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Association of *Proteus mirabilis* with caprine pneumonia in Central India

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Abstract
Goat farming is getting momentum in uplifting socio-economic status of livestock farmers. However, they face the challenging respiratory affections quite often leading to economic losses. The present study places on record the association of pneumonia caused by *Proteus mirabilis* in goats of Chhattisgarh in central India. An overall 398 goats were screened for respiratory diseases in the three agroclimatic zones of Chhattisgarh through stratified random sampling and 212 samples including nasal swabs (85), tracheal swabs (15) lung swabs (56) and lung tissues (56) were collected from September 2019 to July 2022 from goats showing some signs of respiratory ailment/lesions. Out of 212 samples 92 bacterial isolates were obtained. Two (2.17%) isolate of *Proteus mirabilis* were identified using conventional, MALDI-TOF MS analysis and 16s rRNA gene sequencing. Phylogenetic analysis was done. Pathomorphology of pneumonic lungs revealed varying degrees of cranio-ventral consolidation, oedema and abscess formation. Histopathology of affected lungs showed areas of atelectasis, emphysema together with accumulation of polymorphonuclear cells delimited by a pyogenic membrane. This study shows that the opportunistic *Proteus mirabilis* could also result in respiratory infections of goats.

Keywords: Goat, lung, MALDI-TOF MS, *Proteus mirabilis*, swarming bacteria

Introduction
Goat farming is one of the finest and well established livestock sector in India. Being a highly profitable business, progressively it is gaining widespread popularity in the country. In the present scenario of changing agro-climatic conditions, goats have incredible prospective to be projected as the "Future Animal" for rural and urban wealth and value addition. Owing to this fact, goat rearing is steadily turning as the fast growing "livestock industry" in the country. Due to its good economic prospects, goat rearing under intensive and semi-intensive system for commercial production has been gaining momentum for the past couple of years in the country as well as in the state of Chhattisgarh. For a profitable goat production, following good management practices and keeping an eye over goat health is essential. The most common health crisis in goat husbandry is pneumonia (an inflammatory response of the lung parenchyma). This respiratory disease is still the major bottleneck for the development of animal production in the tropics. It is regarded as a disease complex. Caprine pneumonia is multifactorial and many etiological agents like bacteria, virus, fungus, parasites, physiological stress, housing pattern, changing atmospheric factors etc. lead to this fatal pathological condition (Minka *et al.*, 2009) [12].

Proteus mirabilis, a Gram-negative bacterium, is well-known for its ability to swarm across surfaces on bacteriological media. It forms prominent bulls- eye pattern on blood agar producing typical fish smell. *Proteus mirabilis* normally dwells in the intestinal tract of humans, dogs, monkeys, pigs, sheep, cattle, raccoons, cats, rats, and other mammals and has also been isolated from soil, water, sewage and feces (Guentzel, 1991; Rozalski *et al.*, 1997) [7, 16]. *Proteus mirabilis* is an opportunistic pathogen that infect the upper urinary tract. Besides urinary tract infection, it is also associated with respiratory tract infection, wounds, burns, skin, eyes, ears, nose, throat and gastroenteritis. Clinically, this organism is most frequently a pathogen of the urinary tract, particularly in patients undergoing long-term catheterization (Armbruster *et al.*, 2018) [2]. *Proteus mirabilis* uses a diverse set of virulence factors to access and colonize the host urinary tract, including urease and stone formation, fimbriae and other adhesins, iron and zinc acquisition, proteases and toxins such as hemolysin and its function of pore formation, biofilm formation, and regulation of pathogenesis. Abrupt and irrational usage of antibiotics in farm animals is posing challenges in the form of increased prevalence of

antibiotic resistance and development of antibiotic resistance genes particularly, within gram negative organisms and this is considered as the most serious problems in the field of medicine. In *Proteus mirabilis* the antimicrobial resistance is increasing, which causes epidemiologic effect of *Proteus mirabilis* bacteremia (Sohn *et al*., 2011, Al-Jumaily and Zgaer, 2016) [19, 1].

References relating to the isolation of *Proteus spp.* are limited mainly to the urinary system and digestive tract infection in current medical and veterinary texts, in which it has been proven to play a primary or a secondary role (Manos and Belas, 2005; Singh *et al*., 2013, Al-Jumaily and Zgaer, 2016) [11, 18, 1]. There is a paucity of information regarding respiratory tract infection by *Proteus* sp in veterinary science. Keeping in-view the above facts, the present study places on record association of *Proteus mirabilis* as a cause of pneumonia in goats of Chhattisgarh. Moreover, the present report appears to be the maiden report of *Proteus mirabilis* associated pneumonia from goats of central India.

