

# Evaluating the Role of Wastewaters as Reservoirs of Antibiotic-Resistant ESKAPEE Bacteria Using Phenotypic and Molecular Methods

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**Introduction:** Wastewaters carrying thousands of human specimens from the community and representing the diversity of antibiotic-resistant bacteria (ARB) and antibiotic-resistance genes (ARGs) directly from the community mirror the extent of AR spread in the community and environment. This study aimed to investigate the occurrence and distribution of antibiotic-resistant ESKAPEE bacteria in the community versus clinical settings through monitoring nonclinical and clinical wastewaters.

**Methodology:** Seven wastewater samples were collected from different environmental sources. Isolates were obtained on general and selective media, biochemically characterized and antimicrobial-susceptibility tests performed by disk diffusion against 13 antibiotics according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines using MastDisc disk cartridges, and 16S rRNA metagenomic analysis was performed for two water samples.

**Results:** Of 43 isolates, all representatives of the ESKAPEE group were recovered from clinical wastewaters, but Gram-positive cocci were not obtained from nonclinical wastewaters. The most predominant isolate was *Pseudomonas aeruginosa* (n=15; 33%), followed by *Escherichia coli* (n=9; 20%). Complete (100%) resistance to eleven of the tested antibiotics was observed, with only a few isolates being susceptible to clarithromycin, amikacin, and gentamicin. The lowest (79%) resistance rate was observed for linezolid. The multiple antibiotic resistance (MAR) index was calculated, and the resistance phenotype was independent of the wastewater source, indicated by  $\chi^2$  ( $P=0.766$ ). Metagenomic analysis replicated the results, as *Pseudomonas* spp., *Acinetobacter* spp., and *Escherichia* spp. were found to be predominant. The integrase gene (*IntI1*) was also amplified in *Escherichia coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*.

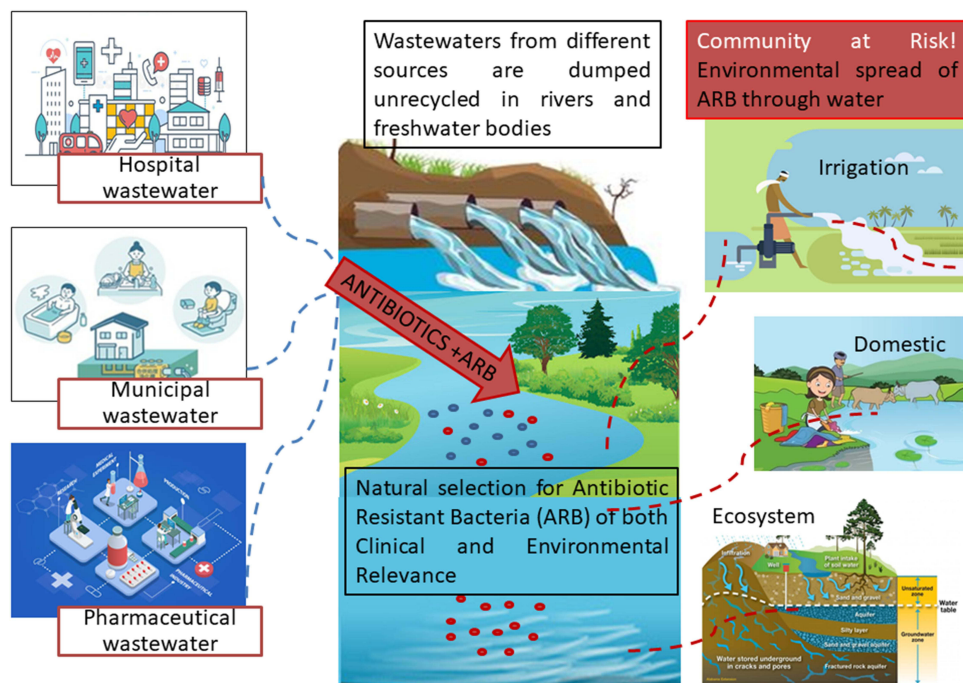
**Conclusion:** Wastewaters are significant carriers of drug-resistant ESKAPEE bacteria and play an important role in their dissemination. This study endorses the periodic surveillance of water systems to evaluate the presence and burden of antibiotic-resistant pathogens.

**Keywords:** resistance profiling, ESKAPEE, wastewater, metagenome, *IntI1*

## Introduction

Antimicrobial resistance has taken the form of a global pandemic in recent years and requires continuous and concerted efforts to mitigate its risks. Antibiotic-resistant bacteria (ARB) especially the multidrug resistant (MDR) ones, are widely spread in the environment.<sup>1</sup> In 2017, a list of AR priority pathogens was published by World Health Organization (WHO), indicating the bacteria that pose a serious threat to mankind and to which new antibiotics are urgently needed. Depending on urgency, these bacteria have been given priority levels as critical, medium, and low.<sup>2-4</sup> Most of these resistant pathogens include Gram-negative bacteria owing to their structure, which renders them more resistant than Gram-positive bacteria.<sup>4</sup> Those at the critical level included *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and Enterobacteriaceae (resistant to carbapenems and third-generation cephalosporins), while vancomycin-resistant enterococci (VRE) and methicillin-resistant *Staphylococcus aureus* were considered high priority ARB.<sup>2</sup> ESKAPEE is an

## Graphical Abstract



acronym for seven bacterial species — *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter* spp., and *Escherichia coli*<sup>5</sup> — that show high levels of antibiotic resistance. Overuse and misuse of antibiotics results in continual increase in antibiotic-resistant bacteria and environmental spread of antibiotic-resistance genes in environmental samples like wastewater of both clinical<sup>6–9</sup> and nonclinical<sup>10–12</sup> origin. Previous studies have shown that hospital wastewater acts as a selective environment for the development of ARB and then as a major contributor to increasing the burden of ARB in natural environments.<sup>8</sup> Antibiotics exert a selection of ARB by killing or inhibiting the growth of susceptible bacteria. The huge amount of antibiotics used in hospitals and private households and their subsequent release into hospital and municipal wastes places a selective pressure on bacteria.<sup>13,14</sup> Antibiotic-resistance genes, via horizontal gene transfer (HGT), are not only present in pathogens but also in environmental bacteria in terrestrial and aquatic environments,<sup>15</sup> and they can be enriched through multiple human activities.<sup>16</sup> Integrons are significant contributors to the acquisition and spread of ARGs, with the class 1 integron-integrase gene being the most important. The dissemination of this gene could be used as a proxy for anthropogenic contamination for a variety of reasons, including their association with antibiotic-resistance genes, their presence in pathogenic and nonpathogenic bacteria, their relative abundances being able to be altered through environmental factors, and playing a significant role in HGT, with the *IntI1* genetic variant being present on diverse mobile genetic elements.<sup>17,18</sup> Initially, class 1 integrons were reported from clinical isolates, but metagenomic and qPCR analyses have revealed their presence in diverse environments.<sup>19</sup>

