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Genomic investigation unveils high-risk ESBL producing *Enterobacteriaceae* within a rural environmental water body

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A R T I C L E I N F O

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A B S T R A C T

Antimicrobial resistance is regarded as a global threat to public health, animals, and the environment, emerging in response to extensive utilization of antimicrobials. The determinants of antimicrobial resistance are transported to susceptible bacterial populations through genetic recombination or through gene transfer, mediated by bacteriophages, plasmids, transposons, and insertion sequences. To determine the penetration of antimicrobial resistance into the bacterial population of the Thiruvandarkoil Lake, a water body located in the rural settings of Puducherry, India, culture-based microbiological and genomic approaches were used. Resistant bacterial isolates obtained from microbiological screening were subjected to whole genome sequencing and the genetic determinants of antimicrobial resistance were identified using *in silico* genomic tools. Cephalosporin-resistant isolates were found to produce extended spectrum beta lactamases, encoded by $bla_{VEB\text{-}6}$ (in *Proteus mirabilis* PS01), $bla_{SHV\text{-}12}$ and *ompK*36 mutation (in *Klebsiella quasipneumoniae* PS02) and $bla_{SHV\text{-}12}$, $bla_{ACT\text{-}16}$, bla_{CTX-M} and $bla_{NDM\text{-}1}$ in (*Enterobacter hormaechei* PS03). Genes encoding heavy metal resistance, virulence and resistance to detergents were also detected in these resistant isolates. Among ESBL-producing organisms, one *mcr-9*-positive *Enterobacter hormaechei* was also identified in this study. To our knowledge, this is the first report of *mcr-9* carrying bacterium in the environment in India. This study seeks the immediate attention of policy makers, researchers, government officials and environmental activists in India, to develop surveillance programs to monitor the dissemination of antimicrobial resistance in the environment.

1. Introduction

Infections caused antimicrobial resistant bacteria has emerged as one of the most pressing challenges in modern healthcare (Laxminarayan et al., 2013). It refers to the ability of bacteria to resist the effects of antibiotics, rendering standard treatments ineffective and infections persisting and spreading. From a clinical point of view bacteria belonging to the ESKAPE category are of major concern as they have developed resistance to many vital antibiotics and have disseminated rapidly across the globe (Boucher et al., 2009). Latest high throughput sequencing technologies have revealed that natural environments also harbour many resistant bacteria (Nesme et al., 2014). Antimicrobial resistance (AMR) in natural environments emerges either due to the dispersal of resistant bacteria from humans and animals or by the release of antimicrobials into the environment (Quinteira and Peixe 2006). Several mechanisms have been discovered in bacteria that causes transmission of AMR and this includes porin alterations, penicillin binding proteins (PBPs) modifications, efflux systems and production of enzymes like extended spectrum β-lactamases (ESBLs), AmpC β-lactamases and metallo-β-lactamases (Batchelor et al., 2005)

Water provides an ideal environment for the evolution and spread of AMR. Bacteria from diverse origins such as humans, animals, and various environmental sources can converge in water, facilitating the exchange of antimicrobial resistant genes (ARGs) (Baquero et al., 2008). World Health Organisation (WHO) has recommended this global concern in its action plan, insisting more research to be conducted on identifying AMR and antimicrobial residues in the environment (Bhattacharyya et al., 2022). The One Health concept emphasizes the interconnectedness of human, animal, and environmental health. In the context of AMR, this holistic approach recognizes that the health of humans, animals, and ecosystems is intricately linked and that AMR knows no boundaries. Resistant bacteria can move between humans,

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animals, and the environment, posing a shared risk to all.

Numerous studies have highlighted the existence of antimicrobial resistant bacteria within pathogenic strains sourced from aquatic systems (Li et al., 2020; Gomes et al., 2021; Conte et al., 2021), stressing the need for surveillance of AMR in the environment. Of most concern is the presence of Extended Spectrum β-Lactamase (ESBL) producing *Enterobacteriaceae* in environmental waters. The Enterobacteriaceae family comprises of bacteria such as *Escherichia coli, Klebsiella pneumoniae, Enterobacter cloacae, Citrobacter freundii*, and *Proteus mirabilis*, which are ubiquitous bacteria found in diverse environments, including the human gut, animals, and natural habitats. Their ability to develop resistance to multiple antimicrobial agents has serious implications for public health, leading to increased morbidity, mortality, and healthcare costs (Richards et al., 2000; Baldelli et al., 2021).

The presence ESBL genes in bacteria, significantly limits treatment options, leading to infections that are difficult to manage and control as these enzymes hydrolyses the β-lactam ring of cephalosporins rendering them inactive. Furthermore, ESBL genes are situated on transferrable conjugative plasmids that promote their transfer in both identical and different species of bacteria (Mahomed and Coovadia, 2015). In most cases the ESBL genes encoded by the bacteria co-exist with quinolone-resistant genes in the same mobile genetic element which imparts co-resistance to fluoroquinolones and cephalosporins (Salah et al., 2019). This existing threat of ESBL producing bacteria is amplified by current reports identifying even the co-existence of carbapenem and colistin resistance determinants within a single transposon (Kieffer et al., 2019). This clearly marks the dawn of antimicrobial chemotherapy.

Insufficient monitoring efforts in non-clinical environments, particularly in developing nations, have significantly contributed to the spread of AMR (Chokshi et al., 2019; Seethalakshmi et al., 2021). The importance of surveillance in monitoring the spread of AMR cannot be overstated. Surveillance programs provide valuable data on the prevalence and distribution of resistant strains, aiding in the formulation of targeted interventions and policies. In this context, whole genome sequencing has emerged as a robust tool as it allows for high-resolution analysis of bacterial genomes, enabling the identification of specific resistance genes, genetic variations, and evolutionary relationships among strains (Bertelli and Greub 2013; Köser et al., 2014). The detailed genomic information obtained through WGS is instrumental in understanding the mechanisms underlying AMR and devising tailored approaches to combat its dissemination. In the subsequent sections of this paper, we examine a specific case study focused on the Thiruvandarkoil Lake, a crucial water source in rural Puducherry, India. By employing a combination of culture-based methods and genomic analyses, we investigated the prevalence and genetic determinants of antimicrobial resistance in Enterobacteriaceae within this environmental niche.

2. Experimental procedures

2.1. Description of sampling site

Thiruvandarkoil Lake near Thirubhuvanai is a freshwater lake located in the Villupuram district of Tamil Nadu, India. The lake covers an area of approximately 16.45 Ha and is a source of water for irrigational purposes in the surrounding villages. The lake is fed by rainfall and supports a variety of aquatic life. The lake and its surrounding area are also home to a variety of bird species, including egrets, herons, and kingfishers. The surrounding area is primarily agricultural, with few

