

Urban Wastewater as Hotspot for Antibiotic and Antibiotic Resistant Bacteria Spread into the Aquatic Environment

MAQSOOD AHMAD^{1,*}, AMIN U. KHAN¹, ABDUL WAHID², MUHAMMAD FARHAN¹, ZAHID ALI BUTT³ and FAROOQ AHMAD¹

¹Sustainable Development Study Centre, Government College University, Lahore, Pakistan

²Department of Environmental Sciences, Bahauddin Zakariya University, Multan, Pakistan

³Abdul Wali Khan University, Mardan, Pakistan

*Corresponding author: Email: maqsood_ahmad92@yahoo.com

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The present study was under taken to investigate the occurrence of ofloxacin in hospital wastewater, municipal wastewater and in river water receiving the sewage; along with the assessment of antibiotic resistant *Salmonella typhimurium* (*S. typhi*) in these waters. Transfer of antibiotic resistance between two different strains of bacteria was also studied. Untreated sewage from the three hospitals (Mayo, Services and Jinnah) directly entering into municipal wastewater and ultimately flows into the river Ravi without any prior treatment. The concentration of ofloxacin in wastewater of the three hospitals was ranged between 10.69 to 30.55 $\mu\text{g mL}^{-1}$. However, before mixing of the hospital wastewater, concentration of ofloxacin in the three municipal wastewater drains (receiving the wastewater) was ranged between 0.26 to 0.43 $\mu\text{g mL}^{-1}$. Downstream to the hospitals, increase in concentration was observed and concentration was ranged between 0.54 to 1.29 $\mu\text{g mL}^{-1}$. The river water was not contaminated with ofloxacin upstream to the city, while a concentration of the antibiotic downstream to the city was 0.067 $\mu\text{g mL}^{-1}$. All the water samples collected from hospital and municipal wastewater drains were contaminated with *S. typhi*. It was noted that hospital isolates were more resistant (50 %) to ofloxacin as compare to municipal wastewater isolates (16.7 %). No *S. typhi* was detected from the river water before entering first municipal wastewater drain from the city. Downstream to first municipal wastewater drain *S. typhi* were detected and 33 % of them were resistant to ofloxacin. The bacterial isolates isolated from river water downstream to the city were 100 % resistant to the antibiotic. The results of co-culture of ofloxacin resistant *S. typhi* and ofloxacin sensitive *Escherichia coli* revealed that the resistant *S. typhi* were able to transfer the antibiotic resistance to another species.

Keywords: Ofloxacin, *Salmonella typhimurium*, Wastewater, Antibiotic resistance.

INTRODUCTION

Antibiotics belong to fluoroquinolones, macrolides and sulfonamides groups are widely using to treat bacterial infections of humans and animals¹. Most of the antibiotics cannot be completely metabolized by humans or animals, so excreted to environment through different excretions like urine and feces². Other sources of antibiotics in the environment are Hospital, municipal and industrial wastewaters³. Most of antibiotics decomposed when enter into aquatic environment at a certain rate, but they become pseudopersistent due to continuous intake into the environment⁴. In the field of environmental science, antibiotic are new type of pollutants and their impacts on humans and animals are also not well know⁵. Common antibiotics like ciprofloxacin, norfloxacin and ofloxacin have been found in hospital wastewater⁶, municipal wastewater⁷, river sediments and water⁸. Antibiotic pollution is now become a serious problem for many rivers and oceans⁴.

The wastewaters also carry human and animal pathogenic bacteria to water environment and many of them carrying antibiotic resistant genes. Once bacteria with the resistant genes enter into the environment, they become able to spread the genes into water and soil bacteria⁹. Water environment not only responsible for the dissemination of antibiotic resistant bacteria among humans and animals through drinking water and food, but also facilitate the resistant bacteria to transfer the resistant genes to nonpathogenic natural bacterial ecosystem. In this way, the nonpathogenic bacteria could be act as reservoir of antibiotic resistant genes¹⁰. This is the reason that antibiotic and antibiotic resistant genes becoming a global health issue. The problem can only solve by developing specific treatment procedures for the removal of antibiotics and pathogenic bacteria from hospital and municipal wastewaters, because conventional wastewater treatment plants are not able to completely remove the pollutants^{9,11}. The problem is more severe in developing countries because no wastewater treatment facility is available in most of the cases⁶.

