Antibiotic Resistance Bacteria and Revealing Resistant Genes from Surface Water

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ABSTRACT

Objectives: To detect antibiotic resistance genes (ARG) genes and bacteria from surface water in Kathmandu.

Methods: Bacteria were isolated on MacConkey agar and Eosin Methylene Blue (EMB) agar, further identified by biochemical test. Antibiotic susceptibility tests, and ESBL production was detected following CLSI guidelines. Plasmid was extracted by phenol and chloroform method and *bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{CTX-M}$, *bla*$_{NDM-1}$ and *bla*$_{OXA-48}$ genes were amplified by PCR.

Results: Out of 100 isolates of *Escherichia coli* (60) and *Klebsiella* spp (40), 15 strains were ESBL producers. Of isolated ESBL positive isolates, total isolated strains were sensitive to imipenem and ofloxacin, whereas, from 85 non ESBL producers, all strains were sensitive to imipenem, ofloxacin, cefotaxime, ceftazidime, and ceftriaxone. The most prevalent gene distributed in surface water includes *bla*$_{TEM,}$ followed by *bla*$_{NDM-1}$ and *bla*$_{CTX-M}$ genes. The combination of *bla*$_{CTX-M}$ and *bla*$_{TEM}$ (97.7%) was the most prevalent, followed by *bla*$_{SHV}$ and *bla*$_{TEM}$ (5.4%) genes.

Conclusion: The detection of ESBL positive genes and bacteria in surface water indicates the growing challenge of antibiotic resistance. It requires a comprehensive response involving environmental management, public health strategies, policy changes, and global cooperation.

Keywords: ESBL, bla$_{TEM}$, bla$_{SHV}$, bla$_{CTX-M}$, bla$_{NDM-1}$ and bla$_{OXA-48}$ genes.

INTRODUCTION

Isolating antibiotic-resistant genes (ARGs) from environmental samples (Schwartz et al., 2003) like surface water contributes to understanding antibiotic resistance's prevalence, diversity, and distribution in environmental bacteria (Larsson and Flach, 2022). ARGs in surface water can indicate environmental contamination with antibiotic-resistant bacteria, and high levels of ARGs may reflect the presence of antibiotics or other pollutants in the water (Amarasiri et al., 2022). Antibiotic resistance in environmental bacteria can affect ecological balances within aquatic ecosystems (Larsson and Flach, 2022, Polianciuc et al., 2020). It may alter microbial communities and potentially impact the health of aquatic organisms (Singh et al., 2022). Surface water containing ARGs can be a reservoir of resistance genes for antibiotics that may transfer to human pathogens via the gene transfer route (Singh et al., 2022, Polianciuc et al., 2020, Dunning Hotopp, 2011). These genes can spread via waterborne routes and contribute to human infections that

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that are difficult to treat with antibiotics (Singh et al., 2022, Schwartz et al., 2003, Majumder et al., 2020).

Various ARGs in surface water indicate the broad range of resistance mechanisms developed by bacteria (Singh et al., 2022). Different classes of antibiotics have specific resistance genes associated with them, and these genes can be found in surface water due to various sources and environmental factors (Koch et al., 2021). The most common types of β-lactam resistance genes include *bla*$_{CTX-M}$, *bla*$_{SHV}$, *bla*$_{TEM}$, *tet*(A), *tet*(B), *tet*(C), *aac*(6')-Ib, *erm*(B), *qnr*, *sul*1, *sul*2, *sul*3, *cat*A1,*cat*B2, *van*A, *van*B, etc (Amarasiri et al., 2022, Larsson and Flach, 2022). At present, more than 500 different ESBL types have been reported and major being TEM (Temoniera) and SHV (sulphydryl variable) and CTX-M (CefoTaxime-Munich) type (Rawat and Nair, 2010). Carbapenems are the treatment of choice for serious infections due to ESBL producing organisms, yet carbapenem-resistant isolates have been reported (Rawat and Nair, 2010). The most common type of carbapenem resistant genes includes KPC (*Klebsiella pneumoniae* carbapenemase), NDM (New Delhi Metallo-β-lactamase), OXA-48, VIM (Verona Integron-encoded Metallo-β-lactamase) and IMP (Imipenemase Metallo-β-lactamase) (Hammoudi Halat and Ayoub Moubareck, 2020). The NDM-1 gene was first identified in a *K. pneumoniae* strain isolated from a patient who was hospitalized in New Delhi, India, in 2008 (Hammoudi Halat and Ayoub Moubareck, 2020). Since then, it has been found in a variety of bacterial species and has spread globally. Originally identified in Turkey, OXA-48-like enzymes do not have a high level of carbapenemase activity but are still effective in conferring resistance to carbapenems, especially in combination with other resistance mechanisms (Poirel et al., 2012).