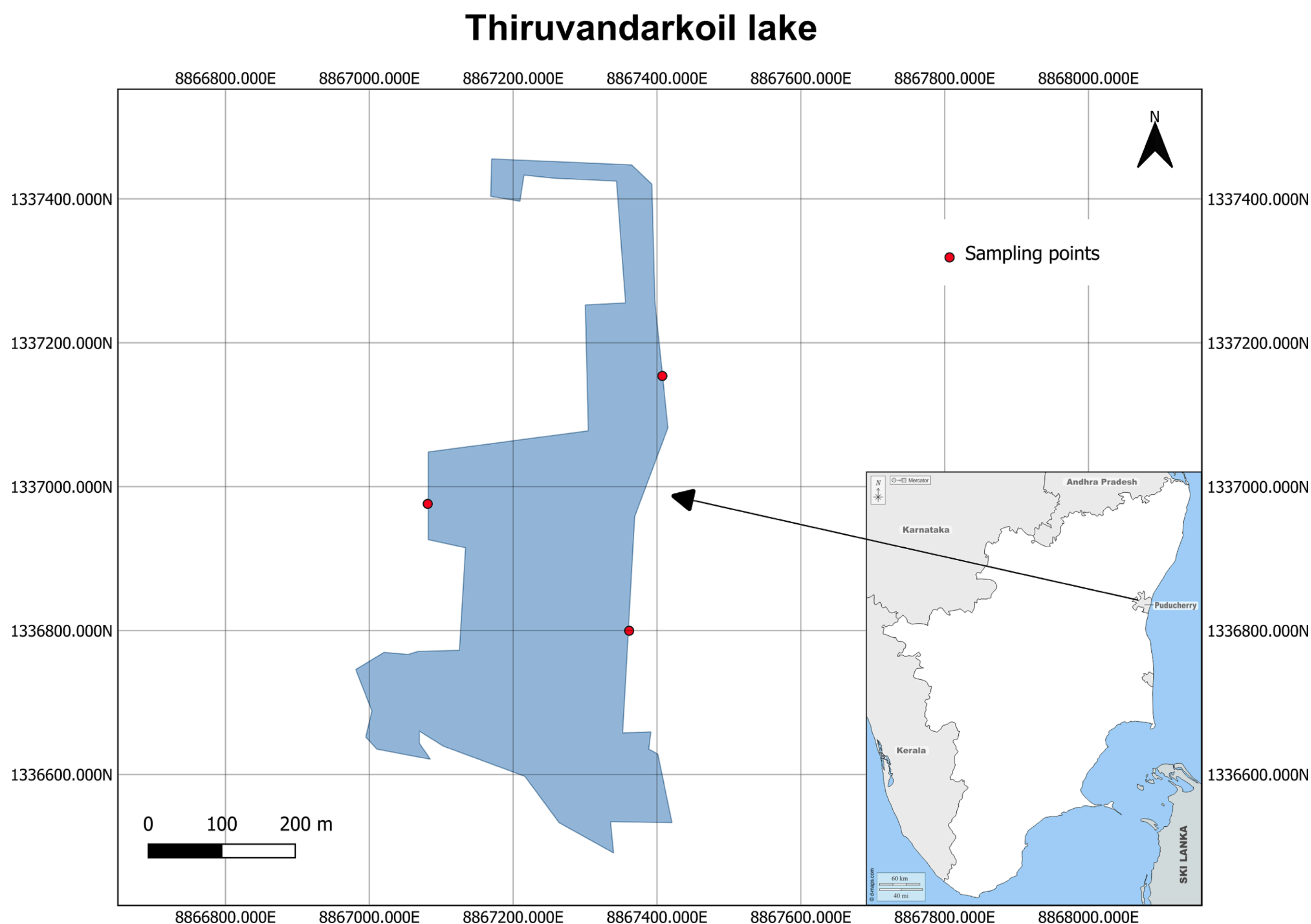


Fig. 1. Overview of the sampling points chosen for collection of samples from the lake.

small-scale industries and local businesses operating in the vicinity of the lake (Fig. 1).

2.2. Physiochemical characterisation of water samples

For determining the physicochemical parameters, water samples were collected in sterile bottles and brought to lab. The pH, electrical conductivity, oxidation reduction potential, dissolved oxygen content, total dissolved solids and salinity of the solution was examined using hand held probe as per manufacturer's instruction (HANNA Multiparameter Water Quality Meter, USA) and was recorded.

2.3. Zebrafish embryo acute toxicity assay of water samples

The experiment was conducted using the standardised zebrafish Embryo Acute Toxicity (FET) from the Organization for Economic Cooperation and Development (OECD) Test-236 (Espíndola et al., 2019). Newly fertilized zebrafish eggs were distributed into 24-well plates, with each well containing 10 embryos. These embryos were then exposed to lake water samples (concentration ranging from 1 % to 100 %) for a duration ranging from 24 to 96 h post-fertilization with experimental controls in triplicates. The 24-well plates were kept for incubation at a temperature of 26 ± 1 °C for 96-hours under the same lighting conditions as the zebrafish stock, with each replicate consisting of 10 embryosand the water being changed every day. Four observations within intervals of 24 h, were recorded as indicators of lethality, sub lethality or coagulation of fertilised eggs. In addition to the apical endpoints, developmental abnormalities were also monitored (Torres et al., 2016). Mortality was monitored daily throughout the entire duration of the exposure period, and coagulated eggs or dead embryos were promptly removed from the study. The impact of the exposure was assessed at four specific time points: 24-, 48-, 72-, and 96-hours post-fertilization. These observation periods were chosen based on specific developmental characteristics exhibited by embryos at these stages of development (Kimmel et al., 1995). The embryo development and abnormalities were observed and recorded using an inverted microscope (Carl Zeiss, Axio imager A2 LED).

2.4. Sample collection and isolation of antibiotic resistant bacteria

50 mL of surface water samples in were collected in triplicates using sterile conical tubes from a rural water body, Thiruvandarkoil Lake in March 2021 (location cordinates: 11.924806 N 79.657278 E, 11.552389 N 79.391574 E, and 11.551831 N 79.392479 E). Samples were brought to laboratory under cold conditions and processed within 12 h for microbiological analysis. Water samples were pooled together under aseptic conditions and were serially diluted to 1:1000. 0.1 mL of each dilution was plated on sterile MacConkey agar and were incubated for 24 h at 37 °C under aerobic conditions. The colonies from each dilution were counted, recorded, and preserved as glycerol stocks for further analysis.

2.5. Identification of the isolates, antibiotic susceptibilities, and determination of minimum inhibitory concentration (MIC)

Gram-negative isolates obtained from the plates were identified by biochemical tests such as Methyl red, Vogues-Proskauer, Indole, Citrate utilisation test and urease test. Resistance profiling of all the isolates were done by Kirby-Bauer disc diffusion method, against ceftriaxone, ceftazidime, cefotaxime, cefepime, ciprofloxacin and the results were interpreted according to CLSI guidelines (2021). The antibiotic resistant isolates were further screened for E-test to determine the MIC of the antibiotics ceftriaxone, ceftazidime, cefotaxime, cefepime, and ciprofloxacin against them (CLSI 2021). The isolates which exhibited resistance to resistance to both 3rd generation cephalosporins were screened for extended spectrum β-lactamase (ESBL) production by double-disc synergy test (CLSI 2021) and confirmed with HiChrome™ ESBL Agar test with 1.5 mg mL^{-1} of ceftazidime, cefotaxime, and 1 mg mL^{-1} ceftriaxone (Nordmann et al., 2012).

