Occurrence and Distribution of Antibiotic-resistant Bacteria and Transfer of Resistance Genes in Lake Taihu

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The overuse of antibiotics has accelerated antibiotic resistance in the natural environment, especially fresh water, generating a potential risk for public health around the world. In this study, antibiotic resistance in Lake Taihu was investigated and this was the first thorough data obtained through culture-dependent methods. High percentages of resistance to streptomycin and ampicillin among bacterial isolates were detected, followed by tetracycline and chloramphenicol. Especially high levels of ampicillin resistance in the western and northern regions were illustrated. Bacterial identification of the isolates selected for further study indicated the prevalence of some opportunistic pathogens and 62.0% of the 78 isolates exhibited multiple antibiotic resistance. The presence of ESBLs genes was in the following sequence: \( \text{bla}_{\text{TEM}} > \text{bla}_{\text{SHV}} > \text{bla}_{\text{CTX-M}} \) and 38.5% of the isolates had a class I integrase gene. Of all tested strains, 80.8% were able to transfer antibiotic resistance through conjugation. We also concluded that some new families of human-associated ESBLs and AmpC genes can be found in natural environmental isolates. The prevalence of antibiotic resistance and the dissemination of transferable antibiotic resistance in bacterial isolates (especially in opportunistic pathogens) was alarming and clearly indicated the urgency of realizing the health risks of antibiotic resistance to human and animal populations who are dependent on Lake Taihu for water consumption.

**Key words:** antibiotic resistance, horizontal gene transfer, integrons, β-lactamase gene, Lake Taihu

During the past few decades, antibiotics have been widely used in human clinics, animal husbandry and aquaculture, aiming to fight bacterial infections. The unmonitored and continued use of antibiotics has led to significant antibiotic contamination of diverse environments, generates an increasing selective pressure on microorganisms and consequently increases the prevalence of antibiotic resistance (AR) among bacteria (41). AR has been recognized as a worldwide ecological problem (33) and a significant concern to public health (58). Antibiotic-resistant bacteria (ARB) and associated antibiotic resistance genes (ARGs) are gradually becoming considered as environmental contaminants (3). ARB and ARGs no longer strictly occur in so-called point sources with antibiotic contamination, e.g., hospitals, sewage, and farms, but can also be detected in other relatively pristine environments, including rivers, lakes and soils (52). Natural water bodies have been reported to act as significant environmental reservoirs for ARB and ARGs owing to the inherent density and diversity of bacterial loading (5, 6, 43).

The distribution and aggregation of AR is not just a case of inheritance or vertical gene transfer, as it occurs mainly due to horizontal gene transfer (HGT) (33, 39, 52). Horizontal transfer of ARGs is always facilitated by vehicles, including plasmids, transposons, integrons, and bacteriophages. Once ARGs are inserted into these mobile genetic platforms, they can be spread among various species and genera (10, 39, 50). There is clear evidence of the exchange of ARGs between environmental and clinical bacteria (52), and natural reservoirs of ARGs have long been considered as an unlimited source of transferable traits for emerging pathogenic organisms (6).

Beta-lactam antibiotic is one of the most broadly used antibiotic compounds (19). The most common mechanism of bacterial resistance to β-lactam antibiotics is the presence of extended-spectrum β-lactamases (ESBLs) along with plasmid-mediated AmpC β-lactamases, which are both capable of hydrolyzing these agents (8). Nearly all ESBLs originate from the common TEM, SHV, OXA, and CTX-M genes. In particular, these genes can also be horizontally transferred with mobile genetic elements. Moreover, ESBLs are even indicated to be strongly correlated with multidrug resistance in Enterobacteriaceae (40, 46).

China has long been considered to be the largest antibiotics producer and consumer in the world and it has been established that about 210,000 tons of antibiotics are produced annually, according to a 2007 survey (29). Additionally, 30% of drugs sold in Chinese hospitals and medical stores are antibiotics, while the proportion is only about 10% in the developed world (11). China also has the highest level of antibiotic resistance and, even worse, a higher rate of resistance development in comparative analysis with Kuwait and the United States (2). In China, many studies have reported the prevalence and characterization of AR in surface water or ground water and resistance appears to be spread rapidly in many regions (6, 18, 44).

