Detection of antibiotic resistance and tetracycline resistance genes in *Enterobacteriaceae* isolated from the Pearl rivers in South China

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**Abstract**

This study investigated antibiotic resistance profiles and tetracycline resistance genes in *Enterobacteriaceae* family isolates from the Pearl rivers. The *Enterobacteriaceae* isolates were tested for susceptibility to seven antibiotics: ampicillin, chloramphenicol, ciprofloxacin, levofloxacin, sulphamethoxazole/trimethoprim, tetracycline and trimethoprim. In Liuxi reservoir, with an exception to ampicillin resistant strains (11%) no other antibiotic resistance bacterial strains were detected. However, multiple drug resistance in bacterial isolates from the other sites of Pearl rivers was observed which is possibly due to sewage discharge and input from other anthropogenic sources along the rivers. Four tetracycline resistance genes *tet* A, *tet* B, *tet* C and *tet* D were detected in the isolates from the rivers. The genes *tet* A and *tet* B were widely detected with the detection frequencies of 43% and 40% respectively. Ciprofloxacin and levofloxacin resistant enteric bacteria were also isolated from the pig and duck manures which suggest a wider distribution of human specific drugs in the environment. This investigation provided a baseline data on antibiotic resistance profiles and tetracycline resistance genes in the Pearl rivers delta.

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1. Introduction

Antibiotics are widely used in human and veterinary medicine to control bacterial infections. They are also used as a feed additive to promote growth and prevent livestock diseases (Sarmah et al., 2006). The world-wide use of antibiotics for animal health purpose in 1996 was estimated at 27 000 tonnes (Schwarz and Chaslus-Dancla, 2001). Antibiotics are poorly absorbed in human and animals gut with majority of them being excreted unchanged in faeces and urine which eventually find there way into the environment through the disposal of sewage, hospital wastewater and animal waste (Schlusener and Bester, 2006). The widespread use of antibiotics in medicine and animal husbandry is the most important factor for the emergence, selection, and dissemination of antibiotic resistant bacteria (Adam, 2002; McDonald et al., 1997; Witte, 1998; Sarmah et al., 2006). Antibiotic resistant bacteria and drug resistance genes have become an important environmental contamination issue which is receiving an increased attention (Kummerer, 2004; Pruden et al., 2006; Sapkota et al., 2007).

The antibiotic resistance genes can be transferred between bacteria in the environment through plasmids, integrons and transposons (Pang et al., 1994; Schwarz and Chaslus-Dancla, 2001; Nordmann and Poirel, 2005; Pruden et al., 2006). Presence of antibiotic resistant genes (ARG), such as *tet* genes, *van* genes and *sul* genes have been reported in wastewater, surface water and sediments (Schwarz et al., 2003; Pei et al., 2006; Ram et al., 2007). Tetracycline class of antibiotics is widely used for the treatment of bacterial infections and also as growth promoters in livestock industry due to their broad-spectrum antimicrobial activity (Col and O’Connor, 1987; Speer et al., 1992). The Animal Health Institute survey in 1999 showed that tetracyclines account for 15.8% of 9.3 million kg of antibiotics used in animal feed in the United States, and nearly 56% of approximately 14 600 kg of antibiotics used in animal food production in Kenya (Mitema et al., 2001). The usage of tetracyclines was approximately 16 268 kg in the UK in 2000...
(Sarmah et al., 2006). In China, tetracyclines are also one of the most widely used antibiotics. The widespread use of tetracyclines can lead to the emergence of drug resistant bacteria and transfer of tetracycline resistant genes between different species of bacteria. At least 38 acquired tetracycline resistance (tet) genes have been reported from a wide variety of bacteria in recent years (Roberts, 2005). Among them 23 genes code for efflux proteins, 11 genes for ribosomal protection proteins and 3 genes for inactivating enzyme and one with unknown resistance mechanism (Roberts, 2005; Zhang et al., 2009). Twenty tet genes have been found in aquatic microbial communities around the world with tet A, B, C, D, E and tet M, O, S, Q, W more frequently detected (Zhang et al., 2009).

The efflux genes (tet A, B, C, D and E) are frequently detected from the members of Enterobacteriaceae family which include Salmonella enterica, Shigella and pathogenic Escherichia coli (Speer et al., 1992; Roberts, 1996). Environmental discharge of untreated sewage and animal waste can lead to contamination of surface and groundwater (Reinthaler et al., 2003; Sapkota et al., 2007). The presence of antibiotic-resistant bacteria in the surface water could pose a potential public health hazard (Messi et al., 2005). Unfortunately, in China, very little is known about the presence of antibiotic-resistant bacteria in the aquatic environment.

