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Antibiotic-Resistant Bacteria in Drinking Water Sources of Lahore, Pakistan: An Emerging Public Health Concern

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A B S T R A C T

The presence of bacteria resistant to antibiotics in Pakistan is causing a decline in clean and fresh drinking water. Transferable genes are causing antibiotic resistance to spread quickly on a larger scale. This study aims to identify the danger posed by bacteria resistant to antibiotics in drinking water from Lahore and Chichawatni. Samples were inoculated on N agar to determine the CFU/ml count. On MacConkey agar, gram-negative bacteria were isolated. Bacterial species were identified using various biochemical and API kit tests. Several antibiotic discs are used in the antibiotic sensitivity test. The *TEM*, *SHV*, and *OXA* genes have been confirmed through the DNA and plasmid isolation. Out of 25 water samples collected from 11 sites of Lahore and Chichawatni, 100 ampicillin-resistant bacterial isolates were obtained. Among 100 beta-lactam-resistant isolates were 64% Pseudomonas, 15% Enterobacter, 7% Klebsiella, 5% Proteus, 4% Alcaligenes, 2% *Salmonella* and *E. coli* and 1% *Serratia*. Ampicillin-resistant strains showed that 43 out of 100 isolates (43%) were positive for production of beta-lactamases.. All the bacterial isolates were 100% resistant to AMP, 56% to CXM, 32% to TZP, 21% to AK and 10% to IPM. The most predominantly detected beta-lactamase was bla*TEM*, which was reported in 25%, followed by bla*OXA* in 22% and bla*SHV* in 12% bacterial isolates. Plasmid extraction and gel electrophoresis revealed the detection of plasmids in 18% isolates.. Studies have shown that ARB and ARGs are common in drinking water samples across the globe, frequently as a result of inadequate water treatment techniques. Effective water treatment methods, such as boiling, sterilisation, and cleaning of pipes, must be developed and put into place to reduce these risks and determine the frequency and dangers of ARB and ARGs in drinking water.

Keywords: Antibiotic resistance, Drinking water, Waterborne pathogens, Public health, Beta-lactamase.

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INTRODUCTION

Antibiotic-resistant bacteria pose a challenging threat to the health of the general public. (Kristanti and Yudiatmaja, 2022). Safe drinking water is inaccessible to 2.1 billion people, and 2.2 million die yearly due to water-related illnesses (Shayo et al., 2023). WHO has estimated that there will be 4,85,000 annual deaths, and half the world population may face the problem of contaminated drinking water by 2025 (Hamad et al., 2022). Discharging wastewater containing human and animal faeces into fresh water significantly contaminates water (Eusebius et al., 2024). *Pseudomonas aeruginosa* is a gram-negative bacterium responsible for causing cystic fibrosis and hospital-acquired infections like soft tissue, eye and ear infections, infections of the urinary tract, pneumonia and bacteremia (Bogiel et al., 2021).

Coliform bacteria belonging to the family *Enterobacteriaceae* (*Enterobacter*, *Escherichia* and

Klebsiella) are considered an indicator for contamination of drinking water (Singh et al., 2020). *Salmonella, Shigella* and other opportunistic pathogens in drinking water can cause infections and pose a significant health risk (Dhengesu et al., 2022). *Alcaligenes* are gram-negative, opportunistic bacteria known for bacteremia, meningitis, pneumonia, soft tissue and urinary tract infection. It is transmitted as a result of contamination of drinking water with hospital wastewater (Huang, 2020). *Proteus mirabilis* is present in the gastrointestinal tract and enters drinking water due to contamination of drinking water with faeces (Shaaban et al., 2022). Antibiotics are widely used in the treatment of various bacterial infections. It is estimated that if antibiotic resistance continues to proliferate at the current rate, it may lead to an annual mortality rate of 10 million people by 2050 (Haenni et al., 2022). Beta-lactamase-producing bacteria lead to difficulty in medical treatment and prolonged infections (Wang et al., 2021).

Penicillin, carbapenem, monobactam and cephalosporin are significant beta-lactam class antibiotics (Bush and Bradford, 2020). *TEM* and *SHV* are widely reported, while *OXA* and other variants of beta-lactamases are less commonly reported in bacterial isolates (Ajuga et al., 2021). Faecal contamination of drinking water is also a significant problem in Lahore; bacteria are becoming increasingly resistant to antibiotics daily (Mir et al., 2021). Lahore is ranked 2nd amongst the populous cities of Pakistan. WASA supplies water to 12 million people of Lahore, which is not pure due to faults in the sewerage system.

