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Klebsiella pneumoniae-Isolation, Identification and Characterization from Naturally Infected Farmed Raised Nile Tilapia *Oreochromis niloticus* (Linnaeus, 1758) in West Bengal, India

Supradhnya Namdeo Meshram and T. Jawahar Abraham

Dept. of Aquatic Animal Health, Faculty of Fishery Sciences, West Bengal University of Animal and Fishery Sciences, 5 Budherhat Road, Chakgaria, Kolkata, West Bengal (700 094), India

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Corresponding sailjameshram@gmail.com

0000-0002-6046-9703

ABSTRACT

The experiment was conducted in 2015 (March–May, 2015) at aquatic animal health laboratory, Faculty of Fishery Sciences, Kolkata, West Bengal, India. *Klebsiella pneumonia* was a rod-shaped, Gram-negative, and facultative anaerobic bacterium. It was widespread in nature and found in many environments such as soil, plants, industrial effluent, sewage, surface water and drinking water. It was one of the important human pathogens, causing most commonly pneumonia, typically bronchopneumonia and bronchitis. However, there were few reports suggesting its potential role as an aquatic pathogen. It was an opportunistic pathogen and was usually present in the normal microbiota of fish but causes diseases in favourable conditions such as low water quality. The present study reported the occurrence of disease in *Oreochromis niloticus* caused by *K. pneumoniae*. The infected fish showed clinical signs such as lethargy, anorexia, gill discoloration, fin/tail rot, subcutaneous haemorrhages and ascites. Bacteria were isolated from the kidney and confirmed as *K. pneumoniae* (Accession no. OQ789963) by morphological evaluation, biochemical tests and nucleotide sequence of 16S rDNA. To observe pathological changes in tissue level, the kidney, brain, liver and spleen were selected for histopathology. The vital organ and kidney sections showed melano-macrophage aggregate, glomerulopathy, inflamed nephritic tubules, degeneration of nephritic tubules, and cellular hypertrophy. The brain exhibits inflamed neurons, haemorrhage, granuloma-like structure and cerebellum with sponge-like appearance. With the findings of this study, the systemic pathogenesis both on the external body parts as well as in internal organs of *O. niloticus* by *K. pneumoniae* infection was elucidated.

KEYWORDS: *Klebsiella pneumoniae, Oreochromis niloticus*, 16s rDNA, histological changes

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1. INTRODUCTION

The aquaculture sector played a significant role in the economy, food and livelihood security by its contribution to income and wealth through the supplied of nutritious food. Tilapia considered to been one of the most productive and internationally traded food fish in the world (Siddik et al., 2014). It was popularly known as aquatic chicken and believed as the food fish for the 21st century. Globally, more than 10 species of tilapias beed cultured and was the second most farmed raised fish specially *Oreochromis niloticus* after carps, which accounts for the production of 5.5 mt (Anonymous, 2022). China was the largest producer of tilapia followed by Indonesia, Egypt, Brazil and Thailand (Anonymous, 2022). India was fourth in captured fisheries and second in aquaculture production. India's total fish production was 0.75 million metric tons (MMT) in 1950–1951, then increased to 9.5 million mt in 2012–2013. Furthermore, due to initiatives and programmes financed by the Indian government, the current production had reached 16.25 million mt (Anonymous, 2022). Pathogens were a major caused of infectious diseases and mortality in wild fish stock and cultured in confined conditions. An expansion of semi-intensive to intensive farming to boost production led to an increased in diseases because of more mass and stressful condition (Das and Mishra, 2014). Among these, bacterial diseases in freshwater fish had emerged as a foremost issue in India (Mishra et al., 2017). Tilapias believed to been resistant to bacterial, parasitic, fungal and viral diseases compared to other cultured fish species (Amal and Zamri-Saad, 2011). But in recent timed, tilapias was reportedly susceptible to several bacterial and viral diseases in aquaculture (Behera et al., 2018). The regular encountered pathogens included *Aeromonas veroni*, *A. hydrophila*, *A. Caviae*, *A. jandaei*, *Edwardsiella tarda*, *Streptococus agalactiae*, *S. iniae*, *Flavobacterium columnare*, *Lactococcus garviae*, *Pseudomonas* spp., *Ichthyophitirius multifiliis*, *Tricodina* sp., Gyrodactylus niloticus, Tilapia lake virus (TiLV) and Tilapia parvovirus (TiPV) (Klesius et al., 2008; Evans et al., 2009; Eyngoret al., 2014; Barony et al., 2015; Assane et al., 2019; Vaneci-Silva et al., 2022; Rajendran et al., 2023). More attention had been paid to bacterial zoonoses of fish since new fish-borne bacterial zoonoses had discovered (via improved molecular diagnostic techniques) (Oliveira et al., 2014; Das et al., 2018; Zhong et al., 2021). *Aeromonas* spp. and *S. iniae* founded to been pathogenic for both fish and humans in which *S. iniae* showed strict fish-born zoozoses (Bomo et al., 2003; Gauthier, 2015).