Surface water often contains diverse microbial communities, facilitating the spread of resistance genes across various types of bacteria. The transfer of ARGs in surface water is a significant concern because it can lead to the emergence and spread of multiple drug resistant (MDR) bacteria, which are more challenging to treat and pose significant risks to public health. This phenomenon underscores the need for careful management of antibiotics and pollutants in the environment (Larsson and Flach, 2022). Low-income countries like Nepal are vulnerable to the antibiotic resistance problem because of limited surveillance, less controlled use of antibiotics in humans and animals, unplanned urbanization, and contamination of river water with sewage. Therefore, this study was conducted to understand the prevalence and distribution of some ARGs in environmental bacteria, which is vital for surveillance and management strategies for future study.

METHODS

Sample Collection and Isolation of Bacteria

Samples were collected from 40 different sites starting from *Gokarneshwor* temple to *Taudaha*, relatively 500 m apart by grab sampling method (Diwan et al., 2013) from Kathmandu Valley and stored at 4^{o}C until analyzed. Bacterial identification was carried out at the Central Department of Microbiology, Tribhuvan University by standard microbiological techniques (Cheesbrough, 2006), and isolates were preserved in Tryptic Soya Broth containing 30% glycerol (Howard, 1956). After that, the isolates were transported to the Annapurna Research Center, Kathmandu for further analysis.

Sample Processing and Antibiotic Susceptibility Test

All the isolates were cultured in the MacConkey agar and nutrient agar, and sub-cultured in nutrient broth. Antibiotic susceptibility test was performed by modified Kirby-Bauer disc diffusion method following CLSI guidelines (CLSI, 2014). The antibiotics such as amikacin (30 µg), gentamycin (10 µg), ciprofloxacin (30 µg), ceftriaxone (30 µg), cefotaxime (30 µg), ceftazidime (30 µg), nitrofurantoin (300 µg), norfloxacin (10 µg), nalidixic acid (30 µg) ofloxacin (5 µg), cotrimoxazole (25 µg), cefixime (5 µg), cefepime (30 µg), tigecycline (15 µg), imipenem (10 µg), meropenem (10 µg), polymyxin B (300 µg), and colistin (10 µg) were used. For ESBL testing, *E. coli* (ATCC 25922), *K. pneumoniae* (ATCC 700603) were taken as negative and positive controls, respectively.

Screening and Confirmation of ESBL Production

For the screening of ESBL, 3rd generation cephalosphorins (cefotaxime 30 µg, ceftazidime 30 µg, and ceftriaxone 30 µg) and monobactam (aztreonam 30 µg) were used. The confirmation was done by combined disc test by using ceftazidime and cefotaxime alone and their combination with clavulanic acid (20 µg/10 µg) following CLSI guidelines (CLSI, 2014).

Plasmid Isolation

Plasmids (pDNA) were isolated by using alkaline lysis method as described by Sambrook and Russel (Green and Sambrook, 2012). Briefly, single isolated colony was inoculated into the Luria-Bertini broth and cultured for 18 h. Bacterial pellet was collected by repeated centrifugation (5000 rpm for 5 min). The pellets were dissolved in 250 µL of Tris-ethylene diamine tetra acetic acid (EDTA) buffer (Tris-HCL 1.0 M, pH 8.0; 3 µL of 0.5 M EDTA, pH 8.0; and 40 µL of 10% sodium dodecyl sulfate) and incubated (65°C for 5 min). Following incubation, 750 µL of isopropanol was added, mixed, and centrifuged (14,000 rpm for 10 min at 15°C). The resulting pellets were

re-suspended in 500 µL of TE buffer followed by addition of phenol-chloroform (1:1). The upper phase was transferred to another clean tube and equal volume of chloroform was added. After mixing, the tube was centrifuged (14,000 rpm for 5 min at 15°C). The supernatant was then treated with 40 µL of 5 M sodium acetate (pH 5.2) and 1 mL of absolute ethanol. The DNA pellet was washed with 70% ethanol and suspended in 50 µL of TE buffer. DNA purity was confirmed using a spectrophotometer (260/280).