2.6. Biofilm formation assay

The ability of the antibiotic resistant isolates to form biofilms was investigated by quantitative adherence assay (Hassan et al., 2011). 24 h old cultures grown at 37 °C under aerobic conditions in trypticase soy broth (TSB) was used for the analysis. 2 μL of cell suspension adjusted to 1×10^6 colony forming units (CFU/mL) was inoculated in sterile 96 well-flat bottom polystyrene microtiter plates that contained 198 μL of sterile TSB. Wells with 200 μL of un-inoculated TSB was used as negative control and the plates were incubated at 37 °C for 24 h. The experiment was carried out with negative control and samples in triplicates. The wells were gently washed 3 times using 200 μL phosphate-buffered saline and were dried by keeping the plates in an inverted position. The wells were stained with 50 μL of 0.1 % crystal violet and was incubated at room temperature for 10 min. Following this crystal violet was discarded and the wells were washed three times with 200 μL of sterile water without disturbing the biofilm. The wells were dried for 30 min at 50 °C, following which 200 μL of 99 % ethanol was added to the wells and biofilm were disrupted by vigorous pipetting. Absorbance measurement values at 570 nm were obtained using a Microplate reader (Synergy HTX Multimode Reader; BioTek).

An average optical density (OD) of negative control + (3× standard deviation (SD) of negative control) was considered as the cut-off (ODc) for tested isolates. Isolates with OD ≤ ODc were considered negative for biofilm production, whereas isolates were classified as weak biofilm producer if the OD value lies below 2 × ODc; moderate biofilm for the range 2 × ODc to 4 × ODc; and strong biofilm producer if OD > 4 × ODc (Hassan et al., 2011).

2.7. Genomic DNA extraction and 16S rDNA sequencing of resistant bacterial isolates

Genomic DNA was isolated from 24 h old culture of antibiotic resistant bacteria in Luria-Bertani broth using HiPer® Bacterial Genomic DNA Extraction Teaching Kit (Column Based) as per manufacturer's instructions (Himedia, Mumbai, India). The quality of DNA (260/280 absorbance ratio) obtained was checked using NanoDrop™ (Thermo Fisher Scientific, Bangalore, India) and quantified using Qubit® 2.0 Fluorometer (Thermo Fisher Scientific, Bangalore, India) by a target specific Qubit assay (dsDNA BR Assay Kit, Thermo Fisher Scientific). The resistant bacterial isolates were subjected to 16S rDNA sequencing following PCR amplification using the primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1392R (5′-GGTTACCTTGTTACGACTT-3′) (Srinivasan et al., 2015). The 16S rDNA partial sequences were deposited in NCBI under the accession numbers: OP474141.1, OP474149.1, OP474147.1 and phylogenetic analysis of the isolates were performed using MEGA v.11.0 (Tamura et al., 2021).

2.8. Whole genome sequencing and analysis

Paired-end sequencing libraries were prepared using genomic DNA using Illumina TruSeq® Nano DNA Library Prep as per manufacturer's protocol (Illumina, San Diego, CA, USA) and paired end sequencing was carried out using (2 × 150 bp) on an Illumina NovaSeq6000 instrument. Quality and quantity of PCR enriched libraries were analysed on Agilent 4200 TapeStation System (Agilent Technologies, CA, USA) using high sensitivity D1000 screen tape as per the instructions provided by the manufacturer. Raw sequence data was generated and FASTQ files were obtained. The quality of obtained data was assessed using FASTQC (v.0.11.5) and low-quality reads were filtered out and adapter regions were removed using Trimmomatic (v.0.36) (Andrews 2010; Bolger et al., 2014). The raw reads obtained from sequencing was assembled *de*

novo using Unicycler (v0.4.8) (Wick et al., 2017) and annotated using RAST tool kit (Brettin et al., 2015). The quality of assembled genome was determined through QUAST v5.0.2 (Gurevich et al., 2013) and the assembled genome were deposited in the NCBI repository under accession numbers: GCA_025290655.1, GCA_025290665.1 and GCA_025290855.1. To confirm our findings from of 16S phylogenetic analysis, the target genome was compared with the type strain genome from TYGS database using the MASH algorithm (Ondov et al., 2016). The genomic distance was calculated based Genome BLAST Distance Phylogeny approach (GBDP) under the 'coverage' algorithm (Meier--Kolthoff et al., 2013). The genome based phylogenetic tree was constructed with 100 pseudo-bootstrap replicates (Farris 1972) and was statistically validated using the δ statistics values (Holland et al., 2002).

Plasmid sequences were identified using PlasmidFinder 2.0 with parameters, 60 % coverage and a 95 % identity threshold (https://cge.food.dtu.dk/services/PlasmidFinder/) and was deposited under accession numbers: JAOCPZ010000084.1, JAOCPZ010000035.1, JAOCPZ010000055.1, JAOCPZ010000048.1, JAOCPZ010000037.1, JAOCPY010000052.1, JAOCPY010000058.1, JAOCPY010000038.1 and JAOCPY010000065.1. Phage DNA was identified using PHASTER (https://phaster.ca/) and acquired antibiotic resistance genes were identified using ResFinder 4.1(https://cge.food.dtu.dk/services/ResFinder/), and Comprehensive Antibiotic Resistance Database (CARD) (https://card.mcmaster.ca/). Acquired virulence genes were identified using VirulenceFinder v2.0 (https://cge.food.dtu.dk/services/VirulenceFinder/) and mobile genetic elements were detected using MobileElementFinder (v1.0.3) (https://cge.food.dtu.dk/services/MobileElementFinder/). The circular image for comparisons between the plasmids identified and other reported plasmids with sequence similarity were visualized using Proksee webserver (https://beta.proksee.ca/) and genomic islands were viewed using IslandViewer4 with the prediction tool, SIGI-HMM (http://www.pathogenomics.sfu.ca/islandviewer/).

3. Results

3.1. Physiochemical properties and toxicity of collected water samples

The physiochemical properties are summarised in (Table 1), where all parameters tested were found to be normal. These findings were further supported in the zebrafish toxicity assay, where no significant changes were observed in embryos exposed to lake water compared to control samples. None of the embryos exposed to the lake water exhibited any lethal or sublethal effects compared to controls, indicating that the presence of any toxic elements may have negligible effect on embryos or may have toxic effects upon chronic exposure.(Fig. 2)

3.2. Bacterial isolation, identification, and antibiotic sensitivity profiling

All 31 isolates collected from three dilutions were identified as Gram-negative, amongst which 28 were lactose fermenting and three were non-lactose fermenting. Of the 31 isolates recovered, different genera of bacteria were identified including *Proteus, Klebsiella* and *Enterobacter*. Antimicrobial sensitivity assay showed that 5/31 (16.1 %) of selected isolates were resistant to ciprofloxacin, tobramycin, ceftriaxone, cefotaxime, and ceftazidime. 3/31 (9.68 %) of bacteria isolated were resistant to the antibiotics meropenem and 1/31 (3.22 %) was resistant to both meropenem and colistin. Double disc synergy test revealed a keyhole phenomenon for all cephalosporin resistant isolates except *Enterobacter* sp. Cephalosporin resistant *Klebsiella* sp., and *Enterobacter* sp. grew on the HiCrome™ ESBL agar producing bluish green coloured colonies whereas *Proteus* sp. formed white colonies, confirming ESBL production. The minimum inhibitory concentration (MIC) of antibiotics exhibited by these resistant isolates is summarised in (Table 2).

Table 1
Overview of the physiochemical parameters of the tested water sample.

