Phenotyping and Genotyping of Antibiotic-Resistant *Escherichia coli* Isolated from a Natural River Basin

JIANYING HU,*,† JIACHEN SHI,† HONG CHANG,† DONG LI,‡ MIN YANG,‡ AND YOICHI KAMAGATA§

College of Urban and Environmental Sciences, Peking University, Beijing, 100871 China, State Key Lab of Environmental Aquatic Chemistry, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China, Research Institute of Genome-based Biofactory, National Institute of Advanced Industrial Science and Technology, 2-17-2-1, Tsukisamu-Higashi, Toyohira, Sapporo 062-8517, Hokkaido, Japan

Received October 23, 2007. Revised manuscript received February 18, 2008. Accepted March 3, 2008.

Scientists have become increasingly concerned about the occurrence of antibacterial resistance in the environment. In this study, *Escherichia coli* resistant to one or more antibiotics among nine antibiotics was screened from Wenyu River Basin in Beijing, China, with mean frequency of 48.7 ± 8.7% of 388 isolates in summer and 47 ± 6% of 236 isolates in winter. The mean multiantibiotic resistance (MAR) index in summer was 0.11 ± 0.03, slightly lower than that (0.14 ± 0.04) in winter. Most frequent resistance appeared for sulfonamides, tetracycline, and ampicillin. The distribution of 20 tetracycline, three sulfonamide, and three β-lactam resistance genes was assessed in the resistant isolates. While 97% of the ampicillin (AMP) resistant mechanism could be explained by the resistance gene *TEM*, 90% of the tetracycline (TC) and 96% of the sulfonamide (SXT) resistances could be explained by *tet*(A), *tet*(B), *tet*(M), and their combinations and *sul*(I), *sul*(II), *sul*(III), and their combinations, respectively. *tet*(M), a tetracycline-resistant gene originally detected in Gram-positive bacteria, and its combinations with *tet*(A) or *tet*(B) were first detected in *E. coli* isolated from a natural river basin, suggesting that *tet*(M) in *E. coli* might have been transferred from other bacterial species through horizontal gene transfer, which was supported by the fact that no *tet*(M) was detected in the isolates of human and chicken sources, except for only one isolate from swine. The source of sulfonamide-resistant *E. coli* in the river was supposed to be mainly from humans, based on a comparison of the sulfonamide resistance genotypes in animals and humans.

Introduction

Overuse in human medicine and agricultural uses, particularly in livestock, contribute significantly toward the problem of antibiotic resistance, which has become a recognized medical problem, and scientists have become increasingly concerned about the occurrence of antibacterial resistance in the environment (*1, 2*). The antibacterial-resistant bacteria together with antibiotics are discharged into the environment through pathways such as domestic sewage and hospital wastewater and are selected and promoted in the environment (*3, 4*), where several antibiotics exist at low levels (*5–7*). Bacteria in the environment can acquire resistance genes from other bacteria in their vicinity in a process known as "horizontal transfer" at chronic low-level exposure to antibiotics (*8, 9*) and therefore contribute significantly to increased gene frequencies and dissemination of resistance genes into other ecosystems.

Several studies have attempted to evaluate the impacts of antibiotic use on antibiotic resistance, and antibiotic resistance genes in the environment have been studied as an emerging contaminant (*10*). Sixteen tetracycline resistance genes (*10–15*), three sulfonamide resistance genes (*10*), and 10 β-lactamase resistance genes (*16*) have been detected in river sediment, seawater, irrigation ditches, dairy lagoons, and the effluents of wastewater recycling and drinking water, indicating the ubiquitous occurrence of these resistance genes. Further studies using *tet*(W) and *tet*(O) for tetracycline, *sul*(I) and *sul*(II) for sulfonamide, and *ere*(A) and *msr*(A) for macrolide show that these genes could not be removed effectively by biological treatment in a dairy lagoon system; in contrast, significant increases of sulfonamide resistance genes were found, especially under antibiotic-spiked conditions (*17*). While these results on the occurrence and fates of antibiotic resistance genes (ARGs) in the environment provide fundamental data for proper risk assessment and environmental management, most previous studies neglected the role of bacteria, which act not only as a reservoir of clinical resistance genes that may provide a source of transferable traits for emerging pathogens but also as a medium for the spread and evolution of resistance genes and their vectors.

The presence of antibiotic-resistant bacteria in freshwater sources has been documented on the basis of the phenotypic resistance testing of antibiotics (*18–20*). In those studies, *Escherichia coli* has been the most widely investigated bacteria (*21, 22*). The resistance to at least two classes of antimicrobial agents in *E. coli* has been frequently found in the environment (*19, 23*), and it has been estimated that 17.6% of the genes in *E. coli* has been acquired by horizontal transfer at a rate of 16 kb/Myr (*24*). The resistance phenotypes may arise from many different genes, and each gene may present specific epidemiological features; however, these studies lacked information about the antibiotic resistance genes that confer the resistance, while several reports have demonstrated an even greater heterogeneity among antibiotic resistance genes and no detectable homology between those found in Gram-negative and -positive species (*25, 26*). To our best knowledge, only one paper reported the occurrence of β-lactam-resistant bacteria in estuarine waters and their resistance mechanism by determining related resistance genes (*16*), and there have been no reports of a detailed study on both the phenotypes and genotypes of *E. coli* resistant to typical antibiotics in a natural river basin and their potential sources. *E. coli* are common bacteria in the intestinal flora of human and other warm-blooded animals and have been widely used as a fecal contamination indicator in environment. Some groups of *E. coli* are the causative agents of many enteric infections worldwide, and animal and human commensal and environmental enteric *E. coli* are supposed to be the natural reservoir of pathogenic strains (*27*). The

* Corresponding author phone and fax: 86-10-62765520; e-mail: hujy@urban.pku.edu.cn.
† Peking University.
‡ Chinese Academy of Sciences.
§ National Institute of Advanced Industrial Science and Technology.