TABLE-1
CONCENTRATION ($\mu\text{g mL}^{-1}$) OF FLOXACIN OF IN HOSPITAL WASTE WATER, MUNICIPAL WASTE WATER AND IN RIVER WATER

Hospital	Mean conc.	Municipal waste water drain receiving hospital waste water		Ravi river	
		Before mixing	After mixing	Upstream to city	Downstream to city
Mayo	27.25	0.26c \pm 0.07	0.85b \pm 0.06		
Services	30.55	0.43a \pm 0.03	1.29a \pm 0.18	ND*	0.067
Jinnah	10.69	0.33b \pm 0.03	0.54c \pm 0.06		

*ND = not detected.

EXPERIMENTAL

Analytical grade chemicals and reagents were used throughout the experiment. In first step, composite wastewater samples were collected from three teaching hospitals (Mayo, Services and Jinnah). Secondly wastewater samples were collected from municipal wastewater drains receiving the hospitals sewage. At last, river water samples were also collected both from upstream and downstream of the city. All the water samples were taken in 4 L amber glass bottles and transfer to laboratory at 4 °C by using ice box. Before analysis of the samples 1 g Na₂EDTA was added, mixed well and stored at 4 °C but not more than 12 h^{12,13}. The samples were concentrated by using solid phase extraction process. Before start of the extraction process, 2 mL methanol followed by 2 mL deionized water was used to activate the cartages. After that, samples were passed through the cartages. The targeted compound was extracted by using 2 mL methanol. The extracted samples were analyzed on HPLC (High performance liquid chromatography) by using reverse phase analytical column (RP-18e 250 mm \times 4.6 mm, 5 μm , manufactured by Merck) at room temperature and the flow rate was 0.8 mL/min^{14,15}.

Bacterial sampling and analysis: For bacterial sampling, sterilized amber glass bottles were used and size of each sample was 4 L. About 2.5 cm of sampling bottles were kept empty for the shaking of samples. The samples were transport to laboratory in ice boxes and processed on same day. When every same day process was not possible, the samples were stored at 4 °C but not more than 2 days¹⁶. Identification and isolation of *S. typhi* was carried out by using Brilliance Salmonella agar by incubating at 37 °C for 24 h¹⁷. Co-culture of ofloxacin resistant *S. typhi* and ofloxacin sensitive *E. coli* was prepared in 1:1 ratio by using nutrient broth. Haemocytometer was used to prepare same inoculum size of each isolate¹⁸ and separately transfer to each antibiotic treatment prepared in iso-sensitest broth. The cultures were incubated at 37 °C for 24 h. Cell density was measured after 0 and 24 h at 600 nm¹⁹.

RESULTS AND DISCUSSION

The antibiotic under study was detected from wastewater samples collected from all the three hospitals, but concentration of the antibiotic was not same in the wastewaters (Table-1). Concentration of ofloxacin in Mayo, Services and Jinnah was 21.25, 30.55 and 10.69 $\mu\text{g mL}^{-1}$, respectively. The difference in concentration was mainly due to different usage of antibiotic, number and type of patients, liking and disliking of physicians²⁰. No wastewater treatment facility was available in the three hospitals. After mixing of hospital wastewater, an increase in concentration of the antibiotic in municipal wastewater was observed. This increase was mainly due to large intake of

antibiotics from the hospitals²¹. Therefore, hospitals must be remembered as vital contributor of different pharmaceutical compounds like antibiotics²².

