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Phenotypic profiling and *bla*$_{TEM}$ gene detection in multidrug resistant gram-negative bacteria isolated from hostel sanitary area

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Author`s Contribution

[WA] Conception and design, [MI] Collection and assembly of data, [II, WA] Analysis and interpretation of the data, Statistical expertise, [SR] Final approval and guarantor of the article

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A B S T R A C T

Background: Antimicrobial resistance (AMR) is a major health concern across the world, with Gram-negative bacteria account for failing the therapeutic options available. Environmental reservoirs, such as sink drains in high-occupancy hostels have been recognized as the neglected hotspots for multidrug-resistant bacteria, has become an imminent health peril because it is associated with biofilm-mediated persistence and transmission of antimicrobial resistance.

Objective: This study aimed to investigate the phenotypic resistance patterns and detect *bla*$_{TEM}$ gene among the Gram-negative strains from the drainage system of hostel sanitary areas.

Methodology: The 80 swab samples were collected and cultured on a selective media to specifically isolate 50 resistant strains followed by performing antibiotic susceptibility testing using Kirby-Bauer disk diffusion method. PCR was conducted to confirm the presence of the *bla*$_{TEM}$ gene.

Results: Biochemical profiling identified various species with *Escherichia coli* and *Acinetobacter spp*. (18% each) were the most common followed by *Pseudomonas spp*. and *Salmonella spp.* (12% each). While *Enterobacter spp*. and *Stenotrophomonas spp*. constituted 10% of isolates. *Proteus spp*. and *Shigella spp*. were identified as 8% and 6% respectively. The less frequently found organisms were *Serratia spp*. and *Aeromonas spp.* Antibiotic susceptibility testing revealed all isolates were resistant to ampicillin, with varying levels of resistance against CZC (98%), CIP (94%), ZOX (88%), CTX (82%), MEM (72%), CN (72%) and TBZ (68%), PCR analysis confirmed the presence of the *bla*$_{TEM}$ gene in 54% (95% CI: 40.4%–67.0%) of the resistant isolates.

Conclusion: In conclusion, the findings indicate that the hostel sanitary drainage system serves as a potential reservoir for MDR Gram-negative bacteria, many of which harbor the *bla*$_{TEM}$ gene. This underscores the urgent need for improved sanitation practices, regular microbial surveillance, strict infection control measures, and rational antibiotic usage to reduce the risk of resistant pathogen transmission in communal living environments.

Key words: Antimicrobial resistance, sink drains, biofilm, lactamase, Kirby-Bauer disk diffusion, and *bla*$_{TEM}$ gene

Introduction

Antimicrobial resistance (AMR) has become one of the top and significant global health threats of the 21st century, negatively affecting the effectiveness of conventional treatment options. World Health Organization (WHO) has listed AMR among the top ten deadly human risks worldwide.[1] According to a systematic analysis in 2021, AMR particularly bacterial resistance has been responsible for about 4.71 million deaths globally.[2] If not overcome timely, it is estimated that by 2050 this trend can increase up to 10 million deaths annually exceeding cancer related death rates. [3] The low- and middle-income countries experience a disproportionate burden of AMR because of resource challenges, inadequate health care, and inaccessibility to diagnostics, misuse and overuse of antimicrobials are intensified.[4]

Many studies have found that beyond the clinical settings, there are some other environmental reservoirs like sink drainage system which home a diverse range of multidrug resistant pathogens which often go neglected.[5,6] Among sanitary system,

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sink drains are considered the primary hotspot for numerous pathogens that become unsusceptible to a large number drugs used against them.[7] During sink usage, bacteria in sink drains can spread to the drain cover area and then expand to the surrounding surfaces and persons through droplet dispersal and cause infectious diseases in them.[8] Hostels with high occupancy and communal facilities, harbor bacteria in their drainage systems and creates a reservoir of the multi-drug resistant gram negative bacteria that endanger the chances of infection among the residents.

