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Antimicrobial Resistance and ARGs Detection in Treated Final Effluent from STPs: An Upcoming Challenge to the Environment

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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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

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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 of antimicrobial resistance towards antibiotics and their genes (ARGs) in the hospital effluent.
Results: In this study, a total of 3 antibiotic resistant bacterial 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. The bacterial 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; Abr genes; final effluent; metagenomics; sewage treatment plants.

1. INTRODUCTION

An antibiotic is basically an antimicrobial agent widely used for the therapy and prohibition of bacterial infections. They will end up the growth of bacteria and limitedthe number which 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 [1]. The occurrences and escalation of multidrug antibiotic resistance among bacterial pathogens may have serious effect on human health [2].

Antimicrobial resistance (AMR) is a worldwide risk to public health and environment, the overuse of antibiotics in 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 [3]. 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 [4]. Hospital effluent are the major suppliers of infection causing bacteria and a variety of substances with antimicrobial activity which will affect in adverse .The effluent from 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 antimicrobial resistance 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. The perseverance of ARBs and their genes in the clinical environment is due to the misusage of antibiotics 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 to analyse the presence of ARGs and more broadly antibiotic resistant bacteria.

2. MATERIALS AND METHODS

2.1 Sample Collection

The current study was carried out to detect the presence of ARBs and genes in final effluent of two hospitals in Thiruvananthapuram 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 Enterobacteriaceae species like *Staphylococcus, Salmonella Shigella* on respective media, Pseudomonas Agar, MSA and XLD agar respectively. *E. coli* was further 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 in SimpliAmp Thermal Cycler, Thermofischer (The Applied Biosystems). A reaction mixture (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 wereused;27F(5′CGGCCCAGACTCCTACGGGAGGCAGCA3′) 1492R(5′GCGTGGACTACCAGGGTATCTAATTC3′).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 the size of amplicons. The PCR products were electrophoresed on 2% agarose gel stained with Ethidium Bromide (1mg/ml), run at constant voltage of 50V in 1XTAE buffer. A100bp DNA ladder was used for the comparative study. The gel documentation was carried 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 were purified 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 from forward sequence data using applied bio system software. The consensus sequence was used to perform BLAST against the NCBI Gene Bank database. The first ten sequences were chosen based on their maximum identity score and aligned using the multiple alignment 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 was identified. The nucleotide sequence of the current isolates was subjected 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. The DNA 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 sets allowing the amplification of short amplicons (160–420 base pairs).

3. RESULTS

3.1 Isolation and Identification of Antibiotic Resistant Bacteria

In this study, a total of 3 antibiotic resistant bacterial 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 (Figs. 1&2)**.**

3.2 Antibiotic Susceptibility Test

Out of the 6 major antibiotics tested, the bacterial strains isolated from final effluent of Hospital 1exhibited highest resistance to Tetracycline and Amoxicillin. Here 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. The bacterial 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. Antibiotic resistant profile of the selected hospitals is shown below (Fig. 4)) of 18% each to th e hospital 2.

3.3 Metagenomic DNA 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). The Gel image of PCR product during DNA isolation is represented in Fig. 5.

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

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 the consecutive primers were obtained. Melt curve of the consecutive primers implies the qPCRamplicon in the samples assessed. The melt curves of 16SrRNA sequence& Sul 1, Sul 2 are represented below in Fig. 6, Melt curves of amp C&vanA are represented in Fig. 7& Melt curves of ermB & tetA are represented in Fig. 8 and further amplification was represented in Fig. 9, 10&11 respectively.