In Ravi river upstream to the city, ofloxacin was not detected while downstream to the city ofloxacin was detected *viz.* 0.067 $\mu\text{g mL}^{-1}$. Source of antibiotics in rivers are treated or untreated municipal wastewater. Therefore, pharmaceutical compounds usually detected from downstream to urban areas and not from upstream, but concentration depends up on amount of antibiotic received and dilution capacity of a river^{8,23,24}.

As given in Table-2, river water upstream to the city before mixing first municipal wastewater drain were not contaminated with *S. typhi*. On the other hand, the samples collected downstream to first municipal wastewater drain were contaminated with the bacteria. The upstream samples were not contaminated because source of pathogenic bacteria in rivers are wastewater drains from urban areas²⁵. All the samples collected downstream to municipal wastewater drains were contaminated with *S. typhi*. This is because, point sources are responsible for contamination of rivers with antibiotic resistant bacteria^{26,27}. These bacteria and antibiotics can reach to human through contaminated drinking water and through food due to plant and animal accumulation, respectively^{4,28}.

TABLE-2
BACTERIA ISOLATED FROM DIFFERENT SITES OF RAVI RIVER

Sampling sites	Bacteria	Status	Resistant (%)
Ravi siphon		-	-
Upstream to first MWW drain	<i>S. typhi</i>	-	-
Downstream to first MWW drain		+	33 %

MWW = Municipal waste water

Resistance level of *S. typhi* isolated from hospital wastewater, municipal wastewater and from the river water was measured at concentration below and above the MIC₉₀ of ofloxacin. The result of the experiment revealed 50 % of hospital wastewater isolates were able to survive at concentrations higher than MIC₉₀ of ofloxacin (Fig. 1). On the other hand, in case of municipal wastewater isolates, 16.7 % were found resistant to the compound (Fig. 2). The results of the study clearly indicate that hospitals isolates were more resistant to ofloxacin as compare to municipal wastewater isolates. This may be due to the fact that patients with severe infection usually hospitalized. As in early studies, it was concluded that hospitals are source of more resistant bacteria as compare to municipal wastewater because of selective type of patients^{29,31}.

When the river isolates exposed to similar concentrations of ofloxacin, it was noted that 100 % were able to survive at concentration higher than MIC₉₀ of ofloxacin (Fig. 3). Previous

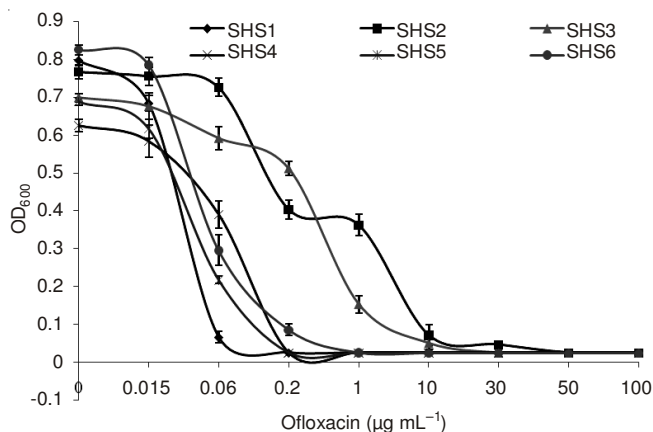


Fig. 1. Effect of ofloxacin on cell growth of *S. typhi* isolated from hospital wastewater

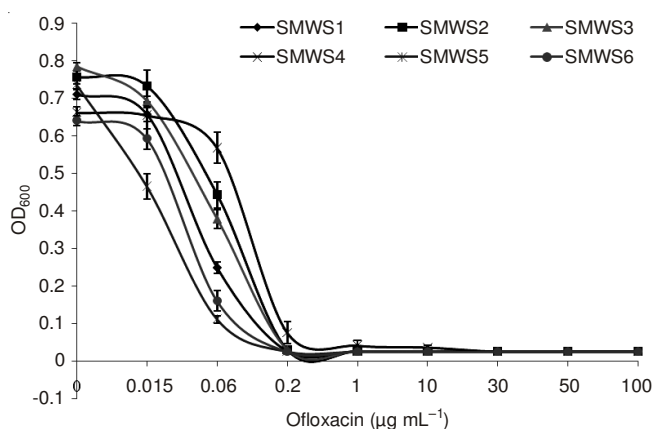


Fig. 2. Effect of ofloxacin on cell growth of *S. typhi* isolated from municipal wastewater