Lake Taihu, a large shallow freshwater lake in China with an area of 2338 km² (47), acts as a main source of drinking, irrigation and fishery water (53). With the extensive growth in agriculture and industry in the past few decades, Lake Taihu has been investigated for eutrophication with high loading of nitrogen and phosphorus, as well as a heavy
density of water bloom (17, 35, 61). However, studies about antibiotic pollution and resistance in the lake are still scarce. Recent studies have demonstrated the wide distribution of antibiotic resistance-associated genes, including tetracycline resistance genes (tet) and the class 1 integron gene (Int I) in the lake (60), and the presence of four ARG concentrations in lake sediments was in the following sequence: \( \text{strB} > \text{qnrB} > \text{strA} > \text{qnrS} \) (57), bringing up important issues for better understanding of the diversity and abundance of antibiotic resistance, and the potential of antibiotic resistance dissemination among the indigenous flora of this aquatic environment. Currently, culture-independent methods, such as metagenomic analysis, are widely applied to detect ARGs in natural water and wastewater treatment plants (WWTP) (20, 49). However, sometimes a discrepancy between genotype and phenotype may be caused by the bias in nucleic acid manipulation, and the isolation of antibiotic-resistant bacteria could help to illustrate pollution with ARB, ARGs expression and transfer potential directly.

In this paper, through a combination of culture-dependent approaches and polymerase chain reaction (PCR) methods, we aim to depict: 1) the antibiotic resistance profiles and the characteristics of AR in isolates recovered from nine disparate areas across Lake Taihu, as well as the correlation of various environment factors with antibiotic resistance; 2) the diversity and distribution of ESBL genes and integrase genes; and 3) the dissemination potential of transferable antibiotic resistance assessed through conjugation mating experiments.

Materials and Methods

Sampling and enumeration of total culturable bacteria and ARB

Nine sites across Lake Taihu were selected for water sampling on October 26, 2011 (Fig. 1): site S1 (31°06′56″ N, 120°00′33″ E), S2 (30°58′14″ N, 120°08′17″ E), S3 (30°58′53″ N, 120°16′21″ E), S4 (31°11′18″ N, 120°10′09″ E), N1 (31°18′14″ N, 119°58′17″ E), N2 (31°16′23″ N, 120°02′55″ E), N3 (31°24′00″ N, 120°20′13″ E), N4 (31°27′52″ N, 120°10′37″ E), N5 (31°31′18″ N, 119°58′17″ E). The Luria-Bertani (LB) and LB supplemented with inhibitory concentrations of antibiotics were used to obtain the total culturable bacteria and ARB, respectively. All plates were incubated at 30°C for 24–48 h and viable cells were enumerated. Antibiotic resistance assessed through conjugation mating experiments. Bacteria isolation and identification of isolates

Isolates were grouped according to sampling sites and anti-microbial resistance patterns. Bacterial identifications were carried out using 16S rRNA gene sequence analysis. Briefly, a boiling method (1) was adopted for extraction of the complete DNA, and the primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTAGCCAGCT-3') (Table 1) were used to amplify the bacterial 16S rRNA gene (16). Then the near-complete 16S rRNA gene was digested with \( HhaI \) (Takara Bio, Otsu, Japan) and grouped through restriction fragment length polymorphism (RFLP) analysis (54). At least one representative isolate from each group was sequenced by Beijing Genomics Institute (BGI). Online similarity searches were conducted with BLAST software at the National Center of Biotechnology Information (NCBI) website.

Antimicrobial sensitivity testing

Antimicrobial susceptibility testing was carried out by the 1% proportion method according to the laboratory’s standard procedure (9). Briefly, isolated colonies of each test strain were first picked from the plates grown overnight, inoculated into 5 mL LB broth and incubated overnight at 30°C, and diluted to a final cell density of about 1×10^8 CFU mL^-1 in phosphate-buffered saline (PBS). Next, 100 μL sample of the suspension was spread on LB agar plates containing inhibitory concentrations of antibiotics just as described above in the Sampling and enumeration of total culturable bacteria and ARB section and controls. The inoculated plates were incubated at 30°C for 24–48 h and viable cells were enumerated. Antibiotic sensitivity/resistance patterns were determined according to the laboratory’s standard procedure (9).

Identification of \( \beta \)-lactamase genes and integrons

Primers used to amplify the major members of the \( \beta \)-lactamase genes, other plasmid-mediated AmpC and specific integrase genes are listed in Table 1. For each isolate, the PCR reaction mixtures (20 μL) contained 0.2 mM of each dNTP, 0.1 μM of forward and reverse primers (BGI, China), 1 U Ex Taq DNA polymerase (Takara Bio), and 40 ng bacterial DNA. All PCR products were visualized by electrophoresis on 1.0% (w/v) agarose gels stained with ethidium bromide.

