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Genetic diversity and prevalence of extended-spectrum beta-lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae* in aquatic environments receiving untreated hospital effluents

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ABSTRACT

The spread of extended-spectrum beta-lactamase (ESBL)-producing bacteria in the environment has been recognized as a challenge to public health. The aim of the present study was to assess the occurrence of ESBL-producing *Escherichia coli* and *Klebsiella pneumoniae* from selected water bodies receiving hospital effluents in Kerala, India. Nearly 69.8% of Enterobacteriaceae isolates were multi-drug resistant by the Kirby–Bauer disc diffusion method. The double disc synergy test was used to detect the ESBL production and the genes responsible for imparting resistance were detected by PCR. Conjugation experiments confirmed the mechanism of plasmid-mediated transfer of resistance. The prevalence of ESBL production in *E. coli* and *K. pneumoniae* was 49.2 and 46.8%, respectively. Among the ESBL-encoding genes, $bla_{\text{CTX-M}}$ was the most prevalent group followed by bla_{TEM}, bla_{OXA}, $bla_{\text{CMY,}}$ and bla_{SHV}. The results suggest that healthcare settings are one of the key contributors to the spread of ESBL-producing bacteria, not only through cross-transmission and ingestion of antibiotics but also through the discharge of waste without a proper treatment, leading to harmful effects on the aquatic environment. The high prevalence of ESBL-producing Enterobacteriaceae with resistance genes in public water bodies even post-treatment poses a serious threat.

Key words: antibiotic resistance, extended-spectrum beta-lactamase, hospital effluent, Kerala, public health

HIGHLIGHTS

- ESBL-producing *E. coli* and *K. pneumoniae* were detected in hospital effluent samples.
- The prevalence of ESBL production in *E. coli* and *K. pneumoniae* was 49.2 and 46.8%.
- $bla_{\text{CTX-M}}$ was the most prevalent among the ESBL-encoding genes.
- The conjugation experiment proved the horizontal mode of gene transfer.
- Hospitals play a significant role in the spread of antibiotics and resistant bacteria.

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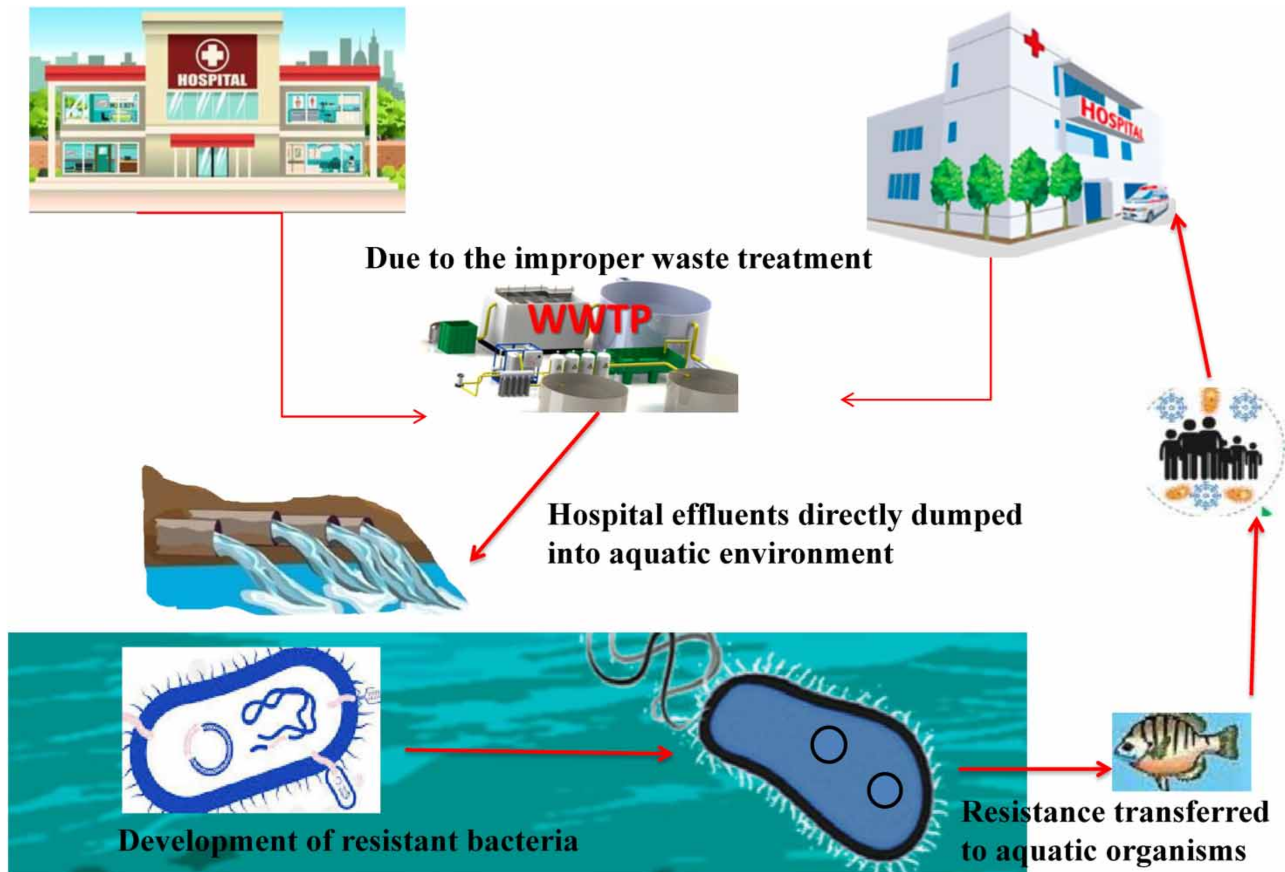
GRAPHICAL ABSTRACT



INTRODUCTION

Antimicrobials are undoubtedly one of the most triumphant chemotherapies in medical history. Human activities since the industrialization of antibiotic production have changed the spread and increased the prevalence of resistance genes. In addition, millions of tons of antibiotics have been released into the environment through wastewater effluents, land use of animal waste, treatment of crop diseases, and aquaculture activities (Kim & Cha 2021; Zheng *et al.* 2021). Antimicrobial resistance (AMR) spreads faster in humans, animals, and aquaculture due to the abuse and overuse of approved antimicrobials. Hospital wastewater treatment plants (WWTPs) are an important AMR driver aggregation path; they do not effectively eliminate all resistant bacterial pathogens and resistance genes and are thus, ultimately released into treated effluents (Osinska *et al.* 2020). Sewage effluent will be diluted when entering the river, estuary, or coastal water, and the resulting pollutant concentration including antibiotics, metals, biocides, and antibiotic resistance genes (ARGs) will interact with the native flora and fauna, causing changes in the microbial community structure and genetic makeup.