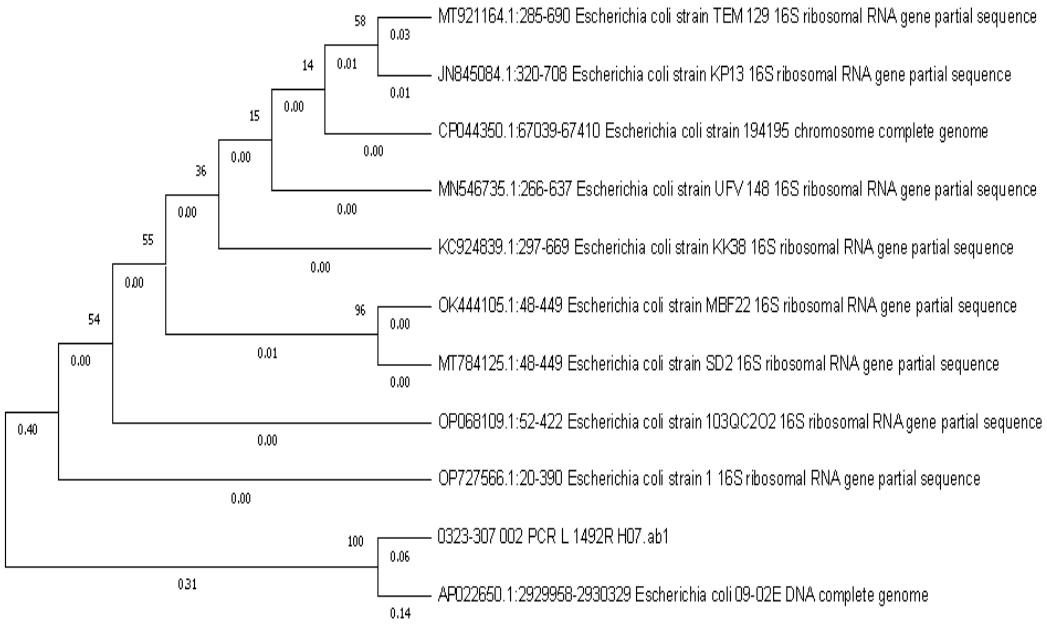

Fig. 1. 16S rDNA phylogeny of *Ecoli* isolate [12,13]
(https://drive.google.com/file/d/1KQ4DBXWcTj0dbb44K-pNb80B1NFxQI-m/view?usp=drive_link)

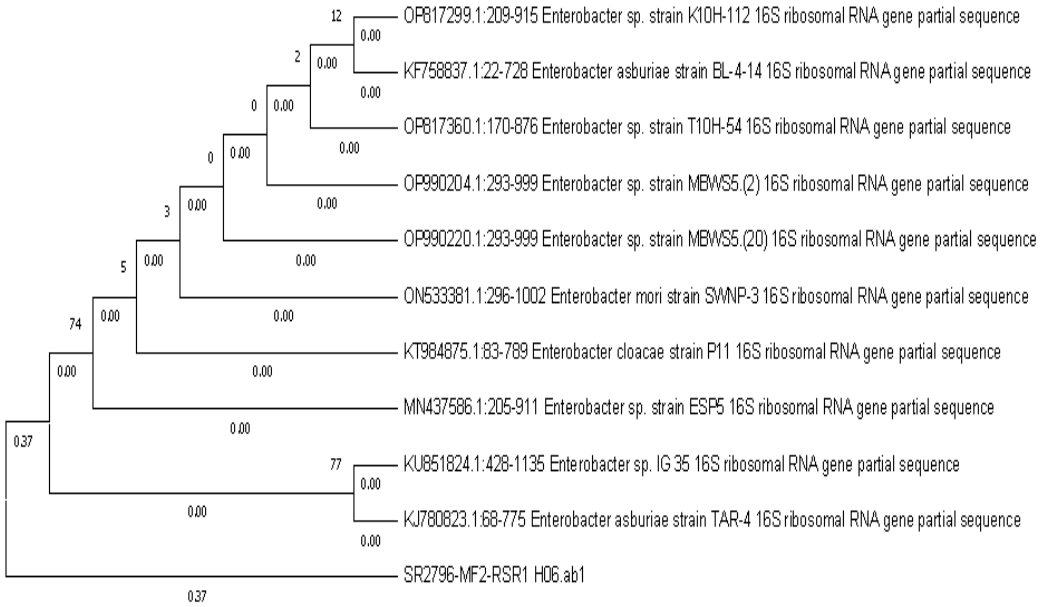

Fig. 2.16S rDNA phylogeny of Enterobacter isolate [12,13]
(https://drive.google.com/file/d/1Uu5eoZweXja-hVMPMQ84WxYcE_P-3VIX/view?usp=drive_link)