Studies have shown the occurrence of ARB in foods and animals,<sup>20,21</sup> and the incidence of ARB and antibiotic-resistance genes (ARGs) of clinical origin, ie, the contaminating resistome in the environment, signifies the increasing AR in humans.<sup>22</sup> Some researchers look at the other side of the picture, ie, clinical AR is an endowment by environmental bacteria and ARGs. They propose that ARGs that have been presented by clinical bacteria evolve from natural antibiotic-producing microbes and their competitors in natural habitats.<sup>23,24</sup> Antibiotic resistance in humans is either hospital-acquired or community-acquired, eg, MRSA and VRE MDR strains of *E. coli* and *A. baumannii* are increasingly being detected in community-acquired infections,<sup>22,25</sup> though ARB occur commonly in hospitals, where they cause nosocomial infections.<sup>26,27</sup> Of particular importance are ESKAPEE

bacteria,<sup>28,29</sup> which are largely environmental or commensal, cause opportunistic infections in immunocompromised people, and are not pathogenic otherwise. They have intrinsic resistance to one or more antibiotics and acquired resistance genes for additional antibiotics as well.<sup>30</sup> Community risk for ARB can be effectively measured by analyzing wastewaters carrying thousands of human specimens from the community, which in turn can lead to the detection of new or rare ARB as well.<sup>31</sup> Municipal wastewater-treatment plants are AMR hot spots, as they harbor numerous fecal and environmental bacteria under the influence of subinhibitory concentrations of antimicrobial compounds.<sup>32,33</sup> In one study, a small number of wastewater samples were used to elucidate diversity in all urban runoffs, endorsing the applicability of a small sample as a screening tool for the existence of ARB in the community.<sup>31</sup>

In Pakistan and other developing countries, hospital wastewater is discharged into the drainage system and subsequently to lakes and river without any treatment.<sup>34</sup> Few studies from Pakistan have been published on the role of wastewaters in the environmental spread of antibiotic resistance.<sup>6,35</sup> This study, therefore, aims to investigate the occurrence and distribution of ARB in community versus clinical settings through monitoring nonclinical (municipal, pharmaceutical, and river) and clinical (hospital) wastewater and their ostensible role in the environmental spread of drug resistance via HGT by detection of the *IntI1* integrase gene.

## Methodology

### Sample Collection

Seven wastewater samples from different environmental sources — hospital (3), municipal (2), pharmaceutical (1), and a river (1) — were collected over a period of 3 months. Hospital wastewater samples were collected from postsurgical, intensive care, and emergency wards. Municipal wastewater samples were collected from the drain-inflow site (where the sewer receives collected water) and near the settling tank (where water is stagnant). For each of the samples, 50 mL was collected in sterile plastic containers and transported within 2 hours of collection to the laboratory using a cooler box with ice packs. For metagenomic DNA isolation, 30 mL in triplicates for two water samples was collected.

### Isolation and Biochemical Characterization of Bacterial Strains

All samples were simultaneously inoculated onto general-purpose (ie, nutrient agar) and selective media (ie, MacConkey and eosin–methylene blue (EMB) agar). Each sample was serially diluted up to a dilution factor of  $10^{-5}$ . Plates were analyzed for total bacterial count and colony morphology. Isolates were subjected to Gram-stain, motility, catalase ([Figure S1](#)), and oxidase tests for preliminary identification to differentiate between members of *Enterobacteriaceae*, *P. aeruginosa*, and *A. baumannii*. Gram-negative isolates were characterized using triple-sugar iron (TSI) and indole, methyl red, Voges–Proskauer, and citrate (IMViC) tests, while *S. aureus* was characterized by selective growth on mannitol salt agar (MSA) ([Figure S2](#)).

### Antibiotic-Susceptibility Testing

Antibiotic-susceptibility testing (AST) was done for each of the bacterial isolates by disk diffusion ([Figure S3](#)). Cultures 24 hours old were transferred to nutrient broth (4 mL for each isolate) for overnight incubation at 37°C. Freshly grown cultures were standardized to turbidity of 0.5 McFarland standard. Bacterial lawns were prepared on Mueller–Hinton Agar, and antibiotic disks were placed aseptically onto the prepared plates. AST was performed against the following antibiotic disks (MastDiscs, Mast Group, Bootle, UK): Piperacillin (PRL, 30 µg), piperacillin–tazobactam (PTZ, 30/6 µg), cefuroxime (CXM, 5 µg), meropenem (MEM, 10 µg), ciprofloxacin (C, 5 µg), levofloxacin (LEV, 1 µg), amikacin (AMK, 30 µg), gentamicin (GM, 10 µg), vancomycin (VA, 5 µg), clarithromycin (CLA, 2 µg), azithromycin (ATH, 15 µg), clindamycin (CD 2 µg), and linezolid (LZD, 10 µg). *E. coli* ATCC 25922 and *K. pneumoniae* ATCC700603 were used as quality controls for disk diffusion testing. These strains were found to be susceptible to all antibiotics used. Diameters of zones of inhibition were measured after 24 hours of incubation and results interpreted according to EUCAST guidelines 2020.<sup>36</sup> MDR strains were defined as resistant to one antibiotic from three or more antibiotic classes,<sup>37</sup> while XDR (extremely drug-resistant) strains were those that were found to be susceptible to one or two classes of antibiotics.<sup>38</sup> The multidrug-resistance index (MARI) was calculated as the ratio of the number of antibiotics to which the bacterial isolate was resistant to the total number of antibiotics included in susceptibility testing ([Table S1](#)).

