mannheimia haemolytica* strains available in NCBI while the related bacterial species formed separate clusters. From the per cent identity matrix, it was found that sample no. 09 shared 97.87% and 97.40% identity with ON715868.1 and KM576848.1 respectively and sample no. 21 shared 97.88% and 97.80% identity with ON715868.1 and JX975411.1 (Fig 12). Similar findings were observed by earlier workers (Mohamed and Abdelsalam 2008; Tan *et al.,* 2016; Tabatabaei and Abdollahi, 2018).

CONCLUSION

Respiratory Mannheimiosis is one of the important causes of goat mortality in young animals. The gross lesions recorded were broncho-interstitial, suppurative and fibrinous pneumonia with hemorrhagic tracheitis. Presence of oat cells, fibrin clots, necrosis and degenerations in the lungs were major histopathological findings. For confirmatory diagnosis of respiratory mannheimiosis PCR could be used as a reliable technique.

Conflict of interest

All authors confirm that there is no conflict of interest related to the manuscript.

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