*International Research Journal of Environmental Sciences*_____ ISSN 2319–1414
Vol. **11(4),** 13-17, October **(2022)** *Int. Res. J. Environmental Sci.*

Isolation of Broad Spectrum Antibiotic-Resistant Bacteria from Waste Water using 16s rDNA Technology

Jyoti Jaglan[1*], Andrew C. Singer[2], Praveen Sharma[1] and Savita Jaglan[3]
[1]Department of Environmental Science & Engineering, Guru Jambheshwar University of Science & Technology, Hisar, Haryana, India
[2]UK Center for Ecology & Hydrology, Benson Lane, Wallingford, United Kingdom
[3]Maharshi Dayanand University, Rohtak, Haryana, India
rs160481003@gjust.org

Available online at: www.isca.in, www.isca.me
Received 27th April 2022, revised 12th July 2022, accepted 26th September 2022

Abstract

This study focuses on multiple drug resistance (MDR) bacteria prevalence in wastewater treatment plants specifically in domestic and medical college sewage treatment plants (STP) in Hisar district of Haryana, India. MDR was identified from wastewater samples using broth dilution and the Kirby Bauer disk diffusion method. Results obtained from these methods showed 100% resistance against Ampicillin (AMP), Vancomycin (VAN), Tetracycline (TET) and Rifampicin (RIF). Variable susceptibility was checked for other antibiotics like 60% to 51.9% for Chloramphenicol (CHL), 53% to 43.2% for Cephotaxime (CTX), 47% to 39% for Streptomycin (STR), 21% to 15.7% for Trimethoprim (TMP) and 27.1% to 13% for Ceftazidime (CAZ). Isolated antibiotic-resistant bacteria were identified by using 16s rDNA technology.

Keywords: MDR, STP, NGS, 16s rDNA.

Introduction

Presently Wastewater treatment plants (WWTPs) are the main issue of concern due to improvement in urban lifestyle that leads to increased production in sewage discharge. To attain sustainable development in urban as well as rural development WWTPs are the main key to protecting local waters, thus playing a vital role in environmental protection also[1]. However, WWTPs act as reservoirs of antibiotic resistance genes (ARGS). Selection pressure imposed on bacterial species is attributed to the hospital environment, disposal, administration and antibiotics released directly into the environment. Among the highest concentrations of antibiotics released by hospitals provide specific evolutionary hotspots to normal bacterial species[2]. Antibiotics released in the hospital environment persist for a long time in the form of residual antibiotics until removed by natural degradation or manual treatment. Previous studies reported that bacteria isolated from environmental conditions give rise to antibiotic resistance genes like qnrA[3]. Every year the number of people who die because of antibiotic resistance increases and this may cost up to $100 trillion by 2050, a cost output greater than the current economy. Serious threat created by hospital-acquired infections (HAI) due to no longer reimbursable and treatment of this infection[4]. To understand disease-causing pathogens it is necessary to understand their whole genome and the mechanism of virulence genes[5]. So technology like whole genome sequencing and next-generation sequencing is implemented for the analysis. Early 1970s DNA sequencing technology came into existence but all initial level sample analysis charges were too costly and it consisted of radioactive or toxic reagents that create a limitation in its use Sanger's sequencing method involves chain termination which made it more practical end it was pioneered study that formed the basis of automatic DNA sequence that is a type of first-generation sequencing[6]. In the meantime, several molecular biology-based methods like polymerase chain reaction (PCR) have been used for microbial community studies. But due to low depth of sequencing and biased PCR method created a limitation in use. In the 1990s a scheme of multi Locus sequence typing evolved by Maiden et al developed a scheme for Neisseria study created its application in public health. Sanger's sequencing method was used for complete genome sequencing of Haemophilus influenza which is a free-living microorganism, it was published in 1995. However, technology that involved whole-genome sequencing WGS was cumbersome, expensive and time-consuming[7].