Physiological parameters	Values with unit
Temperature	33.54 ℃
pH/mV	6.59 pH/ 17.9mVpH
Oxidation Reduction Potential (ORP)	−262.8 mVORP
Dissolved Oxygen	16.4 %, 1.15 mg/L
Conductivity	1776 μS/cm
Resistivity	0.0006 MΩ.cm
Total Dissolved Solids (TDS)	912 ppm
Salinity	0.91 PSU
Atmospheric Pressure	14.617 psi
Sea water sigma	0.0 σt

3.3. Genotypic identification, biofilm formation and phylogenetic relatedness of ESBL producing isolates

16S rDNA sequencing of 3/5 cephalosporin-resistant isolates identified these as *Proteus mirabilis, Klebsiella quasipneumoniae* and *Enterobacter hormaechei*, based on percentage identity to isolates in NCBI. These were designated as PS01, PS02 and PS03, respectively; the remaining two ESBL isolates exhibited phenotypic properties and MIC values that were identical to PS01 and PS02. All ESBL producing isolates were found to be non-biofilm producers based on their OD values with reference to control (**Supplementary file 1**). Phylogenetic tree was constructed using MEGA version 11 (Tamura et al., 2021) by the Maximum Likelihood method with bootstrap value 1000 and the strains closely related to these cephalosporin resistant isolates were identified (Fig. 3).

3.4. Whole genome analysis of ESBL producing bacterial isolates

3.4.1. Genome assemblies

Assembly reports showed that *P. mirabilis* PS01 consisted of 136 contigs, with the total genome length of 4111,496 bp and an average G + C content of 39.06 %, while *K. quasipneumoniae* PS02 comprised of 135 contigs, 5483,121 bp, and 57.61 % GC content. On the other hand, the genome of *E. hormaechei* PS03 had 5352,396 bp with 141 contigs and 54.42 % GC content. The genomic assembly details and distribution of the genome annotations is provided in (**Table S1 and Figure S1, Supplementary file 2**). The taxonomic positions of the strains identified using 16S phylogeny closely matched those determined through whole genome-based phylogenetic analysis, providing strong evidence for the accurate identification of the strains (**Figure S2, Supplementary file 2**).

3.4.2. Plasmids, phage genomes and genomic islands

K. pneumoniae PS02 and *E. hormaechei* PS03 contained five and four plasmids respectively, while plasmids were absent in the *P. mirabilis* strain. PlasmidFinder results showed that *K. pneumoniae* carried the plasmids ColpVC, IncFIB(K), IncFII(K), IncN, IncR and Col440I and *E. hormaechei* PS03 carried IncFIB(pNDM-Mar), IncHI1B(pNDM-MAR), IncHI2, and IncHI2A. The plasmids IncFIB(pNDM-Mar) and IncHI1B (pNDM-MAR) exhibited 100 % identity to the plasmids pMB5632_2 of *Klebsiella aerogenes* strain 4417 (CP103666.1) and pBA813_1 *Klebsiella pneumoniae* strain BA813 (MK649825.1), although neither of them was found to carry ARGs.

Five complete phages including Erwini_vB_EhrS, Entero_mEp390, Phage_Gifsy_2, Salmon_SPN3UB and Escher_HK639; two incomplete phages Salmon_BTP1 and Entero_mEp390 were detected in *E. hormaechei* PS03 genomes. *P. mirabilis* PS01 genome contained five incomplete phages, Burkho_BcepB1A, Escher_500,465_1, Escher_phiV10, Stx2_II and Salmon_118,970_sal3 namely, whereas *K. quasipneumoniae* PS02 comprised of two incomplete phages Edward_GF and Entero_P1.

The prediction of genomic islands (GIs) using Island Viewer 4 led to the identification of 18, 28 and 56 GIs respectively, in the genomes of *P. mirabilis* PS01, *K. quasipneumoniae* PS02 and *E. hormaechei* PS03

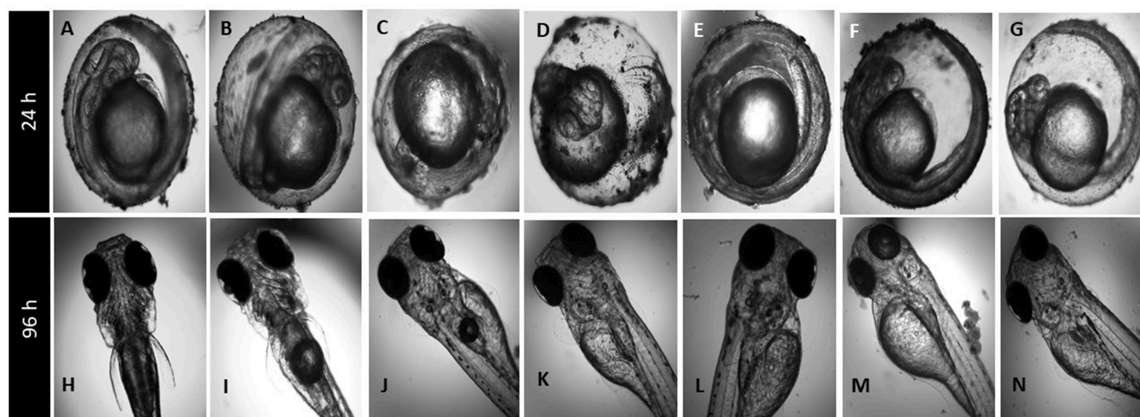


Fig. 2. Toxicity Assessment of Lake water using zebrafish. **A-G** represents 24 h of exposure and **H—N** represents 96 h of exposure. **A, H**—Controls; **B, I**-1 %; C, J-10 %; **D, K**-25 %, E, l-50 %; **F, M**-75 % and **G, N**-100 % (v/v).

Table 2
MIC values exhibited by the resistant isolates obtained in this study.

Name of the isolate	Name of the antibiotic	MIC (μg mL^{-1})
TVL018W (Identified as *P. mirabilis* PS01)	Ceftriaxone	5
	Ceftazidime	5
	Cefotaxime	5
	Cefipime	1
	Ciprofloxacin	3
	Meropenem	0.1
TVL016W	Ceftriaxone	5
	Ceftazidime	5
	Cefotaxime	5
	Cefipime	1
	Ciprofloxacin	3
	Meropenem	0.1
TVL04W	Ceftriaxone	7.5
	Ceftazidime	120
	Cefotaxime	7.5
	Cefipime	1
	Ciprofloxacin	3
	Meropenem	0.1
TVL05W (identified as *K. quasipneumoniae* PS02)	Ceftriaxone	7.5
	Ceftazidime	120
	Cefotaxime	7.5
	Cefipime	1
	Ciprofloxacin	3
	Meropenem	0.1
TVL025W (identified as *E. hormaechei* PS03)	Ceftriaxone	60
	Ceftazidime	120
	Cefotaxime	60
	Cefipime	30
	Ciprofloxacin	30
	Meropenem	5

respectively. The 18 predicted GIs in *P. mirabilis* PS01 ranging from PS01-G01 to PS01-G10 represents a region that mainly encodes integrases, hypothetical proteins and proteins involved in the maintenance of outer membrane integrity, whereas PS01-G11 to PS01-G18 encodes phage proteins, mobile element proteins and antibiotic resistance associated proteins. Similarly, *K. quasipneumoniae* PS02 and *E. hormaechei* PS03 carried GIs encoding mobile genetic elements, phages and ARGs, heavy metal resistant genes from PS02-G16 to PS02-G28 and PS03-G38 to PS03-G56, respectively (**Supplementary file 1**).

