Antimicrobial resistance and ARGsdetection in treated final effluent from STPs: An upcoming challenge to the environment

ABSTRACT

Aim: The present study analysed the presence of antimicrobial resistance organisms and genes in the final effluent from STPs of hospitals.

Study Area and Sampling: Samples for microbiological analysis were collected from two different hospitals in Trivandrum City and carried out further microbiological analysis.

Methodology:In this study,MDRB (Multi Drug Resiatant Bacteria) were detected from the hospital effluent samples collected from two hospitals. Antibiotic susceptibility analysis showed that the 90% screened organisms was resistant to different antibiotics -Tetracycline (30µg), Amikacin (30µg), Gentamycin (10µg), Ciprofloxacin (5µg), Colistin (10µg) and Amoxicillin (30µg)).Metagenomic surveillance of effluent helped to assess the efficacy of STPs, at the same time assessing the local clinical antibiotic resistance condition by detection of the presence ofantimicrobial resistance towards antibioticsand their genes (ARGs) in the hospital effluent.

Results: In this study, a total of 3 antibiotic resistantbacterial strains obtained from hospital STP effluent were identified by16S rRNA sequence analysis. The sequences of *E.coli*, *Klebsiella* and *Enterobacter* were submitted in Genbank with accession number MT784125, OM978270 andMN437586 respectively.The final effluent from Hospital 1 showed100% resistance to Tetracycline and 86% resistance to Amoxicillin followed by sensitivity 28%, 22%, 18% and 10% respectively to Ciprofloxacin, Gentamycin, Amikacin and Colistin. Thebacterial strains isolated from final effluent of Hospital 2 showed highest resistance to Amikacin and Colistin which is 100% and 86% and 82% resistance to Gentamycin and Ciprofloxacin.. The gene primers used for the respective genes above have been amplified in the sample with a higher efficiency of *16 SrRNA*, *ermB* and *amp*C primers showing a lower Cq value. Thus, these three genes were detected in the samples at high amount which showed the prominent use of the consecutive antibiotics in the clinical field

Keywords: Antibiotic resistance, Abrgenes,Final effluent, Metagenomics,Sewage treatment plants

1. INTRODUCTION

An antibiotic isbasically an antimicrobial agent widely used for the therapy and prohibition of bacterial infections.They will end up the growth of bacteria and limitedthe numberwhich possesses antiprotozoal activity and not at all efficacious against viruses,it can be treated only by some anti viral drugs.

Antimicrobial Resistance occurs when microbes swap their character over time period and no longer respond to the medicinesand cause infections harder which will make risker conditions like disease spread, illness etc. The exposure of organisms to various antibiotics will leads to the cumulation of resistance features which helps them to develop cross resistance results in the formation of multidrug-resistant (MDR) bacteria. Multi Drug resistant organisms especially pathogens are a significant threat to global public health and are one of the main clinical concerns [3].The occurrences and escalation of multidrug antibiotic resistance among bacterial pathogens may have serious effect on human health [4].

Antimicrobial resistance (AMR) is a worldwide risk to public health and environment, the overuse of antibioticsin hospital field and human medicine has accelerated the growing worldwide phenomenon of AMR.The medical institutions mainly hospitals also play a significant role in the distribution of antimicrobial condition and rise of ARBs into the environment [1]. Hospital effluents (HEs) are a mixture of different types of waste which may contains special category probably highly hazardous to public health and environment because of their infectious and toxic characteristics [2]. Hospital effluent are the major suppliers of infection causing bacteria and a variety of substances with antimicrobial activity which will affect in adverse .The effluentfrom the hospitals consist of pathogens mostly antibiotic resistant microbes mainly from the sources like research, medicinal excretion from the patients etc.Hospital wastewaters are considered generally as trouble spots for antibiotic resistance, generating an environment for the exchange of antibiotic resistance genes.After treatment from the STPs water is discharged into environment and on water bodies or surface waters. Certain infectious organisms have the capacity to remain in water especially in an aquatic environment for longer periods, creating dissemination routes and environmental reservoirs of antibiotic resistance genes (ARGs) [3].

The acquired new resistance mechanisms are the main cause of the emergence and spread which leads to the occurrence of antimicrobialresistance continues to threaten our ability to treat common infections. Superbugs are the example for this type of pathogens and also known as pan-resistant bacteria.Theperseverance of ARBs and their genes in the clinical environment is due to the misusage ofantibiotics by the human beings which is a whole threat to the environment. The virulence, pathogenicity, disease outbreaks and transmission, leading to prolonged morbidity, hospitalization and even mortality is the result of antibiotic resistance. The developing of resistance to common first-line antibiotics causes the decline over the control of human infections and diseases. [5].

ARGs are not completely removed by the biological treatments but sometimes remain after the chemical treatments also like the disinfection process etc. This causes the discharge of antibiotic resistance into the environment by the effluent from the treatment plants. The effluents from the hospitals are considered as special category because of its hazardous nature and that is due to the presence of biomedical and clinical waste particles which includes the waste materials of patients with infectious diseases. Some research studies have reported that the presence of ARB resistance genes in hospitals effluents is higher compared to wastewater because of the usage of antibiotics in wider level. [6].