In the early 2000s, a parallel sequencing process named Next Generation Sequencing (NGS) technology created a fraction in the cost of Sanger's sequencing and reduced the time of sequencing dramatically. Several outbreaks like Haiti's Cholera epidemic followed by Escherichia coli international outbreak and further fenugreek sprout consumption associated disease created a need to study virulence characteristics and transmission dynamics[8]. In such situations, Academic institutions and government laboratories rapidly responded to NGS technology with crowd sourcing and open data sharing. NGS technology adoption accelerated due to disease outbreaks and its demand in public health laboratories and bigger hospital laboratories. After a few years, this technology was frequently used during outbreaks of several infectious pathogens including Acinetobacter baumannii, Staphylococcus aureus caused by

methicillin resistance and Klebsiella pneumonia due to carbapenem resistance[9]. NGS implementation in public health and food borne disease surveillance was the most extensive and preventable technology. Currently, a bacterial community phylogenetic study in an engineered ecosystem is an easy task due to Illumina Miseq sequencing. The main purpose of the current research work was to discriminate antibiotic-resistant bacterial community profiling from wastewater using Next-Generation Sequencing. The main objective of the study was to isolate and identify bacterial species and to screen out multiple drug-resistant (MDR) using 16s rDNA technology. Multiple drug resistance in bacterial species against multiple antibiotics now become a global threat to animal and human health as well as the environment and food safety. Bacteria use horizontal gene transfer (HGT) for the exchange of genetic material which greatly enhances multiple drug resistance conditions. Ultimately this situation gives rise to "superbugs" that can easily tolerate almost the effect of all antibiotics[10].

Materials and methods

Sampling site: Wastewater sample collected from Hisar City. Two different sewage treatment plants were selected for sampling. A total of 2 years of wastewater samples were collected from the Domestic sewage treatment plant and Medical college sewage treatment plant in Hisar, Haryana, India. Samples were collected in Tarsons - a 500mL wide mouth bottle sample was collected in triplicate and carried in the lab and kept in a deep fridge. For isolation of bacteria, the membrane filtration method was used with the help of 0.45µm cellulose nitrate membrane filter. Aseptically this membrane was transferred on autoclaved culture media plates. For culturing of microorganisms different kinds of culture media were used including Mannitol salt Agar base (M118), R-2A Agar (M962), Lactose blue agar (M1968), Nutrient Agar (M001), BHI (M211), Tryptic Soy Agar (M1968). All culturing and further experimental work was performed in triplicated. 5 fold serial dilution of wastewater samples used for isolation of microorganisms. Purification of bacterial colonies was performed using the streak plate method. All isolates were subjected to Gram staining which results in Gram-negative Bacilli from a majority of tests. Biochemical identification analyses were done at the primary and secondary levels and then subjected to an antimicrobial susceptibility test. The antibiotic susceptibility test was done by using Mueller Hinton Broth and Agar. Initial screening of resistant bacteria was done by using Mueller Hinton Broth and it was spiked with different concentrations of antibiotic - vancomycin hydrochloride (CMS217), ampicillin sodium salt (MB104), tetracycline hydrochloride (CMS219), rifampicin (CMS1889), piperacillin sodium salt (CMS3690), streptomycin sulfate (CMS220), cefotaxime sodium salt (CMS1193), ceftazidime pentahydrate (CMS1194), chloramphenicol (CMS218), erythromycin (CMS528), trimethoprim (CMS216), amoxicillin (CMS646). Kirby Bauer Disk diffusion method was used for identification of antibiotic-resistant bacteria by using Mueller Hinton Agar. Following discs were Hexa G-plus 14 [HX080], Hexa G- plus 4(HX004), Hexa G- plus 24(HX101).

DNA Extraction: Screen out bacterial species from wastewater having maximum multiple drug resistance activity and was subjected to genomic DNA isolation. The selected bacterial sample was picked up and placed in a motor and homogenized with 1ml of extraction buffer then their homogenates mixture was transferred to a 2ml microcentrifuge tube. An equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added to the tubes and mixed well by gently shaking the tubes. Then these tubes were centrifuged at R.T for 15 minutes at 14,000 rpm (MPW-350R). To separate the layer visible after centrifugation which appears an aqueous layer was transferred to a new tube. Any was precipitated from the solution by adding 0.1 volume of 3M sodium acetate pH 7.0 and 0.7 volume of isopropanol. After 15 minutes of incubation at RT, the tubes were centrifuged at 4°C for 15 minutes at 14000 rpm. Pellet was washed twice with 70% ethanol and then very precisely with 100% ethanol and air-dried. The DNA was dissolved in TE (Tris-Cl, 10mM, pH 8.0, EDTA 1mM) for removal of RNA 5µL of DNAse free RNAse a (10mg/mL) was added to DNA.

DNA quantification obtained DNA for the subject for quantification (result - 65.3ng/µL).

