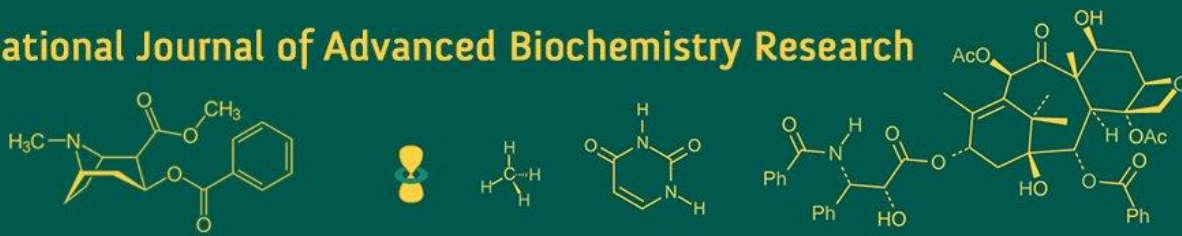


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## Etiopathology of kidney infections in chickens

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### Abstract

**Background:** Although being a major cause of mortality and loss of productivity in poultry, kidney diseases remain among the most unexplored areas of avian pathology. The present study was undertaken with the objectives of detection and molecular characterization of infectious agents causing nephropathies in chickens and to study pathomorphological changes of kidneys infected with infectious agents. The study spanned a duration from Jan 2024 to Dec 2024. A total of 15 poultry farms in Durg, Rajnandgaon, Raipur and Balod districts were surveyed for suspected kidney diseases and in total, 258 necropsied birds were examined for the study. **Methods:** Tissue samples demonstrating distinctive lesions of kidney diseases were gathered from the kidney as representative specimens for pathological examination. Additionally, swab samples from the kidney were collected for bacteriological analysis. The isolation and identification of bacteria were carried out using the previously outlined methods followed by subsequent molecular characterization. **Results:** During the study period, among 258 carcasses, only 44 birds showed renal lesions or nephropathy. The overall period prevalence of nephropathy in four districts of Chhattisgarh was recorded as 17.05%. Forty-four nephropathic kidney samples gave 14 bacterial isolates that were identified through initial phenotypic characterization based on their typical growth patterns on different media and the results of biochemical tests. Further, the bacterial identification was confirmed using MALDI-TOF MS, and the 16S rRNA gene sequencing was outsourced to generate phylogenetic analysis and submitted to Genbank to get accession numbers [*Escherichia coli* (PQ316049), *Klebsiella pneumoniae* (PQ325686) and *Proteus mirabilis* (PQ325687)]. Additionally, they displayed resistance to multiple commonly used antibiotics used in poultry.

**Keywords:** MALDI-TOF MS, 16S rRNA gene, Genbank, Accession number

### Introduction

In terms of consumption, India's main meat source is chicken, in 2020, their proportion of meat eating increased to 65% from roughly 23% twenty years before. It has outperformed buffalo meat and beef, its two competitors (OECD, 2021) [16]. The evolution of the poultry industry in India has been attributed to various factors such as modifications in production and marketing institutions, government policies regarding poultry and feed trade, technological advancements, and ongoing research and development investments aimed at producing higher-quality breeds (Gulati and Juneja, 2023) [9]. Numerous new diseases have emerged recently because of the introduction of new and improved germplasms, intensive rearing on approved scientific lines, and practical breeding policies with the aim of increasing their production. Renal disorders are common in birds and mostly occur because of other diseases, management issues, and toxicity. These pathological lesions in many diseases are manifesting differently, and new syndromes are emerging in many parts of India as well as abroad. It appears that there is an increasing involvement of kidney lesions in several diseases (Lierz, 2003) [10].

The kidneys are an important organ in the chicken body, comprising 1 to 2.6% of body weight compared to an average of 0.5% of body weight in mammals (Rupley, 1997; Lumeij, 2000) [19, 11]. Avian kidneys are paired organs situated in depressions within the pelvic bone of the abdominal cavity. They are reddishbrown in color and consist of three distinct lobes (Frazier et al, 1995; Lumeij, 2000) [7, 11]. Because of their uricotelic nature and lack of uricase enzyme, birds can excrete urine in the form of semi-solid uric acid in their faeces. This ability is further enhanced by their ability to conserve water. Several specific diseases, such as aspergillosis, colibacillosis, salmonellosis, infectious bronchitis, New Castle disease and infectious bursal disease, can impair kidney function. Thus, kidney lesions can be very useful in determining an accurate diagnosis. Usually, bacteria enter the kidneys through the renal portal system or the renal arteries as a subsequent result of a systemic disease, leading to the development of bacterial nephritis (Phalen et al., 1990; Speer, 1997) [17, 23].

Numerous bacteria, including those belonging to the *Enterobacteriaceae* family, *Pasteurella* species, *Pseudomonas* species, *Streptococcus* species, and *Staphylococcus* species, have been associated to bacterial nephritis (Phalen *et al.*, 1990; Speer, 1997; Lierz, 2003; Schmidt *et al.*, 2003) [17, 23, 10, 21].

