Susceptibility for antibiotics among faecal indicators and pathogenic bacteria in sewage treated effluents

Adel A. S. AL-Gheethia, Norli Ismaila,*, J. Lalunga, Azieda Taliba, A. N. Efaqb and Mohd Omar Ab. Kadirb

a Environmental Technology Division, School of Industrial Technology Universiti Sains Malaysia, 11800, USM, Pinang, Malaysia
b Department of Applied Microbiology, School of Applied Science, Taiz University, Yemen

* Corresponding author. E-mail: norlii@usm.my

Abstract

The objective of this work was to study the prevalence of antibiotic resistance phenotypes among total coliforms (TC), E. coli, E. faecalis and Salmonella spp. in the sewage treated effluents generated from three sewage treatment plants in Penang Malaysia. Among the isolates tested, TC and E. coli occurred high resistance for cephalexin (100 and 90.47%), ampicillin (80.93 and 95.23%) and ciprofloxacin (19.06 and 14.3%) compared to E. faecalis (42.86, 71.4 and 4.7%) and Salmonella spp. (59.8, 47.46 and 14.3%) respectively. All E. coli strains, 76.18% of TC, 66.66% of E. faecalis and 35% of Salmonella spp. were multi-resistant.

Key words: antibiotics resistance, faecal indicator bacteria, Salmonella spp., sewage treated effluent

INTRODUCTION

The microbial resistance of antibiotics considers a global problem in human. The main risk factor for the increasing of the antimicrobial resistance is an accepted as consequences of an extensive using of antibiotics which lead to the emergence and spreading of resistant bacteria in animals and humans (Lukaova & Sustakova 2003). β-lactam antibiotics are the most frequently used, because of their effectiveness and low toxicity, β-lactam’s comprise a carboxyl group which makes them weak acids, which make it high water solubility that increases diffusion through membranes (Connor et al. 1994).

Antibiotics enter waste streams through faeces and urine, because the main removal of β-lactam antibiotics from body is by secretion into urine. Thus, sewage effluents from sewage treatment plants (STPs) consider the most important source of antibiotic-resistant bacteria in the environment. Recently, the STPs effluent must meet regulatory limits for faecal indicators bacteria (total coliforms, E. coli, and E. faecalis); however, regulatory limits have not been developed for antibiotic agents and the effect of low antibiotic concentrations and antibiotic resistance development receives limited attention (Walter & Vennes 1985). Reinthaler et al. (2003) reported that presence of antibiotics at low concentrations in the environment might lead to the development of bacterial resistance of antibiotic. The role of STPs on the spread of antibiotic resistance to the natural environment is an important key to the ecological impact of human discharges (Laroche et al. 2009; Servais & Passerat 2009; Garcia-Armisen et al. 2011).

The prevalence of antibiotics resistance among faecal indicators bacteria considers good indicators of the selective pressure of antibiotics usage (Wiggins 1996). Indicators bacteria such as E. coli and E. faecalis are organisms that constitute a natural part of the intestinal flora of humans and animals,
using these species make it feasible to compare level of resistance between populations (Bogaard et al. 2000).

The aim for this paper is to study the prevalence of antibiotic-resistance among faecal indicators (total coliforms, *E. coli*, and *E. faecalis*) and *Salmonella* spp. isolated from three STPs in Penang, Malaysia.

### MATERIALS AND METHODS

#### Collection of sewage treated effluents samples

Twenty-one effluent samples were collected from three STPs (referred to as STP₁, STP₂ and STP₃) in Penang, Malaysia, from May to July 2011 (seven samples from each STP). STP₁ depurate wastewater comes from laboratories, which conduct operations in the pharmaceutical and chemistry sciences in an educational institute, while STP₂ treat the domestic sewage that comes from a medical centre. The treatment at the STP₁ and STP₂ plants includes primary sedimentation and oxidation ponds. STP₃ receives the domestic sewage that comes from different communities areas in Penang. The capacity of this plant is 180,000 m³/day; the size of the population connected to this plant is 1.7 million people. The sewage in STP₃ is treated by primary sedimentation, a secondary process (biological treatment) and UV irradiation in the tertiary treatment stage.