10.1021/es7026746 CCC: $40.75
Published on Web 04/01/2008

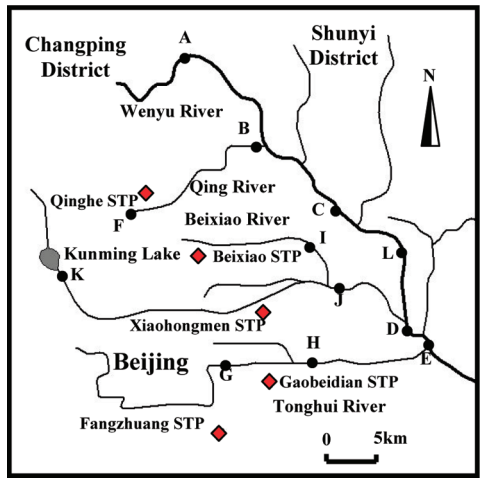

FIGURE 1. Map of sampling points in the Wenyu River Basin.

study on the occurrence of antibiotic-resistant *E. coli* in natural rivers can provide a prototypical view of the effects of antibiotic use on bacterial populations.

In 1999, the annual antibiotic usage in China was about 29 574 tons, including 6716 tons of sulfonamides, 2500 tons of macrolides, 6265 tons of penicillins, 9413 tons of tetracycline, 2360 tons of fluoroquinolones, 702 tons of cephalosporin, and 1618 tons of aminoglycosides (*28*). In this study, we examined the frequency of antibiotic-resistant *E. coli* isolated from the Wenyu River Basin in Beijing, China, based on the phenotypic resistance testing of nine antibiotics [β-lactams including ampicillin (AMP), cefazolin (KZ), cefamandole (MA), cefoperazone (CFP), and imipenem (IPM); tetracycline (TC); sulfamethoxazole−trimethoprim (SXT); levofloxacin (LEV); and gentamicin (CN)]. And then antibiotic resistance genotyping was carried out by examining 20 tetracycline resistance genes (eight genes encoding ribosomal protection protein and 12 tetracycline efflux genes), three sulfonamide resistance genes, and three β-lactamases resistance genes using the polymerase chain reaction (PCR) method. Finally, primary source analysis was carried out by phenotyping and genotyping antibiotic-resistant *E. coli* isolated from swine, chicken, sheep, and human sources. To our knowledge, this is the first study in which the phenotypes and genotypes of *E. coli* isolates from a river basin were comprehensively characterized.

Experimental Section

Sample Collection and *E. coli* Isolation. Figure 1 shows the sampling locations. The Wenyu River flows over a distance of 47.5 km with a catchment area of 2478 km^2. About 55% of the total population of Beijing lives around the Wenyu River Basin, including its tributaries, the Qing, Ba, and Tonghui Rivers. While most of these areas are sewered, there is discharge from untreated wastewater and several stock farms, mainly housing swine. There are some swine, sheep, and chicken stock farms in Shunyi and Changping District located in the suburbs of the Wenyu River Basin. Unfortunately, information on the discharge from untreated wastewater and stock farms into the Wenyu River Basin is unknown.

Sample preparation and bacteriological tests for isolation of *E. coli* were performed by an established membrane filter method (*29, 30*). Briefly, river water samples (about 500−1000 mL) at sites as shown in Figure 1 were collected in sterile Whirl pack bags (Corning) on August 8−11 and on December 21, 2006, respectively. In order to build a source database from animal and human sources to explore the potential sources (animal or human) of antibiotic-resistant *E. coli* in the Wenyu River Basin, wastewater samples from the Fangzhuang Sewage Treatment Plant (STP) in a residential area of Beijing, which receives 100% domestic wastewater, and three lagoons of swine, sheep, and chicken production facilities located in the suburbs of Beijing near the Wenyu River were also taken in this sampling campaign. The detail isolation procedure of *E. coli* is provided in the Supporting Information.

Antibiotic Susceptibility Testing. Isolates were screened for susceptibility to a panel of nine antibiotics on Mueller−Hinton agar (Oxoid) by a disk diffusion method, as described by the CLSI 2005 guidelines (*33*). The following disks (Oxoid, UK) were used: AMP (10 μg), TC (30 μg), SXT (sulfamethoxazole/trimethoprim: 23.75 μg/1.25 μg), LEV (5 μg), KZ (30 μg), MA (30 μg), CFP (75 μg), IPM (10 μg), and CN (10 μg). *E. coli* ATCC 25922 was used as reference strain. The diameter of inhibition zones surrounding the antibiotic disks was interpreted according to the CLSI 2005 guidelines. The isolates that were shown to be resistant to antibiotics were recorded, purified, and collected for subsequent studies. The total frequencies of antibiotic-resistant *E. coli* were estimated by the equation A/B, where A is the numbers resistant to one or more antibiotic, B is the numbers of isolates from the sample. The multiantibiotic resistance (MAR) index of the samples were calculated by the equation $a/(b \times c)$, where a is the aggregate antibiotic resistance score of all isolates from the sample, b is the number of antibiotics, and c is the number of isolates from the sample (*34*).