The preliminary diagnosis of renal diseases can be made based on clinical signs, observable and microscopic pathological lesions, as well as morbidity and mortality patterns. Definitive diagnosis involves isolating and identifying the associated pathogen using conventional and molecular characterization techniques. Although being a major cause of mortality and loss of productivity in poultry, kidney diseases remain among the most unexplored areas of avian pathology. The reports on the prevalence of kidney diseases in chickens vary widely by region. There is currently no baseline data on the prevalence of kidney disorders in Chhattisgarh's poultry population, and no comprehensive study has been conducted on the occurrence of different nephropathies in the state.

Keeping in view all the facts, the present study will be undertaken with the following objectives.

- Detection and Molecular Characterization of infectious agents causing nephropathies in chickens
- To study the pathomorphological changes of kidneys infected with infectious agents

## Materials and Methods

The investigation into the etiopathology of poultry mortality attributed to kidney diseases in chickens was conducted across various poultry farms in Durg, Rajnandgaon, Raipur and Balod districts of the state. The study spanned a duration from Jan 2024 to Dec 2024. A total of 15 poultry farms in the mentioned districts were surveyed for suspected kidney diseases. In total, 258 necropsied birds were examined for this research. All collected samples were transferred to the laboratory located in the Department of Veterinary Pathology, College of Veterinary Science and Animal Husbandry, Anjora. Under aseptic conditions, the collected swabs were inoculated into brain heart infusion broth (BHIB) and incubated aerobically at 37 °C for 6-8 hours. A loopful of the resulting broth was then streaked onto sterile petri plates containing MacConkey agar to initiate the isolation of primary cultures. These plates were further incubated aerobically at 37 °C for a period of 24 hours. For locations at a distance, the swabs were directly streaked onto identified primary culture media, such as brain heart infusion agar and MacConkey agar. The streaked plates were subsequently incubated at 37 °C for 24 hours.

The colonies from the primary culture were identified and subsequently restreaked onto a new petri plate containing 5% calf blood agar/chocolate agar or brain heart infusion agar and MacConkey agar to obtain a pure culture. Representative colonies were initially subjected to Gram's staining method and then restreaked on selective and differential media such as EMB agar. The pure cultures were preserved on nutrient agar/BHI agar/blood agar slant and stored at 4 °C for future investigations.

The identification of bacteria relied on Gram's staining (including staining characteristics, morphology and arrangement), as well as colony features such as color and appearance, along with biochemical tests.

IMViC reactions are a set of four useful reactions that are commonly employed in the identification of members of the family *Enterobacteriaceae*. Indole test, Methyl red test, Voges-Proskauer test and Citrate

Utilization test - These tests were used to differentiate the isolated bacteria from various bacteriological samples collected from dead birds. The test was performed, and the result was interpreted according to the standard procedure described by Cheesbrough (2006) [4].

The antibiotic sensitivity profiles of respiratory pathogens were determined using the Kirby-Bauer disc diffusion method, following the procedure outlined by Bauer *et al.* (1966) [3] and adhering to the guidelines from the Clinical and Laboratory Standards Institute (CLSI, 2023).

The representative isolates from various groups were subcultured onto nutrient agar and submitted for MALDI-TOF MS-based strain identification at NCMR, Pune.

The identification of isolates was carried out at the sequencing facility of National Centre for Microbial Resource (NCMR), National Centre for Cell Science, Pune. At the facility, genomic DNA was isolated by the standard phenol/chloroform extraction method (Sambrook *et al.*, 1989), followed by PCR amplification of the 16S *rRNA* gene using universal primers 16F27 [5'-CCA GAG TTT GAT CMT GGC TCA G-3'] and 16R1492 [5'-TAC GGY TAC CTT GTT ACG ACT T-3'].

The sequences were submitted in NCBI: <https://www.ncbi.nlm.nih.gov>, to get the accession numbers. The comparative sequence analysis of 16S *rRNA* gene of bacterial isolates was carried out along with those of selected sequences available in the GenBank database. Sequence identities of nucleotides were analyzed and aligned using the Clustal W of MEGA XI Software (Morla *et al.*, 2016) [14] and Neighbourjoining (NJ) method (Saitou and Nei, 1987) [20]. Based on maximum identity score, sequences were selected and aligned, sequence distances were generated, and the phylogenetic tree was constructed from derived nucleotide sequences.

Carcasses obtained from various poultry farms underwent a thorough necropsy examination, with particular emphasis on scrutinizing lesions in the kidneys.

Tissue samples from the affected kidney were preserved in 10% buffered formalin for subsequent histopathological analysis. The tissue specimens were sliced into longitudinal and transverse sections with a thickness of 0.5 mm and thoroughly washed in tap water overnight. Dehydration of the tissues was performed using a graded series of acetone. After dehydration, the tissues were clarified with graded benzene and embedded in paraffin wax. The tissue-embedded paraffin blocks were then cut into sections with a thickness of 3-5 µm. These sections were deparaffinized in xylene, stained with Hematoxylin and Eosin (H & E) stain according to Culling (1963) [7] and mounted with DPX. The stained sections were examined under a light microscope.

**Ethics approval consent to participate:** This research followed the guidelines specified by Institutional Animal Ethics Committee (IAEC). All the experimental procedures were carried out as per the recommendations of the IAEC.