## Molecular Detection of *IntI1* Gene

Bacterial strains were subjected to molecular detection of the *IntI1* gene using *IntI1*-F TCTCGGGTAACATCAAGG and *IntI1*-R AGGAGATCCGAAGACCTC primers.<sup>39</sup> For this purpose, DNA was isolated by boiling lysis with suspending loopful of overnight-grown pure bacterial culture in 300  $\mu$ L sterile d.H<sub>2</sub>O and heating the Eppendorf tubes at 95°C for 10 minutes with intermittent vortexing. Tubes were centrifuged at 14,000 rpm for 1 minute to settle cell debris, and supernatant was shifted to a sterile tube. For PCR amplification, initial denaturation was done at 95°C for 5 minutes and subsequent final denaturation at 95°C for 45 seconds. Annealing was done at 55°C for 60 seconds. Initial extension was carried out at 72°C for 60 seconds and final extension done at 72°C for 10 minutes. The total number of cycles was 30. The amplified product was purified by using a GeneJET PCR purification kit (K0701). The purified product was sent for sequencing by Sanger dideoxy method to 1st Base Laboratories, Singapore. Sequencing was carried out using a BigDye Terminator version 3.1 cycle-sequencing kit using an Applied Biosystems thermocycler 9600. Sequenced files were analyzed using Chromas version 2.6.6 and sequences were submitted to the NCBI GenBank after similarity searches using the NCBI BLASTn database.

## Metagenomic Analysis

Two of the water samples (ie, hospital wastewater and river water) were subjected to 16S ribosomal RNA metagenomic sequencing through the Illumina Miseq platform. A paired-end method was used to construct a fragment library for paired-end sequencing. The 16S V3–V4 region was amplified using the primer pair 5'ACTCCTACGGGAGGCAGCAG3' 5'GGACTACNNGGTATCTAAT3'.<sup>40</sup> Raw paired-end reads (FASTQ) from the original DNA fragments were imported to Qiime2 version 2021.4 software.<sup>41</sup> Paired-end reads for all samples were imported using the manifest file method. Quality filtering, denoising, and chimeric sequence removal were done using the DADA2 denoising method.<sup>42</sup> To get constant read length for all reads, read truncation was used. A FASTA file with the sequences to use as reference with QIIME2 data-type feature data (sequence) was used. SILVA (<https://www.arb-silva.de/download/archive/qiime>) for 16S rRNA was used as a reference database. Naïve Bayes classifier and the q2-feature-classifier plugin<sup>43</sup> were used to assign likely taxonomies to each read. This classifier was trained on the 97%-similarity OTU sequences from the reference database.

## Statistical Analysis

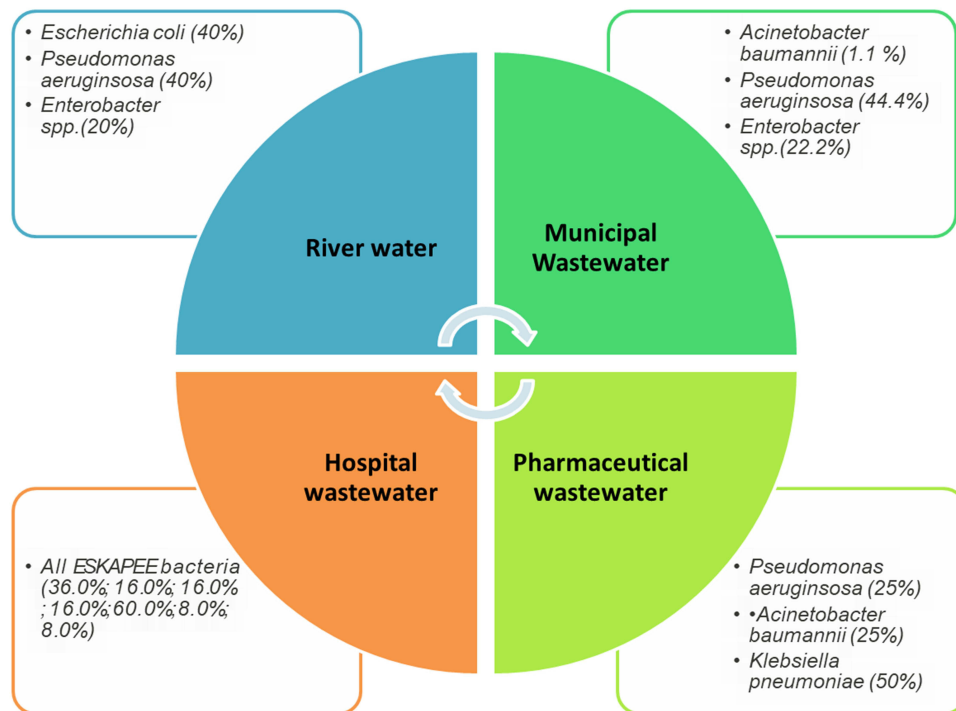
Correlation of resistance status to the isolation source of the isolates was evaluated by  $\chi^2$  tests (Table S2 and S3), and percentage relative abundance of operational taxonomic units (OTUs) assigned to ESKAPEE genera (Table S4) was calculated using Microsoft Excel 2010.