Detection of Antibiotic Resistance Genes. PCR assays were performed in order to determine which antibiotic resistance gene was detectable in antibiotic-resistant *E. coli*. Primers used for the PCR amplification of antibiotic resistance genes were mainly based on published papers (*11, 12, 14, 16, 35*), and their sequences are listed in Table S1 (Supporting Information). The PCR assays were carried out using a Takara Ex Taq kit (Takara) in a 50-μL volume reaction. The PCR mixture consisted of 5 μL of 10× Ex Taq buffer (Mg^{2+} Plus), 4 μL of dNTPs (2.5 mM each), 0.2 μM of each primer, 2 μL of bacteria that were incubated on liquid LB at 37 °C for 24 h before use, and 0.25 μL 5 U/μL Ex Taq DNA polymerase. Each PCR was performed with a S320 Thermal Cycler (Beijing Botong Tech). The PCR was initiated by incubating the reaction mixture at 95 °C for 10 min, followed by 40 cycles of 15 s at 95 °C, 30 s at the annealing temperature (for the annealing temperatures, see Supporting Information Table S1), and 30 s at 72 °C. The reaction was terminated with an extension step consisting of 7 min of incubation at 72 °C (*14*). All PCR experiments contained a negative control (2 μL of *E. coli* ATCC 25922 that was incubated on liquid LB at 37 °C for 24 h before use) and a blank control (no *E. coli*). To generate positive controls, purified PCR products obtained from the resistant isolates from the Wenyu River Basin were cloned and sequenced. If the PCR products were verified as the object resistance genes using the BLAST alignment tool (http://www.ncbi.nlm.nih.gov/blast/), these isolates with such genes were used as the positive control. Both positive and negative controls were included in every run. Each PCR product (6 μL) was mixed with 1.2 μL of 6× loading buffer dye (Takara, Japan) and loaded on a 1.5% horizontal agarose gel (agarose HT, Amresco, America) together with a 100 bp size ladder (Takara, Japan). All gels were run in 50× TAE buffer (Dingguo) for 30 min and 100 V, stained for 20 min in 50× TAE buffer containing 0.5 μg of ethidium bromide per mL, and visualized by UV transillumination (Gel Doc 2000, Bio-Rad Laboratories, Milan, Italy). If the PCR products were not clearly observed after the first PCR, a second, nested PCR was performed with 2 μL of the first PCR mixture as a template and amplification for 40 cycles as described above, and the PCR production was examined by UV transillumination again.

REP-PCR. The *E. coli* isolates with same phenotyping and genotyping from one sampling site were tested by repetitive extragenic palindromic PCR (REP-PCR) with primers BOXA1R

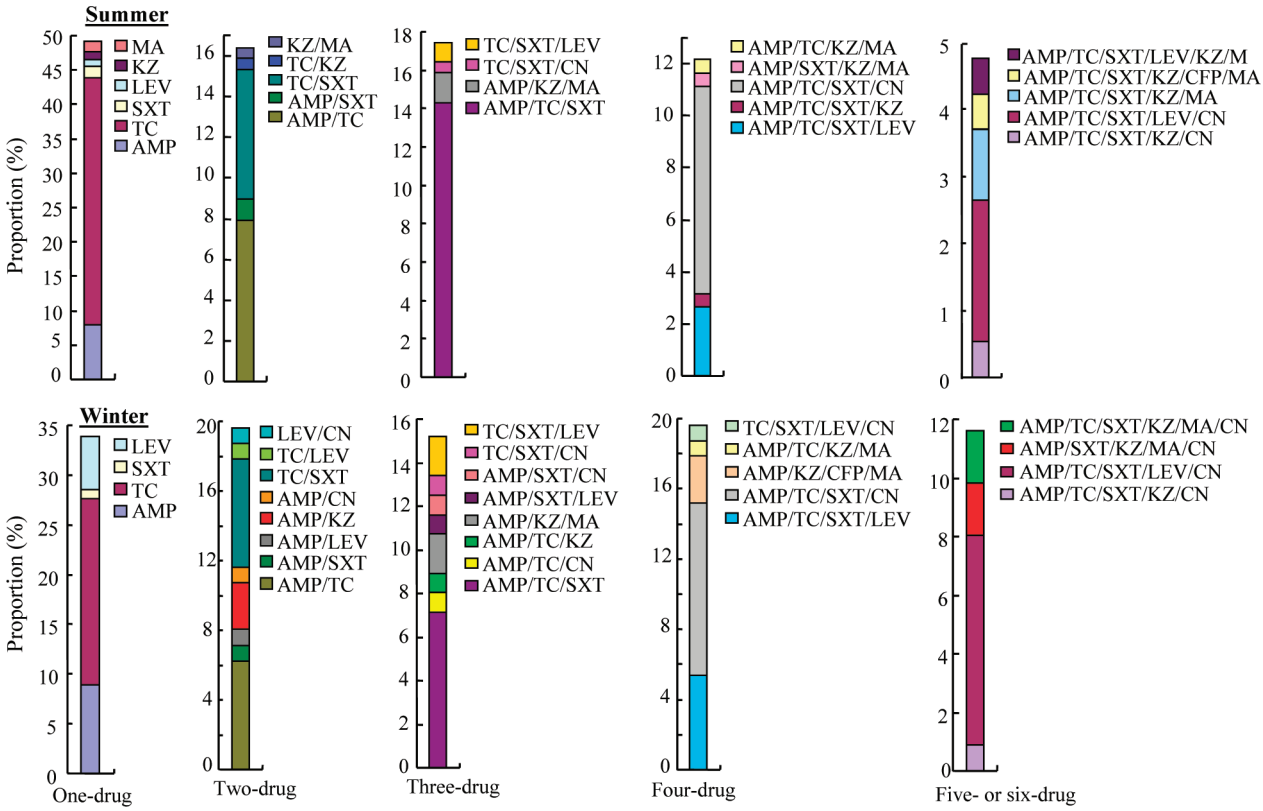

FIGURE 2. Levels of each antibiotic resistance pattern accounting for total antibiotic-resistant *E. coli* isolates from the Wenyu River Basin. Summer: samples taken in August 2006. Winter: samples taken in December 2006. AMP, ampicillin; TC, tetracycline; SXT, sulfamethoxazole−trimethoprim; LEV, levofloxacin; KZ, cefazolin; MA, cefamandole; CFP, cefoperazone; IPM, imipenem; CN, gentamicin.

