Prevalence of antibiotics and antibiotic resistant bacteria in wastewaters - An impact of untreated municipal and hospital wastewater

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Abstract

Present study investigated the prevalence of some antibiotics viz., ciprofloxacin (CIP), levofloxacin (LFO) and ofloxacin (OFL) in hospital wastewater and seasonal drain receiving untreated municipal wastewater from Sialkot city. Bacterial strains including Escherichia coli (E. coli) and Salmonella typhimurium (S. typhi) were isolated from the raw hospital wastewaters and from the drain to investigate antibiotic resistance level. Concentration of CIP, LFO and OFL in hospital wastewater was measured up to 189µg L⁻¹, 142µg L⁻¹ and 116µg L⁻¹ respectively. No antibiotic was detected from the drain water upstream to the first municipal wastewater entering from the city. From the water samples, collected from downstream to the city all the three antibiotics, for instance CIP (0.091µg L⁻¹), LFO (0.078µg L⁻¹) and OFL (0.073µg L⁻¹) were detected. Isolates of E. coli (n=20) collected from hospital wastewater were 90% resistant to CIP, 75% resistant to LFO and 60% resistant to OFL. While the isolates of S. typhi (n=20) were 95%, 85% and 85% resistant to CIP, LFO and OFL respectively. Isolates of E. coli (n=20) collected from the drain downstream to the city were 75%, 70% and 55% were resistant to CIP, LFO and OFL respectively, while the isolates of S. typhi (n=20) were 95%, 65% and 70% resistant to CIP, LFO and OFL respectively. This study revealed the occurrence of antibiotics and antibiotic resistant bacteria in hospital wastewater and receiving water bodies, indicating a potential health threat especially in developing countries with no wastewater treatment facility.

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**Introduction**

Pharmaceuticals has recently been detected from aquatic environment i.e. ground water, surface water, municipal water and also in drinking water. Antibiotics are most important among pharmaceuticals because using for treatment of different type of bacterial infections (Adachi et al. 2013). During the last many years, production of pharmaceuticals has rapidly increased.

Approximately 3000 compounds are in use as medicine and annual production reached up to hundreds of tons (Sim et al. 2011). It was estimated that worldwide annual consumption of antibiotics is about 100,000 to 200,000 tons (Zheng et al. 2011). In 2011 worldwide sale of pharmaceuticals was $ 839 billion (Astra Zeneca 2011) and was $ 950 billion in 2012 (First research 2013). There are about 350 licensed pharmaceutical manufacturers in Pakistan. Annual pharmaceutical market in Pakistan is over $ 1.25 billion (Medinet 2013). Share of antibiotics in the market was approximately $ 140 million during the year 2007 (Shoaib 2007) now it is much more. Antibiotics like β-lactams, streptomycins and others are produced by soil bacteria in natural environment. But concentration of the antibiotics remains very low or usually below the detection limits (Kummerer 2009), but the release of antibiotics from anthropogenic sources can be up to several mg L⁻¹ (Larsson et al. 2007).

Common sources responsible for the presence of pharmaceutical compounds in the environment are hospitals, municipal wastewater, livestock farms and pharmaceutical manufacturing units (Zhang et al. 2008; Matamoros et al. 2008). During the recent years, occurrence, fate and potential toxic effects of antibiotics have become an important research area. Most of the antibiotics are not completely metabolized in human and animals bodies and about 25% to 75% of antibiotics excrete into the environment in active form through urine and feces. So antibiotics used by humans and animals can be found at various concentrations in hospital effluents, municipal wastewater and discharge of wastewater treatment plants. (Brown et al. 2006; Zheng et al. 2011). As a result receiving water bodies like rivers become contaminated with the antibiotics and the compounds become the part of food chain (Jones-Lepp et al. 2012).