Amplification of Target Genes

The primers for amplification of *bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{CTX-M}$, *bla*$_{NDM-1}$ and *bla*$_{OXA-48}$ genes were TEM-F 5′-TCCGCTCATGAGACAATAACC-3′ and TEM-R 3′-ATAATACCGCACCACATAGCAG-5′, SHV-F 5′-ACCATGAGCGATAACAGCG-3′, SHV-R 3′-GATTTGCTGATTTCGCTCGG-5′ (Doosti et al., 2015), CTX-M F 5'-TTGCGATGTGCAGTACCAGTAA-3', CTX-M R 3'-CTATTTTGGCCGTCGCCTC-5' (Varkely et al., 2014), NDM-1 F 5'- GGGCAGTCGCTTCCAACGGT-3', NDM-1 R 3'-TACGGCTGTGACTCGTGATG-5' (Manchanda et al., 2011), OXA-48 F 5'-GCTTGATCGCCCTCGATT-3', OXA-48 R 3'-GATTTGCTCCGTGGCCGAAA-5' (Gurung et al., 2020) respectively.

The amplification was carried out of respective genes by adding 2 µL of respective pDNA (2 -100 ng), 0.5 µL of respective forward and reverse primers (10 picomolar, Macrogen, Korea), 13 µL of Master Mix (Macrogen, Korea) and final volume was maintained 25 µL by adding double distilled water. The optimized condition for amplification of genes has been tabulated in Table 1.

The amplified products were visualized by 2% agarose gel electrophoresis with 100 bp DNA ladder (Macrogen, Korea) and photographed (Uvitec Cambridge, UK).

RESULTS

During this study, *E. coli* (60) and *Klebsiella* spp (40) were isolated from 40 different sites in the Bagmati River. The isolates were characterized by morphological observation and biochemical tests. The colony morphology characteristics were examined on 24 h culture on nutrient agar. The isolates were Gram-negative rod, non-spore-forming, catalase-positive, and oxidase-negative.

It was observed that all of the isolated strains of *E. coli* and *Klebsiella* spp were sensitive to imipenem and ofloxacin, cotrimoxazole (Figure 1A and 1B), whereas isolated *E. coli* strains, 1 strain was resistant to ceftriaxone, 2 strains to cefotaxime, ceftazidime and amikacin and 6 strains to aztreonam and ampicillin, and 5 strains to gentamycin. Similarly, 3 strains of *Klebsiella* spp were resistant to ceftriaxone, 12 strains to cefotaxime, 11 to ceftazidime and 4 strains to aztreonam and amikacin, 5 to ampicillin, and 10 to gentamycin. Of 100 isolated bacteria, 5% of *E. coli* and 30% of *Klebsiella* spp were ESBL producers (Figure 2). Of 15 ESBL producers, 100% of the isolated strains were sensitive to imipenem and ofloxacin (Figure 3A and 3B). However, 93.3%, 86.7%, 26.7%, and 26.7% of ESBL producers were resistant to cefotaxime, ceftazidime, ceftriaxone, and aztreonam, respectively. Additionally, among 85 non ESBL producers, all isolated strains were sensitive to imipenem, ofloxacin, cefotaxime, ceftazidime, and ceftriaxone. However, 7.1% of isolates were resistant to aztreonam.

Table 1: Optimized conditions of different genes

Genes	Initial denaturation	Denaturation	Annealing	Extension	Final extension	Cycles
***bla*$_{TEM}$**	94°C for 5 min	95°C for 45 sec	50°C for 45 sec	72°C for 30 sec	72°C for 10 min	30
***bla*$_{SHV}$**	94°C for 5 min	95°C for 45 sec	56°C for 45 sec	72°C for 30 sec	72°C for 10 min	30
***bla*$_{CTX-M}$**	94°C for 5 min	95°C for 45 sec	62°C for 45 sec	72°C for 30 sec	72°C for 10 min	30
***bla*$_{NDM-1}$**	94°C for 3 min	94°C for 30 sec	60°C for 45 sec	72°C for 30 sec	72°C for 3 min	30
***bla*$_{OXA-48}$**	94°C for 10 min	94°C for 40 sec	60°C for 40 sec	72°C for 1 min	72°C for 7 min	30