Amplification and Sequencing of DNA: Extracted DNA was used for amplification along with 10pM of each primer with a sequence of 16s forward primer 5' GGATGAGCCCGCGGCCT A 3' and primer 5' CGGTGTGTACAAGGCCCGG 3'. For amplification, DNA was run by using BIO-RAD PCR (Alpha unit block assembly for DNA Engine system- ALS1296). To study whether amplification occurred or not agarose gel electrophoresis was run by using a 500 BP ladder and amplified DNA product. For 16s rDNA Sequencing machine (ABI 3130 Genetic analysis) with chemistry cycle sequencing kit (kit due Terminator version 3.1) polymer and capillary array POP_7pd capillary array.

Results and discussion

Microorganisms showed Gram-negative results, from Gram's staining, Biochemical identification analyses were given in Table-1. Wastewater collected from both sites showed different responses to antibiotic susceptibility tests. Domestic sewage treatment plant (STP) showed 100% resistance towards Ampicillin (AMP), Vancomycin (VAN), Tetracycline (TET) while Medical college sewage treatment plant (STP) showed 100% resistance towards Ampicillin (AMP), Vancomycin (VAN), Tetracycline (TET) and Rifampicin (RIF). For other antibiotic Domestic STP showed 60% susceptibility towards Chloroamphenicol (CHL), 53% for Cephotaxime (CTX), 47% for Erythromycin (ERY), 31% for Piperacillin (PIP), 16% for Amoxicillin (AMX), 23% for Streptomycin (STR), 21% for Trimethoprim (TMP), and 27.1% for Ceftazidime (CAZ) (Figure-1 susceptibility test % from Domestic STP). Similarly,

Medical College STP showed 51.9% susceptibility for Chloramphenicol, 43.2% for Cephotaxime, 39% for Erythromycin, 19.6% for piperacillin, 7% for amoxicillin, 17.9% for streptomycin, 15.7% for trimethoprim, 13% for ceftazidime (Figure-2 Susceptibility test % from medical college STP).

16s rDNA analysis outcome: 16s rDNA technology provides specific bacterial strain identification. Out of a total screen out sample Aeromonas species showed maximum resistance towards multiple antibiotics followed by Bacillus sp. *Aeromonas caviae* (Accession no. CDBK01000019), *Aeromonas dhakensis* (Accession no.CDBH01000037), *Bacillus cersus* (Accession no.AE016877), *Aeromonas hydrophila subsp. hydrophila* (Accession no.CP000462) Bacillus paranthrasis (Accession no.MACE1000012).