Conjugation mating experiments

Conjugation was carried out by the membrane filter mating assay (34) using \( E. coli \) Top10 strain (Str+) and \( E. coli \) SM10 strain (Km-) as the recipient strains. Briefly, the donor and recipient bacteria were inoculated in LB broth and grown to the logarithmic phase, mixed at a 1:1 ratio (v/v), spotted on a 0.22 μm filtration membrane and incubated at 37°C for 10 h. Conjugants were selected on LB agar plates supplemented with a combination of 100 μg mL^-1 kanamycin or 100 μg mL^-1 streptomycin and one of the following other antibiotic compounds: ampicillin (Amp) (100 μg mL^-1), gentamicin (Gm) (20 μg mL^-1), tetracycline (Tet) (20 μg mL^-1), or chloramphenicol (Cm) (20 μg mL^-1). Antibiotic sensitivity/resistance patterns of conjunants were determined as described above in the Antimicrobial sensitivity testing section.
Data analysis
Canonical correspondence analysis (CCA) was carried out using Canoco for Windows software (version 4.5) and was used to explore the influence of selected environmental variables on antibiotic resistance rates at different sites. All parameters were log_{10}-transformed to ensure normal distribution and standardized. The significance of the relationship between ARB populations and environmental variables was assessed using Monte Carlo permutation tests.

Nucleotide sequence accession numbers
The 16S rRNA gene nucleotides sequences reported in the current study have been deposited in the GenBank database under accession numbers KC139681–KC139702, and KC161201–KC161203.

Results
Antibiotic resistance profiles of Lake Taihu
Overall, the number of isolates recovered on LB plates from the 9 sites was $3.2 \times 10^3$ CFU mL$^{-1}$ on average and no statistically significant differences were observed among them. These culturable bacteria showed low frequency of resistance to kanamycin and gentamicin, while high levels of resistance to ampicillin (17.0%–61.3%) and streptomycin (43.2–63.1%) were detected, followed by tetracycline and chloramphenicol (Fig. 2). The frequencies of resistance to the three aminoglycosides antibiotics, streptomycin, gentamicin and kanamycin, were relatively constant in all 9 sites, ranging from 43.2% to 63.1%, 1.0% to 6.0% and 6.0% to 21.0%, respectively. By contrast, resistance to ampicillin exhibited obvious spatial heterogeneity. In the present study, over 50.0% of the isolates obtained in N1, N4 and N5 were resistant to ampicillin, whereas only 20.0%–30.0% of isolates from other sites exhibited ampicillin resistance.

CCA analysis was used to correlate the effect of selected water chemical properties on antibiotic resistance patterns, and the results revealed a significant correlation between the AR variation and environmental factors. A total of 57.1% variations could be explained by the selected environmental factors (Fig. 3). N1, N4 and N5 clustered together and were strongly affected by Chl$\alpha$ and TN.

Diversity and antimicrobial susceptibility of the isolated antibiotic-resistant bacteria
Seventy-eight bacterial isolates were randomly selected for further study, were classified into 11 groups based on

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![Fig. 2](image-url). Distribution of antibiotic susceptibility in the isolated strains recovered from nine sampling sites across Lake Taihu.
RFLP analysis of the 16S rRNA gene, and 24 representative isolates were selected for sequencing (Table 2). According to the DNA sequences, representative isolates from each group were affiliated to the same genus, indicating the reliability of RFLP classifications. It was found that the genera of Pseudomonas (35.9%) and Acinetobacter (20.5%) dominated in the 78 isolates, followed by genera of reliability of RFLP classifications. It was found that the most frequent resistance profile among P. aeruginosa isolates was AMP-KM-TET-CHL, indicating the cross resistance of the four antibiotics. Among Acinetobacter isolates, the most frequent resistance pattern was AMP-STR-KM-GEN. In particular, Acinetobacter isolates showed a higher level of resistance to gentamicin.

**Table 2. Distribution of genera among the 78 strains isolated from Lake Taihu and the 24 representative isolates selected for sequencing**

| Genus                        | No. of isolates (%) | No. of representative isolates |
|------------------------------|---------------------|--------------------------------|
| Pseudomonas spp.             | 28 (35.9)           | 7                              |
| Acinetobacter spp.           | 16 (20.5)           | 5                              |
| Agrobacterium spp.           | 7 (9.0)             | 2                              |
| Stenotrophomonas spp.        | 6 (7.7)             | 1                              |
| Bacillus spp.                | 4 (5.1)             | 1                              |
| Brevundimonas spp.           | 4 (5.1)             | 1                              |
| Microbacterium spp.          | 4 (5.1)             | 1                              |
| Comamonas spp.               | 4 (5.1)             | 1                              |
| Cupriavidus spp.             | 2 (2.6)             | 1                              |
| Flavobacterium spp.          | 2 (2.6)             | 1                              |
| Sphingomonas spp.            | 1 (1.3)             | 1                              |

For further study. Multiple antibiotic resistance (MAR), that is, exhibiting resistance to three or more antibiotics, was observed in 62.0% of the isolates. The occurrence of an antibiotic phenotype was mainly related with the taxonomic affiliation of the organisms (Table S1). The genera Brevundimonas and Comamonas showed sensitivity to gentamicin. The most frequent resistance profile among P. aeruginosa isolates was AMP-KM-TET-CHL, indicating the cross resistance of the four antibiotics. Among Acinetobacter isolates, the most frequent resistance pattern was AMP-STR-KM-GEN. In particular, Acinetobacter isolates showed a higher level of resistance to gentamicin.

