Occurrence of antimicrobial-resistant *Escherichia coli* in sewage treatment plants of South India

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**ABSTRACT**

Antibiotics received by sewage treatment plants may be the causative factor in spreading antibiotic resistance bacteria in the aquatic environment. The current study investigates the distribution of antimicrobial-resistant *Escherichia coli* (E. coli) in four sewage treatment plants (STPs) in South India receiving hospital and domestic wastewater in different proportions. A total of 221 E. coli isolates were checked for antimicrobial resistance against 16 antimicrobials. Among the antimicrobials tested, ampicillin (AMP) and cefazolin (CFZ) showed resistance between 20% and 90%, nalidixic acid (NAL) and ciprofloxacin (CIP) showed resistance between 15% and 75% and chloramphenicol (CHL) showed resistance between 2% and 20%. Based on the observations, there is no significant difference between the wastewater inlet and outlet, suggesting that treatment process was not effective in reducing the resistance. In conclusion, the trends of antimicrobial resistance pattern show that the levels of resistance were slightly higher in hospital wastewater than domestic wastewater.

**Key words** | antimicrobial resistance, *E. coli*, sewage treatment plant, wastewater

**INTRODUCTION**

Antimicrobial resistance has become a significant threat to public health, as antibiotics commonly used to treat infections are often becoming ineffective. It is estimated that antimicrobial resistance could lead to ten million deaths by the year 2050 (de Kraker et al. 2016). Broad attention has been given in the medical sciences to the problem of antimicrobial resistance, but less attention has been focused on the spread of antimicrobial resistance in the aquatic environment (Yamashita et al. 2017). Resistance towards the bacteria related to the broad usage of antimicrobial

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drugs in animal and human health is an emerging concern (Prabhasankar et al. 2016). Information about antimicrobial resistance contamination in the Indian environment is scanty (Akiba et al. 2016). Most often, patients with infections are treated with antimicrobials without any microbial tests (Akiba et al. 2015). Bacteria present in wastewater encountering antibiotics respond to develop resistance not only to the single drug but to multiple drugs, which is known as multidrug resistance (Kumar & Pal 2018). In the aquatic environment, Escherichia coli (E. coli) is considered a dominant microorganism in understanding the contamination by antimicrobial-resistant bacteria originating from human and animal faeces. The bacterial communities which include E. coli have been associated with the antibiotic resistance genes that can be transferred to human and animal flora, where they can exert intense pressure for the spread of resistance (Adefisoye & Okoh 2016).

The presence of pathogenic bacteria and their release through human excretion from hospital waste reach sewage treatment plants. The treatment of pathogenic enteric bacteria is difficult due to the transfer of resistance genes by mobile genetic elements and the spread of antimicrobial resistance (Servais & Passerat 2009). These sewage treatment plants represent the major reservoirs of antimicrobial resistance bacteria and genes compared with the aquatic environment (Birosova et al. 2014). After the discharge from sewage treatment plants, the next carrier of the bacterial contaminants are rivers, which are affected by selective pressure due to the release of antibiotic-resistant bacteria and residual antibiotics. Many antibiotics can even persist in the environment and are detected frequently in drinking water (Zanotto et al. 2016).

The spread of antimicrobial resistance genes is facilitated by a horizontal gene transfer mechanism (Bessa et al. 2014). The dissemination of resistance due to microbial density and the divergence of activated sludge may facilitate the transfer of resistance genes (Luczkiewicz et al. 2015). The matrix of microorganisms changes frequently by the continuous attachment and detachment in the sewage system. The resistant bacteria will travel mainly through sewage treatment plants (STPs) or by direct discharge of the drainage system to surface water (Kaeseberg et al. 2014). Hospital wastewater is having a high impact on antimicrobial resistance in Indian sewage treatment plants (Akiba et al. 2015; Guruge et al. 2015).

The patterns of the antimicrobial resistance vary geographically (Mendem et al. 2016). For instance, NDM-1 (New Delhi metallo-β-lactamase) is repeatedly detected in Indian environments and also reported in municipal wastewater in China (Luo et al. 2015; Ahammad et al. 2014). The spread of antimicrobial resistance between nations and continents may be due to trading of food items and lack of ecological barriers (da Costa et al. 2007).

