



Research Article

Assessment of Multidrug-Resistant Bacteria in Karachi Urban Sewage and their Clinical Implications

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Abstract | Antimicrobial resistance (AMR) is a current global public health concern with uncontrolled use of antimicrobials, especially antibiotics, and environmental systems like urban sewage are significant reservoirs of antibiotic-resistant bacteria (ARB). In this study, the prevalence and the antimicrobial susceptibility of bacteria isolated from urban sewage samples from Korangi, Karachi, Pakistan was studied. 9 isolates of bacteria were isolated, stained and subjected to Kirby-Bauer disc diffusion method to determine their sensitivity to Vancomycin (VA), Streptomycin (S), Tetracycline (TE) and Ampicillin (AMP). The resistance to ampicillin, tetracycline, streptomycin and vancomycin were observed in all isolates (100%), 33.3%, 22.2% and 44.4% respectively with HR like activity noted in the remaining isolates. In total, 18 of 36 antibiotic-isolate observations (50%) were resistant, 18 of 36 (50%) were heteroresistant (HR) and no isolates fully susceptible to antibiotics were found. Seven isolates were found to be multidrug resistant (MDR) and two isolates were found to be resistant to three antibiotics, thus giving the highest Multiple Antibiotic Resistance (MAR) index of 0.75, and the average MAR index was 0.50, which was many times the critical value of 0.20. The study suggests that the level of antimicrobial and multidrug resistant bacteria in urban sewerage is considerable and there is a potential risk of contamination of the environment and public health. The findings in this study were limited by the small sample size, geographical location, limited number of antibiotics screened, and lack of molecular identification and resistance gene characterisation and therefore highlight the need for wider surveillance and molecular investigation in future studies.

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Keywords | Antimicrobial resistance, Multidrug resistance, Heteroresistance-like activity, Kirby-Bauer disc diffusion, Sewage



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Introduction

Antimicrobial resistance (AMR) has become one of the most concerning global public health challenges of the century which threatens

the prevention and the treatment of a wide range of infectious diseases (Tiwari *et al.*, 2022). Understanding how individual antibiotic resistance genes (ARGs) emerge, evolve, and spread is crucial for developing sustainable strategies to combat

this threat (Munk *et al.*, 2022). AMR is steadily progressing, with an estimated annual death toll of around 10 million by 2050 (Talat *et al.*, 2025). The quick rise in resistant bacteria is mainly due to the inappropriate use, overuse and misuse of antibiotics in medicine and agriculture (Endalamaw *et al.*, 2024). Consequently, the prevalent treatable infections are becoming increasingly hard to manage which leads to chronic illness, higher healthcare costs and increased mortality rates. The increase in AMR underlines the need to identify and monitor reservoirs of resistant bacteria beyond clinical setting.

Environmental systems, mainly wastewater and urban sewage are increasingly recognized as important reservoirs and pathways for the transmission of antibiotic-resistant bacteria (ARB). Sewage systems contain a mixture of domestic, hospital, and industrial waste, which mostly includes antibiotic-resistant microorganisms and resistance genes (Sarekoski *et al.*, 2024). This environment creates selective pressure that supports the survival and growth of bacteria with resistance to multiple drugs (Tiwari *et al.*, 2022). These organisms can re-enter the human population via contaminated water, food chains or the environment, thus linking environmental sources with clinical AMR.

Testing sewage for bacteria is an important way to monitor AMR and its impact on public health. Identifying resistance patterns in wastewater can be used to develop an early warning system for identifying emerging resistance patterns and understanding the dynamics of how resistance spreads throughout a population. Multidrug resistance (MDR) is the main concern as it makes the treatment of the disease difficult and often results in more serious health outcomes. MDR bacteria pose a significant public health threat on a global scale. They are resistant because of ARGs, which are frequently found on mobile genetic elements like plasmids that can be transferred between bacteria. The genetic changes are very fast in wastewater environments, particularly in hospital wastewater, where ARB, organic matter, extracellular DNA, and antibiotics are present (Stefaniak *et al.*, 2025).

Despite increasing global concern regarding antimicrobial resistance in environmental reservoirs, limited information is available regarding resistance patterns of culturable bacteria present in urban

sewage systems in Karachi, Pakistan. Environmental surveillance of sewage can provide valuable insight into the dissemination of resistant bacteria and emerging resistance phenotypes outside clinical settings. The lack of local data is a major knowledge gap because untreated or partially treated wastewater may be a key source of resistant microorganisms in densely populated urban areas.

Therefore, the objectives of this study were to isolate culturable bacteria from urban sewage, evaluate their susceptibility to four commonly used antibiotics using the Kirby–Bauer disc diffusion method, determine MDR and MAR indices, and assess the occurrence of HR-like activity among the isolates.

HR like activity was identified as visible bacterial growth in a zone of inhibition around an antibiotic disc. This growth implies the presence of multiple subpopulations of bacteria with varying susceptibilities. Since confirmatory population analysis profiling and molecular characterisation were not performed, the term “heteroresistance-like activity” is used throughout this study rather than confirmed HR (El-Halfway and Valvano, 2015).

These results serve as baseline data on the prevalence of antibiotic resistance among the culturable bacteria samples from urban sewage in Karachi and emphasise the critical role of environmental surveillance in integrated AMR monitoring programmes.

Materials and Methods

Sample collection

For sample collection, Korangi, Karachi, Sindh, Pakistan (24.82223° N, 67.101088° E) was selected for this study. A total of three water samples were taken in the morning and obtained in 15ml aseptic falcon tubes along with a small amount of surface soil and sediment to ensure that all microorganisms from the environment were included in the sample. The tubes were closed immediately after the sampling and were then brought back to the lab for further analysis within 24 h of collection to minimize changes in microbial composition.

