U.S.-Pakistan
Centers for Advanced Studies in Water

Identification of Antibiotic Resistant Bacteria in the Different Source Waters of Hyderabad City and its Surroundings

Final Report 2018

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ABBREVIATIONS & ACRONYMS

ARB	Antibiotic-Resistant Bacteria
ARG	Antibiotic-Resistant Genes
CA-SFM	Comitĕ do L`antibiogramma de La Sociētē Française De Microbiologie
CFU	Colony Forming Unit
CLSI	Clinical & Laboratory Standards Institute
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
dl	Deci liter
E. Coli	Escherichia Coli (Bacteria)
EUCAST	European Committee on Antimicrobial Susceptibility Testing
HPC	Heterotrophic Plate Count
M	Molar
MIC	Minimum Inhibitory Concentration
MPN	Most Probable Number
MDR	Multi-Drug Resistant
MUET	Mehran University of Engineering & Technology
MRSA	Methicillin-Resistant *S. Aureus*
n	Number of Samples
PCR	Polymerase Chain Reaction
PSQCA	Pakistan Standards & Quality Control Authority
RO	Reverse Osmosis
S. Aureus	*Staphylococcus Aureus*
SPC	Statistical Process Control

stx	Shiga Toxin
TC	Tetracycline
TDS	Total Dissolved Solids
TEM	Transmission Electron Microscopy
USPCAS-W	U.S.-Pakistan Center for Advanced Studies in Water
USAID	United States Agency for International Development
UV	Ultra Violet
VISA	Vancomycin Intermediate Resistant *S. Aureus*
VRSA	Vancomycin-Resistant *S.Aureus*
w/v	Weight by Volume
WASA	Water & Sanitation Agency
WHO	World Health Organization

EXECUTIVE SUMMARY

Hyderabad is the 2nd largest city of Sindh and 6th most populated city of Pakistan. Like other major cities of Pakistan clean water is major issue for the Hyderabad citizens as well. The groundwater, river water, and municipal water are three major sources for the citizens of this city. Additionally, people also utilize bottled water and in some areas reverse osmosis (RO) plants are also installed for the purification of groundwater. However, majority of Hyderabad population depends on municipal water supply for daily life. In adjacent areas, people directly use river and canal water for washing purposes.

This study was conducted to assess the quality of water in Hyderabad city and its surroundings. A total of 461 samples were collected from different localities of Hyderabad city including Latifabad and Qasimabad and its outskirts (Jamshoro and Kotri). The samples consisted of municipal water (n=277), bore/well water (n=95), bottled mineral water (n=49) and RO plant water (n=40). The samples were analyzed by membrane filtration technique and by most probable number tube method using lactose agar supplemented with a Tergitol 7 TC agar and MacConkey's Broth. Plate count agar was used for heterotrophic plate count (aerobic plate count/ total plate count). Brilliant green lactose bile (BGLB) broth and EC broth were used for confirmation of coliform and fecal coliform bacteria. Selective and differential media, e.g., cetrimide agar, azide dextrose agar, azide dextrose broth and eosin methylene blue were used for isolation and confirmation of *P. aeruginosa*, fecal Enterococci, and *E. coli*. Kirby-Baur antibiotic sensitivity method was used to determine the antibiotics resistance profile of the subject isolates. The polymerase chain reaction (PCR) was used for confirmation of the selected isolates and determination of pathogenicity. The impact of sodium hypochlorite and ultra-violate (UV) radiation on isolated antibiotic-resistant pathogens was also studied.

Microbiological analysis showed that majority of the water samples were not fit for human consumption. According to the World Health Organization (WHO), water used for drinking, cooking, and washing must be free from coliform and fecal coliform bacteria. The present study showed that 39% of municipal water samples carried coliform bacteria whereas 31% samples showed the presence of fecal coliforms. The bore/well water is the other major source of water for Hyderabad and its surrounding population. The results showed that 70% of bore/well water samples were contaminated with total coliform and 49% carried fecal coliforms. For the water samples collected from RO treatment plants; 52% showed the presence of total coliforms and 45% were positive for fecal coliforms. Overall 67% of the RO water samples were also found to carry a high load of heterotrophic bacteria i.e. more than the WHO standard value of <100cfu/

ml. In the case of mineral water samples, 39% of the samples were declared from overleaf not fit for human consumption by the higher load of heterotrophic bacteria. Moreover, these samples also found to carry highly pathogenic and multiple antibiotic-resistant bacteria.

Out of 461 samples, 80 showed the presence of *E. coli*. About 93% of the isolates showed resistance to 3 tested antibiotics, while 67% isolates exhibited resistance to 4 antibiotics, and 51% were resistant to 5 antibiotics. The *P. aeruginosa* strains were recovered from 60 samples. These isolates were confirmed as *Pseudomonas* and *P. aeruginosa* by growth characteristics on differential and selective media, (e.g, cetrimide agar) and reconfirmed by amplification of *oprI* and *oprL* gene. Antibiotic sensitivity profiling showed that majority of these isolates exhibited multidrug resistance. All of these isolates showed resistance to at least one out of 22 tested antibiotics, whereas 76% showed resistance to 4 antibiotics and 47% isolates exhibited resistance to 6 antibiotics. About 55% of isolates exhibited resistance to carbenicillin.

Furthermore, 80 (17.35%) samples showed the presence of fecal Enterococci. One of the fascinating observation of this study was the detection of vancomycin-resistant Enterococci. About 5% (n=4) isolates showed vancomycin resistance. These isolates were resistant to 17 out of 22 tested antibiotics. Moreover, 51% of isolates exhibited multiple antibiotic resistances, and they were also resistant to 13 antibiotics at a time. Other major isolates recovered from water samples of Hyderabad and its surroundings were *Vibro* species (n=90), *Shigella* species (n=70), *Kleb. Pneumonia* species *(*n=45) and *Proteus* species (n= 51). All of these pathogens exhibited resistance to the majority of commonly used antibiotics. In addition to multiple antibiotic resistance, some isolates of *E. coli* were found to harbor genes for *Shiga* toxins (e.g *Stx2)*, which makes it more harmful pathogen. Majority of *P. aeruginosa* isolates and Enterococci species were found to carry genes responsible for biofilm formation, e.g., *pelB, pilT, pilA,* and *rhlI.* The biofilm producers are highly resistant and difficult to control.

Interestingly, the majority of these pathogens were found too sensitive to sodium hypochlorite. About 90% reductions were noticed in colony forming units of *S. aureus*, Enterococci, Shigella, and *E. coli* after exposure to 0.1 mg/ml of sodium hypochlorite. Similarly, UV disinfection was also found to be effective method for water purification.

1. INTRODUCTION

Water is a basic need of life, and according to the new sustainable development agenda of the United Nations, safe drinking water supply must be assured for everyone. This project is designed to evaluate the quality of drinking or tape water in terms of bacterial contamination, antimicrobial resistance, and other physicochemical parameters. Bacterial contamination of water resources and waterborne infections remains a major threat all over Pakistan (Daud *et al*; 2017). It is estimated that 30% of all diseases and 40% of all deaths occur due to poor water quality in Pakistan (Country Report Pakistan, 2000).

Most of the Hyderabad population has no access to clean drinking water, and this is one of the major causes of morbidity and mortality. According to a recent research report majority of the water samples collected from Hyderabad and other cities of Pakistan were found to carry a high load of coliform bacteria (Memon *et al.,* 2010). Guidelines for drinking water published by WHO recommended that *E. coli* or thermo-tolerant coliform bacteria must not be detectable over 1µg/ml in all water used for drinking or cooking purposes (WHO, 2008). According to the study by Jabeen *et al*. (2015), only 13% of water sources are safe for human consumption, and majority (68%) of the water bodies are not safe for human consumption due to bacterial contamination. The studies by Memon *et al*. (2016) and Daud *et al. (*2017) have reported that majority of the water supply for big cities of Pakistan carried a high load of the chemical as well as microbial contamination.

The bacteria have strong ability to resist antibiotics naturally. When antibiotics fail to kill the bacteria it is due to development of the resistance mechanism by adapting the changes in their cell structures and through metabolism for the future antibiotics to resist. These bacteria acquire resistance through genetic mutation or modification of present genetic matter or gaining of new genetic medium (Rai et *al*., 2012). In the present era, antibiotic resistance in bacteria is increasing at a rapid pace, which poses a major challenge for scientists to resolve this problem (Rai *et al.,*. 2012). In the United States, more than 70% of bacterial infections are resistant to one or more antibiotics applied for their disinfection. The people infected by andtibiotic resistante bacteria (ARBs) spend a lot of time in hospitals for their proper treatment, requiring the use of two or three additional antibiotics, which can become more toxic, less effective and expensive (Webb *et al*., 2005). There could be two major reasons for the abundant release of ARBs into the aquatic environment, i.e. excessive usage of antibiotics and the usage of a conventional system for disinfection or absence of disinfection process in the water treatment system. The re-growth of bacteria after disinfection is the main problem in the disinfection process, therefore after the treatment, the residual

persistence of the disinfectants should be observed because the bacteria can recover from an injured phase and re-grow under certain conditions (Florentino *et al*., 2015).

It is because of widespread use of antibiotics through the wrong diagnosis of diseases, improper prescriptions from doctors and usage of antibiotics by the people without consulting the doctors, that even the treatments to common diseases are becoming more difficult. Due to the continuously evolving nature of bacteria and their gaining resistance to antibiotics, the people infected by such bacteria remain ill for longer period (John and McGrowan, 2006). These days, antibiotic resistance is a major human health challenge for the whole world. The bacteria have acquired the ability to survive under antibiotic treatment by having different antibiotic resistance genes (ARGs). The ARGs are of great concern because they have mobile genetic elements which help them to transfer themselves into different microbes through the horizontal gene transfer method and the transformation could occur to convert the non antibiotic resistant into living ARBs. Thus, horizontal gene transfer is the main culprit in aggravating the antibiotic resistance in bacteria (McKinney and Pruden, 2012). When bacteria adopt antibiotic genes, they can exist for longer periods. The ARBs are capable of transferring their genes to the human pathogens, and with time, human pathogens become antibiotic resistant, causing a more dangerous situation which poses a great threat to human health (Xiong and Hu, 2013).

Wastewater treatment plants play a vital role in the protection of human health as well as for aquatic life by treating the highly-contaminated water. The presence of ARBs in wastewater in abundance decreases the efficiency of wastewater treatment plants wherein high concentration of ARBs can increase horizontal gene transfer among them, thus creating multi-drug resistant (MDR) bacteria. Proper disinfection mechanism not only inactivates ARBs, but it can also inhibit the horizontal gene transfer in ARGs (McKinney and Pruden, 2012). As DNA absorbs ultraviolet (UV) radiation, it can disinfect ARBs. UV disinfection process can limit the disinfection by-products; it is non-corrosive for the water treatment system as well as for the water distribution system (McKinney and Pruden, 2012).

We know that the antibiotics are used to inhibit the growth of pathogenic micro-organisms, but due to excessive usage of antibiotic(s) by humans, the bacteria have adjusted themselves against it and decreased the efficiency of antibiotics to cure the infections. These conditions not only change the selectivity and morphology of antimicrobial agents but also change their physiological features. Bacteria can go through mutations when they are exposed to antibiotics for a greater period; they become resistant to those antibiotics and transfer their genes to other colonies of bacteria through horizontal gene transfer, thus making other bacteria and the

environment resistant to antibiotics. These mutations may change the characterization of advantageous bacteria; for example cyanobacteria which can produce more than one-third of free oxygen and are responsible for CO_2 fixation, may change their behavior in the future gene transfer from ARBs (Martinez, 2009).

Different antibiotics that are found in the ecosystem at higher concentrations can also be found in water, i.e. (sewage water and surface water) and soil, i.e. (farm soils and the soils treated with manure). The higher concentration of antibiotics is related to the human activity areas, while the impeccable environments have a low concentration of antibiotics. So the risk assessment should be carried out in the areas with high antibiotics concentration and the areas having a greater number of associated human pathogens (Baquero *et al.,* 2008). According to one study (Livermore, 2005), antibiotics usage for farming and clinical purposes ultimately gives rise to antibiotic-resistant micro-organisms, so it is predicted that the waste and residues from the farm and hospitals would contain ARBs as well as ARGs.

There are lots of natural compounds which serve as antibiotics and have been in contact with the micro-organisms from hundreds of centuries, are biodegradable and become a source of food for other micro-organisms (Dantas *et al.,* 2008). However, synthetic antibiotics, i.e., amoxicillin, quinolones, norfloxacin, etc. can show more refraction towards antibiotics and their degradation rates in the natural environment are also different. For instance, the degradation of oxolinic acid in river water in five months was 20%, while ciprofloxacin took three months to completely degrade in river water (Turiel *et al.,* 2005). When quinolone binds with soils and sediments its biodegradation time increases but the quinolones-polluted water in wastewater treatment does not only undergo biodegradation, but it can also be removed by the photodegradation process (Martinez, 2009). The degradation of antibiotics in the natural environments does not suggest that antibiotics are not relevant pollutants because they degrade at different rates during different seasons of the year and that the moisture content and composition of the soil also impact the degradation of antibiotics in the surface water (Stoob *et al.,* 2007).

Moreover, the ecosystems which continuously suffer from a large amount of antibiotics release, show relatively less amount of antibiotics degradation and are polluted constantly. The presence of excessive antibiotics modifies the metabolic activity of micro-organisms present in the polluted environment. The impact of antibiotics on bacterial colonies will remain even after the degradation of antibiotics has occurred. If the usage of antibiotics is banned, then it will vanish as a pollutant from the environment as stated by Rai *et al*. (2012). The dilution of sewage water causes a reduction in the plasmid-encoded ARGs in *E. coli* when it is thrown into the lakes

and rivers. The reduction in the usage of antibiotics for farming purpose showed a reduction in the antibiotic resistance in animals and its transfer to human beings, but the decline of antibiotic resistance is a very slow process, and some part of the antibiotic resistance remains and causes re-growth of ARBs after a passage of time (Martinez, 2009). Antibiotic resistance can be observed in human pathogens surviving in the environment which has no history of antibiotic contamination, i.e. people and animals living in the remote areas do not receive a significant amount of antibiotics but are exposed to antibiotic resistance. The spread of antibiotics in the soil is low as compared to water and antibiotic-resistant genes form a genetic platform and replicate to move to different ecosystems, so these genes do disappear when their release is stopped and may spread into bacterial populations without reducing their concentration (Martinez, 2009). The antibiotics used to cure infectious diseases in human beings or used for the rapid growth of animals and plants do not metabolize completely, and these are discharged in large quantities along with excreta, either to a waste-water treatment plant or directly to surface water (Dolliver and Gupta, 2008). According to World Health Organization (WHO, 2000), the increment and strength of antibiotic resistance in human pathogens is a strong concern because due to the excessive usage of antibiotics the bacteria are adjusting themselves into the antibiotic contaminated environment and with the passage of time these offer resistances to antibiotics. Once the bacteria become resistant to antibiotics in the ground water and surface water like lakes, rivers, ponds, and streams, the water treatment processes are unable to remove such bacteria, thus leading to harmful health impacts on human beings and animals (Martinez, 2009).

1.1 Objectives of the Study

The main objective of this study was to identify antibiotic-resistant bacteria present in the waters of Hyderabad city and its surroundings and their disinfection mechanism. To achieve this, the following specific objectives were set:

- ☐ To identify antibiotic-resistant bacteria (ARBs) present in the waters of Hyderabad city and its surroundings by using molecular techniques.
- ☐ To disinfect ARBs presence in the water of Hyderabad city by using different disinfectants and their disinfection kinetics.