Bacteria like *Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis, Acinetobacter baumannii*, *Enterobacteriaceae* and other members of gram negative family have been commonly observed in hostel's sanitary system.[9] There are number of ways which help bacteria to become unsusceptible to antimicrobial compounds. One of the most widespread ways by which Gram-negative bacteria avoid the effects of antibiotics is inactivation and alteration, typically through the synthesis of enzymes leading to the irreversible destruction antibiotics.[10] TEM is an enzyme breaking the beta lactam rings, encoded by *bla*TEM gene is frequently present in the approximately every Gram negative antibiotic resistant bacteria.[11] The other mechanism of antibiotic resistance used by different MDR Gram-negative species is the restriction of entry of antibiotics into the cell to inhibit their activity by using efflux pumps.[12] Moreover, gram negative bacteria can also gain resistance against multiple variety of drugs through the alteration of their outer membrane permeability and biofilm formation.[13,14] Bacteria may develop resistance to antibiotics by altering the structures that the medications aim at them.[15]

The purpose of this study was to establish the prevalence of multidrug-resistant Gram-negative bacteria in hostel sanitary environments with special focus on sink drains as possible reservoirs. It is also aimed at characterizing these isolates by means of phenotypic profiling and biochemical identification. Moreover, the research was also interested in identifying the occurrence of antibiotic resistance gene, *bla*TEM among the resistant bacterial isolates.

Materials and Methods

Sample Collection and Transportation:

The study was conducted between November 2024 and May 2025 at the Institute of Microbiology and Molecular Genetics, University of the Punjab, Lahore. To collect the samples, sterile cotton swabs were used to swab about 60 different sink drains of the university hostel, which include the drains of the kitchen and bathrooms. The swabs were inserted inside the drains through the sink holes and rotated at a 45° angle at the sides of the drain pipes to collect the sample. All samples were collected two hours prior to culture and transported to the lab. During transportation, the temperature was maintained in accordance with all standard protocols.

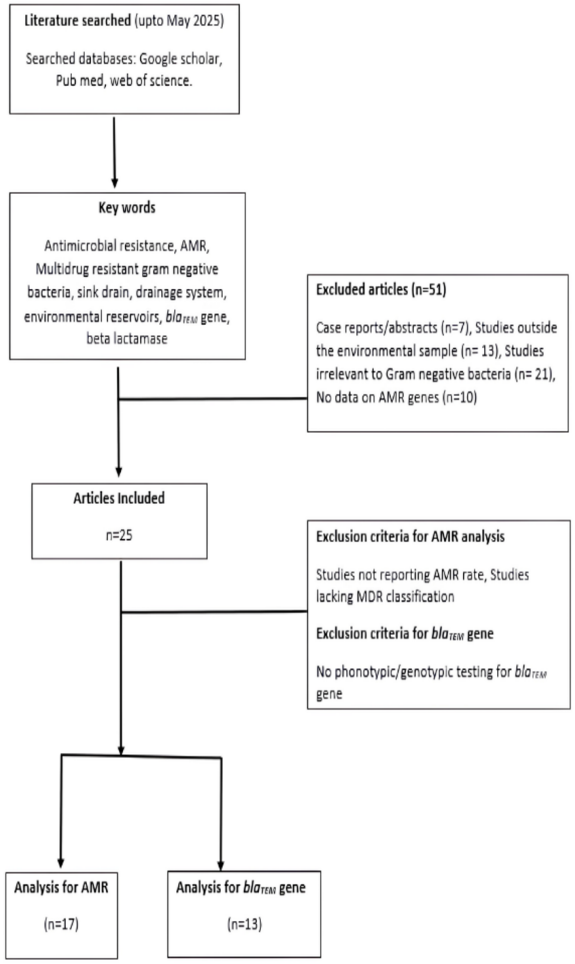

Figure 1: Literature search and study selection process are shown in a flow diagram. Predefined keywords were used in Google Scholar, PubMed, and Web of Science to identify studies up to May 2025. Twenty-five articles were included after applying the inclusion and exclusion criteria of which 17 were analyzed for antimicrobial resistance (AMR) and 13 for the *bla*TEM gene.