Culturing and isolation of the sewage sample

Primary culture was made by spreading 50 µL of the sample onto NA plates with an ethanol-flamed glass spreader. The plates were then incubated at 37 °C

for a 24-hour period (Mannan *et al.*, 2024). A sterile wire loop was then used to transfer morphologically distinct colonies differing in size, shape, elevation, pigmentation, or margin characteristics from primary culture plates to nutrient broth, to create pure cultures. The tubes containing pure isolates were then placed in an incubator overnight at 37 °C.

Identification

Bacterial isolates were identified based on the morphological and staining characteristics through Gram staining. A thin smear of each isolate was prepared on a clean glass slide, air-dried, and heat-fixed. The smear then was stained sequentially with crystal violet for 1 minute and then stained with iodine solution as a mordant for 1 minute. Decolorization was performed using 95% ethanol for approximately 10–20 seconds, and the slide was immediately rinsed with distilled water. Finally, the smear was counterstained with safranin for 1 minute, gently washed and air dried. The stained slides were then viewed in the light microscope with the oil immersion objective (100×) to check for the morphology, arrangement and Gram reaction of bacteria (Gram-positive or Gram-negative) (Gyure, 2010).

Confirmation of *Pseudomonas* spp. by growth on ceftrimide agar

Colony morphology and growth characteristics of the bacterial isolates were similar to those of members of the genus *Pseudomonas* during the characterisation. For further identification it was subcultured onto *Pseudomonas* Ceftrimide Agar which is selective for *Pseudomonas* species. The isolate grew well on the medium and was presumed to be a member of the genus *Pseudomonas*. The isolate is a gram-negative rod and can be grown on Ceftrimide Agar and is likely to be *Pseudomonas aeruginosa* (Tang *et al.*, 2017).

Standardization of bacterial inoculum

All the bacterial inoculums were adjusted to 0.5 McFarland turbidity standard to ensure the uniformity of microbial concentration (Rahim *et al.*, 2024). The isolates were then diluted in sterile saline and the turbidity of the suspensions was adjusted so that each isolate was the same as the new standard (9.95 mL of 1% H₂SO₄ and 0.05 mL of 1% BaCl₂). This procedure resulted in all of the isolates having approximately 1.5 × 10⁸ CFU/mL before the antimicrobial susceptibility analysis.

Antibiotic susceptibility assay

The susceptibility of the bacteria was determined by the Kirby–Bauer disc diffusion method (Abdelgalel *et al.*, 2025). A total of 20 µL of each isolate was deposited onto MHA (Shin *et al.*, 2022), and spread to form a uniform lawn with an ethanol-sterilized glass spreader. Four antibiotic discs were selected based on their common clinical use and representation of different antibiotic classes. A 10 µg of Ampicillin (AMP), 30 µg of Vancomycin (VA), 10 µg of Tetracycline (TE) and 10 µg of Streptomycin (S) were aseptically placed in separate quadrants. The plates were left for overnight incubation at 37°C, which were then examined for the presence and measurement of zones of inhibition.

Determination of antimicrobial activity

The antimicrobial activity was determined by measuring the zones of inhibition formed after the incubation period. The measurements were recorded in millimeters (mm), and the results indicated the presence of both AMR and MDR among the isolates.

Results

Antibiotic susceptibility testing of the nine bacterial isolates, obtained from recruited sewage water samples, was performed against four antibiotics: AMP, TE, S, and VA. The isolates exhibited varying patterns of resistance and HR.

Antibiotic susceptibility pattern of the isolates

AMP showed the highest level of resistance against all isolates, resulting in a 100% resistance rate (Figure 1). For TE, isolates 3, 4, and 5 were resistant (R) while isolates 1, 2, 6, 7, 8 and 9 demonstrated Heteroresistant (HR). This corresponded to 33.3% R, and 66.7% HR isolates (Figure 2). In the case of S, isolates 1 and 3 were resistant and isolates 2, 4, 5, 6, 7, 8, and 9 exhibited HR. Overall, the percentages for R and HR were 22.2% and 77.8%, respectively (Figure 3). For VA, resistance was observed in isolates 5, 6, 7, and 8. Isolates 1, 2, 3, 4 and 9 demonstrated HR. These findings represented 44.4% R, 55.6% HR (Figure 4). The presence of HR in several isolates suggests the coexistence of susceptible and resistant bacterial subpopulations, which may contribute to the emergence and spread of antimicrobial resistance.

The overall findings show clear differences in the effectiveness of the four antibiotics against the

tested bacterial isolates. This comparison highlights the variability in antimicrobial response among the isolates and helps to identify the most and least effective options (Table 1).

