Journal of Water & Health

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Journal of Water and Health Vol 22 No 4, 721 doi: 10.2166/wh.2024.382

Prevalence of antimicrobial resistance, biofilm formation, efflux pump activity, and virulence capabilities in multi-drug-resistant *Klebsiella pneumoniae* isolated from freshwater fish farms

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ABSTRACT

The present study aimed to determine the antibiotic resistance, underlying mechanisms, antibiotic residues, and virulence genes involved in 32 multi-drug-resistant *Klebsiella pneumoniae* isolates from freshwater fishes in Andhra Pradesh, India. Antibiogram studies revealed that all isolates were multi-drug-resistant, harbored *tetA* (96.8%), *tetC* (59.3%), *tetD* (71.9%), *nfsA* (59.3%), *nfsB* (53.1%), *sul2* (68.7%), *qnrC* (43.7%), *qnrD* (50%), bla_{SHV} (75%), bla_{TEM} (68.7%), and $bla_{CTX\text{-}M}$ (93.7%) genes. Multiple antibiotic resistance index was calculated as 0.54. Sixteen isolates were confirmed to be hyper-virulent and harbored *magA* and *rmpA* genes. In total, 46.9, 31.2, and 21.9% of the isolates were categorized as strong, moderate, or weak biofilm formers, respectively. All isolates possessed an active efflux pump and harbored *acrA*, *acrB*, *acrAB*, and *tolC* genes in 94% of the isolates, followed by mdtK (56.2%). Porins such as *ompK35* and *ompK36* were detected in 59.3 and 62.5% of the isolates, respectively. Virulence genes *fimH-1*, *mrkD*, and *entB* were present in 84.3, 81.2, 87.5% of the isolates, respectively. These findings imply a potential threat that multi-drug-resistant bacterial pathogens could transmit to surrounding environments and humans through contaminated water and the aquaculture food chain.

Key words: antimicrobial resistance, aquaculture, biofilms, efflux pump, multi-drug resistance, virulence

HIGHLIGHTS

- Multiple factors contribute to the emergence of MDR *Klebsiella pneumoniae* in aquaculture, raising concerns about public health hazards.
- Hyper-virulent, antibiotics resistance, biofilm, efflux pump gene determinants were identified by PCR.
- Findings suggest that a comprehensive multifaceted approach based on better management practices and biosecurity in aquaculture is essential.

ABBREVIATIONS

AMR antimicrobial resistance
MDR multi-drug-resistance
RND Resistant Nodulation Division
MATE Multi-Antimicrobial Extrusion
TSB tryptic soy broth
TSA tryptic soy agar
AST antibiotic susceptibility test
CLSI Clinical and Laboratory Standards Institute
MAR multiple antibiotic resistance
ESBL extended-spectrum β-lactamase
DDST double disc diffusion test
PCR polymerase chain reaction
ELISA enzyme-linked immunosorbent assay
ARB antibiotic-resistant bacteria
ARGs antibiotic-resistant genes

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INTRODUCTION

Aquaculture is a significant agricultural sector that is rapidly expanding to meet global protein demand (Cabello *et al.* 2013). The modernization of aquaculture practices, comprehensively based on fish feed, seed, production system design, better husbandry, and health management, has contributed to the phenomenal rise of aquaculture over the last decade (Subasinghe *et al.* 2019). As a result, aquatic animals have faced several health issues, including environmental stress, the emergence and introduction of infectious pathogens, and an increase in the frequency of infectious disease outbreaks (Das *et al.* 2018). Aeromoniasis, pseudomoniasis, columnaris, edwardsiellosis, saprolegniasis, fin rot, gill rot, epizootic ulcerative syndrome, trichodiniasis, white spot disease, argulosis, dactylogyrosis, and some environmental factors severely affect farmed fishes (Mishra *et al.* 2017a). Disease outbreaks in aquaculture have been documented in India and other Asian countries, resulting in the widespread use of aqua-medicines and other biological products (Mishra *et al.* 2017b; Suresh & Pillai 2023). However, imprudent use of such products has recently drawn criticism due to the possibility of medication residues and the development of antimicrobial resistance (AMR) among aquatic microorganisms, jeopardizing the safety of food derived from aquaculture (Cabello *et al.* 2013; Watts *et al.* 2017). This problem is exacerbated in nations such as India, where the use of preventive antibiotics for human health, animal husbandry, and aquaculture is on the rise (Mishra *et al.* 2017a, 2017b). Furthermore, extensive antimicrobial use in aquaculture and different environments exerts selective pressure, resulting in reservoirs of MDR bacteria and transferrable genes in fish pathogens and other microorganisms in aquatic environments (Heuer *et al.* 2009).

Andhra Pradesh has seen significant growth in aquaculture in recent years, with huge potential for the development of fish and shrimp farming (Mishra *et al.* 2017a). *Aeromonas hydrophila*, *Aeromonas caviae, Acinetobacter* spp. *Edwardsiella tarda*, *Pseudomonas aeruginosa*, *Pseudomonas putida*, *Pseudomonas fluorescens*, *Pseudomonas putrefaciens*, *Flexibacter columnar*, *Vibrio* spp., *Streptococcus* spp., and the Enterobacteriaceae family are common fish bacterial pathogens affecting commercially important freshwater fish in this area (Mishra *et al.* 2017a; Das *et al.* 2018; Suresh & Pillai 2023; Suresh *et al.* 2023). *Klebsiella pneumoniae,* a member of the *Enterobacteriaceae* family, that is opportunistically pervasive in the natural environment and benignly colonizes the gastrointestinal tracts of humans and animals, can cause a variety of diseases (Das *et al.* 2018). With the widespread use of antibiotics in humans, veterinary medicine, and agricultural operations over the last few decades, the emergence of *K. pneumoniae* strains harboring various resistance genes has increased considerably (Sivaraman *et al.* 2021). It is well known that *K. pneumoniae* can form biofilms on both living and non-living surfaces, inhibiting drug penetration and pathogenicity (Wasfi *et al.* 2016). Efflux pumps are crucial for bacterial survival against chemical exposure, and *acrAB* and *mdtK*, two efflux pump systems found in *K. pneumoniae*, belong to the Resistant Nodulation Division (RND) and Multi-Antimicrobial Extrusion (MATE) efflux pump families, respectively. The uptake of antibiotics into cells requires porins such as *ompk35 a*nd *ompk36,* which contribute to resistance and pathogenicity (Du *et al.* 2014). The most important virulence factors contributing to *K. pneumoniae* pathogenesis in terms of infection severity are type 1 and type 3 fimbriae, which can contribute to biofilm development and virulence (Wasfi *et al.* 2016; Das *et al.* 2018). Several virulence genes, including mucoviscosity-associated gene A (*magA*) and regulator of mucoid phenotype A (*rmpA*), contribute to the hyper-virulent phenotype of *K. pneumoniae* found on the chromosomes and plasmids (Shon *et al.* 2013; Khattab & Hager 2022).