## Results

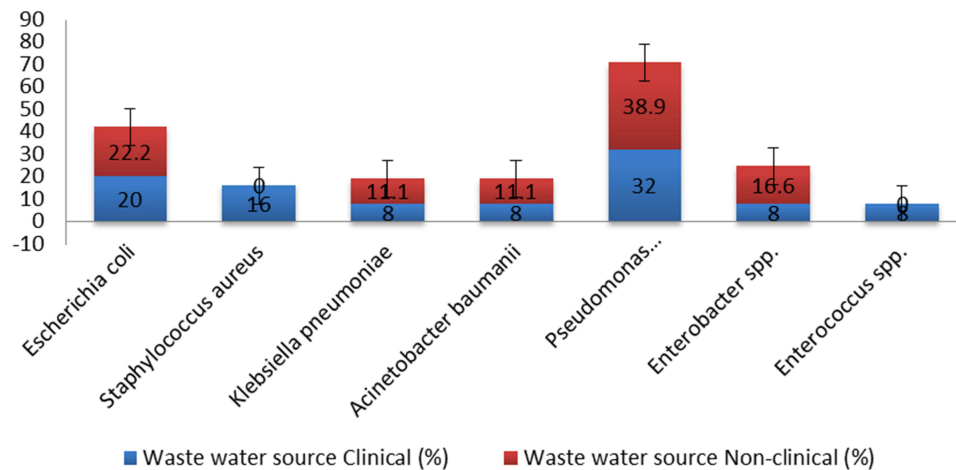
Seven wastewater samples from clinical and nonclinical sources were analyzed for the presence of drug-resistant isolates. A total of 92 isolates were randomly selected based on differences in colony morphology, of which 43 (clinical n=25; nonclinical n=18) were identified as ESKAPEE bacteria based on phenotypic and biochemical characteristics (Table S5). The distribution of bacteria among the wastewater samples showed *Escherichia coli* and *Pseudomonas aeruginosa* to be the most prominent isolates from hospital effluent, while *Pseudomonas aeruginosa* and *Enterobacter* spp. remained predominant in municipal wastewater. Pharmaceutical wastewater was the source of *Klebsiella pneumoniae* primarily. Gram-positive cocci were exclusively obtained from hospital wastewater, among which four isolates were identified as *Staphylococcus aureus* and two as enterococci. River water primarily contained *Pseudomonas aeruginosa* and *Escherichia coli* among the ESKAPEE bacteria (Figure 1). For each of the ESKAPEE isolates, there was no significant difference in numbers from clinical and nonclinical wastewaters, except for *S. aureus* and *Enterococcus* spp., which were recovered from clinical wastewater. Interestingly, all other bacteria were found to be more in number in nonclinical wastewaters (Figure 2).

## Phenotypic Antibiotic-Susceptibility Testing

Susceptibility testing of the 43 isolated bacteria against different antibiotic classes exhibited widespread drug resistance, irrespective of wastewater source (Figure 3). Resistance patterns and MARI showed that most (n=30) isolates were XDRs, with being 100% resistance to all antibiotic categories tested against them. The rest (n=13) showed variable but



**Figure 1** Distribution of ESKAPEE bacteria among wastewater samples according to the present study. The number of each prominent ESKAPEE isolate in a given sample is shown in percentage. For hospital wastewater that contained all ESKAPEE pathogens, the percentages shown are in the same order as that of the acronym.

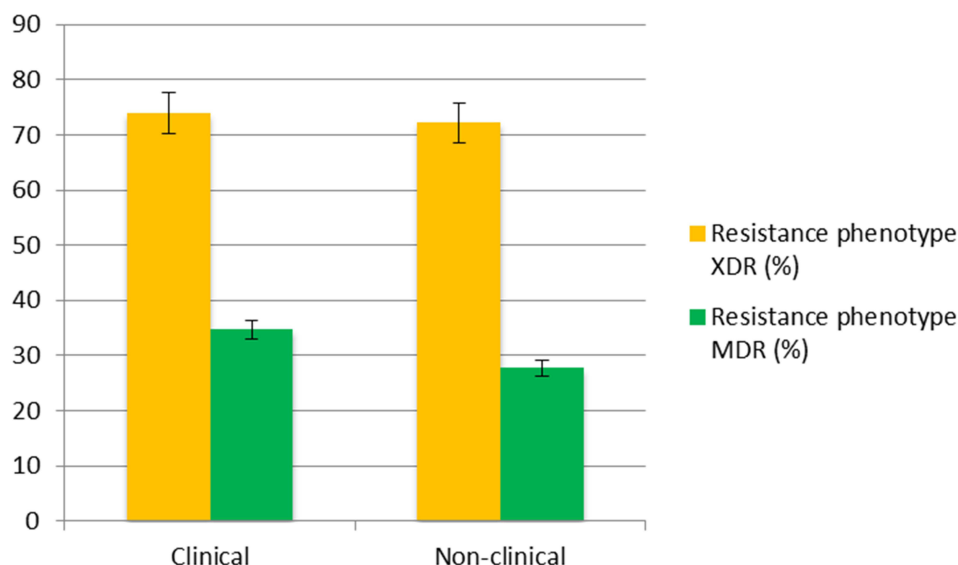


**Figure 2** Percentage of ESKAPEE bacteria in wastewater sources. Gram-negative bacteria were predominant in both clinical and nonclinical wastewater, while Gram positive species were recovered exclusively from clinical wastewater.

large MARI (ranging from 0.7 to 0.9) indicating their MDR status (Table 1). Individual resistance phenotypes for each isolate indicated more XDR from both clinical and nonclinical wastewater sources (Table 2 and Figure 4). MDR was observed for four bacterial species: *E. coli*, *K. pneumoniae*, *A. baumannii*, and *P. aeruginosa*. The rest of the bacterial species were found to be XDR (Table S6).

## Metagenomics

Metagenomic analysis of samples revealed the presence of prominent members of the ESKAPEE group: *Pseudomonas*, *Acinetobacter*, and *Escherichia* spp. Their relative abundances somehow replicated the percentages of these isolates obtained via culturing. Just like phenotypic isolation, genotyping indicated *Pseudomonas* and *Escherichia* spp. as the



**Figure 3** Percentage of drug-resistant isolates from clinical and nonclinical wastewater. Extensive resistance to the 13 drugs used in this study was observed, irrespective of wastewater source, indicating the selection of XDRs in the natural environment.

most abundant in river water, while all *Pseudomonas*, *Acinetobacter*, and *Escherichia* spp. in hospital wastewater (Figure 5). Raw sequence reads have successfully been published with accession PRJNA797209 and SAMN24969811 and SAMN24969812 in SRA and Biosample NCBI databases, respectively.

## Detection of *IntI1* Gene

Nearly 42% (18 of 43) of the isolates were found to be positive for *IntI1*, among which a majority were *E. coli*, *K. pneumoniae*, and *P. aeruginosa* (Figure 6). Sequences for *IntI1* integrase have been submitted to the NCBI GenBank and assigned accession numbers ON230319 and ON230320.