This study is focused on the detection of antimicrobial resistance in wastewater especially from Sewage Treatment plant effluent in hospitals from Trivandrum city and toanalyse the presence of ARGs and more broadly antibiotic resistant bacteria.

2. MATERIALS AND METHODS

2.1 Sample Collection

Thecurrent study was carried out to detect the presence of ARBs andgenes infinal effluentof twohospitals inThiruvananthapuram district,Kerala. Samples for microbiological analysis were collected in sterile glass bottles and placed in icebox and transported to the laboratory for further analysis.

2.2 Isolation and Identification of Antibiotic Resistant bacteria

The presence of coliform bacteria was detected using MPN (Most Probable number) technique.The direct streak plate culture technique was carried out in order to detect the presence of *Pseudomonas* and Enterobacteriaceaespecies like*Staphylococcus*,*Salmonella Shigella*on respective media,Pseudomonas Agar, MSA and XLD agar respectively.*E. coli* wasfurther confirmed from positive MPN tubes by streak plating on Eosin Methylene Blue (EMB) agar or Endo Agar followed by observation for green metallic sheen.

The isolated genomic DNA was checked by agarose gel electrophoresis and was further used for the 16S rDNA amplification .The microbial DNA was amplified using 16srRNA primers. PCR reaction was carried out inSimpliAmp Thermal Cycler, Thermofischer (The Applied Biosystems). A reactionmixture (25 µl reaction volume) was containing 1.5µl of 10µM forward primer, 1.5µl of10µM reverse primer, 12µl of Takara master mix; 5µl of sterile autoclaved water, and 5µlof template DNA samples.The following universal primers were used;27F(5′CGGCCCAGACTCCTACGGGAGGCAGCA3′)1492R(5′GCGTGGACTACCA GGGTATCTAATTC3′).The template DNA was amplified on DNA thermocycler using the PCR conditions 94°Cfor 4 minutes, 94°C for 30 seconds, 64.5°C for 30 seconds, 72°C for 30 seconds. The total numbers of cycles were 35, with final extension at 72°C for 5 minutes,performed using the programmer.Agarose gel electrophoresis was done for the qualitative analysis of PCR products.Horizontal gel electrophoresis unit was used to run the sample on the gel to determine thesize of amplicons. The PCR products were electrophoresed on 2% agarose gel stainedwithEthidium Bromide (1mg/ml), run at constant voltage of 50V in 1XTAE buffer. A100bp DNA ladder was used for the comparative study. The gel documentation wascarried out using Documentation Unit. The remaining PCR product was stored at -20°Cfor sequencing.PCR products of 16S rRNA of the isolate was obtained through amplification and werepurified and sequenced.Sequence results obtained were analyzed using applied bio systems and NCBI-BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi#). A consensus sequence was generated fromforward sequence data using applied bio system software. The consensus sequence wasused to perform BLAST against the NCBI Gene Bank database. The first ten sequenceswere chosen based on their maximum identity score and aligned using the multiplealignment software program.Based on the phylogenetic tree and the pair-wise distance matrix, the closest homolog of each isolate from the NCBI Gene Bank database wasidentified. The nucleotide sequence of the current isolates wassubjected to Blast.

2.3 Antibiotic Susceptibility Test

Antibiotic Susceptibility test for various bacterial isolates was done by antibiotic disks (Himedia) in accordance with the Clinical and Laboratory Standards Institute (CLSI) by Kirby-Bauer disc diffusion method. To carried out the test, the isolates observed were inoculated into Muller Hinton broth and incubated for 12 -24 hrs. The turbidity of the broth for the method was further made equivalent to 0.5 McFarland standards and was used for inoculation over Muller Hinton agar plate by lawn culture. The various antibiotic discs used for the study were Tetracycline (30µg),Amikacin (30µg),Gentamycin (10µg),Ciprofloxacin (5µg),Colistin (10µg) and Amoxicillin(30µg)) .The discs were placed over the lawn culture of MHA plates and were incubated for 24 hrs at 37°C to observe the zone of inhibition inorder to confirm whether the isolates are resistant or not.

2.4 Metagenomic DNA isolation from water samples:

Total DNA was isolated from the water sample using the soil DNA isolation kit from MagGenome. Briefly, 250ml of the water sample was filtered through an autoclaved nitrocellulose membrane with a pore size of 0.2µm using a sterile syringe filter. The membrane was ground in liquid nitrogen and care was taken to avoid thawing of the sample. The ground powder was later transferred to 2ml centrifuge tubes. Following this, DNA was isolated using the manufacturer's protocol (http://www.maggenome.com/wp-content/uploads/2022/04/XpressDNA-Soil-kit.pdf).

The isolated DNA was quantified using nanodrop and further checked for the presence of inhibitions in the PCR amplifications by the amplification of the 16S rRNA gene (27F and 1492R primer pair) using conventional PCR and visualization of gel image. The concentration of the isolated DNA was found to be 314 µg/ml with an absorbance ratio (A260/A280) of 1.8.