The interconnected nature of agri-food systems makes it possible for AMR to spread, posing a serious threat to both human and animal health as well as the structure and sustainability of food production (Lulijwa *et al.* 2020). The occurrence of *K. pneumoniae* in various aquaculture settings is alarming, and its mode of entry and existence may pose a threat to aquaculture sustainability, food derived from aquaculture, and the environment (Yang *et al.* 2023). Thus, we undertook the current investigation to evaluate the prevalence of *K. pneumoniae*, the antibiotic resistance profile, the underlying resistant mechanisms, residue detection, and virulence-encoding genes using phenotypic and molecular methods.

MATERIALS AND METHODS

Study area and sample collection

Sampling was conducted on selected finfish farms ($n = 110$) in Krishna ($n = 60$) (16°36′21.22″N, 80°42′56.39′E) and West Godavari ($n = 50$) (16°53′55.65″N, 81°18′.30″E) districts of Andhra Pradesh, India, from June 2021 to October 2022. The selected fish farms rear Indian (*Labeo rohita*, *Catla catla*) and exotic carps (*Ctenopharyngodon idella*) in polyculture systems,

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whereas pangasius (Pangasianodon *hypophthalmus*) were cultivated in monoculture systems (Mishra *et al.* 2017a). Fish ($n = 110$), water ($n = 110$), and sediment ($n = 110$) samples were collected following the standard protocols. Approximately 100–200 g of sediment was collected from each pond. Water samples were collected in sterile containers (200 mL) from different stretches of the water body (Girijan *et al.* 2020). Approximately 2–3 individual fish were collected, packed in sterile polythene bags, and transported to the laboratory under iced conditions for further analysis.

Isolation and identification of *K. pneumoniae*

Fish were dissected, and samples were collected aseptically following the previously established protocols (Austin 2019). Enterobacteria enrichment (EE) broth Mossel (HiMedia, India), a brilliant green lactose broth, was used for selective enrichment of Enterobacteriaceae. Water (10 mL), sediment (10 g) and fish (10 g) were directly inoculated into 90 ml of EE broth and incubated at 37 °C for 18 h (Sivaraman *et al.* 2021). To isolate *K. pneumoniae* strains, a loopful of enriched culture was streaked onto MacConkey agar (HiMedia, India) and incubated at 37 °C for 18 h. Lactose-fermenting colonies ($n = 150$; 2–3 similar colonies from each sample) that were mucoid and bright pink with the morphological characteristics of *K. pneumoniae* were picked, aseptically inoculated into TSB (HiMedia, India), and transported to the Department of Aquatic Animal Health Management, Kerala University of Fisheries and Ocean Studies, Kochi for bacterial identification and characterization. The suspected *K. pneumoniae* colonies were identified using NMIC/ID-95 panel by automated bacterial identification system BD Phoenix™ M50 (BD Diagnostics, USA). *Escherichia coli* ATCC 25922 was used as the control. The confirmed *K. pneumoniae* strains were preserved in TSB containing 20% glycerol and stored at −80 °C for further use.

String test

The string test was used to determine the hyper-mucoviscosity of *K. pneumoniae* isolates. A positive string test result was defined as the formation of a mucoviscous string measuring more than 5 mm in length when loop is used to stretch the colony on agar plate (Shon *et al.* 2013). If the colony stretch was >5 mm, the strain was classified as hyper-virulent *K. pneumoniae* (hvKP), while negative results were classified as classic *K. pneumoniae* (cKP).

Antimicrobial susceptibility testing

Antibiotic susceptibility testing (AST) was done according to the guidelines of the Clinical and Laboratory Standards Institute guidelines (CLSI 2022) using the disk diffusion method. A loop full of a 24 h TSB culture of isolate was streaked on TSA plate and incubated for 24 h at 37 °C. One or two colonies were picked and adjusted to 0.5 McFarland standard. Using a sterile swab stick, bacterial suspension were applied to the surface of Muller-Hinton agar (HiMedia, India) after which test antibiotics discs were applied and incubated for 24 h at 37 °C. The antibiotic panel containing amikacin (30 μg), ampicillin (10 μg), cefotaxime (30 μg), furazolidone (100 μg), nitrofurantoin (100 μg), oxytetracycline (30 μg), doxycycline hydrochloride (30 μg), co-trimoxazole (25 μg), enrofloxacin (10 μg) and ciprofloxacin (5 μg) were used. Zones of inhibition were measured and interpreted as Resistant (R), Intermediate (I), or Susceptible (S) as per CLSI interpretive categories in represented in CLSI 2022. AST was done in duplicate and *E. coli* ATCC 25922 was used as quality control. MDR isolates were noted for those isolates that were resistant to three or more antibiotic classes (Magiorakos *et al.* 2012). The MAR was calculated as the ratio between the number of antibiotics to which an isolate were resistant and the total number of antibiotics to which the organism had been exposed (Krumperman 1983).

Extended-spectrum β-Lactamase detection by double disc diffusion test (DDDT)

Extended-spectrum β-lactamase (ESBL) detection was performed as recommended by the CLSI confirmatory procedure using ceftazidime (30 μg) alone, a combination of ceftazidime and clavulanic acid (30 μg/10 μg) and cefotaxime (30 μg) alone, and cefotaxime with clavulanic acid (30 μg/10 μg). The isolates showing a higher resistance to cephalosporin antibiotics were selected for checking the ESBL production following the protocols of Linscott & Brown (2005). An increase in the inhibition zone diameter of >5 mm for the combination discs toward ceftazidime or cefotaxime alone was considered positive for ESBL production.