The purpose of this study was to investigate the occurrence and distribution of antibiotic resistant bacteria along with tetracycline resistance genes tet A, tet B, tet C and tet D in the isolates of Enterobacteriaceae family from the Pearl rivers (Liuxi River, Zhujiang River and Shijing River) in South China. Wastewater from sewage treatment plants and animal manure from a farm were also tested in the study to check for animals as potential source of drug resistant bacteria.

2. Materials and methods

2.1. Study site and sample collection

Pearl River system which is the source of drinking water for Guangzhou city is a complex network of interconnected rivers (Fig. 1). Zhujiang River flows through the Guangzhou city, which starts from Yagang and flows to the South China Sea. Shijing River is a small tributary stream of the Zhujiang River. Liuxi River starts from Liuxi reservoir (51) and is connected with the Zhujiang River at Renhe town. Water samples were collected from 15 sampling sites, including 7 samples from Liuxi River (S1–S7), 4 samples from the Zhujiang River (S8–S11) and 4 samples from the Shijing River (S12–S15) (Fig. 1).

Water samples (250 ml) were collected in sterile bottles in triplicates from 15 selected sites at a depth of 50 cm below the water surface in the middle of the river in November 2007. Wastewater samples (influent and secondary effluent) were collected from the four large sewage treatment plants in Guangzhou city (Fig. 1). Animal manure (duck and pig) was also collected from a farm near Guangzhou. After collection, samples were stored at 4 °C in a cooler and transported to the laboratory. The samples were processed for bacterial numbers and isolation of antibiotic resistant bacteria within 24 h.

2.2. Bacterial strains and culturing conditions

E. coli ATCC 25922 (kindly provided by Geng Hui-Na, Southern Hospital, China) was used as quality control in antibiotic susceptibility testing. Same E. coli strain was also used as a negative control for universal PCR assays to detect tetracycline resistant genes. E. coli ATCC 25922 carrying tet A, E. coli B1 carrying tet B, plasmid pBR322 (Takara, Japan) carrying tet C (Ng et al., 2001), and E. coli D24 carrying tet D, were used as positive controls in universal PCR assays for the detection of tetracycline resistant genes. The E. coli strains A11, B1 and D24 were isolated previously from the Pearl rivers and MacKeyconkey agar medium (Oxoid, UK) and then the plate was inverted and incubated at 35 °C for 24 h. The individual colonies on MacKeyconkey agar medium were inoculated onto nutrient agar medium dishes and enriched at 35 °C for 18–24 h (Miranda and Zemelman, 2001, 2002; Jazrawi et al., 1988). Phenotypical characteristics, gram staining, oxidative and fermentation tests were determined for the isolated bacterial colonies to screen for the presence of Enterobacteriaceae which are gram-negative, oxidative-negative, and fermentation-positive. The isolates confirmed as Enterobacteriaceae were tested for antibiotic resistance, and then stored at −20 °C in a store-physiological saline solution (0.85% NaCl) supplemented with 10% dimethyl sulfoxide (DMSO).

2.3. Isolation of Enterobacteriaceae

Ten fold serial dilutions (10^7, 10^4, 10^2, 10^1 and 10^0) of each collected water sample were prepared in sterile saline solution (0.85% NaCl). One hundred microliter of each dilution was spread plated onto nutrient agar plates (Oxoid, UK) in triplicate to determine total bacterial numbers per sample. Inoculated plates were incubated at 35 °C overnight and bacterial numbers were recorded as colony forming units (cfu/ml) to work out total number of bacteria per sample. Based on the bacterial numbers in each water sample, a certain volume of the diluted water sample with 20 ml of antibiotic spread every colony with MacKeyconkey agar medium (Oxoid, UK) and then the plate was inverted and incubated at 35 °C for 24 h. The individual colonies on MacKeyconkey agar medium were inoculated onto nutrient agar medium dishes and enriched at 35 °C for 18–24 h (Miranda and Zemelman, 2001, 2002; Jazrawi et al., 1988).