Selective Isolation:

All the samples were directly inoculated onto the selective medium, MacConkey's agar containing 10 µg/ml of ampicillin antibiotic, using the quadrant streaking to obtain resistant Gram-negative bacteria. Afterwards, for purification, a small inoculum of a strain was picked and sub cultured on MacConkey's agar plates followed by incubation at 37°C for 24-48 hrs.

Microscopic characterization:

All the samples were observed under a light microscope to study their microscopic features.

Biochemical tests:

For the identification of isolates, a series of biochemical tests were performed which includes; Cytochrome Oxidase Test, Indole Production Test, Methyl Red Test and Voges-Proskauer test in accordance with the standard microbiological procedures given by Cappuccino and Sherman (2013).

Antimicrobial Susceptibility Testing:

To determine the antibiotic susceptibility pattern of isolates, Kirby-Bauer disk diffusion method was performed. Multidrug resistance (MDR) was determined based on the criteria suggested by Magiorakos.[16] Following this criteria, multidrug resistant isolates were identified as those that were non-susceptible to at least one antimicrobial agent in 3 or more antimicrobial categories. According to Clinical Laboratory Standard Institute M100, 34th edition (CLSI, 2024) guidelines, antibiogram profiling was done against a number of recommended antibiotics includes: Ampicillin (10) µg, Chloramphenicol (30 µg), Meropenem (10 µg), Azithromycin (15 µg), Amikacin (30µg), Gentamycin (10µg), Clavulanic acid (30/10µg), Ceftizoxime (30µg), Cefotaxime (30µg), Tazobactam (110µg) and Ciprofloxacin (5µg). Muller–Hinton agar (MHA) plates were prepared, by following all aseptic techniques. *Escherichia coli* ATCC 25922 was used for quality control to assure accuracy and reliability of antimicrobial susceptibility testing. The test strains, whose antimicrobial activity were to be determined, mixed into the autoclaved distilled water and made its turbidity equals to 0.5 McFarland Standard. Then, a sterile cotton bud was dipped into the liquid suspension and swabbed onto the MHA plates. Thereafter, antibiotic discs were applied onto the swabbed MHA plates, which were spaced greater than 30 millimeters apart followed by incubation at 37 °C for 18 to 24 hours. The inhibitory zones produced by antimicrobial drug were measured, interpreted and reported the strains as sensitive, intermediate and resistant. According to CLSI 2024 guidelines the isolates with intermediate sensitivity to the antibiotic were reported as a separate category in the AST analysis and not classified as either resistant or susceptible.

Genotypic characterization:

DNA isolation by using the Heat Lysis Method:

The DNA was extracted by suspending 24-hour fresh colony in 50 µL TE buffer, which was then subjected to heat lysis at 95℃ for 10 min in heat block machine followed by centrifugation at maximum speed for 5 min. Supernatant was taken and used as template DNA for further tests.

Singleplex PCR for TEM gene detection:

The selected isolates were screened for the presence of *bla*$_{TEM}$ gene. For this, a total of 15 µL was used, which comprised 2 µL of DNA, 0.3 µL of forward primer, 0.3 µL of reverse primer, 6 µL of master mix, and enough nuclease-free water to reach the desired volume. The sequence of forward primer reverse primer used were 5'ATCAGCAATAAACCAGC'3 and 3'CCCCGAAGAACGTTTTC'5 respectively. A PCR cycle consists of the following steps; initial denaturation at 95°C for 5min, denaturation at 95°C for 1min, annealing at 95°C for about 1min, extension at 72°C for 1 min followed by final extension at 72°C for 10 min. Finally, the PCR product was obtained and run on 1.5% agarose gel at 90V, 400mA for about half an hour and visualized in a UV-illuminator.

Statistical analysis:

Descriptive statistics were used for analyzing data. The Wilson score method was used to calculate 95% confidence intervals (CIs) for the prevalence of bacterial isolates, antibiotic resistance patterns, and the *bla*$_{TEM}$ gene.