2.5 qRT PCR Amplification of Resistant gene

The isolated DNA was quantified using nanodrop.The concentration of the isolated DNA was found to be 314 µg/ml with an absorbance ratio(A260/A280) of 1.8.These samples were further subjected to qPCR amplifications in the previously obtained dilution of DNA (Sample Concentrations 1:4) using the provided gene primers. The PCR reaction was carried out in 10µl reaction mixture containing 5µl 2X SYBER GREENqPCR Mix (G bioscience), 1µl of 2µM forward and reverse primer, 1µl of template DNA and2µl Nuclease free water. TheDNA dilution (1:4) was found to have maximum amplification and minimum inhibition. The dilution 1:4 was used in further amplifications using the primer pairs.The primer sequences (Table: 1) used for qPCR using previously published primer setsallowing the amplification of short amplicons (160–420 basepairs).

3. RESULTS

3.1 Isolation and identification of Antibiotic resistant bacteria

In this study, a total of 3antibiotic resistantbacterial strains obtained from hospital STP effluent were identified by16S rRNA sequence analysis. The sequences of *E.coli, Klebsiella* and *Enterobacter*were submitted in Genbank with accession number MT784125, OM978270 andMN437586 respectively. Here, NCBI BLASTn analysis confirmed the isolates as *E. coli* and *Klebsiella* and *Enterobacter*. The phylogenetic analysis of the isolated strains are represented below (Figure 1&2)

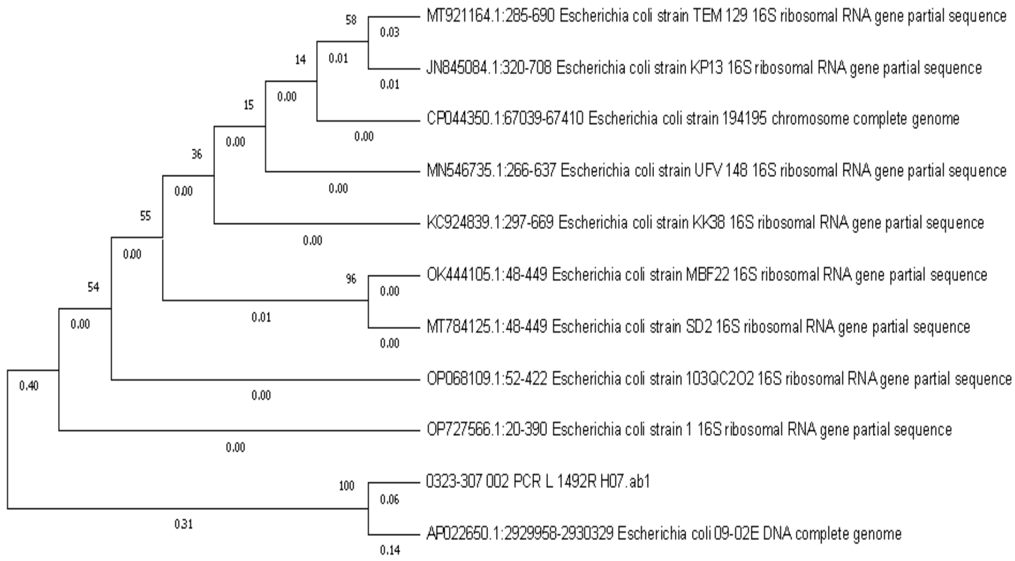

Figure.1.16S rDNA phylogeny of *Ecoli* isolate[12, 13]

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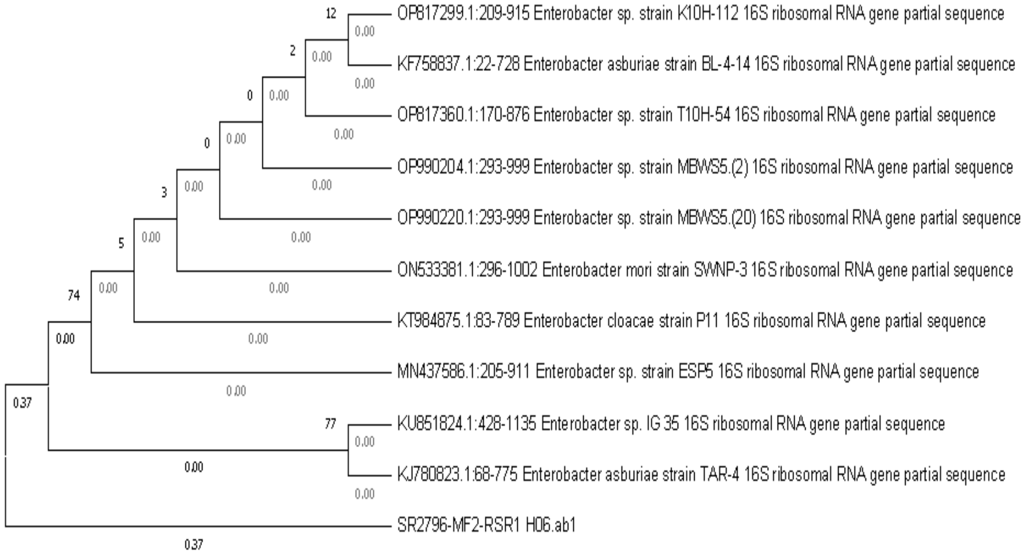

