Vol. 120: 115–123, 2016
doi: 10.3354/dao03013

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published July 7

Incidence of antimicrobial-resistance genes and integrons in antibiotic-resistant bacteria isolated from eels and aquaculture ponds

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ABSTRACT: The overuse of antimicrobials in aquaculture has promoted the selection of antimicrobial-resistant bacteria. Here we investigated the abundance of antimicrobial-resistance genes and integrons in 108 strains of antibiotic-resistant bacteria isolated from eels and aquaculture ponds in China. Conventional PCR was implemented to examine common antibiotic-resistance genes, integrons, and their gene cassette arrays. The results showed that the antibiotic-resistance genes bla_{TEM}, *tetC*, *sulI*, *aadA*, *floR*, and *qnrB* were detected at high percentages, as were a number of other resistance genes. Class I integrons were present in 79.63% of the strains, and 10 out of 108 isolates carried class II integrons. Class III integrons were not detected. Three strains carried both class I and class II integrons, and 73.26% of the class I integron-positive isolates contained the *qacEΔ1/sul1* gene. Fourteen types of integron cassette arrays were found among class I integron-positive isolates. A new array, *dfrB4–catB3–*bla_{OXA-10}*–aadA1*, was discovered in this study. The gene cassette array *dfrA12–orfF–aadA2* was the most widely distributed. In summary, 23 different gene cassettes encoding resistance to 8 classes of antibiotics were identified in the class I integrons, and the main cassettes contained genes encoding resistance to aminoglycosides (*aad*) and trimethoprim (*dfr*). All class II integron-positive strains had only a single gene cassette array, viz. *dfrA1–catB2–sat2–aadA1*. High levels of antimicrobial-resistance genes and integrons in eels and auqauculture ponds suggest that the overuse of antimicrobials should be strictly controlled and that the levels of bacterial antimicrobial-resistance genes in aquaculture should be monitored.

KEY WORDS: *Anguilla rostrata* · Antibiotic-resistant bacteria · Antimicrobial-resistance genes · Integrons

INTRODUCTION

In recent years, aquaculture has developed rapidly in China, with more than 60% of the world's aquaculture volume contributed by this country (Cao et al. 2015). Since 1989, China has been the largest producer of global aquaculture in the world. With the continuing expansion of the aquaculture industry, many domestic fisheries are overexploited, leading to aquatic animal diseases becoming increasingly serious. Bacterial infections are an important type of aquatic animal disease, and antimicrobials are currently the main tools to effectively prevent and treat bacterial infections (Grave et al. 1999).

However, the overuse of antimicrobials has promoted the selection of antimicrobial-resistant bacteria. Bacteria have developed a series of mechanisms to resist the effects of antimicrobials, including changing the permeability of the cell membrane or cell wall, active efflux of antimicrobial agents, inactivation or modification of the antimicrobial to destroy its structure, and modification of antibiotic targets.

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Bacterial resistance to antimicrobials is determined by specific resistance genes located on the chromosome or mobile genetic elements, such as plasmids, transposons, and integrons, which facilitate gene transfer between different bacterial species (Korzeniewska & Harnisz 2013, Harnisz et al. 2015). Integrons are site-specific recombination systems, which can identify, capture, integrate, or shear extracellular free gene segments through a self-encoded integrase (Stokes & Hall 1989, Jacquier et al. 2009). Integrons play an important role in the carriage and dissemination of antibiotic-resistance genes (Fluit & Schmitz 1999, Chang et al. 2007). Generally speaking, on the basis of the sequence conservation, integrons contain 3 parts: the 5′-conserved segment (5′-CS), the 3′-conserved segment (3′-CS), and the variable region between 5′-CS and 3′-CS that may or may not contain 1 or more gene cassettes. Usually gene cassettes contain only a single gene and a recombination site (*attC*). More than 130 different gene cassettes carrying known antibiotic-resistance genes have been identified in integrons (Partridge et al. 2009). Integrons consist of 3 essential elements: the integrase gene (*intI*) encoding a site-specific recombinase, the recombination site (*attI*), and a promoter (Pc). Three elements are present in the 5′-CS of integrons (Rowe-Magnus & Mazel 1999, Ochman et al. 2000, Zhu et al. 2014). On the basis of integrase amino acid sequence similarity, integrons are divided into 6 classes. Most studies have focused on the class I, II, and III integrons that are commonly associated with antibiotic resistance, with the class I integrons being the most prevalent class (Labbate et al. 2009, Uyaguari et al. 2013, Sarria-Guzmán et al. 2014). The 3′-CS of typical class I integrons consists of the genes *qacEΔ1*, *sul1*, and *orf5* (see Fig. S1 in the Supplement, available at www.int-res.com/articles/suppl/d120p115_supp.pdf). In recent years, many studies of integrons have been concerned with the carriage of antibiotic-resistance markers by nosocomial or animal pathogens, while there is relatively limited research on their presence in aquatic animals (Dolejska et al. 2007, Zhu et al. 2011, Spindler et al. 2012, Nguyen et al. 2014).