**Fig. 3.** Canonical correspondence analysis (CCA) compares the abundance of tested resistance bacteria (symbols) and the environmental factors. TP, total phosphorus; TN, total nitrogen; TOC, total organic carbon; Chl a, chlorophyll a. Circles represent different sampling sites.

**Diversity of β-lactamase genes and integron genes**

The β-lactamase genes in the Amp-resistant strains, i.e. bla*SHV*, bla*TEM*, bla*OXA*, and 6 other plasmid-mediated AmpC genes were screened by PCR using specific primers (Table 1). Thirty-one of the 64 Amp-resistant isolates were found to carry at least one of these β-lactamase genes. The most predominant genotype detected was bla*TEM* (22.0%), followed by bla*SHV* (12.5%), bla*CTX* (7.8%) and bla*OXA* (1.6%). As for the plasmid-mediated AmpC β-lactamase genes, only bla*EBC* and bla*OXY* were detected in 4 and 3 isolates, respectively. The distributions of β-lactamase genes were different in each species/genus. Of the 28 Pseudomonas isolates, only bla*TEM* gene and bla*SHV* were detected in 6 and 2 isolates, respectively, although Amp resistance presented as the main phenotype. In the Acinetobacter isolates, 2 bla*TEM*, 2 bla*SHV*, 1 bla*OXA*, and 3 bla*CTX* were detected. For the other minority genera, Agrobacterium and Stenotrophomonas also demonstrated the prevalence of bla*TEM* and bla*SHV*.

Thirty (38.5%) of the 78 isolates were found to carry int I, of which 3 isolates harbored both int I and int II. Int III, representing the class 3 integrons, was not detected in the present study (Table 3). Among all isolates tested, relatively high proportions of integrons were detected in Pseudomonas (16/28, 57.1%), Stenotrophomonas (3/6, 50.0%), Bacillus (2/4, 50.0%), Cupriavidus (1/2, 50.0%) and Comamonas (2/4, 50.0%), followed by 31.3% of Acinetobacter (5/16) and lastly 14.3% (1/7) of Agrobacterium. No integrons were detected in Brevundimonas, Microbacterium, Flavobacterium, and Sphingomonas. In particular, in P. aeruginosa isolates, up to 83.3% (10/12) were observed to carry the int I gene. About 28 (93.3%) of the 30 integron-positive strains were detected to show multiple antibiotic resistance, while the proportion in all the integron-negative strains was only 53.7%. Thus, the presence of integron may enable better prediction of antibiotic resistance.

**Conjugation of antibiotic resistance**

For the use of streptomycin or kanamycin as selective markers, only 63 isolates with only one of the two antibiotic resistances could be subjected to conjugation assays as donor cells. Overall, 40 strains, distributed in 9 genera, were successfully able to transfer antibiotic resistance to *E. coli* SM10 or *E. coli* Top10 through conjugation (Table 4). Among them, only 9 conjugants exhibited all resistance profiles of the donor strains. In terms of the conjugation frequencies of different antimicrobial resistance, the spread of ampicillin (58.3%) and tetracycline resistance (57.1%) was quite high,
### Table 3. Distribution of various ARB genes among different species/genera

| Species/Genus          | No. of Bla Genotypes (%<sup>a</sup>) | No. of Integras (%<sup>b</sup>) |  |
|------------------------|--------------------------------------|---------------------------------|---|
|                        | TEM | SHV | OXA-1-1 | CTXM | ECBM | MOXM | Int I | Int II | Int III |
| P. aeruginosa          | 1 (1.6) | 2 (3.1) | 0 | 0 | 0 | 0 | 10 (12.8) | 1 (1.3) | 0 |
| Pseudomonas            | 6 (9.5) | 2 (3.1) | 0 | 0 | 0 | 0 | 16 (20.5) | 2 (2.6) | 0 |
| Acinetobacter          | 2 (3.1) | 2 (3.1) | 1 (1.6) | 3 (4.7) | 0 | 0 | 5 (6.4) | 0 | 0 |
| Agrobacterium          | 2 (3.1) | 1 (1.6) | 0 | 1 (1.6) | 0 | 0 | 1 (1.3) | 0 | 0 |
| Stenotrophomonas       | 2 (3.1) | 1 (1.6) | 0 | 0 | 1 (16.7) | 1 (1.6) | 3 (3.8) | 0 | 0 |
| Bacillus               | 1 (1.6) | 0 | 0 | 0 | 1 (25.0) | 1 (1.6) | 2 (2.6) | 0 | 0 |
| Brevundimonas          | 0 | 0 | 0 | 1 (1.6) | 0 | 0 | 0 | 0 | 0 |
| Microbacterium         | 0 | 0 | 0 | 0 | 1 (1.6) | 0 | 0 | 0 | 0 |
| Comamonas              | 0 | 0 | 0 | 0 | 2 (50.0) | 0 | 2 (2.6) | 1 (1.3) | 0 |
| Cupriavidus            | 0 | 1 (1.6) | 0 | 0 | 0 | 0 | 1 (1.3) | 0 | 0 |
| Flavobacterium         | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Sphingomonas           | 1 (1.6) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Total                  | 14 (22.0) | 7 (12.5) | 1 (1.6) | 5 (7.9) | 4 (6.3) | 3 (4.8) | 30 (38.5) | 3 (3.9) | 0 |