**Table 1** Antibiotic-resistance profiles of ESKAPEE bacterial isolates. Resistance was found to be 100% in most of the ESKAPEE bacteria. MAR index of 1 for most of the isolates validated the results by indicating that most were resistant to all the antibiotics included in AST

Isolates	Resistance pattern	Resistance; n (%)	MARI
H1A	PRL-PTZ-CXM-MEM-C-LEV-AMK-GM-VA-CLA-ATH-CD-LZD	13 (100)	1
H1B	PRL-PTZ-CXM-MEM-C-LEV-AMK-GM-VA-CLA-ATH-CD-LZD	13 (100)	1
H1C	PRL-PTZ-CXM-MEM-C-LEV-AMK-GM-VA-CLA-ATH-CD-LZD	13 (100)	1
H1D	PRL-PTZ-CXM-MEM-C-LEV-AMK-GM-VA-CLA-ATH-CD-LZD	13 (100)	1
H1E	PRL-PTZ-CXM-MEM-C-LEV-AMK-GM-VA-CLA-ATH-CD-LZD	13 (100)	1
H2A	PRL-PTZ-CXM-MEM-C-LEV-AMK-GM-VA-CLA-ATH-CD-LZD-NOR	*14 (100)	1
H2B	PRL-PTZ-CXM-MEM-C-LEV-AMK-GM-VA-CLA-ATH-CD-LZD-NOR	14 (100)	1
H2C	PRL-PTZ-CXM-MEM-C-LEV-AMK-GM-VA-CLA-ATH-CD-LZD-NOR	14 (100)	1
H2D	PRL-PTZ-CXM-MEM-C-LEV-AMK-GM-VA-CLA-ATH-CD-LZD-NOR	14 (100)	1
H2E	PRL-PTZ-CXM-MEM-C-LEV-AMK-GM-VA-CLA-ATH-CD-LZD-NOR	14 (100)	1
H2F	PRL-PTZ-CXM-MEM-C-LEV-AMK-GM-VA-CLA-ATH-CD-LZD-NOR	14 (100)	1

(Continued)

Table 1 (Continued).

Isolates	Resistance pattern	Resistance; n (%)	MARI
H2G	PRL-PTZ-CXM-MEM-C-LEV-AMK-GM-VA-CLA-ATH-CD-LZD-NOR	14 (100)	1
H2H	PRL-PTZ-CXM-MEM-C-LEV-VA-ATH-CD-LZD	10 (71.4%)	0.71
H2I	PRL-PTZ-CXM-MEM-C-LEV-AMK-GM-VA-CLA-ATH-CD-LZD-NOR	13 (92.8)	0.9
H2J	PRL-PTZ-CXM-MEM-C-LEV-AMK-GM-VA-CLA-ATH-CD-LZD-NOR	13 (92.8)	0.9
H2K	PRL-PTZ-CXM-MEM-C-LEV-VA-ATH-CD-LZD	10 (71.4)	0.71
H3A	PRL-PTZ-CXM-MEM-C-LEV-CLA-ATH-AMK-GM-VA-CD	12 (92.3)	0.9
H3B	PRL-PTZ-CXM-MEM-C-LEV-AMK-GM-VA-CLA-ATH-CD-LZD	13 (100)	1
H3C	PRL-PTZ-CXM-MEM-C-LEV-VA-CLA-CD-LZD	11 (84.6)	0.8
H3D	PRL-PTZ-CXM-MEM-C-LEV-AMK-GM-VA-CLA-ATH-CD	12 (92.3)	0.9
H3E	PRL-PTZ-CXM-MEM-C-LEV-AMK-GM-VA-CLA-ATH-CD	12 (92.3)	0.9
H3F	PRL-PTZ-CXM-MEM-C-LEV-VA-CLA-CD-LZD	13 (100)	1
H3G	PRL-PTZ-CXM-MEM-C-LEV-AMK-GM-VA-CLA-ATH-CD-LZD	13 (100)	1
H3H	PRL-PTZ-CXM-MEM-C-LEV-AMK-GM-VA-CLA-ATH-CD-LZD	13 (100)	1
H3I	PRL-PTZ-CXM-MEM-C-LEV-AMK-GM-VA-CLA-ATH-CD-LZD	13 (100)	1
M1A	PRL-PTZ-CXM-MEM-C-LEV-AMK-GM-VA-CLA-ATH-CD-LZD	13 (100)	1
M1B	PRL-PTZ-CXM-MEM-C-LEV-AMK-GM-VA-CLA-ATH-CD-LZD	13 (100)	1
M1C	PRL-PTZ-CXM-MEM-C-LEV-AMK-GM-VA-CLA-ATH-CD-LZD	13 (100)	1
M2A	PRL-PTZ-CXM-MEM-C-LEV-AMK-GM-VA-CLA-ATH-CD-LZD	13 (100)	1
M2B	PRL-PTZ-CXM-MEM-C-LEV-AMK-GM-VA-CLA-ATH-CD	12 (92.3)	0.9
M2C	PRL-PTZ-CXM-MEM-C-LEV-GM-VA-CLA-ATH-CD	12 (92.3)	0.9
M2D	PRL-PTZ-CXM-MEM-C-LEV-AMK-GM-VA-CLA-ATH-CD-LZD	13 (100)	1
M2E	PRL-PTZ-CXM-MEM-C-LEV-AMK-GM-VA-CLA-ATH-CD-LZD	13 (100)	1
M2F	PRL-PTZ-CXM-MEM-C-LEV-AMK-GM-VA-CLA-ATH-CD-LZD	13 (100)	1
P1A	PRL-PTZ-CXM-MEM-C-LEV-AMK-GM-VA-CLA-ATH-CD-LZD	13 (100)	1
P1B	PRL-PTZ-CXM-MEM-C-LEV-AMK-GM-VA-CLA-ATH-CD-LZD	13 (100)	1
P1C	PRL-PTZ-CXM-MEM-C-LEV-AMK-GM-VA-CLA-ATH-CD-LZD	13 (100)	1
P1D	PRL-PTZ-CXM-MEM-C-LEV-AMK-GM-VA-CLA-ATH-CD-LZD	13 (100)	1
R1A	PRL-PTZ-CXM-MEM-C-LEV-VA-CLA-ATH-CD	10 (76.9)	0.76
R1B	PRL-PTZ-CXM-MEM-C-LEV-AMK-GM-VA-CLA-ATH-CD-LZD	12 (92.3)	0.9
R1C	PRL-PTZ-CXM-MEM-C-LEV-AMK-GM-VA-CLA-ATH-CD-LZD	13 (100)	1
R1D	PRL-PTZ-CXM-MEM-C-LEV-VA-CLA-ATH-CD	10 (76.9)	0.76
R1E	PRL-PTZ-CXM-MEM-C-LEV-AMK-GM-VA-CLA-ATH-CD-LZD	13 (100)	1