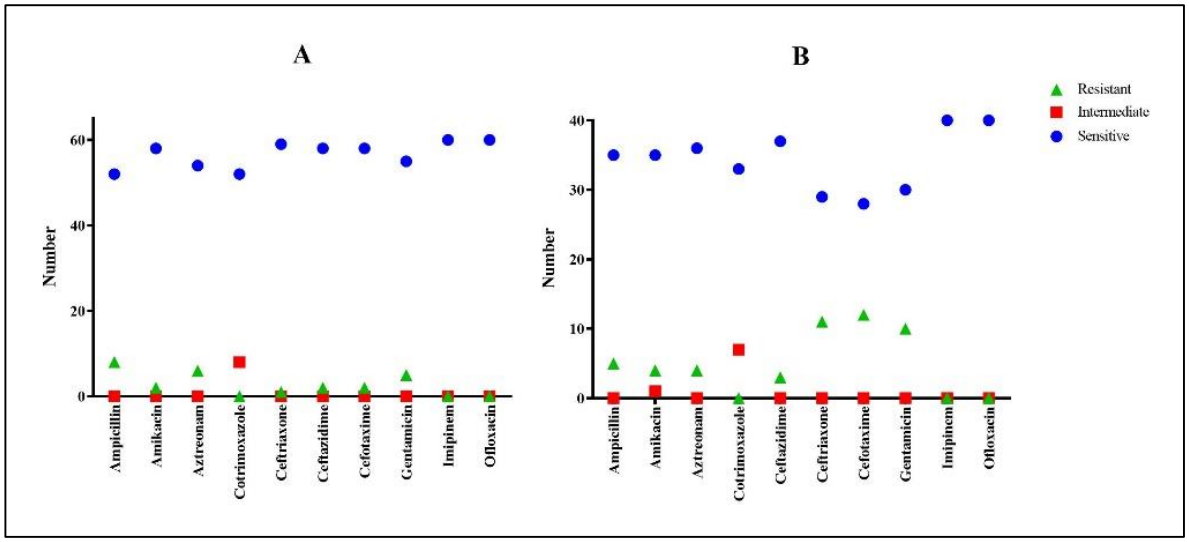

Figure 1: Disk diffusion test with isolates, *E. coli* (A) and *Klebsiella* spp (B) from surface water. The diameters of all zones of inhibition are measured and those values translated to categories of susceptible, intermediate, or resistant following the CLSI guidelines.

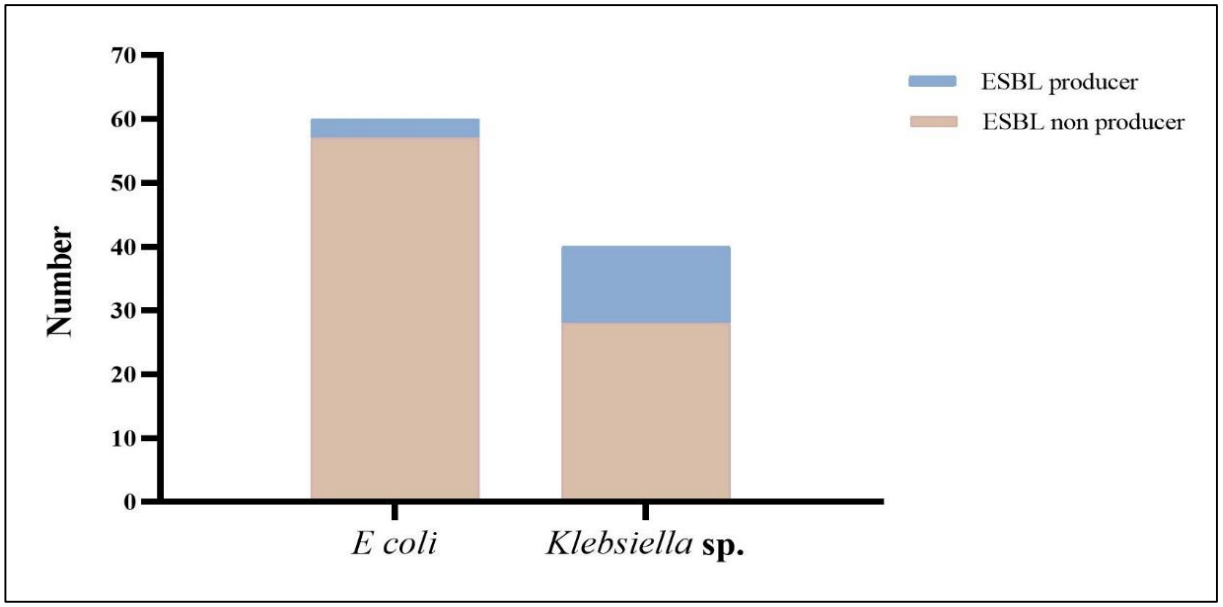

Figure 2: Confirmation of ESBL using combined disc test following CLSI guidelines.