<sup>a</sup> Percentage of strains containing β-lactamase genes in the 64 Amp-resistant isolates.

<sup>b</sup> Percentage of integron-positive strains among all 78 screening isolates.

### Table 4. Antimicrobial resistance patterns of donor strains and conjugants

| Genus            | Isolates<sup>*</sup> | Donor resistance profile | Resistance patterns of the conjugants |
|------------------|-----------------------|--------------------------|--------------------------------------|
| Pseudomonas      | S1-A3                 | AMP-CHL                  | CHL                                  |
|                  | S1-K3                 | STR-KM                   | KM                                   |
|                  | S1-A5                 | AMP-KM-CHL               | AMP-KM-CHL                           |
|                  | S1-T4                 | AMP-KM-TET-CHL           | AMP                                  |
|                  | S2-A6                 | AMP-TET-CHL              | AMP                                  |
|                  | S2-T8                 | AMP-STR-TET-CHL          | AMP-STR                              |
|                  | S3-P1                 | AMP-STR-TET-CHL          | AMP-STR-CHL                          |
|                  | S4-A15                | AMP-STR-CHL              | AMP-STR-CHL                          |
|                  | S4-T13                | AMP-STR-KM-TET-CHL       | AMP-STR                              |
|                  | N1-A24                | AMP-STR-KM              | AMP-KM                               |
|                  | N2-A27                | AMP-KM-TET-CHL           | AMP-KM-TET-CHL                       |
|                  | N2-A29                | AMP-KM-TET-CHL           | AMP-KM-TET-CHL                       |
|                  | N2-A30                | AMP-TET-CHL              | AMP-TET                              |
|                  | N4-A32                | AMP-STR-TET-CHL          | AMP-CHL                              |
|                  | N5-P7                 | AMP-TET-CHL              | AMP-TET                              |
| Acinetobacter    | S1-A2                 | AMP-STR-CHL              | STR-CHL                              |
|                  | S1-K2                 | STR-KM                   | KM                                   |
|                  | S2-A10                | AMP-STR-KM-GEN           | AMP                                  |
|                  | S3-A11                | AMP-STR                  | AMP                                  |
|                  | S3-A12                | AMP-STR-GEN-CHL          | AMP-CHL                              |
|                  | S4-A17                | AMP-GEN-CHL              | GEN-CHL                              |
|                  | N4-A33                | AMP-TET-CHL              | AMP-TET-CHL                          |
|                  | N4-A37                | AMP-STR-TET-GEN-CHL      | STR-TET                              |
| Agrobacterium    | S1-A4                 | AMP-KM-CHL               | CHL                                  |
|                  | S1-K1                 | KM-CHL                   | KM-CHL                               |
|                  | N4-A35                | AMP-KM-TET-GEN-CHL       | AMP-KM-TET-CHL                       |
|                  | N4-A36                | AMP-STR-CHL              | AMP                                  |
| Comamonas        | S2-A9                 | AMP-STR                  | AMP                                  |
|                  | S3-A13                | AMP                      | AMP                                  |
|                  | S3-A14                | AMP-STR-CHL              | CHL                                  |
|                  | S2-A7                 | AMP                      | AMP                                  |
|                  | S4-A19                | AMP-STR-GEN-CHL          | STR-GEN                              |
| Stenotrophomonas | S3-T12                | AMP-TET                  | AMP                                  |
|                  | N5-P1                 | AMP-TET-CHL              | AMP-TET-CHL                          |
| Capriavidus      | N1-A25                | AMP-STR-KM-GEN           | STR-KM-GEN                           |
|                  | N4-A38                | AMP-CHL                  | AMP                                  |
| Microbacterium   | N2-A28                | AMP-CHL                  | AMP-CHL                              |
|                  | N2-K10                | KM                       | KM                                   |
| Bacillus         | N5-P3                 | AMP-CHL                  | AMP-CHL                              |
|                  | N5-P6                 | AMP-KM-TET-GEN-CHL       | AMP                                  |

<sup>*</sup> Isolates were named according to sampling sites.
ment. However, further study on both phenotypic and molecular scales are required to identify to what extent antibiotic resistance is linked to these anthropogenic-driven selective pressures.