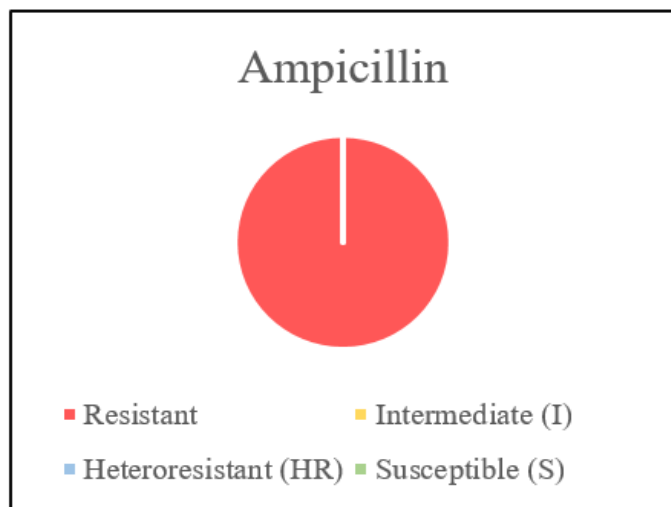


Figure 1: *Ampicillin susceptibility distribution.*

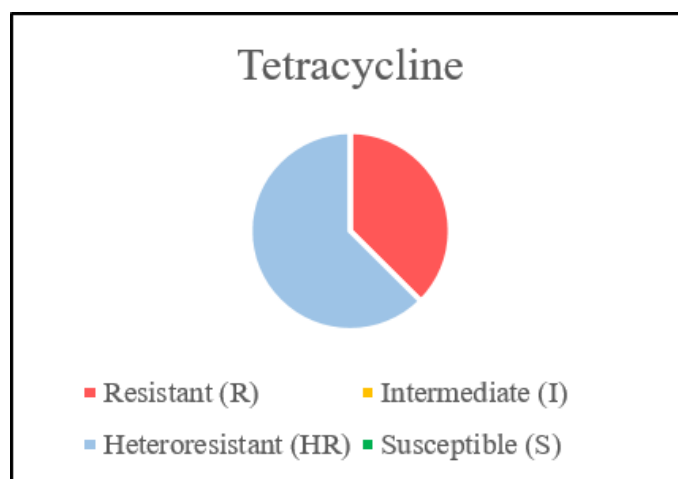


Figure 2: *Tetracycline susceptibility distribution.*

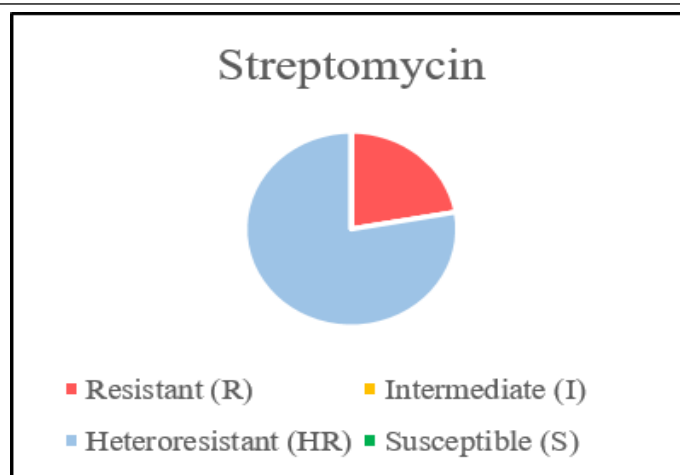


Figure 3: *Streptomycin susceptibility distribution.*

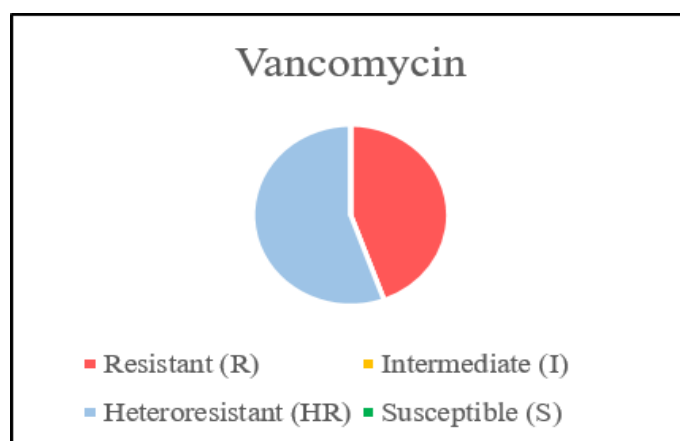


Figure 4: *Vancomycin susceptibility distribution.*

The bar graph illustrates the comparative antimicrobial activity of the four antibiotics against all tested isolates. Differences in the level of susceptibility and resistance among the antibiotics can be visually observed, allowing easier comparison of their effectiveness highlighting differences in how each antibiotic performs (Figure 5).

Table 1: *Zone of inhibition measurements (mm) and antibiotic susceptibility profiles of the nine bacterial isolates against four antibiotics.*

Isolate ID	Identification	Ampicillin (AMP)	Tetracycline (TE)	Streptomycin (S)	Vancomycin (VA)
1	Gram negative Rod	RS (0 mm)	HR (17 mm)	RS (0 mm)	HR (19 mm)
2	Gram positive Rod	RS (0 mm)	HR (15 mm)	HR (15 mm)	HR (19 mm)
3	Gram positive Rod	RS (0 mm)	RS (8 mm)	RS (13 mm)	HR (20 mm)
4	Gram positive Rod	RS (0 mm)	RS (0 mm)	HR (20 mm)	HR (15 mm)
5	Gram negative Rod	RS (0 mm)	RS (0 mm)	HR (19 mm)	RS (14 mm)
6	Gram positive Rod	RS (0 mm)	HR (18 mm)	HR (18 mm)	RS (0 mm)
7	Gram positive Rod	RS (0 mm)	HR (20 mm)	HR (21 mm)	RS (0 mm)
8	Gram negative Rod	RS (0 mm)	HR (22 mm)	HR (17 mm)	RS (0 mm)
9	Gram positive Rod	RS (12 mm)	HR (28 mm)	HR (17 mm)	HR (19 mm)

RS, resistant; I, intermediate; S, sensitive; HR, heteroresistant. Colour shading denotes antimicrobial susceptibility (red, resistant; yellow, intermediate; green, sensitive; blue, heteroresistant). *The susceptibility of the total 9 bacterial isolates was determined by the disk diffusion method against four antibiotic disks, 10 microgram of Ampicillin (AMP), 30 µg of Vancomycin (VA), 10 µg of Tetracycline (TE) and 10 µg of Streptomycin (S), aseptically placed in separate quadrants.