Biofilm formation assay

Biofilm formation was evaluated by the tissue culture plate method (Akinpelu *et al.* 2020). Isolates were inoculated into brain heart infusion broth (HiMedia, India), supplemented with 2% of sucrose and incubated for 18 h at 37 °C. A one in 100 dilution of the culture was made with fresh sterile brain heart infusion broth and 0.2 mL was dispensed into individual

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wells of a 96-well tissue culture plate. Sterile broth serves as negative control. Incubation was done at 37 °C for 24 h. After incubation, wells were tapped and washed thrice with sterile phosphate buffer saline (PBS pH 7.2) to remove free floating planktonic bacteria. The plates were then stained with crystal violet (0.1% w/v) and allowed to stay for 45 min. Excess stain was rinsed off by washing with sterile deionized water thrice and plates were allowed to dry. Crystal violet incorporated by the adherent cells was solubilized by adding 200 μL of 33% glacial acetic acid (HiMedia, India). The optical density (OD) of each well was determined with iMark™ Microplate Reader (Bio-Rad, USA) at wave length 650 nm. The biofilm forming potentials were classified as strong (>0.108), moderate (0.108–0.083), and weak (<0.083) biofilm formers (Hassan *et al.* 2011). The assay was performed in triplicate.

Detection of efflux pump activity by Ethidium bromide agar Cartwheel (EtBrCW) method

The ethidium bromide cartwheel method according to Martins *et al.* (2013) was used in evaluating efflux pump activity of the isolates. Approximately 0.5 McFarland standard (10^6 CFU) of isolates were streaked on tryptic soy agar (HiMedia, India) plates containing 0.5, 1, 1.5, and 2 mg/L concentration of EtBr and incubated at 37 °C for 24 h. After incubation, plates were examined under UV light for fluorescence. Fluorescent isolates showed inactive efflux pumps, whereas non-fluorescent ones showed active efflux pumps.

Molecular detection of antibiotic resistance, efflux pump, porins and virulence-encoding genes in *K. pneumoniae*

Genomic DNA of isolates was extracted according to the method of Kpoda *et al.* (2018). Thirty-two isolates were screened for genes encoding antibiotic resistance, efflux pumps, porins and virulence. A 25 μL PCR reaction was used which contained 8.5 μL nuclease free water, 1 μL of forward primer, 1 μL of reverse primer, 2 μL DNA template and 12.5 μL of PCR master (EmeraldAmp GT PCR Master Mix, Takara, Japan). PCR was carried out in an Applied Biosystems™ Proflex™ thermal cycler (Thermo Fisher Scientific, USA). In all the cases, nuclease free water was used as negative control. Specific primer sequences (Sigma-Aldrich, USA), expected amplicon sizes, and PCR conditions followed according to the respective references given in Table 1. PCR products were electrophoresed at 85 V for 40 min in 1.5% agarose gel stained with ethidium bromide and visualized under a gel image system (Bio-Rad, USA). A 100 bp DNA ladder (EmeraldAmp, Takara, Japan) was used as a molecular weight marker.

Antibiotic residue detection

Commercially available enzyme-linked immunosorbent assay (ELISA) kits were used to identify antibiotic residues of tetracyclines, sulfonamides, and quinolones (Randox Laboratories Ltd, UK). Sample (muscle) collection, processing, analysis, and interpretation were performed according to the manufacturer's instructions ((Kit No. TCS10117A, SZ3471, and QL3454). Duplicates were maintained to ensure the reliability of the findings.

RESULTS

Bacterial identification and antimicrobial susceptibility testing

Based on Gram-staining, phenotypic characteristics on MacConkey agar, and identification by the BD Phoenix™ M50, 56, 32, 30 and 32 numbers were confirmed to be *E. coli, K. pneumoniae, Klebsiella oxytoca* and *Serratia marcescens*, respectively, from fish, water and sediment (Table 2). All 32 isolates confirmed to be *K. pneumoniae* were included in the present study. The isolates were resistant to oxytetracycline (31/32; 96.8%), co-trimoxazole (23/32; 71.8%), doxycycline (22/32; 68.7%), amikacin (21/32; 65.6%), ampicillin (20/32; 62.5%), cefotaxime (20/32; 62.5%), ceftadizime (21/32; 65.6%), furazolidone (17/32; 53.1%), nitrofurantoin (16/32; 50%), enrofloxacin (14/32; 43.7%), and ciprofloxacin (13/32; 40.6%). The mean MAR index was 0.54. Furthermore, the string test confirmed that 50% of the isolates were *hvKP*, and the remaining isolates were *cKP* (Figure 1; Table 3).

ESBL production

K. pneumoniae has been reported to often produce ESBLs, which are linked to antibiotic resistance (Sivaraman *et al.* 2021). The DDST revealed that ESBL production was observed in 78.1% (25/32) of MDR *K. pneumoniae* strains. The zone of inhibition produced by ceftazidime or cefotaxime was clearly extended toward cefotaxime-clavulanate acid.

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Table 1 | List of primers, expected amplicon size, and annealing temperatures used in the present study

Primer	Sequence (5′–3′)	Product size (bp)	T_m (°C)	PCR cycles	Reference
tetA	F- GGCGGTCTTCTTCATCATGC R- CGGCAGGCAGAGCAAGTAGA	502	58	35	Jahantigh *et al.* (2020)
tetC	F- TTCAACCCAGTCAGCTCCTT R- GGGAGGCAGACAAGGTATAGG	560	55	32	
tetD	F- GAGCGTACCGCCTGGTTC R- TCTGATCAGCAGACAGATTGC	780	55	30	
sul2	F- CGGCATCGTCAACATAACCT R- TGTGCGGATGAAGTCAGCTC	721	62	35	Jiang *et al.* (2019)
bla_{SHV}	F- TTAACTCCCTGTTAGCCA R- GATTTGCTGATTTCGCCC	795	52	30	Girijan *et al.* (2020)
bla_{TEM}	F- ATAAAATTCTTGAAGACGAAA R- GACAGTTACCAATGCTTAATC	1,080	50	32	
$bla_{CTX\text{-}M}$	F- CGCTTTGCGATGTGCAG R- ACCGCGATATCGTTGGT	550	55	35	
qnrD	F- GCAAGTTCATTGAACAGGCT R- TCTAAACCGTCGAGTTCGGCG	582	57	32	
qnrC	F- GGGTTGTACATTTATTGAATC R- TCCACTTTACGAGGTTCT	477	50	32	
nfsA	F- ATTTTCTCGGCCAGAAGTGC R- AGAATTTCAACCAGGTGACC	1,036	56	35	Mottaghizadeh *et al.* (2020)
nfsB	F- CCCGCTAAATCTTCAACCTG R- AAAAGAGTGCGTCCAGGCTA	913	61	35	
acrA	F- TGATGCTCTCAGGCAGCTTA R- GCCTGGATATCGCTACCTTC	226	58	32	Wasfi *et al.* (2016)
acrB	F- CGTCTCCATCAGCGACATTAAC R- GAACCGTATTCCCAACGCGA	219	59	32	
acrAB	F- ATCAGCGGCCGGATTGGTAAA R- CGGGTTCGGGAAAATAGCGCG	312	53	30	
tolC	F- ATCAGCAACCCCGATCTGCGT R- CCGGTGACTTGACGCAGTCCT	527	51	32	
Ompk35	F- CTCCAGCTCTAACCGTAGCG R- GGTCTGTACGTAGCCGATGG	241	51	28	
Ompk36	F- GAAATTTATAACAAAGACGGC R- GACGTTACGTCGTATACTACG	305	43	30	
mdtK	F- GCGCTTAACTTCAGCTCA R-GATGATAAATCCACACCAGAA	453	58	32	
fimH	F-GCCAACGTCTACGTTAACCTG R- ATATTTCACGGTGCCTGAAAA	180	59	28	
entB	F- CTGCTGGGAAAAGCGATTGTC R- AAGGCGACTCAGGAGTGGCTT	385	61	30	
mrkD	F- CCACCAACTATTCCCTCGAA R- ATGGAACCCACATCGACATT	226	56	32	
magA	F- GGTGCTCTTTACATCATTGC R- GCAATGGCCATTTGCGTTAG	1,283	58	32	Khattab & Hager (2022)
rmpA	F- ACTGGGCTACCTCTGCTTCA R- CTTGCATGAGCCATCTTTCA	516	58	35	