Materials and Methods

Location and Duration of Work: Commencing from September 2019 to July 2022, a total of 398 goats of either sex and of different age groups were screened for respiratory diseases from organized and unorganised goat herds kept in semi-intensive to extensive (backyard) housing conditions and at slaughter houses situated in various districts divisible in 5 divisions (Durg, Raipur, Bilaspur, Bastar and Surguja) of three agroclimatic zones of Chhattisgarh viz. Chhattisgarh plains, Bastar plateau and Northern hills.

Collection of samples: Samples were collected using stratified random sampling method by randomly selected goat of at least 2-3 locations of the divisions of respective agroclimatic zone.

Nasal Swabs: Nasal swabs (n = 85) were collected from goats suffering with respiratory diseases and showing clinical signs *viz*. nasal and ocular discharge, coughing, fever (105 - 107 °F) and respiratory distress. Briefly, the nostrils were washed and cleaned with a paper towel to remove adhering nasal discharge after humanely securing the animal. The head of animal was raised. A dry, sterile cotton swab was inserted inside the nasal cavity. It was gently rolled five to six times inside the nasal cavity for collection of nasal swab in transport media. The swabs were brought to the laboratory over cool packs.

Tracheal Swabs and Lung Swabs/Lung Tissues: Swabs form trachea (n = 56) and lungs (n = 56) showing gross pathological changes were collected from naturally died/slaughtered animals. For collecting the lung swabs, the surface of the affected portion of lung was seared with hot spatula, incised with sterile BP blade, the incised portion was opened with sterile forceps and the swab was gently rolled within the opened lung parenchyma. The swab was immediately placed in the pre marked swab holder. In a few cases, the affected portions of lungs were brought to the laboratory and opened within the laminar hood aseptically. Representative tissues (3x3x2 mm) from lungs (n=56) were collected from dead goats and goats slaughtered at slaughter houses in separate sterile containers and transported to the laboratory at 4 - 8 ºC for bacterial isolation studies.

Isolation and Phenotypic characterization: The samples were processed to isolate and identify *Proteus sp*. The aseptically collected swabs and lung tissues were streaked onto 5% Calf blood agar and Mc Conkey agar incubated aerobically at 37 °C for 24 hours. Identification of bacteria was based on Gram's staining, colony characteristics and biochemical tests (Quinn, 2002) [14]. The identified bacteria were validated on the basis of matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDl-TOF MS) at National Centre for Microbial Resource, NCCS, Pune (Maharashtra, India).

Molecular characterization of *Proteus mirabilis*

Nucleotide sequencing: The 16S rRNA gene sequencing was outsourced to get both forward and reverse sequences to generate consensus sequences. The sequences were initially analyzed using NCBI online BLAST server to identify the sequence specificity. Based on the BLAST results, sequences were further compared with other nucleotide sequences of *Proteus mirabilis* in GenBank database. The sequences of 16S rRNA were submitted to NCBI GenBank under accession number OP107266.1.

Phylogenetic analysis: The sequence was aligned, analysed and compared using CLUSTAL W with other publicly available sequences of isolates from various countries like India (MK110486.1, KU378106.1, KF917137.1, ON460264.1), Pakistan (KT216590.1), UAE (MZ169439.1), China (KP152656.1, MZ067157.1, JF775415.1, EF091150.1, OL629230.1, OL629226.1, OL629225.1, OL629222.1, OL629188.1), Japan (JF946781.1, JF946778.1), Iran (MF399345.1), Venezuela (JF947362.1) and Nigeria (LC385636.1). Phylogenetic tree was constructed using neighbor -joining algorithm with 1000 bootstrap replicate to represent the evolutionary history of the taxa analyzed. The analysis involved 21 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1289 positions in the final dataset. Evolutionary analyses were conducted in MEGA-11 software (Tamura *et al.*, 2021) [20].

Pathomorphological study: For pathomorphological studies, tissue (3x3x2 mm) samples from lungs and kidneys were collected in 10% neutral buffered formal saline solution processed routinely, embedded in paraffin, sectioned at 4–5 µm thickness and stained with haematoxylin and eosin (Bancroft and Stevens, 1990) [3].