2. MATERIALS AND METHODS

2.1 Collection of Water Samples

A total of 461 samples were collected from different localities of Hyderabad city including Qasimabad & Latifabad, and its outskirts (i.e., Jamshoro and Kotri) from July 2016 to December 2017. The samples consisted of tap water (n=277), bore/well water (n=95), RO plant water (n=40) and mineral bottled water (n=49). Samples of 2.5 liters each were collected from the sampling sites in sterile bottles and transported to the laboratory in the icebox and analyzed within 6 hours of the collection. The chlorinated samples were obtained from the municipal water supply and 2.5 ml of 10% (w/v) solution of sodium thiosulfate ($Na_2S_2O_3$), (Merck-Darmstadt, Germany) was added to each sampling bottle to prevent the chlorination process during transportation.

2.2 Qualitative Analysis

Water samples were processed as described by Rand *et al.* (1976) and ISO-9308 (1998). In brief, three tubes of double strength MacConkey's broth (OXOID Ltd, Basingstoke, UK) with Durham tubes were inoculated with 10 ml of water sample separately and two sets of three tubes of single strength with 1.0 ml and 0.1 ml respectively. These samples were later incubated at 35°C for 48 hrs. The growth of coliform in the water samples are indicated by the production of acid along with gas in the Durham test tubes. Tubes showing a sign of coliform were identified, and the most probable number (MPN) was calculated according to MPN tables in accordance with the WHO guidelines (WHO, 2008).

2.2.1 Confirmatory test for coliform and fecal coliforms

For coliform and fecal coliforms test, 1 ml from each positive tube of presumptive Coliforms and fecal coliforms was inoculated in Brilliant Green Lactose Bile Broth (BGLB) (OXOID-Ltd, Basingstoke-UK) tube and EC broth (OXOID-Ltd, Basingstoke-UK) tubes and incubated in water bath for 24 hrs at 35°C and at 44.5°C respectively. Tubes with gas and turbidity were considered positive. BGLB tubes indicated the presence of coliforms and EC broth tubes indicated the presence of thermotolerant fecal coliforms (ISO-9308, 1998).

2.2.2 Isolation of *E. coli* from water samples

Each sample of 100 ml was filtered through a 0.45 µm cellulose membrane filter (Durapore-Cork-Ireland) and placed on Lactose TTC agar (bioMérieux, Italia), and the plates were incubated at 35°C for 24 hrs. Yellow colonies on Lactose (TTC) agar were picked and streaked on EMB agar for confirmation of the presence of *E. coli* (ISO 9308-1, 2000).

2.2.3 Isolation of *Enterococcus* spp. from water samples

Azide dextrose broth was used for the standard presumptive test of fecal *enterococcus* species. Three tubes of double strength azide dextrose broth (OXOID-Ltd, Basingstoke-UK) with Durham tubes were inoculated with 10 ml water sample (in each tube) and two sets of three tubes of single strength with 1.0 ml and 0.1 ml, respectively. After 48 h incubation at 35°C, production of acid and the presence of gas in any of the Durham tube indicated the presence of Enterococci. The inoculums from positive tubes were sub-cultured in Bile Esculin Azide Agar (OXOID-Ltd, Basingstoke-UK) and incubated at 37°C. The brown-black colonies indicated the growth of Enterococci (Pinto *et al.,* 1999).

2.2.4 Isolation of *Pseudomonas aeruginosa* from water samples

100 ml of each sample was filtered through a 0.45µm cellulose membrane filter, placed on Pseudomonas Cetrimide agar (OXOID-Ltd, Basingstoke-UK); and the plates were incubated at 35°C for 48 hrs. The green colonies were isolated on Plate Count Agar (bioMérieux Italia) at 37°C for 24 hrs (Tsoraeva and Martinez, 2000). After the oxidase test (bioMérieux Italia), the species identification was confirmed through PCR.

2.2.5 Isolation of *Vibrio spp.* from water samples

Presumptive tests for recovery of *Vibrio spp.* and *V. cholera* were performed with 1 ml of each dilution (10^{-1} to 10^{-3}) that was inoculated in alkaline peptone water (OXOID-Ltd, Basingstoke-UK) containing 1% NaCl having pH of 8.5, followed by incubation for 24 hrs at 35°C. Spread-plating of 0.1 ml made the confirmatory tests by inoculation from tubes with positive growth onto thiosulfate citrate-bile salts-sucrose (TCBS) agar (OXOID-Ltd, Basingstoke-UK), and followed by the incubation for 24 hrs at 35ºC. Orange-yellow colonies were streaked on TSA (OXOID-Ltd, Basingstoke-UK) and incubated at 35°C for further 24 hrs. After 24 hrs single isolated colonies were transferred into 1, 3 and 6 % sodium chloride tryptone water, and incubated at 35°C for seven days. After every 24 hrs, tubes were checked for turbidity. These analyses were based on the phenomena that, if the growth is positive in 0 and 3 % and negative in 6% NaCl solution, it indicated the presence of *V. cholera* (Costa *et al.,* 2010).

2.3 Antibiotic Sensitivity Testing (Kirby Bauer-Disk Diffusion Method)

Antibiotic sensitivity testing was performed using commercially available antibiotic discs in accordance with Kirby-Bauer methods chartered by the Clinical & Laboratory Standards Institute (CLSI-VET01-A4, 2013). The identified strains were tested for antibiotics resistance to the following antibiotics; piperacillin (TPZ), ciprofloxacin (CIP), erythromycin (E), polymixin B (PB), cefuroxime Na (CXM), ampicillin (AMP), bacitracin (B), colistin sulphate (CT), imipenem (IPM), chloramphenicol (C), gentamycin (CN), clarithromycin (CLR), meropenem (MEM), linezolid (LZD) and tetracycline (TC) (CLSI-VET01-A4, 2013).

2.4 Disinfection

2.4.1 Disinfection by sodium hypochlorite (Chlorination)

Initially sodium hypochlorite doses 0.05, 0.1, 0.2, 0.5 and 5 mg/L were adjusted in de-ionized distilled water. Then a known concentration of overnight grown cells of subject isolates was added to 100 ml sterilized de-ionized distilled water solution containing a known concentration of chlorine. After inoculation (final concentration 1×10^5 CFU/ml), inactivation kinetics was studied for 1 hr at different intervals (0.5, 1, 5, 10, 30, and 60 mins). Immediately after the sampling, residual chlorine was neutralized with an equal volume of 0.02 M sodium thiosulfate ($Na_2S_2O_3$). Each experiment was performed thrice. All the results were expressed in milligrams of chlorine as Cl_2 per liter (Gomes *et al.,* 2016).

2.4.2 Disinfection by ultraviolet (UV) radiation

The subject isolates were inoculated into pre-sterilized water at **a** final concentration of 1×10^5 CFU/ml and exposed to UV lamp for 1hr. The samples were collected from the outlet at different intervals, i.e. 0.5, 1, 5, 10, 30 and 60 minutes. The standard plate count was done by pour plate technique using 10-fold dilutions. 1 ml of each dilution was poured (in duplicates) in empty and sterilized Petri-dishes. About 15 ml of plate count agar (kept at 45°C in a water bath) was then added to each plate. Plates after solidification were incubated at 35°C for 48 hrs. Plates containing 30-300 colonies were counted to determine the specific plate count (SPC) per ml of the sample tested (Johnson *et al.,* 2010).

2.4.3 Disinfection by silver nanoparticles

Antibiotic-resistant bacteria were disinfected by using silver nano-particles of 10 nm particle size observed through Transmission Electron Microscopy (TEM) in 0.02 mg/ml in the aqueous buffer containing sodium citrate as a stabilizer (Sigma Aldrich USA).

Silver nanoparticles were washed with saline water thrice, vortexed for 10 mins and centrifuged at 10,000 rpm. This procedure was repeated three times. The particles were suspended in dimethyl sulfoxide (DMSO) at 0.02 mg/ml for stock solution. The antimicrobial activities were determined by modified agar well diffusion assay. Tryptone soya agar was poured in pre-sterilized Petri dishes, and the bacteria were inoculated on those plates. The media on the plates were punched with 6 mm diameter hole and filled with different dilutions ranging from 8 - 128 μg/ml of silver nanoparticles from the stock solution. DMSO was used as negative control and streptomycin discs (10 μg/ disc) were used as positive controls. These Petri dishes were incubated at 37^0C for 24 hours and observed for growth of bacteria.

2.5 Molecular Identification by PCR

2.5.1 Genomic DNA extraction

Bacterial genomic DNA was extracted from phenotypical and biochemical tested subject isolates by using a colony PCR method as described by Mirhendi *et al. (*2007). In brief, single isolated colonies from TSA were picked and suspended in 200 µl sterilized distilled water in a 1.5 ml Eppendorf Tube to obtain a suspension of 1-2× 10^9 cells/ml. This suspension was vortexed and subjected to heat treatment at 80°C for 10 mins and centrifuged at 10,000 rpm at 4°C for 10 mins. The supernatant was discarded, and the pellet DNA was collected in a sterile tube and stored at -20°C.

2.5.2 Polymerase chain reaction (PCR)

PCR reaction comprised of 25 µl reaction mixture, 12.5 µl Taq Master Mix, 0.5 µl each of reverse and forward primer, 9 µl sterile MilliQ water and 2.5 µl of Genomic DNA. The DNA was amplified at different annealing temperatures depending upon the nature of primer/gene.

2.5.3 Molecular characterization of *P. aeruginosa*

The molecular characterization of *P. aeruginosa* isolates was carried out according to the methods described by Al-Ahmad and Roodsari (2016) using *oprI* and *oprL* genes specific primers and *rhlI, pilT, pilA,* and *pelB* genes as described by Galil *et al.* (2013). Primer list and sequences are mentioned in Table 2.1, whereas details of PCR conditions are mentioned in Table 2.2.

2.5.4 Molecular characterization of *E. coli*

For the molecular characterization and identification *stx1, stx2, Eae, Hlya*, and *espP* genes were targeted as described by Schmidt *et al. (*2000). Primer sequences and PCR conditions are described in Tables 2.3 and 2.4, respectively.

Table 2.1: List of selected primers used for characterization of *P. aeruginosa* isolates

Gene	Rev. Primer sequence	Fwd. Primer sequence	Amplicon size (bp)
oprI*	5'-CTT GCG GCT GGC TTT TTC CAG-3'	5'-ATG AAC AAC GTT CTG AAA TTC TCT GCT-3'	249
oprL*	5'-CTT CTT CAG CTC GAC GCG ACG-3'	5'-ATG GAA ATG CTG AAA TTC GGC-3'	504
rhlI**	5'-GCG AAG ACT TCC TTG AGC AG-3'	5'-CTC TCT GAA TCG CTG GAA GG -3'	245
pilT**	5'-GTC CTG GAT GGT GAG GAT GT-3'	5'-CTT GGC ATG GGA GTG TT -3'	156
pilA**	5'-CCG TCC TAC CAG GGT TAC CT – 3'	5'-ACT GTT GGT CGT CTT CC-3'	160
pelB**	5'-AGT CGT TGG GAT TGG ACT TG-3'	5'-CGC CTG CTC TGG TTC TAC AT-3'	190

* Al Ahmad and Roodsari (2016)

** Ghalil *et al.* (2013)

Table 2.2: The primers and conditions of PCR for *P. aeruginosa*

Targets	Initial Denaturation		Denaturation		Annealing		Extension		Final extension	
	Temp (°C)	Time (mins)	Temp (°C)	Time (mins)	Temp (°C)	Time (mins)	Temp (°C)	Time (mins)	Temp (°C)	Time (mins)
oprI	94	5	94	1	55	1	72	1	72	1
oprL	94	5	94	1	55	1	72	1	72	1
RhlI	94	5	94	30	55.5	30	72	1	72	10
pilT	94	5	94	30	55.5	30	72	1	72	10
pilA	94	5	94	30	55.5	30	72	1	72	10
pelB	94	5	94	30	55.5	30	72	1	72	10

2.5.5 Molecular characterization of fecal Enterococci isolates

The molecular characterization of fecal Enterococci isolates was carried out according to the methods described by Asmat *et al.* (2014) using specific primers for *rpoA* for the confirmation of Enterococci. According to Naser *et al.* (2005), *rpoA* genes can be used as reliable tools for identification of clinical and environmental species of *Enterococcus* and are efficient screening methods for the detection of novel species. Moreover, *espP* and *gel* gene amplification were carried out to know the origin or source of isolates and its biofilm formation capacity. The *espP* gene is responsible for biofilm formation and adhesion of Enterococci, whereas *gel* is used to identify environmental and clinical isolates (Asmat *et al.,* 2014). Details of the primer sequences and PCR conditions are mentioned in Tables 2.5 and 2.6, respectively.

Table 2.3: Molecular characterization of *E. coli* (Ref: Schmidt *et al.*, 2000)

Gene	Name	Sequence
stx1	*Shiga* toxin 1	5'-CAGTTAATGTGGTGGCGAAGC-3' 5'-CACCAGACAATGTAACCGCTG-3'
stx2	*Shiga* toxin 2	5'-ATCCTATTCCCGGGAGTTTACG-3' 5'-GCGTCATCGTATACACAGGAGC-3'
Eae	Intimin	5'-CCCGAATTCGGCACAAGCATAAGC-3' 5'-CCCGGATCCGTCTCGCCAGTATTCG-3'
Hlya	α hemolysin	5'-GGTGCAGCAGAAAAAGTTGTAG-3' 5'-TCTCGCCTGATAGTGTTTGGTA-3'
espP	Extracellular serine protease	5'-AAACAGCAGGCACTTGAACG-3' 5'- GGAGTCGTCAGTCAGTAGAT-3'

Table 2.4: The primers and conditions of PCR for *E. coli*

Primer designation	Target gene	Length	PCR conditions					
			Denaturation		Annealing		Extension	
			Temp (ºC)	Time (mins)	Temp (ºC)	Time (mins)	Temp (ºC)	Time (mins)
lp30/lp31	*stx1*	348	94	0.5	57	1	72	1
lp43/lp44	*stx2*	584	95	0.5	57	1	72	1
sk1/sk2	*eae*	863	96	0.5	52	1	72	1
hlyA1/hlyA4	*hlya*	1551	97	0.5	57	1	72	1.5
espA/espB	*espP*	1830	98	0.5	56	1	72	2.5

Table 2.5: Molecular characterization of fecal Enterococci isolates (Asmat *et al.*, 2014)

Gene	Forward primer sequence	Reverse primer sequence	Amplicon size (bp)
rpoA	5'- ACHGTRTTRATDCCDG-CRCG-3	5'-ATGATYGARTTT-GAAAAACC-3'	*
gel	5-TATGACAATGCTTTTTGG-GAT-3	5-AGATGCACCCGAAATAATA-TA-3	213
espP	5-AGATTTCATCTTTGAT-TCTTGG-3	5-AATTGATTCTTTAGCATCT-GG-3	510

* As per standard

Table 2.6: Conditions for DNA amplifications

Target gene	Initial denaturation		Denaturation		Annealing		Extension		Final extension		Gel %
	Temp (°C)	Time (min)	Temp (°C)	Time (min)	Temp (°C)	Time (min)	Temp (°C)	Time (min)	Temp (°C)	Time (min)	
rpoA	95	4	94	1	56	1	72	1	72	7	2
gel	95	5	94	1	56	1	72	1	72	7	2
espP	95	5	94	1	56	1	72	1	72	7	1.5

3. RESULTS AND DISCUSSION

3.1 Quality of Water Samples Collected from Hyderabad and its Surroundings

The present study showed that majority of the water samples collected from different localities of Hyderabad city carried a high load of coliforms and fecal coliforms along with heterotrophic bacteria (Tables 3.1 and 3.2).