2.4. Antimicrobial susceptibility test

Antibiotic resistance of the Enterobacteriaceae isolates was determined by the Kirby–Bauer disk diffusion method using the standard procedure of the Clinical and Laboratory Standards Institute (CLSI, 2007). The isolates were screened for susceptibility to a panel of seven antibiotics including Mueller–Hinton agar (Oxoid, UK) media. The antibiotic discs (Oxoid, UK) containing the following antibiotics were used: ampicillin (AMP, 10 μg), chloramphenicol (C, 30 μg), ciprofloxacin (CIP, 5 μg), levofloxacin (LEV, 5 μg), sulphamethoxazole/trimethoprim (SXT, 25 μg), tetracycline (TE, 30 μg) and trimethoprim (W, 5 μg).

The inoculum for antibiotic resistance pattern testing was prepared in stroke-physiological saline solution by dispensing a single colony picked up with a sterile cotton swab. The turbidity of the resulting solution was adjusted to 0.5 McFarland standard. One hundred microliter of solution was spread plated onto Mueller–Hinton agar plates. The antibiotic discs were placed 30 mm apart on the inoculated plates by using a disc dispensing apparatus. Fifteen minutes after the discs were applied, the plates were inverted and incubated at 35 °C for 16–20 h. The inhibition zone diameters were measured to the nearest millimeter and recorded. Each bacterial isolate was classified as susceptible (S), intermediate (I), and resistant (R) to antibiotics according to the zone diameter interpretation standard recommended by the Clinical and Laboratory Standards Institute (CLSI, 2007). E. coli ATCC 25922 was used as a quality control strain to check the media and antibiotic discs quality and accuracy of the testing procedure.

The frequency of antibiotic-resistant Enterobacteriaceae isolates was calculated by the equation: A/B × 100%, where A is the number of isolates resistant to an antibiotic and B is the total number of isolates from the sample.

The multiple antibiotic resistance (MAR) index of each samples was estimated by the equation: α/(α + β), where α represents the aggregate antibiotic resistance score of all isolates from the sample, β represents the number of antibiotics, and c represents the number of isolates from the sample as outlined in Krumperman (1983) and Miranda and Zemelman (2002).

2.5. Identification of tetracycline resistant Enterobacteriaceae

The tetracycline resistant Enterobacteriaceae isolates were identified with an Enterobacteriaceae identification kit (Tianhe, China). The identification kit consists of 10 miniaturized biochemical tests (d-glucose fermentation/oxidation, l-lysine decarboxylase, f-orciniline decarboxylase, H2S production, indole production, lactose fermentation/oxidation, galactitol fermentation/oxidation, phenylalanine deaminase, urea, and citrate). Purified single colonies were inoculated into the microtubes and incubation was carried out overnight at 36 °C. The results from all biochemical tests were recorded and the genus and species names of the bacteria was positively confirmed with the help of handbook supplied with the kit. E. coli ATCC 25922 was used as the quality control strain.

2.6. Detection of tetracycline resistance genes

Genomic DNA was extracted from the isolates by a boiling (Gueimonde et al., 2004; Naas et al., 2007). Briefly, a few colonies of each bacterial strain was suspended in 100 μl of distilled water and heated at 100 °C for 20 min followed by cooling for 10 min. The lysed cell suspension was centrifuged at 12,000 × g for 2 min and the recovered supernatant was frozen at −20 °C until use.

PCR assays were performed to determine the presence of tetracycline resistance genes in tetracycline-resistant Enterobacteriaceae. The sequences of primers used for PCR amplification of antibiotic resistance genes are listed in Table 1. Amplification of the DNA was performed in a PCR apparatus with TaKaRa Ex Taq kit (TaKaRa, Japan). The 20 μl reaction mixture contained 2 μl of 10 × Ex Taq buffer, 1 μl of dNTPs (2.5 mM each), 1.2 μl of MgCl2 (25 mM), 0.4 μl of each primer (10 μM each), 0.1 μl Ex Taq DNA polymerase (5 U/μl), and 1 μl template of DNA extraction and 13 μl ddH2O. The PCR was initiated by incubating the reaction mixture at 94 °C for 2 min, followed by 30 cycles of 30 s at 94 °C, 30 s at the annealing temperature of 58 or 60 °C (Table 1), and extension for 30 s at 72 °C. The reaction was terminated after a final extension step for 10 min at 72 °C. All PCR experiments contained a positive control (2 μl of DNA extraction of corresponding reference strain or 5 pg/μl of plasmid.}
Fig. 1. Location of sampling sites along the Pearl rivers (Liuxi River, Zhujiang River and Shijing River). Site S1 is located at the reservoir; sites S2–S7 are located at Liuxi River; sites S8–S11 are located at Zhujiang River; and sites S12–S15 are located at Shijing River.