K. pneumoniae recognized as the most common multidrug-resistant bacterial pathogen in humans, and little was known about its pathogenicity in aquatic animals. In humans, it caused neonatal sepsis, nosocomial pneumonia, liver abscess, and chronic intestinal diseases 4 (Martin and Bachman, 2018.) in terrestrial animals, liver abscesses in nonhuman primates, subclinical mastitis in dairy cows, and respiratory infections in cats and dogs (Cheng et al., 2021; Silva et al., 2022). In aquatic animals, it had reported to caused pleuritis and suppurative bronchopneumonia in sea lions (Jang et al., 2010), spasm, splenomegaly, congestive hepatic and intestinal edema in American bullfrogs, *Rana catesbeiana* (Lin et al., 2023), liver and kidney necrosis, peritoneal hemorrhages in Indian major carp (Das et al., 2018) and edematous bodies and hyperaemia in leeches (Yibin et al., 2021). In Brazil, it was responsible for caused disease outbreak in ornamental Nishikigoi carp, *Cyprinus carpio* (Oliveira et al., 2014).

Very few studied had published so far on the infection in fish with *K. pneumoniae*. In the current studied, *K. pneumoniae* was isolated from naturally infected tilapia, *Oreochromis niloticus* which was the ultimate food source. The purpose of the studied was to identified the etiological agent of the bacterial disease outbreak in cultivated Nile tilapia, *Oreochromis niloticus* by analyzing its morphological, biochemical, and 16S rDNA gene sequence analysis.

2. MATERIALS AND METHODS

2.1. Sample collection

Infected Nile tilapia, *O. niloticus*, juvenile (n=5, weight 50±5.0 g) collected from the Serampur (N 22°44'55.30", E 88°20'23.02") fish farm, in 2015, West Bengal, India. The clinical signed and symptoms recorded and brought to the laboratory of aquatic Animal Health Management, Faculty of Fishery Science, Kolkata for microbiological, histopathology and molecular analysis.

2.2. Bacteria isolation and characterization

For bacterial isolation, aseptically, fish dissected, and samples streaked from the target organ (kidney) on brain heart infusion (BHI) agar (HiMedia). The plates incubated for 24 h at 37°C, and dominant colonies were re-streaked on soybean casein digest agar (trypticase soya agar, TSA) (HiMedia) to obtained a pure colony. Pure colonies were subjected to Gram stained, oxidase, catalase and a series of biochemical reactions described by Lechevallier et al. (1980) and Collins et al. (2004). Further, the bacterial isolates were characterized with biochemical kit-KB002 (HiMedia).

2.3. Bacterial DNA extraction and 16S rDNA gene amplification

The genomic DNA of *Klebsiella pneumoniae* SMT4K extracted by used a Genomic DNA isolation kit (Macherey-Nagel, Germany) as per the manufacturer's protocol. A partial sequence of the highly conserved 16S rDNA gene amplified and sequenced used set of universal prokaryotic

primer 8F, 5'-AGAGTTTGATCCTGGCTCAG-3', and 1492R, 5'GGTTACCTTGTTACGACTT-3' (Eden et al., 1991). The PCR master mixed contained 50 ng of genomic DNA, 10 µM of each primer and 2X PCR Taq Mixture (HiMedia, India). The amplification was done by initial denaturation at 95°C for 5 min, followed by 35 cycled of denaturation at 95°C for 30s, annealing at 44°C for 30 s, and extension at 72°C for 60s. The final extension was at 72°C for 5 min. The amplified PCR products was analyzed on a 1.5% agarose gel stained with 0.5 µg ml^{-1} ethidium bromide in 1X Tris-acetate-EDTA (TAE) and visualized under UV light.