The objective of the present study is to understand the distribution of antimicrobial resistance in E. coli for the selected antibiotics in four different treatment plants at three different time intervals.

**MATERIALS AND METHODS**

**Sample collection and description of sewage treatment plants**

In the current study, samples were collected from the inlet and the outlet of four sewage treatment plants (STPs). STP 1 and STP 2 have a capacity of 2,000 and 1,500 m³ per day, respectively. They receive a combination of hospital and domestic wastewater. STP 3, which receives domestic waste, has a capacity of 43,500 m³ per day using upflow anaerobic sludge blanket reactor (USABR) technique followed by biological treatment with activated sludge and chlorine disinfection. STP 4 has a capacity of 50 m³ per day to treat hospital wastewater exclusively. The treatment scheme used in STP 1, STP 2 and STP 4 is conventional biological treatment followed by clarification and chlorine disinfection. The water samples were collected from the selected STPs at three specific time intervals of the day (8 am, 1 pm and 7 pm). A total of 24 samples were collected to analyse antimicrobial resistance.

**Isolation of bacteria**

The isolation of bacteria was carried out in the Division of Bacterial and Parasitic Disease, National Institute of Animal Health, NARO, Japan. The collected samples were kept with 25% glycerol at −80 °C until used. Later, the samples were diluted with sterile phosphate buffer saline (PBS) and spread on Chromocult phosphate buffer saline (PBS) and spread on Chromocult agar plates to isolate violet colonies which were tested for β-galactosidase and β-glucuronidase positive.
The isolated colonies were then spread on MacConkey agar plates to enumerate the total viable counts of bacteria. Among the violet colonies, the indole positive and oxidase negative were identified as *E. coli* bacteria. The isolated and identified *E. coli* were stored in LB broth with 25% glycerol at −80 °C for future use (Akiba et al. 2015).

**Antimicrobial susceptibility testing**

A total of 221 isolates of *E. coli* were picked randomly and checked for antimicrobial resistance against 16 antimicrobials using Kirby-Bauer disk diffusion test recommended by Clinical & Laboratory Standards Institute CLSI (2007) (Table 1). The selected antimicrobials were ampicillin (AMP) (10 μg), cefazolin (CFZ) (30 μg), cefoxitin (FOX) (30 μg), cefotaxime (CTX) (30 μg), cefepime (FEP) (30 μg), imipenem (IMP) (10 μg), meropenem (MEM) (10 μg), streptomycin (STR) (10 μg), kanamycin (KAN) (30 μg), gentamycin (GEN) (10 μg), tetracycline (TET) (30 μg), chloramphenicol (CHL) (30 μg), sulfisoxazole (SUL) (250 μg), sulfamethoxazole-trimethoprim (SXT) (23.75/1.25 μg), nalidixic acid (NAL) (30 μg) and ciprofloxacin (CIP) (5 μg) (Becton, Dickinson and Company) (Akiba et al. 2015).

**Statistical analysis**

A two-sample t-test was performed to evaluate the significance of the differences between the percentage levels of the antimicrobial resistance between the inlet and the outlet, timely variations and between the different STPs.

**RESULTS AND DISCUSSION**

The prevalence of antimicrobial resistance of *E. coli* was tested against 16 antimicrobials. As observed from Figure 1, in STP 1, AMP, CFZ, CTX and FEP showed >80% of resistance whereas STR, NAL, CIP showed >60% resistance and other antimicrobials showed resistance between 10 and 60%. In STP 2, AMP and CFZ exhibited >60% of resistance whereas others showed resistance in the range of 5–50%. In STP 3, AMP, CFZ, TET and NAL displayed >20% of resistance and the other antimicrobials showed <20%. In STP 4, AMP and CFZ exhibited >80% resistance and CTX, FEP, NAL and CIP showed >60% resistance. It is interesting to observe that AMP, CFZ and CTX exhibited >80% resistance in both STP 1 and STP 4. STP 1 and STP 4 are highly resistant to maximum antimicrobials compared to STP 2 and STP 3. The highest prevalence of resistance was recorded in STP 1, STP 2 and STP 4, respectively, for AMP, CFZ, CTX, FEP, TET, NAL, SUL and CIP antimicrobials.