Table 3.1: Microbiological quality of water samples collected from different localities of Hyderabad.

Types of drinking water tested					
Parameters	**RO plant water**	**Mineral bottled water**	**Municipal water***	**Bore/well water**	***P* value**
	(n=40)	(n=49)	(n=277)	(n=95)	
Mean ± SD					
HPC (cfu/ml)**	1.67 ± 0.47	1.38 ± 0.49	1.69 ± 0.46	1.87 ± 0.33	<0.05
Total coliform/dl	1.52 ± 0.52	1.28 ± 0.45	1.39 ± 0.49	1.68 ± 0.46	<0.05
Fecal coliform/dl	1.45 ± 0.50	1.16 ± 0.37	1.32 ± 0.46	1.49 ± 0.46	<0.05

* Municipal water includes water directly collected from households water coolers, treatment plants and taps.

** Heterotrophic plate count

Table 3.2: Water samples (%) not fit for drinking according to WHO criteria based on heterotrophic plate count (HPC), total coliform and fecal coliform

Types of drinking water tested and result according to WHO criteria					
Parameters	**RO plant water**	**Mineral bottled water**	**Municipal water***	**Bore/well water**	**WHO Criteria+**
	(n=40)	(n=49)	(n=277)	(n=95)	
HPC(**) (cfu/ml)	67%	39%	69%	87%	<100cfu/ml
Total coliform/dl	52%	28%	39%	68%	<1/dl
Fecal coliform/dl	45%	16%	31%	49%	<1/dl

* Municipal water includes water directly collected from households water coolers, treatment plants and taps.

** Heterotrophic plate count

\+ WHO (2008)

The municipal water samples (n=277) were found to carry a high load of heterotrophic bacteria, 39% of the samples showed the presence of coliforms and 31% were contaminated with fecal-coliforms. Overall, 69% of the municipal water samples from Hyderabad were not fit for human consumption according to WHO guidelines (Table 3.2). These findings are also in agreement with the previous study by Nabeela *et al. (*2014). A study by Kalhoro *et al.* (2014) reported that water samples collected from Hyderabad and its surroundings were highly contaminated and carried a high load of *E. coli*, Enterococci*,* and *Salmonella spp.* and that all of the municipal water samples were unfit for human consumption. Similarly, Patoli *et al.* (2010) also reported that municipal water of Hyderabad city was highly contaminated with *E. coli* and that it is not recommended for human consumption in its present state. In a survey report published by Ahmed *et al*. (2013), they reported a similar situation in the nearby city of Badin. They described that all of the samples collected from municipal water supply carried a high load of coliform and fecal coliform bacteria.

In case of the samples collected from RO processing units, 67% samples were rejected by the total bacterial count whereas 52% showed the presence of coliforms and 45% samples carried fecal-coliforms (Table 3.2). Similarly, 39% of bottled or commercial water samples were also found to carry a high load of heterotrophic bacteria with 28% of the samples showing the presence of coliforms (Table 3.2). This is a very alarming situation because RO plant water and bottled water is always considered as a safe option and majority of the hospitals and patients prefer to use it. Khatoon and Pirzada (2010) reported that 67 out of 187 bottled water samples collected from Karachi were found to be unfit for human consumption while 39 samples were contaminated with *P. aeruginosa*.

It is of utmost interest to know the reasons for the poor quality of the water from RO plants. In this regard, few RO plants were visited in the vicinity and found that most of these were not following the WHO guidelines and that bottled water was exposed to elevated temperatures for a long time which could be a possible reason for bacterial growth. In a study by Rind *et al.* (2014), it was reported that 14 water filtration units were operational in Hyderabad and that the samples from 13 of these units were contaminated with coliform bacteria and were declared adulterated.

Furthermore, it was found that most of the RO and other commercial water treatment plants were violating the set guidelines for equipment maintenance and calibration. Majority of the treatment plants were installed in small shops with open doors, where all activities were performed, e.g., washing and filling of bottles, and storage of processed and unprocessed water. Besides that, all the RO plants were operated by non-professionals and only total dissolved salts (TDS) status is used to predict

the water quality. Unfortunately, none of the RO plants had written documents for membrane checkup. According to Pihlajakuja *et al.* (2017), RO membrane is a favorable surface for bacterial growth as initially, the nutrient concentration is higher near the membrane due to concentration polarization; secondly the flow of water in the spiral membrane module is laminar leading to no excess shear forces; and lastly the water flow continuously brings new nutrients for the bacteria to feed on.

To sum up, all these factors are responsible for bacterial growth in RO treated water. In addition to this, all the RO system operators were not using pretreated water. They just use the municipal or groundwater without any pretreatment, which consequently leads to impurities like organic carbon, polysaccharides and bio-fouling agents' deposits on the membrane and results in damage and high load of bacteria in the product water. It may be noted that RO system is very effective and reliable, but it requires a change in the attitude and behavior of plant operators/managers to apply required safety protocols and follow the effective working guidelines of WHO or Pakistan Standard Quality Control Authority (PSQCA) to avoid any bacterial contamination. Most of the water supply of big cities of Pakistan was found to carry a high load of the chemical as well as microbial contamination.

According to the observations made during this study, there are many sources of contamination of municipal water supply systems. One of the major causes is the sewerage system which runs parallel to the drinking water supply lines. Poor monitoring and maintenance of old pipelines causes leakages and intermixing of water regularly. The leakage and overflow of the sewerage system are also responsible for the introduction of microbial and chemical contamination in groundwater bodies. Another source of bacterial contamination of water supplies is the animals. Hyderabad city is located on left bank of the Indus River, which is the only major source of water for the city. It was observed that people use rivers and canals for animals bathing as well as for vehicle and cloth washing as a routine practice. Also, the domestic wastes from households and the sewage are also dumped to the rivers/canals without any treatment which increases the pollution and bacterial load. In a similar study on Ravi River in Pakistan, Qureshi *et al.* (2011) reported that only 4 out of 50 (8%) samples were found to be fit for drinking while the rest were polluted and contaminated with high bacterial load in accordance with the WHO standards. Despite this situation, most of the Hyderabad population is still using this water without any proper treatment. In a survey report by Baig *et al.* (2017), they reported that a clear majority of the citizens (42.8%) were using treated water (mostly boiled water) while 34% were utilizing untreated drinking water, 14.2% used bottled water and the rest 9% of the respondents were using the groundwater as a source of drinking water.

3.1.1 Common pathogens in drinking water

Pathogen assay of the water samples collected from Hyderabad showed a high load of major pathogens, i.e., *E. coli, P. aeruginosa, Vibrio spp., Shigella* spp., & fecal Streptococci or Enterococci (Table 3.3). These pathogens are a major source of waterborne infections. Various studies reported that water samples collected from Karachi were also found to carry a high level of pathogens like Klebsiella, *P. aeruginosa, E. coli,* and *S. aureus* (Amin, 2014; Yousuf *et al.,* 2014). This suggests that waterborne pathogens may be a common problem in big cities all over Pakistan.

Table 3.3: Common pathogens recovered from water samples collected from Hyderabad.

Bacteria	Drinking water samples with bacteria (%)
***E. coli* (n=80)**	17.35
***P. aeruginosa* (n=60)**	13.02
***Vibrio spp.*(n=90)**	19.52
***Staphylococcus spp.* (n=25).**	5.42
***Enterococci spp.* (n=80)**	17.35
***Shigella* spp. (n=70)**	15.18
***Kleb. Pneumoniae* (n=45)**	9.76
***Proteus* spp. (n= 51)**	11.06

Over 17% of the samples (17.35% to 19.52%) carried pathogens like *E. coli*, *Vibrio spp. and* Enterococci *spp.* Other pathogens found in the water samples included *Staphylococcus spp.* (5.42%), *Proteus* species (11.06%), *Kleb*. *Pneumoniae* (9.76%) and *P. aeruginosa (*13.02%). These results were also confirmed by PCR through amplification of target primers (Table 3.3).

Overall, majority of the pathogens stated in literature were recovered from the municipal water samples. In a study conducted by Rasheed *et al*. (2009), vast majority of the water samples collected from different cities of Azad Kashmir, Pakistan showed the presence of highly pathogenic strains of gram positive and negative bacteria. Moreover, antibiotic sensitivity profile of these isolates suggested that all of these pathogens exhibited multi-drug resistance. Normally a waterborne bacterium exhibits a low level of antibiotic resistance as compared to hospital-acquired pathogens. However, the present study showed that majority of *P. aeruginosa* and *E. coli* isolates were resistant to more than six major antibiotics.

Similarly, Enterococci were also resistant to majority of the available antibiotics. The highlight of the present study is the recovery of methicillin-resistant *S. aureus* (MRSA)

and vancomycin-resistant *S. aureus* (VRSA). Out of 25 isolates, one strain exhibited oxacillin and vancomycin resistance (Table 3.4). Normally, this pathogen is restricted to hospitals. This isolate was harboring with *mecA* gene responsible for methicillin/ oxacillin resistance in *S. aureus* and other staphylococci (Wielders *et al.,* 2002; Wiersma *et al.,* 2009). However, it is *vanA* negative. The *vanA* gene is responsible for vancomycin resistance stated by Perichon and Courvalin (2009). Although MRSA and VRSA are normally restricted to clinical setup, there are some reports about community-acquired MRSA and VRSA (Icgen, 2016; Nakipoğlu *et al.,* 2017). The other isolates also exhibited multidrug resistance but not to a critical level.

Table 3.4: Antibiotics sensitivity profile of *Staphylococcus aureus*

Antimicrobial agent	Disc content	Resistant isolates	Zone diameter interpretive criteria	MIC interpretation criteria
Ertapenem[(b)]	*	4	*	*
Ampicillin[(b)]	*	3	18	-
Gentamycin[(a)]	*	1	12	16
Carbenicillin	*	5	*	*
Erythromycin[(a)]	*	3	13	8
Optochin	*	2	*	*
Chloramphenicol[(a)]	30µg	1	12	32
Colistin sulphate[(b)]	*	3	*	*
Cefoxitin[(b)]	*	1	*	*
Clarithromycin[(a)]	*	3	13	8
Vancomycin[(a)]	30µg	1	-	16
Amoxycillin / Clavulanic acid 2:1 (AMC)[(b)]	30µg	1	*	*
Cefpirome	30µg	1	*	*

- Indicates that interpretive criteria are not applicable

* As per standard.

a CLSI

b EUCAST

3.2 *P. aeruginosa*

P. aeruginosa is a gram-negative pathogen. It may survive in a wide range of environments from hospital to natural waters. It is normally present in natural water, e.g. lakes and rivers; however, it is not common indweller of drinking water (Meena and Gerba, 2009). Moreover, antibiotic sensitive strains of waterborne *P. aeruginosa* are not a serious problem. Unfortunately, majority of *P. aeruginosa* isolates recovered from Hyderabad water samples exhibited multidrug resistance (Table 3.5). Out of 461 samples, 60 showed the presence of *P. aeruginosa* strains. These isolates were confirmed as Pseudomonas and *P. aeruginosa* by growth characteristics on differential and selective media, (e.g. cetrimide agar, Fig. 3.1-B) and reconfirmed by amplification of *oprI* and *oprL* (Table 3.6) genes as described by Al-Ahmad and Roodsari (2016). The *oprI* gene is targeting I lipoproteins specific for *Pseudomonas* genus and *oprL* targeting L lipoproteins specific for *P. aeruginosa* (Al-Ahmad and Roodsari, 2016). Antibiotic sensitivity profiling showed that majority of these isolates exhibited multidrug resistance. All of these isolates recovered from Hyderabad water system showed resistance to at least one out of 22 tested antibiotics (Table 3.5). Whereas, 76% showed resistance to 4 antibiotics while 47% isolates resisted 6 antibiotics and 55% of isolates resisted carbenicillin antibiotic (a carboxypenicillin semi synthetic penicillin) (Table 3.5).

Ertapenem is a member of carbapenem group of antibiotics (Codjoe and Donkor; 2017). It is used for the treatment of severe, life-threatening infections. Unfortunately, 35% of isolates of *P. aeruginosa* recovered from water samples of Hyderabad city were found resistant to ertapenem (Table 3.5). It is possible that ertapenem resistant *P. aeruginosa* isolates may also exhibit cross-resistance to carbenicillin and other beta-lactam antibiotics (Livermore et al., 2005). It was noted that 31% of these isolates exhibited resistance to ampicillin while 41% to clarithromycin. 21% of the subject isolates also exhibited resistance to cefpirome, a 4th generation cephalosporin (Table 3.5). It is a general concept that environmental isolates of *P. aeruginosa* are comparatively less pathogenic and sensitive to common antibiotics (Alonso et al., 1999). It is not anticipated for the subject isolates of *P. aeruginosa* to exhibit multidrug resistance. Interestingly, these isolates were resistant to second-line antibiotics like carbenicillin, ertapenem, and clarithromycin. 41% of these isolates exhibited resistance to clarithromycin (Table 3.5), which is a semi-synthetic macrolide antibiotic. Majority (31%) of these isolates also exhibited resistance to chloramphenicol and bacitracin (32%). The most effective antibiotics against subject isolates of *P. aeruginosa* were levofloxacin, and cefuroxime. Only one isolate showed resistance to levofloxacin and two isolates were resistant to cefuroxime (Table 3.5). This study suggested that antibiotic resistance is a common phenomenon of waterborne isolates of *P. aeruginosa*. This is also in agreement with

Table 3.5: Antibiotic resistance profile of *Pseudomonas aeruginosa*

Antimicrobial agent	Disc content	Resistant isolates	Zone diameter interpretive criteria	MIC interpretation criteria
Tetracycline[a]	30µg	7	-	≥16
Aztreonam[a]	*	6	-	≥32
Polymyxin B[a]	*	7	≤11	≥8
Ciprofloxacin[a]	5µg	8	≤15	≥4
Levofloxacin[a]	*	1	≤13	≥8
Impenem[a]	*	8	-	≥16
Amoxycillin / Clavulanic acid (2:1) (AMC)[b]	30µg	8	-	-
Cefpirome	*	13	*	*
Cefoxitin		13	*	*
Ertapenem[b]	*	21	-	-
Ampicillin[b]	*	19	-	-
Gentamycin[a]	*	7	≤12	≥16
Carbenicillin[a]	*	33	-	≥64
Erythromycin[b]	*	18	-	-
Meropenem[a]	*	6	≤15	≥8
Chloramphenicol[a]	*	19	-	≥32
Bacitracin	*	14	*	*
Cefuroxime Na[a]	*	2	-	≥64
Colistin sulphate[a]	*	4	≤10	≥8
Cefpriome	*	13	*	*
Clarithromycin[b]	*	25	-	-
Cephradin[c]	*	9	*	*

- Indicates that interpretive criteria are not applicable

* As per standard.