Figure.2.16S rDNA phylogeny of *Enterobacter* isolate[12, 13]

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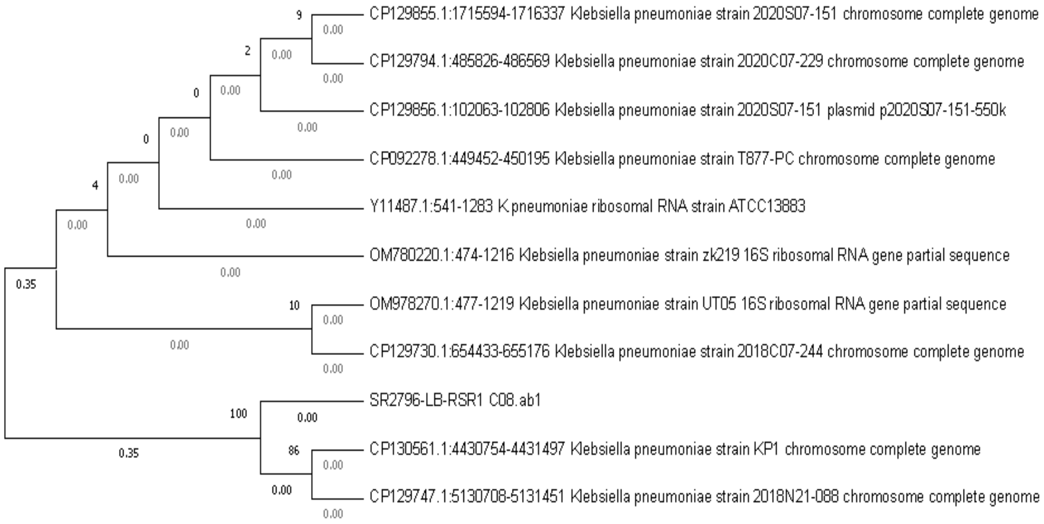

Figure.3.16S rDNA phylogeny of *Klebsiella* isolate[12, 13]

(https://drive.google.com/file/d/1q4n6Owo7uG_Ghr4fALRHDIgFCiszd6Et/view?usp=drive_link)

3.2 Antibiotic Susceptibility Test

Out of the 6 major antibiotics tested, the bacterial strains isolated from final effluentof Hospital 1exhibitedhighest resistance to Tetracycline and Amoxicillin. Here the final effluent fromHospital1 showed100% resistance to Tetracycline and 86% resistance to Amoxicillin followed by sensitivity 28%, 22%, 18% and 10% respectively to Ciprofloxacin,Gentamycin,Amikacin and Colistin.Thebacterial strainsisolated from final effluent of Hospital 2 showed highest resistance to Amikacin and Colistin which is 100% and 86% and 82% resistance to Gentamycin and Ciprofloxacin.Antibiotic resistant profileof the selected hospitals is shown below (Figure:4)