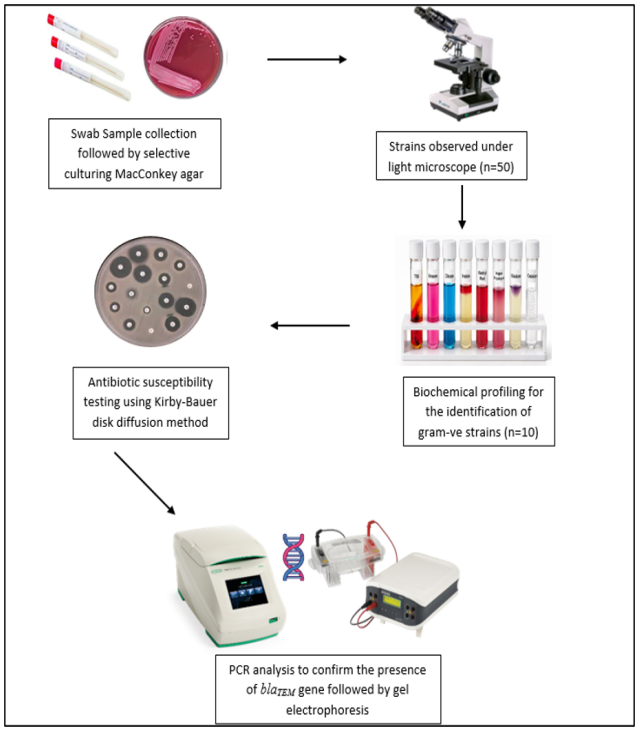

Figure 2: Flowchart of the experimental procedure to isolate, identify and characterize bacterial isolates from swab samples of sink drains. Sterile swabs were used to collect samples that were selectively cultured on MacConkey's agar. Isolated colonies were analyzed in the light microscope (n = 50), and biochemical profiling of Gram-negative strains (n = 10) was done. The Kirby Bauer disk diffusion was used to carry out antibiotic susceptibility testing. PCR was used to confirm target

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resistance gene *bla*TEM and the visualization of the PCR product was done by gel electrophoresis.

Results

Sample Collection: The total of 80 samples were collected. Out of which, 56 (70%) were taken from toilet sink drains, and 24 (30%) were taken from kitchen sink drains at The Girls Hostel, University of the Punjab, Lahore. The strain was given different name codes from W1 to W50.

Selective Isolation:

The selective bacterial isolation was done in all samples. Of these, 50 bacterial isolates that were antibiotic resistant were further phenotypically identified and proceed while excluded the rest sensitive isolates. The growth was observed on MacConkey's agar, of which 36 (72%) strains were colorless colonies, indicating non-lactose fermenters (NLF), and 14 (28%) were reddish pink/rose color colonies, indicating lactose fermenters (LF). Colony morphology was also observed for all strains.

Microscopic characterization: All the isolated strains were observed as gram negative rods under microscope.

Biochemical tests: Strains were identified on the bases of respective biochemical tests. The frequency of the isolated strains showed widespread range of Gram-negative bacterial species with varying percentages. *Escherichia coli* and *Acinetobacter spp*. were the most common ones with 18% each. Both *Pseudomonas spp.* and *Salmonella spp.* were observed as 12% followed by *Enterobacter spp*. and *Stenotrophomonas spp*. 10% each. *Proteus spp*. and *Shigella spp*. were isolated to 8% and 6% respectively. The bacteria which were not observed frequently were *Serratia spp*., 4% and *Aeromonas spp*. 2%. (Table 1)

Antimicrobial Susceptibility Testing: Antibiotic susceptibility test on the isolated bacterial strains showed different levels of resistance against given antibiotics. Notably all the isolated strains were AMP (10µg) resistant. It was found that the 16 strains (32%) exhibited resistance to C (30µg). Resistance against MEM (10µg) and CN (10µg) was found in 36 strains (72%). Moreover, 49 strains (98%) were non susceptible to CZC (30/10µg). For ZOX (30µg), 44 isolates (88%) were resistant. The 41 strains (82%) were resistant against CTX (30µg). Resistance against TPZ (110µg) and CIP (5µg) was found to be in 34 strains (68%) and 47 strains (94%) respectively. The resistance rate was relatively low at a rate of (18%) 9 isolates against AZM (15µg), and 10 isolates (20%) against AK (30µg). (Table 2) (Figure 3)