3.4.3. Resistome, virulome and mobile genetic elements

The resistome analysis of *P. mirabilis* PS01 revealed the presence of various acquired antibiotic resistance genes (ARGs), including a quinolone resistance gene (aac(6′)-Ib-cr), aminoglycoside resistance genes (*aac(6′)-Ib, aadA1, ant(2′′)-Ia, armA*, and *aac(3)-IId*), a broad spectrum β-lactamase gene encoded by $bla_{\text{TEM-1-B}}$, an extended-spectrum β-lactamase (ESBL) gene ($bla_{\text{VEB-6}}$), phenicol resistant genes (*catA1*), and a trimethoprim resistance gene (*dfrA1*). Similarly, *K. quasipneumoniae* PS02 carried broad spectrum β-lactamase genes ($bla_{\text{TEM-1B}}$ and $bla_{\text{OKP-B-8}}$), an ESBL gene ($bla_{\text{SHV-12}}$), and ARGs such as *sul1*, c*atA2, qnrB-1, aph (3′′)-Ib*, and *aph(6)-Id*. Interestingly, mutations were observed in efflux pumps *acrR, ompK*, and *gyrA*, which have the potential to increase resistance to quinolones, cephalosporins, and carbapenems. *E. hormaechei* PS03 contained multiple ESBL genes in the genome, including $bla_{\text{NDM-1}}$, $bla_{\text{CTX-M}}$, $bla_{\text{ACT-16}}$, and $bla_{\text{SHV-12}}$, along with the colistin resistance gene, *mcr-9*. Furthermore, this isolate carried genes that encoded resistance to antibiotics such as aminoglycosides, quinolones, sulpha drugs, and chloramphenicol. The *qacE* gene, associated with class 1 integrons was present in all three isolates, with *K. quasipneumoniae* bearing *OqxA* and *OqxB*, indicating resistance to quaternary ammonium compounds. Multiple point mutations were observed in genes *ompK, acrR, gyrA, gyrB, ParC*, and *ramR*, with mutations in *gyrA* and *acrR* potentially contributing to increased resistance to quinolones (Table 3).

In addition to the acquired ARGs, the isolate *K. quasipneumoniae* PS02 carried the hypervirulent gene *iutA*, which encodes an aerobactin receptor, and *traT*, an invasin that inhibits the complement cascade. The *E. hormaechei* PS03 isolate carried two copies of the tellurium ion resistant gene, *terC*. Insertion sequences were detected in all three ESBL isolates, linked to both ARGs and virulence genes. The chromosome of *E. hormaechei* PS03 contained a total of 19 mobile genetic elements, with transposon Tn*6196* observed in the IncHI1B plasmid. Tn*3000*, carrying $bla_{\text{NDM-1}}$ and *qnrB1*, was present in the genome of *E. hormaechei* PS03, suggesting co-selection for carbapenem and quinolone resistance. The *sul2* gene, which conferred resistance to sulfamethoxazole, was flanked by IS*Vsa3* and IS*5075*, while the heavy metal resistance gene *terC* was linked to IS*Kpn50* and IS*Kpn21*. The genome of *K. quasipneumoniae* PS02 contained a total of eight mobile genetic elements in the chromosome, with IS*Ec33* found to be associated with the *iutA* gene. Tn*6082*, carrying *aph(3′′)-Ib* and *aph(6)-Id*, was also detected. *P. mirabilis* PS01 carried a total of five mobile genetic elements, with IS*Cfr1* observed in conjunction with the aminoglycoside resistance gene, *aac(3)-IId*, and IS*629* and IS*5* linked to the hemolysin expression modulator, *hha* (Fig. 4).

3.4.4. In-silico MLST and pMLST results

MLST typing results showed that *E. hormaechei* PS03 belonged to ST144 with 100 % identity to the alleles *dnaA*_53, *fusA*_35, *gyrB*_20, *leuS*_44, *pyrG*_45, *rplB*_4 and *rpoB*_6 of the sequence type. *K. quasipneumoniae* PS02 did not match a specific ST as novel alleles of *phoE*_266 and *tonB*_208 were detected. However, *K. quasipneumoniae* PS02 matched 100 % to other alleles like *gapA*_18, *infB*_15, *mdh*_26,

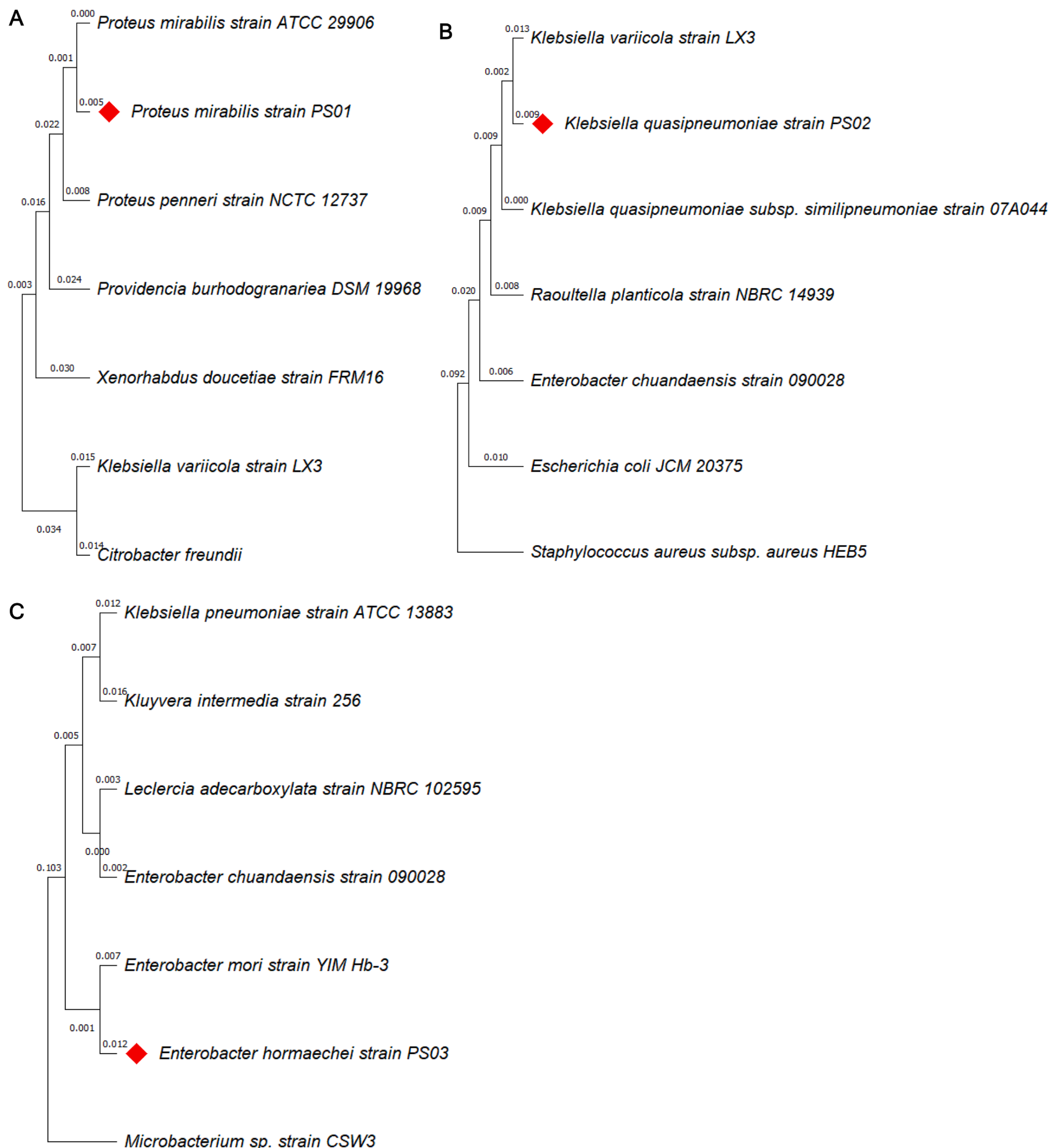


Fig. 3. Phylogenetic tree constructed from 16S rDNA sequences of **A.** *P. mirabilis* strain PS01 **B.** *K. quasipneumoniae* PS02 **C.** *E. hormaechei* strain PS03 using maximum likelihood method.