Most common types of β-lactam resistance genes, which include *bla*$_{CTX-M}$, *bla*$_{SHV}$, and *bla*$_{TEM}$, were detected, and NDM-1 and OXA-48 genes were also detected. Of 60 isolated strains of *E. coli*, the TEM gene was the most prevalent, followed by NDM-1 and CTX-M genes. OXA-48 gene was not detected in the isolated *E. coli* strains (Figure 4). Additionally, out of 40 *Klebsiella* spp, TEM and CTX-M genes were the most prevalent, followed by OXA-48 and NDM-1 genes. In the case of 85 non ESBL producers, TEM and CTX-M genes were the most prevalent, followed by NDM-1, SHV, and OXA-48 genes. Among 15 ESBL producers, CTX-M and TEM genes were the most prevalent, followed by SHV, OXA-48, and NDM-1 genes.

Out of 100 isolated bacteria, the TEM gene was the most prevalent. However, the combination of CTX-M and TEM was the most prevalent (97.7%), followed by SHV and TEM (5.4%). Only 2 strains were positive for TEM, SHV, and CTX-M genes. Only one strain was positive for NDM-1 and OXA-48 genes (Figure 5).

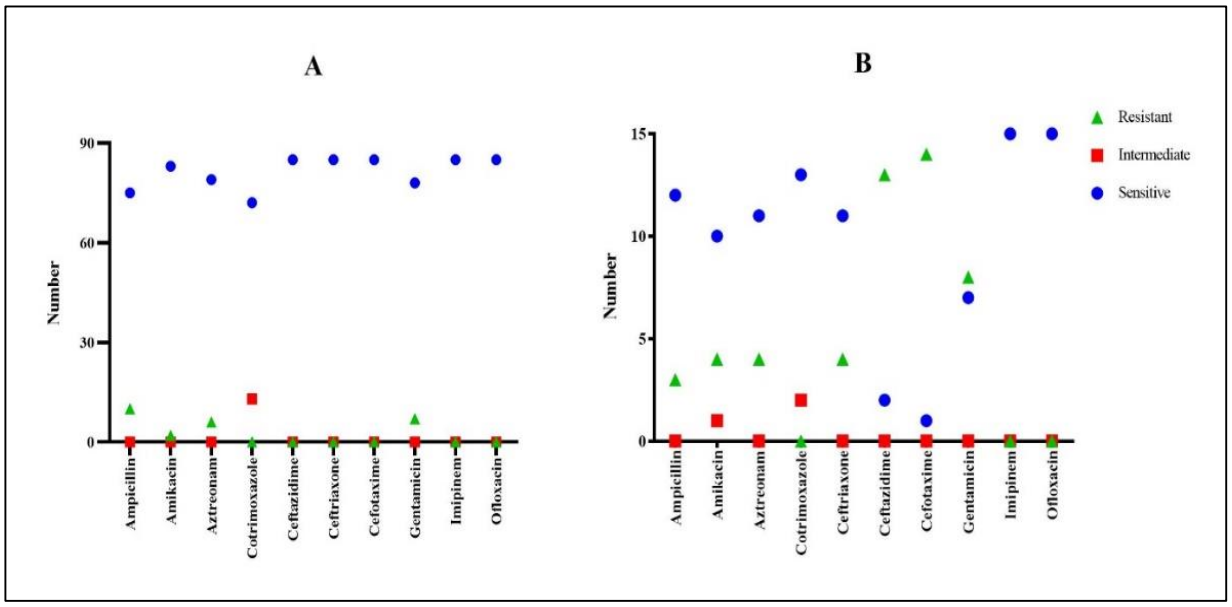

Figure 3: Disk diffusion test with isolates ESBL (A) and *E. coli* and *Klebsiella* spp (B) from surface water. The diameters of all zones of inhibition are measured and those values translated to categories of susceptible, intermediate, or resistant following the CLSI guidelines.