2.4. Sequencing and phylogenetic analysis

The sequence of amplified 16S rDNA gene was obtained by automated sequencer from Genomics Division, Xcelris Labs Ltd, ahmedabad, India. The sequence edited used Clustal×MEGA 11 software. Closely related sequences determined by Basic Local Alignment Searched Tool (BLAST) (http://blast.ncbi.nlm.nih.gov) to found out the nearest neighbor of the amplified sequence. Phylogenetic analysis was performed on a selection of 16S rDNA gene sequences that comprised the new consensus sequence and the tree was inferred used the neighbor-joined method.

2.5. Histopathology

After observed and recorded the external and internal signed of disease, the tissues collected from infected fish for histological analysis. The liver, spleen, kidney and brain fixed in Bouin's solution for 24–48 h. The fixed organs were processed by standard techniques and embedded in paraffin wax. A thin (5 µm) section was prepared and stained with haematoxylin and eosin to observed the pathological changed under the light microscope (Roberts, 2012).

2.6. Antimicrobial resistance assay

The disc diffusion method used on Muller-Hinton agar (MHA) to determine the bacterium's antibiotic susceptibility as given by the Clinical and Laboratory Standards Institute (Oxoid, Hampshire, UK), which sets the standards (Bauer et al., 1966; CLSI, 2006). Young culture of bacteria (24 h old) grown on a BHI agar plate, single picked with a sterile cotton swab, and spread onto MHA plates. Antibiotic impregnated discs was placed aseptically onto the inoculated agar plates at least 15 mm away from the edge, at an equal distance and sufficiently separated from each other to avoided overlapping of the zone of inhibition. The plates were then incubated for 24 h at 30±2°C and the diameter of the zone of inhibition in mm was measured. Interpretation of sensitivity was based on the zone size interpretation chart provided by the manufacturer of the antibiotic impregnated discs. The antibiotic agents (disc content indicated in parentheses) used in the presented studied, viz., amoxyclav (30 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), Polymyxin B (300 µg), clindamycin (2 µg), Colistin (10 µg) co-trimoxazole (25 µg), Tobramycin (10 µg), erythromycin (15 µg), gentamycin (10 µg), gatifloxacin (5 µg), nitrofurantoin (300 µg), oxytetracycline (30 µg), sulphafurazole (300 µg),Trimethoprim (5 µg), vancomycin (30 µg), Doxycycline (30 µg).

3. RESULTS AND DISCUSSION

3.1. Pathological signs of diseased fish

The observed clinical signs in infected fish was lethargy, swimming abnormality, anorexia, gill discoloration, tail/fin rot, hemorrhages in different parts of the body like base of the dorsal, pectoral and pelvic fin; mouth and opercular area, pale liver, accumulation of fluid in body cavity. The major pathological signs observed was depicted in Figure 1.

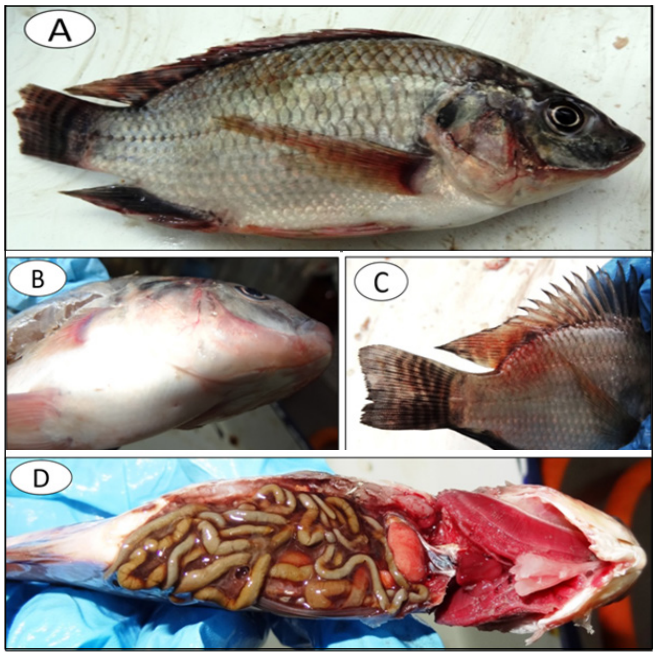

Figure 1: External and internal pathological signed of diseased Nile tilapia showed tail/fin rotted, hemorrhagic fins (A), hemorrhages at pectoral fin, pelvic fin and opercular area (B), hemorrhages at base of dorsal fin and body (C), ascites in body cavity (D)