Table 1
Primers used in this study for detection of tetracycline resistance genes.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer pair</th>
<th>Sequences (5' → 3')</th>
<th>Annealing temp (°C)</th>
<th>Amplicon size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>tet A</td>
<td>tet A-FW</td>
<td>GCGCGATCTGGTTCACTCG</td>
<td>60</td>
<td>164</td>
<td>Aminov et al. (2002)</td>
</tr>
<tr>
<td></td>
<td>tet A-RV</td>
<td>AGTCGACAGYRGCGCCGGC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tet B</td>
<td>tet B-FW</td>
<td>TACGTGAATTTATTGCTTCGG</td>
<td>58</td>
<td>206</td>
<td>Aminov et al. (2002)</td>
</tr>
<tr>
<td></td>
<td>tet B-RV</td>
<td>ATACAGCATTTTTAATGGCTTCCG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tet C</td>
<td>tet C-FW</td>
<td>GCGGGATATCGTCCATTCCG</td>
<td>60</td>
<td>207</td>
<td>Aminov et al. (2002)</td>
</tr>
<tr>
<td></td>
<td>tet C-RV</td>
<td>GCGTAGAGGATCCACAGGACG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tet D</td>
<td>tet D-FW</td>
<td>GGAATATCTCCCCGGAAGCGG</td>
<td>60</td>
<td>187</td>
<td>Aminov et al. (2002)</td>
</tr>
<tr>
<td></td>
<td>tet D-RV</td>
<td>CACATTGGACAGTGCCGCCAG</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a FW, forward; RV, reverse.
solution), a negative control (2 μl of DNA extraction of E. coli ATCC 25922) and a blank control (pure water instead of DNA extraction).

Amplified DNA from each sample (5 μl) was mixed with 1 μl of 6× loading buffer dye and loaded onto a 0.8% horizontal agarose gel containing 0.5 μg/ml of ethidium bromide. A DNA ladder ranging from 20 to 500 bp (TaKaRa, Japan) was also added on each gel to confirm the size of amplified DNA bands. All gels were run in TAE buffer at 5 V/cm for 30 min, and visualized by UV transillumination.

2.7. Statistical analysis

Two way analysis of variance (ANOVA) was performed to test the significant difference in the antibiotic resistance frequency at different sampling sites and critical P-value was set at 0.05.

3. Results

3.1. Antibiotics resistance

A total of 361 Enterobacteriaceae strains were isolated from surface water samples of the Pearl rivers (Liuxi River, Zhujiang River and Shijing River) and all the isolates were tested for resistance to seven antibiotics. The frequencies of antibiotic resistance for the isolates of the Pearl rivers are presented in Table 2 and Fig. 2. The antibiotic resistance data in Table 2 show that more than half (55%) of the Enterobacteriaceae family isolates in the three Pearl rivers were found to be resistant to AMP, 10% were resistant to C, 7% to CIP, 5% to LEV, 15% to SXT, 19% to TE, and 20% to W, respectively. The resistance frequencies of all the isolates from the different sampling sites in the Pearl rivers were: 11% to 100% for AMP, 0% to 43% for C, 0% to 25% for CIP, 0% to 21% for LEV, 0% to 62% for SXT, 0% to 58% for TE, and 0% to 62% for W (Fig. 2).

Similar resistance profiles were found in the isolates from the wastewater and animal manure samples (Fig. 3). The isolates from the wastewater had higher resistance frequencies for AMP, SXT, TE and W than for C, CIP and LEV.

The total bacterial numbers in water samples from Liuxi River increased from 56 cfu/ml at the reservoir centre to 104 cfu/ml in the river (Table 2). The strains isolated from the Liuxi reservoir water (S1) showed a resistance frequency of 11% to ampicillin but no resistance to the other six antibiotics tested. The Enterobacteriaceae isolates from Liuxi River had a resistance frequency varying from 11% to 100% for AMP, while lower or no resistance frequencies were found in the isolated strains for the antibiotics SXT, TE and W (Table 2). No isolated strains were found to be resistant to two fluoroquinolones (CIP and LEV) in the water samples collected at the seven sites of Liuxi River.

The isolates from the Zhujiang River showed high resistance (71–100%) to AMP (Table 2). The frequencies of resistance to CIP and LEV in the bacterial strains isolated from Zhujiang River were found to be lower than other antibiotics. Low to medium resistance frequencies were found for the antibiotics SXT, TE and W. Significant differences in resistance frequencies were found among the four sites at Zhujiang River (ANOVA, F = 6.9, p < 0.05).