In Gram-negative bacteria, drug resistance is present as a serious global problem (Gniadkowski 2001). The beta-lactam antibiotics are the first-line therapeutic option for the treatment of Enterobacteriaceae infections, but resistance to these compounds has increased in recent decades (Rossolini & Mantengoli 2005). An epidemiological study reported that extended-spectrum beta-lactamase (ESBL)-producing Enterobacteriaceae, in particular, *Escherichia coli* and *Klebsiella pneumoniae* are the major causes of community-acquired infections (Stadler *et al.* 2018). ESBL enzymes have hydrolytic capacities for a range of beta-lactams including penicillins, monobactams, and extended-spectrum cephalosporins (except cephamycins) and are inactivated by beta-lactamase inhibitors such as clavulanic acid (Gniadkowski 2001). Resistance to beta-lactams in Enterobacteriaceae is primarily due to the production of beta-lactamases, the genes for which are encoded on plasmids or chromosomally (Bush & Bradford 2016). Several different mechanisms of resistance to this antibiotic

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family have been detected, such as porin alterations, penicillin-binding proteins (PBPs) modifications, efflux systems, and the production of enzymes like ESBLs, AmpC beta-lactamases, and metallo-beta-lactamases (MBLs) (Batchelor *et al.* 2005).

Since the first study of the ESBLs reported in 1979, the occurrence of ESBL-producing bacteria was frequently identified worldwide from clinical isolates attributed to the cumulative use of beta-lactam antibiotics and carbapenems; the latter is generally used as the last choice for most severe bacterial infections (Sanders & Sanders 1979). According to their functional and structural characteristics, over 200 beta-lactamases are classified into four main groups and eight subgroups (Bush & Jacoby 2010). When compared to ARGs from other antibiotic classes, beta-lactamase genes have a higher number and diversity (Piotrowska *et al.* 2019). As the largest group of varied and unique determinants of resistance in bacteria, beta-lactamase genes are studied in detail, in terms of distribution mechanisms in the environment. The ESBLs are mainly derived from plasmid-mediated TEM or SHV beta-lactamases by a mutation that results in one or more amino acid sequence alterations and results in the change of binding to the active substrate site (Paterson & Bonomo 2005). Enterobacteriaceae isolates have long been known to recruit narrow-spectrum ambler class A beta-lactamases of the TEM and SHV lineages, which were linked to nosocomial epidemics in the 1980s (Quinn *et al.* 1989). Being mediated with plasmid and transposon, it spreads rapidly to various bacterial species, and it was found that up to 90% of the resistance to ampicillin in *E. coli* is due to the production of TEM beta-lactamases (Livermore 1995). A relatively new type of CTX-M ESBLs has a high affinity for cefotaxime and has been divided into five categories, with more than 40 variants distributed worldwide (Bush 2018). Additionally, AmpC beta-lactamases confer resistance to a wide range of beta-lactam antibiotics, primarily cephamycins such as cefoxitin and cefotetan, less frequently used with cefotaxime, ceftazidime, cefpodoxime, ceftriaxone, and sometimes monobactams such as aztreonam and they are chromosomally, or plasmid-encoded. The plasmid-mediated AmpC beta-lactamase (pAmpC) now poses a complex danger to the activities of penicillins, cephalosporins, monobactams, and carbapenems (Ahmed & Shimamoto 2008).

The aim of the present study was to assess the role of hospital effluents on the accumulation of microbial contaminants in the receiving public water bodies and aquaculture farms in their vicinity using the same water as an inlet source. In particular, the study aimed to determine the prevalence, resistance pattern, identification of the major ESBL-encoding genes in *E. coli* and *K. pneumoniae* isolates, and their mode of transfer between bacteria since they can cause serious public health problems.

METHODS

Study area

The methods are described in brief here. A detailed description of the method is available in previously published papers (Kalasseril *et al.* 2020; Sneha *et al.* 2020; Sneha & Devika 2021). Three tertiary care hospitals (H1, H2, and H3) located near water bodies and five aquaculture farms (F1, F2, F3, F4, and F5) in their vicinity in three districts (Ernakulam, Kollam, and Kannur) of Kerala, India were selected for the study. Hospital 1 (H1) is a multi-super specialty hospital with 350 patient beds and 43 ICU beds located in Ernakulam city near to Vembanad backwaters of Kerala (N0 9°55.160′ E0 76°19.277′). Hospital 2 is a government medical college with 500 patient beds located in the Kollam district near to Ashtamudi backwaters (N0 8°90108′ E0 76°.58865′ E). Hospital 3 is located in the Kannur district near to Kuyyali River with 250 patient beds (N0 11°764102′ E0 75°.4796778′). Two aquaculture farms were selected in the district of Ernakulam; adjacent to Hospital 1 (N 9°.919500′ E 76°.324444′ and N 9°.920528′ E 76°.322667′). Only one farm was selected in the Kollam district; adjacent to Hospital 2 (N 8°.90781′ E 76°.6028′). Two farms were selected in the Kannur district; adjacent to Hospital 3 (N 11°.764930′ E 75°.480421′ and N 11°.765277 E 75°.481462). The study was conducted from August to December 2020–2021. The three hospitals released their sewage effluents into water bodies that were used for various purposes.

Sample collection and processing

From the sampling points, sediment, water, and fish/shrimp/clams were collected following standard procedures (Walsh *et al.* 2011). The animal samples (fish/shrimp/clams) collected during the study period, were from the river downstream of the hospitals. All the mentioned hospitals are located in crowded cities and these hospitals release sewage directly into open water bodies. The same water is used for irrigation and aquaculture purposes, apart from fishing activities. Two sampling locations were chosen: (i) the point where hospital effluents enter public water bodies and (ii) farms downstream of hospital sites. Water samples were taken from the outlet pipes of the hospitals in amber-colored sterile bottles and transported to the microbiology laboratory on ice within 2 h of collection. From each site, approximately 100–200 g of sediment and 50 g of animal tissue were taken and homogenized. The samples were diluted serially and a volume of 100 μl from each

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sample was inoculated onto Trypticase soy agar (TSA) (Himedia, India) in duplicate plates and incubated at 37 °C for 24 h. Colonies were enumerated and different morphotypes were randomly selected and transferred to a fresh plate to ensure their purity.