Table 2: Multiple antibiotic resistance (MAR) index values of the bacterial isolates.

Antibiotic	Isolate 1	Isolate 2	Isolate 3	Isolate 4	Isolate 5	Isolate 6	Isolate 7	Isolate 8	Isolate 9
Ampicillin	RS	RS	RS	RS	RS	RS	RS	RS	RS
Tetracycline	HR	HR	RS	RS	RS	HR	HR	HR	HR
Streptomycin	RS	HR	RS	HR	HR	HR	HR	HR	HR
Vancomycin	HR	HR	HR	HR	RS	RS	RS	RS	HR
No of Resistant	2	1	3	2	3	2	2	2	1
MAR Index Calculation (Number of R/4)	2/4	1/4	3/4	2/4	3/4	2/4	2/4	2/4	1/4
MAR Index Vale	0.50	0.25	0.75	0.50	0.75	0.50	0.50	0.50	0.25

≈MAR index > 0.2 indicates contamination from a high-risk source with frequent antibiotic exposure; Total 9 bacterial isolates ranging from 0.25 to 0.75. *RS=Resistant; HR= Heteroresistant.

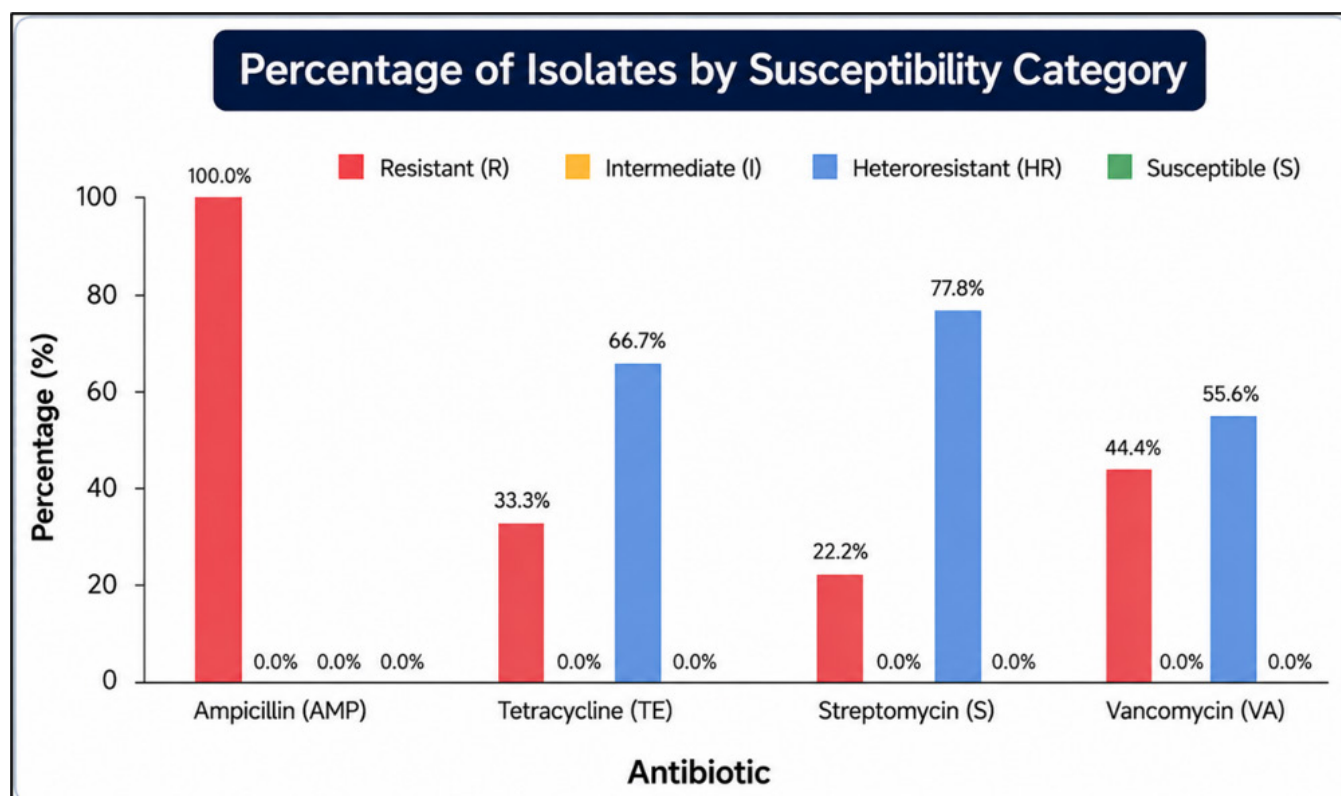


Figure 5: Percentage activity of four antibiotics against bacterial isolates: Ampicillin showed 100% resistance; Tetracycline showed 33.3% resistance and 66.7% HR; Streptomycin showed 22.2% resistance and 77.8% HR; and Vancomycin showed 44.4% resistance and 55.6% HR. No susceptibility or intermediate response was observed for any antibiotic.