3.2. Isolation and characterization

In the presented study, 5 different typed of bacterial colonies were isolated from the kidney. Based on the colony morphology (size, shape and colour) most predominant bacterial colony (SMT4K) was chosen and re-streaked on TSA to got pure colonies. Biochemical characteristics of pathogen SMT4K were Gram negative, rod-shaped, non-motile, catalase, vogus-proskauer, citrate, nitrate, urease was positive and methyl red, indole, and hydrogen sulfide were negative. The capsule was not observed from the selected pathogen.

3.3. Sequenced and phylogenetic analysis

The bacterial pathogen isolated from diseased Nile tilapia, O. niloticus was initially confirmed as *K. pneumoniae* at the genus leveled through a biochemical test and finally confirmed by 16s rDNA sequence analysis. The universal prokaryotic primer 8F and 1492R successfully amplified ≈ the 1500 bp sequence (Figure 2) of the 16s rDNA gene from *K. pneumoniae*. The edited sequence was finally confirmed by 16S rDNA gene sequence strain of SMT4K as *K. pneumoniae* at 1199 bp. Phylogenetic tree constructed based on the alignment of fifteen 1200-1500 bp rDNA gene sequences which included 11 Klebsiella spp., *Edwardsiella tarda* same group and *Aeromonas* sp. and *Pseudomonas* sp. from other groups (Figure 3). The *K. pneumoniae*, SMT4K nucleotide sequence information had deposited in NCBI Genbank under the accession numbered OQ789963.

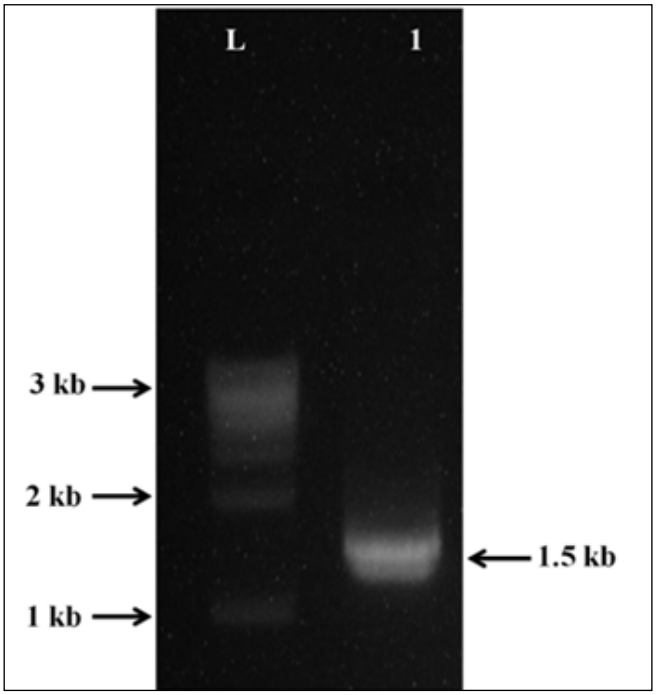

Figure 2: Agarose gel (1.5%) showing 16S rDNA gene amplification of *K. pneumoniae* SMT4K from *O. niloticus*. Lane L: 1kb DNA Ladder (Takara Bio Inc., Japan); Lane 1: *K. pneumoniae*

3.4. Histopathological analysis

Besides microbiology and molecular identification, pathological identification also revealed the caused of infection by observed the changed in different organs ofNile tilapia. The kidney tissue exhibits melanomacrophage aggregate, dilation of the blood vessel, glomerulopathy, inflamed nephritic tubules, necrosis, degeneration of nephritic tubules, proteinaceous casts in the tubular lumen, cellular hypertrophy, nuclear hypertrophy, widened lumen