The effluent samples were collected in glass bottles. Bottles were rinsed three times with the effluent sample before collection of the actual sample, transferred to the laboratory and stored in the refrigerator (the maximum storage time was overnight) for bacterial analysis.

#### Isolation of bacteria from effluent samples

Samples were thoroughly mixed to distribute the bacteria uniformly prior to analysis. TC, *E. coli*, *E. faecalis* and *Salmonella* spp. were isolated by direct plate techniques on appropriate selective media according to internationally accepted techniques and principles (Eaton et al. 2005; U.S. EPA 2006a and b) with some modifications.

Thirty millilitres of each sample was suspended in 270 mL of sterilized distilled water. Serial dilutions (10⁻¹–10⁻⁴) were prepared, and then 1 mL of each dilution was seeded on EMB (Merck-Germany) to count TC, Endo agar media (M029) for *E. coli*, bile esculin iron agar (BEIA, M972) for *E. faecalis* and xylose lysine deoxycholate agar (XLD) for *Salmonella* spp. (Endo agar, BEIA and XLD media were supplied by HiMedia Laboratories, Pvt. Ltd., India).

Media was sterilized by autoclave (Model ES-315, Tomy-Japan) at 121 °C for 15 min except of XLD medium was sterilized by the boiling. Experiments were carried out inside a horizontal laminar flow cabinet (Model AHC-4A1-ESCO). Plates were incubated (Memert incubator-Germany) for 24–48 h at 37 °C for TC and *Salmonella* spp. and at 44.5 °C for *E. coli* and *E. faecalis*. Colonies of the bacteria were determined based on the appearance of the morphology and colour of the colonies.

TC was identified as the aerobic and facultative anaerobic, gram-negative, nonspore-forming, rod-shaped bacteria that ferment lactose with gas production within 48 h at 35 °C (APHA 1989) with greenish metallic sheen or pink colour colonies on the EMB agar. These characteristics are enough for identified of TC and no confirmatory tests are required (Edberg et al. 1990). *E. coli* was identified as colony with greenish metallic sheen on Endo agar at 44.5 °C, while the colonies of *E. faecalis* grown on BEIA had brown centres were confirmed by a catalase test. *Salmonella* spp. were red in colour with black centres on XLD medium.
Purification and identification of bacterial isolates

For the purpose of purification, the isolated bacterial colony was streaked on the agar surface of the isolation medium. After 48 h incubation at 37 °C, a single colony was transferred to another plate again on the agar surface of the same medium in a similar manner. This technique was repeated for about four consecutive times for all isolated colonies. The transferred loop was rubbed on the agar medium surface of such a plate by means of sterilized glass spreader, which was made in order to distribute and separate the bacterial cells as much as possible. One of the developed bacteria colonies after 24 h was checked for its purity by mean of Gram stain and identified based on morphological and cultural characteristics and biochemical tests by using API 20 Strep (Ref 07226 B), API 20NE (Ref 07224 B) and RapiD 20E (Ref 12134 A) (BioMerieux, SA-France) according to Kenner & Clark (1974), Hart & Shears (2001) and Brenner et al. (2005).

Disk diffusion susceptibility test

The disk diffusion susceptibility test was performed to determine the antibiotic resistance of faecal indicators and pathogenic bacteria in the sewage treated effluents samples collected from three STPs at Penang Malaysia. The susceptibility test was performed by placing paper disks containing the antibiotic on a lawn of bacteria grown on agar. To create a lawn of bacteria 0.1 mL of the suspension of interest was spread on the agar. After an incubation period, the diameter for zone of inhibition, the area around the disk without bacterial growth, was measured. The size of the zone of inhibition determined the susceptibility of the organisms to the antibiotic.