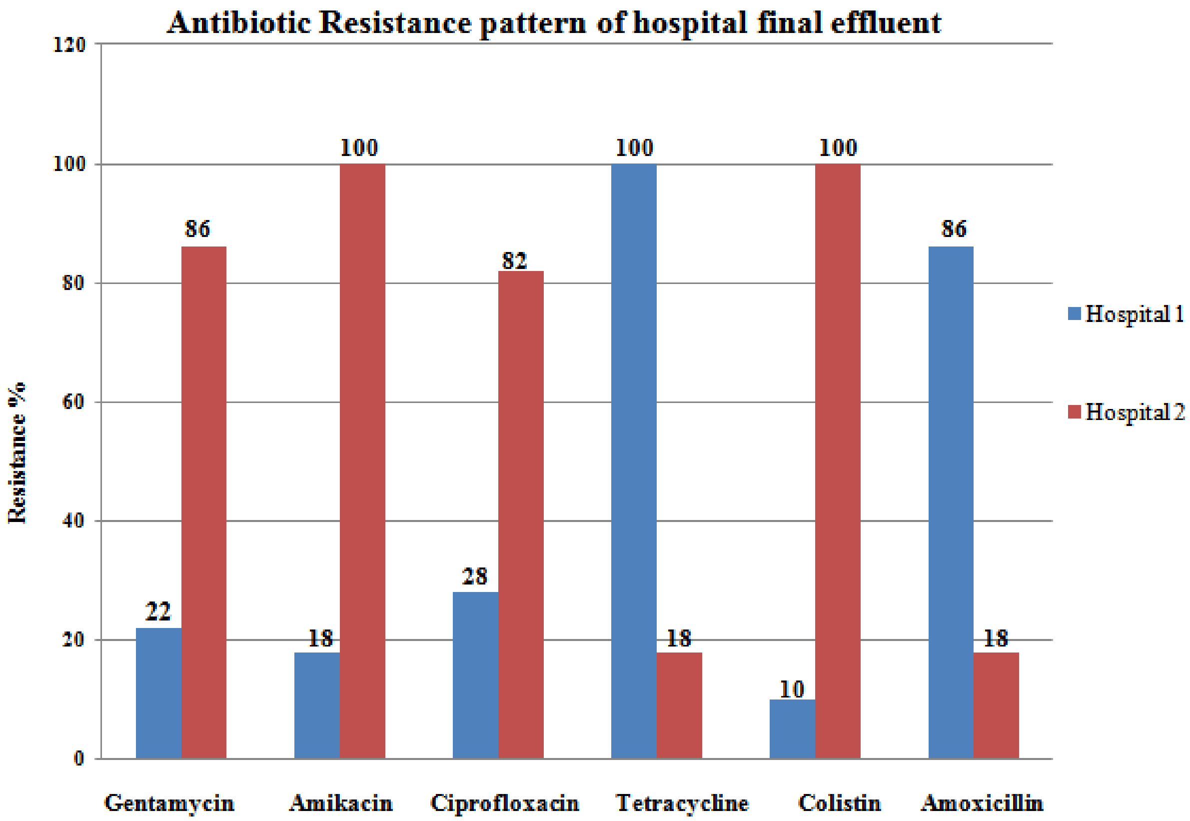

Figure.4. **Antibioticresistance pattern of hospital final effluent**

3.3 MetagenomicDNA isolation from water samples

For Metagenomic analysis, DNA bands were obtained and the concentration of the isolated DNA was found to be 314 µg/ml with an absorbance ratio of 1.8 (A260/A280). TheGel image of PCR product during DNA isolation is represented in Figure 5

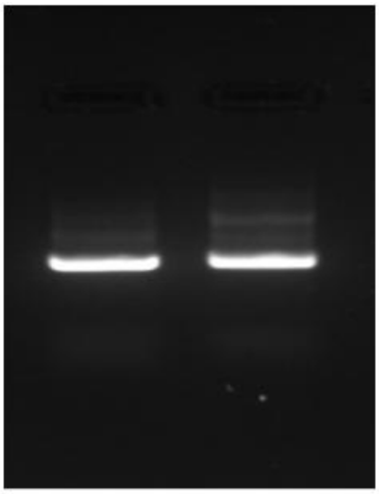

Fig.5.**Gel image of PCR product during DNA isolation**

The primer sequences used for the metagenomic analysis of the sample is shown below (Table 1).

Table 1: Primersequences [14]

Gene	Sequence	Annealing temperature (°C)
sul1	F: CGCACCGGAAACATCGCTGCAC	68
	R: TGAAGTTCCGCCGCAAGGCTCG	
Sul2	F: TCCGGTGGAGGCCGGTATCTGG	60
	R: CGGGAATGCCATCTGCCTTGAG	
ermB	F: CATTTAACGACGAAACTGGC	63
	R: GGAACATCTGTGGTATGGCG	
ampC/UKSAL1	F: TTCTATCAAMACTGGCARCC	55
	R: CCYTTTTATGTACCCAYGA	
tet(A)	F: CCTGCGCGATCTGGTTCACT	55
	R: GCCAGCGAGACGAGCAAGA	
vanA	F: ATGAATAGATAAAAGTTGCAATAC	55
	R. GGAGTAGCTATCCCAGCATT	
16S rRNA	F: CCTACGGGAGGCAGCAG	68
	R: ATTACCGCGGCTGCTGGC	

The qPCR assay allowed the quantification and detection of Abr genes present in the sample. When testing water samples with this assay, melting curves and amplification curves for theconsecutive primers were obtained. Melt curve of the consecutive primers implies the qPCRamplicon in the samplesassessed. The melt curves of 16SrRNA sequence& Sul 1, Sul 2are represented below in Figure 6,Melt curves of amp C&vanA are represented in Figure 7& Melt curves of ermB & tetA are represented in Figure 8 and further amplification was represented in Figure 9, 10&11respectively.

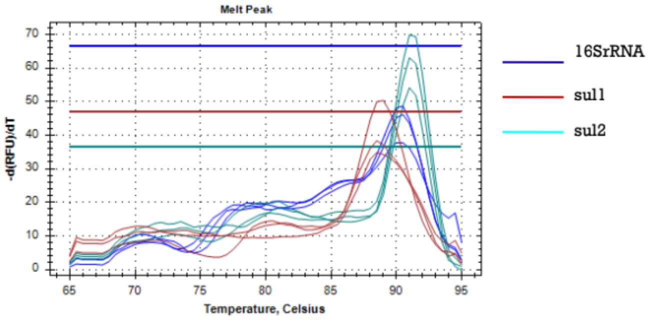

Figure.6. Melt curve of 16SrRNA sequence& Sul 1, Sul 2 shows the melt profile for PCR amplicons

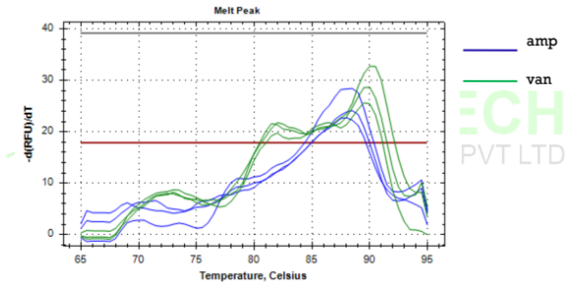

Figure.7. Melt curve of amp C&vanAsequenceshows the melt profile for PCR amplicons.