**Table 1 | Number of isolates from the selected sewage treatment plants (STPs)**

| Origin | S. no | Process | Time of sampling | No. of isolates | Origin | S. no | Process | Time of sampling | No. of isolates |
|--------|------|---------|-----------------|----------------|--------|------|---------|-----------------|----------------|
| STP 1  | 1    | Inlet   | 8:00 am         | 10             | STP 3  | 13   | Inlet   | 8:00 am         | 10             |
|        | 2    | Outlet  | 8:00 am         | 1              |        | 14   | Outlet  | 8:00 am         | 10             |
|        | 3    | Inlet   | 13:00 pm        | 10             |        | 15   | Inlet   | 13:00 pm        | 10             |
|        | 4    | Outlet  | 13:00 pm        | 10             |        | 16   | Outlet  | 13:00 pm        | 10             |
|        | 5    | Inlet   | 19:00 pm        | 10             |        | 17   | Inlet   | 19:00 pm        | 10             |
|        | 6    | Outlet  | 19:00 pm        | 10             |        | 18   | Outlet  | 19:00 pm        | 10             |
| STP 2  | 7    | Inlet   | 8:00 am         | 10             | STP 4  | 19   | Inlet   | 8:00 am         | 10             |
|        | 8    | Outlet  | 8:00 am         | 6              |        | 20   | Outlet  | 8:00 am         | 4              |
|        | 9    | Inlet   | 13:00 pm        | 10             |        | 21   | Inlet   | 13:00 pm        | 10             |
|        | 10   | Outlet  | 13:00 pm        | 10             |        | 22   | Outlet  | 13:00 pm        | 10             |
|        | 11   | Inlet   | 19:00 pm        | 10             |        | 23   | Inlet   | 19:00 pm        | 10             |
|        | 12   | Outlet  | 19:00 pm        | 10             |        | 24   | Outlet  | 19:00 pm        | 10             |

Pearson’s bivariate correlation analysis evaluated the correlations of the antimicrobial resistance between the treatment plants. The significance level of this study is alpha = 0.05, XLSTAT (Addinsoft 2019), Version 2018.7

The two-tailed t-test showed that there is no significant difference between the mean and variance of the antimicrobial resistance between the samples obtained at the inlet and the outlet. The same is observed in the data of the timely variations of the antimicrobial resistance.
When comparing between the STPs, it was observed that there was a significant difference ($\alpha = 0.05$) between the antimicrobial resistance values obtained from STP 1 and STP 2, STP 2 and STP 3, and STP 3 and STP 4 ($p$-value is 0.029, 0.004 and 0.025, respectively). However, the difference in variance was not found to be statistically significant. For other pairs of STPs, the difference between the mean or variance was not found to be statistically significant.

The average response of antimicrobial resistance in the inlet and the outlet of all the STPs is displayed in Figure 2. It was found that there was a slight difference in the trend of antimicrobial resistance observed by FOX, CTX, MEM, KAN, GEN, CHL, SUL, SXT, NAL and CIP to the treatment process, but was not statistically significant.

Figure 3 explains the comparative data at three different sampling times, morning (8 am), noon (1 pm), evening (7 pm), to understand the pattern of resistance from the isolates. It was observed that the maximum level of resistance was recorded in the morning compared to noon and evening. It is interesting to note that the antimicrobials CTX and NAL show uniform resistance throughout the day, whereas CIP exhibits the same level of resistance in the morning and evening. The antimicrobials AMP, CIP, STR, SUL and SXT show high resistance in the morning. The presence of resistant bacteria in clinical settings, as well as the human community, continues to be a serious issue to be addressed. Many studies have been carried out to identify the presence of antibiotic-resistant bacteria in hospital wastewater as well as in the aquatic environment.
Due to the favourable environment, the density of bacteria and the duration of antibiotics increases the resistance. The continuous distribution of antimicrobials for over longer periods provides favourable conditions for the movement of resistance genes (Kümmerer 2009).