## Results and Discussion

Feed samples were analyzed for multi-toxin estimation from Animal Feed Analytical and Quality Assurance Laboratory, Veterinary College and Research Institute, Namakkal - 637 002 before doing a necropsy of birds to rule out mycotoxin-related nephropathy, where the result shows that Aflatoxin-B1, Aflatoxin-B2, Aflatoxin-G1 are in a reference range and Aflatoxin-G2, Ochratoxin, T-2 Toxin, Citrinin, Zearalenone were not detected (Table 1).

**Table 1:** Result of Multi-toxins estimations

| Sr no | Test         | Result (ppb) |     |     |      |
|-------|--------------|--------------|-----|-----|------|
|       |              | DF1          | RF1 | BF1 | RiF1 |
| 1     | Aflatoxin-B1 | 28           | 19  | 32  | 26   |
| 2     | Aflatoxin-B2 | 5            | 9   | 8   | 8    |
| 3     | Aflatoxin-G1 | ND           | ND  | 11  | ND   |
| 4     | Aflatoxin-G2 | ND           | ND  | ND  | ND   |
| 5     | Ochratoxin   | ND           | ND  | ND  | ND   |
| 6     | T-2 Toxin    | ND           | ND  | ND  | ND   |
| 7     | Citrinin     | ND           | ND  | ND  | ND   |
| 8     | Zearalenone  | ND           | ND  | ND  | ND   |

Unit ppb – parts per billion, ND – Not detected

### 1.1 Isolation and Phenotypic Characterization of Bacterial Kidney Pathogens

The isolation and identification for the *enterobacteriaceae* family were done as per standard protocol. A total of 44 birds showing kidney lesions suspected of bacterial nephritis encountered at post-mortem examination were subjected to isolation and identification of bacteria.

Pooled tissue samples from all divisions of kidneys and their kidney swabs were initially placed in nutrient broth and brain heart infusion broth at 37 °C overnight. One combined infection of two bacteria in a single case has been identified i.e. mixed infection of *E coli* with *Proteus mirabilis*.

In the present study, 11 isolates of *Escherichia coli* were retrieved from 14 isolates (78.57%). Three *E coli* isolates were associated with concurrent infection with *Infectious bronchitis*. In all cases, kidney lesions were observed as swollen, enlarged and congested of various divisions. The bacteria appear pink to Gram's staining, arranged singly or in pairs as small rods (Fig. 1 c) and produced circular, convex, smooth greyish white, translucent opaque colonies on nutrient agar; pink coloured, circular, convex colonies on MacConkey agar (Fig. 1 a) and typical green metallic sheen on eosin methylene blue (EMB) agar (Fig. 1 b) were characterized as *Escherichia coli*. The isolates tested positive for indole and methyl red. However, they were negative for Voges-Proskauer and citrate utilization tests.



**Fig 1:** Phenotypic characters of *Escherichia coli* on MacConkey (a) and EMB agar (b) with Gram's staining morphology (c)

In the present study, a total of 01 isolates of *Klebsiella* spp. was retrieved from 14 isolates (7.14%). The bacteria showing negative reaction to Gram's staining, arranged singly or in pairs arranged in short chains or clusters as short rods (Fig. 2 c) and produced circular, dome-shaped, mucoid, greyish white, translucent opaque colonies on nutrient agar plates. The bacteria were late lactose fermenter (pale pink coloured), circular,

convex, opaque mucoid colonies on MacConkey agar plates (Fig. 2 a). The bacteria formed translucent opaque, purple, mucoid colonies (typical fisheye shaped) without a green metallic sheen on EMB agar (Fig. 2 b). The isolated strains were indole-negative, methyl red-negative, Voges-Proskauer positive and citrate-positive.



**Fig 2:** Phenotypic characters of *Klebsiella* spp on MacConkey (a) and EMB agar (b) with Gram's staining morphology (c)

In the present study a total of 02 isolates of *Proteus mirabilis* were retrieved from 14 isolates (14.28%). These bacteria appeared as Gram-negative rods upon Gram's staining (Fig. 3 c). The bacteria produced characteristic successive waves to form a thin filmy layer of concentric rings (swarming) producing a striking bulls'-eye pattern on blood agar after 24 - 48 hours' aerobic incubation at 37 °C (Fig. 3 b). On nutrient agar, the characteristic swarming could not be appreciated and

bacteria produced irregular, effuse, 1-2 mm, glistening and translucent greyish-white colonies. On MacConkey agar the bacteria produced smooth, circular, 2-3 mm low convex, pale colourless transparent colonies without swarming (Fig. 3 a) with a characteristic fishy odour. The colonies were strong methyl red positive and positive for citrate utilization. However, they were negative for the indole test and Voges-Proskauer.