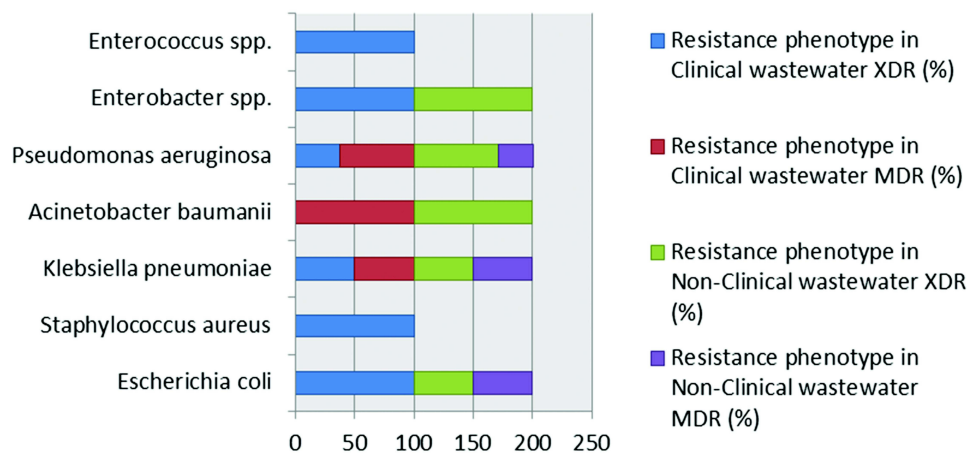
**Abbreviations:** \*PRL, piperacillin; PTZ, piperacillin-tazobactam; CXM, cefuroxime; MEM, meropenem; C, ciprofloxacin; LEV, levofloxacin; AMK, amikacin; GM, gentamicin; VA, vancomycin; CLA, clarithromycin; ATH, azithromycin; CD, clindamycin.

**Table 2** Antibiogram of the isolated bacterial species from wastewater sources. Species-wise resistance rate against 13 antibiotics showed only a few isolates

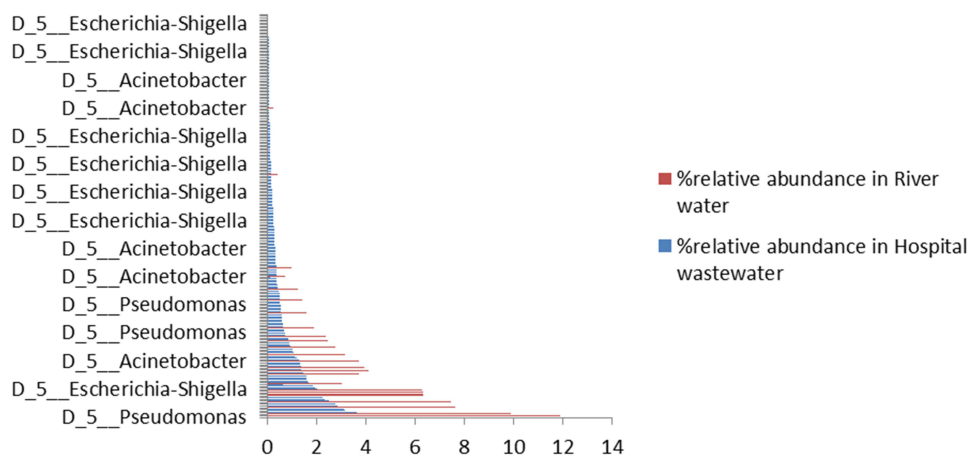
	Antibiotic Resistance No. (%)												
	Piperacillin (PRL)	Piperacillin-tazobactam (PTZ)	Cefuroxime (CXM)	Meropenem (MEM)	Clarithromycin (CLA)	Azithromycin (ATH)	Clindamycin (CD)	Ciprofloxacin (C)	Levofloxacin (LEV)	Amikacin (AK)	Gentamicin (GM)	Vancomycin (VA)	Linezolid (LZD)
<i>Escherichia coli</i>	9 (100)	9 (100)	9 (100)	9 (100)	9 (100)	9 (100)	9 (100)	9 (100)	9 (100)	9 (100)	9 (100)	9 (100)	7 (77.7)
<i>Staphylococcus aureus</i>	4 (100)	4 (100)	4 (100)	4 (100)	4 (100)	4 (100)	4 (100)	4 (100)	4 (100)	4 (100)	4 (100)	4 (100)	2 (50)
<i>Klebsiella pneumoniae</i>	4 (100)	4 (100)	4 (100)	4 (100)	4 (100)	4 (100)	4 (100)	4 (100)	4 (100)	3 (75)	3 (75)	4 (100)	4 (100)
<i>Acinetobacter baumannii</i>	4 (100)	4 (100)	4 (100)	4 (100)	3 (75)	4 (100)	4 (100)	4 (100)	4 (100)	3 (75)	3 (75)	4 (100)	3 (75)
<i>Pseudomonas aeruginosa</i>	15 (100)	15 (100)	15 (100)	15 (100)	14 (93.3)	15 (100)	15 (100)	15 (100)	15 (100)	13 (86.6)	12 (80)	15 (100)	13 (86.6)
<i>Enterobacter spp.</i>	5 (100)	5 (100)	5 (100)	5 (100)	5 (100)	5 (100)	5 (100)	5 (100)	5 (100)	4 (80)	4 (80)	5 (100)	4 (80)
<i>Enterococcus spp.</i>	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	1 (50)
Total (n=43)	43 (100)	43 (100)	43 (100)	43 (100)	41 (95.3)	43 (100)	43 (100)	43 (100)	43 (100)	38 (88.3)	37 (86)	43 (100)	34 (79)

**Notes:** ESKAPEE bacteria were susceptible to just clarithromycin, amikacin and gentamicin. The highest susceptibility observed was to linezolid.