Results and Discussion

Occurrence, Isolation and Phenotypic characterisation: Based on the results of the present study out of 212 samples 92 bacterial isolates were obtained. Two (2.17%) lung samples were positive for *Proteus* mirabilis from Bastar division. No *Proteus* sp. could be isolated from Raipur division, Durg division, Surguja division and Bilaspur division. The number might be appearing very meagre but it's evident that this opportunistic bacterium was involved in respiratory infections apart from urinary and digestive system. These bacteria appeared as Gram negative rods and produced characteristic successive waves to form a thin filmy layer of concentric rings (swarming) and produced a prominent bulls'-eye pattern. The growth had distinctive fish odor on blood agar after 24-48 hours aerobic incubation at 37 °C. The edge of the swarm colony advanced fairly continuously along with

some crowding of the growth behind the edge. The rings seemed to form within the colony well behind the leading edge, producing a ripple in the surface which later developed into one of the concentric rings. Further, the isolates did not produce a line of demarcation (Dienes line) when streaked together on blood agar plate (Fig. 1). On Mac Conkey agar the bacteria produced smooth, pale colourless colonies without swarming (Fig. 2). The colonies were strong urease positive, methyl red positive and positive for citrate utilization. However, they were negative for oxidase test and indole test.

Similar results of bacterial swarming have been reported previously (Guentzel, 1991; Rozalski *et al.*, 1997, O'hara *et al*., 2000, Singh *et al.*, 2013, Al-Jumaily and Zgaer, 2016) [7, 16, 13, 18, 1]. *Proteus mirabilis* is well known for its ability to differentiate into hyperflagellated, motile and elongated swarmer cells that rapidly spreads over a surface. When two different strains of *Proteus mirabilis* swarm on the same agar plate, a visible demarcation line with lower cell density forms at the intersection known as Dienes line. However, when two identical isolates meet, the swarming edges merge without formation of a Dienes line. Thus, the isolates of the present study appear to be similar in nature. According to classical phenomenon of swarming, Doughlas and Bisset (1976) [6] described that a thin even film of growth first progressed over the medium. Motion at the leading edge then slowed and ceased but growth continued, resulting in a thickening over the whole area. The edge of this thickened layer comprised the edge of the ring, from which a renewed thin film emanated to repeat the cycle, the growth being composed of sharply defined step.

The results of biochemical tests viz. indole negative, oxidase negative and strong urease positive isolates of *Proteus mirabilis* corroborates well with earlier reports (Kamga *et al*., Kishore, 2012; 2012; Al-Jumaily and Zgaer, 2016) [9, 10, 1].

MALDI-TOF MS of the bacterial isolate yielded good quality MALDI-TOF MS spectra (Fig. 3) with score of 2.413 and was identified as *Proteus mirabilis* DSM 18254 on the basis of Bruker taxonomy database using Biotyper 3.1 software. The strains with more than 2.0 score values means reliable species-level identification, strains with score value ranging from 1.7 to 1.99 indicate genus-level identification, and the strains with score values less than 1.7 could not be identified by MALDI biotyper database. MALDI-TOF MS has gained popularity as a microbial biotyping tool due to its speed, low-cost, simplicity, and applicability for a wide range of microbes including bacteria, archaea, and fungi. It is especially useful in clinical set-ups due to lesser turn-around time. The ability of this technique to differentiate at the species level has profound implication. This technique is based on fingerprinting analyses of primarily ribosomal proteins, which are synthesized under all growth conditions and are the most abundant cellular proteins (Ryzhov and Fenselau, 2001; Rahi *et al.*, 2016) [17, 15].

Isolation of *Proteus mirabilis* from pneumonic lungs of goat corroborates well with earlier reports from lungs of rats (Singh *et al.*, 2013) [18]. *Proteus sp.* has been implicated in a variety of sporadic infections of dogs, cats and cattle. Cystitis and urinary infections are the most common, particularly in dogs and ponies. Occasionally, *Proteus sp.* is thought to be involved in diarrhea in young mink, calves, goats and puppies (Carter *et al.*, 1995, Dewangan *et al.*, 2012) [4, 5]. However, the present report appears to be the maiden report of *Proteus mirabilis* associated pneumonia from goats of central India.

Sequence and phylogenetic analysis of *Proteus mirabilis*: Sequences of 16S rRNA gene of the bacterium showed 96.95%-97.03% nucleotide similarities with other previously published sequences. Nucleotide sequences of the present study showed a high degree of similarity with *Proteus mirabilis* 16S rRNA gene sequences detected in Pakistan (97.03%), China (96.95% - 97.03%), Venezuela, UAE and Japan (97.03%), Hungary, Turkey and Switzerland (98.38%). Phylogenetic analysis showed that the present isolate from central India is grouped with Pakistan (Fig. 4).