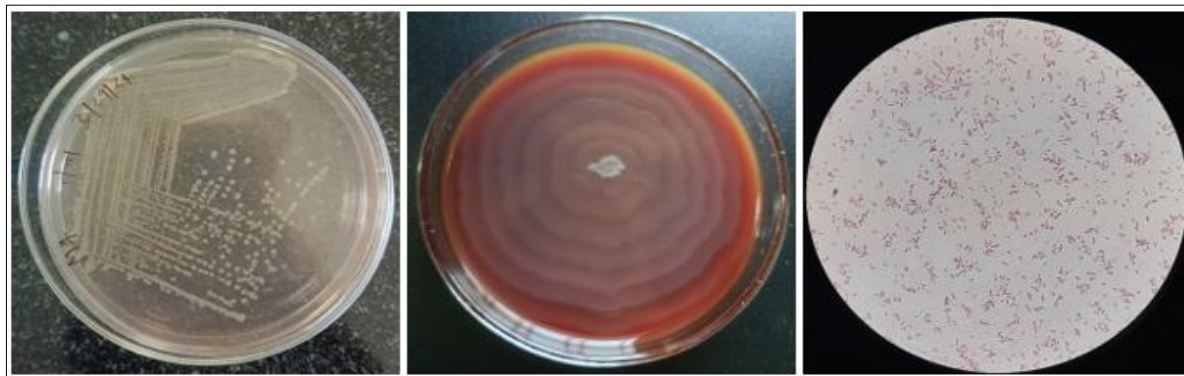


**Fig 3:** Phenotypic characters of *Proteus mirabilis* on MacConkey (a) and blood agar (b) with Gram's staining morphology (c)

The prevalence of present study is in close agreement with preceding reports who observed several outbreaks in the year 2013-14, involving baby chick nephropathy in Indian commercial broilers resulting in growth retardation and mortality up to 20% (Gogoi *et al.*, 2017) [8]. Rafiq *et al.* (2024) [18] recorded a higher incidence of 35% of nephropathy associated with bacterial diseases in the broiler birds of Kashmir valley.

Colony characteristics of *E. coli*, *Klebsiella* spp. and *Proteus mirabilis* observed in nutrient agar, MacConkey agar and EMB agar were similar to the findings which is an agreement with the report of Rafiq *et al.* (2024) [18], Ameen *et al.* (2015) [2] and Mohamed and Shehata, (2009) [13].

### 1.2 Antibiotic Sensitivity Profiles of Kidney Pathogens

Antibacterial susceptibility tests (AST) were performed to detect the susceptibility of the isolated bacteria to antibiotics which included, tetracycline 30 mcg, ciprofloxacin 5 mcg, gentamicin 10 mcg, amoxycylav 30 mcg, ampicillin 10 mcg, chloramphenicol 30 mcg, levofloxacin 5 mcg, meropenem 10 mcg, co-trimoxazole 25 mcg, cefotaxime 10 mcg. In the present study, antimicrobial sensitivity was performed by disc diffusion method. In the present study, the antimicrobial sensitivity test of the *E. coli* isolates of infected birds revealed the highest susceptibility to gentamicin, amoxycylav, chloramphenicol, meropenem and cefotaxime followed by tetracycline and levofloxacin. *E. coli* isolates exhibited the highest resistance to ciprofloxacin, followed by ampicillin and co-trimoxazole. This agrees with the findings of Ameen *et al.* (2015) [2] who reported that *E. coli* isolates were sensitive to ciprofloxacin and co-trimoxazole but resistant to doxycycline, ampicillin, and chloramphenicol. Mohamed and Shehata (2009) [13] also revealed that neomycin, gentamycin, and trimethoprim are the most effective drugs for *E. coli*.

In the present study the isolate of *Klebsiella* spp. was subjected to antibiotic sensitivity test. The results indicated the highest susceptibility of *Klebsiella* spp. isolate towards tetracycline, ciprofloxacin, gentamicin, amoxycylav, chloramphenicol, levofloxacin, meropenem, co-trimoxazole and cefotaxime. *Klebsiella* isolates showed the highest resistance towards ampicillin only. This resistance pattern is a known feature of *Klebsiella* spp. attributed to beta-lactamase production, which

renders beta-lactam antibiotics, including ampicillin, ineffective (Cheng *et al.*, 2018; Nirwati *et al.*, 2019) [15]. Manikandan and Asmath (2013) [12] observed high sensitivity to imipenem (86.1%) and gentamicin (80.6%), with resistance rates for ampicillin exceeding 90% in isolates from cattle, aligning with findings in poultry.

The results of antimicrobial sensitivity testing indicated the highest susceptibility of *Proteus mirabilis* to gentamicin, amoxycylav, levofloxacin, meropenem and cefotaxime followed by ciprofloxacin. On the other hand, the *Proteus mirabilis* isolate was most resistant to tetracycline, ampicillin, and chloramphenicol, followed by co-trimoxazole. These findings are similar with Chen *et al.* (2012) [5] demonstrate that *Proteus mirabilis* exhibits multidrug resistance due to a combination of intrinsic and acquired mechanisms isolated from chicken.

### 1.3 Characterisation of Bacterial Kidney Pathogens

Based on the provided MALDI TOF MS results of representative isolates, *Escherichia coli*, *Klebsiella pneumoniae* ssp pneumoniae and *Proteus mirabilis* were identified. Additionally, the 16S rRNA gene sequencing of characteristic isolate was done which yielded good quality coloured electropherograms sequences. The consensus sequences were used for phylogenetic analysis and submitted to Genbank to get accession numbers [*Escherichia coli* (PQ316049), *Klebsiella pneumoniae* (PQ325686) and *Proteus mirabilis* (PQ325687)].