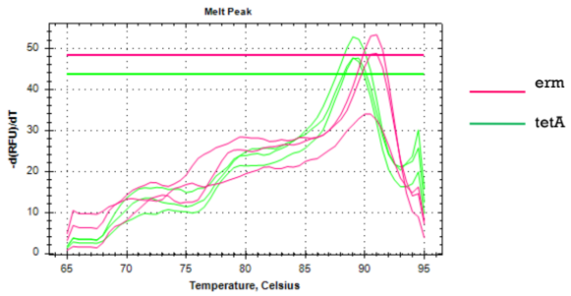

Figure.8. Melt curve of ermB&tetAsequence shows the melt profile for PCR amplicons

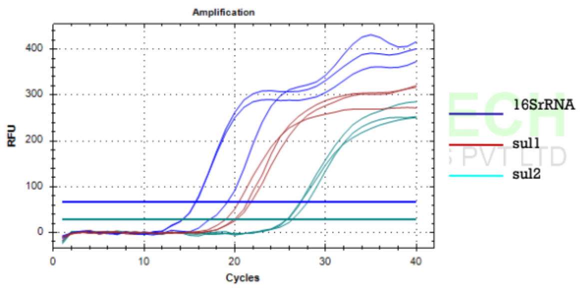

Figure.9.Amplification of 16 S rRNASequence, Sul1&Sul2

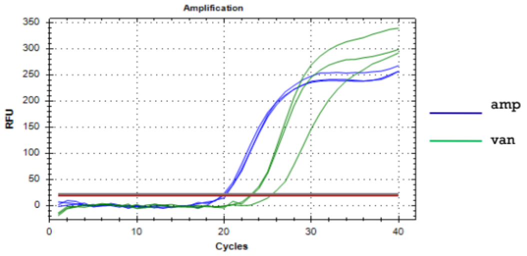

Figure .10.Amplification of ampC&van A

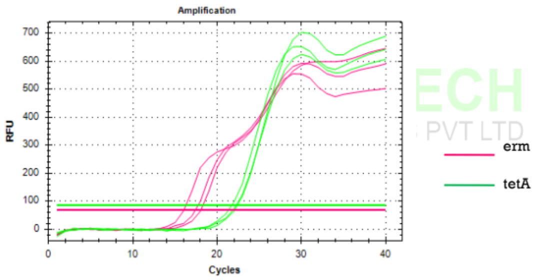

Figure.11.Amplification of ermB&tet A

.Quantification data of sample replicates and its Quantification cycle mean representsthe amount of genes present in the sample (Table 2).

Table 2:Quantification data

Gene	**Sample replicates**	**Cq Mean**
Sul 1	Hospital 2a	21.80
Sul 1	Hospital 2b	21.41
Sul 1	Hospital 2c	20.46
16SrRNA	Hospital 2a	19.14
16SrRNA	Hospital 2b	15.67
16SrRNA	Hospital 2c	15.64
Sul 2	Hospital 2a	26.37
Sul 2	Hospital 2b	25.82
Sul 2	Hospital 2c	25.76
ermB	Hospital 2a	16.03
ermB	Hospital 2b	18.05
ermB	Hospital 2c	17.39
Tet A	Hospital 2a	21.72

Tet A	Hospital 2b	22.39
Tet A	Hospital 2c	22.23
amp C	Hospital 2a	19.70
amp C	Hospital 2b	20.19
amp C	Hospital 2c	19.92
van A	Hospital 2a	25.48
van A	Hospital 2b	23.13
van A	Hospital 2c	23.25

For Analysis, metagenomic DNA isolated from wastewater samples was used to determine ARGs profiles.The gene primers used for the respective genes above have been amplified in the sample with a higher efficiency of *16 SrRNA, ermB* and *amp*C primers showing a lower Cq value. Cq value is inversely proportional to the amount of genes in the sample, Cq mean value for 16 SrRNA was 19.14,15.67,15.64 and for ermB was 16.03,18.05,17.39 and for ampC was 19.70,20.19 and 19.92 respectively ,thereby indicating higher relative quantification. Thus, these three genes were detected in the samples at high amount which showed the prominent use of the consecutive antibiotics in the clinical field.While ,*Sul 1* ,*Sul 2, Tet A* and *van A*had higher Cq value of about 21,25, 22 and 23 respectively indicating , lower quantification compared to *16 SrRNA, ermB* and *amp*C.