Genotypic characterization:

DNA isolation by using the Heat Lysis Method:

Both the genetic material and plasmid DNA with different confurigations of strains were isolated. Plasmid DNA was further proceeded because it supposed to have ESBL genes on it.

Table 1: Distribution of Gram-negative bacterial isolates 95% confidence intervals from hostel sanitary systems.

Bacterial isolates	Frequency (n)	Percentage (%)	0.95 CI (%)
Escherichia coli	9	18	9.8–30.8
Acinetobacter spp.	9	18	9.8–30.8
Pseudomonas spp.	6	12	5.6 – 23.8
Enterobacter spp.	5	10	4.3–21.4
Salmonella spp.	6	12	5.6 – 23.8
Proteus spp.	4	8	3.2 – 18.8
Shigella spp.	3	6	2.1 – 16.2
Stenotrophomonas spp.	5	10	4.3 – 21.4
Serratia spp.	2	4	1.1 – 13.5
Aeromonas spp.	1	2	0.4 – 10.5

Table 2: The frequency distribution of antibiotic susceptibility among isolates with 95% confidence intervals for resistance recovered from hostel sink drains samples

Antibiotics	Sensitive (n)	Intermediate (n)	Resistance (n)	0.95 CI (%)
AMP	0	0	50	92.9–100.0
C	29	5	16	20.8–45.8
MEM	24	0	26	38.5–65.2
AZM	39	0	11	12.8–35.2
AK	19	21	10	11.2–33.0
CN	17	15	18	24.1–49.9
CZC	0	1	49	89.5–99.6
ZOX	6	0	44	76.2–94.4
CTX	11	0	39	64.8–87.3
TPZ	16	0	34	54.2–79.2
CIP	3	0	47	83.8–97.9

Singleplex PCR for TEM gene detection:

All the isolated bacterial strains were tested for the presence of *bla*$_{TEM}$ gene using optimized Singleplex PCR protocol. Of all the isolates, 27 (54.0%; 95% CI: 40.4%–67.0%) had the *bla*$_{TEM}$ gene present and this was detected by distinct 516 bp bands. (Figure 4) This gene was found in *Escherichia coli*, *Klebsiella oxytoca*, *Salmonella typhimurium*, *Acinetobacter baumannii*, *Klebsiella pneumonia*, *Salmonella maltophila*, *Pseudomonas aeruginosa*, and *Shigella flexneri*.

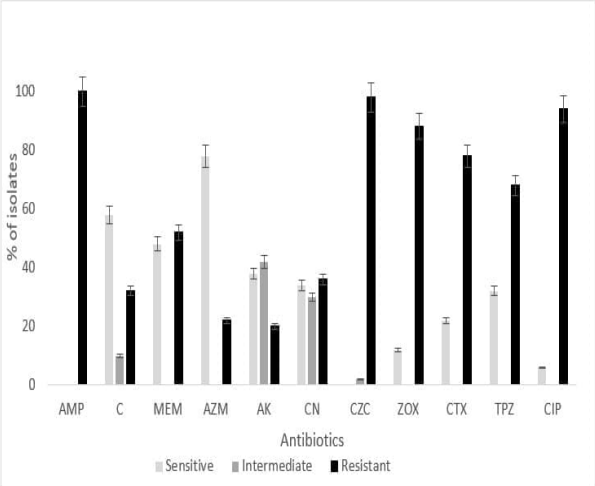

Figure 3: Antibiotic susceptibility patterns among Gram-negative bacterial isolates obtained from hostel's drainage system. The distribution of resistant, intermediate and susceptible has been shown in the form of percentages against each antibacterial drug used in accordance with Clinical Laboratory Standard Institute (CLSI, 2024) guidelines.