### 1.4 Gross pathology and Histopathology

Postmortem examination of collected chickens with kidney lesions was observed as swollen, enlarged and congested kidneys (Fig. 7). A few birds revealed haemorrhages on the surface of the kidney with whitish fluid material in dilated ureters resulted severe atrophy of left division ( Fig. 8). Some cases also revealed the presence of necrotic foci and pinpoint haemorrhages on the surface of the kidney besides enlargement (Fig. 9). In severe cases, kidneys were swollen, enlarged and light pale in colour with severe dilated ureters filled with urate deposits (Fig. 10).

The present findings closely supported the findings of the Ameen *et al.* (2015) [2], Al-Hiyali *et al.* (2005) [24] and Rafiq *et al.* (2024) [18].



**Fig 7:** Swollen, enlarged and congested kidney of chicken



**Fig 8:** Hemorrhagic kidney showing whitish fluid material in dilated ureter resulting severe atrophy of left division



**Fig 9:** Necrotic foci and petechial haemorrhages on the surface of kidney



**Fig 10:** Swollen and enlarged kidney with severe dilated ureters filled with urate deposits

The histopathological examination of the affected kidney included the dilation of renal tubules along with mononuclear cell infiltration in interstitium and glomerular shrinkage (Fig 11). Kidneys revealed glomerular and periglomerular cellular infiltration with complete cease of Bowman's space (Fig. 12). Degenerative changes in renal tubules with shrunken glomeruli were an evident feature (Fig. 13). Marked congestion and haemorrhages in intertubular spaces were common in most

cases (Fig. 14). Glomerular lesions were observed in the form of glomerular hypercellularity with focal haemorrhages in between renal tubules (Fig. 15). Some of the kidneys revealed desquamation and necrosis of tubular epithelium along with marked congestion (Fig. 16).

The results of the histopathological examination of the current study were identical with those mentioned by Sokker *et al.* (1998) <sup>[22]</sup>, Ameen *et al.* (2015) <sup>[2]</sup> and Rafiq *et al.* (2024) <sup>[18]</sup>.

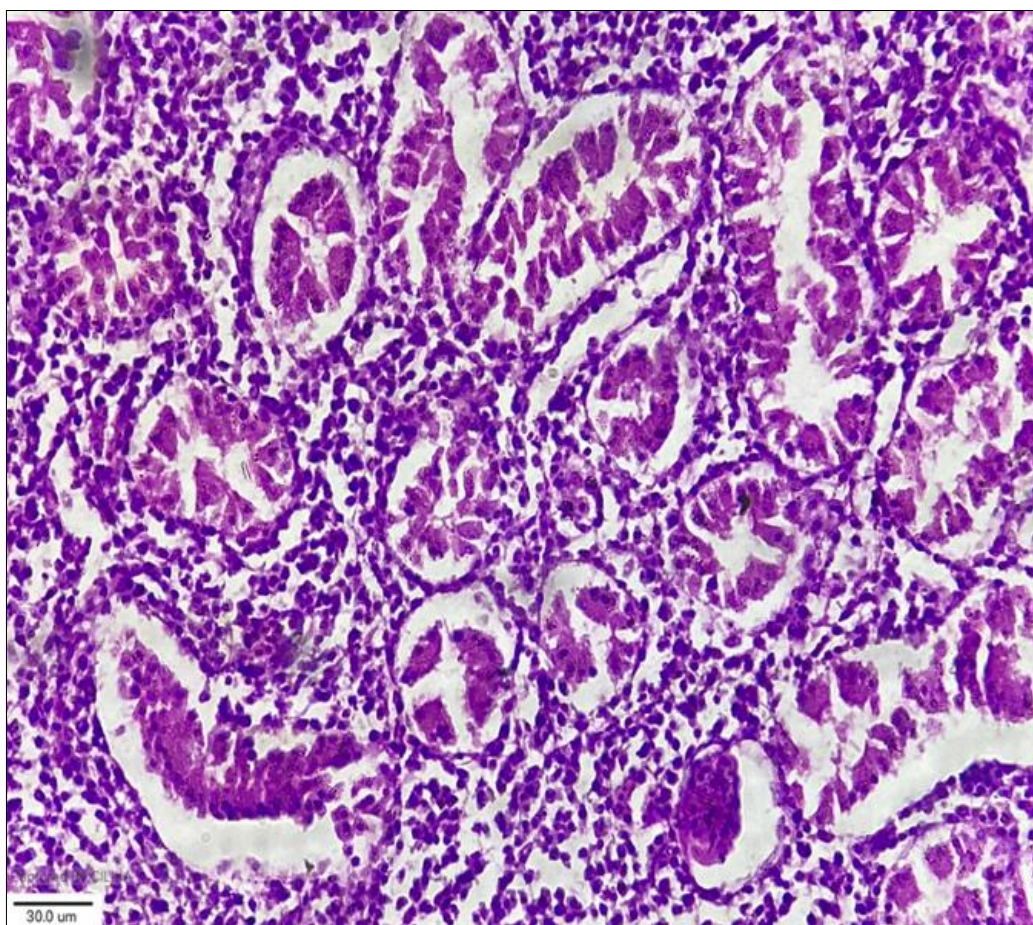


**Fig 11:** Photomicrograph of a section of kidney revealing dilation of renal tubules along with enlargement of epithelial cells with mononuclear cell infiltration in interstitium and glomerular shrinkage (arrow) (H&E X 400)