Identification of *E. coli* and *K. pneumoniae* isolates from samples

For descriptive analysis, total colony-forming units per plate within the range of 30–300 were included. Single colonies were then transferred to MacConkey agar (Himedia, India) and Hicrome Klebsiella selective agar base followed by incubation for 24–48 h at ambient temperature until visible colonies were observed. Isolates that appeared bright pink and purple mucoid colonies on the selective agar were confirmed as *E. coli* and *K. pneumoniae* respectively. The isolates were further confirmed by biochemical characterization (Holt *et al*. 1994) and 16S rDNA sequencing.

Antimicrobial susceptibility testing

According to the Clinical and Laboratory Standards Institute (CLSI 2019), antibiotic resistance phenotypes were determined based on the disc diffusion method (Bauer *et al*. 1966). All the antibiotic discs and the media were purchased from Himedia, India. Pure colonies of *isolates* were grown in Mueller–Hinton broth for 8–12 h, the turbidity was adjusted to 0.5 McFarland's standard and was spread plated on well-dried Mueller–Hinton agar using a sterile cotton swab. Discs were placed on the agar media with the help of sterile forceps ensuring sufficient distance between discs and the plates were incubated at 35 °C for 24 h. The following antibiotic discs were used for antimicrobial susceptibility testing: ampicillin (10 μg), amoxicillin (10 μg), amoxycillin/clavulanate (20/10 μg), cefotaxime (30 μg), cefotetan (30 μg), cefoxitin (30 μg), ceftazidime (30 μg), ceftriaxone (30 μg), cefpodoxime (10 μg), cefepime (10 μg), aztreonam (30 μg), chloramphenicol (30 μg), azithromycin (15 μg), gentamicin (10 μg), amikacin (30 μg), ciprofloxacin (5 μg), ofloxacin (5 μg), nalidixic acid (30 μg), trimethoprim/sulfamethoxazole (1.25/23.75 μg), imipenem (10 μg), and meropenem (10 μg). Using the strip (Himedia, India) method, the minimum inhibitory concentration (MIC) for selected isolates was determined.

Phenotypic detection of ESBL production

The isolates showing a higher resistance to the cephalosporin class of antibiotics were selected for checking the ESBL production. A Double Disc Synergy test was used for the detection of ESBL production in which a disc of ceftazidime (30 μg) alone and a combination of ceftazidime with clavulanic acid (30 μg/10 μg), and cefotaxime (30 μg) alone and cefotaxime with clavulanic acid (30 μg/10 μg) were used (Linscott & Brown 2005). A lawn culture of the test organism was plated on Mueller–Hinton agar and the discs were carefully placed with centers at least 24 mm apart. The plates were incubated overnight at ambient temperature. Any decrease or increase in the inhibition zone diameter of $\geq$5 mm for the combination discs compared to ceftazidime or cefotaxime alone was considered as positive for ESBL production.

Molecular detection of ESBL-encoding genes

The presence of ESBL-encoding genes was detected as described by Villegas *et al.* (2004); Sharma *et al.* (2010) and Ibrahim *et al.* (2014). Details of the primers used and PCR conditions are given in Table 1. The PCR was carried out in a 25-μl reaction mixture containing 15.75 μl of nuclease-free water, 10X Taq buffer (2.5 μl) (100 mM Tris HCl, pH 8.3, 20 mM $MgCl_2$, 500 mM KCl, 0.1% gelatin), 200 mM dNTP's (0.5 μl) (dATP, dTTP, dGTP, dCTP), 10 pmol each of forward and reverse primers (2 μl), 1.0 unit of Taq DNA polymerase (0.25 μl) and 2 μl of the template. All the reagents were purchased from Origin Diagnostics and Research, Bangalore. The PCR was carried out in a My Cycler thermal cycler (Biorad, USA). The PCR products were purified and sequencing was performed with an automated ABI 3100 Genetic analyzer using ABI BigDYE terminator method (M/s Agrigenome Pvt Ltd, Kerala, India). A Basic Local Alignment Search Tool (BLAST) algorithm was used to analyze the nucleotide sequences in the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/BLAST). The nucleic acid sequences were submitted to GenBank (GenBank accession numbers MN715313, MN715314, MW642081, and MW642080).

Bacterial plasmid isolation and conjugation

Using a plasmid extraction kit (GeNei, Bangalore), the bacterial plasmid was extracted. The purified plasmid DNA was dissolved and stored at 4 °C in TE buffer. Conjugation experiments were carried out by a broth mating method using azide-resistant (AzR) *E. coli* J53 as the recipient and $bla_{\text{CTX-M-15}}$-positive *E. coli* as the donor (Wang *et al*. 2008). Pure colonies of donor and recipient cells were inoculated separately and incubated overnight at 37 °C with shaking. These overnight

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Table 1 | List of primers used for PCR amplification

Specific gene for amplification	Primer sequence (5′-3′)		Amplicon size	Reference
bla$_{OXA\text{-}48}$	Forward Reverse	TCAACTTTCAAGATCGCA GTGTGTTTAGAATGGTGA	591 bp	Ibrahim *et al.* (2014)
bla$_{SHV}$	Forward Reverse	TTAACTCCCTGTTAGCCA GATTTGCTGATTTCGCCC	795 bp	Sharma *et al.* (2010)
bla$_{TEM}$	Forward Reverse	ATAAAATTCTTGAAGACGAAA GACAGTTACCAATGCTTAATC	1,080 bp	Sharma *et al.* (2010)
bla$_{CMY}$	Forward Reverse	TGGAACGAAGGCTACGTA GACAGCCTCTTTCTCCACA	1,007 bp	Ibrahim *et al.* (2014)
bla$_{CTX\text{-}M}$	Forward Reverse	CGCTTTGCGATGTGCAG ACCGCGATATCGTTGGT3	550 bp	Villegas *et al.* (2004)

cultures were diluted in a fresh medium at 1:100 and each was grown to the early exponential phase. The mating combination was prepared by adding 0.1 ml of donor cells to 0.9 ml of recipient cells. The mixture was gently whirled for a few minutes and incubated at 37 °C for 6 h (without shaking). This was followed by plating on Luria–Bertani (LB) agar medium (Himedia, India) containing cefotaxime (2 mg/l). Trans-conjugants carrying the same *bla*$_{CTX\text{-}M\text{-}15}$ gene as their donor were verified by PCR.