*pgi*_332, and *rpoB*_74 of ST1312 making it the nearest sequence type. This could be attributed to the unavailability of an MLST scheme specific for *K. quasipneumoniae* and the MLST scheme of *Klebsiella pnuemoniae* was used instead. MLST was not performed for *P. mirabilis* PS01 as a scheme for *in silico* MLST is not available yet. pMLST of IncHI2 plasmid indicated that plasmid belonged to ST1 since it yielded 100 % similarity with the alleles *smr*0018_1 and *smr*0199_1. However, typing of IncN plasmid yielded a match with only *repN*_2 allele, and did not find any *korA* and *traJ* alleles, due to which the sequence type of the plasmid could not be confirmed.

4. Discussion

Freshwater is an important resource for industrial, agricultural, and domestic purposes and the quality of freshwater resources are in a constant decline due to the urbanisation and expansion of population (Ercin and Hoekstra 2014). Inputs of antimicrobial residues in freshwater environment from agricultural, hospital, pharmaceutical and

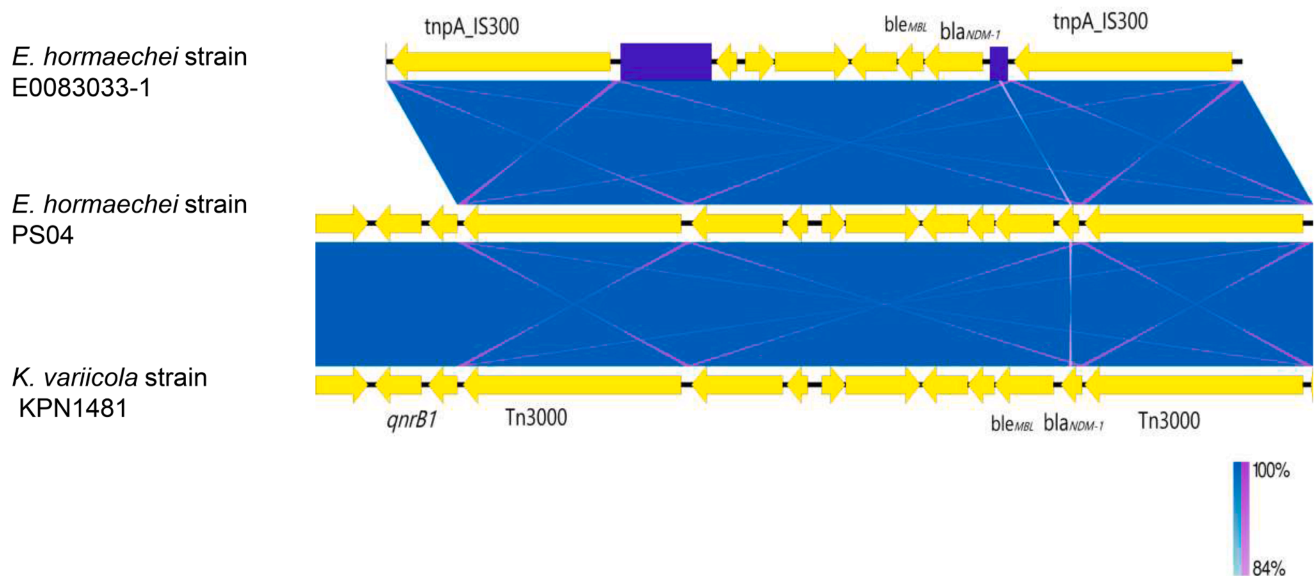
Table 3
An overview of the antimicrobial resistant genes, chromosomal mutations, resistance phenotype and the mobile genetic elements in the resistant isolates.

Isolate	Antimicrobial resistant genes	Chromosomal mutations encoding resistance	Mobile genetic elements	Resistance phenotype
P. mirabilis PS01	**Intrinsic -** *msr*(E), *mph*(E), *tet*(A), *tet*(J), bla_{TEM-1B} **Acquired -***ant(2′′)-Ia, aac(6′)-Ib-cr, aadA1, armA, aadA5, aac(3)-IId, aac(6′)-Ib, sul1, dfrA17, dfrA1,* bla_{VEB-6}, *qacE, cat, catA1*		IS*629*, IS*5*, ISE*c29*, ISC*fr1*, IS*26*	erythromycin,azithromycin, gentamicin, tobramycin, ciprofloxacin, spectinomycin, streptomycin, tobramycin,isepamicin, netilmicin, apramycin, dibekacin, sisomicin, sulfamethoxazole, trimethoprim, doxycycline,tetracycline, chloramphenicol, benzylkonium chloride, ethidium bromide,chlorhexidine amd cetylpyridinium chloride
K. quasipneumoniae PS02	**Intrinsic -***msr*(E), *fos*A, $bla_{OKP-B-8}$, bla_{TEM-1B}, **Acquired -** *aph(6)-Id, aph(3′′)-Ib, mph* (E), *mph*(A), *OqxA, OqxB, qnrB1, sul1,* bla_{SHV-12}, *catA2*	*acrR* (p.R173G), *acrR* (p.G164_None 491del), *acrR* (p.F172S), *acrR* (p.K201M), *acrR* (p.F197I), *acrR* (p.L195V), *acrR* (p. P161R), *OqxA* (*OqxA*_EU370913), *OqxB* (*OqxB*_EU370913), *ompK*36 (p.N218H), *ompK*37 (p.I70M), *ompK*36 (p.A217S), *ompK*37 (p.I128M), *ramR*:p.K194*, *parC*:p. A171G, *parC*:p.S172I, *parC*:p.L88Q, *parC*:p. P170T, *gyrA* (p.S83Y)	IS*426*, Tn*6170* ISE*c33*, Tn*6082*, ISK*pn25*, ISE*c9*, ISK*pn38*, IS*6100*, ISK*pn14*	erythromycin,azithromycin,quinupristin, pristinamycin ia, virginiamycin, streptomycin, chloramphenicol,benzylkonium chloride, cetylpyridinium chloride,nalidixic acid, ciprofloxacin,trimethoprim, sulfamethoxazole, amoxicillin,ampicillin,aztreonam,cefepime, cefotaxime,ceftazidime,ceftriaxone,piperacillin, ticarcillin and carbapenem
E. hormaechei PS03	**Intrinsic -***msr*(E), *fos*A, *ere*(A), *mph*(A), **Acquired -** *aph(6)-Id, aph(3′′)-Ib, armA, aac(6′)-Ib-cr, aadA1, aac(6′)-Ib3, aadA2, aac(6′)-IIc, mcr-9, mph*(E), *msr* (E), *qnr*B, *sul2, sul1, dfrA1, dfrA12, tet* (D), bla_{NDM-1}, bla_{CTX-M}, bla_{SHV-12}, bla_{ACT-16}, bla_{TEM-1B}, *qacE, catA1* and *catA2*		ISK*pn41*, ISK*pn50*, ISK*pn21*, ISK*pn25*, ISE*sa2*, ISE*sa1*, Tn*3000*, ISE*c29*, Tn*6196*, IS*Vsa3*, IS*5075*, ISK*pn38*, ISK*pn8*, ISE*cl1*, ISK*ox3*, ISK*pn34*, ISK*pn28*, IS*102* IS*6100*	erythromycin,azithromycin,quinupristin, pristinamycin,virginiamycin, streptomycin, amikacin,gentamicin,tobramycin, isepamicin,netilmicin, ciprofloxacin, streptomycin, amikacin,tobramycin, Fosfomycin, trimethoprim, sulfamethoxazole, doxycycline,tetracycline, amoxicillin, amoxicillin+clavulanic acid,ampicillin, ampicillin+clavulanic acid,cefepime,cefixime, cefotaxime,cefoxitin,ceftazidime,ertapenem, imipenem,meropenem,piperacillin, piperacillin+tazobactam,temocillin, benzylkonium chloride,ethidium bromide, chlorhexidine,cetylpyridinium chloride and chloramphenicol