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Table 2 | Prevalence and distribution of *K. pneumoniae* isolates from different samples

Sample type	Number of samples	Presumed *K. pneumoniae*	Confirmed *K. pneumoniae*
Fish	110	70	14
Water	110	60	14
Sediment	110	20	4

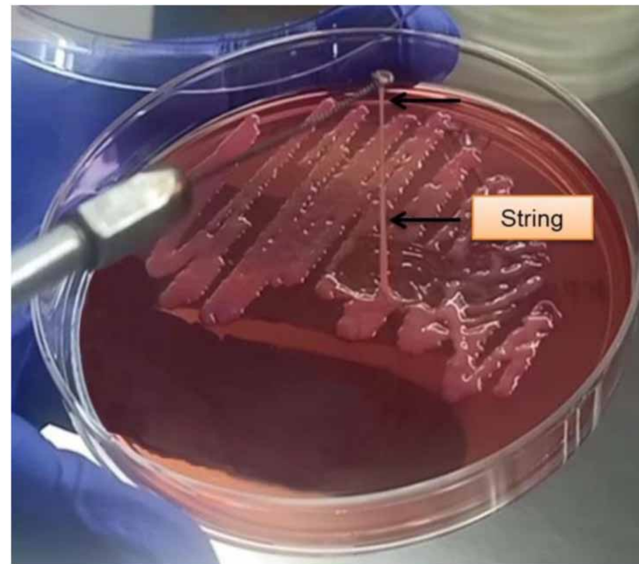

Figure 1 | Confirmation of hyper-virulent *K. pneumoniae* by string test (black arrows).

Biofilm formation

The present study confirmed that all ($n = 32$) isolates were altered as biofilm producers. Among the total biofilm formers, 46.9% (15/32), 31.2% (10/32), and 21.9% (7/32) were categorized as strong, moderate, and weak biofilm formers, respectively.

Efflux pump activity

In the present study, the efflux pump activity of 32 resistant *K. pneumoniae* isolates from different freshwater fish farms was assessed. Following incubation, a variety of fluorescent bacterial masses were detected on all EtBr-coated agar plates. A unique efflux pump activity was detected in 100% (32/32) of isolates at EtBr concentrations of 1 and 1.5 mg/L, whereas 81% (26/32) of the isolates had an active efflux pump at 2 mg/L of EtBr.

Molecular detection of antibiotic resistance, efflux pumps, virulence-encoding genes

In the present study, 32 multi-drug-resistant isolates from different freshwater fish farms were screened for different antibiotic resistance, efflux pump, porins, and virulence-encoding genes. Regarding antibiotic resistance, isolates were positive for *tetA* (31/32; 96.8%), *tetC* (19/32; 59.3%), *tetD* (23/32; 71.9%), *nfsA1* (19/32; 59.3%), *nfsB2* (17/32; 53.1%), *sul2* (22/32; 68.7%), *qnrC* (14/32; 43.7%), *qnrD* (16/32; 50.0%), bla_{SHV} (24/32; 75.0%), bla_{TEM} (22/32; 68.7%), and $bla_{CTX\text{-}M}$ (30/32; 93.7%), (Figure 2; Table 3). Surprisingly, the majority of the isolates with *tetA* and *tetD* genes were positive for the *sul2* gene. Concerning the efflux pump encoding genes, the prevalence for *acrA*, *acrB*, *acrAB*, and *tolC* was detected in 94% (30/32) of the isolates, followed by *mdtK* 56.2% (18/32), respectively. Genes encoding porins *ompk35* and *ompk36* were detected in 59.3% (19/32) and 62.5% (20/32) of the isolates, respectively (Figure 2; Table 4). The *fimH-1* and *mrkD* genes, encoding type-1 and type-3 fimbrial adhesins, were present in 84.3% (27/32) and

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Table 3 | Source, AMR profiles, hyper-virulence and virulence-encoding genes of *K. pneumoniae* isolated from freshwater fish farms