In the present study, the data were collected from a hospital sewage treatment plant (STP 4), a domestic water treatment plant (STP 3) and a combination of hospital and domestic waste water treatment plants (STP 1 and STP 2), which may help us in understanding the distribution of antimicrobials in the hospital and domestic environment. Since hospitals are the primary source of antimicrobials, we found that in STP 1, STP 2 and STP 4, E. coli showed higher resistance to antimicrobials. A similar pattern has been observed for a few antibiotics in the hospital wastewater collected between 9:30 am and 11:00 am (Le et al. 2016). To study the prevalence of antimicrobial resistance of E. coli in the domestic environment, we have collected samples from STP 3 and analysed them. We observed that in STP 3 E. coli shows the least order of resistance to antimicrobials compared to other STPs. The antibiotics FOX, IMP, MEM, STR, KAN, GEN, SXT and SUL show negligible resistance to E. coli. There is not much difference observed between the inlet and the outlet of the treatment plants in decreasing antimicrobial resistance. A slight difference is observed in the trend of antimicrobial resistance for some antimicrobials such as FOX, CTX, MEM, KAN, GEN, CHL, SUL, SXT, NAL and CIP in the treatment process.
The results of sampling at the same point at three different intervals of time in a day indicate that there were variations in the response of antimicrobials to \textit{E. coli}. Such variation may be due to the water usage, administration of antibiotic use, water flow during the day and could be attributed to pharmacokinetic factors such as metabolism, excretion rate and half-life (Diwan \textit{et al.} \textcopyright 2013). In addition to these, other processes such as hydrolysis and photolysis may also result in the temporal changes in the resistance values.

Several reports are available on the presence of antimicrobials in sewage treatment plants in India (Prabhasankar \textit{et al.} \textcopyright 2009; Balakrishna \textit{et al.} \textcopyright 2011). However, the development of resistance in natural bacteria communities owing to the presence of antimicrobial residues in the environment is not clearly established. There are no guidelines to understand the spread of antimicrobial resistance. In the current case, we found that CHL showed negligible resistance in all STPs, whereas IMP and MEM showed less than 20% resistance. This could be attributed to the minimal usage of the CHL drug in India (Sood \textcopyright 2009). The treatment plants in the present study showed nearly similar resistance levels for CIP (60%), as observed in a study in South Africa (Igwaran \textit{et al.} 2018). Two sewage treatment plants from South India received both domestic and hospital effluents and had maximum concentrations of sulfamethoxazole, ciprofloxacin and ofloxacin (Balakrishna \textit{et al.} 2017). Whereas, in the present case, we tested 16 antimicrobials and found that the hospital effluents offered maximum resistance to antimicrobials AMP, CFZ, CTX and FEP.
intermediate resistance to FOX, STR, TET, SXT, SUL and CIP and minimum resistance to IPM, MEM and CHL. Servais & Passerat (2009) reported higher prevalence of antimicrobial resistance (71%) in hospital wastewater than in rivers and municipal wastewater. The current study showed the presence of antibiotic resistance bacteria in sewage treatment plants which is a serious issue that must be addressed as this water may find a path to the nearby ecosystem. This suggests that there is a lack of treatment efficiency for reducing the bacterial growth. Thus, we consider that the present study will be helpful in designing novel methodologies to remove antimicrobials from wastewater treatment plants. Antimicrobial resistance is a global concern and the source of resistance was found in many countries including India. Due to the poor sanitation and low infection control rate, misuse of antibiotics was common in most tropical countries (Raoult 2019). This study could serve as the basis for further scientific understanding in the spread of antimicrobials, resistance and their impact on public health.

CONCLUSION

The occurrence of a resistant pattern for 16 antimicrobials of *E. coli* bacteria in sewage treatment plants is demonstrated. Antimicrobial resistance testing among the isolates has shown high resistance to many antimicrobials like AMP, CFZ, CTX, FEP, STR, NAL and CIP in the treatment plants. The results showed that those treatment plants having the inflow of hospital wastewater and a combination of hospital and domestic wastewater showed higher prevalence of antimicrobial resistance in *E. coli*. The samples collected in the morning tend to have a higher rate of antimicrobial resistance for the maximum number of antibiotics compared to evening and noon. It could be suggested that limiting the usage of such highly resistant antimicrobials without proper prescription may minimize the distribution of antimicrobial resistance. Thus, in conclusion, hospital wastewater needs to be carefully treated to avoid contamination with antimicrobial resistance bacteria.

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