domestic wastes can select for antimicrobial resistant bacteria (Seethalakshmi et al., 2023). Even though sunlight can kill antimicrobial resistant bacteria, the ARGs remains intact and can be acquired by other bacterial communities in the water microbiome (Nnadozie and Odume 2019).

Circulation of resistant genes in water environments especially, ESBL producing *Enterobacteriaceae*, is of major concern as they can result in highly epidemic infections. bla_{CTX-M} is the most dominant ESBL genotype observed in India with *Escherichia coli* and *K. pneumoniae* being the most frequently isolated bacteria bearing the gene (Hawkey 2008). In this study, bla_{CTX-M} was found to be integrated into the genome of *E. hormaechei* PS03 strain in the chromosome rather than a plasmid. A study by Yoon et al. (2020) have pointed out that under conditions surrounded by an abundance of antibiotics, bacteria may assign ARGs as residential genes to ensure propagation and this might explain why bla_{CTX-M}, and bla_{SHV-12} were encoded by the chromosome. Unlike this, bla_{NDM-1}, was carried by Tn*3000* along with quinolone resistant gene, *qnrB1* in *E. hormaechei* PS03. Tn*3000*, has been reported to facilitate the transfer of bla_{NDM-1} between plasmids from different incompatibility groups in India, Brazil, Morocco, and Nepal (Campos et al., 2015). Besides its clinical prevalence in India, bla_{NDM-1} has also been detected in water bodies (Ahammad et al., 2014; Kalasseril et al., 2020), sewage treatment plants (Akiba et al., 2016) and hospital effluents (Lamba et al., 2017). Oral- faecal dissemination of bacteria is a global issue and the circulation of bacteria carrying bla_{NDM-1} in water environments can be a health hazard for public, especially in countries with improper sanitation (Walsh et al., 2011).

Following the first report of *mcr-1* gene in raw meat samples from India (Ghafur et al., 2019), the Indian government declared a complete ban on the use of colistin as a growth promoting agent in poultry (Davies and Stockton 2018). Since then, much attention has been given to monitor the transmission of colistin resistance in clinical settings, food products and environment. *mcr-1* has been detected in urban sewage waters (Gogry and Siddiqui 2019) and very recently *mcr 5.1* has been found in hospital sewage waters in India (Talat et al., 2022). To our knowledge, this is the first report highlighting the transmission of *mcr-9* in India. It is to be noted that *mcr-9* is not always linked to colistin resistance as it is an inducible attribute in Enterobacteriace. Few studies have reported that despite bearing *mcr-9/10* gene, bacterial isolates can be sensitive to colistin unlike other *mcr* genes (Tyson et al., 2020; Liao et al., 2022). The two-component system *QseCB*, located downstream of the *mcr-9* gene, at sub inhibitory concentrations of colistin, induces the expression of the resistant gene and exhibits resistance to the antibiotic, colistin (Yuan et al., 2019; Kieffer et al., 2019). In the isolate *E. hormaechei* PS03 chromosome, *QseCB* was not identified downstream to *mcr-9*, which explains the sensitivity of the strain to colistin. *quasipneumoniae* is an inhabitant of gastrointestinal tract and is considered as a less virulent opportunistic pathogen compared to *K. pneumoniae* (Long et al., 2017). However, recent studies have identified the propagation of ESBL and carbapenem resistance encoding ARGs in *K. quasipneumoniae* (Arabaghian et al., 2019; Sajeev et al., 2022; Sakai and Maesaki 2022). In general, beta lactam genes such as bla_{SHV}, bla_{LEN} and bla_{OKP} genes are closely associated with the chromosomes of *K. pneumoniae, K. variicola* and K. *quasipneumoniae*, respectively, although these species of *Klebsiella* can acquire beta lactam resistant genes from one another through homologous recombination (Long et al.,



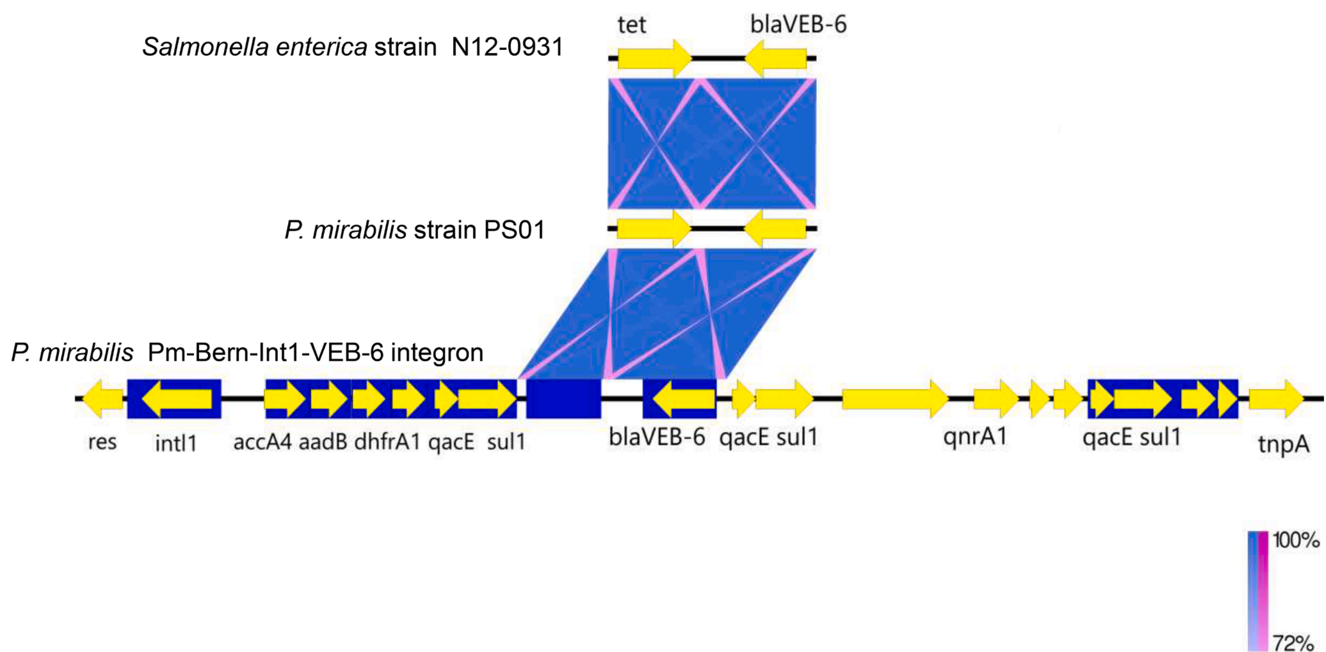


Fig. 4. Comparison of genetic environments of **A.** Tn3000 of *E. hormaechei* PS03 and **B.** $bla_{VEB\text{-}6}$ regions of *P. mirabilis* PS01 with similar plasmids or chromosome. Yellow arrows denote the positions and transcriptional directions of ORFs and blue rectangular blocks indicate miscellaneous features. Regions of homology are highlighted by shading.