4. DISCUSSION

The advancement and growth of Antimicrobial resistance associated with the STP sector is an emerging threat to health and social well being of humans and the environment.Antimicrobial resistance in wastewater and hospital effluent has been noted as one of theincreasing microbial threats of this century. Recent studies show the external validity of the resistant organisms and resistance genes from effluent to humans through water bodies which is an emerging risk factor for public health [7]. Many studies have spotlighted the role of wastewater as a significant environmental reservoir of AMR as it represents a supreme environment for AMR bacteria and antimicrobial resistant genes to persist [8].Many researchworks have focus attention on the role of sewageeffluent as a major environmental reservoir of AMR, as it represents an ideal environment for AMR microorganisms and ARGs to persevere. The situation of ARGs is composite, because they are not degradable and can even be spread among microbial communities in the environment through the mechanism of horizontal gene transfer, which is the main resistance mechanism in *Enterobacteriaceae family.*

Ampicilin, Sulfamethoxazole, and Ciprofloxacin, or closely related drugs (Amoxicillin), are among the top 5 antibiotics prescribed for use for adults in the United States [9], and all have been found to occur in WWTPs in varying concentrations and design conditions. Aftertheusage of the antibiotic drugs and related substances, they are released into effluent through patient excreta. Unused drugs are sometimes junked of down drains and mixed up with the wastewater .The drugs were released into the effluent where they are not eliminated through the process of sewage treatment method and enter the aquatic environment and eventually reach drinking water. If the concentrations are high enough, effluent from hospitals, municipal sewage and sewage treatment plants (STPs) may become a reservoir for the selection of resistant bacteria [10].

The predominant organism detected being Escherichia coli, Klebsiella pneumoniae), Citrobacter freundii, Alcaligenes faecalis, and Pseudomonas mendocina which is commonly found in this effluents. Besides Escherichia coli recording the highest prevalence among all others[11]In the current study, the samples (final effluent) collected from two hospitals were found to have thepresence of antibiotic resistant bacteria, E. *Coli*MT784125, *Klebsiella* OM978270 and *Enterobacter sp*MN437586, which were resistant to multiple drugs. Theattendance of antibiotics in hospitals,clinical samples and treatment plants may help the bacterial genes to persist in the micro biome for a long time and helps to develop resistance.The presence of antibiotics even in small amount in the final effluent after treatment ensures the sustainability of such resistant genes and may dominantly express in the microbial community.

Commonly theeffluents from hospitals mainly in hospital wastewater contain a high number of multidrug-resistant coliform bacteria and *E. coli*. These strains can transfer multiple resistance genes through the conjugative plasmid and spread them to susceptible bacterial species. Hospital wastewaters frequently contain significant amounts of fecal coliforms, which exhibit resistance or multi-resistance towards various types of antibiotics [12]. Theexistence of AMR bacteria and antimicrobial resistance genes in the hospital environment,clinal samples and its associated wastewater poses a potential cross-transmission threat to patients who are more vulnerable to opportunistic and cross infections, healthcare staff and the public in the wider community setting [13].

Metagenomic analysis benefits the ability to quantify thousands of genes in the sample and the data can be reanalysed if novel genes of interest are identified.Here, in this work, qPCRtechnique objectively and independently document the abundance of ARG in wastewater effluentand quantified the absolute abundance of target genes in rawwastewater samples..The DNA was isolated from final effluent samples collected from 2 hospitals on different sampling days for metagenomic analysis.In this study, STP effluent was found as a potential source of AMR by detecting ARGs using a Metagenomics DNA-seq approach. Metagenomic DNA-seq analysis showed that the genes amplified in the sample with a higher efficiency were, *16 SrRNA*, *ermB* and *ampC* primers showing a lower Cq and higher relative quantification values which denotes higher abundance of these 3 genes in the sample. This is an indication of heavy usage of the antibiotics like Erythromycin and Ampicilin in the clinical field.Overall, we found that the STP can reduce the amount of ARB but still releases a significant amount of ARGs. The limitation regarding the metagenomic analysis is that in most environments, ARGs are rare in number in comparison to other functional genes, and therefore deep sequencing is needed to capture the whole diversity .Most metagenomic sequencing platforms produce short reads that, as such, give only limited information about the sequenced genes [14]

5. CONCLUSION

The over usage of Antibiotics inhospitals for disease prevention may cause the evolutionof antimicrobial resistance. *Enterobacter*,*E. coli* and *Klebsiella pneumoniae*species*isolated* in the study shows high resistance to Tetracycline, Amoxicillin and Colistin which might be the after effect of over exploitation of these antibiotics in clinical field as colistin is a polycationic peptide which shows narrow spectrum of activity. Also, the resistance genes are suspected to beemanate through the species and its continuous evolution may causecross resistance to other antibiotics.The metagenomic analysis alsoconfirms the presence of resistant genes in the effluent sample. Hence antibioticuse inhospitals and its final disposal should be monitored properly toavoid the development ofresistance.The study showed a correlation between the antibiotics used in the hospitals and the emergence of antimicrobial resistance among the treated effluent. The results of the study indicate the need for strict control over use of antibiotic in the environment to limit the rapid evolution and spread of antimicrobial resistance to different antibiotics.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. DidierHocquet, Marie Petitjean, Laurence Rohmer, BenoîtValot, Hemantha D Kulasekara, Lucas R Hoffman, Samuel I Miller (2016)"Pyomelanin-producing Pseudomonas aeruginosa selected during chronic infections have a large chromosomal deletion which confers resistance to pyocins", Society for Applied Microbiology and John Wiley & Sons Ltd Environmental Microbiology Volume18(10):3482-3493