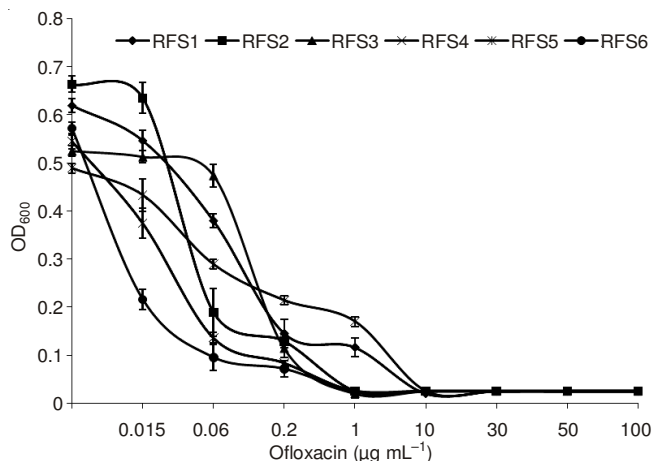


Fig. 3. Effect of ofloxacin on cell growth of *S. typhi* isolated from Ravi river downstream to Hudiara drain

studies reported that river isolated from downstream to point sources were 90 to 100 % resistant to fluoroquinolones^{32,33}. Most of clinical isolates of *S. typhi* were resistant to frequently using antibiotic including fluoroquinolones³⁴. Many *S. typhi* isolates which were sensitive to fluoroquinolones are now resistant to the antibiotics³⁵. Bacteria gain antibiotic resistance when exposed to the compounds for longer period of time³⁶ and through horizontal transfer by the transfer of plasmids³⁷.

Transfer of antibiotic resistance between bacteria of two different species was studied by developing co-culture of antibiotic resistant and resistance *S. typhi* and *E. coli*, respectively. After 24 h of co-culture when the sensitive of *E. coli* was analyzed, it was found that 20 % of isolates were resistant to ofloxacin. So it can be stated that antibiotic resistance characteristics can transfer among two different species of bacteria³⁸. Transfer of antibiotic resistance between two different species of bacteria can also occur in river water³⁹.

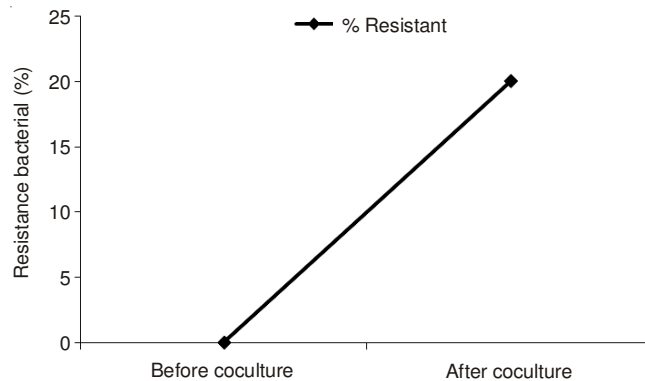


Fig. 4. Transfer of antibiotic resistance after coculture

Conclusion

It was concluded that river water was not contaminated with the antibiotic and antibiotic resistance bacteria upstream to the city before the introduction of first municipal wastewater drain. Untreated hospital and municipal wastewaters are responsible for dissemination of antibiotics and antibiotic resistant bacteria in the rivers. Concentrations of the antibiotics are increasing in river because of continuous input from hospitals and municipal wastewaters. Antibiotic resistance is also increasing in the aquatic environment due to contamination of antibiotics and horizontal transfer among different species.

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