 Seventy-eight strains, dominated by Gammaproteobacteria of Pseudomonas and Acinetobacter, were randomly selected for further analysis. In our other study of culture-independent analysis of the bacterioplankton community through 16S rRNA gene clone library and T-RFLP, Betaproteobacteria, Actinobacteria and Alphaproteobacteria were the three major groups (unpublished data), indicating the significant superiority of Pseudomonas and Acinetobacter in the ARB population. Pseudomonas, colonizers of the aquatic environment, possess pronounced capacity for the acquisition and dissemination of resistance genes, and strains belonging to this genus are in fact frequently resistant to several antimicrobial agents, with susceptibility patterns similar to those of clinical strains (38). It is noticeable that P. aeruginosa and Acinetobacter are both important opportunistic pathogens responsible for a variety of nosocomial infections, and other minority isolates are also phylogenetically related to opportunistic or nosocomial pathogens, including Cupriavidus respiraculi (14), Sphingomonas (45) and Flavobacterium (23). Considering that 62.0% of the 78 isolates were identified as multiple ARBs, the presence of opportunistic pathogens that exhibit resistance to diverse antibiotics may be serious hazards for consumers of lake water (48).

Amp resistance was overwhelming in culturable ARB across Lake Taihu and also among the 78 isolates. Beta-lactamases are ancient enzymes originally encoded in bacterial chromosomes (8). Recently, ESBL-producing bacteria have been rapidly spreading throughout the world (8) and constitute a serious threat to human health (40, 46). Our results showed that the ESBL genes blaTEM and blaSHV had significantly higher frequency among the 78 isolates, followed by blacTXM and blaoXA-1, indicating the prevalence of ESBLs in Lake Taihu isolates. Plasmid-mediated blacTXM, mostly found in E. coli and K. pneumoniae (31, 32), has been reported to dominate the lactam-resistant genes and is frequently detected in natural aquatic systems (4, 27). Our results also revealed that blacTEM can be detected in Gram-positive Bacillus, probably due to gene transfer in the aquatic environment. New families of ESBLs, such as CTXM and OXA-1 types, were found in our study. For example, CTXM4 were also found in our environmental isolates, including Acinetobacter, Agrobacterium and Brevundimonas. In recent years, blacTEM has gradually become the most widespread class (15) and it has mainly been found in strains of Salmonella enterica serovar Typhimurium, E. coli and other species of Enterobacteriaceae (8). The detected AmpC genes in our environmental isolate have been reported to be mobilized to a substantial degree on plasmids only in the last few decades in clinical settings (30). In general, it can be concluded that new families of human-associated ESBLs and AmpC genes can also be found in natural environmental isolates.

Consistent with a previous study (55), the observation of antibiotic- and beta-lactamase class-specific resistance genes distribution in the culturable bacteria in this study also indicates that integron-positive isolates are more likely to be antibiotic resistant and even multidrug resistant. In particular,
the predominantly class 1 integrons were found in all integron-positive ARB. Tacão et al. (51) showed that >50% of the environmental bacterial isolates contained class 1 integron. Zhang et al. (60) also reported that each water sample contained a significant number of the class 1 integron (10^3 copies mL^-1) in Lake Taihu. Integrons are known to play a major role in the introduction and spread of antibiotic resistance genes in environmental bacteria due to their ability to capture and exchange genes via site-specific recombination (55, 25, 36). For further investigation, conjugation, a gene horizontal transfer path, was examined. In the conjugation study, 63.5% of the bacteria were able to transfer an antibiotic-resistant gene to E. coli, highlighting the high frequency of antibiotic resistance-associated gene dissemination in Lake Taihu. These results provide evidence that a wide variety of clinically important antibiotic resistance genes are mobile within aquatic bacterial communities. However, a few conjugants shared the same antibiotic resistance profiles with the donor strains, while the others just acquired part of the antibiotic resistances of donors. This can be interpreted that only ARGs that are situated in mobile genetic platforms, such as plasmids, transposons, integrons, or bacteriophages, can be horizontally transferred across cells (36). All of the examined Int I-positive isolates could transfer antibiotic resistance. Given the high level of Int I detected in Lake Taihu in a previous study (60), it is assumed that uncultured bacteria also constitute reservoirs for antibiotic resistance genes in natural systems. Additionally, aquatic organisms from phytoplankton to large aquatic mammals could act as vectors to further facilitate the transmission of microorganisms and meanwhile represent an important environmental matrix within which HGT can take place (7, 24). Combined together, the frequency of HGT in freshwater, for example Lake Taihu, may be higher than expected. Moreover, considering that E. coli strains acted as recipients in the assay, our results confirmed the flow of resistance genes between native and foreign organisms and indicated the possibility of ARG transfer from environmental reservoirs to clinical pathogenic strains, which should be underlined in the future. In summary, we have reported a comprehensive study of antibiotic resistance in Lake Taihu and the results revealed that a pool of antibiotic-resistant bacteria and associated genes existed in the surface water. Resistance against ampicillin and streptomycin occurred in high frequency, especially in western and northern regions of Lake Taihu. CCA analysis revealed that some environmental factors, including Chl a and TN, might be important to exert positive influences on the variations in antibiotic-resistant populations. New families of human-associated ESBLs and AmpC genes can be found in natural environmental isolates. The distribution of integrons and the horizontal transfer of ARGs across different genera indicated the prevalence of the promiscuous exchange and communication of genes within the large, shallow lake. The prevalence of AR and the dissemination of transferable antibiotic resistance in bacterial isolates (especially pathogenic bacteria) call for further studies to determine the extent to which the dissemination of antibiotic-resistant bacteria occurs and the health risks that this dissemination poses by invading human and animal populations who are dependent on the lake for water consumption.