2. Sara Rodriguez-Mozaz, Sara Chamorro, Elisabet Marti, Belinda Huerta, MeritxellGros, Damià Barceló, Jose Luis Balcázar (2015) "Occurrence of antibiotics and antibiotic resistance genes in hospital and urban wastewaters and their impact on the receiving river" Journal of Water, Volume 69:234-242

3. Nikola Roulová, PetraMot'ková, IvetaBrožková, MarcelaPejchalováJ Water Health (2022)"Antibiotic resistance of Pseudomonas aeruginosa isolated from hospital wastewater in theCzech Republic" Journal of Water health, Volume 20 (4): 692–701

4. Magdalena Pazda, JolantaKumirska, PiotrStepnowski, EwaMulkiewicz 2019 Dec "Antibiotic resistance genes identified in wastewater treatment plant systems" Journal of Science of The Total Environment, A review Volume 697: 134023.

5. Kristina E Rudd, Sarah Charlotte Johnson, Kareha M Agesa, Katya Anne Shackelford, Derrick Tsoi, R Scott Watson, Mohsen Naghavi 2020 "Global, regional, and national sepsis

incidence and mortality, 1990-2017: analysis for the Global Burden of Disease Study" The Lancet, Volume 395(10219):200-211.

6. U.Rozman, Darja Duh; M.Cimerman; Sonja Sostar Turk "Hospital wastewater effluent: hot spot for antibiotic resistant bacteria", Journal of Water, Sanitation and Hygiene for Development Volume10 (2): 171–178.
7. S. Sreejith, ShamnaShajahan, P.R. Prathiush, V.M. Anjana, ArathyViswanathan, VishnuChandran, G.S. Ajith Kumar, R. Jayachandran, JyothisMathew, E.K.Radhakrishnan (2020) "Healthy broilers disseminate antibiotic resistance in response to tetracycline input infeed concentrates", Microbial Pathogenesis., Volume 149, 104562 149:104562
8. NourFouz, Krisna N. A. Pangesti, Muhammad Yasir, Abdulrahman L. Al-Malki, Esam I. Azhar, Grant A. Hill-Cawthorne and MoatazAbd El Ghany (2020) "The Contribution of Wastewater to the Transmission of Antimicrobial Resistance in the Environment: Implications of Mass Gathering Settings", Tropical Medicine and Infectious disease,2020, *5*(1), 33; https://doi.org/10.3390/tropicalmed5010033.
9. Maitreyee Mukherjee, Edward Laird, Terry J. Gentry, John P. Brooksand Raghupathy Karthikeyan "Increased Antimicrobial and Multidrug Resistance Downstream of Wastewater Treatment Plants in an Urban Watershed", Front. Microbial, 24 May 2021Sec.Antimicrobials, Resistance and Chemotherapy, Volume 12 – 2021 https://doi.org/10.3389/fmicb.2021.657353.
10. K. Kummerer, "Significance of antibiotics in the environment", *Journal of Antimicrobial Chemotherapy*, Volume 52, Issue 1, July 2003, Pages 5–7, https://doi.org/10.1093/jac/dkg293.
11. Addae-Nuku, Daisy S, Kotey, Fleischer CN, Dayie, Nicholas TKD, Osei, Mary-Magdalene, Tette, Edem MA, et al "Multidrug-Resistant Bacteria in Hospital Wastewater of the Korle Bu Teaching Hospital in Accra, Ghana" Environmental Health Insights Volume 16: 1–9
12. T.Mackuak, K.Cverenkarova,M.Feher,MichalTamas , MiroslavGal, Monika Naumowicz, VieraSpalkova,and Lucia Birosova "Hospital Wastewater—Source of Specific Micro pollutants, Antibiotic-Resistant Microorganisms, Viruses, and Their Elimination", Antibiotics (Basel). 2021 Sep; 10(9)
13. University of Limerick. "Bacteria resistant to antibiotics in hospital wastewater system."

ScienceDaily.ScienceDaily,13September2023<www.sciencedaily.com/releases/2023/09/230913122711.htm>.

14. Antti Karkman, Thi Thuy Do, Fiona Walsh, and Marko P.J. Virta "Antibiotic-Resistance Genes in Waste Water" Trends in Microbiology, March 2018, Vol. 26, No. 3
15. Kimura M. (**1980**). "A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences". *Journal of Molecular Evolution* **16**:111-120.
16. Tamura K., Stecher G., and Kumar S. (**2021**). MEGA 11: Molecular Evolutionary Genetics Analysis Version 11. *Molecular Biology and Evolution*https://doi.org/10.1093/molbev/msab120.
17. C. Stangea, J.P.S. Sidhub, S. Tozec**,** A. Tiehma (2019) "Comparative removal of antibiotic resistance genes during chlorination, ozonation, and UV treatment" International Journal of Hygiene and Environmental Health222 (2019) 541–548.