Overall susceptibility distribution

The overall susceptibility distribution analysis included a total of 36 antibiotic–isolate observations. Among these, 18 observations were classified as resistant, accounting for 50% of the total observations and 18 observations (50%) exhibited HR, indicating mixed susceptibility patterns or emerging resistance (Figure 6).

Multiple antibiotic resistance (MAR) index analysis

MAR testing showed that isolates 1, 3, 4, 5, 6, 7, and 8 were multi-resistant to at least two antibiotics, while

isolate 2 and 9 were only resistant to one antibiotic. Isolates 3 and 5 had the highest MDR pattern, with each showing resistance to three drugs. Specifically, isolate 3 was AMP, TE, and S resistant, and isolate 4 was AMP, TE, and VA resistant. The index of the MAR score ranged from 0.25 to 0.75 and was estimated by dividing the number of antibiotics an organism was R to by the total number of antibiotics tested. Isolates 1, 4, 6, 7 and 8 had a MAR index value of 0.50, and isolates 3 and 5 had the highest value of 0.75. Isolate 2 and 9 showed the lowest MAR index value of 0.25 (Table 2).

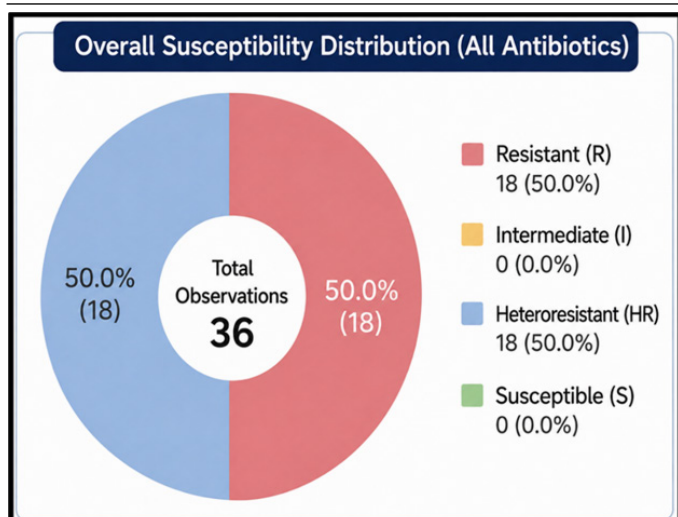


Figure 6: Antibiotic susceptibility profile of 36 observations showing mainly resistance and heteroresistance.

The average MAR of all isolates was determined as 0.5 which is significantly high from the critical value of MAR index 0.20. Isolates with a MAR more than 0.20 are likely from high-risk settings, where frequent exposure to antimicrobial agents occurs (Mooney *et al.*, 2025). This means that there are important selective pressure and antibiotic contamination in the sewage water environment, which promotes the emergence and persistence of multidrug resistant bacteria.

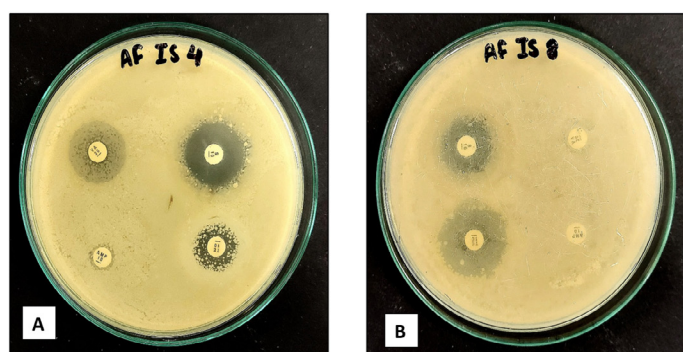


Figure 7: Isolate 4 (A) and Isolate 8 (B) exhibiting resistance (RS) against Ampicillin (AMP) and Tetracycline (TE) and Heteroresistant (HR) against Vancomycin (VA) and Streptomycin (S).

Phenotypic characterisation of sewage bacterial isolates

All nine of the bacterial isolates were gram stained to observe their gram reaction and cellular morphology. The results showed that six isolates were Gram-positive; that is, they stain purple/violet and three were Gram-negative; they stain pink/red (Figure 8). Most isolates exhibited a rod-shaped (bacillary) morphology. The majority of sewage isolates were Gram-positive bacteria, indicating that there is a

wide variety of bacteria in environments that are exposed to antimicrobial treatment. The findings will give preliminary phenotypic characterisation of the isolates and will add to the antimicrobial susceptibility results of this study (Figure 9).

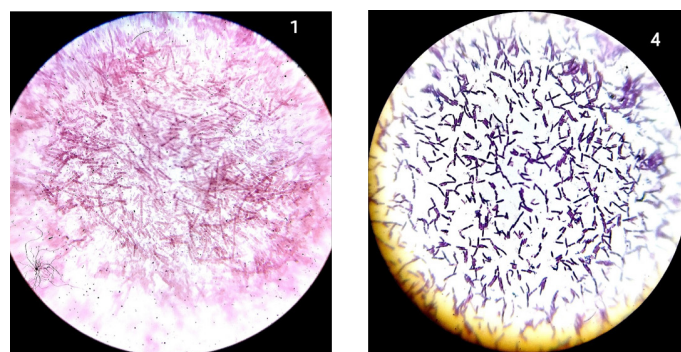


Figure 8: Gram staining of Isolate 1 (A) and Isolate 4 (B) demonstrating Gram-negative bacilli under light microscopy (100× magnification).