a CLSI

b EUCAST

c CA-SFM

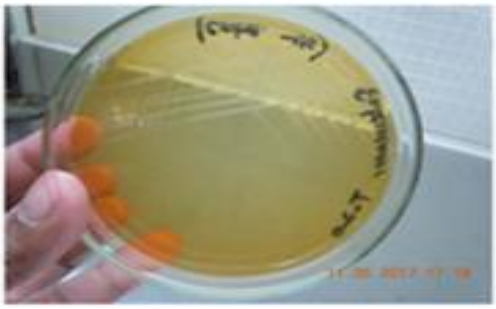

Fig. A. Selective media for enterococci

Fig.B. Selective media for Pseudomonas

Fig. C. Selective media for vibrio

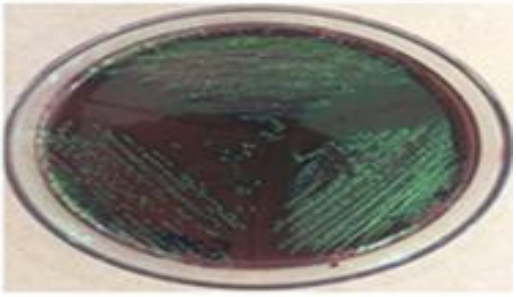

Fig. D. Selective media for E.coli

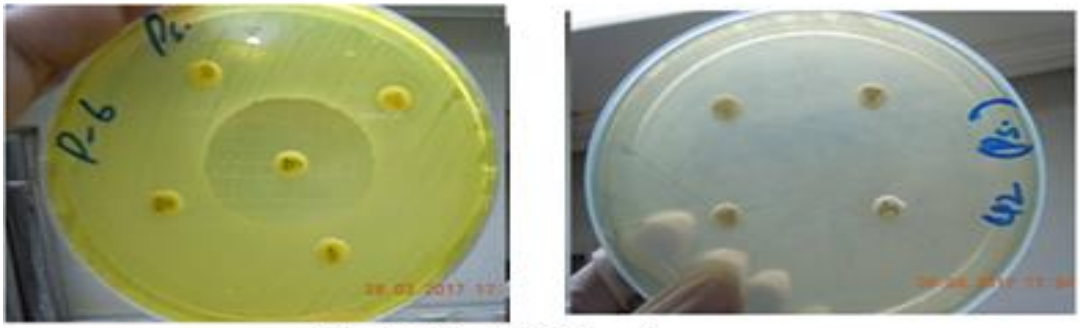

Fig. E. Disc Diffusion Assay

Fig. 3.1: A-D represent bacterial pathogens cultured on the selective media. Disc diffusion assay plates are shown in E.

the work proposed by Kittinger *et al.* (2016). Blasco *et al*. (2009) reported that a total of 60 isolates of *P. aeruginosa* recovered from natural water reservoirs and four from industrial cooling towers exhibited multiple antibiotic resistance. In this regard, Kittinger *et al*. (2016) suggested that antibiotic resistance can be acquired by and persists even in *Pseudomonas* species that are normally not in direct contact with the humans. A possible scenario is that these bacteria provide a reservoir of antibiotic resistance genes that can spread to the related human pathogens by horizontal gene transfer. Furthermore, majority of the subject *P. aeruginosa* were biofilm producer, which could be the possible reason for multidrug resistance in these isolates. It was

further confirmed by the amplification of *pelB*, *pilT*, and *rhlI* genes (Table 3.6). Majority of these isolates were found to harbor the *pelB* gene. It is also reported that *pelB* provides protection against antibacterial agents and increases the survival capability of *P. aeruginosa* isolates in harsh environments by adhesion or biofilm formation (Colvin et al., 2011). The other property of these multi-drug resistant isolates of *P. aeruginosa* is the presence of *oprL* gene (Fig. 3.2). Majority of the isolates harboring *pelB* were also found to carry *rhlI* gene (Table 3.6). The *rhlI* gene along with *pilT* and *pilA* has a major role in survival and persistence of *P. aeruginosa* infections and found traces of *pilT* in this research (Fig. 3.3). According to Galil *et al*. (2013) *rhlI*, *pelB*, *pilT*, *pilA* gene of *P. aeruginosa* are working in a coordinated manner; *rhlI* gene coding for quorum sensing, *pilA* and *pilT* are responsible for motility and *pelB* is suggested to be involved in adhesion and biofilm formation. The present study suggested that the isolates recovered from different water samples exhibited multidrug resistance as well as these are equipped with various tools to survive in low nutrient environment prevailing in drinking water.

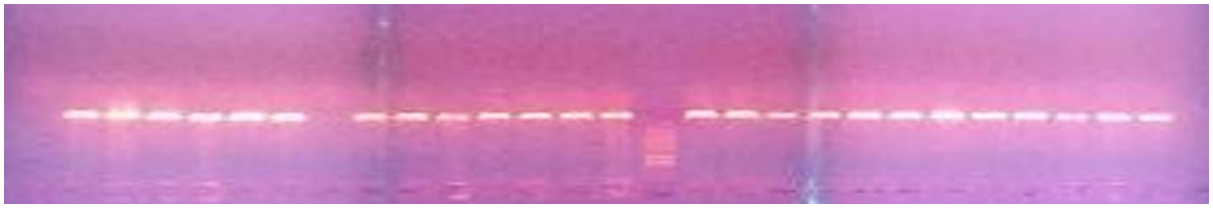

Fig. 3.2: PCR results depicted the presence of oprL gene for confirmation of *Pseudomonas and P. aeruginosa*

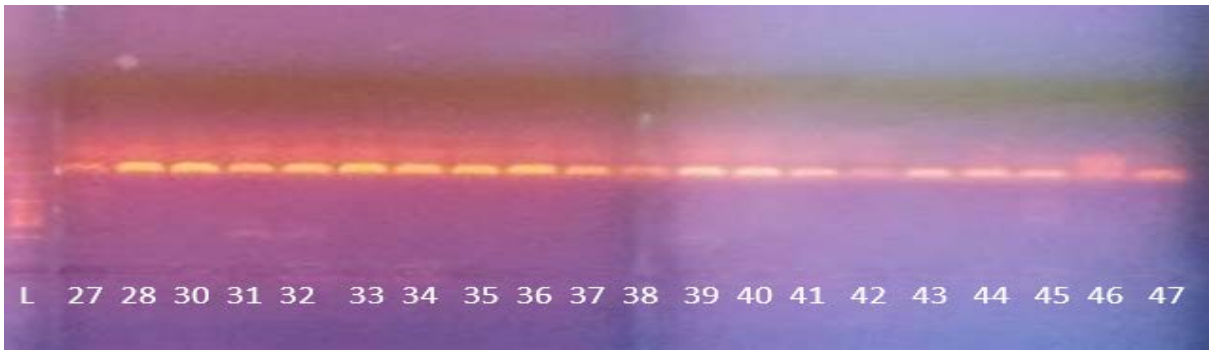

Fig. 3.3: PCR results indicate the presence of *pilT* gene responsible for multidrug resistance and biofilm formation in *P. aeruginosa.*

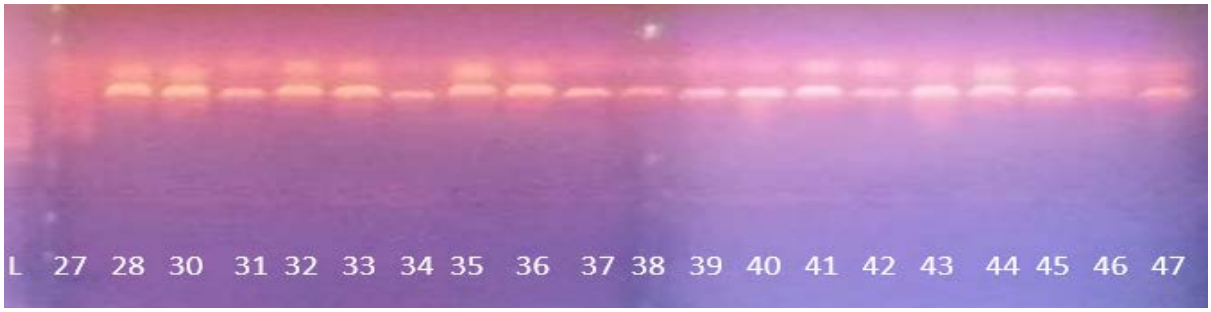

Fig. 3.4: **PCR results indicate the presence of *rhlI* gene responsible for biofilm formation in *P. aeruginosa.***

Table 3.6: **Molecular identification of Pseudomonas and *P. aeruginosa***

Type of water	*oprI*	*oprL*	*pelB*	*rhlI*	*pilT*	*pilA*
Tap Water	+	+	+	+	+	+
Well water	+	+	+	+	+	+
Well water	+	+	+	+	+	+
Well water	+	+	+	+	+/-	+
Well water	+	+	+	+	+	+
Well water	+	-	-	+	+	+/-
Well water	+	+	+	+/-	+	+
Tap Water	+	+	+	+/-	+/-	-
Tap Water	+	+	+	+	+	+
Tap Water	+	+	+	+	+	+
River Water	+	+	+	+	+	+
River Water	+	+	+	+	+	+
River Water	+	+	+	-	+	-
River Water	+	-	+	-	+/-	+
River Water	+	+	+	+	+	+
River Water	+	+/-	-	-	-	-
Bottle water	+	+	+/-	+	-	-
Well water	-	-	-	-	+	-
Well water	+	+	+	+	+	+
Well water	-	-	-	-	+	-
Tap Water	+	+	+	+	+	+
Bottle water	+	+	+	-	-	-
Tap Water	+	+	+	+	+	+
Tap Water	+	+	+	+	+	+
Tap Water	+	+	+	+	+	+
Bottle water	+	+	+	+	+	-

Tap Water	+	+	-	-	+	-
Tap Water	+	+	+	+	+	+
Tap Water	Neglected	Neglected	Neglected	Neglected	Neglected	Neglected
River Water	+	+	+	+	+	+
River Water	+	+	+	+	+	-
Bottle water	+	+	+	+	+	-
Tap Water	+	+	+	+	+	+
Tap Water	+	-	+	+	+	-
Bottle water	+	+	+	+	+	+
Tap Water	+	+	+	+	+	+
Tap Water	+	+	+	+	+	+
Tap Water	+	+	+	+	+	+
River Water	+	+	+	+	+	-
River Water	-	+	+	+	+	+
River Water	+	+	+	+	+	+
Tap Water	+	+	+	+	+	+
Tap Water	+	+	+	+	+	+
River Water	+	+	-	-	+	-
River Water	+	+	+	+	+	+
River Water	+	+	+	*	*	+
Bottle water	+	+	-	*	*	-
Tap Water	+	+	-	*	*	-
Tap Water	+	+	+	*	*	+
Tap Water	+	+	-	*	*	-
Tap Water	+	+	-	*	*	-
Tap Water	+	+	-	*	*	-

+ Shows the presence of subject isolates

- Represents no trace of subject isolates

+/- Shows weak relationship (cannot be identified)

* Not applicable

3.3 *E. coli* Isolates

E. coli is a useful indicator of fecal conamination and is not considered as a human pathogen being a part of healthy human's intestinal flora (Davis, 1996). *E. coli* has an important role in food, water microbiology and diagnostics. The presence of coliforms and *E. coli* indicates the presence of pathogenic organisms which brings in questions about the efficiency and integrity of the water supply system. In this study, 461 water samples of Hyderabad and its surroundings were analyzed according to WHO guidelines (Table 3.1). The *E. coli*, coliform, fecal coliforms and fecal Enterococci were used as an indicator.

These samples were divided into four categories, e.g., water purified through reverse osmosis, bottle or commercial water, tap water and bore/well water. The presence of total coliforms and fecal coliforms in these samples were observed in Lauryl sulfate broth tubes by the growth and gas production. The BGLB was used for the confirmation of coliform at 35^{o}C, and EC broth was used for detection of fecal coliforms.

Although, E. coli and fecal-coliforms are usually nonpathogenic, however, they can cause significant diarrhea and various intestinal diseases (Carbal, 2010). Recent reports showed that member of Enterobacteriaceae family *E. coli* have acquired or developed resistance to various important antibiotics (Munita & Arias, 2016). Out of 461 samples, 80 (17.35%) showed the presence of *E. coli.* Eosin methylene blue (EMB) agar media was used for the identification and confirmation of *E. coli.* The typical colonies of *E. col*i showed characteristics of green metallic sheen on EMB agar due to rapid fermentation of lactose (Fig 3.1). Oxidase and other biochemical reaction tests were used for further confirmation. Antimicrobial sensitivity profile showed that majority of these isolates exhibited multidrug resistance i.e. 93% isolates showed resistance to 3 of the tested antibiotics, while 67% of isolates exhibited resistance to 4 antibiotics and 51% were resistant to 5 antibiotics (Table 3.7). About 67% of the subject isolates of *E. coli* isolates showed resistance to carbenicillin, and 48% were resistant to ampicillin (Table 3.7). The other least effective antibiotics were cefpirome, bacitracin, and ertapenem (Table 3.7). In a study, Chen et al. (2017) also reported that multidrug-resistant *E. coli* prevails in water. Most of the isolates were resistant to tetracycline, followed by ampicillin, piperacillin, trimethoprim/sulfamethoxazole, and chloramphenicol (Chen et al., 2017). The results of this study showed that majority of the Hyderabad waterborne isolates were also resistant to gentamycin (22.5%), erythromycin (20%), ciprofloxacin (12.5%) and tetracycline (15%).

Similarly, Shah and Zehra (2014) showed that water samples collected from Islamabad and its surroundings carried multidrug-resistant bacteria. The studies from other parts of Pakistan also support these findings. Samra *et al.* (2009) reported that drinking water samples of Lahore city carried a high load of multidrug-resistant *E. coli.* This seems to be a chronic issue as Patoli *et al*. (2010) also reported that a vast majority of *E. coli* recovered from water samples collected from Hyderabad were multidrug resistant.

In this study, the isolates exhibited not only multiple antibiotic resistances but also carried various pathogenic markers. Molecular characterization of these isolates indicates that this isolate belongs to different pathogenic groups of *E. coli* (Table 3.8). It is a well-known fact that *Shiga* toxin *Stx* producing strains of *E. coli* O157: H7 and *E. coli* O157: NM are major human pathogens causing a variety of human diseases, e.g.