Farmed eels are an important agricultural product exported by China. The recent development of this industry has led to a significant growth in the global demand for edible eels. The increase of aquaculture density frequently results in diseases of these animals, which are a major source of economic loss in the aquaculture industry. Above all, bacterial diseases are the most frequent and major cause of mass death in fish.

The aim of this study was to investigate (1) the prevalence of selected antimicrobial-resistance genes in eels and antibiotic-resistant bacteria isolated from aquaculture ponds in China and (2) the occurrence and distribution of class I, II, and III integrons and their gene cassette arrays in these isolates.

MATERIALS AND METHODS

Sample collection, isolation, and identification of antibiotic-resistant bacteria, and antibiotic susceptibility testing

Wu et al. (2015) isolated 108 strains from eels *Anguilla rostrata* and aquaculture ponds in Fujian province, China, and screened these on Luria-Bertani nutrient agar medium containing 1 of 5 antimicrobials. The phylogeny of these isolates was determined by 16S rDNA sequencing, and their antibiotic resistance was determined by the Kirby-Bauer disc diffusion method, demonstrating that these 108 strains consist of many highly antibiotic-resistant isolates.

DNA extraction

Total DNA of the 108 resistant strains was extracted using the TIANamp Bacteria DNA kit (Tiangen Biotech). Plasmid DNA from the 108 strains was extracted with the E.Z.N.A.^{TM} Plasmid Mini Kit (Omega). Template DNA was stored at –20°C.

Detection of antimicrobial-resistance genes

In total, 108 resistant isolates were analyzed for the presence of 29 different antimicrobial-resistance genes using polymerase chain reaction (PCR). The primers used in this study have been reported in previous studies and are shown in Table S1 in the Supplement. Each 25 µl PCR mixture contained 2.5 µl 10× *Taq* DNA buffer (with Mg^{2+}), 0.5 µl dNTPs (10 mM each), 0.5 µl forward primer and reverse primer (10 µM each), 0.125 µl *Taq* polymerase (5 U μl^{-1}), 0.5–1 µg total DNA, and double-distilled water (ddH_2O).

The conditions used for PCR amplification were as follows: pre-denaturation at 95°C for 5 min; 30 cycles of 94°C for 40 s, annealing for 40 s at 54–62°C (Table S1), and extension at 72°C for 50 s; followed by final extension at 72°C for 5 min. Amplification products were analyzed on 0.8–1.2% agarose gels.

The antimicrobial-resistance genes that were detected were sequenced at GenScript Biotechnology (Nanjing, China) to verify the gene-specific primers. Sequences were compared with the GenBank database using NCBI BLAST (www.ncbi.nlm.nih.gov/blast/).

Detection of three integrons and gene cassette array characterization

Primers used for the detection of integrons and their variable regions are shown in Table S2 in the Supplement. Total DNA and plasmid DNA from all isolates were screened for class I, II, and III integrons using the primers *intI* I F/R, *intI* II F/R, and *intI* III F/R, respectively. The variable regions of the integrons were amplified with the primers hep58/59 (for the class I integrons) and hep74/51 (for the class II integrons). The gene cassette arrays were characterized for the corresponding positive isolates in the previous step. Each 50 µl PCR mixture included 5 µl 10× *Taq* DNA buffer (with Mg^{2+}), 1 µl dNTPs (10 mM each), 1 µl of forward primer and reverse primer (10 µM each), 0.25 µl *Taq* polymerase (5 U μl^{-1}), 0.5–1 µg total DNA, and ddH_2O.

The conditions used for PCR amplification were as follows: pre-denaturation at 95°C for 10 min; 30 cycles of 94°C for 30 s, annealing for 30 s at 55°C, and extension at 72°C for 1 min (3 min for the variable regions); followed by the final extension at 72°C for 10 min. Amplification products were then analyzed on 0.8–1.2% agarose gels.