(5′-CTACGGCAAGGCGACGCTGACG-3′), and the composition of reaction mixtures and PCR program were as described previously (*31*). All PCR experiments contained a positive control (*E. coli* ATCC 25922) and a negative control (2 μL of water instead of *E. coli*). PCR products were electrophoresed in 1.5% agarose gels, stained with ethidium bromide, and visualized by UV transillumination (Gel Doc 2000, Bio-Rad Laboratories, Milan, Italy). Gel images were analyzed with the Quantity One 4.3.0 software (Bio-Rad Laboratories, Milan, Italy). Isolates representing similar fingerprint were discarded and only one of them would be recorded as a result.

Results and Discussion

Phenotyping of Antibiotic-Resistant *E. coli* Isolates. Eleven and six water samples were taken from the Wenyu River Basin in the summer season (August 2006) and winter season (December 2006), respectively, as shown in Figure 1. Median values for *E. coli* in samples taken from the Wenyu River in summer and winter were in the range from 22 (site L) to 1.7 $\times$ 10^4 cfu/mL (site E) and 18 (site D) to 3.6 $\times$ 10^3 cfu/mL (site B), respectively. It should be noted that the *E. coli* levels in the samples from sites A, B, E were much higher than those in the effluents of STPs located along the river basin (Figure 1) due to the inputs of untreated domestic waste, as found in our previous paper (*36*). The 388 isolates were screened from sites A−K in summer with regard to their resistances against the nine antibiotics used. While the total frequencies of antibiotic-resistant *E. coli* ranged from 40% (site A) to 67% (site C), the spectra of antibiotic-resistant *E. coli* are distinct among sites. As shown in Figure S1 (Supporting Information), the frequency of single-drug resistance in total *E. coli* at site C is as high as 41%, followed by three-drug (10%) and four-drug resistances (10%), while its frequency at site A is only 13%, followed by three-drug resistance (15%) and double resistance (10%). It should be noted that relatively high total frequency (58%), with 8% of five-drug and 3% of six-drug resistance were detected at site E, which was located downstream of the Wenyu River. Profiling of resistance phenotypes in the anabranches of the Wenyu River (sites F−K) was basically similar to those in the Wenyu River except for site K, where a relatively high frequency of four-drug resistance (19%) was found. In addition, two-drug resistance with high frequency (14–19%) was found at sites G−I, and relatively high three-drug resistance was found at sites F and J, where five-drug (5%) and six-drug (3%) resistances were detected. To investigate the seasonal variation of antibiotic resistance profiles, 236 isolates were obtained from sites A−E and L in winter. The antibiotic resistance frequency ranged from 41% to 55%, a variation that is not significant compared with the summer samples. However, variations in the antibiotic resistance spectrum were obvious: the frequencies of three-, four-, and five-drug resistance increased. Overall, the mean frequency of antibiotic-resistant *E. coli* in summer and winter were 48.7 $\pm$ 8.7% and 47 $\pm$ 6%, respectively, indicating no obvious seasonal variation, which is similar with observations (*18*) in Japan. It should be noted that the mean multiantibiotic resistance (MAR) index in summer was estimated to be 0.11 $\pm$ 0.03, a little lower than that (0.14 $\pm$ 0.04) in winter.

Figure 2 shows the resistance spectra of *E. coli* isolates against the nine antibiotics. Twenty-five different resistance patterns (six single-drug, five two-drug, four three-drug, five four-drug, three five-drug, and two six-drug resistance patterns with the proportions of 49.5, 16.5, 17, 12.2, 3.7, and 1.1%, respectively) were observed for the isolates in summer, while 29 different patterns were found in winter (four single-drug, eight two-drug, eight three-drug, five four-drug, three five-drug, and two six-drug resistance patterns with the proportions of 34.0, 19.6, 15.2, 19.6, 9.8, and 1.8%, respectively). Among single-drug-resistant isolates in summer, the

highest proportion of resistance was against TC (36%), followed by AMP (7.9%), SXT (1.6%), MA (1.6%), LEV (1.1%), and KZ (1.1%), and no single-drug-resistant *E. coli* against IPM, CN, or CFP was detected, which is similar to reports in previous papers (*18*). Of the multiresistant isolates in summer, the most common resistance pattern was AMP/TC/SXT, which accounted for 13.8% of the 188 resistant isolates, followed by AMP/TC/SXT/CN (8%) and AMP/TC (8%), TC/SXT (6.4%), and AMP/TC/SXT/LEV/CN (2.1%). Only one six-drug-resistant isolate showed resistance against CFP. Among the antibiotic-resistant isolates in winter, the most abundant resistance pattern was also the single TC resistance with a much lower proportion (18.8%) of the 112 resistant isolates, and the most abundant multiresistant pattern was a four-drug one, AMP/TC/SXT/CN (9.8%), followed by AMP/TC/SXT/LEV/CN (7.1%) and AMP/TC/SXT (7.1%), TC/SXT (6.3%), and AMP/TC/SXT/LEV (5.4%), suggesting that the multiresistant *E. coli* might be more persistent or be discharged at a higher rate in winter. It should be noted that among the nine antibiotic agents tested, the resistances for AMP, TC, and SXT were the most frequent in both summer and winter.