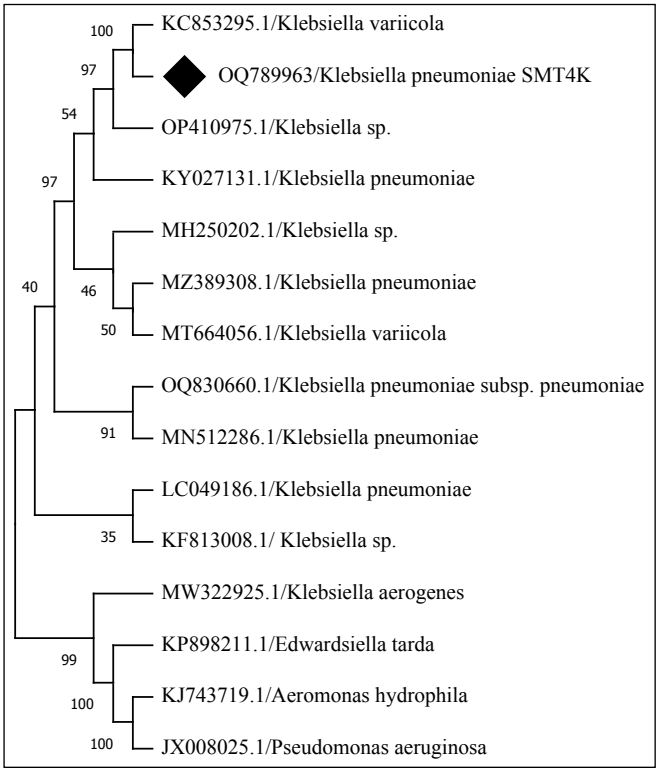

Figure 3: Phylogenetic tree constructed through Kimura 2-parameter model using the Neighbor-joining method of 16S rDNA gene sequence of *K. pneumoniae* SMT4K. Numbers at nodes indicate bootstrap confidence valueed (1000 replicates). The GenBank accession numbers are provided for each species

and hypoplastic haematopoietic tissue (Figure 4 A-B). The brain tissue showed lesion development, inflammation, haemorrhage, inflamed neurons and granuloma-liked structure (Figure 4 C-D). The changed in liver tissue depict cellular degeneration, severe congestion and mild congestion of blood cells, haemocyte infiltration into sinusoids and fatty changed (Figure 4E) and spleen showed densely packed red pulp and loosely packed white pulp, melanomacrophage aggregate and necrosis (Figure 4F).

3.5. Antmicrobial resistance assay

Particularly when it came to harmful bacteria like *K. pneumoniae*, antibiotic resistance is a serious concerned. An antibiogram studied of SMT4K revealed that Ampicilin (AMP), Ciprofloxacin (CIP), Clindamycin (CD), Tobramycin (TOB), Erythromycin (E), Nitrofurantoin (NIT), Trimethoprim (TR) and Vancomycin (VA) was resistant (Table 1). The majority of the results of the antibiogram susceptibility tested found to been comparable to those reported by previous researchers (Das et al., 2018).

The major obstacle to effective aquaculture practiced was diseases. In general, *K. pneumoniae* founded everywhere in nature. They invade the mucosal surfaces of mammals like humans, horses, swine, amphibians and are also founded