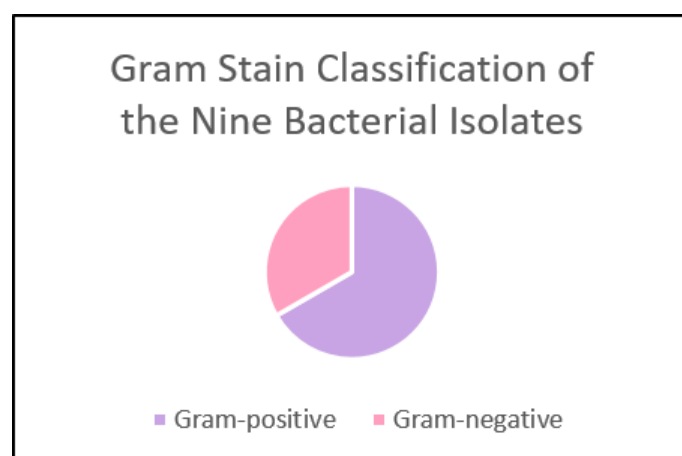


Figure 9: Distribution of antibiotic resistance among gram-positive and gram-negative bacterial isolates.

Isolate 1 (Figure 8a) was further characterised by growing it on Cetrimide Agar, a selective media generally used for the isolation of *Pseudomonas* spp. The isolate grew well on Cetrimide Agar and was therefore confirmed to be a *Pseudomonas* species. The isolate is a gram-negative rod that grows on selective media; its morphology, origin, and growth confirmed by selective media suggests that the organism may be *Pseudomonas aeruginosa*. An opportunistic pathogen with significant clinical importance that has been linked with serious infections such as pneumonia, bloodstream infections, urinary tract infections, and wound infections especially in immunocompromised patients. It is clinically significant by virtue of its intrinsic resistance to a number of antibiotics and its capacity to gain further resistance mechanisms, which makes treatment difficult. However, the species-

level identification of *Pseudomonas aeruginosa* is not always possible by conventional culture-based methods, and needs to be confirmed by molecular characterisation, such as sequencing of the *16S rRNA* gene and whole genome sequencing.

Comparative antimicrobial activity of tested antibiotics

The distribution and consistency of the zone of inhibition data are clearly different for each of the four antibiotics. The median zone diameter was also relatively high for streptomycin and tetracycline (17 mm for both), which means that the majority of the isolates had moderate to high zone diameters for these two antibiotics. There was much more variation ($x \pm 9.68$ mm) in tetracycline than in streptomycin ($x \pm 6.33$ mm), which indicates that there is more variability in response between the isolates in the former. Vancomycin had an intermediate median (15 mm) and high variability (± 9.05 mm) which suggest varying responses in samples. The median zone diameter for ampicillin was 0 mm and the mean was very low (1.33 ± 4.00 mm), the range was 0–12 mm indicating that most isolates displayed either no zone or a very small zone of activity. Overall, there is strong consistency and spread to the various antibiotics, with ampicillin having a very skewed distribution towards the minimum values and the others a broader but more even distribution (Table 3).

Table 3: Descriptive statistics of inhibition zone diameters for different antibiotics.

Antibiotic	Mean $\pm$ SD (mm)	Median (mm)	Range (mm)
Ampicillin	1.33 ± 4.00	0	0–12
Tetracycline	14.22 ± 9.68	17	0–28
Streptomycin	15.56 ± 6.33	17	0–21
Vancomycin	11.78 ± 9.05	15	0–20

Descriptive statistics showing the mean $\pm$ standard deviation (SD), median, and range of inhibition zone diameters (mm) produced by Ampicillin, Tetracycline, Streptomycin, and Vancomycin against the tested bacterial isolates.

The Friedman test indicated a statistically significant difference in zone of inhibition for the four antibiotics (χ^2 (3) = 11.01, $p = 0.0117$), which meant that at least one of the antibiotics is different from each other in its antimicrobial effectiveness. This means that there is a variation in susceptibility patterns among treatments. But the test does not tell us what kinds of differences there are among groups (Table 4).

Using a Bonferroni correction ($\alpha = 0.05/6 = 0.0083$),

only, AMP vs S remained statistically significant ($p = 0.0078$). The other pairwise comparisons did not remain significant after correction. Bonferroni corrected ($\alpha = 0.0083$) post-hoc Wilcoxon signed-rank test were applied. All the differences between the groups were not statistically significant after adjustment, except for the comparison ampicillin versus streptomycin ($p = 0.0078$). This indicates that most antibiotics have a similar activity against the isolates tested and that a large difference exists between the activity of ampicillin and streptomycin (Table 5).

Table 4: Friedman test analysis of differences in antimicrobial effectiveness among four antibiotics.

Statistical test	Test statistic (χ^2)	Degrees of freedom (df)	p-value
Friedman test	11.01	3	0.0117

A significant result ($p < 0.05$) indicates differences in antimicrobial effectiveness among the antibiotics.

Table 5: Post-hoc pairwise comparisons of antibiotic activities using the Wilcoxon signed-rank test.

Comparison	p-value
AMP vs TE	0.0156
AMP vs S	0.0078*
AMP vs VA	0.0313
TE vs S	0.703
TE vs VA	0.652
S vs VA	0.480

Results of post-hoc Wilcoxon signed-rank tests comparing inhibition zone diameters among the tested antibiotics. Bonferroni correction was applied to adjust for multiple comparisons (adjusted significance level, $\alpha = 0.0083$). *Significant.