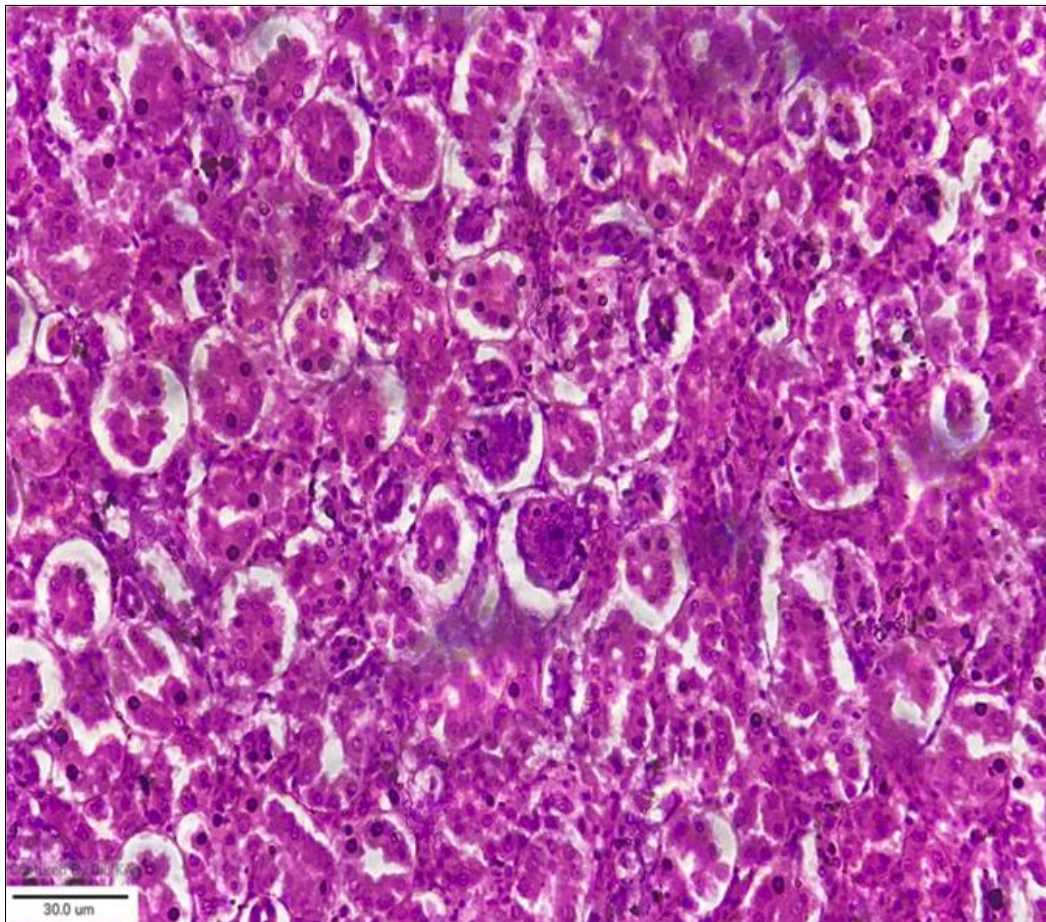


**Fig 13:** Photomicrograph of a section of kidney revealing degenerative changes in renal tubules with shrunken glomeruli (Arrow) (H&E X 400)