Amplification products of the variable regions were purified using the E.Z.N.A.™ Gel Extraction Kit (Omega), cloned using the pMD™19-T vector Cloning Kit (TaKaRa), and sequenced. Using the primers hep58/59 or hep74/51, we sequenced the variable regions of approximately 1500 bp. When sequences were longer than 1500 bp, primer walking was used until the full sequence was obtained. All sequencing was performed by GenScript Biotechnology. More than 130 different gene cassettes carrying known antibiotic-resistance genes have been identified in integrons, and almost all antibiotic-resistance genes have been identified. Therefore, the majority of integron gene cassette arrays could be identified directly with NCBI BLAST. While a few new arrays could be identified with low sequence identity, we cut them into several segments to identify every segment of the gene cassettes, and then assembled gene cassettes according to the original sequence.

RESULTS

Diversity and antimicrobial resistance of the drug-resistant bacteria

We successfully isolated 108 resistant strains and classified them into 20 genera, with high detection rates of bacteria in the genera *Aeromonas*, *Citrobacter*, and *Acinetobacter*. The percentage of bacteria with resistance to 3 or more antibiotics was 93.5%. The frequency of resistance to amoxicillin (90.7%) was high, as was resistance to tetracycline, rifampicin, sulfonamides, and amphenicols (60–80%; Fig. S2 in the Supplement).

Abundance of antimicrobial-resistance genes

Eight main classes of antimicrobial-resistance genes were detected from total DNA of the 108 antibiotic-resistant isolates in this study (Fig. 1). The aminoglycoside-resistance genes mainly included those that encode aminoglycoside acetyltransferases (*aac*), aminoglycoside adenyltransferases (*aad*), and aminoglycoside phosphotransferases (*aph*). Among all genes of this class that were investigated, the *aadA* gene was the most prevalent (74.07%), whilst *aphA1* was found in 48 strains, *strA* (or *strB*) in 24 strains, and *aac*(*3*)-*IV* in only 7 strains. The *aac*(*3*)-*I* gene was not detected in any of the isolates.

The bla_{TEM} gene was found in all tested isolates. Other β-lactamase-resistance genes were present at lower rates. The *DHA* gene was found in 39 strains. *EBC*, bla_{SHV}, and bla_{OXA} genes were found in 17, 16, and 5 strains, respectively, while the *MOX* gene was not detected in any of the 108 strains.

Among the resistance genes for antifolates, *sulI* had the highest detection rate (96.30%), followed by the dihydrofolate reductase genes *dfrA12* > *dfrA5* > *dfrA1* > *dfrA7*.

The tetracycline-resistance genes *tetA*, *tetB*, *tetC*, *tetD*, and *tetE* were detected in all 108 strains in this study. Our results showed that only 13 strains carried *tetE* and 17 strains were positive for *tetB*, while *tetA* and *tetD* were found in 27 and 40 of the 108 strains, respectively. The *tetC* gene was found in almost all of the isolates (104 of 108 strains).

The quinolone-resistance genes *qnrA*, *qnrB*, and *qnrS* were relatively common in previous studies. Of the strains having quinolone resistance in our study, 5 were positive for *qnrS* (4.63% of the total). The *qnrB* gene was observed in over half of the isolates