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References

1. Afghani, B., and H.R. Stutman. 1996. Polymerase chain reaction for diagnosis of M. tuberculosis: Comparison of simple boiling and a conventional method for DNA extraction. Biochem. Mol. Med. 57:14–18.
2. Ali, S.Q., A. Zehra, B.S. Naqvi, S. Shah, and R. Bushra. 2010. Resistance pattern of ciprofloxacin against different pathogens. Oman Med. J. 25:294–298.
3. Allen, H.K., J. Donato, H.H. Wang, K.A. Cloud-Hansen, J. Davies, and J. Handelsman. 2010. Call of the wild: antibiotic resistance genes in natural environments. Nat. Rev. Microbiol. 8:251–259.
4. Alpay-Karaoglu, S., O.B. Ozgunus, E. Sevim, F. Kolayli, A. Sevim, and P. Yesilug. 2007. Investigation of antibiotic resistance profile and TEM-type β-lactamase gene carriage of ampicillin-resistant Escherichia coli strains isolated from drinking water. Ann. Microbiol. 57:281–288.
5. Ash, R.J., B. Mauck, M. Morgan, R. Ash, B. Mauck, and M. Morgan. 2002. Antibiotic resistance of gram-negative bacteria in rivers, United States. Emerg. Infect. Dis. 8:713–716.
6. Baquero, F., J.-L. Martinez, and R. Cantón. 2008. Antibiotics and antibiotic resistance in water environments. Curr. Opin. Biotechnol. 19:260–265.
7. Bogomolni, A.L., R.J. Gast, J.C. Ellis, M.R. Dennett, K.R. Pugliarese, B.J. Lentell, and M.J. Moore. 2008. Victims or vectors: a survey of marine vertebrate zoonoses from coastal waters of the Northwest Atlantic. Dis. Aquat. Organ. 81:13–38.
8. Bradford, P.A. 2001. Extended-spectrum β-lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. Clin. Microbiol. Rev. 14:933–951.
9. Canetti, G., S. Froman, J. Grotes, P. Hauduroy, M. Langerová, H. Mahler, G. Meissner, D. Mitchison, and L. Šula. 1963. Mycobacteria: laboratory methods for testing drug sensitivity and resistance. Bull. World Health Organ. 29:565–578.
10. Carattoli, A. 2009. Resistance plasmid families in Enterobacteriaceae. Antimicrob. Agents Chemother. 53:2227–2238.
11. Chen, B., W. Zheng, Y. Yu, W. Huang, S. Zheng, Y. Zhang, X. Guan, Y. Zhuang, N. Chen, and E. Topp. 2011. Class 1 integrons, selected virulence genes, and antibiotic resistance in Escherichia coli isolates from the Minjiang River, Fujian Province, China. Appl. Environ. Microbiol. 77:148–155.
12. Christensen, F. 1998. Pharmaceuticals in the environment—a human risk? Regul. Toxicol. Pharmacol. 28:212–221.
13. Christian, T., R.J. Schneider, H.A. Färber, D. Skurlatek, M.T. Meyer, and H.E. Goldbach. 2003. Determination of antibiotic residues in manure, soil, and surface waters. Acta Hydroch. Hydrob. 31:36–44.
14. Coenye, T., T. Spilker, R. Reik, P. Vandamme, and J.J. Lipuma. 1963. Mycobacteria: laboratory methods for testing drug sensitivity and resistance. Bull. World Health Organ. 29:565–578.
15. Collet, A.M., A. Novais, A. Carattoli, L. Poirel, J. Pitout, L. Peixe, F. Baquero, R. Cantón, and P. Nordmann. 2008. Dissemination of clonal related Escherichia coli strains expressing extended-spectrum β-lactamase CTX-M-15. Emerg. Infect. Dis. 14:195–200.
16. Dong, X., and C.R. Lovell. 2000. Bacterial primary colonization and early succession on surfaces in marine waters as determined by amplified RNA gene restriction analysis and sequence analysis of 16S rRNA genes. Appl. Environ. Microbiol. 66:467–475.
17. Dong, X., H. Bennion, R. Battarbee, X. Yang, H. Yang, and E. Liu. 2008. Tracking eutrophication in Taihu Lake using the diatom record: potential and problems. J. Paleolimnol. 40:413–429.
Antibiotic resistance among fish surface associated bacterial populations in non-aquaculture freshwater environment. Water Res. 46:6382–6390.