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![Fig. 2. Frequencies of antibiotic resistance of the Enterobacteriaceae isolates in surface water from the Pearl river (Liuxi River, Zhujiang River and Shijing River, excepting the reservoir site S1). AMP: ampicillin; C: chloramphenicol; CIP: ciprofloxacin; LEV: levofloxacin; SXT: sulphamethoxazole/trimethoprim; TE: tetracycline; W: trimethoprim. Error bars indicate the standard deviations.](image1)

![Fig. 3. Antibiotic resistance frequencies of the Enterobacteriaceae isolates in wastewater from the four sewage treatment plants and animal manure from a farm in Guangzhou. AMP: ampicillin; C: chloramphenicol; CIP: ciprofloxacin; LEV: levofloxacin; SXT: sulphamethoxazole/trimethoprim; TE: tetracycline; W: trimethoprim.](image2)

---

Table 2

<table>
<thead>
<tr>
<th>Site</th>
<th>cfu/ml</th>
<th>Frequency of resistant Enterobacteriaceae isolates (%)</th>
<th>MAR index</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AMP</td>
<td>C</td>
<td>LEV</td>
</tr>
<tr>
<td>S1</td>
<td>5.60E+1</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td>S2</td>
<td>0.21E+4</td>
<td>50</td>
<td>17</td>
</tr>
<tr>
<td>S3</td>
<td>0.80E+4</td>
<td>53</td>
<td>16</td>
</tr>
<tr>
<td>S4</td>
<td>0.49E+4</td>
<td>41</td>
<td>18</td>
</tr>
<tr>
<td>S5</td>
<td>3.12E+4</td>
<td>19</td>
<td>0</td>
</tr>
<tr>
<td>S6</td>
<td>0.91E+4</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>S7</td>
<td>0.95E+4</td>
<td>100</td>
<td>8</td>
</tr>
<tr>
<td>S8</td>
<td>1.40E+4</td>
<td>100</td>
<td>43</td>
</tr>
<tr>
<td>S9</td>
<td>1.34E+4</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>S10</td>
<td>1.32E+4</td>
<td>92</td>
<td>3</td>
</tr>
<tr>
<td>S11</td>
<td>1.94E+5</td>
<td>71</td>
<td>8</td>
</tr>
<tr>
<td>S12</td>
<td>2.16E+5</td>
<td>57</td>
<td>13</td>
</tr>
<tr>
<td>S13</td>
<td>1.51E+5</td>
<td>26</td>
<td>4</td>
</tr>
<tr>
<td>S14</td>
<td>2.82E+5</td>
<td>27</td>
<td>14</td>
</tr>
<tr>
<td>S15</td>
<td>4.83E+5</td>
<td>63</td>
<td>17</td>
</tr>
<tr>
<td>All sites</td>
<td>55</td>
<td>10</td>
<td>7</td>
</tr>
</tbody>
</table>

---

*a* cfu: colony forming units of total culturable bacteria in water.

*b* AMP: ampicillin; C: chloramphenicol; CIP: ciprofloxacin; LEV: levofloxacin; SXT: sulphamethoxazole/trimethoprim; TE: tetracycline; W: trimethoprim.

*c* MAR index: multiple antibiotic resistance index.
Shijing River was found to have the highest number of total culturable bacteria (up to $4.83 \times 10^5$ cfu/ml). The frequencies of antibiotic resistant strains in Shijing River were found to range from 26% to 63% for AMP, 4% to 17% for C, 13% to 25% for CIP, 9% to 21% for LEV, 13% to 42% for SXT, 26% to 58% for TE and 13% to 42% for W (Table 2). Antibiotic resistant bacteria have been found in the water samples collected from every site of Shijing River. The levels of Enterobacteriaceae resistance to each antibiotic had a decreasing trend from upstream to downstream of Shijing River (from S15 to S13; ANOVA $F = 6.5, p < 0.05$), but the levels of AMP, C, SXT and W resistance increased at the site S12, at the entrance point of Shijing River to Pearl River (Table 2).

The MAR index ranged from 0.02 to 0.45 with a mean value of 0.19 in the water samples collected from the three Pearl rivers. The highest value of the MAR index was observed at the site S8 of Zhujiang River (MAR 0.45), followed by the site S15 of Shijing River (MAR 0.42). The median MAR index for the influent and effluent samples from the sewage treatment plants were 0.4 and 0.3, respectively, while those for the duck and pig manure were 0.61 and 0.64, respectively.