For non-clinical research applications, the zone of inhibition was classified as resistant, moderately resistant, and susceptible according to Handal & Olsen (2000). The diameter of the paper disks were 0.7 cm. The zone of inhibition around the antibiotic disks was measured. If the diameter of the zone of inhibition was between 0.7 and 1.0 cm, the organisms were considered resistant. If the zone was between 1.0 and 1.2 cm, the bacteria were considered moderately susceptible and if the zone was greater than 1.2 cm, the microorganisms were considered susceptible.

The antibiotics that used in the test were amoxicillin (50 μg mL⁻¹), ampicillin (50 μg mL⁻¹), cephalaxin (50 μg mL⁻¹), cefuroxime (50 μg mL⁻¹) and ciprofloxacin (50 μg mL⁻¹). Nutrient agar was used in the disk diffusion tests performed in this research. Plates were incubated for 24 h at 37 °C.

RESULTS AND DISCUSSION

The prevalence of antibiotic-resistant phenotypes among faecal indicators bacteria as well as *Salmonella* spp. isolated from the sewage treated effluent samples was investigated in this study. Five antibiotics were selected: amoxicillin, ampicillin, cephalaxin, cefuroxime and ciprofloxacin. These antibiotics are the most commonly used in the treatment of infections caused by enteric pathogens under study. β-lactam antibiotics are the most widely used antibiotics and account for almost 50% of universal antibiotic consumption in the world, and the extensive usage of such antibiotics have exerted considerable selection for microbial resistance (Livermore & Brown 2001).

Results showed that antibiotic resistance for TC was cephalaxin, 100; cefuroxime, 85.7; ampicillin, 80.95; ciprofloxacin, 19.06 and amoxicillin, 71.4%, for *E. coli*; cephalaxin, 90.47; cefuroxime, 85.7; ampicillin, 95.23; ciprofloxacin, 14.3 and amoxicillin, 19.06 for *E. faecalis* was cephalaxin, 42.86; cefuroxime, 95.23; ampicillin, 71.4; ciprofloxacin, 4.76; and amoxicillin 80.93% and for *Salmonella* spp. cephalaxin, 59.8; cefuroxime, 51.9; ampicillin, 47.49; ciprofloxacin, 14.3 and amoxicillin, 56.53%.
TC and *E. coli* occurred high resistance for cephalaxin (100 and 90.47%) ampicillin (80.93 and 95.23%) and ciprofloxacin (19.06 and 14.3%) compared to *E. faecalis* (42.86, 71.4 and 4.76%) and *Salmonella* spp. (59.8, 47.46 and 14.3%) respectively. However, *E. faecalis* has more resistance for amoxicillin than TC (80.93 vs. 71.4%) and for cefuroxime (95.23%) compared to both TC and *E. coli* (85.7%). *Salmonella* spp. were less resistance for all antibiotics under study than faecal indicators bacteria.

Similar results was reported by Wose et al. (2010) who found that a large proportion (70 – 95%) of *E. coli* isolated from wastewater samples obtained from the different sampling sites was resistant to ampicillin. The Enterobacteriaceae family has been linked to well-known antibiotic-resistant gene pools; these genes are transferred into the normal flora of humans and animals (Lin & Biyela 2005) where they exert a strong selective pressure for the emergence and spread of resistance in both pathogenic and commensal bacteria (Aminov 2009) and leads to occurrence of multi-resistance of antibiotics. It was found that 90% of faecal bacteria in treated wastewater had antimicrobial resistance patterns, and 29% of enterococci were multi-antibiotic resistant (Luczkiewicz et al. 2010).