The problem of antibiotic resistant bacteria was emerged after the first clinical use of penicillin during the early 1940 (Hiramatsu et al. 2012). On the base of research work, Sir Alexander Fleming in an interview (1945) warned that improper use of penicillin could lead to resistant *Staphylococcus aureus* that may cause more severe infections (Alanis 2005). Presence of antibiotics in the environment causes selective pressure on bacterial populations, which lead to resistant bacteria even at low concentrations (Stepanauskas et al. 2006). Antibiotic resistant strains belong to pathogenic bacteria can be found from aquatic environment and because of long time exposure to different clinical antibiotics they are resistant to more than one clinical antibiotics (Munir et al. 2011). The problem of antibiotic resistant bacteria and their resistant genes is becoming a major global health issue because antibiotic resistant bacteria can distribute resistant genes among microbial community (Schmieder and Edwards 2012). Research is underway to solve the problem by developing new antibiotics. However, soon the bacteria started acquiring resistance to the newly developed drugs and begin to survive as multi drug resistant organism (Hori et al. 1993). The situation is more serious in developing countries where water bodies receive untreated wastewater (Ahmad et al. 2014) and even in developed countries where rivers are receiving treated effluents contaminated with antibiotics and antibiotic resistant bacteria (Adachi et al. 2013). In Pakistan, excess use of antibiotics and antibiotic resistance is sever (Ahmad et al. 2017). In developing countries average growth rate of antibiotic resistance is very high, as in China it was 22% in during 1994 to 2000 and it could be the most rapid growth rate of the resistance all over the world (Zhang et al. 2006). Wastewater from hospitals are entering into MWW without any treatment and ultimately into receiving water bodies like rivers and lakes etc.
The main aim behind the study was to investigate impact of untreated hospital and municipal wastewater on natural water bodies of Sialkot city. For this purpose wastewater samples from hospital and the drain receiving the combined wastewater were taken to investigate the prevalence of three antibiotics (CIP, LFO and OFL) and bacteria including E. coli and S. typhi and resistance of these bacteria against the antibiotics under study.

**Materials and methods**

Analytical grade chemicals and reagents used in this study were purchased from Merck (Germany), Sigma-Aldrich (USA), BDH (UK) and Oxide (UK). Separation columns used in high performance liquid chromatography (HPLC) were also purchased from Merck (Germany).

**Solid phase extraction**

Before the extraction process the samples were filtered through 0.45µm membrane filter and acidify to pH 3 by adding H₂SO₄. Solid phase extraction (SPE) was carried out by using SPE cartridges (C18) to purify and concentrate the samples. The samples were passed though the cartridges.

To extract the compound of interest 2ml of methanol was passed through the cartridges and collected in a tube. Evaporation of the extracted sample was carried out at 40°C. At the last step of the extraction process the 1.0ml of 5% aqueous acetonitrile was added to reconstitute the sample. (Lindberg et al., 2004; Brown et al. 2006; Shao et al. 2009).

**Sample analysis**

Analysis of the extracted sample was carried out by using high performance liquid chromatography (UFLC Shimadzu, Japan) equipped with diode array detector (SPD M20A).

The analytical column RP-18e (250mm×4.6mm, 5µm) was used at room temperature for analysis. Acetonitrile and H₂O with 1% (v/v) formic acid were used as mobile phase at flow rate of 0.8 ml min⁻¹ (Lindberg et al. 2004; Seifrtova et al. 2009).

**Bacterial analysis**

For bacteria identification water samples were diluted up to 10² to 10⁴ times by serial dilution method. From prepared samples 0.1ml was transferred to two types of petriplates containing Brilliance Salmonella agar and Brilliance E. coli/coli form selective agar for identification and isolation of, S. typh and E. coli respectively. To spread the sample properly on selective growth media, the plates were rotated clockwise and anti-clockwise and the plates were incubated at 37°C for 24h. In the petriplates containing Brilliance E. coli/coliiform selective agar, number of purple colored E. coli colonies appeared and S. typhi on Brilliance Salmonella agar were also appeared as purple colored colonies. (Clark et al. 1958; Luczkiewicz et al. 2010; Koczura et al. 2012).

Different concentrations of CIP, LFO and OFL were prepared by using Iso-sensitst broth. One ml (10⁴ CFU/ml) of each bacterium inoculum was transfer to each concentration. The cultures were incubated at 37°C for 24h. Optical density (OD) of the culture was measured by adjusting the wavelength at 600nm after 0 and 24h. Values of minimum inhibitory concentration (MIC₉₀) for the three bacteria according to specific antibiotics were used to study the tolerance level of the pathogens. The isolates which were able to increase cell population in antibiotic concentrations equal to or higher than the MIC₉₀ were considered to be resistant (Roychoudhury et al. 2001; Joosten et al. 2008; Bidlas et al. 2008; Huang et al. 2012). Each experiment was run in duplicate.