Genotyping of Antibiotic-Resistant *E. coli*. About 109 isolates displaying various resistance patterns such as AMP/TC/SXT, TC/SXT, AMP/TC, and SXT/TC were selected to detect their genotypes (Supporting Information Table S2). It was observed that of 26 resistance genes [*tet*(B/P), *tet*(M), *tet*(O), *tet*(Q), *tet*(S), *tet*(T), *tet*(W), *Otr*(A), *tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E), *tet*(G), *tet*(H), *tet*(J), *tet*(L), *tet*(Y), *tet*(Z), *tet* (*30*), *sul*(I), *sul*(II), *sul*(III), *TEM*, *SHV*, *CARB*] selected, seven genes [*tet*(A), *tet*(B), *tet*(M), *sul*(I), *sul*(II), *sul*(III), and *TEM*] were detected in these resistant isolates.

At first, 12 efflux genes and eight of the ribosomal protection tetracycline resistance genes listed in Table S1 (Supporting Information) were analyzed in 91 tetracycline-resistant isolates. Of the 12 efflux genes, only *tet*(A) and *tet*(B) were detected, but *tet*(E), which had been originally isolated from *E. coli*, was not detected. The frequencies of *tet*(A) and *tet*(B) were 66% and 41% (Supporting Information Table S2), respectively. This result is different from that in lactose-fermenting coliforms from humans and animals (*26*), where the majority of wild-type tetracycline-resistant coliforms carried *tet*(B) with a frequency of 73.3%, while *tet*(A) was detected in only about one-fifth (21.7%), together with the relatively low detection frequency of *tet*(C). Such differences may be due to the species−specific gene patterns, because coliforms included not only *E. coli* but also *Enterobacter* and *Klebsiella*, where *tet*(B) may be more prevalent. In fact, the *tet*(B) has been reported to have the widest host range among Gram-negative species (*37*). The gene profile in this study was also different from those in Gram-negative bacteria isolated from polluted and unpolluted marine sediments (*38*). In that study, the most dominant resistance determinant was *tet*(E) (66–70%), which was speculated to be the indigenous tetracycline resistance gene for microorganisms in marine sediments, because *tet*(E) is associated with large plasmid, that are neither mobile nor conjugative (*39*). The majority of Gram-negative isolates described in the literature carry a single type of *tet* gene, except for one study that showed that 3.5% of the lactose-fermenting coliforms simultaneously carried two different *tet* genes, *tet*(A) and *tet*(B) [*tet*(A,B)] (*26*). However, a recent study of polluted marine sediments from Norway (*38*) found 26% of tetracycline-resistant isolates carried both *tet*(D) and *tet*(E). In this study, 15 isolates among 91 tetracycline-resistant *E. coli* isolates carried *tet*(A,B) with a frequency of 16.5%, which is much higher than that (3.5%) in lactose-fermenting coliforms from humans and animals (*26*). It should be noted that of the ribosomal protection tetracycline resistance genes, only *tet*(M) was detected in 15 isolates (16%), of which 8.8% and 5.5% were combined with *tet*(A) [*tet*(A,M)] or *tet*(B) [*tet*(B,M)], respectively, and 1% combined with both *tet*(A) and *tet*(B) [*tet*(A,B,M)]. Although *tet*(M) had been detected in *E. coli* strains from clinic (*40*) and pig and chicken isolates (*41*), to our best knowledge, this is the first report on the presence of *tet*(M) in *E. coli* strains from a river basin. It should be noted that none of the 20 tetracycline-resistance genes in this study were detected in the nine tetracycline-resistant *E. coli* isolates (10%), suggesting that there is a need for further study to determine the comprehensive distribution of tetracycline resistance genes or to explore new tetracycline resistance genes. Thus, 90% tetracycline (TC) resistance could be explained by *tet*(A), *tet*(B), *tet*(M), and their combinations.

Resistance to sulfonamides in *E. coli* frequently results from the acquisition of an alternative dihydropteroate synthase (DHPS) gene (*sul*) (*42*). Three alternative sulfonamide resistance DHPS genes [*sul*(I), *sul*(II), and *sul*(III)] in Gram-negative bacteria have been documented, and all of them were present in this study. *sul*(I) and *sul*(II) were detected almost at equal frequency: 60 isolates of the 73 isolates (82%) carried *sul*(I), and 56 isolates (77%) carried *sul*(II), of which the combination of *sul*(I) and *sul*(II) was detected in 49 isolates (67%). *sul*(III) was detected in only 12 isolates with 16.4% frequency, which consisted of 4% *sul*(III) (three isolates), 1.4% combination of *sul*(I) and *sul*(III) [*sul*(I,III)], 1.4% combination of *sul*(II) and *sul*(III) [*sul*(II,III)], and 9.6% combination of *sul*(I), *sul*(II), and *sul*(III). Thus, 96% of the sulfonamide-resistant isolates can be explained by the presence of *sul*(I), *sul*(II), and *sul*(III). This result was similar to that in sulfonamide-resistant *E. coli* isolates of clinical origin (*43*).

β-Lactams are the most commonly used antibacterial agents for treating infectious diseases in humans and veterinary practice, and resistance to these compounds among Gram-negative bacteria is most frequently related to the production of β-lactamases with an increased spectrum of activity (*44*). *TEM*, *SHV*, and *CARB*-type β-lactamases, which are most often found in *E. coli* were analyzed in this study. While *TEM* with 97% frequency was detected in the 78 isolates, no *SHV* and *CARB* were detected, which is similar to that reported in the previous paper, where the frequencies of *TEM*, *SHV*, and *CARB* in *E. coli* were 95% (33/35), 1% (1/35), 1% (1/35), respectively (*16*).