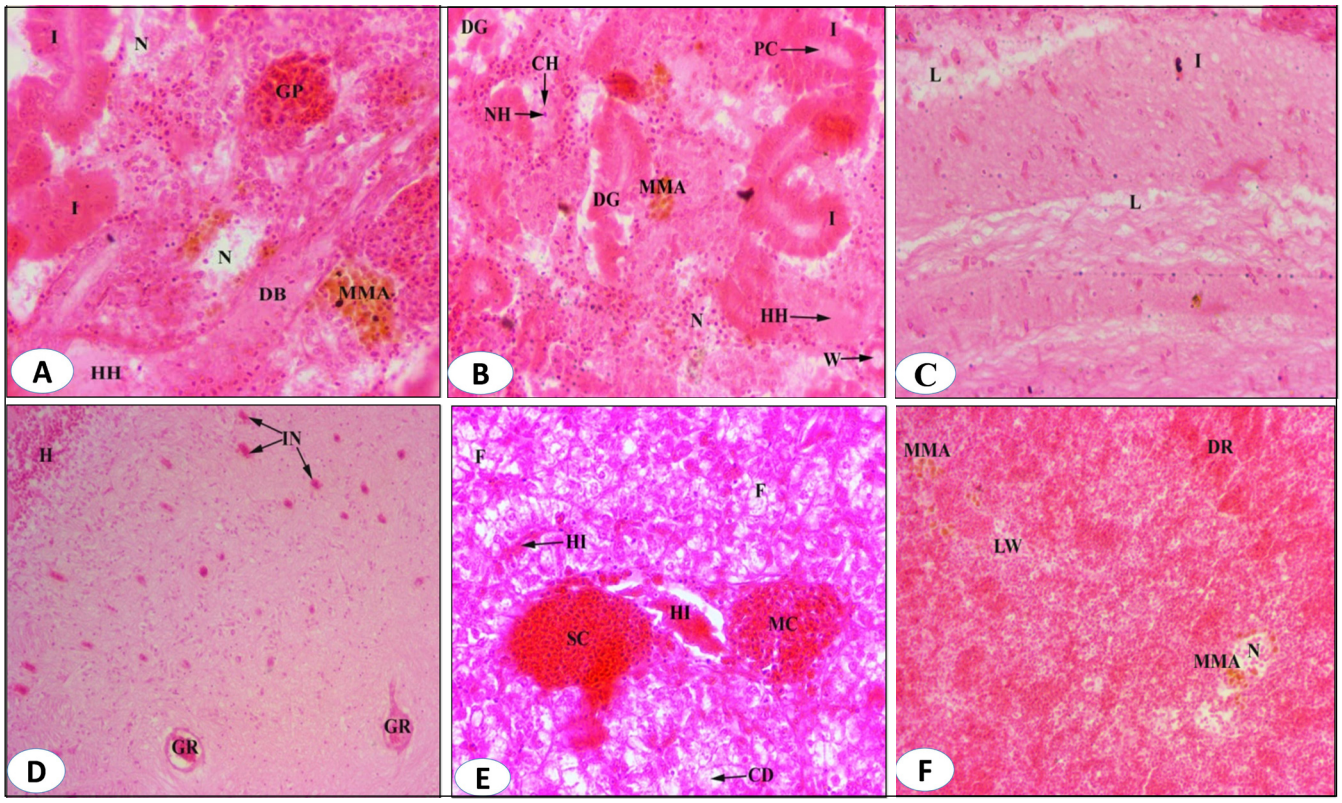

Figure 4: Histopathological changed in the kidney (A-B), brain (C-D), Liver (E) spleen (F) tissue of naturally infected Nile tilapia *Oreochromis niloticus* by *K. pneumoniae* showed melanomacrophage aggregate (MMA), dilation of blood vessel (DB), glomerulopathy (GP), inflamed nephritic tubules (I), necrosis (N), degeneration of nephritic tubules (DG), proteinaceous casts in the tubular lumen (PC), cellular hypertrophy (CH), nuclear hypertrophy (HN), widened lumen (W), hypoplastic haematopoietic tissue (HH), lesion development (L), inflammation (I), haemorrhage (H), inflamed neurons (IN) and granuloma-like structure (GR), cellular degeneration (CD), severe congestion (SC) and mild congestion (MC) of blood cells, haemocyte infiltration into sinusoids (HI) and fatty changed (F), the densely packed red pulp (DR) and loosely packed white pulp (LW) X200 and X100 H&E

in some fish species such as *Nemipterus japonicus*, *Cyprinus carpio, Labeo rohita* (Das et al., 2018). Bacterial diseases had a significant impact on farmed raised tilapia culture. There are different bacterial species had identified in commercial Nile tilapia aquaculture disease outbreaks and mass mortalities (*A. caviae, A. hydrophila, A. jandaei, A. veronii, Edwardsiellat arda, Francisella orientalis, Flavobacterium columnare, Lactococcus garviae, Streptococcus agalactiae, S. dysgalactiae,* and *S. iniae*) (Vaneci-Silva et al., 2022). In the presented studied the isolated pathogen SMT4K was confirmed as *K. pneumoniae* through biochemical tested and 16s rDNA sequence analysis. It confirmed behind the disease outbreak in Nile tilapia mortalities. The clinical signed of the disease (Figure 1) founded more or less similar to the worked done by Vaneci-Silva et al. (2022) in Brazil. Non-specific clinical signed was also reported in Indian major carp, *Labeo rohita* in natural *K. pneumoniae* infection by Das et al. (2018). The microbiological analysis is important for proper identification of disease in fish so the series of biochemical tested done to identified the bacteria in the presented investigation was founded similar to the work done by Gopi et al. (2016) and Das et al. (2018).