Table 3.7: Antibiotic sensitivity profile of *E. coli* and *Shigella spp.*

Antimicrobial agent	Disc Content	Test results (no. of isolates)		Zone diameter interpretive criteria	MIC interpretation criteria
		E. coli	*Shigella* spp.		
Tetracycline[a]	30µg	12	17	≤11	≥16
Doxycline[a]	*	7	0	≤10	≥16
Ertapenem[b]	*	23	65	≤18	≥2
Ampicillin[a]	10µg	39	40	≤13	≥32
Gentamycin[a]	*	18	15	≤12	≥16
Carbenicillin	*	54	80	*	*
Erythromycin[b]	*	16	14	-	*
Meropenem[a]	*	8	39	≤ 19	≥16
Chloramphenicol[a]	*	15	45	≤12	≥32
Bacitracin	*	23	15	*	*
Cefuroxime[a]	*	6	0	≤14	≥32
Colistin sulphate[b]	*	4	0	-	≥2
Cephradin[c]	*	10	22	*	*
Impenem[a]	*	8	14	≤19	≥4
Cefpirome	*	33	39	*	*
Ciprofloxacin[a]	5µg	10	16	≤15	≥4
Polymixin B [b]	*	4	4	*	*
Aztreonam[a]	30µg	7	8	≤17	≥16
Levofloxacin[a]	*	10	8	≤13	≥8
Clarithromycin [b]	15µg	20	37	*	-
Amoxycillin / Clavulanic acid 2:1 (AMC)[b]	30µg	9	6	*	≥8

- \- Interpretive criteria are not applicable
- * As per standard.
- a CLSI
- b EUCAST
- c CA-SFM

Table 3.8: Pathogenic genes *Shiga Toxins (Stx)* detection *in E. coli* isolates recovered from Hyderabad water samples

S#	Water type	*LT*(ETEC)	*pet* (EAEC)	*astA*(EAEC)	*Stx2*(EHEC)
1	Bottled drinking water	-	-	+	-
2	Bottled drinking water	-	-	-	-
3	Bottled drinking water	-	-	+	-
4	Tap drinking water	-	-	-	-
5	Bottled drinking water	-	+	-	-
6	River Water	-	+	-	-
7	River Water	-	-	-	-
8	River Water	-	-	-	-
9	River Water	-	-	-	-
10	River Water	-	-	+	-
11	River Water	-	-	+	-
12	River Water	-	-	-	-
13	Tap Water	-	-	-	-
14	Tap Water	-	-	-	-
15	Tap Water	-	-	-	-
16	Tap Water	-	-	-	-
17	Tap Water	-	-	-	-
18	Tap Water	-	-	-	-
19	Tap Water	-	-	+	-
20	Tap Water	-	-	-	-
21	RO plant water	-	-	-	-
22	Tap Water	-	+	+	-
23	Tap Water	-	-	-	-
24	Bottled Water	-	-	-	-
25	Tank water	-	-	-	+
26	Tap Water	-	-	-	+
27	Tap Water	-	-	-	+
28	Tap Water	-	+	+	+
29	Tap Water	-	-	-	-
30	Tap Water	-	+	+	-
31	Tap Water	-	+	+	-
32	Tap Water	-	-	-	-
33	Tap Water	-	-	-	-
34	River Water	-	-	-	-
35	River Water	-	+	+	+

36	River Water	-	-	+	-
37	River Water	-	-	-	-
38	Tap Water	-	+	+	+
39	Well Water	-	-	-	-
40	Well Water	-	-	-	-
41	Well Water	-	+	+	-
42	Tap Water	-	-	-	-
43	RO Water	-	-	-	-
44	RO Water	+	-	-	-
45	Tab Water	-	+	-	-

\+ Shows the presence of subject isolates

\- Represents no trace of subject isolate

diarrhea. *Stx* is one of the most potent bacterial toxins known. It is found in *Shigella* dysenteriae 1 and in some serogroups of *E. coli* called *Stx1* in *E. coli*. In addition to or instead of *Stx1*, some *E. coli* strains produce a second type of *Stx*, *Stx2*, that has the same mode of action as *Stx* or *Stx1* but that is anti-genically distinct (Villaseca *et al.,* 2000). Therefore, in this study, the Stx gene was targeted. Six out of 45 isolates were found to carry the *Stx2* gene (Fig 3.6). This indicates that these isolates can be lethal for humans, particularly for infants. All these isolates were found to be negative for Stx1.

Interestingly, these isolates exhibited multiple antibiotic resistances that may further complicate its treatment. Another characteristic feature of some of these isolates is the presence of *pet* toxin. Pet toxin is a serine protease from entero-aggregative *E. coli,* and it has been described as causing neurotoxic and cytotoxic effects (Villaseca *et al.,* 2000). However, as mentioned above, majority of these isolates were *Stx* negative (Fig 3.6) which suggests that these virulence factors are not correlated.

Some of these isolates also carry heat stable entero aggregative heat stable toxin, which is confirmed by the amplification *astA* gene (Fig 3.5). Further, 10 isolates were found to carry *pet* gene where as 13 isolates harbor astA gene, indicating that these toxins are major pathogenic factors of entero aggregative *E. coli*. These results indicate that drinking water of Hyderabad is contaminated and carries highly pathogenic strains of *E. coli*. Likewise, the majority of *Shigella spp.* was also multidrug resistant. This finding suggests that the majority of waterborne pathogens carry multiple antibiotic resistances and could be fatal for the consumers exposed to polluted water in this area.

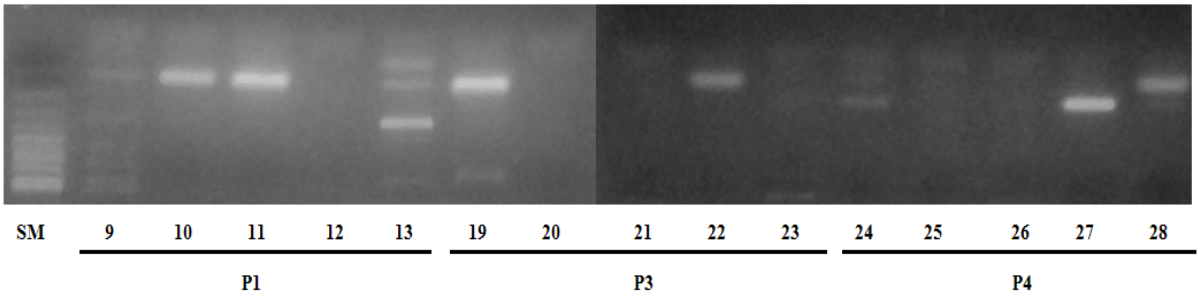

Fig. 3.5: PCR results showing astA gene positive isolates of *E. coli.*

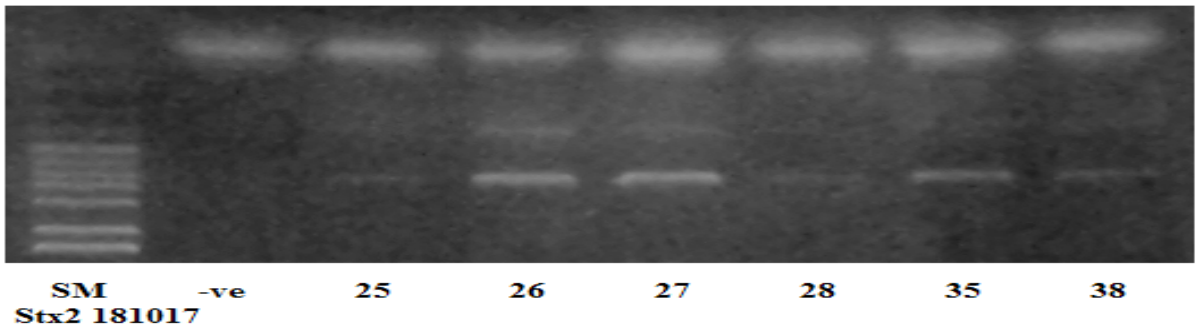

Fig. 3.6: PCR results showed Stx2 gene-positive isolates of *E. coli*

3.4 Fecal Enterococci species

In addition to *E. coli*, fecal Enterococci are another commonly used indicator of fecal contamination in water (Ben et al., 2015). In this study, 17.35% of the samples showed the presence of fecal Enterococci as confirmed by growth on selective and differential media, e.g. azide dextrose broth which was reconfirmed by the amplification of *rpoA* gene as described by Asmat et al. (2014). Antibiotic sensitivity assay showed that like *P. aeruginosa* and *E. coli*, enterococci species also exhibited multiple antibiotic resistance (Table 3.9). The interesting observation of this study is the detection of vancomycin-resistance "Enterococci" (VRE) in 5% (n=4) isolates (Table 3.9). These isolates were resistant to 17 antibiotics out of 22 tested.

Moreover, 51% isolates exhibited multiple antibiotic resistance to 13 antibiotics at a time, 71% isolates exhibited resistance to 9 antibiotics whereas; 83% isolates were resistant to 6 antibiotics. Out of 80 isolates, 91% isolates were resistant to 2 antibiotics at a time (Table 3.9). Most effective antibiotics against the isolates of Enterococci were linezolid, colistin sulfate, cephradine, aztreonam & optochin. The least effective antibiotics were ertapenem, carbenicillin, and cefpirome, with 27.5% isolates resistant to these antibiotics.

Furthermore, 23.7% isolates were resistant to gentamycin, 20% to erythromycin, 22.5% to cefoxitin, 16.25% to clarithromycin and 12.5% showed resistance to levofloxacin

(Table 3.9). Similar to this research, the majority of the waterborne Enterococci were also recovered from other parts of Pakistan and were also resistant to multiple antibiotics (Livermore and Woodford, 2010). Our findings are also in agreement with the Abbas *et al*. (2007) and Ali *et al.* (2013). Additionally, *espP* gene amplification studies were carried out to know the source of Enterococci. Scott *et al*. (2005) reported that *espP* is normally related to human feces. However, the majority of the isolates were *espP* gene negative (Table 3.10, Fig. 3.7) which suggests that the fecal Enterococci introduced in Hyderabad water supply are from non-human sources. 90% of the isolates of fecal Enterococci indicated the presence of *gel* gene (Fig 3.8). The *gel* is considered as virulence marker for Enterococci (Table 3.10), which suggests that most of these isolates recovered from Hyderabad water sources exhibited multi-drug resistance and harbor virulence factors and could be harmful to consumers.

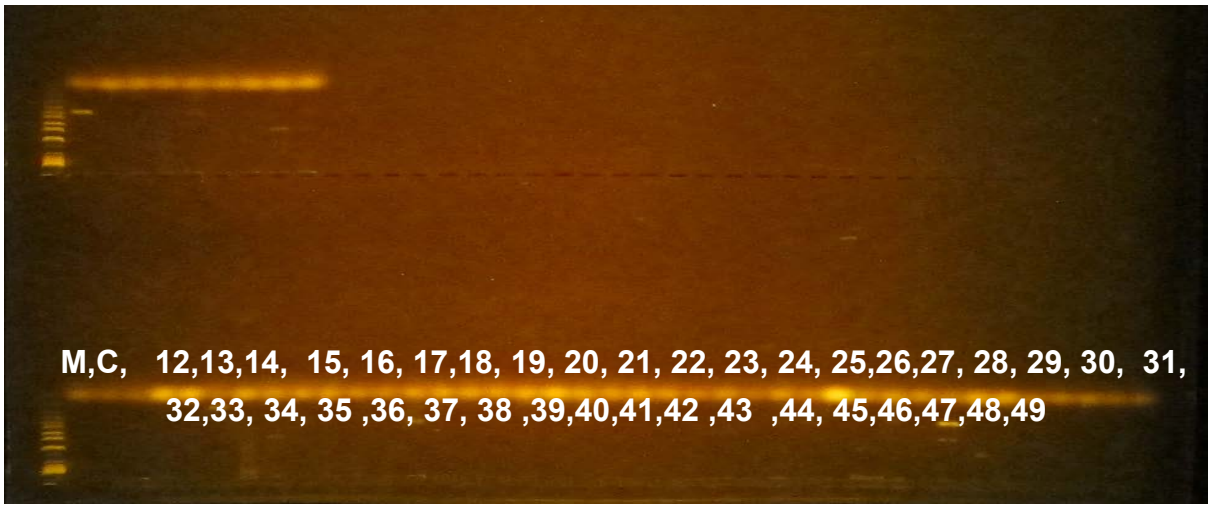

Fig. 3.7: PCR *r*esults indicate the presence and absence of *espP* gene responsible for biofilm formation and multidrug resistance in subject isolates of Enterococci

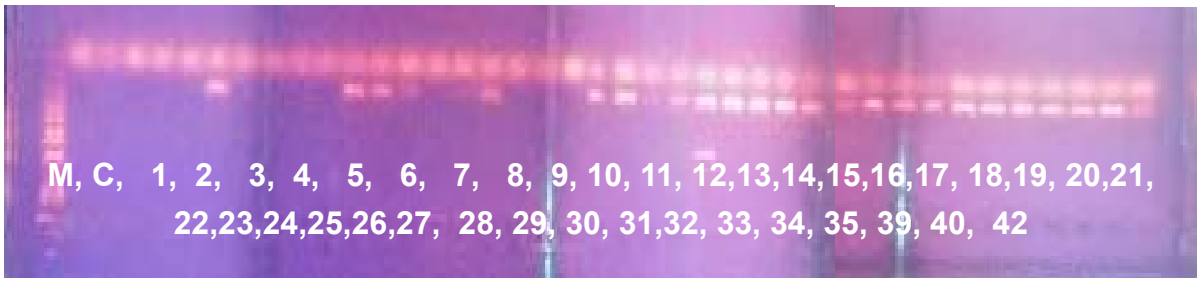

Fig. 3.8: PCR Results indicate the presence and absence of gel gene responsible for biofilm formation and multidrug resistance in subject isolates of Enterococci

Table 3.9: Antibiotic sensitivity profile of Enterococci

Antimicrobial agent	Disc content	Resistant Isolates	Zone diameter interpretive criteria	MIC interpretation criteria
Tetracycline[a]	30µg	4	≤14	≥16
Ertapenem[b]	*	22	-	-
Ampicillin[a]	*	4	≤16	≥16
Gentamycin[b]	*	19	*	*
Carbenicillin	*	22	*	*
Erythromycin[a]	*	16	≤13	≥8
Optochin	*	3	*	*
Linezolid[a]	*	2	≤20	≥8
Chloramphenicol[a]	*	7	12	32
Colistin sulphate[b]	*	2	-	-
Cephradin[c]	*	2	*	*
Impenem[b]	*	11	18	8
Cefpriome	*	22	*	*
Cefoxitin[b]	*	18	-	-
Clarithromycin[b]	15µg	13	-	-
Ampicillin	10µg	12	*	*
Amoxycillin/Clavulanic acid (2:1)[b]	30µg	4	*	-
Levofloxacin[a]	5µg	10	13	8
Vancomycin[a]	30µg	4	14	32
Ciprofloxacin[a]	5 µg	5	15	4
Polymixin B[b]	300 U	4	*	*
Aztreonam[b]	30µg	2	-	-

- Indicates that interpretive criteria are not applicable

* As per standard.

a CLSI

b EUCAST

c CA-SFM

Table 3.10: Molecular characterization of fecal Enterococci recovered from Hyderabad water samples

S#	Water Type	*rpo-A*	gel	*espP*
1	Tap water	+	-	-
2	Tap water	+	-	-
3	Tap water	+	-	-
4	Tap water	+	-	-
5	Tap water	+	+	-
6	Tap water	+	-	-
7	Tap water	+	-	-
8	Tap water	+	-	-
9	River/Canal Water	+	-	-
10	River/Canal Water	+	+	-
11	Tank water	+	+	-
12	River/Canal Water	+	+	-
13	River/Canal Water	+	-	-
14	River/Canal Water	+	-	-
15	Bottled water	+	+	-
16	River/Canal Water	+	-	-
17	Tap water	+	-	-
18	Tap water	+	-	-
19	Bottled water	+	+	-
20	Tap water	+	+	-
21	Tap water	+	+	-
22	River/Canal Water	+	+	-
23	Bottled water	+	+	+
24	RO water	+	+	-
25	Tap water	+	+	-
26	Tap water	+	+	-
27	Tap water	+	+	-
28	Tap water	+	+	-
29	Tap water	+	+	-
30	Tap water	+	+	-
31	Tap water	+	+	-
32	Tap water	+	+	-
33	Bottled water	+	+	-
34	Tap water	+	+	-
35	Tap water	+	+	-
36	Tap water	+	+	-
37	Tap water	+	+	-

38	Tap water	+	+	-
39	Tap water	+	+	-
40	River/Canal Water	+	+	-
41	River/Canal Water	+	+	-
42	River/Canal Water	+	+	+
43	River/Canal Water	+	-	-
44	River/Canal Water	+	-	-
45	River/Canal Water	+	-	-
46	Well water	+	+	-
47	Well water	+	+	-
48	Bottled water	+	-	-
49	Tap water	+	-	-
50	Tap water	+	+	-
51	Well water	+	+	-
53	Tap water	+	-	-
53	Well water	+	+	-
54	Tap water	+	+	-
55	Tap water	+	-	-
56	Tap water	+	+	-
57	Tap water	+	-	+
58	Tap water	+	+	-

\+ Shows the presence of subject isolates

\- Represents no trace of subject isolate

3.5 Other Isolates

Other major isolates recovered from water samples of Hyderabad and its surroundings were *Vibrio* species (n=90), *Shigella* species (n=70), *Kleb. Pneumonia* (n=45) and *Proteus* species (n= 51) (Table 3.3). All of these pathogens exhibited resistance to the majority of commonly used antibiotics, e.g. antibiotic sensitivity profile of *Vibrio* species is shown in Table 3.11. These are major pathogens responsible for various infectious diseases, e.g., cholera and dysentery in the community as well as in-hospital setups (Cabral, 2010). Alam *et al*. (2018) reported that member of family *Enterobacteriaceae* present in water could transfer the antibiotic-resistant character to normal flora of the body or other pathogens by conjugation. In a large-scale study, Din *et al*. (2014) reported that water samples collected from Quetta city were found to carry a high load of multidrug-resistant bacteria, e.g., *E. coli*, Enterobacteria, Klebsiella, Pseudomonas, and Salmonella. These pathogens expressed high-level resistance to commonly used antibiotics, e.g., tetracycline, gentamycin, sulphamethoxazole, piperacillin, ampicillin, Augmentin, imipenem, etc. which is in agreement with the findings of this research.