Pathomorphological study: On detailed post mortem examination of goat cadaver, it was noticed that the lungs were grossly pneumonic showing right cranio-ventral consolidation, diffused areas of atelectasis and emphysema in various lung lobes. However the diaphragmatic lobes appeared to be normal. The microscopic examination of lungs revealed moderate degenerative to necrotic changes in bronchiolar mucosa with occasional sloughing of mucous membrane together with lysis of bronchiolar wall. There were severe thickening of alveolar septum. The alveolar capillaries were congested. At places, there were diffused escape of erythrocytes in lung parenchyma. Few sections also revealed presence of haemosiderin pigments. Apart from this, there was huge accumulation of polymorphonuclear neutrophils delimited by a fibrous pyogenic membrane (Fig. 5, 6) Gross examination of kidneys revealed diffused haemorrhages on cortical surface. Microscopically, there were degenerative and necrotic changes in glomeruli, and tubular epithelium together with occasional desquamation of tubular epithelium (Fig. 7) were recorded. Pathomorphology of *Proteus mirabilis* pneumonic lungs of goat corroborates well with earlier reports from lungs of rats (Singh *et al.*, 2013) [18].

Proteus mirabilis has a diverse mode of transmission. It can cause infection in different anatomical sites of the body. An overpowering infection, possibly of feeding materials and /or nosocomial contamination due to hygienic flaws coupled with environmental stress might have been responsible for the culmination of respiratory infection. *Proteus* species are considered potential pathogenic bacteria in human gastrointestinal or urinary tract infections. It is postulated that human guts are the reservoir of *Proteus* spp., especially for the *Proteus mirabilis* species. However, the interactions between *Proteus* spp. and hosts may lead to the pathogenicity of this genus resulting from population expansion. Isolation of *Proteus mirabilis* from Bastar division, in the present study could be attributed to unhygienic housing and lack of adequate managemental practices. As it is evident that, *Proteus* species are known to colonize the urinary tract; bacterial excretion can contaminate the feeding/bedding materials, which might serve as potential source of respiratory infection. Equally, haematogenous route of infection cannot be ruled out. *Proteus mirabilis* induced lung infection may cause complications as the organism is known to produce a biofilm, which is very difficult to treat (Jacobsen and Shirtliff, 2011) [8]. Several potential *Proteus mirabilis* virulence factors including fimbrial-mediated adherence to the uroepithelium, swarming motility mediated by flagella, outer-membrane protein expression, cell invasiveness, urease production, haemolysin production and iron acquisition related to urinary infections have been described (Singh *et al.*, 2013) [18]. The most well studied fimbria is the mannose-resistant Proteus-like (MR/P) fimbria, whose expression is phase-variable. Similar to other members of the *Enterobacteriaceae, Proteus*

mirabilis carries numerous secretion systems including types I, III, IV, V, and VI. Lastly, the bacterium carries an integrative and conjugative element that can self-replicate and self-transfer to other strains and species, transferring virulence genes and antibiotic resistance (Armbruster *et al.*, 2018) [2].

It could be concluded that although *Proteus mirabilis* is linked with inflammatory conditions of urinary system and gastrointestinal tract, the bacteria may lead to pneumonia due to stress and unhygienic conditions. Moreover, the present report appears to be the maiden report of *Proteus mirabilis* associated pneumonia from goats of central India. Further monitoring on etiopathogenesis of pneumonia due to *Proteus mirabilis* in animals is suggested.

Fig 1: *Proteus mirabilis* showing characteristic bull's eye swarming colonies on blood agar.

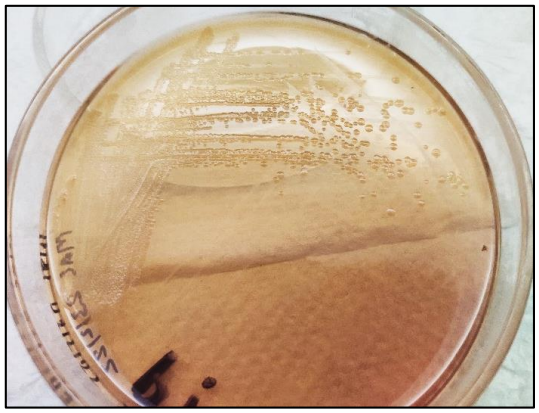

Fig 2: *Proteus mirabilis* showing pale, colourless (non-lactose fermenting) colonies on Mac Conkey's agar.

