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Impact of wastewater from different sources on the prevalence of antimicrobial-resistant *Escherichia coli* in sewage treatment plants in South India

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ABSTRACT

The sewage treatment plant (STP) is one of the most important interfaces between the human population and the aquatic environment, leading to contamination of the latter by antimicrobial-resistant bacteria. To identify factors affecting the prevalence of antimicrobial-resistant bacteria, water samples were collected from three different STPs in South India. STP1 exclusively treats sewage generated by a domestic population. STP2 predominantly treats sewage generated by a domestic population with a mix of hospital effluent. STP3 treats effluents generated exclusively by a hospital. The water samples were collected between three intermediate treatment steps including equalization, aeration, and clarification, in addition to the outlet to assess the removal rates of bacteria as the effluent passed through the treatment plant. The samples were collected in three different seasons to study the effect of seasonal variation. *Escherichia coli* isolated from the water samples were tested for susceptibility to 12 antimicrobials. The results of logistic regression analysis suggest that the hospital wastewater inflow significantly increased the prevalence of antimicrobial-resistant *E. coli*, whereas the treatment processes and sampling seasons did not affect the prevalence of these isolates. A bias in the genotype distribution of *E. coli* was observed among the isolates obtained from STP3. In conclusion, hospital wastewaters should be carefully treated to prevent the contamination of Indian environment with antimicrobial-resistant bacteria.

1. Introduction

Bacterial infections pose a threat to public health, especially in developing countries, including India. Although approximately 44 million cases of pneumonia are reported in India each year, the estimated case-fatality rate was 0.93% (Mathew, 2009). The relatively low fatality rate suggests that antimicrobial therapy is common and effective. In fact, >80% of patients with symptoms

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of acute respiratory infections and diarrhea are likely to receive antimicrobials without any microbiological tests (Kumar et al., 2008). The consumption of antimicrobials in India is steadily increasing. The units of antimicrobials sold in India increased by about 40% between 2005 and 2009. In particular, the sales of extended-spectrum cephalosporins (ESCs) had markedly increased by 60% over the five-year period (Ganguly et al., 2011).

Antimicrobials place a high selective pressure on bacteria, thus eliminating the antimicrobial-susceptible strains and subsequently causing the proliferation and dissemination of resistant bacteria. In fact, "superbugs" that show resistance to a broad range of β-lactam antimicrobials, including ESCs, pose a serious threat to both the clinical settings and general community in India (Kumarasamy et al., 2010; Zhang, 2010; Berrazeg et al., 2014). The antimicrobial-resistant bacteria excreted by patients flow into a hospital sewage system or directly into a municipal wastewater stream, which is then treated in a sewage treatment plant (STP). After treatment in STP, the water is discharged into surface waters or is used for irrigation. Thus, STP is one of the most important interfaces of environmental contamination with antimicrobial-resistant bacteria (Kummerer, 2004; Bouki et al., 2013).

Despite the increasing consumption of antimicrobials and dissemination of superbugs, little is known about the contamination of the Indian environment with antimicrobial-resistant bacteria (Ganguly et al., 2011). To the best of our knowledge, no data are available regarding the presence of antimicrobial-resistant bacteria in STPs in India. In the present study, systematic sampling of wastewater was performed in three different STPs in South India for the investigation of the distribution of antimicrobial-resistant *Escherichia coli*. The findings revealed, for the first time, a strong influence of hospital wastewaters on the prevalence of antimicrobial-resistant *E. coli* in Indian STPs.

2. Materials and methods

2.1. Characteristics of the STPs and sample collection

In this study, the water samples were collected from three different STPs. STP1 and STP2 are typical plants designed to treat sewage as well as mixed wastewaters catering to a combined population of approximately 15,000 residents, including a large hospital located in southern India. The treatment scheme used in these STPs is the conventional extended aeration activated sludge process. The actual inflow into each of these STPs is approximately 1600–1800 m^3/day. STP3 treats the effluent from a hospital with an actual flow of 50 m^3/day by the conventional activated sludge process. The sources of sewage for STP1, STP2, and STP3 are domestic wastewater, hospital and domestic wastewaters, and hospital wastewater, respectively. The treated water from STP1 and STP2 is used for inland irrigation, whereas that from STP3 is discharged into the drain, which is connected to a municipal STP. The first step of wastewater treatment is equalization, followed by aeration where bacteria degrade the organic matter. This is followed by settlement of the degraded organic matter in a clarifier and filtration through a sand and activated carbon filter. The treated water is released after chlorination. The water samples were collected between three intermediate treatment steps including equalization, aeration, and clarification, in addition to the outlet. STP3 lacks the equalization samples due to the absence of an accessible sampling point. The wastewater sampling was performed in three different seasons, including post-monsoon (February), pre-monsoon (May), and monsoon (September), in 2013. A total of 28 wastewater samples was collected as shown in Table S1, and analyzed.