Primary Analysis of Sources. To explore the potential sources of antibiotic-resistant *E. coli* in the Wenyu River Basin, we also analyzed the phenotyping and genotyping of antibiotic-resistant *E. coli* in the sources of swine, sheep, chicken, and human (domestic wastewater from the Fangzhuang STP in Figure 1). Figure S2 (Supporting Information) compares the spectrum of resistant *E. coli* isolates in the river basin with those of swine, chicken, and human sources, except for sheep, because only AMP- and TC-resistant *E. coli* were isolated from sheep. The frequency of LEV-resistant isolates from human source increased with increasing drug number of antibiotic resistance, which is largely different from the spectra of both swine and chicken sources, where four- and five-drug-resistant isolates were mainly detected together with a relatively low frequency of three-drug resistance. As for AMP, TC, and SXT, the three-drug-resistant isolates are most dominant in both swine and chicken, but the three- and four-drug-resistant isolates in human are at a similar level. However, all of these spectra lack obvious similarity with that in the river basin to judge the potential source.

Resistance genes among AMP-, TC-, and SXT-resistant *E. coli* isolates from animal and human sources were also examined (Supporting Information Table S3). *TEM* was detected in all 53 AMP-resistant isolates from swine and human source, but no *SHV* and *CARB* were found, which was similar to that in the river basin. However, only 50% of the 32 AMP-resistant isolates from chicken source were

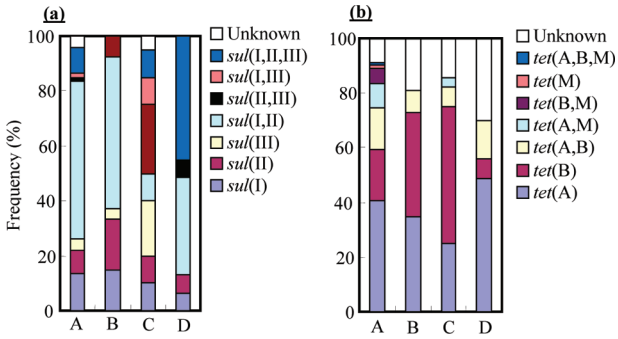

FIGURE 3. Comparison of genotypic resistance pattern among sulfonamide- and tetracycline-resistant *E. coli* isolates from the Wenyu River Basin with animal and human. (a) Resistance genes coding for sulfonamide resistance and (b) resistant genes coding for tetracycline resistance: A, samples from the Wenyu River Basin; B, domestic wastewater samples from the Fangzhuang STP; C, swine stock farm; D, chicken stock farms.

explained by *TEM*, excluding the possibility of chicken stock farm as the potential sources of AMP-resistant isolates in the river basin. Figure 3a shows the profiles of resistance genes coding for sulfonamide resistance in *E. coli* isolates from swine, chicken, and human sources together with the results for the Wenyu River Basin. It can be found that the profile in the river basin is very similar to that in human except for *sul*(I,II,III) and *sul*(I,III), but distinctly different from those in swine and chicken. Isolates with *sul*(I,II) were dominant among the resistant isolates from both the Wenyu River Basin and human with the frequencies of 58% and 56%, respectively, much higher than those from swine (10%) and chicken (35%). And *sul*(III) was highly detected in animal: 65% frequency for swine [20% of *sul*(III), 25% of *sul*(II,III), 10% of *sul*(I,II,III), and 10% of *sul*(I,III)] and 51% for chicken [6% of *sul*(II,III), 45% of *sul*(I,II,III)]. These results indicated that the sulfonamide-resistant *E. coli* isolates that accounted for 45.6% of total antibiotic resistant isolates in the Wenyu River Basin mainly stemmed from human source. It should be noted that, in the river basin, we detected isolates carrying *sul*(I,II,III), which was not observed in human but found in animal, especially in chicken, indicating that a part of resistant isolates in the river basin possibly stemmed from animal sources.

Figure 3b compares the genotypic profiles of tetracycline resistance genes in the river basin with those in human, swine, and chicken sources. While *tet*(A), *tet*(B), and their combination *tet*(A,B) were detected in river basin, human, swine, and chicken, *tet*(M) and its combination with *tet*(A) or/and *tet*(B) were detected with 15% frequency in the river basin, and *tet*(M) combined with *tet*(A) was detected only in swine with a relatively low frequency (3.5%, one isolate of 28 TC-resistant isolates). These results indicate that there should be other *E. coli* discharging sources such as wild animals, as reported in previous papers (*32, 44*), or other antibiotic resistance formation mechanism, such as horizontal transfer in the river basin besides human and animal sources. *tet*(M) was hypothesized to have Gram-positive origin and can be introduced into a significant number of other genera, including Gram-negative and Gram-positive organisms and species lacking cell walls (*37*). Thus, the finding of the presence of *tet*(M) and its combined genes *tet*(A,M), *tet*(B,M), and *tet*(A,B,M) in *E. coli* from the river basin suggested that the horizontal gene transfer of *tet*(M) would occur in the river basin, and further investigation is necessary.

There are several *E. coli* source identification methods based on either the DNA fingerprinting techniques such as ribotyping and REP-PCR or on physiological characteristics such as resistance to various antibiotics, although there is concern that these methods may not be as robust as previously thought (*45*). In this study, the source for sulfonamide-resistant *E. coli* isolates in the natural river basin was supposed mainly to be human by comparing the antibiotic resistance genotypes of *E. coli* isolates with those from the domestic animals and human. And the method based on the antibiotic resistance genotypes would be expected to provide meaningful results for source identification of *E. coli* in the environment (*46*). Further work is going on in our laboratory to characterize the resistance formation mechanism, especially for the occurrence of *tet*(M) in *E. coli*.

Acknowledgments

Financial support from the National Natural Science Foundation of China [20610103], the Ministry of Science and Technology [2006DFA91130], and the National Basic Research Program of China [2007CB407304] is gratefully acknowledged.

Supporting Information Available

Additional information as noted in the text. This information is available free of charge via the Internet at http://pubs.acs.org.