Relationship between bacterial characteristics and antibiotic response

The Mann–Whitney U test revealed no statistically significant differences in zone of inhibition for any of the antibiotics tested (AMP $p = 0.637$, TE $p = 1.000$, S $p = 0.604$, VA $p = 0.688$) suggesting that there was no effect of Gram classification on antimicrobial susceptibility in this dataset. This indicates that the responses of both groups of bacteria to the antibiotics were similar with overlapping distribution of inhibition zone than the susceptibility pattern. Biologically it means that most of the isolates are not resistant due to structural changes of the cell wall (such as modification of outer membrane of Gram-negative), but rather due to enzymatic degradation,

target modification or efflux pump activity which can be present in both groups. In general, these results indicate that the efficacy of these antibiotics is not related to Gram reaction and that the differences in susceptibility are more likely to be due to strain-specific resistance characteristics than to the Gram reaction (Table 6).

Table 6: Comparison of antibiotic activity between gram-positive and gram-negative isolates using the mann–whitney U test.

Antibiotic	p-value
AMP	0.637
TE	1.000
S	0.604
VA	0.688

Results of the Mann–Whitney U test comparing inhibition zone diameters between Gram-positive and Gram-negative bacterial isolates for each antibiotic tested.

Table 7: Spearman's rank correlation analysis of zones of inhibition among four antibiotics.

Comparison	Spearman ρ	p value	Interpretation
AMP vs TE	0.550	0.125	Moderate positive correlation, not significant
AMP vs S	-0.069	0.860	Very weak negative correlation, not significant
AMP vs VA	0.283	0.460	Weak positive correlation, not significant
TE vs S	-0.067	0.864	Very weak negative correlation, not significant
TE vs VA	-0.286	0.456	Weak negative correlation, not significant
S vs VA	-0.702	0.035*	Strong negative correlation, statistically significant

Spearman's rank correlation coefficient (ρ) was used to assess the relationship between antibiotic inhibition zones. * Correlation is significant at the 0.05 level (2-tailed).

Spearman correlation analysis showed that there was only one significant relationship between the zone diameters of all the antibiotics. There was a moderate to strong negative correlation between the zone of inhibition for streptomycin with vancomycin ($\rho = -0.702$, $p = 0.035$), meaning that isolates with larger zones of inhibition for streptomycin tended to have smaller zones on vancomycin while isolates with smaller zones on streptomycin tended to have larger zones on vancomycin. This inverse relationship implies there may be some trade-off in susceptibility patterns

such that bacterial isolates which are more susceptible to one antibiotic may have some relative resistance to the other. Biological, this may be due to a difference in the mode of action of the two drugs, streptomycin acting on protein synthesis and vancomycin acting on cell wall synthesis, as well as a heterogeneity in resistance properties of the tested isolates. Overall, however, because only one correlation was significant, there is limited interdependence between most antibiotic responses; susceptibility to the antibiotics tested appear largely independent of each other except for this inverse correlation between susceptibility to 16S and 23S.

Discussion

The present study revealed that there is a high prevalence of AMR in bacterial isolates from sewage water, which has led to the increasing concern that environmental systems may be reservoirs and transporters of ARB. The results support the One Health concept, which focuses on the close linkage of human health, environmental pollution and the transmission of AMR (Rahim *et al.*, 2024; Tirwari *et al.*, 2022). In sewage environments, waste from homes, hospitals, agriculture and industry exposes bacteria constantly to antibiotics, disinfectants, heavy metals and other chemicals. This exposure exerts selective pressure that promotes the emergence, persistence, and dissemination of resistant bacterial populations. The Gram staining results also showed that the sewage microbiota comprised Gram-positive ($n = 6$) and Gram-negative ($n = 3$) bacterial isolates, indicating that sewage systems are host to diverse bacterial populations which could serve as reservoirs of antimicrobial resistance determinants.

The AMP showed no observable activity against the tested isolates and this may be due to the high number of β -lactam use, the most commonly prescribed an antibiotic drug in human and animal health over long time periods, extensive environmental contamination, and selection pressures that have promoted resistant strains (Mannan *et al.*, 2024). The widespread ampicillin resistance in this study suggests that the sewage environment contains bacteria that have developed resistance through mechanisms commonly associated with β -lactam antibiotics. This interpretation is supported by the statistical analysis, where ampicillin showed the lowest mean zone of inhibition (1.33 ± 4.00 mm) and a median value of 0

mm, indicating that most isolates exhibited little or no susceptibility. In addition, post hoc Wilcoxon analysis showed that ampicillin was significantly different from streptomycin (after Bonferroni correction), as its activity was considerably less than that of other antibiotics tested.

Resistance patterns for TE, S, and VA varied among isolates, indicating the presence of diverse bacterial populations with different resistance patterns. The MAR indices of isolates 3 and 5 are particularly significant because MDR bacteria pose a major public health concern, as it reduces available treatment options and increases the risk of treatment failure in humans and animals if transmitted. The descriptive statistical analysis also revealed high inter-isolate resistance heterogeneity for tetracycline and vancomycin. This is typical of sewage where bacteria from a variety of sources are constantly subjected to various selection pressures.

Some bacterial isolates demonstrated HR-like activity which is the presence of susceptible and resistant subpopulations in a bacterial isolate. HR-like patterns may be due to the heterogeneous susceptibility response in the environmental bacterial population, and may be a subject of further investigation for understanding the dynamics of resistance in the sewage environment. This is clinically significant as bacteria may be initially seen as susceptible when performing routine susceptibility testing, but then become resistant when exposed to antibiotics. The bacterial isolates were not identified to the species level and therefore the observed HR-like patterns should be interpreted with caution and considered to be preliminary evidence of heterogeneous susceptibility, rather than to be the definitive evidence of HR.