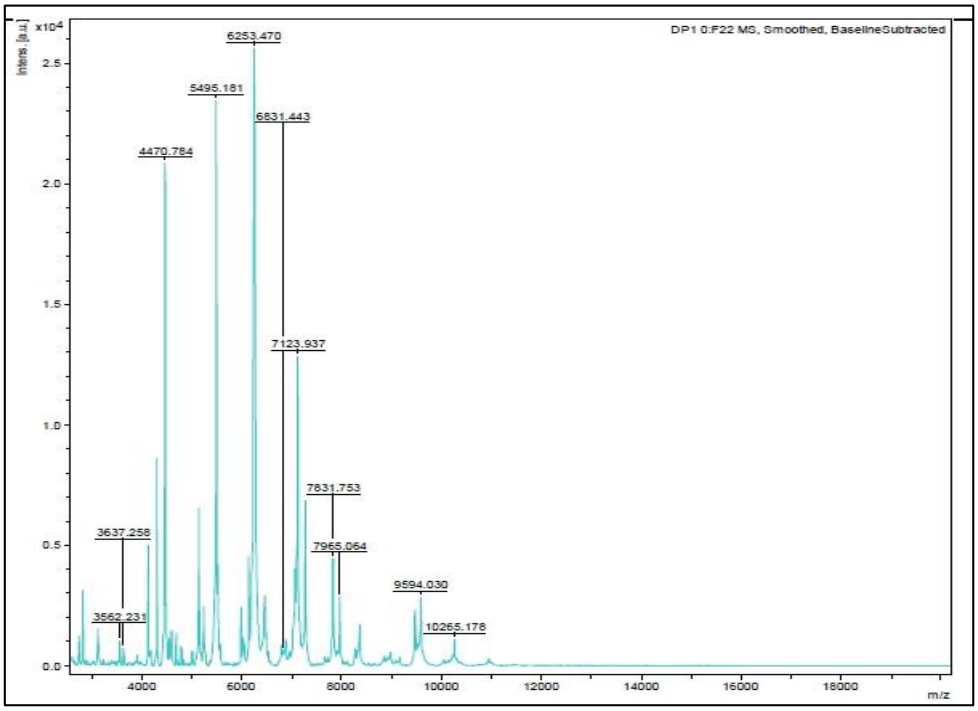

Fig 3: MALDI TOF MS spectra of *Proteus mirabilis* showing good quality spectra.

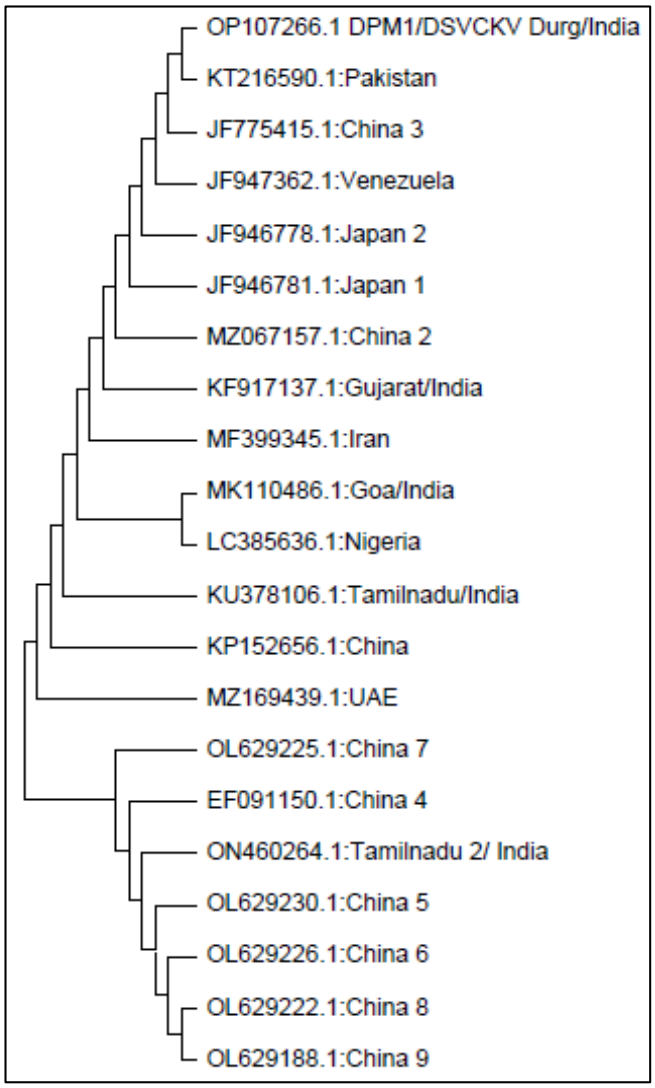

Fig 3: Cladogram of *Proteus mirabilis* using neighbour-joining algorithm with 1000 bootstrap replicate to represent the evolutionary history of the taxa analyzed. (OP107266.1 DPM1/DSVCKV Durg/India from present study)