MATERIAL AND METHODS

Collection of samples

Water samples were collected from 18 filter plants in Lahore and five filter plants in Chichawatni in sterile, labelled Eppendorf tubes and transferred to the Institute of Microbiology and Molecular Genetics, University of the Punjab, Lahore, for further processing. Petri plates containing nutrient agar and MacConkey agar were poured with 100µl of water from each labelled Eppendorf. A Glass spreader was used to spread water onto the entire surface of the plate. These plates were incubated at 37ºC for 24 hours. MacConkey agar allowed the growth of gram-negative bacteria only, while nutrient agar plates permitted the growth of bacteria and fungi. CFU/ml was calculated by dividing the number of colonies by the volume of the water sample plated on the petri plate. Isolated colonies were individually picked up by sterile inoculating loop from plates of MacConkey agar and quadrantally streaked on MacConkey agar plates. This helped us obtain pure, isolated colonies of bacterial strains.

Screening of ampicillin-resistant strains (Lee et al., 2021)

Ampicillin was inoculated into MacConkey agar at a concentration of 100µg per ml, and this growth medium was poured onto petri plates to allow growth of only ampicillin-resistant gram-negative bacteria. Bacterial colonies were individually streaked on petri plates and placed in an incubator for one day at 37ºC. Only ampicillin-resistant strains were processed further for identification. Gram-staining was done to further confirm the characteristics of gram-negative bacteria by using crystal violet, Gram iodine and safranin dyes on a fixed smear of bacterial isolates on glass slides. Biochemical tests like triple sugar iron (TSI), indole, methyl red, Voges-Proskauer, citrate, and API-20e helped accurately identify gram-negative isolates.

Antimicrobial Susceptibility Testing

Kirby-Bauer's disc diffusion method was used to evaluate bacterial isolates' sensitivity and resistance patterns. Bacterial colonies were individually inoculated into TSB broth, and their turbidities were compared with a 0.5 McFarland standard. Sterile cotton swabs were used to swab labelled plates of Mueller-Hinton agar (MHA) with that bacterial growth medium. Discs of ampicillin (AMP=10µg), imipenem (IPM=10µg), amikacin (AK=30µg), cefuroxime (CXM=30µg) and tazobactam-piperacillin (TZP=110µg) were placed at a certain distance from one another on the surface of MHA petri plates inoculated with bacteria. These plates were then incubated for one day, and a vernier calliper measured the inhibition zones after 24 hours. The formula calculated the multiple antibiotic resistance (MAR) index of antibiotics to which bacteria are resistant/ Total no. of antibiotics against which it is tested.

Bacteria that resisted at least three antibiotics were classified as multidrug resistant (MDR). MAR index was calculated for all the bacterial isolates. DNA was extracted from bacterial colonies using the boiling lysis method to perform PCR. For this purpose, an isolated colony was placed in an Eppendorf containing 50µl of ampoule water and in a heat block for 10 minutes. Then it was centrifuged for 5 minutes, the supernatant was taken, and the pellet was discarded. Then gel electrophoresis was performed to confirm the presence of DNA bands for each bacterial isolate. Forward and reverse primers of all three variants of beta-lactamases were added along with the master mix to the PCR tubes in which the DNA was previously inoculated. Then, PCR was performed, and gel electrophoresis helped detect bacterial isolates capable of producing either of three beta-lactamases.

RESULTS

Identification of ampicillin-resistant isolates

Out of 25 water samples collected from 11 sites of Lahore and Chichawatni, 100 ampicillin-resistant bacterial isolates were obtained. Among 100 beta-lactam-resistant isolates were 64% *Pseudomonas*, 15% *Enterobacter*, 7% *Klebsiella*, 5% *Proteus*, 4% *Alcaligenes*, 2% *Salmonella* and *E. coli* and 1% *Serratia* (Figure 1). Bacteria were highly frequent in the filter plant of 'CR' and 'B1' Filter plant. No bacterial isolates survived upon inoculating ampicillin into the media from water samples of 'AR', 'B3', and 'B7' water plants. Comprehensive details of contamination of filter plants with antibiotic-resistant bacteria have been given in Table 1.