Isolate	Source	AMR gene profile	hvKP	Hyper-virulent genes	Virulence gene
KP1	Fish	*tetA, tetC, tetD, nfsB, sul2, qnrC,* bla_{SHV}, bla_{CTX-M}	+	*magA, rmpA*	*entB, fimH, mrkD*
KP2	Water	*tetA, tetC, tetD, nfsB, sul2, qnrC,* bla_{SHV}, bla_{CTX-M}	−	–	*entB, fimH*
KP4	Fish	*tetA, tetC, tetD, nfsA, nfsB, sul2, qnrD,* bla_{TEM}, bla_{CTX-M}	−	–	*entB, fimH*
KP5	Fish	*tetA, tetC, tetD, nfsB, qnrC, qnrD,* bla_{SHV}, bla_{CTX-M}	+	*magA, rmpA*	*entB, fimH, mrkD*
KP7	Water	*tetA, tetC, tetD, nfsB, qnrC,* bla_{SHV}, bla_{TEM}, bla_{CTX-M}	−	–	*fimH, mrkD*
KP8	Soil	*tetA, tetC, tetD, sul2, qnrC,* bla_{SHV}, bla_{TEM}	−	–	*entB, fimH*
KP9	Water	*tetA, tetC, tetD, nfsA, nfsB, qnrD,* bla_{SHV}, bla_{CTX-M}	+	*magA, rmpA*	*entB, fimH, mrkD*
KP10	Fish	*tetA, tetC, tetD, nfsA, sul2, qnrD,* bla_{SHV}, bla_{CTX-M}	+	*magA, rmpA*	*entB, fimH, mrkD*
KP11	Fish	*tetA, tetD, nfsA, nfsB, sul2, qnrD,* bla_{SHV}, bla_{CTX-M}	+	*magA, rmpA*	*entB, fimH, mrkD*
KP13	Water	*tetA, tetC, tetD, nfsA, sul2, qnrD,* bla_{TEM}, bla_{CTX-M}	+	*magA, rmpA*	*fimH, mrkD*
KP14	Fish	*tetA, tetC, tetD, nfsA, qnrC,* bla_{SHV}, bla_{CTX-M}	+	*magA, rmpA*	*entB, mrkD*
KP16	Water	*tetA, tetD, nfsA, nfsB, qnrC,* bla_{SHV}, bla_{CTX-M}	−	–	*entB, mrkD*
KP17	Soil	*tetA, tetC, tetD, nfsB, qnrC,* bla_{SHV}, bla_{CTX-M}	−	–	*entB, mrkD*
KP18	Water	*tetA, tetC, tetD, sul2, qnrC,* bla_{SHV}, bla_{CTX-M}	+	*magA, rmpA*	*entB, fimH, mrkD*
KP19	Fish	*tetA, tetC, tetD, nfsB, qnrD,* bla_{SHV}, bla_{TEM}, bla_{CTX-M}	−	–	*entB, fimH*
KP21	Water	*tetA, tetD, nfsA, nfsB, qnrD,* bla_{SHV}, bla_{TEM}	+	*magA, rmpA*	*fimH, mrkD*
KP23	Water	*tetA, tetD, nfsA, sul2, qnrD,* bla_{TEM}, bla_{CTX-M}	−	–	*entB, fimH*
KP25	Fish	*tetA, tetC, nfsA, sul2, qnrD,* bla_{SHV}, bla_{TEM}, bla_{CTX-M}	−	–	*entB, mrkD*
KP26	Fish	*tetA, tetC, sul2, qnrC,* bla_{SHV}, bla_{TEM}, bla_{CTX-M}	+	*magA, rmpA*	*entB, fimH, mrkD*
KP27	Fish	*tetA, tetD, nfsB, sul2, qnrD,* bla_{SHV}, bla_{TEM}, bla_{CTX-M}	−	–	*fimH, mrkD*
KP28	Water	*tetA, tetC, nfsA, sul2, qnrD,* bla_{TEM}, bla_{CTX-M}	−	–	*entB, mrkD*
KP29	Fish	*tetA, tetC, nfsA, qnrC,* bla_{SHV}, bla_{TEM}, bla_{CTX-M}	+	*magA, rmpA*	*entB, fimH, mrkD*
KP31	Water	*tetA, tetC, nfsA, sul2, qnrD,* bla_{TEM}, bla_{CTX-M}	+	*magA, rmpA*	*entB, fimH, mrkD*
KP32	Water	*tetA, nfsB, Sul2, qnrD,* bla_{SHV}, bla_{TEM}, bla_{CTX-M}	+	*magA, rmpA*	*entB, fimH, mrkD*
KP35	Soil	*tetA, tetD, nfsA, sul2, qnrD,* bla_{TEM}, bla_{CTX-M}	+	*magA, rmpA*	*entB, fimH, mrkD*
KP36	Water	*tetA, tetD, nfsB, sul2, qnrD,* bla_{TEM}, bla_{CTX-M}	−	–	*entB, fimH*
KP37	Soil	*tetA, tetD, sul2, qnrC,* bla_{SHV}, bla_{TEM}, bla_{CTX-M}	−	–	*entB, fimH, mrkD*
KP38	Fish	*tetA, nfsA, nfsB, sul2,* bla_{SHV}, bla_{TEM}, bla_{CTX-M}	+	*magA, rmpA*	*entB, fimH, mrkD*
KP39	Water	*tetA, tetD, nfsA, qnrC,* bla_{SHV}, bla_{TEM}, bla_{CTX-M}	−	–	*entB, fimH, mrkD*
KP40	Fish	*tetA, tetD, nfsA, sul2,* bla_{SHV}, bla_{TEM}, bla_{CTX-M}	+	*magA, rmpA*	*entB, fimH, mrkD*
KP41	Fish	*tetA, nfsA, nfsB, sul2,* bla_{SHV}, bla_{TEM}, bla_{CTX-M}	−	–	*entB, fimH, mrkD*
KP42	Water	*tetC, nfsA, nfsB, sul2, qnrC,* bla_{TEM}, bla_{CTX-M}	−	–	*entB, mrkD, fimH*

81.2% (26/32) of the isolates, respectively. The enterobactin biosynthesis gene (*entB*) encoding for iron siderophores was present in 87.5% (28/32) of isolates. 50% (16/32) of the isolates were confirmed to be hyper-virulent (*hvKp*) and all the *hvKP* isolates possessed *magA* and *rmpA* hyper-mucoviscosity genes (Figure 3).

Antibiotic residue detection

A total of 110 fish muscle samples were examined for the presence of tetracycline, sulfonamide, and quinolone residues to establish corroborating evidence of the persistence of antibiotic residues in the fish muscles. Only six samples from *P. hypophthalmus* were found to be positive for antibiotic residue, while the remaining samples were not. Among the six samples, five, four, and five had detectable ranges of tetracycline, sulfonamides, and quinolone residues, respectively. The average levels of tetracyclines, sulfonamides, and quinolone residues detected were 30, 35, and 12 ppb, respectively (Table 5).