The overall susceptibility distribution also highlighted the high proportion of sewage that was resistant. Half of the antibiotic-isolate interactions were resistant and alarmingly none were susceptible. The significant rate of HR observations also suggests that in the environmental bacteria, resistance can be more complex than simple susceptible/resistant distinctions. These results are consistent with earlier reports indicating that sewage systems are reservoirs of resistant organisms, as well as a dynamic environment where resistant traits can be generated and disseminated among microorganisms. A difference between the efficacy of the four antibiotics used was

also shown by Friedman test ($p = 0.0117$), suggesting that efficacy was not the same for each antibiotic. However, multiple testing was applied in the pairwise comparisons and majority of the differences were not significant, which means that the variability observed should be considered conservatively.

The Multiple Drug Resistance (MAR) index analysis also showed that there was considerable exposure to antibiotics in the sewage environment. All isolates exhibited MAR index values above the critical threshold of 0.20 ([Mooney et al., 2025](#)), while isolates 3 and 5 showed particularly high values of 0.75. The higher the MAR index, the more likely that bacteria from the environment have been exposed to a high amount of antibiotics, which is typically found in environments that are exposed to repeated antibiotic contamination. The average MAR index value of ~ 0.5 strongly indicates that the sewage source is a high-risk environment and responsible for the maintenance and spread of multidrug resistant bacteria.

The Gram-negative isolates of this study were not very sensitive to vancomycin. This is in line with the natural resistance of Gram-negative bacteria to glycopeptides, which are antibiotics that target the peptidoglycan layer of the cell wall; the outer membrane of Gram-negative bacteria creates a permeability barrier that prevents vancomycin from penetrating to the peptidoglycan layer. Thus, vancomycin is more effective against Gram-positive bacteria than Gram-negative bacteria ([Maher and Hassan, 2023](#)). However, statistical comparison between Gram-positive and Gram-negative isolates revealed no significant differences in susceptibility for any of the antibiotics tested ($p > 0.05$). The resistance patterns observed in the isolates were not completely explained by Gram classification, indicating that other resistance mechanisms may have contributed more to the antimicrobial susceptibility of the isolates.

This study illustrates the importance of sewage water as an environmental source of antimicrobial resistance genes and the need for better antibiotic stewardship, wastewater management, and environmental surveillance programs. Environmental AMR monitoring can help to increase knowledge of resistance distribution and aid evidence-based antimicrobial stewardship and wastewater management strategies. However, one of the drawbacks of the present study is that the bacterial

identification was limited to Gram-staining and not genus or species level characterization which does not allow easy comparison with the clinical pathogens and limits the epidemiological interpretation.

Limitations

The present study gives some preliminary knowledge about the presence of MDR bacteria in urban sewage and some limitations should be taken into consideration. It is only a small sample of isolates from one environmental sample that was used for the study, which may limit the generalizability of the results. Furthermore, the lack of biological replicates could affect the robustness and reproduction of the results. A small number of four antibiotics were used for antibiotic susceptibility testing and this might not reflect the full resistance profile of the isolates. Additionally, the species-level identification was carried out using only phenotypic and culture-based techniques and not molecular characterization, such as 16S rRNA gene sequencing, which limits definitive species-level identification. These drawbacks notwithstanding, the study offers a good baseline data of the antimicrobial resistance patterns of the sewage-associated bacteria and calls for further wider investigations.

Conclusion

In this study, nine bacterial isolates were found from an urban sewage sample in Karachi, Pakistan and nine isolates were found to be resistant to antimicrobial agents. The isolates were all ampicillin resistant, and showed different degrees of resistance and/or heteroresistance to tetracycline, streptomycin and vancomycin. Multidrug resistance was observed in several isolates, and the average MAR index of 0.50 indicated that they were exposed to environments with high antimicrobial selection pressure. The results have shown the presence of antimicrobial resistant bacteria in the sewage sampled and the importance of wastewater monitoring for AMR surveillance. The conclusions of the study must be taken with caution because of the limitations such as small sample size, point source sampling location, limited number of antibiotics tested, and the absence of molecular identification of the organisms and resistance gene analysis. Additional antimicrobials, molecular characterization and larger-scale sampling are needed to fully characterize antimicrobial resistance in sewage systems and its public health implications.

Recommendations

Due to the large release of antibiotics, sources such as hospitals, farms, wastewater and pharmaceutical industries contribute to the contamination of environmental systems, including water and soil, which promotes the spread of ARGs in aquatic environment. These environments may act as reservoirs for AMR posing potential risks to human health and ecosystems. To detect and study ARGs in environmental samples, advanced molecular techniques such as PCR, qPCR and metagenomics are being used (Mutuku *et al.*, 2022).

Our aim in the future is to purify the isolates further and to find the exact species by sequencing. Further microbiological tests such as Gram staining will also be done to examine the morphological features of the bacteria. These analyses could offer a greater understanding of the antimicrobial resistance pattern and possible clinical significance of the isolates identified. In addition, further studies, better wastewater treatment systems and more stringent management of antibiotic discharge are required to fight the rise of antibiotic resistance.

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Novelty Statement

It is based on the preliminary evidence of the pattern of AMR and HR-like activity of culturable bacteria present in sewage samples. Due to the exiguous environmental data availability in Karachi samples, MAR and phenotypic susceptibility provides the odds of samples as reservoir of MDR bacteria.

Author's Contribution

All authors have equal contributions.

Generative AI and AI assisted technology statement

The authors declare that no generative AI and AI assisted technology was used in the creation of this manuscript.

Conflict of interest

The authors have declared no conflict of interest.

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