3.2. Antibiotic resistance patterns

There are 128 different resistance patterns of 7 antibiotics possible, based on the theoretical combinatorial formula of $C_7^2 + C_7^3 + C_7^4 + C_7^5 + C_7^6 + C_7^7 = 516$, but only 30 different resistant patterns were observed among all 361 isolates in surface water from the Pearl rivers (Table 3).

3.3. Detection of tetracycline resistance genes

Out of total 361 Enterobacteriaceae isolates, 58 were tetracycline resistant. The majority of them, 42 isolates (72%) were E. coli; 12 isolates (21%) were S. enterica, 3 isolates (5%) were Enterobacter aerogenes; and only one isolate (2%) was Serratia marcescens (Table 4).

The 58 Enterobacteriaceae isolates resistant to tetracycline were analyzed for tetracycline resistance genes tet A, tet B, tet C and tet D. The distribution of tetracycline resistant genes in the tested strains were: 43% for tet A (25 out of 58 isolates), 40% for tet B (23 of 58), 21% for tet C (12 of 58), and 21% for tet D (12 of 58).
tested positive for tet B/C genes. Out of remaining eight isolates, four *E. coli*, three *Salmonella* spp. and one *S. marcescens* tested positive for tet B/D genes.

The tet A gene was detected in *Enterobacteriaceae* isolates from 11 sampling sites (S3, S4, S5, S7, S9, S10, S11, S12, S13, S14, and S15), tet B was detected in isolates from 9 sampling sites (S2, S8, S9, S10, S11, S12, S13, S14, and S15), tet C was detected from 4 sampling sites (S3, S11, S13, and S14), and tet D was only detected in isolates from 2 sampling sites (S8 and S12). It is noticeable that all 8 isolates in the site S8 were detected only with tet gene combination tet B/D, which was not found in the other sites.

### 4. Discussion

Contaminated drinking water is a major source of gastrointestinal microbial pathogens and cause of numerous waterborne disease outbreaks in the developing world. The presence of drug resistant bacteria in surface water and groundwater is a major public health concern as drug resistant bacteria could be transferred to humans via consumption of contaminated drinking water which then contributes to the spread and persistence of antibiotic resistance bacteria in general population and environment. The present investigation showed a wide presence of antibiotic resistant bacteria in the Pearl rivers which is drinking water source for Guangzhou city. The antibiotic resistance patterns of the *Enterobacteriaceae* isolates in the three rivers include no-drug resistance (1), single-drug (5), two-drug (9), three-drug (6), four-drug (4), five-drug (2), six-drug (2), and seven-drug resistance (1). The drug resistance pattern suggests that most of the isolates had multiple drug resistance. The average MAR index was 0.19 for all the isolates from the three rivers, with sites S8 and S15 having the MAR index of 0.42 and 0.45, respectively. This may indicate heavy contamination of the two sites (S8 and S15) by wastewaters. Presence of multiple drug resistant enteric bacteria isolates from aquatic environment has been reported previously (Akinbowale et al., 2006; Olaniran et al., 2009). Olaniran et al. (2009) investigated antibiotic resistance profiles of *E. coli* isolates from two rivers in Durban, South Africa and found that 71–97% of the isolates were resistant to the antibiotics tested. Multiple antibiotic resistances were also found in strains of *Enterobacteriaceae* isolated from rivers of Turkey and Bangladesh (Toroglu et al., 2005; Zahid et al., 2009). The observed similarity for the drug resistance profiles for the seven antibiotics between river water and wastewater (Figs. 2 and 3), suggest that wastewater is a possible source of antibiotic resistant bacteria. Human activities especially discharge of wastewater are probably the main contributor to the multi-drug resistance patterns since rivers are the primary receptacle of sewage effluents. 