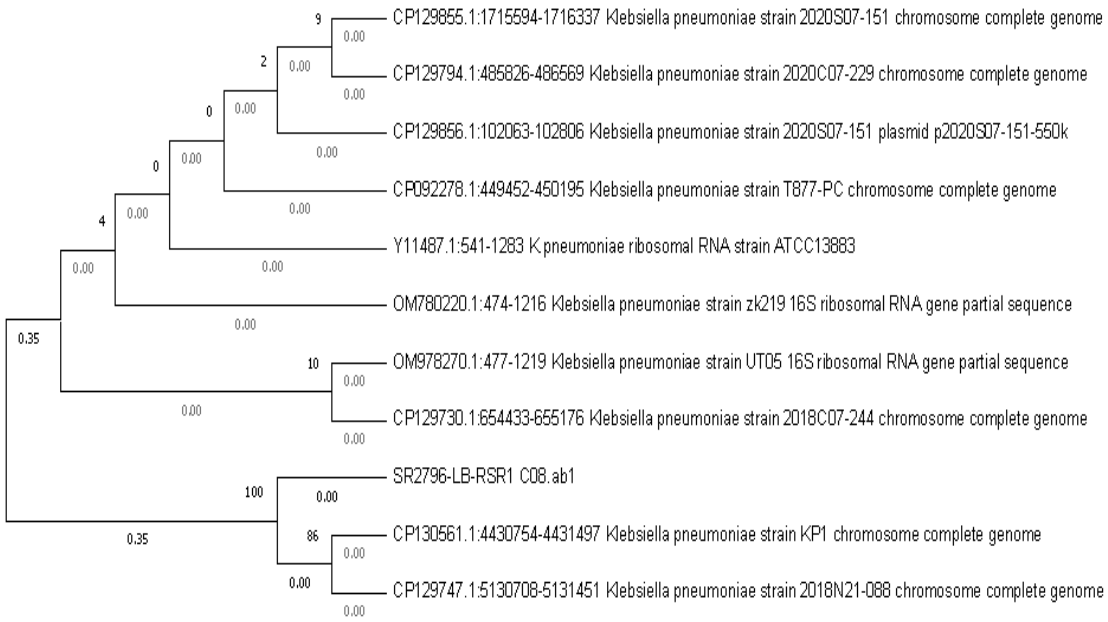

Fig. 3. 16S rDNA phylogeny of *Klebsiella* isolate [12,13]
(https://drive.google.com/file/d/1q4n6Owo7uG_Ghr4fALRHDIgFCiszd6Et/view?usp=drive_link)