Literature Cited

(1) Schmidt, C. W. Antibiotic resistance in livestock: More at stake than steak. *Environ. Health Persp.* **2002**, *110*, 396–402A.
(2) D'Costa, V. M.; McGram, K. M.; Hughes, D. W.; Wright, G. D. Sampling the antibiotic resistome. *Science* **2006**, *311*, 374–377.
(3) Kim, S.; Jensen, J. N.; Aga, D. S.; Weber, A. S. Teracycline as a selector for resistant bacteria in activated sludge. *Chemosphere* **2007**, *66*, 1643–1651.
(4) Cook, M.; Moloto, E.; Anderson, C. Fluorochrome labeling in Roman period skeletons from Dakhleh Oasis. *Egypt. Am. J. Phys. Anthropol.* **1989**, *38*, 144–1451.
(5) Kim, S. C.; Carlson, K. Temporal and spatial trends in the occurrence of human and veterinary antibiotics in aqueous and river sediment matrices. *Environ. Sci. Technol.* **2007**, *41*, 50–57.
(6) Miao, X. S.; Bishay, F.; Chen, M.; Metcalfe, C. D. Occurrence of antimicrobials in the final effluents of wastewater treatment plants in Canada. *Environ. Sci. Technol.* **2004**, *38*, 3533–3541.
(7) Blackwell, P. A.; Lutzhoft, H. C. H.; Ma, H. P.; Halling-Sorensen, B.; Boxall, A. B. A.; Kay, P. Fast and robust simultaneous determination of three veterinary antibiotics in groundwater and surface water using a tandem solid-phase extraction with high-performance liquid chromatography−UV detection. *J. Chromatogr. A* **2004**, *1045*, 111–117.
(8) Shoemaker, N. B.; Vlamakis, H.; Hayes, K.; Salyers, A. A. Evidence for extensive resistance gene transfer among *Bacteroides* spp. and among *Bacteroides* and other genera in the human colon. *Appl. Environ. Microbiol.* **2001**, *67*, 561–568.
(9) Davison, J. Genetic exchange between bacteria in the environment. *Plasmid* **1999**, *42*, 73–91.
(10) Pruden, A.; Pei, R.; Storteboom, H.; Carlson, K. H. Antibiotic resistance genes as emerging contaminants: Studies in Northern Colorado. *Environ. Sci. Technol.* **2006**, *40*, 7445–7450.
(11) Aminov, R. I. Molecular ecology of tetracycline resistance: Development and validation of primers for detection of tetracycline resistance genes encoding ribosomal protection protein. *Appl. Environ. Microbiol.* **2001**, *67*, 22–32.
(12) Aminov, R. I.; Chee-Sanford, J. C.; Garrigues, N.; Tegeredegne, B.; Krapac, I. J.; White, B. A.; Mackie, R. I. Development, validation and application of PCR of Gram-negative bacteria. *Appl. Environ. Microbiol.* **2002**, *68*, 1786–1793.
(13) Smith, M. S.; Yang, R. K.; Knapp, C. W.; Niu, Y.; Peak, N.; Hanfelt, M. M.; Galland, J. C.; Graham, D. W. Quantification of tetracycline resistance genes in feedlot lagoons by real-time PCR. *Appl. Environ. Microbiol.* **2004**, *70*, 7372–7377.
(14) Pei, R.; Kim, S. C.; Carlson, K. H.; Pruden, A. Effect of river landscape on the sediment concentrations of antibiotics and corresponding antibiotic resistance genes (ARG). *Water Res.* **2006**, *40*, 2427–2435.
(15) Kim, S. R.; Nonaka, L.; Suzuki, S. Occurrence of tetracycline resistance genes *tet*(M) and *tet*(S) in bacteria from marine aquaculture sites. *FEMS Microbiol. Lett.* **2004**, *237*, 147–156.
(16) Henriques, I. S.; Fonseca, F.; Alves, A.; Saavedra, M. J.; Correia, A. Occurrence and diversity of integrons and β-lactamase genes among ampicillin-resistant isolates from estuarine waters. *Res. Microbiol.* **2006**, *157*, 938–947.