**Figure 4** Resistance phenotypes of resistant bacteria from clinical and nonclinical wastewaters. Four of seven bacterial species showed multiple drug resistance in addition to extensive drug resistance (red and purple areas), while strains of Gram positive cocci and *Enterobacter* spp. were found to be extensively drug-resistant (blue and green areas).



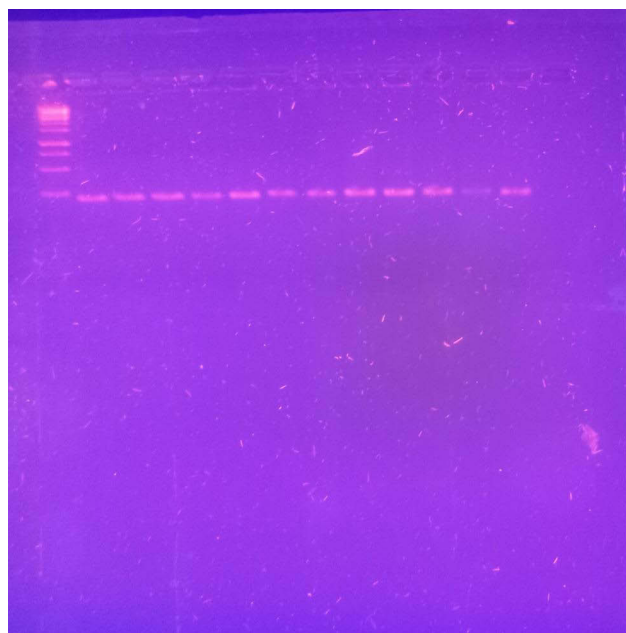
**Figure 5** Relative abundance of three major genera of ESKAPEE bacteria in water samples. Relative abundance was calculated based on operational taxonomic units (OTUs) assigned to the given genera indicating *Pseudomonas* spp. as the most abundant genus.

## Statistical Analysis

Using  $\chi^2$  tests, drug-resistance status and wastewater source were found to be independent variables, ie, resistant strains were widespread throughout the water systems ( $P=0.766$ ).

## Discussion

In recent years, one of the most important pandemics is antimicrobial resistance in general and bacterial antibiotic resistance in particular after the emergence of MDR and XDR strains. In the present study, antibiotic-resistance patterns for ESKAPEE bacteria were analyzed, as the WHO has enlisted most of them as “priority level 1: critical group” bacteria. Wastewater is the most important vehicle for the environmental dissemination of drug-resistant bacteria and antibiotic-resistance genes (ARGs). Bacterial isolates from different sources were obtained and ESKAPEE bacteria identified by biochemical profiling. Of 92 isolates, 43 (46.7%) from wastewater sources belonged to the ESKAPEE group pointing to their spread in the environment. Of these 43 isolates, 25 (58.1%) were recovered from clinical wastewater sources and 18 (41.8%) from nonclinical wastewater. With respect to the total bacterial count, no significant difference was found among the samples. Such results are due to the fact that not only hospital wastewaters have high concentrations of antibiotics and disinfectants but also municipal, pharmaceutical, and industrial effluents carry heavy loads of antimicrobial compounds that allow resistant bacteria to outnumber nonresistant phenotypes in such environments.



**Figure 6** PCR amplification with *Int1* primers. Positive amplification was observed for three isolates of *E. coli*, *K. pneumoniae*, and *P. aeruginosa*. Lane 1, 1 kb DNA ladder (Thermo Fisher Scientific); lanes 2–5, PCR amplification with *E. coli* strains (H1C, H2D, M2B, R1B); lanes 6–8, PCR amplification with *K. pneumoniae* strains (H1E, H3C, P1D); lanes 9–10, PCR amplification with *P. aeruginosa* strains (H2A, H3B, R1A); lanes 11–12, PCR amplification with *A. baumannii* strains (H2K, M2D).

Widespread resistance to multiple antibiotics was also reported in a study from Bangladesh that compared the rate of resistance among ESKAPEE bacteria from clinical and nonclinical sources.<sup>9</sup>

The most frequent isolate was *Pseudomonas aeruginosa* (n=15) in both clinical and nonclinical effluent, followed by *Escherichia coli* (n=9). This frequency can be attributed to the extraordinary potential of *Pseudomonas aeruginosa* and *E. coli* in biofilm formation in sewerage systems, as many studies have correlated the extent of biofilm formation and drug resistance in isolates from wastewaters.<sup>44,45</sup> Several studies have reported these Gram-negative rods as predominant drug-resistant species in hospital runoffs in addition to *Klebsiella* and *Enterobacter* spp.<sup>46–48</sup> All of the seven ESKAPEE representatives were recovered from hospital wastewater, but *S. aureus* and enterococci were not obtained from nonclinical wastewaters. Somewhat similar results were reported in a study where the percentage of MRSA and VRE in different wastewater samples was found to be lowest compared to Gram-negative bacteria from the ESKAPEE group, but in contrast to the present study, they recovered very low methicillin-resistant *S. aureus* (MRSA) (37.5%) from hospital effluent compared to municipal wastewater (75%) and no vancomycin-resistant Enterococci (VRE) from hospital water (89.3%).<sup>12</sup>