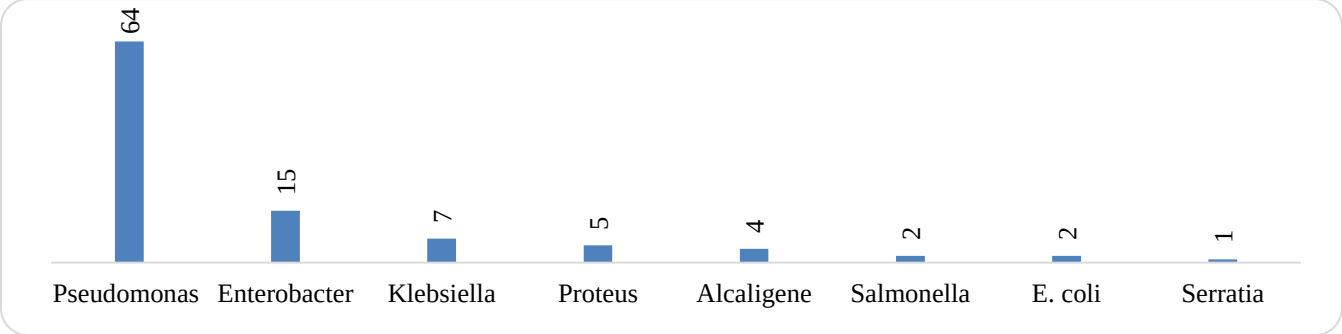

Figure 1: Distribution of ampicillin-resistant bacteria in drinking water.

Table 1: Bacterial isolates recovered from samples and isolates positive for bla_{TEM}, bla_{SHV} and bla_{OXA}.

Sr. No.	Sampling Site	No. of Samples collected	Ampicillin resistant Bacterial isolates	No. of isolates positive for bla_{TEM}	No. of isolates positive for bla_{SHV}	No. of isolates positive for bla_{OXA}
1.	CR	10	64	15	8	16
2.	GB	1	2	0	1	0
3.	BT	2	3	0	0	0
4.	IL	1	4	1	1	1
5.	AR	3	0	0	0	0
6.	RF	3	3	1	0	1
7.	B1	1	18	6	2	4
8.	Block3	1	0	0	0	0
9.	BS	1	0	0	0	0
10.	KM	1	2	1	0	1
11.	B7	1	0	0	0	0

Identification of beta-lactamase producers in water samples

PCR and gel electrophoresis results on all ampicillin-resistant strains showed that 43 out of 100 isolates (43%) were positive for production of beta-lactamases. 'CR' Filter Plant reported production of bla_{OXA} in 25% isolates, bla_{TEM} in 23.4% and bla_{SHV} in 12.5% isolates out of 64 ampicillin-resistant gram-negative bacteria. Water samples from 'B1' Filter plant also demonstrated bla_{TEM} in 6/18 (33%), bla_{SHV} in 2/18 (11%) and bla_{OXA} in 4/18 (22%) of ampicillin-resistant isolates. This trend of beta-lactamase production in bacteria from drinking water shows high transmission of antibiotic resistance in the environment.

Trends of antibiotic resistance

Bacterial isolates demonstrated 10-100% resistance to antibiotics. All the bacterial isolates were 100% resistant to AMP, 56% to CXM, 32% to TZP, 21% to AK and 10% to IPM. MAR index with a value of ≥0.6 was calculated in 22 out of 100 isolates. So, 22% bacterial strains turned out to be multi-drug resistant (MDR) (Figure 2).

Frequency and distribution of bla_{TEM}, bla_{SHV} and bla_{OXA}

The most predominantly detected beta-lactamase was $bla_{TEM,}$ which was reported in 25%, followed by bla_{OXA} in 22% and bla_{SHV} in 12% bacterial isolates. The combination of bla_{TEM} and bla_{OXA} was detected in 10%, bla_{TEM} and bla_{SHV} in 4%, and bla_{SHV} and bla_{OXA} in 3% strains. None of the

three beta-lactamases has been detected in 57% isolates. Plasmid extraction and gel electrophoresis revealed the detection of plasmids in 18% isolates. Plasmid produced circular bands on gel electrophoresis.

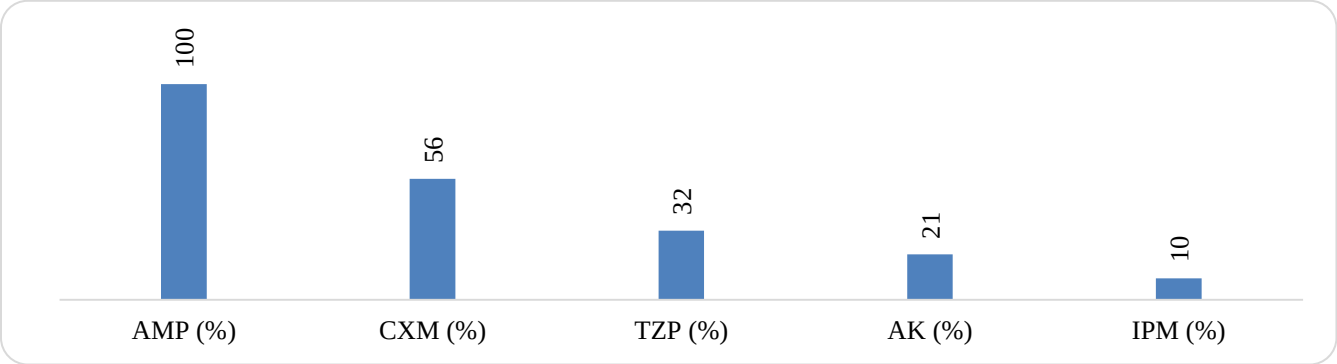

Figure 2: Percentage resistance of bacterial isolates to 5 classes of antibiotics (AMP=Ampicillin, CXM=Cefuroxime, TZP=Tazobactam-piperacillin, AK=Amikacin, IPM=Imipenem).