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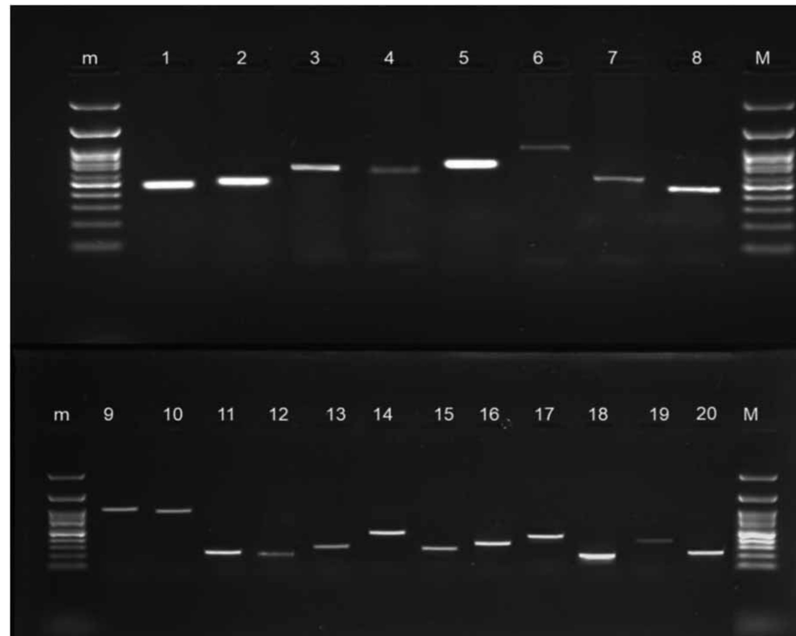

Figure 2 | PCR amplification of antibiotic resistance, efflux pump, porin and virulence genes in *K. pneumoniae* strains. Lane m: molecular marker (100 bp); Lane 1: *tetA* gene (502 bp); Lane 2: *tetC* gene (560 bp); Lane 3: *tetD* gene (780 bp); Lane 4: *sul2* gene (721 bp); Lane 5: bla_{SHV} gene (795 bp); Lane 6: bla_{TEM} gene (1,080 bp); Lane 7: *qnrD* gene (582 bp); Lane 8: *qnrC* gene (477 bp); Lane 9: *nfsA* gene (1,036 bp); Lane 10: *nfsB* gene (913 bp); Lane 11: *acrA* gene (226 bp); Lane 12: *acrB* gene (219 bp); Lane 13: *acrAB* gene (312 bp); Lane 14: *tolC* gene (527 bp); Lane 15: *ompK35* gene (241 bp); Lane 16: *ompk36* gene (305 bp); Lane17: *mdtK* gene (453 bp); Lane 18: *fimH* gene (180 bp); Lane 19: *entB* gene (385 bp); Lane 20: *mrkD* gene (226 bp).

DISCUSSION

The indiscriminate use of antimicrobials across all sectors has led to the development and spread of AMR in aquatic pathogens (Schar *et al.* 2020; Vaiyapuri *et al.* 2021; Lassen *et al.* 2022). Therefore, the spread of antibiotic-resistant bacteria (ARB) from aquatic animals to humans and the environment is a major global health concern. A recent study from 40 low- and middle-income countries has shown that the MAR index of aquaculture-derived bacteria strongly correlates with human clinical bacterial isolates (Reverter *et al.* 2020). *K. pneumoniae* found could be a fish pathogen; however, it is not the most common bacterial pathogen found in fish. *K. pneumoniae* is an opportunistic pathogen that is present everywhere in nature and can infect a wide range of organisms, including humans, animals, and fish (Das *et al.* 2018; Sivaraman *et al.* 2021). In the present study, 32 *K. pneumoniae* strains were isolated from freshwater fish farms, which indicate that their prevalence and distribution in aquaculture settings may pose a threat to aquatic animals and food derived from aquaculture. Antibiogram studies revealed that 32 isolates were multi-drug-resistant, and the resistance pattern was similar to that reported in a previous study (Das *et al.* 2018). The multiple antibiotic resistance index was 0.54, clearly indicating that the isolates originated from high-risk sources. Our findings on *K. pneumoniae* retrieved from aquaculture settings are supported by the previous research reports (Das *et al.* 2018; Sivaraman *et al.* 2021). Previous studies highlighted that inappropriate prescription and residue persistence in the sediment will create selective pressure in favor of AMR development in the target and non-target microbes (Lulijwa *et al.* 2020; Reverter *et al.* 2020). Additionally, hyper-virulent (*hvKp*) strains possess hyper-mucoviscosity and are responsible for MDR and infectious diseases (Holden *et al.* 2016; Khattab & Hager 2022). In the present study, 50% of the resistant isolates were confirmed to be *hvKp* and harbored *magA* and *rmpA* hyper-mucoviscosity encoding genes. A previous study reviewed the hyper-virulent lineages with acquired antimicrobial resistances, which have been increasing striking in recent years across sectors (Lan *et al.* 2021).

The prophylactic and therapeutic use of tetracycline in aquaculture has demonstrated tetracycline-mediated resistance in Gram-negative bacteria by efflux pumps, ribosomal protection and enzymatic degradation. In the present study, the majority of the isolates that were resistant to oxytetracycline and doxycycline harbored *tetA*, *tetC*, and *tetD* genes belonging to major facilitator superfamily (MFS) efflux pumps. Our findings are consistent with (Gao *et al.* 2012; Grossman 2016) who found

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Table 4 | Biofilm production, efflux pump activity and porin-encoding genes of *K. pneumoniae* isolated from freshwater fish farms