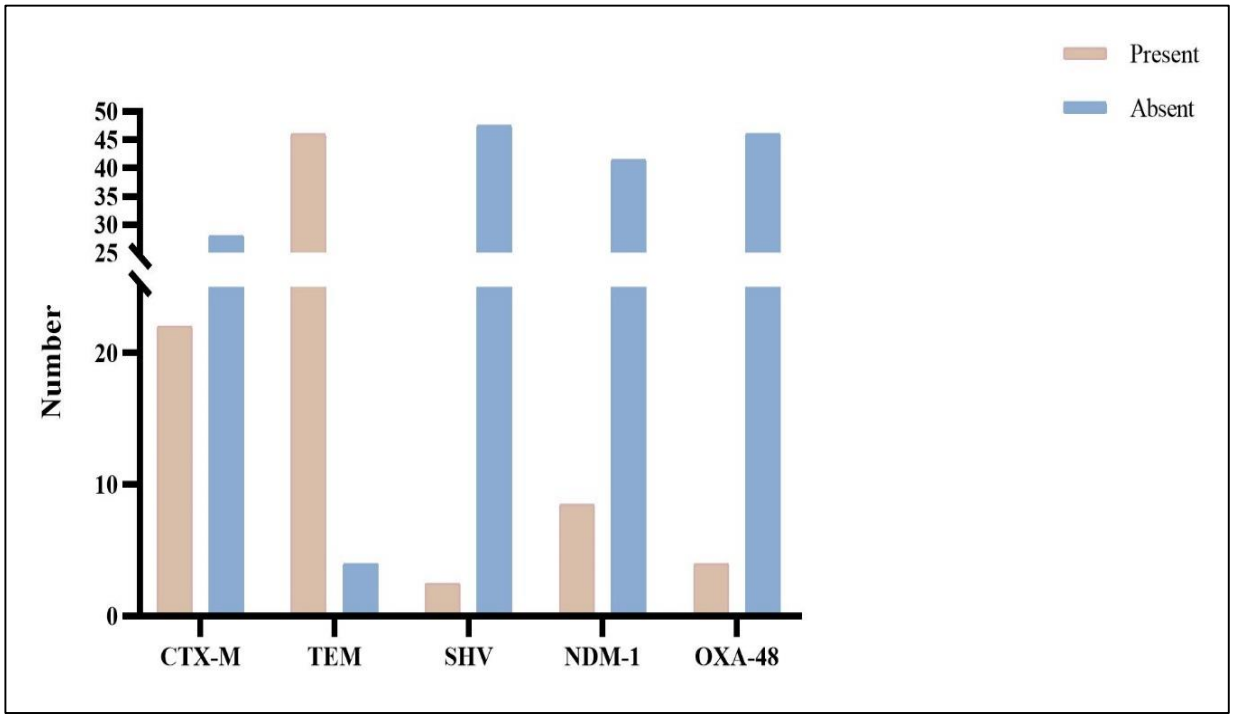

Figure 4: Antibiotic resistant genes CTX-M, TEM, SHV, NDM-1, OXA-48 by conventional PCR.