Table 3.11: Antibiotics sensitivity profile of *Vibrio spp.*

Antimicrobial agent	Disc content	Resistant Isolates	Zone diameter interpretive criteria	MIC interpretation criteria
Clarithromycin[a]	*	10	*	*
Ertapenem[b]	*	28	*	*
Ampicillin[b]	*	24	13(3)	39(2)
Gentamycin[a]	*	19	12(3)	16(2)
Carbenicillin	*	12	*	*
Erythromycin[a]	*	2	13(1)	-
Optochin	*	3	*	*
Linezolid[a]	*	8	*	*
Meropenem	*	2	*	*
Chloramphenicol[a]	*	1	15(1)	32(2)
Cephradin[c]	*	2	*	*
Impenem[b]	*	2	*	16(2)
Cefpriome[b]	*	2	*	*
Cefoxitin[b]	*	1	*	*
Ciprofloxacin (CIP)[a]	5µg	3	15(3)	4(2)
Polymixin B (PB)[b]	300 U	2	-	-

- Indicates that interpretive criteria are not applicable

* As per standard.

a CLSI

b EUCAST

c CA-SFM

3.6 Disinfection Processes

3.6.1 Chlorination

Sodium hypochlorite is considered as one of the best antibacterial agents for the waterborne bacteria. It is widely used for water purification all over the world (Collivignarelli *et al.,* 2018). In the present study, different doses of sodium hypochlorite, i.e. 0.1, 0.5, 1.0, 1.5 and 2 .0 mg/l were applied on multidrug-resistant bacteria recovered from Hyderabad water samples. The in-vitro study showed that majority of waterborne bacteria were sensitive to 1 mg/l of sodium hypochlorite. However, some isolates of *P. aeruginosa* (n=3) were found to tolerate the toxic effect of sodium hypochlorite, and

they grew even after 15-minutes of exposure. These hypochlorite resistant isolates were recovered from open water tanks of Qasimabad and Jamshoro areas (Fig 3.9). All three isolates were multidrug resistant.

Contrary to *P. aeruginosa,* all of the *E. coli* isolates were sensitive to 0.5 to 1.0 mg/l of sodium hypochlorite and killing time was 1 to 5 mins. Like *E. coli* and other isolates, e.g., *Vibrio* species (n=90), *Shigella* species (n=70), *Kleb. pneumonia (*n=45) and *Proteus* species (n= 51) were also sensitive to 0.5 mg/l of sodium hypochlorite. Only two isolates of *E. coli* and four isolates of Vibrio survived for 1 min in the presence of the antibacterial agent. The gram-positive isolates of the subject study were comparatively more sensitive, and all the *S. aureus* isolates and fecal Enterococci isolates were sensitive to 0.1 mg/l and killing time for these isolates was 1 min. This is in line with the findings of Estrela *et al*. (2003) who also reported that 1 mg/l of sodium hypochlorite inhibited the isolates of *S. aureus*, E. faecalis, *P. aeruginosa,* C. albicans and B. subtilis. These results are in line with the studies reported by D'Arcangelo *et al.* (1999) and Silva *et al.* (2002). This concludes that sodium hypochlorite is an effective disinfectant against most of the waterborne bacteria.

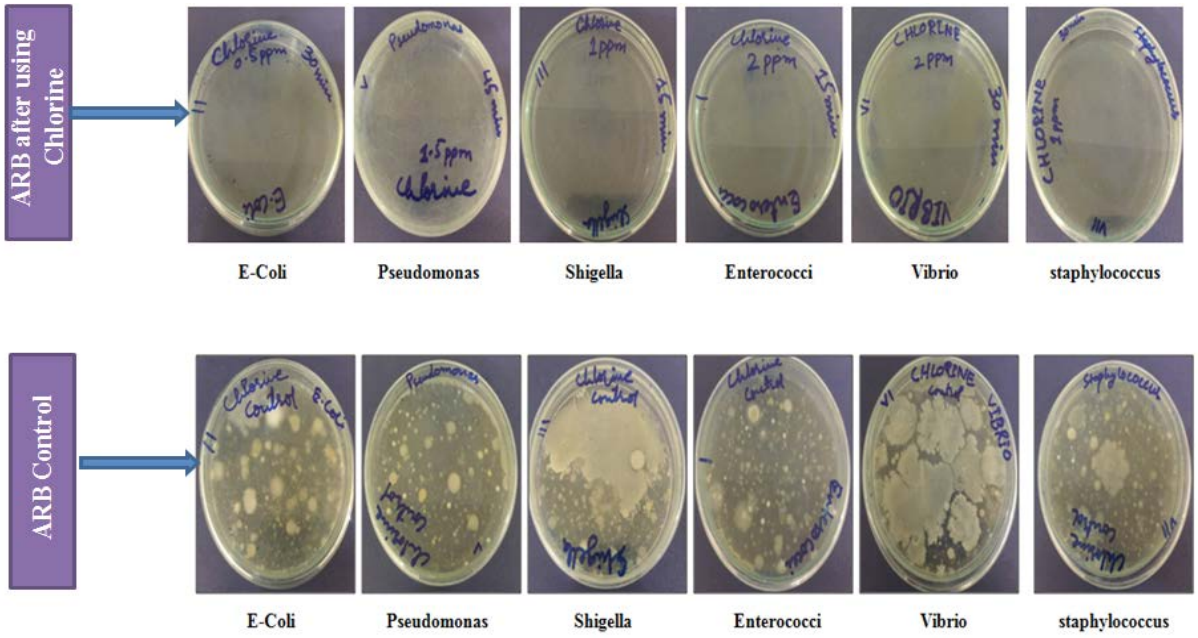

Fig. 3.9: Impact of sodium hypochlorite on the population of the subject isolates.

3.6.2 UV radiations

When UV light (11 W, 254 nm and 30,000 µW/cm^2/sec) was applied for 30 seconds, major reduction was noticed in the growth of subject isolates. A reduction by 95 % in cfu was noticed in *P. aeruginosa*. The control showed 117 cfu/ml where as a reduction to 98 cfu/ml was observed after 30 sec. Other isolates (n=9) of P. aeruginosa also

showed a reduction by 90% in cfu values in the growth media after exposure to UV radiations. Similarly, other isolates tested also showed similar results (Fig 3.10).

Numerous UV disinfection tests were compared against the chlorine disinfection process by working on antibiotic-resistant *E. coli* present in urban wastewater treatment effluent plant. Effects were also checked for *E. coli* strain resistant to ciprofloxacin, sulphamethoxazole, and amoxicillin. The concentration of UV radiations for 60 mins was ($1.25*10^4$ μW/cm^2/sec) while chlorine (2 mg/L) was used for 120 mins. Results showed that chlorine did not disinfect ciprofloxacin, sulphamethoxazole, and amoxicillin-resistant bacteria. The UV rays were only effected for ciprofloxacin-resistant bacteria to an extent of 33% when applied for 60 minutes and 50% for 120 mins, respectively, but sulphamethoxazole and amoxicillin-resistant bacteria were not disinfected by UV disinfection process (Rizzo *et al.,*. 2013).

E. coli in synthetic wastewater was completely disinfected by using UV/C (290 nm – 100 nm wavelength and 11 W lamp), within 3 mins of photolytic treatment, while UV/A (400 nm – 320 nm wavelength and 9 W lamp) required 60 mins to completely inactivate *E. coli* in synthetic wastewater (Chatzisymeon *et al.,* 2011).

The effects of UV disinfectant on both, the heterotrophic bacteria and the ARBs (erythromycin, sulphadiazine, chloramphenicol, ciprofloxacin, gentamicin, rifampicin, tetracycline, cephalexin and vancomycin-resistant bacteria) were examined, and these effects were checked in secondary effluent coming from the municipal wastewater treatment plant. Wastewater effluent was exposed to the UV dose of 5, 10, 20, 50 and 80 mJ/cm^2. Vancomycin rifampicin-, chloramphenicol tetracycline- and sulphadiazine- resistant bacteria did not show any significant impact of UV disinfection but erythromycin, ciprofloxacin, gentamicin, and cephalexin resistant bacteria were completely disinfected by UV disinfection process (Guo *et al.,*. 2013).

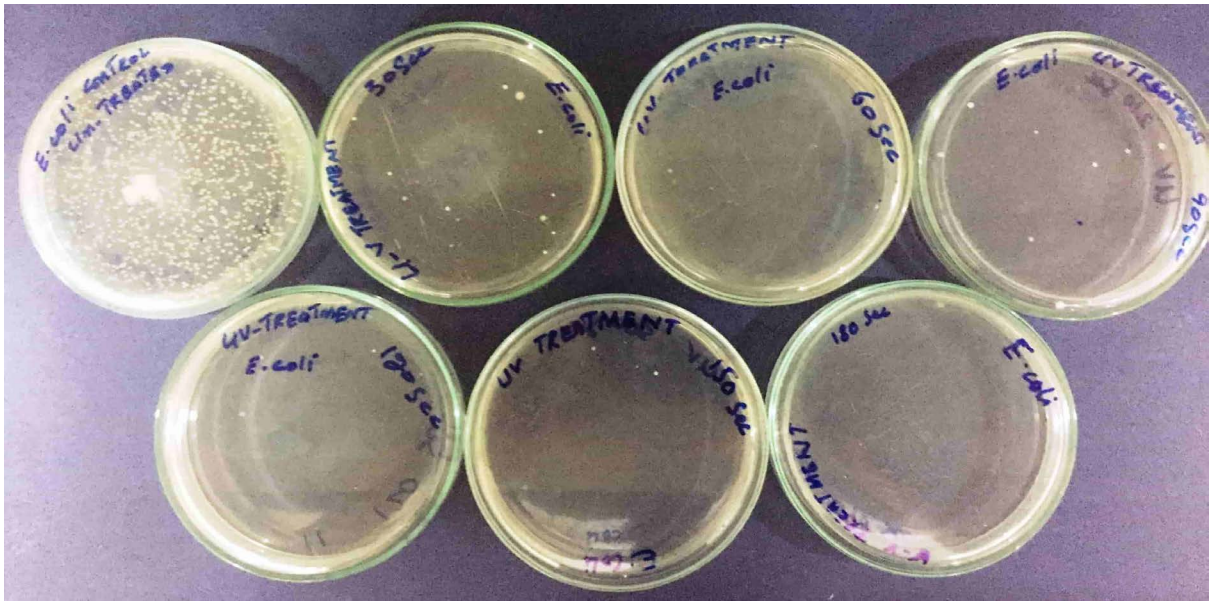

Fig. 3.10: Reduction in colony forming units (CFU) after exposure to UV radiation.

3.6.3 Silver nano particles

A similar behavior was observed when ARB's were exposed to silver nanoparticles for the disinfection process. Different minimum inhibitory concentration (MIC) values of silver nanoparticles were observed for the same bacteria from different areas i.e. for Pseudomonas, MIC value of 128 µg/ml was observed for the samples collected from Sindh University, Jamshoro and Latifabad No. 07, Hyderabad areas while MIC value of 64 µg/ml was observed for LUMHS, Jamshoro and Latifabad No. 02, Hyderabad areas. For Vibrio, a MIC value of 128 µg/ml was observed for Hyderabad city and Jamshoro areas, and 64 µg/ml for Qasimabad, Kotri and Latifabad areas. For Shigella, MIC value of 64 µg/ml was observed for Sindh University and Naseem Nagar Choak areas, and 32 µg/ml for MUET. *E. coli* MIC value of 64 µg/ml was observed for Resham Bazar and Latifabad No. 09 areas and 32 µg/ml for Hyder Choak and Latifabad No: 07 areas. For Enterococci and *S. aureus*, respective MIC values of 32 and 64 µg/ml were observed for Latifabad No. 02 (Fig 3.11).

Fig. 3.11: Effect of silver nanoparticles

3.7 Dissemination of Research Results

The research results were disseminated by publishing articles in newspaper (Appendix 1) and by organizing a National Seminar titled “Identification of Antibiotic Resistant Bacteria in the Different Source Waters of Hyderabad City and its Surroundings” at the Center in October 2018. Main purpose of the seminar was to bring government, policymakers, and relevant stakeholders together to share, deliberate, and brainstorm about the Antibiotic Resistant Bacteria (ARBs) in the different source waters of Hyderabad and to suggest protective measures. The seminar was attended by stakeholders from different government and non-government organizations, officials from Water and Sanitation Authority (WASA) Hyderabad, Public Health Engineering Department, Government of Sindh, civil society activists, academia, research scientists and students. The seminar was chaired by Dr. Muhammad Aslam Uqaili, Vice Chancellor, MUET, Jamshoro. The seminar announcement brochure, photographs and media coverage are given in Appendix 2 and 3.

3.8 Research Output

The details of research output are given in Appendix 4 in terms of research papers presented in conferences (Appendix 4a), papers submitted in research journals, (Appendix 4b) and M.Sc. thesis completed as a part of this project (Appendix 4c).

3.9 Building Research Partnership

This research project provided an opportunity to work in collaboration with Dr. Ramesh Goel, Professor of Civil Engineering Department at the University of Utah during the visit of Dr. Rasool Bux Mahar, Professor of Environmental Engineering, USPCAS-W, MUET, Jamshoro under faculty exchange program. Goel and Mahar have sustained this research partnership towards resolving drinking water challenges in Pakistan. A brief on this partnership prepared by University of Utah *(https://water.utah.edu/2019/05/20/getting-to-know-bad-bugs-in-pakistans-drinking-water/)* is attached as Appendix 5.