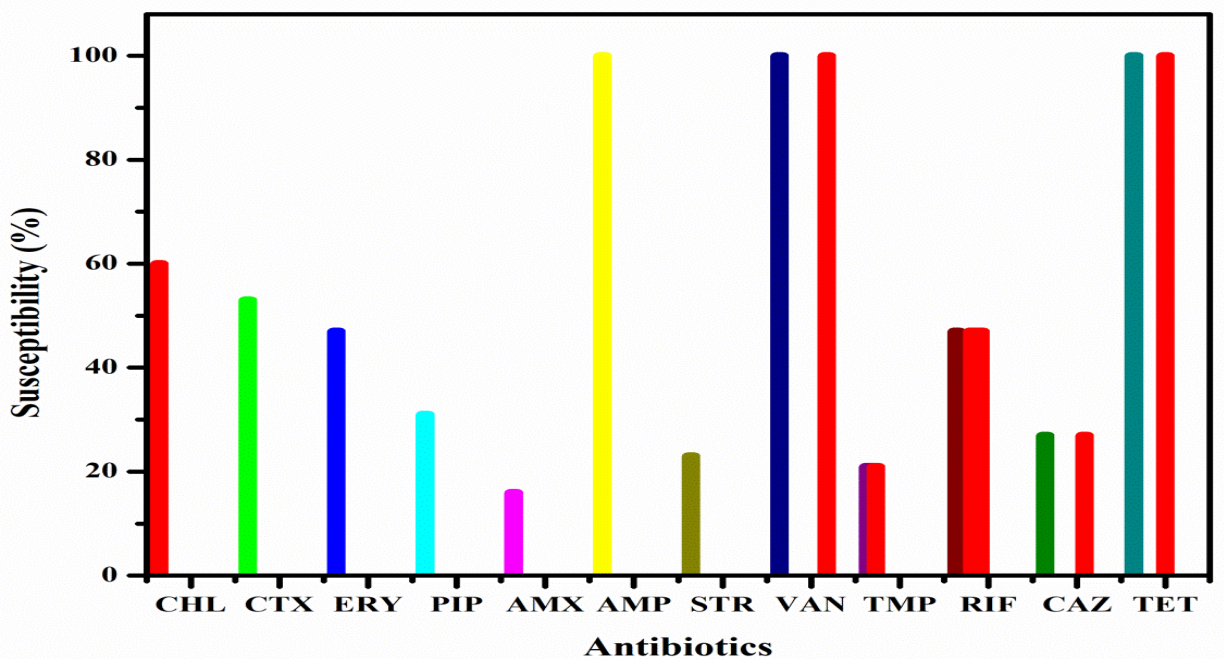

Figure-1: Susceptibility test % from Domestic STP.

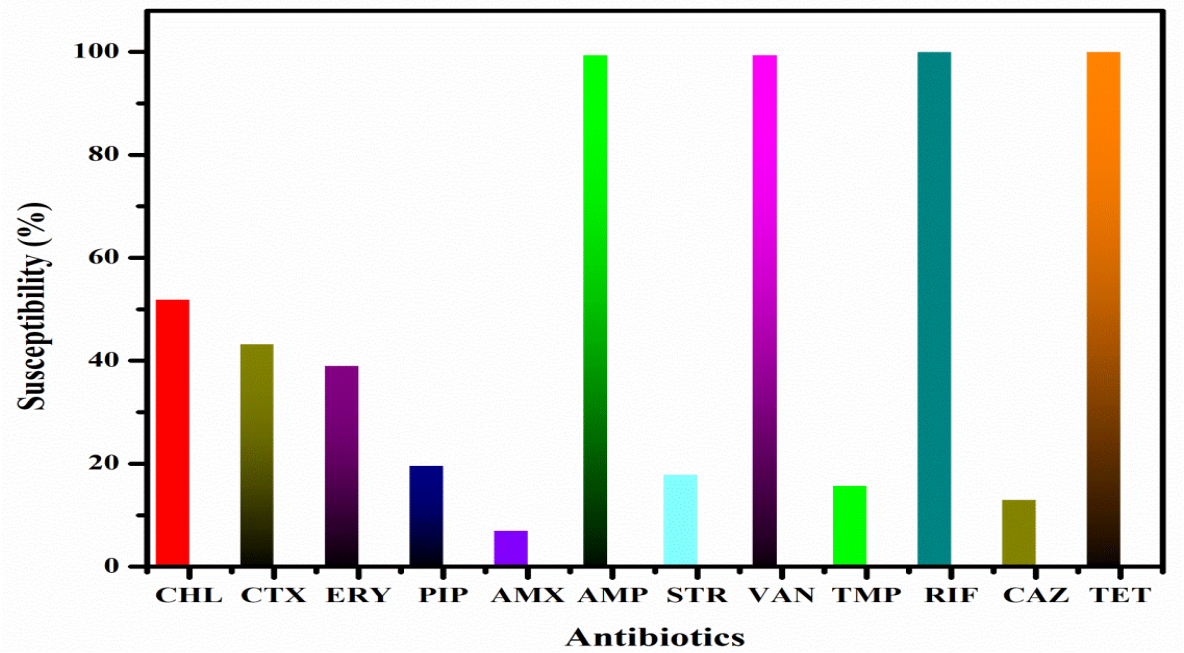

Figure-2: Susceptibility test % from medical college STP.

Table-1: Biochemical identification analyses.

Biochemical identification	Oxidase	Urea	VP	Catalase	Citrate	KCN	LDC	ODC	Amp^R
A	+	-	+	+	+	+	+	-	R
B	+	-	-	+	+	+	-	-	R
C	+	-	-	+	+	+	+	+	R

The main objective of the study was to isolate and characterize antibiotic resistance microorganisms from environmental samples, specifically wastewater treatment. The presence of pathogenic bacteria in the sample poses a severe health-related risk to consumer's mainly immuno-compromised individuals as well as general people. Antimicrobial profiling of wastewater reveals that isolates were 100% resistant to ampicillin, vancomycin and tetracycline antibiotics. Previous studies reported the presence of tetA, tetE, genes coded for tetracycline resistance located in the plasmid region. For streptomycin, there are sat1, aadA1, and aadA2 genes that provide resistance against streptomycin. For chloramphenicol resistance, there are specific genes like cat, catB2, catB3, catB8, reported in *Aeromonas caviae* as well as other Aeromonads.