RESULTS AND DISCUSSION

Bacterial identification and ESBL detection

From the study area, a total of 524 Gram-negative bacteria were isolated, of which 366 isolates (69.8%) showed multi-drug resistance (MDR) to a different class of antibiotics tested. The highest number of bacterial isolates were retrieved from sediment and animal samples. The number of cultivable bacteria was much higher in Kollam effluent samples (H2) followed by H1 (Ernakulam) and H3 (Kannur). In aquaculture farms, the number of cultivable bacteria was found to be highest in F3 (Kollam) followed by F1, F2, (Ernakulam) F4, and F5 (Kannur). The occurrence and distribution of different bacterial isolates from different locations are shown in Table 2. Various samples screened from direct hospital effluents, *E. coli* was found to be the dominant isolate ($n = 135$) followed by *K. pneumoniae* ($n = 49$), *Klebsiella oxytoca* ($n = 32$), *Aeromonas hydrophila* ($n = 18$), *Enterobacter aerogenes* ($n = 15$), *Pseudomonas aeruginosa* ($n = 15$), *Enterobacter cloacae* ($n = 12$), *Pseudomonas putida* ($n = 12$), *Citrobacter* sp. ($n = 11$), *Salmonella* sp. ($n = 9$) and *Acinetobacter baumannii* ($n = 7$). In comparison, *E. coli* ($n = 74$), *K. pneumoniae* (17), *E. aerogenes* (13), *P. aeruginosa* (7), and *A. baumannii* ($n = 3$) were obtained from aquaculture farms downstream of the above sites.

The CLSI recommends screening of *E. coli*, *K. pneumoniae*, *K. oxytoca*, and *Proteus mirabilis* isolates for ESBL production by the use of cefpodoxime, ceftazidime, aztreonam, cefotaxime, or ceftriaxone, followed by phenotypic confirmation with clavulanate. Only *E. coli* and *K. pneumoniae* isolates that showed resistance to the cephalosporin class of antibiotics were subjected to ESBL detection by the Double Disc Synergy test (Figure 1). Of the 209 *E. coli* isolated from different points, 103 isolates (49.2%) were positive for ESBL production; 76 isolates from direct hospital effluents (36.3%) (H1 = 23, H2 = 41, H3 = 12) and 27 isolates (12.9%) from the F3 site. Among the 66 *K. pneumoniae* isolates, 25 (37.8%) isolates from hospital points (H1 = 10, H2 = 15) and 6 isolates (9%) from the F3 site were found to be ESBL producers. None of the other bacterial isolates from direct hospital and aquaculture farm samples were positive for ESBL production.

In soil and aquatic ecosystems, anthropogenic activities affect water quality and, as a result, its long-term use is frequently threatened. Considering aquatic systems as reactors for diverse biological interactions with major genetic consequences, the study of aquatic antibiotic resistance including ARGs, pathogenic and non-pathogenic antibiotic-resistant bacteria (ARBs) is important, as it may indicate the degree of alteration of water environments by different types of pollutions. In Kerala, the southernmost state of India, the majority of hospitals and factories are located on heavily populated riversides, near cities and towns, which causes MDR isolates, to disseminate quickly through these water bodies. In this study, public water bodies receiving hospital effluents yielded a total of 69.8% MDR isolates, including 48.6% ESBL producers. Total coliforms were found to be substantially higher in samples taken from direct hospital effluent than in samples collected from

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Table 2 | Occurrence and distribution of *Escherichia coli* and *Klebsiella pneumoniae* from different locations

Sampling area	Location and distance from main discharge to aquaculture farms	Types of samples	Total Gram-negative isolates from different source	Total number of multi-drug isolates	No. of *E. coli* isolates and ESBL-positive isolates	No. of *K. pneumoniae* isolates and ESBL-positive isolates
Ernakulam	Direct hospital effluent (H1)	Water Soil Fish clam	16 42 31 25 } 114	85	40 ESBL-positive – 23	15 ESBL-positive – 10
Ernakulam	Aquaculture Farm 1 (F1 – 1 km)	Water Soil Fish clam	9 21 16 9 } 55	36	16 ESBL-positive – 0	4 ESBL-positive – 0
Ernakulam	Aquaculture Farm 1 (F2 – 2 km)	Water Soil Fish clam	8 12 10 6 } 36	27	9 ESBL-positive – 0	3 ESBL-positive – 0
Kollam	Direct hospital effluent (H2)	Water Soil Fish clam	24 51 41 38 } 154	116	67 ESBL-positive – 41	26 ESBL-positive – 15
Kollam	Aquaculture farm 3 (F3 – 800 m)	Water Soil Fish clam	9 24 33 13 } 79	45	31 ESBL-positive – 27	8 ESBL-positive – 6
Kannur	Direct hospital effluent (H3)	Water soil clam	11 30 22 } 63	49	28 ESBL-positive – 12	8 ESBL-positive – 0
Kannur	Aquaculture farm (F4 – 2 Km)	Water soil shrimp	2 8 5 } 15	5	11 ESBL-positive – 0	1 ESBL-positive – 0
Kannur	Aquaculture farm (F5 – 3.5 Km)	Water soil shrimp	1 4 3 } 8	3	7 ESBL-positive – 0	1 ESBL-positive – 0

aquaculture farms located downstream of the main discharge. This result could be explained by the fact that WWTP units in Kerala have not been fully functional, with 94% of sewage being dumped in open fields and water bodies (Kanth 2020). The existence of a large number of Gram-negative isolates indicated that the water quality had deteriorated, altering the river's ecology.

Antimicrobial susceptibility testing and MIC of ESBL isolates

Among the 76 ESBL-positive *E. coli* isolates from direct hospital effluents, all the isolates showed complete resistance to ampicillin, amoxicillin, cefoxitin, cefotetan, cefepime, cefpodoxime, amoxycillin/clavulanate, ceftazidime, cefotaxime, ceftriaxone, ciprofloxacin, ofloxacin, nalidixic acid, aztreonam, trimethoprim/sulfamethoxazole, amikacin, gentamicin, and imipenem whereas all the strains were susceptible to azithromycin, meropenem, and chloramphenicol. In comparison, out of the 49 hospital effluent, *K. pneumoniae* isolates, 25 ESBL-positive isolates ($H1 = 10$, $H2 = 15$) showed complete resistance to ampicillin, amoxicillin, amoxycillin/clavulanate, cefotaxime, cefotetan, cefoxitin, ceftazidime, ceftriaxone, cefpodoxime, and cefepime, whereas *K. pneumoniae* isolates from H2 ($n = 15$) showed resistance to ciprofloxacin, ofloxacin, nalidixic acid, imipenem, meropenem, aztreonam, and trimethoprim/sulfamethoxazole. However, they were sensitive to amikacin, gentamicin, azithromycin, and chloramphenicol. In aquaculture farms (F3 site), 27 *E. coli* and 6 *K. pneumoniae* isolates showed high resistance to cephalosporin and quinolone class of antibiotics, the farm which was adjacent to the hospital discharge site. The pattern of antibiotic resistance of *E. coli* and *K. pneumoniae* from F3 was similar to that found in the direct hospital (H2) effluent