4. CONCLUSION AND RECOMMENDATIONS

4.1 Conclusion

This study suggested that majority of water samples collected from Hyderabad and its surroundings are microbiologically unfit for human consumption. About 70% of Municipal water samples and 87% of bore/water samples were declared unfit for cooking, washing and drinking purposes in accordance to the WHO guidelines. These samples were found to carry high load of heterotrophic bacteria along with coliform and fecal coliforms. The presence of potential pathogens e.g. *P. aeruginosa*, *E. coli*, *S. aureus*, Shigella and fecal Enterococci depicts a horrible picture of Hyderabad water sources. The Shiga toxins-positive, *E. coli* species and *Vibrio* species are among the major causes of diarrhea and dysentery. *P. aeruginosa* and *S. aureus* are major pathogens responsible for variety of infections in community and hospital setups. Our results suggested that majority of these pathogens are resistant to multiple antibiotics. Moreover, vancomyicn resistant Enterococci were also recovered from four water samples along with vancomyicn resistant *S. aureus*. The overall findings suggest that consumers exposed to such water are always at stake for acquiring multidrug resistant infections.

The causes of the microbial contamination and ARB could be listed as: disposal of untreated wastewater in the water sources like rivers, canals and open ponds etc.; drinking water supply lines are old and deteriorated; drinking water supply lines are parallel and closer to sewerage lines, consumers fetch water through pumps, water supply system is intermittent, timing of supply water is not regular and improper disinfection of water supply. All above reasons could cause the intrusion of sewage and microbial contamination in the water supply lines and resultantly it contaminates the drinking water supply. Besides, over use and self-medication are also a caus of the ARBs in the water bodies. Therefore, it is suggested to educate the consumers of this water to carry out precautionary measures such as boiling before utilizing it to avoid direct usage of river and canal water due to contamination.

The chlorination is an effective method to remove most of the waterborne pathogens. However, it is not being applied on regular basis and also UV rays treatment are also not practiced in most parts of Pakistan. The present study showed that majority of the waterborne pathogens were completely disinfected, when chlorine dose of 1.5 mg/l was applied for 1 to 5 mins. Similarly, application of UV (11 W, 254 nm and 30,000 $\mu W/cm^2/sec$) radiation for 30 sec killed 90% growth of subject pathogens. The silver nanoparticles of 10 nm size showed complete disinfection of the isolated ARBs when their MIC value reached 128 µg/ml.

The RO water treatment is considered to be very effective and is widely used in some parts of Hyderabad. However, due to lack of trained personals it has lost its efficacy. It was also noticed during this study that, majority of RO water samples were not fit due to high bacterial load.

4.2 Recommendations

The study of the whole drinking water treatment plant, water distribution network and discussions with the WASA staff, following recommendation are formulated:

1. The water supply sources may be protected by stopping the untreated wastewater discharges into rivers, canals and open depressions.
2. The old deteriorated and leaky water supply pipes may be replaced.
3. Fetching of water with pumps from water supply lines may be stopped.
4. The pressurized and continuous water supply system may be maintained.
5. The proper disinfection of water supply should be done.
6. Over use and self-medication may be stopped by raising awareness.
7. Drinking water quality may be monitored regularly.
8. Public should be informed in case of microbial contamination in drinking water supply and guided how to use water.

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Appendix 1. Newspaper Articles

THE EXPRESS TRIBUNE

May 24, 2017

Alarming results

High presence of antibiotic resistant bacterium detected in water

Bacterium found on surface, underground and in tap water in Hyderabad and Jamshoro

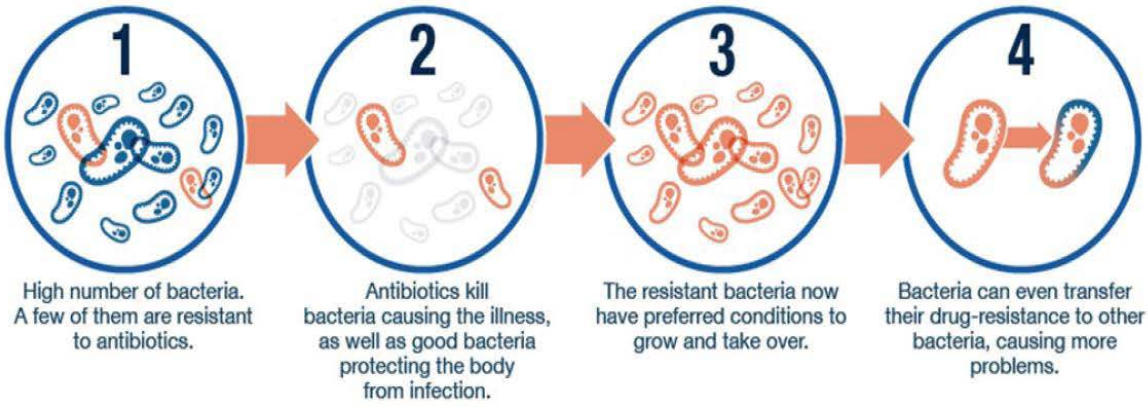


PHOTO: COURTESY RESEARCHER ASAD ALI LAGHARI

OUR CORRESPONDENT
HYDERABAD

A research on microbes found in water of Hyderabad and Jamshoro has unearthed an alarming public health risk pertaining to unresponsive medical treatment or slow health recovery. A considerably high presence of antibiotic resistant bacterium has been detected in the surface, underground and tap water of the two cities.

For the people who suffer from infectious diseases or those with low immunity, the disclosure seems to portend some serious health risks. "These micro-organisms threaten the communities with new strains of infectious diseases that are difficult to cure," said Asad Ali Laghari, a final year Master's of Science student at US-Pakistan Centre for Advanced Studies in Water (USPCASW) at Mehran University of Engineering and Technology. "I believe the situation won't be different in other parts of Sindh and even in Pakistan owing to the same poor standards of water management."

Laghari shared findings of his research at the two-day '1st Young Researchers National Conference on Water and Environment', which commenced on Monday at USPCASW, Jamshoro. He was the first student to have been given the opportunity to present his research among 52 researchers who read their papers during the two-day event.

Antibiotics are an antimicrobial drug that kill or inhibit growth of bacterial infections. But their inappropriate use is known to lead to the emergence of resistant organisms.

According to Laghari, whose research has been supervised by environmental scientist Prof Dr Rasool Bux Mahar, 62 samples of surface, subsoil and filtered tap water were collected from Hyderabad and Jamshoro for analysis. In a horde of bacterium, the researcher found seven common ones such as E Coli, Shigella, Staphylococcus Aures, Vibrio and Pseudomonas, which infect humans with a range of infections, including the fatal ones.

> Two separate researches revealed that despite being a technological hub, the faculty, staff and students at MUET are being supplied impure drinking water

"In the lab tests, I applied 16 different and commonly prescribed antibiotics on each of the pathogen," he said. "What surprised us was that a majority of the bacterium resisted these drugs [that] are meant to kill them." Among the seven bacteria, Pseudomonas and Vibrio survived 12 antibiotics, Shigella and E Coli 10 each and Entercococci nine, he added.

Most of these strains affect the tract that involves stomach and intestines. But they also infect open wounds and can cause septicaemia - blood infection.

Release of untreated municipal and industrial waste water, improper filtration by means of chlorination and faulty water supply system in urban areas have been identified as the sources of such bacterial spread. "But the major reason for the microbial resistance is the consumption of antibiotics itself," Laghari said, citing foreign researches.

"These antibiotics ... are producing antimicrobial resistant genes and bacteria, which have caused greater risk to the health of humans and animals."

He explained that the human and animal bodies release up to 70% of a consumed medicine through urine and defecation. And these drugs reach the microbes through the unbridled, as well as unlawful, practice of releasing municipal and industrial waste water in rivers and canals.

For the researcher, the remedy lies in the sparing prescription of antibiotics and by bringing a complete end to discharging untreated waste water in rivers and canals.

Plenary session

Prof Dr Rasool Bux Mahar, the centre's director, informed at the inaugural session that as many as 105 abstracts and 72 detailed research papers were submitted for the conference. Finally, only 52 research papers and 11 posters were selected for presentation.

MUET Vice-Chancellor Dr Mohammad Aslam Uqaili said the newly established centre aims to produce 250 scholars who will contribute to extensive research on water issues. The centre is providing free Master's of Science and Masters of Engineering Education along with Rs15,000 monthly stipend to the students. Currently, 80 students are enrolled in two separate batches.

During the session, urban planner and architect Arif Hassan delivered a talk on 'urbanisation trends and their environmental repercussions', especially on water supply.

Nadeem Ahmad, the manager of policy and advocacy of Water Aid Pakistan, gave a presentation on Sustainable Development Goals (SDGs) and water.

Researchers from Karachi University, Peshawar University, Sindh University, MUET, National University of Science and Technology, Islamabad, Sindh Agriculture University and Federal Urdu University of Arts, Science and Technology, Karachi, also read their papers.

Heavy metallic concentration in water

A research conducted by Maheen Saeed of USPCASW found high concentration of lead, copper and zinc in Indus River near Kotri Barrage. "... concentration in water were higher than WHO's permissible limits," the study noted. Other metals such as cadmium, iron and manganese were also found beyond the health standards mainly during the non-monsoon period, which lasts for a long part of a year.

Hydel generation

Research conducted by Rubab Saher of USPCASW challenged the theory that Sindh's plain terrain is ill-suited for the hydel power production unlike the northern parts of Pakistan where dams have been built. She claimed of evaluating 3.2 megawatt (MW) generation capacity at just one point of Nara canal, which springs from Sukkur barrage.

NOx and CO2 emissions

As the government plans to increase manifold the coal power generation capacity of an existing plant near Jamshoro to 1,320 MW, a study has found already existing high level of nitrogen oxide (NOx) and carbon dioxide (CO_2) emissions.

> "I believe the situation won't be different in other parts of Sindh and even in Pakistan owing to the same poor standards of water management
>
> Master's of Science final year student at MUET **Asad Ali Laghari**

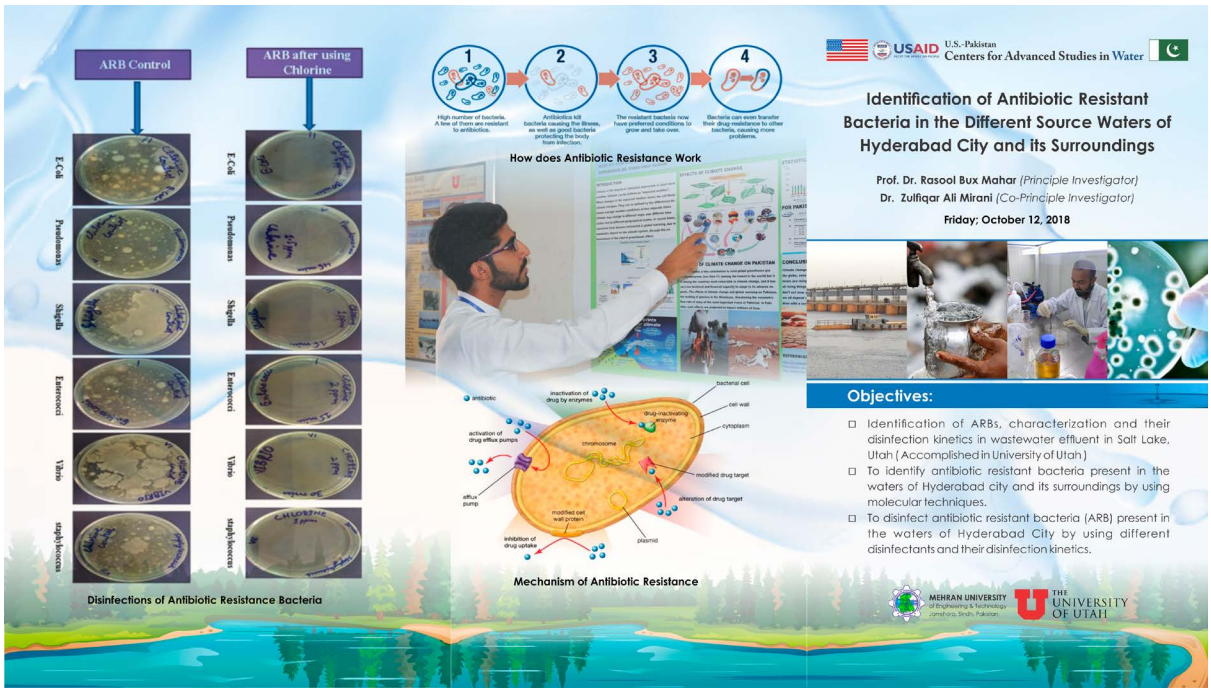
Kaleemullah Shaikh of USPCASW applied air dispersion model to assess the ground level concentration of NOx and CO_2 in 50 kilometres radius.

Water in MUET

Meanwhile, two separate researches revealed that despite being a technological hub, the faculty, staff and students at MUET are being supplied impure drinking water. Findings of Azizullah Gabol, who checked heavy metals like chromium, zinc, copper and iron from the KB Feeder Canal to the end distribution point in the varsity, found a high concentration of the latter.

Another study assessed the physical parameters, including temperature, turbidity, electrical conductivity, total dissolved solids, dissolved oxygen and pH level of water. "The current water treatment system doesn't offer adequate disinfection," the study concluded.

Appendix 2a. Seminar Brochure



Seminar Objectives

Main purpose of the seminar is to bring Government, policymakers, and relevant stakeholders together to share, deliberate and brainstorm about the Antibiotic Resistant Bacteria in the different Source Waters of Hyderabad City and its Surroundings "and suggest protective measures. The U.S.-Pakistan Center for Advanced Studies in Water, Mehran University of Engineering & Technology, Jamshoro, Pakistan, is gratefully acknowledged for funding the project" Identification of Antibiotic Resistant Bacteria in the Different Source Waters of Hyderabad City and its Surroundings ". Following are the specific objectives of the seminar.

- To share outcome/results of the project on Identification of Antibiotic Resistant Bacteria in the Different Source Waters of Hyderabad City and its Surroundings ". Funded by USAID through U.S.-Pakistan Center for Advanced Studies.
- To raise awareness about the Antibiotic Resistance Bacteria and their presence in water Environment.
- To provide a platform to share knowledge and discuss, deliberate and recommend solutions to control Antibiotic Resistance Bacteria and mitigate its impacts for the sustainable, healthy water environment

Background

Resistance offered by bacteria to the antibiotics is a global public health issue today. The problem is the greatest in areas where the usage of antibiotics is the greatest. These bacteria make their way into the aquatic ecosystem quite easily. Scientists have applied different methods to kill the bacterial contaminants present in the water. However, this has been a difficult task. These bacteria enter the groundwater, surface water and wastewater through animal and human feces. Once these bacteria reach the aquatic ecosystem, they start to replicate under suitable conditions, as they possess the quality of gene transfer from one bacterium to another. As the gene gets transferred to the other bacterium, it also starts to offer resistance to the antibiotics. So due to this quality of them, they start to become a threat to the world as these bacteria start to cause more public health issues worldwide. The objective of this research is to find which antibiotic resistant bacteria are present in the waters of district Hyderabad and disinfect them by using different disinfectants.