**Results**

During the study temporal variation in the concentration of antibiotics was observed in the wastewater of the hospital. Minimum concentration of antibiotics was observed during 15:00 to 19:00 hours and maximum concentration was detected during 11:00 to 15:00 hours (Table 1). Temporal variation in concentration of pharmaceutical compounds in wastewaters is common phenomenon, and it is due to different factors, including retention time in body, used amount of a medicine, and excretion rate (Roig 2010; Gerrity et al. 2011). Concentration of the three antibiotics in the hospital
wastewater was ranged from 63µg L\(^{-1}\) to 189µg L\(^{-1}\) and concentration of CIP, LFO and OFL was not same. This variation in concentration is due to different factors including number of patients, liking and disliking of physicians, availability and usage of a drug etc. (Schuster et al. 2008; ort et al. 2010). No antibiotic under study was detected from the seasonal drain (locally known as Aik) upstream to first MWW drain but all the three antibiotics were detected downstream to the first MWW drain entering from the city, and concentration was ranged from 0.073 to 0.091µg L\(^{-1}\). The concentration of the pollutants was increased up to 0.18µg L\(^{-1}\) downstream to the city. This is because main source of pharmaceuticals are MWW drains (Zhou et al. 2011) so antibiotics not usually detected from rivers upstream to point sources (Brown et al. 2006; Jones-Lepp et al. 2012) and cities (Yang and Carlson 2003).

In second portion of the study two bacteria E. coli and S. typhi were isolated from hospital effluents and from the drain water understudy. It is worthwhile that E. coli and S. typhi were not detected from the water samples collected from upstream to the city while both were present in the samples collected downstream to first MWW drain from the city. Hospital effluent isolates (n=20) of E. coli (60% to 90%) and S. typhi (85% to 95%) were resistant to concentration equal to MIC\(_{90}\) of CIP, LFO and OFL (Fig. 1 to 6). Raw hospital wastewater isolates are usually more resistant to antibiotics (Duong et al. 2008) because of patients with severe infections. (Prado et al. 2008; Rosenblatt-Farrell 2009). The drain isolates (n=20) of E. coli (53% to 75%) and S. typhi (70% to 95%) were resistant to CIP, LFO and OFL were able to survive at concentration higher than MIC\(_{90}\) (Fig. 6 to 12). Main source of the resistant bacteria for water bodies are treated and untreated wastewater (Huang et al. 2012) and the problem of antibiotic resistant pathogenic bacteria is more severe in less developed countries (Isturiz and Carbon 2000). From the results it is evident that situation is quite serious, so ongoing studies in local areas should be conducted to investigate further sources and faith of antibiotics and antibiotic resistant bacteria in the environment.

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<tr>
<th>Antibiotic in hospital wastewater</th>
<th>Antibiotics in MWW/seasonal drain</th>
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<tbody>
<tr>
<td>11:00 to 15:00</td>
<td>15:00 to 19:00</td>
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<tr>
<td>CIP</td>
<td>Downstream to first MWW drain</td>
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<tr>
<td>189±0.26</td>
<td>0.09±0.002</td>
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<td>LFO</td>
<td>0.07±0.0015</td>
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<td>142±0.38</td>
<td>0.19±0.094</td>
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<td>OFL</td>
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**Table 1.** Concentration of antibiotics in hospital wastewater and seasonal drain receiving from Sialkot city.

![Fig. 1. Resistance of E. coli isolates against CIP isolated from hospital effluent.](image1)

![Fig. 2. Resistance of E. coli isolates against LFO isolated from hospital effluent.](image2)
Fig. 3. Resistance of *E. coli* isolates against OFL isolated from hospital effluent.

Fig. 4. Resistance of *S. typhi* isolates against CIP isolated from hospital effluent.

Fig. 5. Resistance of *S. typhi* isolates against LFO isolated from hospital effluent.

Fig. 6. Resistance of *S. typhi* isolates against OFL isolated from hospital effluent.

Fig. 7. Resistance of *E. coli* isolates against CIP isolated from the drain.

Fig. 8. Resistance of *E. coli* isolates against LFO isolated from the drain.
Fig. 9. Resistance of *E. coli* isolates against OFL isolated from the drain.

Fig. 10. Resistance of *S. typi* isolates against CIP isolated from the drain.

Fig. 11. Resistance of *S. typhi* isolates against LFO isolated from the drain.

Fig. 12. Resistance of *S. typhi* isolates against OFL isolated from the drain.

References