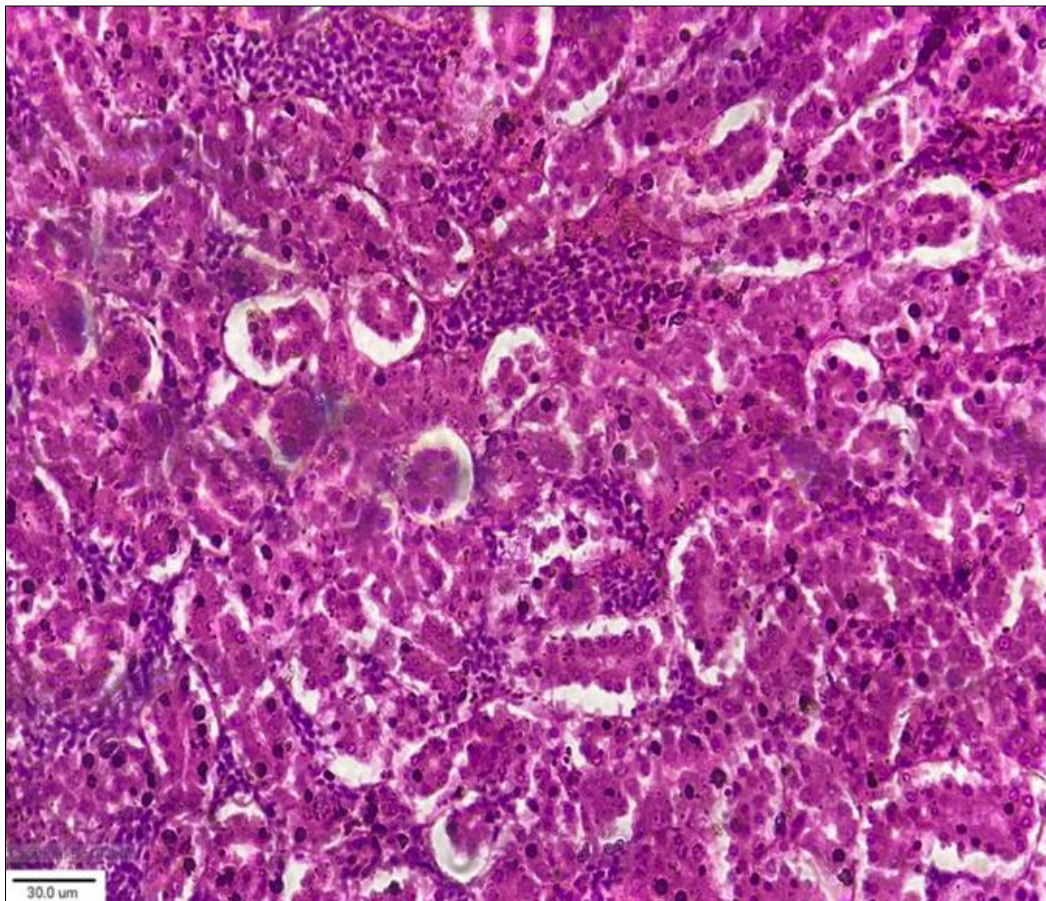


**Fig 14:** Photomicrograph of a section of kidney revealing cortical area showed marked intertubular congestion and haemorrhages (H&E X 400)

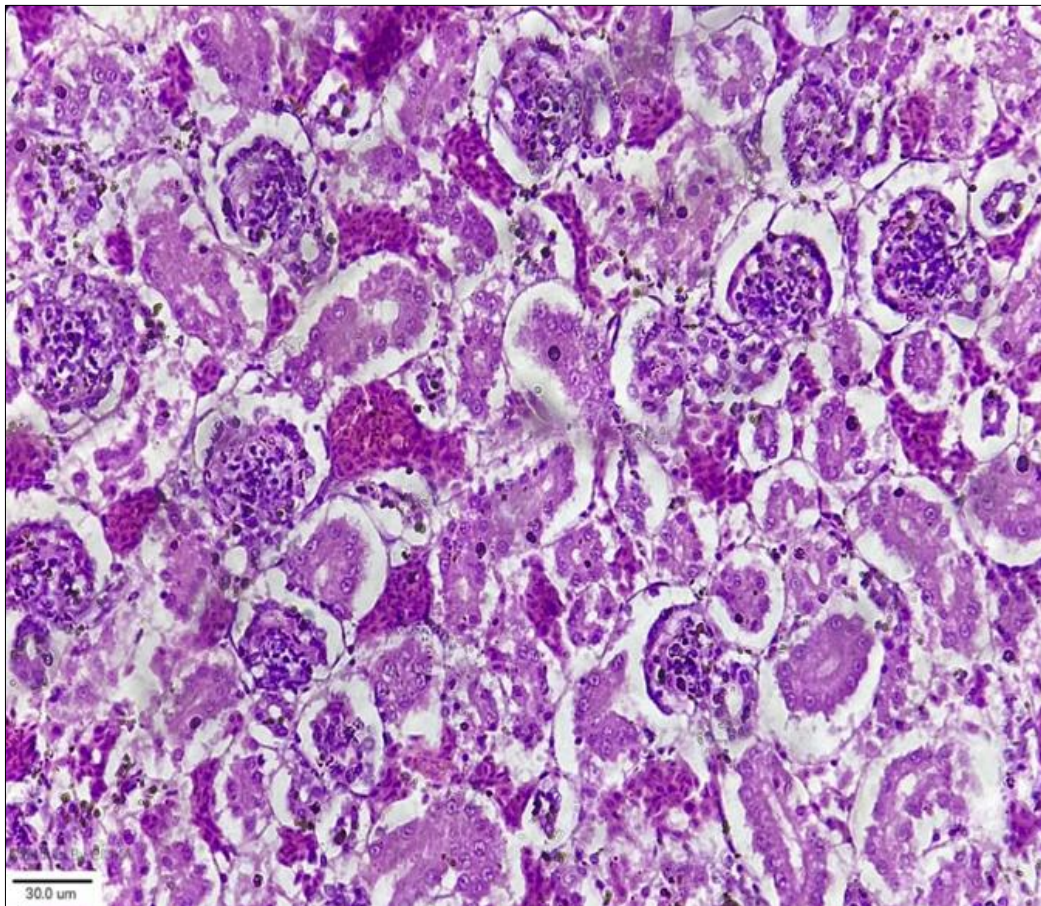


**Fig 15:** Photomicrograph of a section of kidney revealing focal intertubular haemorrhages with hypercellularity in glomeruli (H&E X 400) Fig.