2.2. Enumeration and isolation of bacteria

The wastewater samples were appropriately diluted with sterilized phosphate buffered saline (PBS) and spread onto nutrient agar (Nissui Pharmaceutical, Tokyo, Japan) and MacConkey agar (Eiken Chemical, Tokyo, Japan) plates to enumerate the total viable counts of bacteria (TVC) and total coliforms (TC), respectively. The diluted wastewater samples were also spread on Chromocult coliform agar (Merck KGaA, Darmstadt, Germany) plates to randomly isolate violet colonies (positive for both β-galactosidase and β-glucuronidase). Up to 10 violet colonies were picked up from each sample. To detect oxidase and indole production, cytochrome oxidase test strip (Nissui Pharmaceutical) and dimethylaminocinnamaldehyde (DMACA) indole reagent (Becton, Dickinson and Company, Sparks, MD) were used. Among the violet colonies, the oxidase-negative and indole-positive isolates were identified as *E. coli* and were stored in LB broth (Becton, Dickinson and Company) with 25% glycerol at −80 °C until further use.

2.3. Antimicrobial susceptibility testing

A Kirby–Bauer disk diffusion test was performed using Muller-Hinton agar plates (Becton, Dickenson and Company), according to the recommendation of the Clinical and Laboratory Standards Institute (2007a). The following antimicrobials were tested: ampicillin (10 μg), cefazolin (30 μg), cefoxitin (30 μg), cefotaxime (30 μg), imipenem (10 μg), chloramphenicol (30 μg), tetracycline (30 μg), streptomycin (10 μg), kanamycin (30 μg), sulfamethoxazole–trimethoprim (23.75/1.25 μg), nalidixic acid (30 μg), and ciprofloxacin (5 μg). The minimum inhibitory concentration (MIC) of ampicillin, cefotaxime, nalidixic acid, ciprofloxacin, and ofloxacin was determined for all the isolates by agar dilution methods, according to the recommendation of Clinical and Laboratory Standards Institute (2007b).

2.4. Genotyping of the isolates by pulsed-field gel electrophoresis

A plug of each strain was prepared using previously described methods (Rice et al., 1999). The plugs were digested with 60 U/ml of *Xba*I (Takara Bio, Shiga, Japan) at 37 °C for 6 h. The digested fragments were separated by pulsed-field gel electrophoresis (PFGE; 6.0 V/cm for 22 h with a pulsing time linearly ramped from 5 to 50 s) on a 1% pulsed-field certified agarose gel (Bio-Rad Laboratories, Hercules, CA). *Salmonella enterica* subsp. *enterica* serovar Braenderup (ATCC BAA-664) was used as the DNA size marker, and the PFGE profiles were scanned and analyzed using BioNumerics software version 5.1 (Applied Maths, Sint-Martens-Latem, Belgium). Pairwise comparisons were made between all the isolates in terms of the Dice similarity coefficient with the position tolerance set at 0.5%. Cluster analysis was performed using unweighted pair arithmetic average algorithm (UPGMA), and a dendrogram was prepared.

2.5. Quantification of the antimicrobials in the wastewater samples

For the solid-phase extraction, Oasis® hydrophilic–lipophilic balanced (HLB) cartridges purchased from Waters (Milford, MA, USA) were used. Furthermore, Milli-Q water was used. Disodium ethylene diamine tetraacetate, chloramphenicol, trimethoprim, sulfamethoxazole, and ofloxacin were purchased from Wako Pure Chemical Industries Ltd. (Tokyo, Japan). The sample extraction involved a multi-step procedure. The procedure started with preconditioning of the sorbent with 5 ml of methanol, followed by 5 ml of Milli-Q water. Then, the sample was loaded onto the HLB cartridges with a flow rate of 3 ml/min, and the target analytes were retained on the cartridges. The cartridges were washed with