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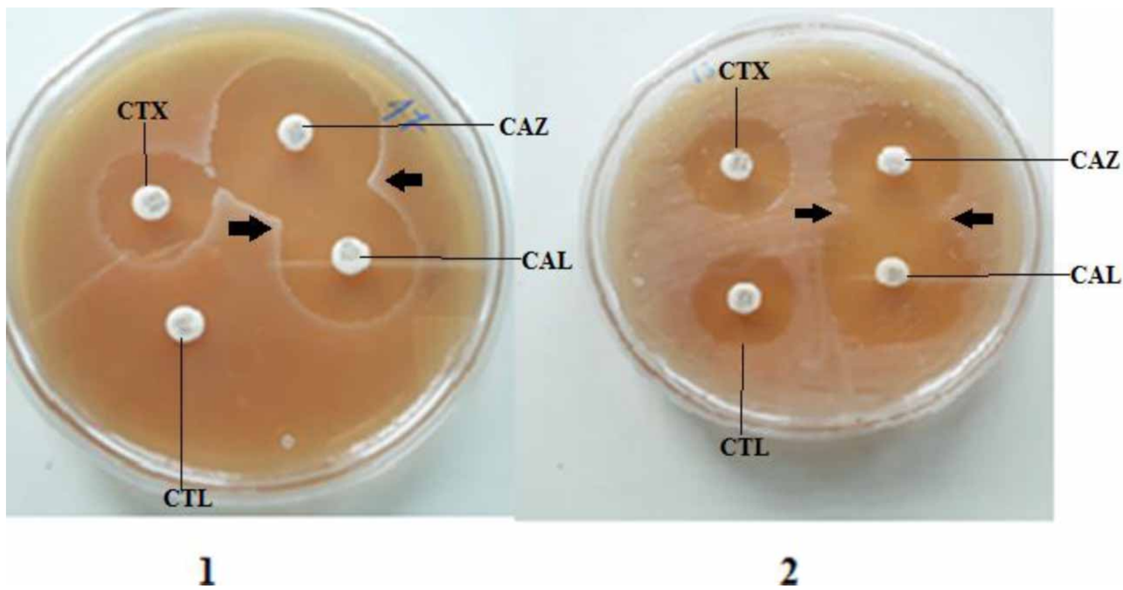

Figure 1 | ESBL production of (1) *Escherichia coli* and (2) *Klebsiella pneumoniae* (2) by the Double Disc Synergy test (DDST). A positive synergy was observed only between ceftazidime (CAZ – 30 μg) and ceftazidime with clavulanic acid (CAL – 30 + 10 μg) is indicated by arrows. Negative result was observed in cefotaxime (CTX – 30 μg) and cefotaxime with clavulanic acid (CTL – 30 + 10 μg).

samples. The F3 site, located about 800 m from the hospital effluent discharge site H2, was the only site among the aquaculture farms which harbored multi-drug resistant bacterial isolates with ESBL production. The similarity in the occurrence and pattern of MDR bacteria in H2 and F3 suggests that the close proximity of the farm to the hospital effluent discharge site could be the reason for the occurrence of MDR bacteria in the farm. It must be pointed out that there was no antibiotic use in the aquaculture farms selected for the study during the cultural activities. The location of the aquaculture farm appeared to be a critical factor. As the distance between the aquaculture farm and the point of discharge of hospital effluent increased, there was a significant decrease in the number of MDR isolates. This may be attributed to the increased dilution under flowing water conditions. The isolates from the farms F1, F2, F4, and F5 associated with H1 and H3 were sensitive to the tested antibiotics. These farms were located at a greater distance from the hospital location. The antibiotic resistance patterns of ESBL-positive isolates collected from direct hospital effluents and aquaculture farms (F3) are shown in Figure 2.

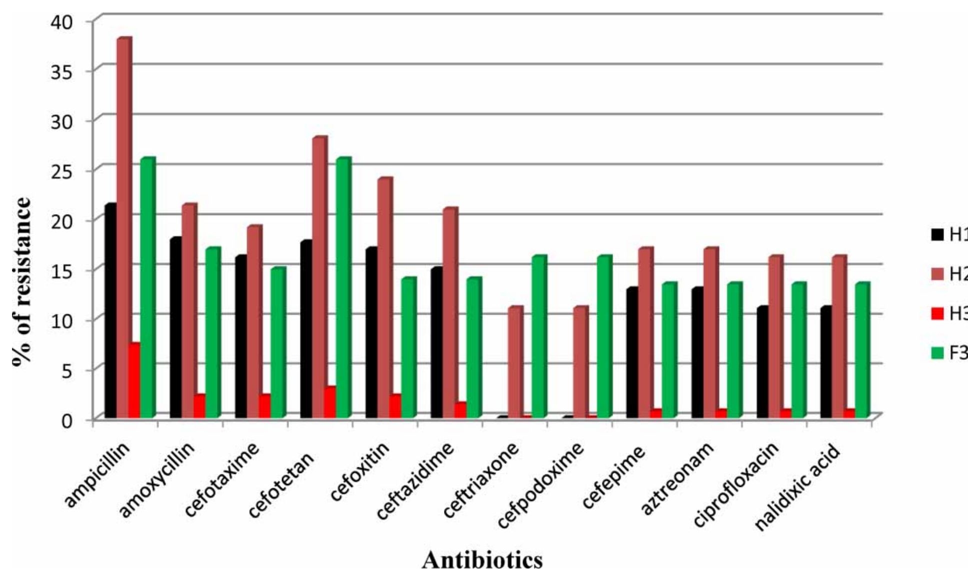

Figure 2 | Antibiotic resistance percentage of isolates from different direct hospital effluents (H1, H2, H3) and the aquaculture farm (F3).

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Overall, the resistance trends of ESBL-producing bacteria analyzed in our study were similar to those reported in other studies, i.e., the ESBL producers were resistant to a variety of antibiotic groups, including fluoroquinolones, aminoglycosides, and trimethoprim/sulfamethoxazole, in addition to beta-lactams which lead to the selection and persistence of MDR ESBL strains and plasmids in both clinical and community settings. This indicates that the organisms were well exposed and developed resistance to these antibiotics. A study by Conte *et al.* (2017) isolated MDR bacterial strains from hospital wastewaters in Brazil, primarily *E. coli*, *K. pneumoniae*, and *K. oxytoca,* which showed high resistance to cefotaxime and ceftazidime. Resistance to these two antibiotics indicates the development of ESBLs, which are characteristic of bacteria found in hospital effluents. In our study, the cephalosporin resistant ESBL-positive isolates also showed high resistance to the quinolone class of antibiotics. ESBL-producing *E. coli* with ciprofloxacin-resistance was isolated from Ireland's hospital effluent samples, signifying that these genes can coexist (Galvin *et al.* 2010). Co-selection of phenotypes of cephalosporin and quinolone resistance was previously reported in Enterobacteriaceae in the clinical setting, possibly leading to MDR bacteria (Lavilla *et al.* 2008). Research on ESBL genes indicated that their levels are significantly associated with other AMR genes especially quinolone genes and their mode of mechanism was regulated by the same promoter (Wu *et al.* 2016).