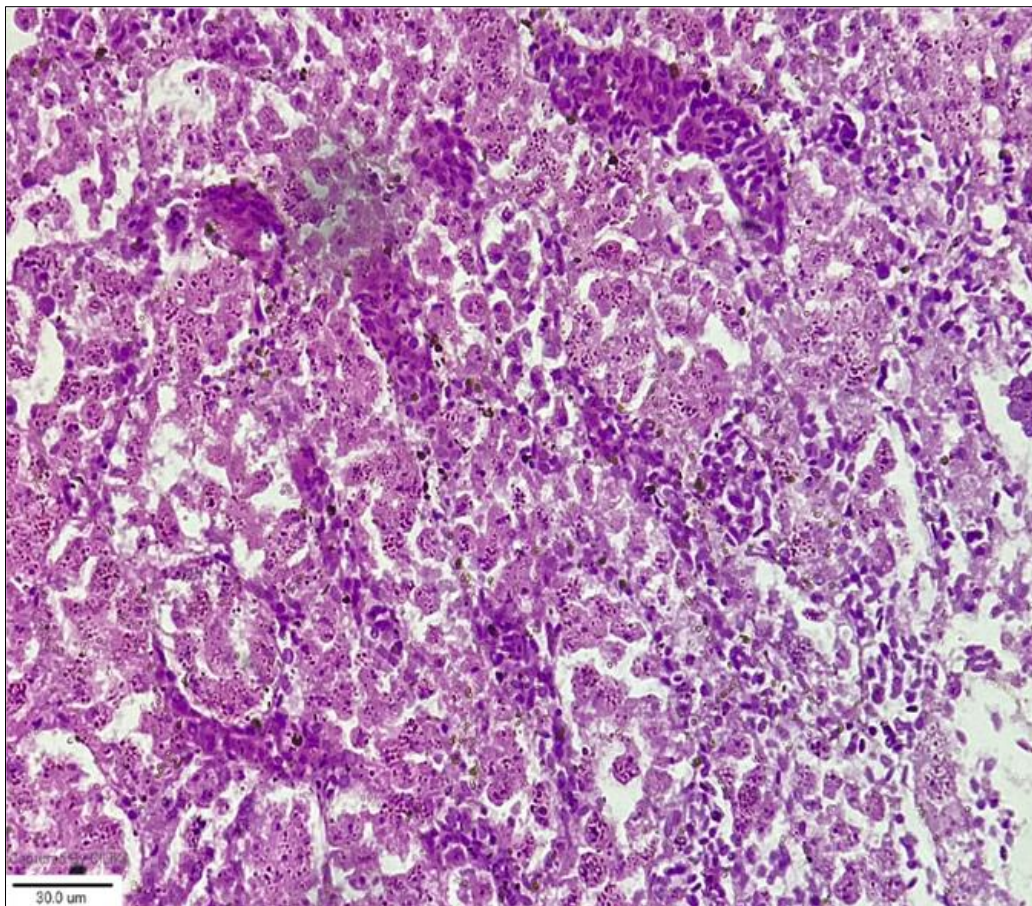


**Fig. 16** Photomicrograph of a section of kidney revealing tubular degeneration with marked severe congestion (H&E X 400)

## Conclusion

In this study out of 258 dead birds examined, 44 nephropathic kidney samples gave 14 bacterial isolates that were identified through initial phenotypic characterization based on their typical growth patterns on different media and the results of biochemical tests. Additionally, they displayed resistance to multiple commonly used antibiotics used in poultry. They were found to be multi-drug resistant to the commonly used antibiotics like ampicillin and co-trimoxazol. The prevalence of *E. coli*, *Klebsiella* spp., *Proteus mirabilis*, were 11 (78.57%), 01 (7.14%), 02 (14.28%), respectively in poultry. Further, the bacterial identification was confirmed using MALDI-TOF MS. Additionally, the 16S rRNA gene sequencing of characteristic isolate was done which yielded good quality coloured electropherograms sequences. The consensus sequences were used for phylogenetic analysis and submitted to Genbank to get accession numbers [*Escherichia coli* (PQ316049), *Klebsiella pneumoniae* (PQ325686) and *Proteus mirabilis* (PQ325687)]. The gross lesions observed in kidneys were enlargement and congestion with presence of necrotic foci and petechial hemorrhages on the surface of kidneys of chicken. The histopathological examination of affected kidneys included Marked tubular necrosis and glomerular degeneration along with cellular infiltration in glomeruli and peritubular space. Additionally, the lesions in kidney were characterized by marked cortical congestion and haemorrhages in interstitial spaces between renal tubules.

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**Consent for publication:** All the authors consent to the publication of this manuscript.

**Ethics approval consent to participate:** This research followed the guidelines specified by Institutional Animal Ethics Committee (IAEC). All the experimental procedures were carried out as per the recommendations of the IAEC.

Use of Artificial Intelligence (AI)-Assisted Technology for manuscript preparation: The authors confirm that there was no use of AI-assisted technology for assisting in the writing of the manuscript and no images were manipulated using AI.

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