Park, J., J. Lee, J. Oh, Y. Jeong, J. Cho, H. Joo, W. Lee, and W. Lee. 2003. Antibiotic selective pressure for the maintenance of antibiotic resistant genes in coliform bacteria isolated from the aquatic environment. Water Sci. Technol. 47:249–253.

Peng, X., J. Tan, C. Tang, Y. Yu, and Z. Tang. 2008. Multiresidue determination of fluoroquinolone, sulfonamide, trimethoprim, and chloramphenicol antibiotics in urban waters in China. Environ. Toxicol. Chem. 27:73–79.

Perkins, S.D., J. Mayfield, V. Fraser, and L.T. Angelent. 2009. Potentially pathogenic bacteria in shower water and air of a stem cell transplant unit. Appl. Environ. Microbiol. 75:5363–5372.

Philippon, A., G. Arlet, and G.A. Jacoby. 2002. Plasmid-determined AmpC-type \( \beta \)-lactamases. Antimicrob. Agents Chemother. 46:1–11.

Qin, B. 1999. Hydrodynamics of Lake Taihu, China. Ambio. 28:669–673.

Rusin, P.A.J.B. Rose, C.N. Haas, and C.P. Gerba. 1997. Risk assessment of opportunistic bacterial pathogens in drinking water. Rev. Environ. Contam. Toxicol. 152:57–83.

Shi, P., S. Jia, X-X. Zhang, T. Zhang, S. Cheng, and A. Li. 2012. Metagenomic insights into chlorination effects on microbial antibiotic resistance in drinking water. Water Res. 47:111–120.

Stokes, H., and R.M. Hall. 1989. A novel family of potentially mobile DNA elements encoding site-specific gene-integration functions: integrons. Mol. Microbiol. 3:1669–1683.

Tácão, M., A. Correia, and I. Henriques. 2012. Resistance to broad-spectrum antibiotics in aquatic systems: anthropogenic activities modulate the dissemination of blaCTX-M-like genes. Appl. Environ. Microbiol. 78:4134–4140.

Taylor, N.G., D.W. Verner-Jeffreys, and C. Baker-Austin. 2011. Aquatic systems: maintaining, mixing and mobilising antimicrobial resistance? Trends Ecol. Evol. 26:278–284.

Townsend-Small, A., M.J. McCarrthy, J.A. Brandes, L. Yang, L. Zhang, and W.S. Gardner. 2007. Stable isotopic composition of nitrate in Lake Taihu, China, and major inflow rivers. Hydrobiologia 581:135–140.

Unkawa, H., K. Kita-Tsukamoto, and K. Ohwada. 1997. 16S rDNA genotyping using PCR/RFLP (restriction fragment length polymorphism) analysis among the family Vibrionaceae. FEMS Microbiol. Lett. 152:125–132.

Wang, C., H. Dang, and Y. Ding. 2008. Incidence of diverse integrons and \( \beta \)-lactamase genes in environmental Enterobacteriaceae isolates from Jiaozhou Bay, China. World J. Microbiol. Biotechnol. 24:2889–2896.

Wang, P., Y.-L. He, and C.-H. Huang. 2011. Reactions of tetracycline antibiotics with chlorine dioxide and free chlorine. Water Res. 45:1838–1846.

Wang, P.-F., C. Wang, N.-N. Han, and S.-H. Zhang. 2013. Characterization of antibiotic resistance \( E. coli \) and antibiotic resistance genes in aquatic environment of Taihu Lake, China. Appl. Mech. Mater. 290:630–634.

Wise, R., T. Hart, O. Cars, M. Streulens, R. Helmhut, P. Huovinen, and M. Sprenger. 1998. Antibiotic resistance: is a major threat to public health. B. M. J. 317:609–610.

X. Zhang, B. Wu, Y. Zhang, T. Zhang, L. Yang, H.-H. Fang, T. Ford, and S. Cheng. 2009. Class 1 integronase gene and tetracycline resistance genes tetA and tetC in different water environments of Jiangsu Province, China. Environ. Microbiol. 11:364–369.

Xu, Z.-G. 2008. Eutrophic status and causing factors for a large, shallow and subtropical Lake Taihu, China. J. Lake Sci. 20:21–26.