The majority of the ESBL-positive isolates from direct hospital effluent and aquaculture samples had MIC values of >32–256 μg/ml to each of the third-generation cephalosporin tested, thus confirming high resistance. All the ESBL-positive isolates exhibited high-level resistance to penicillins (>256 μg/ml). The frequency of antibiotic resistance was high in those isolates of *E. coli* and *K. pneumoniae* which had TEM, SHV, and CTX-M genes. *E. coli* isolates carrying CTX-M ESBL confer high-level resistance to ceftazidime (MIC range 64–256 μg/ml) and they had co-resistance to quinolone class of antibiotics and showed an elevated level of MIC value ranging from 8 to 64 μg/ml. However, in the case of *K. pneumoniae* isolates at the concentration of 128 μg/ml of ceftazidime, only twelve isolates showed resistance, while the remaining thirteen isolates had MIC value of >64 μg/ml toward ceftazidime. At the concentration of ≥128–256 μg/ml of cefotaxime and ceftriaxone, all the ESBL-positive *E. coli* isolates were resistant. On the other hand, ESBL-positive *K. pneumoniae* isolates had MIC levels of 64–128 μg/ml of cefotaxime and ceftriaxone; while the MIC range of 32–> 256 μg/ml was observed among *E. coli* and *K. pneumoniae* isolates for cefepime. In aquaculture farms, specifically ESBL-positive *E. coli* and *K. pneumoniae* from the F3 site showed an elevated level of MIC toward ceftazidime, ceftriaxone, and cefotaxime (Table 3).

According to recent findings, a combination of decreased outer membrane permeability and the hydrolytic impact of TEM-1 and SHV-1 beta-lactamases enhanced cefotaxime's MIC significantly (Drawz & Bonomo 2010). This difference suggests the presence of CTX-M-type ESBLs that hydrolyze cefotaxime and ceftriaxone more efficiently than ceftazidime (Tzouvelekis *et al.* 2000). All the ESBL-positive *E. coli* and *K. pneumoniae* showed an elevated level of MIC for ceftazidime (128–256 μg/ml). Previous studies have shown that the enzyme SHV-1 hyper production is one reason for the increased MIC of ceftazidime in Enterobacteriaceae (Rice *et al.* 2000). Resistance to cefoxitin, which is a cephamycin, has been observed in both *E. coli* and *K. pneumoniae* isolates, is not generally hydrolyzed by ESBLs, but resistance to cefoxitin has been increasing among ESBL-producing isolates due to altered membrane porins (Subha & Ananthan 2002). The MDR isolates in this study showed high MIC value to cefepime also due to the high prevalence of CTX-M-type ESBLs (Yu *et al.* 2002).

Table 3 | MIC values for cephalosporin-resistant *Escherichia coli* isolates from different sampling points (expressed in μg/ml)

Antibiotics	H1	H2	H3	F3
Ampicillin	128	256	128	256
Ciprofloxacin	32	64	8–16	32
Cefepime	>128	>256	32	256
Ceftazidime	128	256	128	128
Cefotaxime	>128	256	128	128
Ceftriaxone	>128	256	128	128

MIC, minimum inhibitory concentration; tested range of antibiotics is 0.125– >512 μg/ml.

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Table 4 | Distribution of Enterobacteriaceae isolates with ESBL production with resistance-encoding genes

Location and distance from main discharge to aquaculture farms	No. of *E. coli* isolates	No. of ESBL isolates	No. of isolates with resistance genes	No. of *K. pneumoniae* isolates	No. of ESBL isolates	No. of isolates with resistance genes
Direct hospital effluent (H1)	40	23	19	15	10	7
Aquaculture Farm 1 (F1 – 1 Km)	16	0	0	4	0	0
Aquaculture Farm 1 (F2 – 2 Km)	9	0	0	3	0	0
Direct hospital effluent (H2)	67	41	40	26	15	12
Aquaculture farm 3 (F3 – 800 m)	31	27	14	8	6	6
Direct hospital effluent (H3)	28	12	0	8	0	0
Aquaculture farm (F4 – 2 Km)	11	0	0	1	0	0
Aquaculture farm (F5 – 3.5 Km)	7	0	0	1	0	0

Molecular characterization of ESBL-encoding gene

For a reliable epidemiological investigation of AMR, molecular detection, and identification of resistance-encoding genes would be necessary. The distribution of Enterobacteriaceae isolates with ESBL production with resistance-encoding genes is illustrated in Table 4.

For ESBL resistance-encoding genes, $bla_{\text{CTX-M}}$ was found to be more prevalent ($n = 38$) followed by bla_{TEM} ($n = 21$) and bla_{SHV} ($n = 18$). The plasmid-mediated cephalosporin resistance coding genes of the bla_{SHV} group were detected in 18 *E. coli* isolates in combination with the $bla_{\text{CTX-M}}$ resistance gene (Figure 3(a)). Two SHV genotypes such as $bla_{\text{SHV-1}}$ ($n = 11$) and $bla_{\text{SHV-61}}$ ($n = 7$) were found among *E. coli* isolates in the H2 site (Figure 3(c)). It is noteworthy that the number of beta-lactamase genes in *E. coli* was clearly associated with the frequency of the ESBL producer at each sampling site, specifically at the H2 site. The 'Hospital Size' has been observed as an important factor where bigger hospitals contributed greater bacterial loads to their surroundings relative to smaller hospitals (Lamba *et al.* 2017). A similar observation was made in the present study as well, with a higher bacterial load as well as a greater number of resistant isolates being observed in the discharge sites of bigger hospitals. More number of highly resistant isolates were found in H2, the hospital with the largest number of beds selected in the present study, as compared to H1. The least number of resistant isolates were found in H3, the smaller hospital in the study with only 250-bed capacity. In hospital effluent samples, for $bla_{\text{CTX-M}}$ positive isolates, $bla_{\text{CTX-M-15}}$ was the predominant genotype ($n = 38$). For bla_{TEM}, only the TEM-95b variant was detected from H1 and H2 *E. coli* isolates (Figure 3(b)). However, bla_{OXA} (Figure 3(d)) and the most numerous and diverse AmpC gene type $bla_{\text{CMY-6}}$ was detected in six and five *E. coli* isolates from the H2 site, respectively. It is interesting to note that in none of the ESBL-positive *K. pneumoniae* isolates, the $bla_{\text{CTX-M}}$, bla_{TEM}, $bla_{\text{OXA,}}$ and bla_{CMY} genes were detected; however, three types of bla_{SHV} genotypes like $bla_{\text{SHV-266}}$ ($n = 7$), $bla_{\text{SHV-211}}$ ($n = 7$) and $bla_{\text{SHV-148}}$ ($n = 5$) were detected in *K. pneumoniae* isolates from direct hospital effluent samples. In an aquaculture farm (F3 site), ESBL-positive *E. coli* isolates with the encoding genes $bla_{\text{CMY-6}}$ ($n = 5$), $bla_{\text{CTX-M-15}}$ ($n = 6$), and $bla_{\text{OXA-48}}$ ($n = 3$) and ESBL-positive *K. pneumoniae* isolates with $bla_{\text{SHV-148}}$ ($n = 6$) were detected.