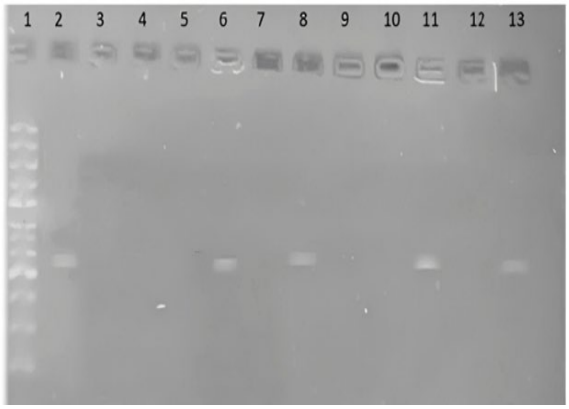

Figure 4: Agarose gel electrophoresis showing singleplex PCR amplification of the *bla*$_{TEM}$ gene (516 bp). Lane 1: DNA molecular weight marker; Lane 6: positive control; Lanes 6, 8, 11, and 13 show positive amplification bands at 516 bp, indicating the presence of the *bla*$_{TEM}$ gene

Discussion

The outcomes of this study emphasize the crucial role of drainage system in spreading the antimicrobial resistance, as the sink drains serve as an important reservoir for multi-drug resistant gram negative bacteria. [17] The high distribution of *Escherichia coli* and *Acinetobacter* (18% each) and other *Enterobacteriacea* in this study reflect their high adaptability to wet drainage conditions of the sinks. This parallel with the recent sink drain surveillance reports in the UK hospitals, e.g., a genomic study in UK hospitals revealed that sink drains are continuously predominant with *Escherichia coli* together with *Klebsiella spp*., and both resistant and susceptible lineages are present.[18] The prevalence of the pathogens in this study is proportional to the global findings of sink drains as a long-term reservoir of drug resistant pathogens, where the biofilms of microbes help them to survive in hostile environments and frequent contamination of various settings. [19]

The observed antibiotic-susceptibility pattern of this study, showed alarming resistance against multiple beta lactamase drugs including 100% resistance to AMP, high resistance to cephalosporins (CZC 98%, ZOX 88%, CTX 82%), and prominent resistance to ciprofloxacin (94%) and meropenem (72%) are congruent with a cross sectional sink drain studies in Sindh, Pakistan reported that carbapenem-resistant *Enterobacteriaceae* were frequently recovered and that resistance rates were high to cephalosporins and fluoroquinolones, which confirms our observation that plumbing biofilms encourage multidrug-resistant strains.[20] Further corroboration of our findings provided by systematic reviews and outbreak studies indicate that drain-related outbreaks are commonly associated with the presence of carbapenemase-producing organisms and the presence of high cephalosporin/fluoroquinolone resistance. [21]

The multi-drug-resistant pathogens reside inside the drainage system are of clinical importance causing several lethal infections. There have been reports of outbreaks of infections due to antibiotic resistant pathogens , include *Klebsiella oxytoca* and *Pseudomonas aeruginosa*, which also have been isolated in this study, associated with contaminated sink drains and plumbing systems.[22] In clinical environments, handwashing sinks contaminated with carbapenem-resistant *Pseudomonas aeruginosa* and *Enterobacteriaceae* have been identified as the direct cause of outbreaks that fail to be eliminated by the structural control measures of sink replacement.[23]