Conclusion

An increased amount of antibiotics in day to day life creates serious conditions like multiple drug resistance. According to WHO, industrialized countries have about 30% of the population that is affected by food-related disorders every year. Similarly, by 2050 multiple drug resistance will be the main reason for death all over the world. This situation became worse due to antibiotic abuse and misuse widely even if it's educated or not. This situation leads to gain resistance in bacteria through different mechanisms like Horizontal and vertical gene transfer. Both activities support bacteria to become resistant to multiple drugs. Environment behaves as a resistome for these genes. Commensal bacteria such as Proteobacteria, Bacteroidetes, Acidobacteria, Firmicutes, Chloroflexi, and Actinobacteria are the main dominating phyla in wastewater treatment plants carrying multiple drug resistance genes with them. Multiple drug resistance in bacterial species against multiple antibiotics now become a global threat to animal and human health as well as the environment and food safety. Bacteria use horizontal gene transfer (HGT) for the exchange of genetic material which greatly enhances multiple drug resistance conditions. Ultimately this situation gives rise to "superbugs" that can easily tolerate almost all antibiotics.

References

1. Greay, T. L., Gofton, A. W., Zahedi, A., Paparini, A., Linge, K. L., Joll, C. A., & Ryan, U. M. (2019). Evaluation of 16s next-generation sequencing of hypervariable region 4 in wastewater samples: an unsuitable approach for bacterial enteric pathogen identification. *The Science of the Total Environment*, 670, 1111–1124.

2. Sun, S.-J., Zhao, Z.-B., Li, B., Ma, L.-X., Fu, D.-L., Sun, X.-Z., Thapa, S., Shen, J.-M., Qi, H., & Wu, Y.-N. (2019). Occurrence, composition profiles and risk assessment of polycyclic aromatic hydrocarbons in municipal sewage sludge in China. *Environmental Pollution*, 245, 764–770.

3. Wang, M., Shen, W., Yan, L., Wang, X.-H., & Xu, H. (2017). Stepwise impact of urban wastewater treatment on the bacterial community structure, antibiotic contents, and prevalence of antimicrobial resistance. *Environmental Pollution*, 231(Pt 2), 1578–1585.

4. Zhang, L., Shen, Z., Fang, W., & Gao, G. (2019). Composition of bacterial communities in municipal wastewater treatment plant. *The Science of the Total Environment*, 689, 1181–1191.

5. Jünemann, S., Kleinbölting, N., Jaenicke, S., Henke, C., Hassa, J., Nelkner, J., Stolze, Y., Albaum, S. P., Schlüter, A., Goesmann, A., Sczyrba, A., & Stoye, J. (2017). Bioinformatics for NGS-based metagenomics and the application to biogas research. *Journal of Biotechnology*, 261, 10–23.

6. Rasmussen-Ivey, C. R., Figueras, M. J., McGarey, D., & Liles, M. R. (2016). Virulence factors of Aeromonas hydrophila: in the wake of reclassification. *Frontiers in Microbiology*, 7, 1337.

7. Amador, P. P., Fernandes, R. M., Prudêncio, M. C., Barreto, M. P., & Duarte, I. M. (2015). Antibiotic resistance in wastewater: occurrence and fate of Enterobacteriaceae producers of class A and class C β-lactamases. *Journal of Environmental Science and Health. Part A, Toxic/Hazardous Substances & Environmental Engineering*, 50(1), 26–39.

8. Stachurová, T., Sýkorová, N., Semerád, J., & Malachová, K. (2022). Resistant Genes and Multidrug-Resistant Bacteria in Wastewater: A Study of Their Transfer to the Water Reservoir in the Czech Republic. *Life (Basel, Switzerland)*, 12(2), 147.

9. Fadare, F. T., Adefisoye, M. A., & Okoh, A. I. (2020). Occurrence, identification, and antibiogram signatures of selected Enterobacteriaceae from Tsomo and Tyhume

rivers in the Eastern Cape Province, Republic of South Africa. *PloS One*, 15(12).

10. Fouz, N., Pangesti, K. N. A., Yasir, M., Al-Malki, A. L., Azhar, E. I., Hill-Cawthorne, G. A., & Abd El Ghany, M. (2020). The Contribution of Wastewater to the Transmission of Antimicrobial Resistance in the Environment: Implications of Mass Gathering Settings. *Tropical Medicine and Infectious Disease*, 5(1), 33.