Liuxi River is protected as the drinking water source for Guangzhou and surrounding towns, but the river water has still been affected by non-point and point pollution along the river with the development of industry and agriculture in the river catchment (Liu et al., 2004; Zhang et al., 2004; Song et al., 2007). The present study predominantly found no antibiotic resistance with an exception of AMP (only 11% resistance frequency) for the *Enterobacteriaceae* isolated from the central part (S1) of Liuxi reservoir. Ampicillin resistance found among the isolates at this site might be due to the presence of natural populations of beta-lactam resistant bacteria. As low levels of beta-lactam drug resistance is known to be intrinsically present in some gram-negative environment isolates (Esiohu et al., 2002). The water sample from the reservoir centre (S1) also exhibited the lowest total bacterial counts (56 cfu/ml). Therefore, this site can be used as the reference site with little human activity. Human activities at the upstream of Liuxi River near the reservoir (S2), Liangkou town (S3) and Conghua city (S4) increased the total bacterial counts and resistance frequencies.
for AMP, C, SXT, TE and W (Table 2). The resistance frequencies for trimethoprim (W) ranged from 18% at the site S2 to 53% at the site S4, while those for chloramphenicol (C) varied between 16% and 18%. The presence of more drug resistant bacteria is possibly due to increased human activity downstream of river. However, lower detection of antibiotic resistant bacteria (SXT and TE) at the downstream sites of the river (S5, S6 and S7) is primarily due to protection of the drinking water source from domestic and agricultural input using green zones along the river section. The present study showed ubiquitous bacterial resistance to ampicillin in the Pearl rivers with some sites even having 100% resistance frequencies. This suggests a relatively high prevalence of ampicillin resistant bacteria as compared to other tested antibiotics. In contrast, Goni-Urriza et al. (2000) found that only 20% of enterobacteria resistant species as compared to other tested antibiotics. In the present study showed ubiquitous bacterial resistance to ampicillin in the Pearl rivers with some sites even having 100% resistance frequencies. This suggests a relatively high prevalence of ampicillin resistant bacteria as compared to other tested antibiotics. In contrast, Goni-Urriza et al. (2000) found that only 20% of enterobacteria were resistant to ampicillin at the downstream of urban effluent discharge point in Arga River, Spain. Since concentration of ampicillin in the river water was not determined, it is difficult to conclusively suggest that presence of higher AMP resistant bacteria is linked to the presence of high AMP levels in the river water. No bacterial isolate resistant to the two fluoroquinolones (CIP and LEV) was observed in the water samples from the Liuxi River. These two fluoroquinolones are primarily used as human medicines, since there is no point discharge source from the wastewater treatment plants in this area to river. Absence of CIP and LEV resistance in isolated bacteria is not unexpected.

Zhujiang is a river that passes through the metropolitan city Guangzhou and receives discharge of domestic wastewater (Dong and Mei, 2008). Treated effluent from the four sewage treatment plants in Guangzhou city is also discharged into the river. Moreover, the tidal wave has a significant influence on the water quality of Zhujiang River, especially in the sections of the two water channels (S10 and S11) (Wang et al., 1997). The water in the channels is diluted by the water from the downstream of Zhujiang River through tidal action. The water quality in the upstream of Zhujiang River (at Yagang, S8) is less influenced by tidal wave but affected by untreated wastewater from nearby villages. This is reflected by the highest bacterial resistance frequencies to AMP (100%), C (43%), SXT (62%) and W (62%) and highest MAR index (0.45) found in the water at the site S8 of Zhujiang River (Table 1). Low CIP resistance frequency (10%) and no LEV resistance in Enterobacteriaceae isolates along with the presence of only tet B/D gene combination at site S8 suggest that source of contamination other than the sewage is also present at this site. The bacterial resistance for AMP, SXT, TE and W was found in all water samples from the Zhujiang River at various frequencies. Harakeh et al. (2006) also reported high SXT resistance frequency (75%) of E. coli isolated from the Lebanese aquatic environment. Up to 11% resistance frequencies for both CIP and LEV were found at the site S11, which is located at a sewage effluent discharge point. High resistance (53%) to ciprofloxacin and norfloxacin was reported in E. coli strains isolated from a hospital wastewater in Vietnam (Duong et al., 2008). This suggests that high resistance frequencies for CIP and LEV are possibly due to wastewater discharge and human activities along the river in this area. Ciprofloxacin and levofloxacin resistant enteric bacteria were also isolated from the pig and duck manures which suggest a wider distribution of human specific drugs in the environment and animals are a potential reservoir of these drug resistant bacteria.

Shijing River is a heavily polluted river because it receives large quantities of untreated sewage (>100 million Liters) and industrial wastewater (Luo, 2002). The river used to be an important drinking water source for Guangzhou city and now its water quality is even worse than treated sewage effluent and its BOD5 value was more than 14 mg/l (Luo, 2002; Sheng, 2007). The dissolved oxygen levels ranged between 0.99 and 4.67 mg/l, which were measured during the sampling of river water. Shijing River could also affect the water quality of Zhujiang River since it is connected at the upstream of Zhujiang River. As expected, the present study found resistance to all seven tested antibiotics in the Enterobacteriaceae isolates at each site of Shijing River. The resistance frequencies for CIP and LEV in the river were 13–25% and 9–21%, respectively, reflecting high domestic sewage contribution. High numbers of culturable bacteria (up to 4.83 × 10^3 cfu/ml), more than 8 resistance patterns and high levels of the MAR index were observed in the water samples at each site of Shijing River suggests heavy pollution of river from wastewater and various other sources of contamination. Contamination of Shijing River by domestic wastewater was also demonstrated by detection of high concentrations of various human pharmaceuticals in the river (Zhao et al., 2009).