The presence of *bla*$_{TEM}$ gene in 54% of the drain isolates demonstrates a high rate of plasmid-mediated β-lactamase genes in the hostel drainage system. Our results are consistent with a recent cross-sectional investigation of environmental reservoirs of antimicrobial resistance in Lahore, Pakistan, which

found that 44% of sink drain isolates carried *bla*TEM gene with extensive β lactam and carbapenem resistance.[15] There are several possible explanations for the high proportion of antimicrobial resistance of bacterial isolates in this study. Improper and excessive use of antibiotics in the community and health care environments selects for the growth and proliferation of resistant microorganisms. Moreover, sink drains are considered as stable reservoir for the bacteria to grow and colonize in the form of biofilms, which further increases their survival as they shield the them from potent antimicrobial agents and environmental stresses and provide horizontal gene transfer, which contributes to the persistence and dissemination of multidrug-resistant (MDR) bacteria.[24] Biofilm resistomes in hospital sink systems have been reported to be recalcitrant to traditional disinfection methods, implying that these microbial communities cannot be easily removed by standard cleaning protocols.[25]

Several environmental factors may be responsible for the persistence of multi-drug resistance (MDR) bacteria in the hostel drainage system. Constant moisture, accumulated organic residue and continuous flow of wastewater provides favorable conditions for the survival of resistant microorganisms. Furthermore, the use of shared sanitation facilities is often heavily loaded and used regularly, leading to increased spread of resistant microbes into the sink drainage system.

The presence of multidrug resistant bacteria in the drainage system of university hostels is of a major concern with respect to public safety. These organisms could be a potential threat to those residing in the hostels, and make infections more difficult to control and treat. The spread of antimicrobial resistant bacteria in such hostels with large numbers of students living together may have an impact on community health. Hence, it is important to see that these findings should bring to the fore the need for the consideration of university hostel as a significant environment for antimicrobial resistance surveillance and infection control measures.

Limitations:

This study has some limited points including a small sample size of 50 strains in a certain region, which may not adequately represent diversity and resistance patterns of bacteria in other drainage systems. Additionally, *bla*TEM gene alone was screened, and no additional ESBL and carbapenemase genes were investigated. Future research ought to incorporate more varied sampling of various hostels and institutions to represent a wider variety of resistant bacteria. Detecting other resistance genes, like *bla*CTX-M, *bla*SHV, *bla*VIM and carbapenemases, would also offer a better depiction of antimicrobial resistance. Whole genome sequencing, examining the environmental conditions as well as studying biofilm dynamics and plasmid-mediated gene transfer, could be useful in creating specific interventions to reduce the dissemination of multidrug-resistant pathogens in built environments. Furthermore, bacterial identification was done on the basis of conventional biochemical tests, but these routine tests have less discriminatory power for bacterial species. However, molecular identification techniques like 16S rRNA sequencing or MALDI-TOF MS provide more accurate and precise identification of bacterial species. These methods were not performed in this study due to limited resources and funds. But future studies should include these techniques for the validated identification of bacterial isolates.

Recommendations:

Several steps should be taken to prevent the spread of infections cause by multidrug resistant pathogen, and battle drug resistance. The most critical step is encouraging students and staff in hostels to practice regular handwashing and other appropriate hygiene habits. Additionally, it is essential to clean sinks frequently and appropriately with the proper disinfectants to prevent the dissemination and the development of MDR pathogens. Furthermore, implementing antimicrobial stewardship activities in university healthcare systems can help to ensure the responsible use of antibiotics. Last but not the least, regular monitoring and surveys of sink drains and other high-risk areas are necessary to identify emerging resistance strains early and take prompt action to limit infections.

Conclusion

The study concluded that the sanitary system of the hostel is a potential reservoir for the growth and development of gram-negative, multidrug-resistant bacteria which may cause health risk among residents. This combination of phenotypic antimicrobial susceptibility testing and molecular detection of antimicrobial resistance genes can be used to characterize environmental bacterial isolates, thereby providing a comprehensive profile of antimicrobial resistance in non-clinical settings where such information is rarely available. The findings have valuable implications regarding the prevalence of antimicrobial resistance in the environment of university hostels in Pakistan where monitoring of such resistance is limited and also serve as a call to include such environments in antimicrobial resistance surveillance programs. So, regular monitoring of drainage systems, better sanitary practices, infection prevention measures and responsible use of antimicrobials drugs should be emphasized to limit the environmental dissemination of these resistant microorganisms in the community.

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