Among the different genes coding for ESBL production, $bla_{\text{CTX-M}}$ was found to be the most prevalent ESBL-encoding gene in *E. coli* isolates and the prevalence was 32.8%. CTX-M subtype of $bla_{\text{CTX-M-15}}$ has emerged as the most common ESBL-encoding genes among *E. coli* from clinical and aquatic environments in recent years (Hu *et al.* 2013). Because of the worldwide use of cefotaxime and ceftriaxone for urinary tract infections, it is not surprising that CTX-M-type ESBLs are now present in many countries. However, no epidemiological studies have yet been conducted that have correlated cefepime use with CTX-M-type ESBL infection. It is notable that elevated cefepime MICs are typical for isolates of Enterobacteriaceae producing CTX-M-type ESBLs, as seen in our study. Previous research has shown that $bla_{\text{CTX-M-15}}$ is the most commonly distributed genotype linked to significant infections in both public and hospital settings (Canton *et al.* 2012). Recent studies in Africa and Europe have observed a significant increase in Gram-negative bacteria that

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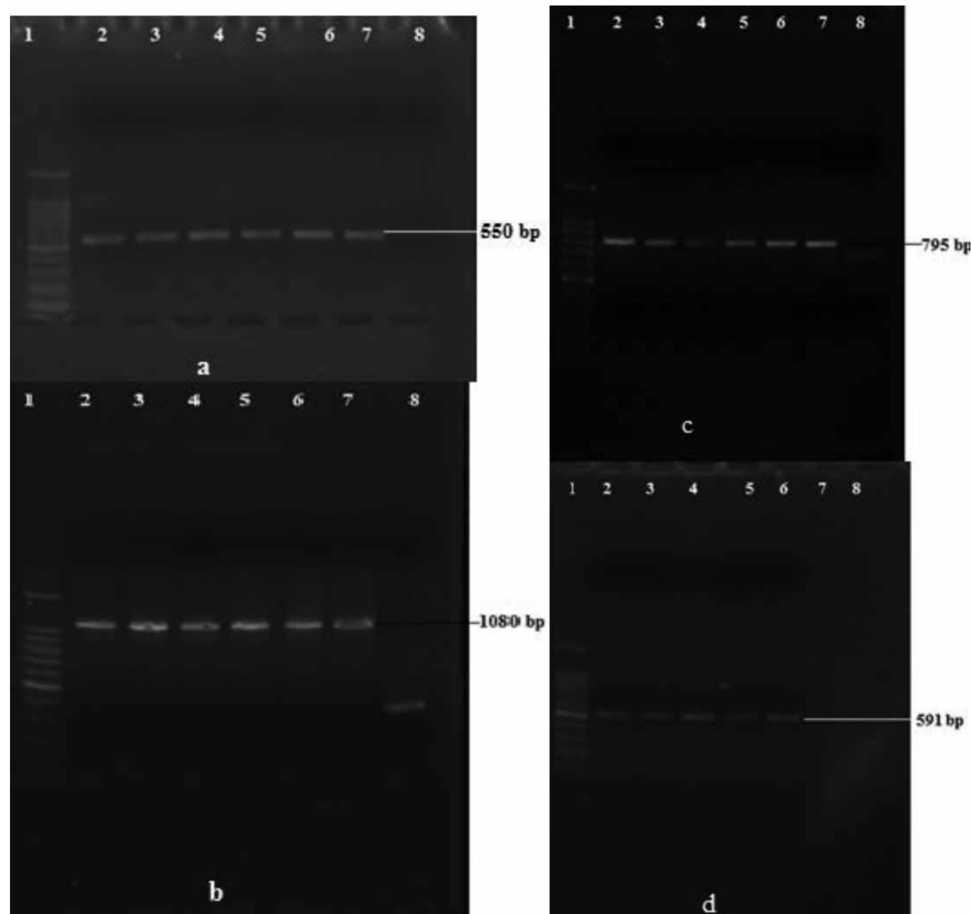

Figure 3 | Amplification of *bla*$_{\text{CTX-M}}$ (a), *bla*$_{\text{TEM}}$ (b), *bla*$_{\text{SHV}}$ (c) and *bla*$_{\text{OXA}}$ (d) gene lane with 1:100 bp molecular weight marker; lanes 2–8 showing samples from Kollam (H2).

produce ESBLs, especially those with *bla*$_{\text{CTX-M-15}}$ genes, which cause community urinary tract infections (Ibrahimagic *et al.* 2015).

The second major prevalent group of ESBL-encoding gene observed in our study was *bla*$_{\text{TEM}}$ and its prevalence was 15.6%. Among the variants of TEM gene, *bla*$_{\text{TEM-95b}}$ was detected in *E. coli* isolates with high resistance to ampicillin from direct hospital effluent samples. There are very few reports on the genetic determinants of *E. coli* with *bla*$_{\text{TEM-95b}}$ variant isolated from hospital wastewater. The *bla*$_{\text{TEM-95b}}$ gene renders resistance to beta-lactam antibiotics similar to those isolates with TEM-1-type beta-lactamase. The TEM beta-lactamases are one of the most clinically relevant beta-lactamase families. TEM-1, the first of this group to be discovered, is a broad-spectrum enzyme that hydrolyzes early cephalosporins as well as several penicillins. The gene-encoding *bla*$_{\text{TEM-95b}}$ shows a mutation at nucleotide 635, causing a change in amino acid 145 converting proline to alanine (Brinas *et al.* 2002). The roles played by specific mutations in the action spectra of TEM beta-lactamase have been previously analyzed and described in several studies (Vakulenko *et al.* 1998; Sideraki *et al.* 2001). *E. coli* isolates carrying *bla*$_{\text{TEM-95b}}$ gene render ampicillin resistance phenotype similar to those of other isolates carrying the *bla*$_{\text{TEM-1}}$ gene.