High antibiotic resistance patterns (>8) are often associated with contaminated sites with sewage and other anthropogenic sources, sites S2–4 of Liuxi River, S11 of Zhujiang River and S12–15 of Shijing River fall in this category (Table 3). Previous studies also suggest that sewage pollution contribute to the dissemination of antibiotic resistant bacteria in the environment (Reinthaler et al., 2003; Martins da Costa et al., 2006). In our previous investigation (Yang et al., in press), the antibiotic concentrations in different sites in general followed the following pattern: sewage influent > Shijing River > sewage effluents > Zhujiang River > Liuxi River. The concentration of the antibiotics in Shijing River and wastewaters were at least one order of magnitude higher than those in Zhujiang River and Liuxi River. The results of the current study on bacterial resistance profiles are consistent with the previous study on antibiotic levels in the three rivers. Long term exposure of bacteria to trace levels of antibiotics is known to increase antibiotic resistance. The presence of tet genes in different members of Enterobacteriaceae family is known to occur due to transfer of drug resistant genes between bacteria through plasmids, transposons and integrons (Pang et al., 1994; Nordmann and Poirel, 2005; Pruden et al., 2006).

Tetracycline resistance genes tet A and tet B were widely detected in the Enterobacteriaceae strains in the Pearl rivers, but genes tet C and tet D were only detected in the isolates from a few sites of the rivers. The tet genes including tet A (Ageros and Sandvang, 2005; Srinivasan et al., 2005), tet B (Ageros and Sandvang, 2005; Dang et al., 2007), tet C (Ageros and Sandvang, 2005; Akinbowale et al., 2007), and tet D (Schmidt et al., 2001) have been previously reported in bacterial isolates from aquatic environment.

This study shows that about 28% of the Enterobacteriaceae isolates from the Pearl rivers carried two different tet genes, which is consistent with previous reports (Schmidt et al., 2001; Bryan et al., 2004). Some previous studies suggest that multiple tet genes can be present in more than 20% frequency within gram-negative bacteria in some ecosystems, for example tet genes for aemorodactis isolated at Danish rainbow trout farms (Schmidt et al., 2001). In animals over 30% of E. coli isolated from pigs, turkeys and horses have been reported to carry 2–3 different tet genes and about 20% E. coli isolates from E. coli carried multiple tet genes (Bryan et al., 2004). It appears that drug resistant bacteria are widely distributed in the Pearl rivers, sewage inflow and animal manures contribute towards the distribution of drug resistant genes in environment. Wastewater disposal along with antibiotic use must be carefully regulated and monitored to control the spread of drug resistant bacteria.

5. Conclusions

This study provides a baseline data on antibiotic resistance profiles and patterns in drinking water sources of the three Pearl (Liuxi River, Zhujiang River and Shijing River). In general, water in
Liuxi River exhibited the lowest antibiotic resistance, especially at the reservoir centre and at the downstream, whereas the water at the sites from Liangkou to Conghua showed increased resistance patterns due to the influences caused by human activities in villages and towns along the river. Elevated antibiotic resistance rates were observed in the *Enterobacteriaceae* isolated from Zhujiang River, which receives treated sewage effluent in Guangzhou. Highest antibiotic resistance rates were found in the water samples from Shijing River, which receives untreated domestic wastewater from nearby towns. Based on the antibiotic resistance profiles found in wastewater and animal manure, untreated sewage and manure are most probably the main cause of widespread presence of antibiotic resistant bacteria in the rivers. Isolation of Ciprofloxacin and levofloxacin resistant enteric bacteria from the pig and duck manures suggest animal manures being a potential reservoir of these drug resistant bacteria.

The PCR analysis of tet genes demonstrated the existence of tetracycline resistance genes in the rivers. Among the four tet genes detected, the tetA and B had higher detection rates than tetC and D. Further research is needed to determine the occurrence and distribution of drug resistant bacteria and other antibiotic resistance genes in *Enterobacteriaceae* family and other aquatic bacteria.

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