Pan-drug resistance (PDR) refers to resistance of the isolate to all the compounds in all the categories of antibiotics, while some studies use PDR and XDR synonymously.<sup>38,49,50</sup> However, for the current study, isolates showing 100% resistance to all included drugs were considered XDR to overcome study bias, as AST was performed only for the available classes of antibiotics. Overall, XDR phenotypes were commonly observed irrespective of the wastewater source: 73.9% (clinical) and 72.2% (nonclinical) mainly showing susceptibility toward a few antibiotics, including AMK, GM, and LZD. Resistance to gentamicin was also found to be uncommon (3.5%) in another study carried out on *E. coli* from hospital and community wastewaters in Norway.<sup>31</sup> Percentages of MDR phenotypes in clinical and nonclinical sources were 34.7% and 27.7%, respectively (Figure 3). The results were validated with  $\chi^2$  tests that indicated no statistical significance between the two variables, ie, resistance phenotype and wastewater source (P=0.766). Further MDR strains were observed for *E. coli*, *P. aeruginosa*, *A. baumannii*, and *K. pneumoniae*, while the three other species were almost exclusively found to be XDRs (Figure 4). Several studies have reported MDR Gram-negative bacteria from environmental sources where their resistance pattern was established using disk diffusion according to either EUCAST<sup>51,52</sup> or Clinical and Laboratory Standards Institute (CLSI) guidelines.<sup>53–56</sup> The high incidence of extensively

drug-resistant strains in both clinical and nonclinical wastewaters may be attributed to the constant selective pressure placed on the bacteria not only by the excessive use of antibiotics but also by other antimicrobial agents that are released from hospitals and other urban runoffs, eg, industrial and agricultural effluent. In Pakistan, the situation is worsened by the collection of hospital effluent into main drains, eg, wastewater from Jinnah Hospital is dumped into the Sattukatla drain, while the Shadman drain receives effluent from the Services Institute of Medical Sciences (SIMS) and Mayo Hospital.<sup>6</sup> Such mixing of wastewaters promotes the selection of more resistant strains of diverse bacterial species by putting overwhelming selective pressure on antimicrobials. All these main sewers fall into the river Ravi without any treatment. One substantial contributor to nonclinical wastewater is pharmaceutical effluent,<sup>57</sup> and this may favor the selection of resistant bacteria, among which the fitter ones are XDR strains. All isolates were found to be completely resistant to the following categories of antibiotics:  $\beta$ -lactams, including penicillin (PRL and PTZ), cephalosporin (CXM), and carbapenem (MEM); fluoroquinolones, including ciprofloxacin (C) and levofloxacin (LEV); macrolides, including azithromycin (ATH) and clindamycin (CD); and glycopeptides, including vancomycin (VA). A study conducted on wastewaters in Pakistan also reported high rates of resistance (58%–89%) for all the tested  $\beta$ -tactams, while high MIC values were observed for ciprofloxacin (256  $\mu\text{g}/\text{mL}$ ) and Gram-negative bacteria, including *E. coli* (28%) and *Pseudomonas* spp. (7%),<sup>35</sup> among ESKAPEE members. A few isolates of *P. aeruginosa* and *A. baumannii* showed susceptibility to clarithromycin (macrolide). Most bacterial species showed susceptibility towards aminoglycosides, including amikacin (AK), and gentamicin (GM) and oxazolidinone, including linezolid (LZD) (Table 2). Sensitivity towards aminoglycosides has been reported from isolates of wastewater systems in Ethiopia.<sup>33</sup>

Both of the Gram-positive species were XDR, with some strains being susceptible to just linezolid. Among Gram-negative bacteria, *Enterobacter* spp. from both hospital and nonhospital wastewater were found to be exclusively XDR. Another study reported *Enterobacter* spp. were resistant to all the 12 antibiotics tested against them, while *Klebsiella* and *Pseudomonas* spp. followed, showing resistance to 11 and 10 antibiotics, respectively.<sup>48</sup>

Metagenomic analysis was done to evaluate the extent of spread of ESKAPEE bacteria in freshwater bodies through wastewater contamination by selecting two termini of the wastewater-collection system. Impact of anthropogenic activities on river-water quality and microbiome was reported in a study from Malaysia, where 100% of *E. coli*, *K. pneumoniae*, and *E. cloacae* and nearly 85% of *E. faecalis* were found to be MDR in the river Melayu, indicating the presence of MDR ESKAPEE members in water systems.<sup>55</sup> Persistence of most important opportunistic pathogens that bear both environmental and clinical significance like *Escherichia* spp., *Pseudomonas* spp., and *Acinetobacter* spp., may clearly be attributed to their structural and functional virulence factors<sup>9,57</sup> that lead to their selection through the course of the water-collection system and final dumping into the river. Integrons are classified as classes 1, 2, and 3 based on the integrase gene (*intI*), with class 1 being the first detected and most frequent type in antibiotic-resistant bacteria.<sup>5</sup> They can easily attain many genes to acquire resistance in bacteria. This study showed the widespread drug resistance among ESKAPEE through AST and that they are involved in HGT of drug-resistance genes as indicated by the presence of *IntI* gene, so we can say that these bacteria are important contributors to environmental dissemination of drug resistance. Class 1 integron (*intI*) genes were found to be abundantly present in a wastewater-treatment plant in Texas in addition to the presence of MDR *E. coli*.<sup>54</sup> A positive-feedback loop is established for long-term sustenance of ARB if such contaminated runoffs are used for agricultural, industrial, or domestic purposes, as in third-world countries. Most significantly, the presence of XDRs in both clinical and nonclinical wastewaters indicate a constant pressure that has resulted from indiscriminate use of antibiotics and other antimicrobials and their subsequent release into the environment, paving a way to the development and/or selection of MDR and XDR strains among bacterial species. This study provides a dimension to check the abundance of pathogenic bacteria in water systems, and such studies must be conducted periodically for the environmental surveillance of ARB and ARGs, and the factors that result in their enrichment in any given environment must be evaluated subsequently.

## Data Sharing

Data sets related to the current study can be found in [Tables S1–S6](#) and [Figures S1–S3](#).

## Ethics Approval and Consent to Participate

Not applicable, as the study did not include any human participants, human data, or human tissue.

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## Author Contributions

All authors made a significant contribution to the work reported, whether in the conception, study design, execution, acquisition of data, analysis and interpretation, or all these areas, took part in drafting, revising, or critically reviewing the article, gave final approval to the version to be published, have agreed on the journal to which the article has been submitted, and agree to be accountable for all aspects of the work.

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## Disclosure

The authors declare that they have no conflicts of interest in relation to this work.

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