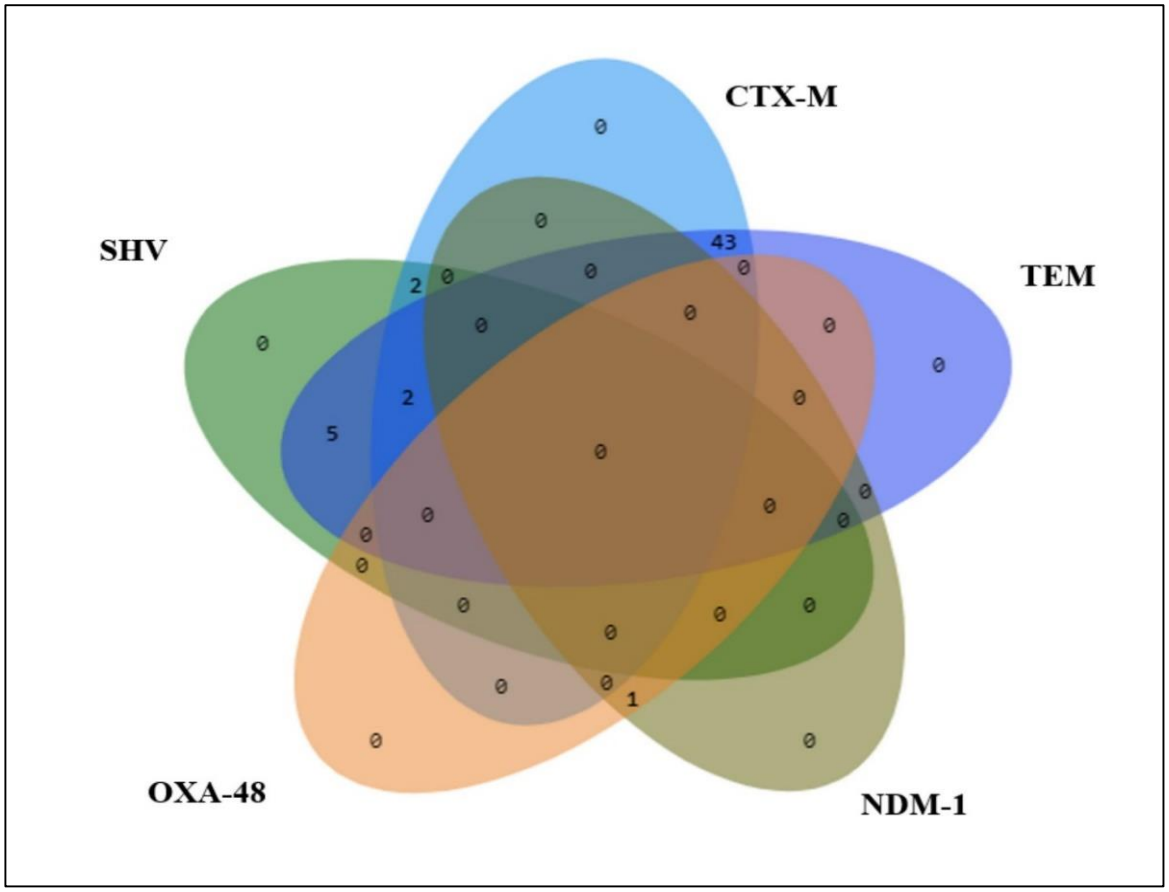

Figure 5: Representing antibiotic resistant genes; *bla*$_{SHV}$, *bla*$_{CTX-M}$, *bla*$_{TEM}$, *bla*$_{NDM-1}$ and *bla*$_{OXA-48}$ isolated from surface water.

DISCUSSION

Surface water is most prone to contamination due to human activities (Khatri and Tyagi, 2014). This study isolated antibiotic-resistant *E. coli* and *Klebsiella* spp, showing different antibiotic resistance (Figures 1A and 1B). Of the isolated strains, 5% of *E. coli* and 30% of *Klebsiella* spp were ESBL positive phenotypically (Figure 2).

ARGs were reported in source and drinking water samples from Canada, in which the TEM gene was the most prevalent, followed by SHV, CTX-M, OXA-48, GES, and NDM genes (Fernando et al., 2016). Similarly, in Mexico, *K. oxytoca*, *E. coli*, and *E. cloacae* were isolated, exhibiting resistance to cefazoline, cefuroxime, ampicillin, and ampicillin-sulbactam reflecting possible transmission mechanism for diarrheal outbreaks (Delgado-Gardea et al., 2016). In the USA, antibiotic resistance *E. coli, Enterobacter cloacae, Pseudomonas aeruginosa,* and *K. pneumoniae* were isolated along with ARGs, which include *tet*A and *sul*1 from raw water source of a drinking water treatment plant in a rural community (Bergeron et al., 2017).

The isolation of resistant bacteria from surface water reflects its spread and colonization and finally infects humans (Falgenhauer et al., 2021). Netherlands (Duarte et al., 2023, Sabri et al., 2020), Switzerland (Czekalski et al., 2012), Sweden (Larsson and Flach, 2022), Romania (Polianciuc et al., 2020), Belgium (Crettels et al., 2023), UK (Hayes et al., 2022) as well as Turkey (Delik et al., 2024) also have reported antibiotic-resistant bacteria in surface water.

On genotypic and phenotypic examination of 199 clinical isolates from Syria, 125 isolates were ESBL positive. Of isolated *E. coli*, 98 were positive for ESBL, and 88 of them harbored CTX-M genes. Similarly, of 27 ESBL positive *K. pneumoniae*, all isolates were ESBL positive. The most predominant gene was the CTX-M-1 type in *E. coli* and *K. pneumoniae* isolates (Al-Subol and Youssef, 2015). In Iran, SHV, CTX-M, and TEM resistance genes were detected in 49

isolates, with 39 isolates being positive for ESBL producing strains from infected wounds, with the frequency of CTX-M and TEM genes being significantly higher than that of SHV genes (Komijani et al., 2017). The ARGs have been also reported from Jordan (Swedan and Abu Alrub, 2019) as well as Saudi Arabia (Alotaibi and Ghafarifarsani, 2023).