Isolate	Biofilm	Efflux pump	Efflux pump encoding genes	Porin-encoding genes
KP1	+	+	*acrA, acrB, acrAB, tolC, mdtK*	*ompk35*
KP2	+	+	*acrA, acrB, acrAB, tolC, mdtK*	–
KP4	+	+	*acrA, acrB, acrAB, tolC, mdtK*	–
KP5	+	+	*acrA, acrB, acrAB, tolC*	–
KP7	+	+	*acrA, acrB, acrAB, tolC*	*ompk35*
KP8	+	+	*acrA, acrB, acrAB, tolC, mdtK*	*ompk35*
KP9	+	+	*acrA, acrB, acrAB, tolC, mdtK*	*ompk35, ompk36*
KP10	+	+	*acrA, acrB, acrAB, tolC*	*ompk35,*
KP11	+	+	*acrAB, tolC, mdtK*	*ompk36*
KP13	+	+	*acrA, acrB, acrAB, tolC*	*ompk35, ompk36*
KP14	−	+	*acrA, acrB, acrAB, tolC*	–
KP16	+	+	*acrA, acrB, acrAB, tolC*	*ompk35, ompk36*
KP17	+	+	*acrA, acrB, acrAB, tolC, mdtK*	*ompk35, ompk36*
KP18	+	+	*acrA, acrB, acrAB, tolC, mdtK*	–
KP19	+	+	*acrA, acrB, acrAB, tolC*	*ompk35, ompk36*
KP21	+	+	*acrA, acrB, acrAB, tolC, mdtK*	*ompk35, ompk36*
KP23	+	+	*acrA, acrB, acrAB, tolC*	*ompk36*
KP25	+	+	*acrA, acrB, acrAB, tolC*	*ompk36*
KP26	+	+	*acrA, acrB, acrAB, tolC, mdtK*	*ompk36*
KP27	+	+	*acrA, acrB, acrAB, mdtK*	*ompk35, ompk36*
KP28	−	+	*acrA, acrB, acrAB, tolC, mdtK*	*ompk35, ompk36*
KP29	+	+	*acrA, acrB, acrAB, tolC, mdtK*	––
KP31	+	+	*acrA, acrB, acrAB, tolC, mdtK*	*ompk35, ompk36*
KP32	+	+	*acrA, acrB, acrAB, tolC, mdtK*	–
KP35	+	+	*acrA, acrB, acrAB, tolC, mdtK*	–
KP36	+	+	*acrA, acrB, acrAB, tolC*	*ompk35, ompk36*
KP37	−	+	*acrA, acrB, tolC, mdtK*	*ompk35, ompk36*
KP38	+	+	*acrA, acrB, acrAB, tolC*	*ompk35, ompk36*
KP39	+	+	*acrA, acrB, acrAB, tolC*	*ompk36*
KP40	+	+	*acrA, acrB, acrAB, tolC*	*ompk35, ompk36*
KP41	+	+	*acrAB, tolC*	*ompk35, ompk36*
KP42	+	+	*acrA, acrB, mdtK*	*ompk35, ompk36*

that bacteria linked to fish farms possessed members of the *tet* family of resistant determinants. Similarly, sulfonamides were also given high priority for usage in veterinary animals and had great potential to enter the environment. Resistance to sulfonamides are mediated by the presence of *sul1*, *sul2*, and *sul3* genes, which encode dihydropteroate synthase (DHPS), which has a low affinity for sulfonamides (Wang *et al.* 2014; Jiang *et al.* 2019). However, only the *sul2* gene was found in 68.7% of the isolates in this study. Surprisingly, the majority of the isolates with tetracycline resistance were positive for the *sul2* gene. Several studies have also found that tetracycline and sulfonamide genes are correlated and mediate multidrug resistance in *Acinetobacter* spp. *E. coli*, *P. aeruginosa*, *K. pneumoniae*, *Bacillus* spp. and many more (Su *et al.* 2011; Girijan *et al.* 2020; Suresh *et al.* 2023). Further, nitrofurans possess a broad antibacterial and anti-parasitic spectrum; however, their use in veterinary and aquaculture is prohibited due to their potentially harmful effects on humans and animals (Mottaghizadeh *et al.* 2020). In the present study, nitrofuran resistance-encoding genes such as *nfsA* and *nfsB* were found in 59.3 and 53.1% of the isolates, respectively. Use of cephalosporin antibiotics in aquaculture practices is unlikely, based

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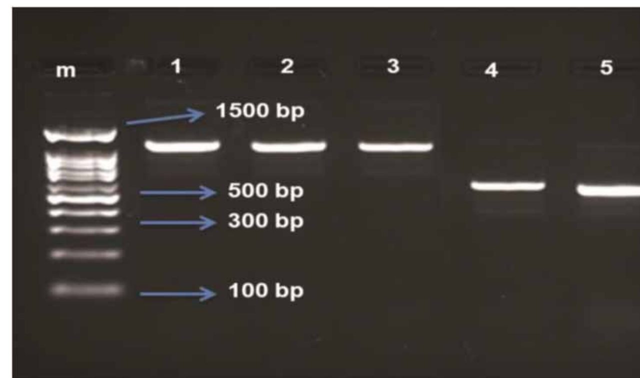

Figure 3 | PCR amplification of antibiotic resistance and hyper-virulent genes in *K. pneumoniae*. Lane m: molecular marker (100 bp); Lanes 1–3: *magA* gene (1,283 bp); Lane 4: $bla_{CTX\text{-}M}$ gene (550 bp); Lane 5: *rmpA* gene (516 bp).

Table 5 | Presence of antibiotic residues and average residue fractions in fish samples

Total number of test sample	Number of positive samples	Antibiotic compound	Average antibiotic residue fraction (ppb)
$n = 110$	$n = 6$	Tetracyclines ($n = 5$)	30
		Sulfonamides ($n = 4$)	35
		Quinolone ($n = 5$)	12

on the initial survey. However, the double disc synergy test revealed that ESBL production observed in 78% of MDR *K. pneumoniae* strains possessed bla_{SHV} (75%), bla_{TEM} (68.7%) and $bla_{CTX\text{-}M}$ (93.7%) genes. Our findings raise the question of how cephalosporin-resistant strains emerge in aquaculture systems. Previous study reports explained that co-resistance to other antibiotics as a result of selective pressure from overuse of other antibiotics, the determinants of which are co-localized with carbapenemase on the same plasmid (Dang *et al.* 2011). Another possible explanation could be the unregulated use of carbapenems to treat fish. Unfortunately, this cannot be clarified, as there is no available data on the use of such antibiotics in aquaculture. A metagenomic approach has explored that ARBs and ARGs may gain access to aquaculture through integrated aquaculture systems and other agriculture-related sectors through animal manures, human, animals, sediment and water during aquaculture practices (Dang *et al.* 2011; Wang *et al.* 2014; Yang *et al.* 2023). This could explain the emergence and spread of *K. pneumoniae* in fish culture farms, as the majority of fish farms were found to use poultry and cow manure to boost phytoplankton and zooplankton production. Furthermore, quinolone resistance in Enterobacteriaceae has emerged several times independently and is associated with chromosome mutations or plasmid-borne genes. In the current study, quinolone resistance was consistently observed in 43.7 and 50% of isolates harboring *qnrC* and *qnrD* genes, respectively. Previous studies (Wu *et al.* 2019; Girijan *et al.* 2020) found that plasmid-mediated quinolone resistance is common in bacterial pathogens in aquatic environments with high antibiotic pressure, lending support to the current study's findings. In addition, *OqxAB*, a plasmid-borne efflux pump gene belonging to the RND-type multi-drug efflux pump that confers resistance to trimethoprim and quinolones, which was not screened in this study, could have also contributed to the final phenotype. Many studies have also demonstrated that horizontal gene transfer is initiated by excess nutrients, the diversity and density of microorganisms, sludge, and biofilms (Kelly *et al.* 2009; Cabello *et al.* 2013; Lassen *et al.* 2022). This assertion is closely related to our findings since the majority of farms have been in operation for more than three years with no regular water exchange and typically contain high microbial load, organic waste, and vigorous use of disinfectants and pesticides, which can promote the creation of biofilms and encourage horizontal gene transfer between the mixed bacterial populations in the aquatic environment. In addition, pesticides act as mediators for the appearance of AMR and as a route for ARB and antibiotic-resistant genes (ARGs) to enter the environment (Malagon-Rojas *et al.* 2020). Hence, proper farm management, such as regular water exchange and the use of probiotics rather than antibiotics and other chemicals, can significantly mitigate the chemical use and disease outbreaks, proliferation, and dissemination of antibiotic resistance genes (Lassen *et al.* 2022).