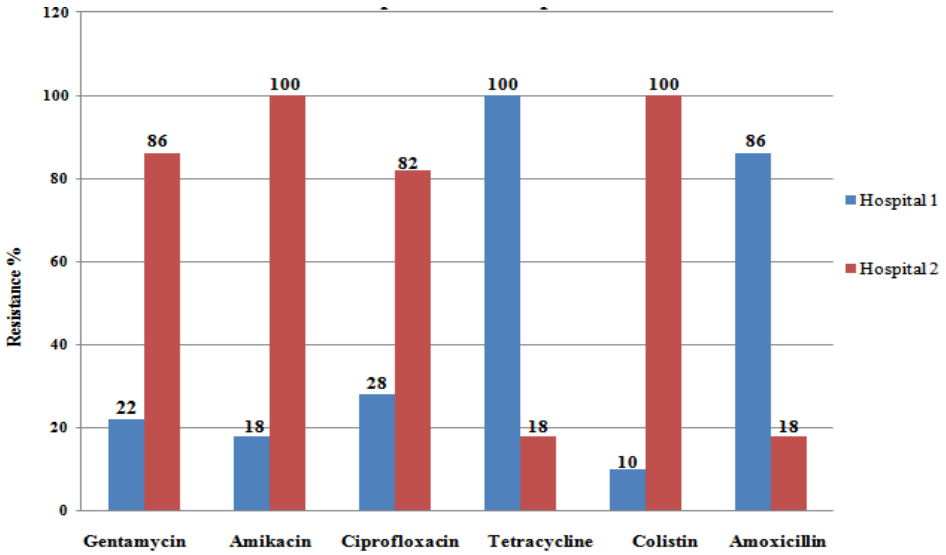

Fig. 4. Antibiotic resistance pattern of hospital final effluent

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.

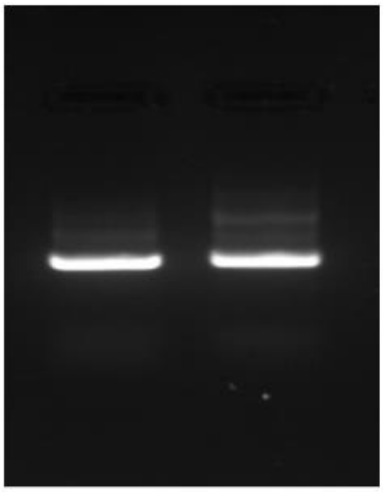

Fig. 5. Gel image of PCR product during DNA isolation

Table 1. Primer sequences [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	

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 the increasing 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 research works have focus attention on the role of sewage effluent 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.*

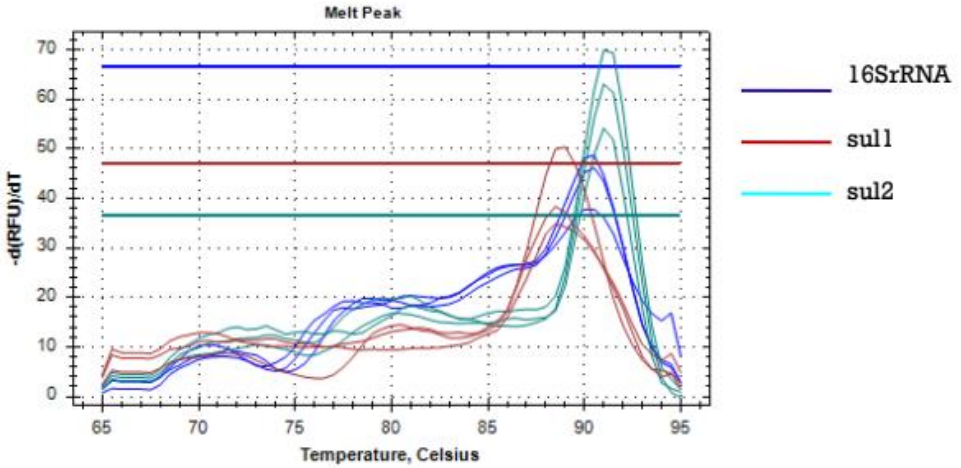

Fig. 6. Melt curve of 16SrRNA sequence& Sul 1, Sul 2 shows the melt profile for PCR amplicons