DISCUSSION

Waterborne infections are responsible for 12.8% of the infant mortality rate, as 27.2 million people don't have access to clean drinking water in Pakistan (Ashraf et al., 2023). Flood-affected areas in Sindh reported 17% diarrhoea and 10% typhoid cases due to drinking contaminated water in 2022 (Ali et al., 2023). Low socioeconomic conditions and overpopulation are the major contributing factors toward the alarming rise in waterborne infections and deaths in the country. Antibiotic-resistant bacteria are highly prevalent in clinical and environmental settings. This study explores antibiotic resistance trends in gram-negative bacteria collected from filtered drinking water in Chichawatni and Lahore, Pakistan. Another study was also conducted to investigate the rate of multidrug-resistant *E. coli* in drinking water (Mir et al., 2021). This study is focused on isolating ampicillin-resistant gram-negative bacteria in drinking water. A similar study also reported a high trend of ampicillin resistance in bacteria isolated from freshwater (Ghadigaonkar and Rath, 2023).

Findings of another study reported 100% resistance in bacteria from drinking water collected from South Benin (Koudokpon et al., 2021). *Pseudomonas* was frequently isolated as a bacteria in water samples, with 64% of all bacterial isolates. Another study conducted on microbiological investigation of water samples in Karachi supported findings of this study by reporting isolation of 66.6% *P. aeruginosa* (Afshan et al., 2021). Spain's Surface water samples confirmed *Pseudomonas aeruginosa's* prevalence in 45.5% samples (Rojo-Bezares et al., 2024). Other findings reported the isolation of 43.3% of Pseudomonas spp. in drinking water samples (Adesoji and Call, 2020). This shows that *Pseudomonas* is more frequently isolated from water samples in this study than in previous research conducted in other countries. This study reported a prevalence of 15% *Enterobacter spp.,* which is somewhat correlated to the results of another research where analysis of household drinking water in South Ethiopia reported isolation of 12% *Enterobacter spp.* (Dhengesu et al., 2022).

Klebsiella was prevalent in 7% of water samples, supported by another research representing the distribution of 7.6% *Klebsiella spp.* among bacteria isolated from stored household water (Chibuike et al., 2023). showing isolation of 5.8% *Klebsiella* in human drinking water collected from India (Jindal et al., 2021). Water samples tested in this study demonstrated 5% *Proteus spp.* which shows same results as another study which reported isolation of 6.45% *Proteus spp.* in water samples of Lahore (Zahra et al., 2022). *Alcaligene spp.* accounted 4% of all resistant bacteria isolated in this study. Another study is correlated to our findings demonstrating prevalence of 5% *Alcaligene spp.* from well water samples of Nigeria (Adeoyo and Omaku, 2022). Low frequency of *Salmonella* and *E. coli* has been observed in water samples of this study. Other studies report a high percentage of *Salmonella* (53%) and *E. coli* (49%) from drinking water samples of Government Schools of Sindh Province (Ahmed et al., 2020). Low prevalence of *Salmonella* and *E. coli* can be attributed to lower contamination of drinking water samples with faecal components. Water samples of Southwest Nigeria demonstrated a prevalence of 2.65% *Serratia spp.,* which exhibits almost the same results as findings of this study reporting 2% *Serratia* in water samples (Aromolaran et al.,

2023).

This study reported 100% resistance to ampicillin among all the bacteria isolated from drinking water. Similar studies support this study's findingdy, as isolates recovered from water samples in Lahore exhibited 100% ampicillin resistance (Hashmi and Jamil, 2023). The resistance rate in bacterial isolates to cefuroxime is 56% in this study, which is similar to the trend of antibiotic resistance in bacteria isolated from potable water in Nigeria, reporting 64% resistance against cefuroxime (Eze et al., 2023). Resistance has been reported in 31% isolates to TZP, while another study investigating the antibiotic resistance profile exhibits a high level of resistance (55%) to TZP in drinking and surface water samples of Odisha, India (Das et al., 2024).

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