Acknowledgement:

The US-Pakistan Center for Advanced Studies in Water, Mehran University of Engineering & Technology, Jamshoro, Pakistan, is gratefully acknowledged for funding the project " Identification of Antibiotic Resistant Bacteria in the Different Source Waters of Hyderabad City and its Surroundings". And also thankful for PCSIR Laboratory Karachi for providing the access of the microbiology lab.

Venue

Auditorium hall, U.S.-Pakistan Center for Advanced Studies in Water, Opposite New Administration Building MUET, Jamshoro.

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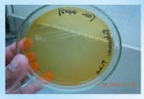


Fig. 3.1: Selective media for Enterococci


Fig. 3.2: Selective media for Pseudomonas

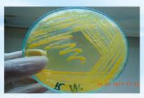


Fig. 3.3: Selective media for Vibrio

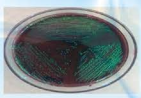
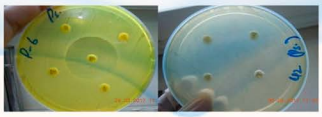
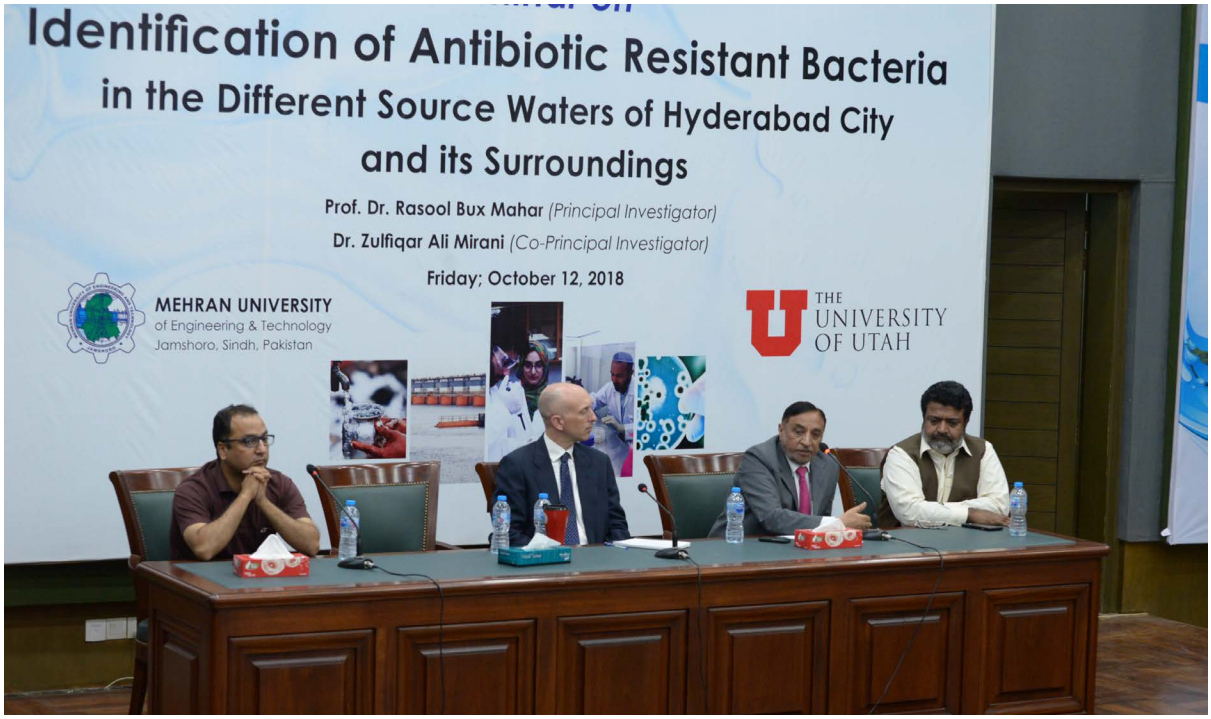


Fig. 3.4: Selective media for E.coli


Disc Diffusion Assay


Water Sampling Point

Appendix 2b. Seminar Photographs



Appendix 3. Media Coverage

THE EXPRESS TRIBUNE

Oct 14, 2018

Threat to life

Most water samples unfit for human consumption: study

Alarming presence of antibiotic resistant bacteria detected in Hyderabad

OUR CORRESPONDENT

HYDERABAD

A study conducted by the researchers of US-Pakistan Centre for Advanced Studies in Water (USPCASW) and Pakistan Council of Scientific and Industrial Research (PCSIR) has found alarming presence of antibiotic resistant bacteria (ARB) in water in Hyderabad. The findings were shared at a seminar held in USPCASW at Mehran University of Engineering and Technology (MUET), Jamshoro, on Saturday.

"... the presence of ARB is found in the groundwater, surface water and wastewater in Hyderabad and its surroundings," said USPCASW Deputy Director and principle investigator of the study Dr Rasool Bux Mahar. He identified the industrial waste, agricultural runoff and the waste of humans and animals as sources of the ARB.

"Bacterial isolates identified in the study's findings pose a potential threat to the people living in Hyderabad," he highlighted. According to Dr Mahar, the water distribution network in Hyderabad, being operated by the Water and Sanitation Agency, brimmed with technical flaws, deteriorating the water quality and also providing ripe conditions for the bacterial growth.

The Heterotrophic Plate Count, a method of measuring colony formation of bacteria in drinking water, found pseudomonas, shigella, vibrio and e-coli bacteria in the water samples. The ARBs reduce the efficacy of antibiotic medicines, making treatment of life-threatening illnesses difficult, if not impossible. Dr Mahar also illustrated the disinfection processes of ARB including the chlorine, ultraviolet and silver nano particles.

PCSIR senior scientific officer and co-investigator of study Dr Zulfiqar Ali Mirani informed that a total of 501 isolates of escherichia coli, pseudomonas aeruginosa, staphylococcus aureus, klebsiella pneumoniae, proteusp., shigella, vibrio and enterococci were identified in the study. The research, he added, screened them against commonly prescribed antibiotics.

"The microbiological analysis showed that the majority of water samples were not fit for human consumption," he concluded. Dr Mirani said that water used for drinking, cooking and washing should be free from coliform and fecal coliform bacteria.

The study, he told, found 70% of water samples, taken from the Water and Sanitation Agency's system, and 87% of river, canal and groundwater samples are unfit for drinking, cooking and washing purposes. "Majority of reverse osmosis (RO) plants' water samples were also not fit due to high bacterial load."

Isra University Prof Dr Hussain Bux Kolachi, while sharing his research over typhoid, told the seminar that antibiotic resistant strains of Salmonella Typhi are showing a rapid increase. "We are running out of the treatment options, which makes [preventive] vaccination against typhoid even more imperative," he underscored.

"Typhoid infection prevention is more crucial than treatment," he added. He cited World Health Organization's figures according to which up to 33 million cases typhoid resistant cases are reported globally, claiming between 500,000 to 600,000 lives due to typhoid fever annually.

University of Utah, USA, Prof Dr Jeff Ulman said that the revealed study paved the way for the global health networks to tackle such health issues. He suggested that lifestyle changes are indispensable as a preventive measure.

The seminar recommended that the communities, government and relevant authorities should be sensitised about the health issues through awareness campaigns. The people should be discouraged from self-medication and excessive use of antibiotics. It also suggested disposal of hospital waste and treatment of wastewater before it is released in the fresh waterways. The recommendations also emphasised on removing the technical flaws in the water distribution network.

INTERNATIONAL THE NEWS
Mon Jul 15, 2019

USAID research: Agriculture runoffs creating drug-resistant bacteria in freshwater

IH
Imtiaz Hussain

October 14, 2018

Listen

HYDERABAD: Industrial effluents and agricultural runoffs are begetting antibiotic resistant bacteria in ground and surface water, terminating the efficacy of medicines to fight off diseases, researchers and academicians said.

Rasool Bux Mahar, deputy director of US-funded water centre at Mehran University of Engineering and Technology, Jamshoro said the presence of antibiotic resistant bacteria (ARB) in different water sources lessen the efficacy of antibiotic medicines prescribed by doctors to the patients.

"The presence of ARB is found in the groundwater, surface water, and wastewater in Hyderabad and its surroundings due to industrial wastes, agricultural runoff, and wastes of humans and animals," Mahar said, sharing findings of a research study at a seminar.

UrduPoint
18 Successful Years of Publication

Water In Hyderabad Contains Antibiotic Resistant Bacteria: A Research Reveals

Rukhshan Mir (@rukhshanmirpk) 9 months ago

Sat 13th October 2018 | 09:29 PM

A study by US-Pakistan Center for Advanced Studies in Water (USPCAS-W), Mehran University of Engineering and Technology (MUET) Jamshoro, has found significant quantity of antibiotic resistant bacteria in water in Hyderabad district.

Appendix 4. Research Output

Appendix 4a. Papers Presented in Conferences:

1. Laghari, A. A., **Mahar, R. B.,** and Mirani, Z. A. (2017). Identification of antibiotic resistant bacteria in the drinking water sources of Hyderabad city and its surroundings. 1st Young Researchers National Conference on Water and Environment NCWE-17, Organized by USPCAS-W, MUET, Jamshoro. 22-23 May, 2017.

2. Muhammad, A., **Mahar**, **R. B**., and Mirani, Z.A. (2018). Disinfection of antibiotic resistant bacteria in the water of Hyderabad city by using different disinfectants. 2nd International Conference on Chemical Engineering (ICCE2018). Organized by Chemical Engineering, MUET, Jamshoro. 22-23 January, 2018.

Appendix 4b. Papers in Research Journals:

1. Mirani, Z. A., Shaista U., and **Rasool B. Mahar, R. B.**. Prevalence of multidrug resistant *E. coli* and faecal Enterococci in drinking water of Hyderabad, Sindh. *Pakistan Journal of Scientific and Industrial Research* (Submitted).

Appendix 4c. MS Theses Produced (two):

1. Asad Ali Laghari. Identification of Antibiotic Resistant Bacteria in the Drinking Water Sources of Hyderabad City and its Surroundings. 2017

2. Awais Magsi. Disinfection of Antibiotic Resistant Bacteria in Drinking Water of Hyderabad City by using Different Disinfectants. 2018

Appendix 5. Getting to Know "Bad Bugs" in Pakistan's Drinking Water: USPCAS-W Faculty Exchange Fosters Long-term Research Partnership

It was Spring of 2016 when Dr. Rasool Bux Mahar left Pakistan to spend a semester at the University of Utah. At the time, the one-year-old USAID-funded U.S.-Pakistan Center for Advanced Studies of Water was busy constructing what would become a well-equipped hub for the country's water research at Mehran University of Engineering & Technology.

His new lab still rising from the sandy soil of Jamshoro, Mahar traveled nearly 24 hours to first meet his peer mentor, Dr. Ramesh Goel, in the foothills of Utah's Wasatch mountains. As part of a train-the-trainers strategy, USPCASW connects faculty in Pakistan with partners in the U.S. to advance research skills, improve course content, modernize teaching methodology, and establish best practices of successful professors. Mahar was one of the first to participate in the Exchange.

Back in Pakistan, just over 10 miles from Mahar's home university, lies Hyderabad City–a key field site for his research into antibiotic-resistant bacterial contamination. The second biggest city in Pakistan's Sindh province, Hyderabad has wrestled with drinking water contamination and remains ground zero for an ongoing outbreak of highly drug-resistant Typhoid that has sickened over 5,200 people since 2016. A

2018 study, to which Mahar contributed, found that 70 percent of samples taken from Hyderabad's Water and Sanitation Agency's system and 87 percent of the river, canal, and groundwater samples are unfit for drinking, cooking and washing purposes due to the presence of antibiotic-resistant bacteria.

The city is not alone in its plight. The emergence of bacterial antibiotic resistance is a global problem and one of the grand challenges of the 21st century. Pakistan is no exception from this curse and, country-wide, drinking water infrastructure suffers greatly from the presence of ARBs. Human, animal and industrial waste, plus agricultural runoff combine all too often with drinking water into a sometimes-fatal cocktail.

One of the challenges researchers face is not only detecting, but properly identifying and quantifying the presence of "bad bugs." Which is why Mahar needed to build his skills halfway around the world; the specialized training he obtained at the University of Utah will bolster his ability to address this important research frontier.

He describes his exchange experience and working with Goel as "a big shift in my career." Their work together embraced molecular biology as a complement to Mahar's background as an environmental engineer. It was an interdisciplinary dance familiar to Goel, but "this was a new field for me," noted Mahar. "Albeit a challenge, it was quite exciting and interesting to push my limits and learn new things."

Goel, a Civil and Environmental Engineering professor, had experience in applying molecular diagnostic techniques to environmental engineering problems. That informed a new research approach for Mahar, and the challenges in Pakistan provided ample opportunity for field study. "After working in Dr. Goel's lab, I committed within myself that on return I would establish a very similar lab in Pakistan," said Mahar.

With the assistance of USAID funding, he set to work acquiring the necessary instrumentation to better define not only which pathogens are present in the water supply and the biofilms that line distribution systems, but their specific resistance

factors. While public health experts agree that prevention is critical in controlling the spread of ARBs, Mahar noted that “without adequately knowing the main problem, preventative measures are hard to take.” He hopes his lab will open the door to a deeper understanding of the factors that are sickening so many so that government response can be tailored accordingly.

“Our laboratory is now fully equipped with state-of-the-art facilities and students are being trained to use the resources to provide solutions and services that address water issues in Pakistan,” said Mahar. His lab is leading partnerships with local industries to better manage wastewater and providing quality assessment and monitoring of surface drinking water in Hyderabad and Karachi.

Goel and Mahar have sustained the research partnership first established by the USPCASW program. The two are currently mid-stream on a quarter million dollar joint research project titled “Capacity building at Mehran University of Engineering and Technology to address Drinking Water Issues in Pakistan.” They aim to train the next generation of water professionals to address ARBs in addition to their independent research in antibiotic-resistant bacteria in Pakistan. Goel notes “the success has been truly inspiring with research publications already emerging.”

About the Authors

Dr. Rasool Bux Mahar is working as Professor (Environmental Engineering) and Deputy Director (Academic and Research) in U.S.-Pakistan Center for Advanced Studies in Water at Mehran University of Engineering and Technology (MUET), Jamshoro. He is also an Editor of Mehran University Research Journal of Engineering & Technology. He did his PhD from Tsinghua University, Beijing, China. and post-Doctorate from the University of Utah, USA. He has more than 25 years teaching and research experience. He has published more than 100 research papers in the journals of international repute and presented more than 30 papers in National and International conferences and symposia. He has supervised six PhD and more than 60 MS/ME students. He has remained as a Co-Director/HoD of Environmental Engineering Department, in the Institute of Environmental Engineering & Management, MUET, Jamshoro. He has also worked as a Project Coordinator/P.I of various research projects funded by HEC, DFID, UNEP, etc.

Dr. Zulfiqar Ali Mirani is Senior Scientific Officer of Microbiology at PCSIR Laboratories Complex Karachi since 2008. Dr. Mirani has supervised 10 MS/ M.Phil and Ph.D. students in the field of Food and Water Microbiology. He has published 30 research papers in various reputed journals. At present Dr. Zulfiqar is serving as Deputy Section In-charge of Microbiology section at PCSIR Laboratories Complex Karachi.

Main thrust of Applied Research component of the Water Center is to stimulate an environment that promotes multi-disciplinary research within the broader context of water-development nexus to support evidence-based policy making in the water sector. This is pursued using the framework provided by the six targets of the Sustainable Development Goal on Water i.e. SDG-6.

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