The phylogenetic analysis of different *Klebsiella* species used in the studied revealed the 16S rDNA gene sequence of SMT4K similarity with KC853295.1. They were clusters with the highest bootstrap valued. Vaneci-Silva et al. (2022) also identified the infection of *K. pneumoniae* pathogenic strain from Nile tilapia through 16S rRNA gene sequence analysis.

Histopathology was a well established tool to considered the qualitative changed of the affected organs and the patterns of recovery. In the presented studied, changed observed in the kidney of *O. niloticus* (Figure 4 A-B) similar typed of changed noted by Vaneci-Silva et al. (2022). Also, Gaafar et al. (2015) observations from the kidney revealed glomerulotubular necrosis with activation of melanomacrophage centres. This was about *K. pneumoniae*, but other bacteria from Enterobacteriaceae family was able to caused infection in *O. niloticus* and several pathological

Table 1: Antimicrobial susceptibility tested result of *K. pneumoniae* (SMT4K) isolate from naturally infected Nile tilapia

Antibiotics	Results
Amoxyclav (AMC30)	I
Ampicilin (AMP10)	R
Chloramphenicol (C30)	S
Ciprofloxacin (CIP5)	R
Polymyxin B (300)	S
Clindamycin (CD2)	R
Colistin (CL10)	S
Co-trimoxazole (COT25)	S
Tobramycin (TOB10)	R
Erythromycin (E15)	R
Gentamycin (GEN10)	I
Gatifloxacin (GAT5)	S
Nitrofurantoin (NIT300)	R
Oxytetracycline (OTC30)	S
Sulphafurazole (SF300)	S
Trimethoprim (TR5)	R
Vancomycin (VA30)	R
Doxycycline (DO30)	S

changed were documented by Hassan et al. (2012) such as glomerular congestion, vacuolation and necrosis of some renal tubular epithelium with pyknotic nuclei in the kidney. Abraham et al. (2015) reported histological changed in the kidney of african catfish *Clarias gariepinus* infected by *Edwardsiella tarda* matched the presented studied. The pathological changed observed in liver tissue (Figure 4E) also studied by Ramkumar et al. (2014) in the experimentally challenged *Labeo rohita* with *Providencia vermicola*, showed hepatic necrosis and irregular cytoplasmic vacuolation with converging sinusoids. Likewise, the liver of infected fishes (*Aphanopus carbo*, *Hoplostethus atlanticus*, *Phycis blennoides*, *Coryphaenoides rupestris*) exhibits the trabecular arrangement of hepatocytes and significant variation in the individual hepatocytes reported by Feist et al. (2015). Gopi et al. (2016) had observed similar alterations in tissue structure of the liver and kidney of *Amphiprion nigripes* due to infection with *K. pneumoniae*. Melanomacrophage aggregates (MMAs) in the spleen and kidney observed in the presented studied, exhibiting the defense mechanism which supported the earlier surveyed of Vaneci-Silva et al. (2022) in Nile tilapia infected with *K. pneumoniae*. The brain pathology of the presented studied exhibited a granuloma-liked structure (Figure 4 C-D) due to *K. pneumoniae*. Miyazaki and Kaige (1985) recorded granuloma formation in the kidney, liver and spleen of an *E. tarda* infected tilapia but not the brain. Later on, Iregui et al. (2012) studied the other changed, inflammatory necrotic, proliferative changed affecting the walls of the blood vessels of the meninges, brain parenchyma with no evidence of the formation of granuloma in the brain of affected tilapia by *E. tarda*.

The prevalence of antibiotic-resistant bacteria in aquaculture had dramatically increased recently in several regions of the world. Antibiotics were widely used in aquaculture and animal husbandry systems to controlled bacterial infections (Praveen et al., 2014). Due to their frequent exposure to various antibiotics in the intestinal tract, members of the *Enterobacteriaceae* family had the ability to spread genes that confer antibiotic resistance (Goldstein et al., 2001). In our studied *K. pneumoniae*, SMT4K founded to been resistant against several third generation antibiotics.

4. CONCLUSION

In aquaculture, active surveillance method was crucial for prevented the development of pathogenic and multidrug-resistant strains of *K. pneumonia* and for measured the financial and health impacts of infection. Infection with *K. pneumoniae* caused *O. niloticus* to developed systemic pathogenesis, which could impact both its internal organs and external body part. It could have concluded that the limited numbered of samples had screened and restricted area covered in the presented studied, extensive investigation required to determine the prevalence and epidemiological factors.

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