(17) Pei, R.; Cha, J.; Carlson, K. H.; Pruden, A. Response of antibiotic resistance genes (ARG) to biological treatment in dairy lagoon water. *Environ. Sci. Technol.* **2007**, *41*, 5108–5113.
(18) Seino, A.; Hasegawa, Y.; Masunaga, S. Distribution of antibiotic-resistant *E. coli* in Kaname, Tsurumi, and Tama Rivers. *J. Jpn. Soc. Water Environ.* **2004**, *27*, 693–698.
(19) Young, H. K. Antimicrobial resistance spread in aquatic environments. *J. Antimibrob. Chemother.* **1993**, *31*, 627–35.
(20) Ronald, J. A. Antibiotic resistance of Gram-negative bacteria in rivers, United States. *Emerg. Infect. Dis.* **2002**, *8*, 713–716.
(21) Reinthaler, F. F.; Posch, J.; Feierl, G.; Wust, G.; Haas, D.; Ruckenbauer, G.; Mascher, F.; Marth, E. Antibiotic resistance of *E. coli* in sewage and sludge. *Water Res.* **2003**, *37*, 1685–1690.
(22) Alekshun, M. N.; Levy, S. B. Molecular mechanisms of antibacterial multi-drug resistance. *Cell* **2007**, *128*, 1037–1050.
(23) Baum, H. V.; Marre, R. Antimicrobial resistance of *Escherichia coli* and therapeutic implications. *Int. J. Med. Microbiol.* **2005**, *295*, 503–511.
(24) Lawrence, J. G.; Ochman, H. Molecular archaeology of the *Escherichia coli* genome. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 9413–9417.
(25) Burdett, V.; Inamine, J.; Rajagopalan, S. Heterogeneity of tetracycline resistance determinants in *Streptococcus. J. Bacteriol.* **1982**, *149*, 995–1004.
(26) Marshall, B.; Tachibana, C.; Levy, S. Frequency of tetracycline resistance determinant classes among lactose-fermenting coliforms. *Antimicrob. Agents Chemother.* **1983**, *24*, 835–840.
(27) Falkow, S. The evolution of pathogenicity in *Escherichia coli, Shigella,* and *Salmonella.* In *Escherichia coli and Salmonella: Cellular and Molecular Biology*, 2nd ed.; Neidhardt, F. C., Ed.; American Society for Microbiology: Washington, DC, 1996; pp 27232769.
(28) *1999' Statistics Information of Chemial and Pharmaceutical Industry.* China Chemical and Industry Association of China: China, 2000.
(29) Parveen, S.; Murphree, R. L.; Edmiston, L.; Kaspar, C. W.; Portier, K. M.; Tanplin, M. L. Association of multiple-antibiotic resistance profiles with point and nonpoint sources of *Escherichia coli* in Apalachicola Bay. *Appl. Environ. Microbiol.* **1997**, *63*, 2606–2612.
(30) Whitlock, J. E.; Jone, D. T.; Harwood, V. J. Identification of the sources of fecal coliforms in an urban watershed using antibiotic resistance analysis. *Water Res.* **2002**, *36*, 4273–4282.
(31) Dombek, P. E.; Johnson, L. K.; Zimmerley, S. T.; Sanowsky, M. J. Use of repetitive DNA sequences and the PCR to differentiate *Escherichia coli* isolates from human and animal sources. *Appl. Environ. Microbiol.* **2000**, *66*, 2572–2577.
(32) Seurinck, S.; Versrtaete, W.; Siciliano, S. D. Use of 16S-23S rRNA intergenic spacer region PCR and repetitive extragenic palindromic PCR analyses of *Escherichia coli* isolates to Identify nonpoint Fecal Sources. *Appl. Environ. Microbiol.* **2003**, *69*, 4942–4950.
(33) CLSI. 2005. Performance Standards for Antimicrobial Susceptibility Testing; Sixteenth Informational Supplement, M100-S16. Clinical and Laboratory Standards Institute (National Committee for Clinical Laboratory Standards), Wayne, PA.
(34) Krumperman, P. H. Multiple antibiotic resistance indexing of *Escherichia coli* to identify high-risk sources of fecal contamination of foods. *Appl. Environ. Microbiol.* **1983**, *46*, 165–175.
(35) Miranda, C. D.; Kehrenberg, C.; Ulep, C.; Schwar, S.; Roberts, M. C. Diversity of tetracycline resistance genes in bacteria from Chilean salmon farms. *Antimicrob. Agents Chemother.* **2003**, *47*, 883–888.
(36) Chang, H.; Hu, J. Y.; Shao, B. Occurrence of natural and synthetic glucocorticoids in sewage treatment plants and receiving river waters. *Environ. Sci. Technol.* **2007**, *41*, 3462–3468.
(37) Chopra, I.; Roberts, M. Tetracycline antibiotics: Mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiol. Mol. Biol. Rev.* **2001**, *65*, 232–260.
(38) Andersen, S. R.; Sandaa, R. A. Distribution of tetracycline resistance determinants among Gram-negative bacteria isolated from polluted and unpolluted marine sediments. *Appl. Environ. Microbiol.* **1994**, *60*, 908–912.
(39) DePaola, A.; Roberts, M. C. Class D and E tetracycline resistance determinants in Gram-negative catfish pond bacteria. *Mol. Cell. Probes* **1995**, *9*, 311–313.
(40) Jones, C. H.; Tuckman, M.; Murphy, E.; Bradford, P. A. Identification and sequence of a *tet*(M) tetracycline resistance determinant homologue in clinical isolates of *Escherichia coli. J. Bacteriol.* **2006**, *188*, 7151–7164.
(41) Bryan, A.; Shapir, N.; Sadowsky, M. J. Frequency and distribution of tetracycline resistance genes in genetically diverse, nonselected, and nonclinical *Escherichia coli* strains isolated from diverse human and animal sources. *Appl. Environ. Microbiol.* **2004**, *70*, 2503–2507.
(42) Perreten, V.; Boerlin, P. A new sulfonamide resistance gene (sul3) in *Escherichia coli* is widespread in the pig population of Switzerland. *Antimicrob. Agents Chemother.* **2003**, *47*, 1169–1172.
(43) Radstrom, P.; Swedberg, G.; Skold, O. Genetic analyses of sulfonamide resistance and its dissemination in Gram-negative bacteria illustrate new aspects of R plasmid evolution. *Antimicrob. Agents Chemother.* **1991**, *35*, 1840–1848.
(44) Bradford, P. A. Extended-spectrum β-lactamases in the 21st century: Characterization, epidemiology, and detection of this important resistance threat. *Clin. Microbiol. Rev.* **2001**, *14*, 933–951.
(45) Somarellia, J. A.; Makarewicz, J. C.; Siab, R.; Simonc, R. Wildlife identified as major source of *Escherichia coli* in agriculturally dominated watersheds by BOXA1R-derived genetic fingerprints. *J. Environ. Manage.* **2007**, *82*, 60–65.
(46) Hamelin, K.; Bruant, G.; El-Shaarawi, A.; Hill, S.; Edge, T. A.; Fairbrother, J.; Harel, J.; Maynard, C.; Masson, L.; Brousseau, R. Occurrence of virulence and antimicrobial resistance genes in *Escherichia coli* isolates from different aquatic ecosystems within the St. Clair River and Detroit River areas. *Appl. Environ. Microbiol.* **2007**, *73*, 477–484.

ES7026746