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K. pneumoniae has emerged as a major public health threat owing to its multi-drug resistance to a wide range of antibiotic compounds mediated by biofilms, active efflux pumps, proteins encoded by chromosomal and plasmid-borne genes, and mutated porins (Martins *et al.* 2013; Wasfi *et al.* 2016; Akinpelu *et al.* 2020; Girijan *et al.* 2020). Biofilm formation is a major virulence factor in *K. pneumoniae*, allowing the bacteria to cling to both living and non-living surfaces and inevitably contributing to drug resistance and virulence (Holden *et al.* 2016). In the current study, 100% of isolates were altered as biofilm formers responsible for biofilm development, antibiotic resistance and virulence. In addition, active efflux pumps help reduce the intracellular concentrations of antimicrobial compounds and assist bacterial survival (Girijan *et al.* 2020). In the present study, *acrAB* and *mdtK* efflux pumps were identified; however, *acrAB* efflux pumps are more common than *mdtK* efflux pumps. In the present study, 94% of the isolates possessed *acrAB-TolC*, while the remaining 6% lacked either the *acrAB* efflux pump or the *tolC* outer membrane proteins, or both. Likewise, the *mdtK* gene is present in 58% of the isolates, conferring resistance to tetracycline and quinolones when overexpressed. These findings are in line with Lan *et al.* (2021) and Al-Dahmoshi *et al.* (2022) who found a strong correlation between antibiotic resistance and efflux pumps, which may be reflected in biofilm formation in *E. coli* and *K. pneumoniae*.

Having observed a consistently high prevalence of resistance to tetracycline, sulfonamide, and quinolones in the *K. pneumoniae* isolates from finfish farms, a cursory analysis of fish samples was carried out for the presence of antibiotic residues by ELISA methods (Randox Laboratories Ltd, UK). The amount of residue fractions did not exceed the maximum residue limits (MRL) recommended by the European Union Commission (Commission Regulation 2009; Okocha *et al.* 2018). The MRL values for tetracycline, oxytetracycline, quinolones and sulfonamides antibiotics are not to exceed 100 μg/kg (Okocha *et al.* 2018). According to previous reports, antibiotics at low, sub-inhibitory concentrations for long periods may exert selective pressure on the spread of antibiotic resistance, affect cell function and virulence, and promote the transfer of antibiotic resistance (Kummerer 2009).

Porins, including *ompk35* and *ompk36*, are crucial in the development of antibiotic resistance and virulence in *K. pneumoniae*, and impede the activity of neutrophil phagocytes (Wasfi *et al.* 2016; Khattab & Hager 2022). In this investigation, 59.3 and 62.5% of the isolates had the *ompK35* and *ompK36* porin-encoding genes, respectively. Efflux pumps, porins, and the pathogenicity of pathogenic bacteria are directly correlated (Padilla *et al.* 2010; Girijan *et al.* 2020), and our observations corroborate their findings. In the current study, *fimH1* and *mrkD* fimbrial-encoding and iron-binding siderophore enterobactin biosynthesis (*entB*) genes were identified. It is well known that *K. pneumoniae* possess fimbrial-encoding and iron-binding siderophore which contribute significantly to pathogenicity and disease outbreaks in humans and animals (Wasfi *et al.* 2016; Das *et al.* 2018). A previous study has also identified a potential risk of the presence of highly virulent and antimicrobial-resistant *P. aeruginosa* and *K. pneumoniae* in farm workers and farmed fishes (Suresh *et al.* 2023; Yang *et al.* 2023). Hence, care should be taken while performing routine activities in aquaculture settings to overcome the health risks associated with them.

CONCLUSION

The present study recognized that multiple factors contribute to the emergence and spread of MDR *K. pneumoniae* in aquaculture facilities, raising concerns about the safety of food derived from aquaculture owing to public health hazards. The findings of the current study provide insights into the distribution of *K. pneumoniae* in the fish culture facilities, resistance patterns, underlying mechanisms, virulence profiles and antibiotic residues and public health risks for future research. However, further studies are needed to evaluate the extent of *K. pneumoniae* emergence and distribution in different aquaculture settings, sources and sinks, propagation strategies, and the role of antibiotic residues and AMR development mechanism in the aquatic environments. To address AMR and disease-related issues, a comprehensive multifaceted approach based on proper farm management, biosecurity, healthy breeds and seeds, risk identification and analysis, regular disease surveillance, AST, and effective policies with proper guidelines from all stakeholder groups in the sector is required.

ACKNOWLEDGEMENTS

The authors acknowledge the authorities of Kerala University of Fisheries and Ocean Studies, Kochi, Kerala, and Rajiv Gandhi Centre for Aquaculture, Tamil Nadu, and India for providing facilities to carry out the work.

FUNDING SOURCES

This research did not receive any specific grant from funding agencies in the public, commercial or not-for-profit sectors.

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AUTHOR CONTRIBUTIONS

K.S. was involved in conception, planning, sample collection, analysis, writing an original draft, review, and editing. D.P. was involved in supervision, planning, critical review, and editing of the article.

ETHICAL APPROVAL

All methods were carried out in accordance with the applicable guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), registration number: 1174/ac/08/CPCSEA.

DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

CONFLICT OF INTEREST

The authors declare there is no conflict.

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First received 19 December 2023; accepted in revised form 25 March 2024. Available online 2 April 2024

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