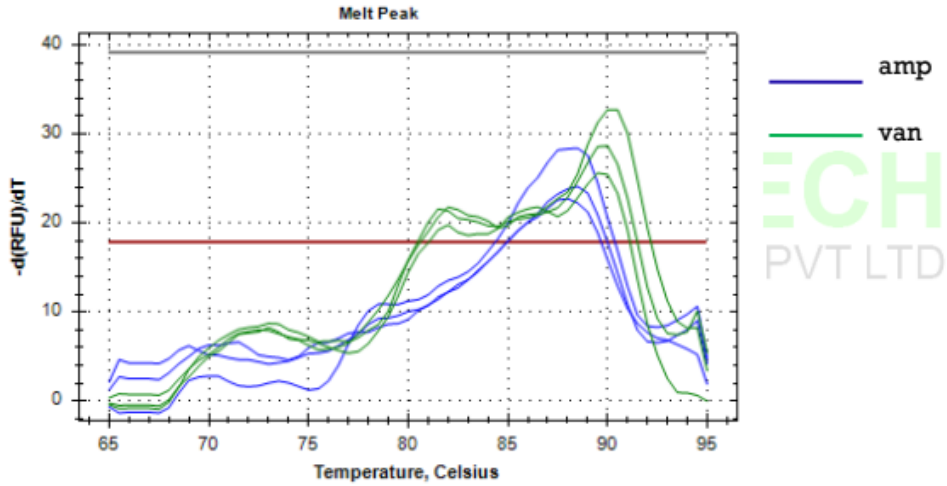

Fig. 7. Melt curve of amp C & vanA sequence shows the melt profile for PCR amplicons

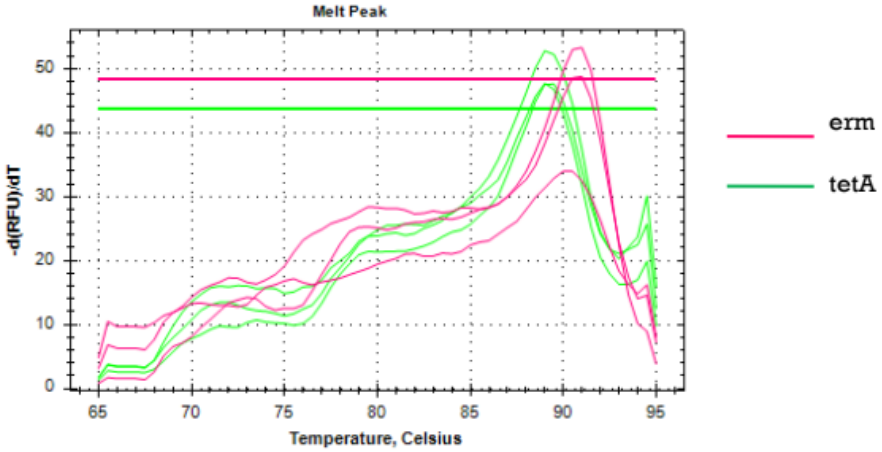

Fig. 8. Melt curve of ermB & tetAsequence shows the melt profile for PCR amplicons