The results of the distribution of anti-microbial multi-resistance show that all *E. coli*, 76.18% of TC, 66.66% of *E. faecalis* and 35% of *Salmonella* spp. were multi-resistant to antibiotics (resistant to 3 or 4 antibiotics under study). Reinthaler et al. (2003) found that 40% of the cultivable *E. coli* strains isolated from sewage were resistant to one or more antibiotics, and 9.8% were resistant to more than three antibiotics. The transfer of multiple-antibiotic-resistant bacteria from effluents of STPs into natural waters would pose a serious problem and a secondary pollutant of drinking water (Huang et al. 2012).

On the other hand, the percentage of resistance of amoxicillin by TC obtained from STP2 was more than that from STP1 and STP3 (Table 1). *E. coli* obtained from STP1 and STP3 showed more resistance for cephalaxin and cefuroxime compared to that from STP2, whereas *E. coli* obtained from STP1 and STP2 had more resistance percentage for ampicillin and amoxicillin compared to that from STP3 (Table 2). For *E. faecalis* the cephalaxin, ampicillin and ciprofloxacin resistance pattern was the most frequent in STP3 (85.7, 100 and 14.3%, respectively) compared to that obtained from STP1 and STP2 which occurred more resistance for cefuroxime (Table 3). *Salmonella* spp. obtained from STP3 were significantly more resistant for all antibiotics under study compared to these from STP2 and STP1 respectively except for amoxicillin which didn’t occur significant differences between *Salmonella* spp. obtained from STP1 and STP3 (Table 4).

### Table 1 | Prevalence of antibiotics resistance among total coliforms (TC) obtained from the sewage treated effluents of three sewage treatment plants (STPs) at Penang Malaysia.

<table>
<thead>
<tr>
<th>STPs</th>
<th>Cephalaxin (%)</th>
<th>Cefuroxime (%)</th>
<th>Ampicillin (%)</th>
<th>Ciprofloxacin (%)</th>
<th>Amoxicillin (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>STP1</td>
<td>100</td>
<td>57.1</td>
<td>85.7</td>
<td>0</td>
<td>57.1</td>
</tr>
<tr>
<td>STP2</td>
<td>100</td>
<td>100</td>
<td>85.7</td>
<td>14.3</td>
<td>100</td>
</tr>
<tr>
<td>STP3</td>
<td>100</td>
<td>100</td>
<td>71.4</td>
<td>42.9</td>
<td>57.1</td>
</tr>
</tbody>
</table>

### Table 2 | Prevalence of antibiotics resistance among *E. coli* obtained from the sewage treated effluents of three sewage treatment plants (STPs) at Penang Malaysia.

<table>
<thead>
<tr>
<th>STPs</th>
<th>Cephalaxin (%)</th>
<th>Cefuroxime (%)</th>
<th>Ampicillin (%)</th>
<th>Ciprofloxacin (%)</th>
<th>Amoxicillin (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>STP1</td>
<td>100</td>
<td>85.7</td>
<td>100</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>STP2</td>
<td>71.4</td>
<td>71.4</td>
<td>100</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>STP3</td>
<td>100</td>
<td>100</td>
<td>85.7</td>
<td>42.9</td>
<td>85.7</td>
</tr>
</tbody>
</table>
The distribution of antimicrobial resistance among faecal indicators and pathogenic bacteria most commonly present in domestic sewage from a particular municipality and eventually in the waste-water produced during treatment depend on the public health and size of the local community, as well as on the presence of hospitals in the same area and class of antibiotics used in treatment of bacterial infection. Bacteria from less contaminated water present less antibiotic resistance than those isolated from highly contaminated water (Gonzalo et al. 1989). Velickovic-Radovanovic et al. (2009) found that the resistance of amoxicillin and clavulanic acid by *E. coli* correlated significantly with their utilization.

**CONCLUSIONS**

It can be concluded that, presence of pathogenic bacteria resisted for antibiotics in sewage treated effluents increase the risk for the incidence of microbial resistance to antibiotics in the environment during final disposal or reuse of sewage effluents.

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