2017). The strain *K. quasipneumoniae* PS02 isolated in this study, carried $bla_{SHV\text{-}12}$, in the chromosome in addition to $bla_{OKP\text{-}8\text{-}B}$. $bla_{SHV\text{-}12}$ is an ESBL encoding ARG commonly carried by plasmids and therefore it can be presumed that $bla_{SHV\text{-}12}$ may have integrated into the chromosome from a plasmid rather than a resultant of homologous recombination with *K. pneumoniae,* as the latter would have resulted in acquisition of putative beta lactam genes $bla_{SHV\text{-}1}$ and $bla_{SHV\text{-}11}$ (Lee et al., 2006). *traT* identified in this strain is an invasin and a cellular infection promoter that can also cause tissue dissemination and sepsis (Li et al., 2022). *iutA*, an outer membrane transporter of the aerobactin system, observed in hypervirulent strains of *Klebsiella* sp. is linked to community associated infections such as liver abscess, pneumonia, and increased mortality (Lam et al., 2018). Virulence genes in isolates from natural environment can enter into human populations through contamination of water sources used for drinking, recreation, or irrigation, or via interactions with aquatic animals and can cause severe infections that can be fatal.

Vietnamese ESBL or VEB was initially reported in France from a Vietnamese patient in 1999, and is often seen together with class 1 integron along the gene cassettes *arr-2,* bla_{OXA-10} and *aadB* (Poirel et al., 1999). *P. mirabilis* has also been reported to carry $bla_{VEB\text{-}6}$ in class-1 integron with other ARGs such as *aadB, aacA4, dfrA1, tet(A), sul1*, and *qnrA1* (Nordmann and Naas 1994) and in some cases was found to be

present on Salmonella genomic island 1 (Schultz et al., 2015). However, in this study the ESBL producing *P. mirabilis* strain PS01 was found to carry *bla*$_{VEB\text{-}6}$ in class-1 integrons along with *tet(A)* gene, only.

Several factors could contribute to the dissemination of ARGs in aquatic ecosystems. This includes the vectors for horizontal gene transfers like bacteriophages, transposons, insertion elements and plasmids in antimicrobial resistant bacteria which can propagate ARGs to other bacterial communities in water. Another potential factor that enables proliferation of AMR is bacterial biofilms, that are formed upon adhesion of bacterial cells to suspended particles in surface waters. Bacterial biofilms provide a microenvironment which brings cells close to one another and facilitate transfer of ARGs at much higher rates than their planktonic forms (Fang et al., 2018). Though none of the resistant isolates screened in this study formed biofilms under controlled conditions in the laboratory, it is important to keep in mind that the results may not fully reflect the behaviour of these bacteria in their natural environments. It has also been reported that biofilm formation act as mechanism for survival in bacterial isolates that do not have a high level of resistance to antibiotics (Qi et al., 2016). Hence it is also possible that the isolates were weak biofilm producers due their increased resistance to antibiotics.

Thiruvandarkoil lake located at Villainur Taluk of Puducherry is benefited by two villages namely Thiruvandarkoil and Kothapurinatham, and is a chief source of water for agricultural activities in the area. Through irrigation these resistant bacteria can enter food systems and can advance the dissemination of ARGs into the human intestinal microbiota. The lake is also a hotspot for many migratory birds, indicating another possible route for global dissemination of AMR. The presence of genes imparting resistance to quaternary ammonium compounds, heavy metals and point mutations associated antibiotic resistance in the isolated bacteria hints the possibility of selective pressure exerted by antimicrobial and heavy metal residues in the water.

The absence of adequate wastewater treatment plants leads to the direct release of household sewage into aquatic ecosystems. AMR is not included in India's water quality standards and guidelines precisely from this situation (Bureau of Indian Standards (BIS), New Delhi 2012; Kumar et al., 2021). In this study, our findings of high-risk MDR pathogens in lake water emphasize the necessity of AMR as a critical quality parameter, particularly for drinking water sources. This is further supported by the evidence in our study, where despite the presence of hazardous bacteria in the freshwater samples, our analysis revealed that all physicochemical parameters remained within normal ranges. Even in the presence of these potentially harmful bacteria, the water exhibited no signs of toxicity. Zebrafish embryos, often used as sensitive indicators of aquatic health, displayed normal development without any observable anomalies. These results highlight the broader concern of water security, underlining the importance of addressing AMR in the context of both lake water quality and the overall safety of drinking water supplies.

This study, while contributing valuable insights into the genomic construct of ESBL producing *Enterobacteriaceae*, is not without limitations. As such, the findings offer preliminary insights rather than exhaustive conclusions about the AMR in entire microbial community and the study's design as a pilot investigation necessitates a cautious interpretation of the results.

5. Conclusion

As antibiotics are consistently used in the health, livestock, and food sectors, the chances of antimicrobial residues reaching the environment is high and this can intensify the development of AMR. The AMR crisis in India is still largely underexplored, especially in environmental settings and this limits our understanding of the burden it poses to the country. Our study has identified the presence of ESBL-producing bacteria in a rural lake water sample. The detection of these resistant strains in the environment is concerning, as they pose a potential threat to public health by contributing to the spread of antimicrobial resistance. The results of our study emphasize the importance of monitoring environmental reservoirs for antimicrobial-resistant bacteria, particularly in areas where there is a high risk of contamination from human or animal waste.

Further investigation is needed to better understand the extent and mechanisms of antimicrobial resistance in environmental ESBL-producing bacteria, as well as the potential for these strains to cause infections in humans and animals. This could include additional surveillance studies to assess the prevalence and distribution of ESBL-producing bacteria in the environment, as well as molecular and genetic analyses to characterize the resistance mechanisms and genetic traits of these strains. Overall, our findings highlight the need for a multi-faceted approach to combat antimicrobial resistance, including the prudent use of antibiotics in both human and veterinary medicine, improved sanitation and hygiene practices, and enhanced monitoring and surveillance of antimicrobial-resistant bacteria in the environment.

Compliance with ethical standards

Research involving human participants and/or animals: This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent: This article does not contain any studies with human participants performed by any of the authors.

CRediT authorship contribution statement

P S Seethalakshmi: Investigation, Writing – original draft, Methodology, Data curation. **Vishnu Prasad Nair RU:** Methodology, Writing – review & editing. **Anushara Prabhakaran:** Methodology, Writing – review & editing. **Ragothaman Prathiviraj:** Methodology, Writing – review & editing. **Rajesh Pamanji:** Methodology, Writing – review & editing. **George Seghal Kiran:** Supervision, Writing – review & editing. **Joseph Selvin:** Conceptualization, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

All data is included in the manuscript and supplementary files

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.crmicr.2023.100216.

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