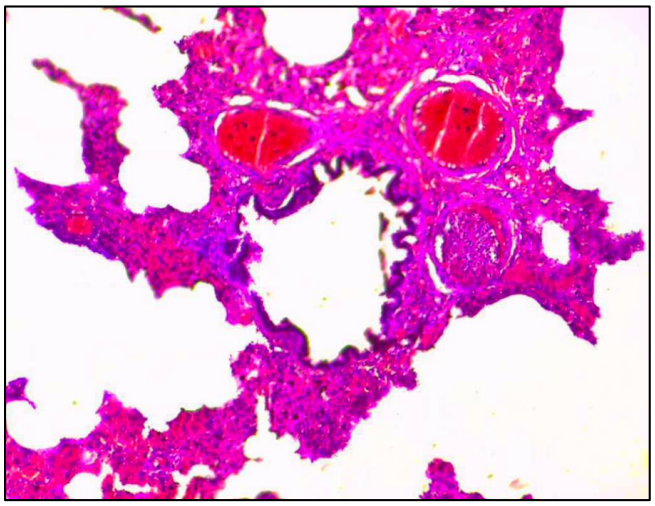

Fig 4: Photomicrograph of goat lung showing thickening of alveolar septa, congestion and accumulation of inflammatory polymorphonuclear cells. (H & E x100).

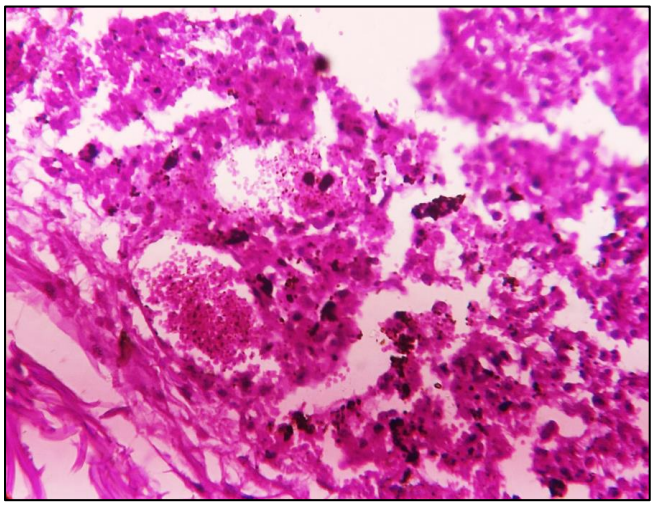

Fig 5: Photomicrograph of goat lung showing, congestion, haemorrhages, haemosiderosis and accumulation of inflammatory cells. (H & E x400).

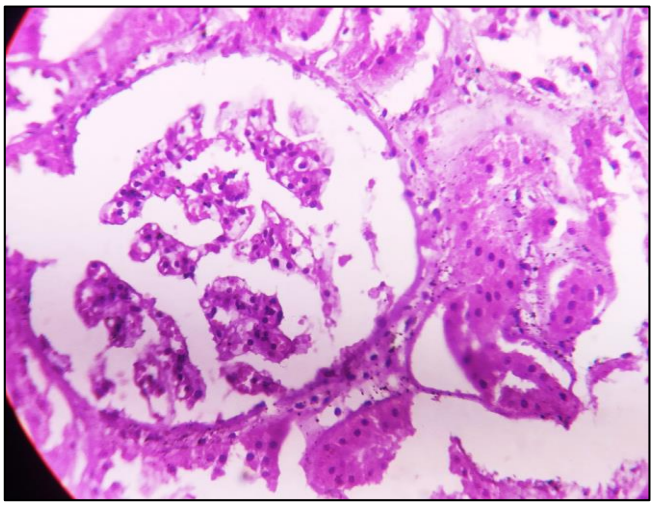

Fig 6: Photomicrograph of goat kidney showing severe degenerative and necrotic changes in glomerulus and tubular epithelium (H & E x400).

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