The most prevalent carbapenemase gene found in *E. coli* from hospital discharge points was *bla*$_{\text{OXA-48}}$ and the prevalence was found to be 6.7%. To date, 11 OXA-48-like forms have been reported, the most frequent of which is classical OXA-48 (Hendrickx *et al.* 2020). OXA-48 and its variants are special carbapenemases with low rates of hydrolysis against carbapenems but no intrinsic action toward extended-spectrum cephalosporins. Despite the inability of OXA-48-like carbapenemases to hydrolyze the cephalosporin class of antibiotics, pooled isolates showed high variable resistance to ceftazidime and cefepime, which is likely to represent high levels of ESBL co-production (Stewart *et al.* 2018). After the first OXA-48-like

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carbapenemase report in Turkey, the enzyme-producing bacterial strains have been extensively investigated in nosocomial and community outbreaks in several parts of the world, especially in the Mediterranean region and in European countries (Gulmez *et al.* 2008). The observation made in the present study is supported by the findings of other researchers and suggests that *bla*$_{OXA-48}$ is one of the prevalent groups of carbapenemase in Enterobacteriaceae found in hospital effluents and municipal waters highlighting its widespread distribution (Nasri *et al.* 2017; Parvez & Khan 2018; Ebomah & Okoh 2020). AmpC enzyme production is less common than ESBL production. However, the prevalence of cefoxitin-resistant *E. coli* has increased worldwide in recent years, reflecting the spread of isolates with plasmid-mediated AmpC beta-lactamase. However, in the current study, only five *E. coli* isolates showed the presence of *bla*$_{CMY-6}$ gene, and the prevalence was merely 7.4%. A high prevalence of *bla*$_{CMY-6}$ detected in clinical isolates of *E. coli* and *K. pneumoniae* from Myanmar and northeast Iran suggests its rapid spread and may become an important public health issue (Rizi *et al.* 2020; San *et al.* 2020).

In recent years, SHV has been found in several Enterobacteriaceae groups mainly in *K. pneumoniae* and *E. coli* in different environmental niches. In our study, *E. coli* isolates with *bla*$_{SHV-1}$ and *bla*$_{SHV-61}$ variants and *K. pneumoniae* with *bla*$_{SHV-266}$, *bla*$_{SHV-211}$ and *bla*$_{SHV-148}$ were obtained from hospital effluent discharge points and aquaculture farms also. The prevalence of *E. coli* and *K. pneumoniae* isolates with *bla*$_{SHV}$ was found to be 13.4 and 18.6% respectively can be considered as a proof for the inappropriate waste treatment management system in the hospitals and its spread through water. A high prevalence of *bla*$_{SHV}$ alleles was observed in untreated hospital WWTPs in Australia (Gundogdu *et al.* 2013) and Bangladesh (Yesmin *et al.* 2014). The study detected TEM, SHV, and CTX-M genes in 36.0, 24.2, and 21.1% from Enterobacteriaceae isolates. Marti *et al.* (2013) reported that isolates with SHV genes can transfer to surface water through WWTPs. Studies have shown that SHV types can be significantly reduced together with CTX-M and OXA genes through biological treatments such as activated sludge processing and anaerobic digestion, although not all of them can be effectively eliminated (Yi *et al.* 2015). SHV-producing Enterobacteriaceae isolates found in public water bodies and aquaculture farms can be considered the proof for the inappropriate waste treatment management system in hospitals.

Bacterial plasmid conjugation

The plasmid-mediated ESBL resistance was successfully transferred from *E. coli* with *bla*$_{CTX-M-15}$ gene to azide-resistant *E. coli* J53 isolate. Plasmid isolated from the trans-conjugants and analyzed by PCR showed the specific amplification of *bla*$_{CTX-M-15}$ gene (550 bp) in the trans-conjugants. All the trans-conjugant strains tested showed resistance to extended-spectrum cephalosporins. They were more resistant to cefotaxime than to ceftazidime and were susceptible to imipenem. MIC of trans-conjugants for cefotaxime and ceftazidime were related to those for each wild-type isolate and showed decreased ciprofloxacin sensitivity (MIC 0.2–1 μg/ml). Antibiotic resistance in pathogenic organisms is a result of prolonged exposure of microbes to low antibiotic concentrations. Additionally, the presence of antibiotic residues in hospital wastewater treatment facilities and hospital effluents increases the selection pressure, which causes common aquatic microbes to develop resistance through several transfer mechanisms. In addition to hospital wastewater, these resistant bacteria also spread in municipal wastewater, urban water, agricultural, and aquaculture systems (Allen *et al.* 2010). The spread of antibiotic resistance plasmids in human pathogens is especially well studied and shows that once resistance genes have become established on successful plasmids, they may rapidly spread across different strains, species, or even genera. These genes are currently found in humans, animals, and the environment (Hartmann *et al.* 2012; Woerther *et al.* 2013). The global spread of several ARGs encoding resistance to beta-lactams, quinolones, aminoglycosides, tetracyclines, sulfonamides, and other drug classes has been attributed to the transfer of plasmids in pathogens, which has resulted in the development of MDR. Studies showed that most of the ESBL-encoding genes are located on mobile plasmids which can promote the transfer of resistance genes between Enterobacteriaceae and nonEnterobacteriaceae groups. More than one beta-lactamase gene is known to be carried in ESBL-positive plasmids. They can, therefore, simultaneously transfer more than one antibiotic resistant gene.

CONCLUSIONS

The findings of this study revealed the presence of MDR isolates with ESBL-encoding genes in direct hospital effluent samples and aquaculture farms, demonstrating the potential for clinical pathogens to spread into aquatic environments from untreated hospital discharges. The large diversity of ARGs harbored in the bacteria found in hospital wastewaters in Kerala clearly

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indicates the risk of the spread of AMR to the bacteria in the aquatic environment. Although only a small fraction of emergent AMR variants may sufficiently fit for broader dissemination, the spread of Gram-negative hospital pathogens with AMR genes can begin as localized expansions, which can rapidly progress to global dissemination from such tropical hotspots. Therefore the presence of mobile genetic elements (MGEs), ARGs, and antibiotic residues in the effluents put human and environmental health at risk. Existing WWTPs should be augmented by novel treatment methods and government policies should adequately address and give priority to implementation of hospital stewardship programmes as well as regulations for effluent discharge into water bodies.

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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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