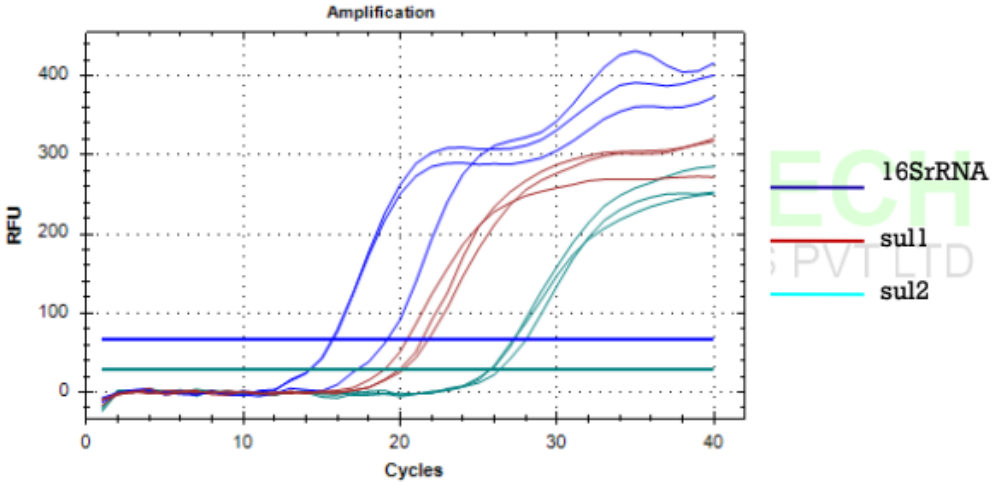

Fig. 9. Amplification of 16 S rRNASequence, Sul1 & Sul2

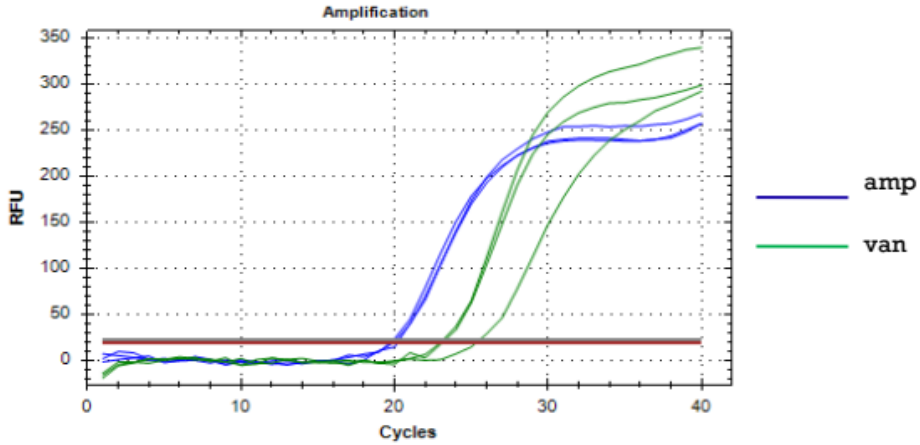

Fig. 10. Amplification of ampC & van A

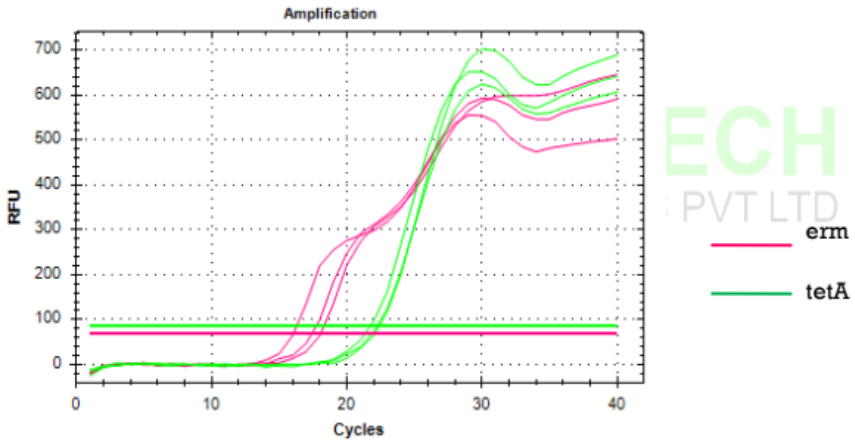

Fig. 11. Amplification of ermB & tet A

Quantification data of sample replicates and its Quantification cycle mean represents the 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

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. After the usage 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 the presence of antibiotic resistant bacteria, E. *Coli* MT784125, *Klebsiella* OM978270 and *Enterobacter sp* MN437586, which were resistant to multiple drugs. The attendance 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 the effluents 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]. The existence 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, qPCR technique objectively and independently document the abundance of ARG in wastewater effluent and quantified the absolute abundance of target genes in raw wastewater 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 [14-17].

5. CONCLUSION

The over usage of Antibiotics in hospitals for disease prevention may cause the evolution of 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 be emanate through the species and its continuous evolution may cause cross resistance to other antibiotics. The metagenomic analysis also confirms the presence of resistant genes in the effluent sample. Hence antibiotic use in hospitals and its final disposal should be monitored properly to avoid the development of resistance. 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.

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