In India, water sources have been reported to be contaminated with antimicrobial-resistant bacteria and their genes, primary sources being inadequate treatment of pharmaceutical and hospital effluents. The *bla*$_{\text{NDM-1}}$ gene was reported in 2 drinking water samples and 51 seepage samples from New Delhi (Walsh et al., 2011). Drug-resistant bacteria were also isolated from the 2 longest rivers of India, and resistant genes such as *bla*$_{\text{NDM-1}}$ and *bla*$_{\text{OXA-48}}$ genes were detected. Rivers, ponds, lakes, springs, hand pumps, and tube wells were also contaminated with the resistant bacteria (Taneja and Sharma, 2019). In another study from South India, pDNA was isolated from *E. coli* from hospitalized patients, healthy individuals, and the environment in South India. CTX-M was predominant in clinical samples (88%), healthy individuals (95.3%), and environmental isolates (77.8%), TEM gene was present in clinical isolates (10%), healthy individuals (16.9%) and environment (33.3%) and SHV gene was present in healthy individuals (1.5%), environment (22.2%). The high rate of CTX-M genes from healthy individuals reflects a high rate of intestinal carriage with wide dissemination into the community (George et al., 2015). NDM-1 positive isolates as well as genes were detected in 2010 in surface water from New Delhi. On evaluation of surface water from Yamuna River in Delhi, concentration of *bla*$_{\text{NDM-1}}$, *bla*$_{\text{OXA}}$ gene was in high percentage in New Delhi as compared to upper catchment of river (Ahammad et al., 2014).

In Bangladesh, drinking water in rural households (5%), drinking water in poultry farms (10%), and wastewater (90%) were positive for ESBL positive *E. coli* (Asaduzzaman et al., 2022). Similarly, in Pakistan, various MDR bacteria were identified from water sources and the surface with prevalence of *bla*$_{\text{SHV}}$, *bla*$_{\text{TEM}}$, and *bla*$_{\text{NDM}}$ genes (Aleem et al., 2021). In Vietnam, antibiotics (78%) were detected in surface water at about 67.9% detection frequency (Tran et al., 2019).

Above studies reflect that β-lactam resistant genes along with NDM-1 and OXA-48 genes are widely distributed in our community, restricting treatment options and prolonging hospitalization. The *bla*$_{\text{NDM-1}}$ genes are located on self-transmissible plasmids, and their isolation from surface water reflects the unsafe disposal of untreated sewage samples into the surface water. During this study, despite ESBL positive isolates, non ESBL positive isolates from surface water were also positive for ESBL positive genes. The distribution of ARGs in non ESBL positive bacteria was a critical aspect of the more significant ARGs problem, possibly due to the gene transfer from antibiotic-resistant bacteria to environmental bacteria. This could worsen the treatment problem, limiting the treatment options and increasing the patients' hospitalization time.

Of 332 non-repetitive clinical samples processed in Nepal, 25.8% were phenotypically confirmed as ESBL *E. coli* (Pokhrel et al., 2014). Different researchers have reported the presence of antibiotic-resistant bacteria as well as ESBL positive bacteria in the clinical samples (Kayastha et al., 2020, Larsson and Flach, 2022, Lohani et al., 2019). The TEM gene was the most prevalent in *E. coli*, followed by NDM-1 and CTX-M genes. The most prevalent gene in clinical isolates was CTX-M gene, reported by many studies (Shilpakar et al., 2021, Lohani et al., 2019, Amarasiri et al., 2022). This study reflects that the distribution of the CTX-M gene was less in the environment than the TEM genes.

Conclusion

Antibiotics resistance are growing concern worldwide due to unrestricted use of antibiotics and its effect on the treatment of disease. The contamination of river water with the antibiotic resistant bacteria serves as the reservoir for the dissemination of resistant bacteria into the biotic and abiotic factors enriching the inter and intra species of ARG transfer with scaling up of the antibiotic tolerance. The detection of β-lactam resistant as well as the carbapenemase resistant bacteria has already impacted on the treatment regimen and national policies should be developed to mitigate the trend of antibiotic resistant development.

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CONFLICT OF INTEREST

The authors declared no conflict of interest.

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