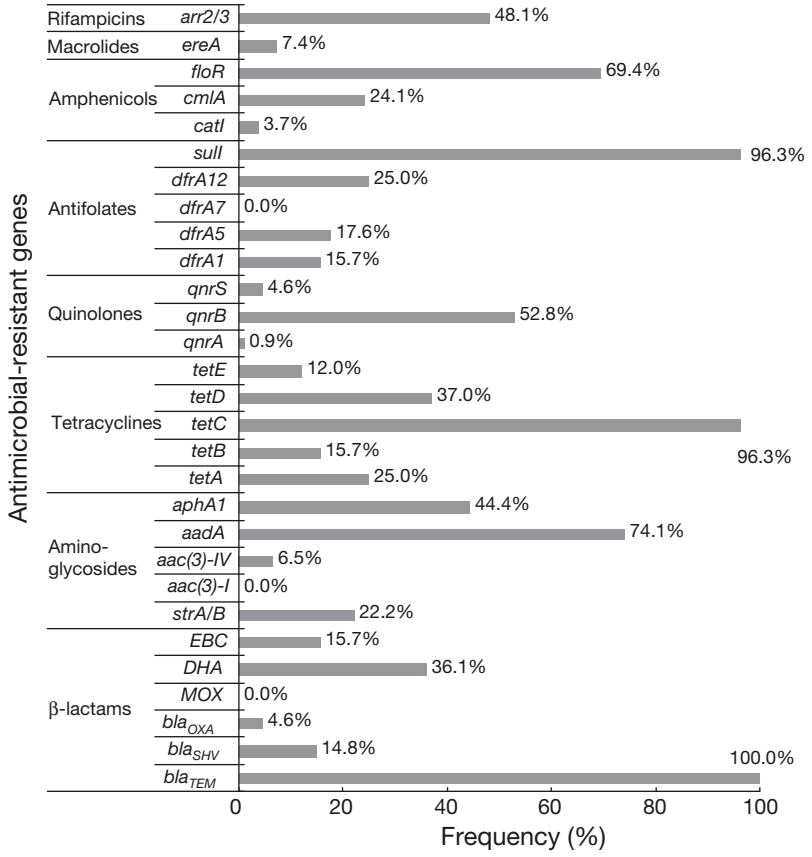

Fig. 1. Distribution of 29 different antimicrobial-resistance genes in 108 strains of antibiotic-resistant bacteria isolated from eels *Anguilla rostrata* and aquaculture ponds

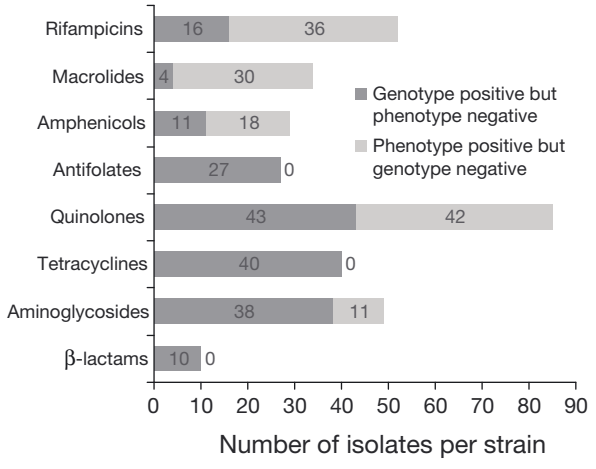

Fig. 2. Inconsistency of phenotype and genotype associated with antibiotic resistance in bacterial strains isolated from eels *Anguilla rostrata* and aquaculture ponds

in this study (52.78%), while only 1 strain possessed the *qnrA* gene.

The amphenicol-resistance genes *floR*, *cmlA*, and *catI* encode resistance to florfenicol and chloramphenicol. The *floR* gene was present in a large proportion of the isolates (75 out of 108, 69.44%), while *cmlA* was detected by PCR in 26 strains, and only 4 strains carried the *catI* gene.

The *arr2/3* gene encodes a rifampicin ADP-ribosylating transferase, conferring resistance to rifampicin. Almost half of the strains in the study were positive for *arr2/3*. The *ereA* gene, which confers resistance to erythromycin, was only present in 8 strains.

The phenotype and genotype of the antibiotic-resistance markers were analyzed in each of the isolates (Fig. 2). The results showed that isolates with inconsistent antibiotic-resistance phenotype and genotype mainly showed resistance to rifampicins, quinolones, and aminoglycosides.

Distribution of integrons and characterization of gene cassette arrays

PCR reactions using total DNA of the 108 strains as a template identified class I integrons in 86 of the antibiotic-resistant strains (79.63%), while only 10 isolates (9.26%) carried class II integrons. Class III integrons were not detected. Three strains carried both class I and class II integrons. The variable regions from 41 of the class I integron-positive isolates were successfully amplified and sequenced (Table S3 in the Supplement). Of the class I integron-positive isolates, 33 had 'empty' class I integrons, i.e. no gene cassette was present between the 5′-CS and 3′-CS. Additionally, gene cassette arrays could not be amplified from 12 strains with class I integrons. Meanwhile, 73.26% (63/86) of the class I integron-positive isolates contained the *qacEΔ1/sul1* gene as part of the 3′-CS of the class I integrons. The variable regions could be amplified from all 10 isolates containing class II integrons. By testing for the presence of the integrons (both class I and class II) in plasmid DNA from each strain, we found that all integrons were located on plasmids in these strains.

Fourteen types of gene cassette arrays were found among class I integron-positive isolates (Fig. 3). Furthermore, a new array, viz. *dfrB4–catB3–bla*$_{OXA-10}$*–aadA1*, was discovered in this study. The gene cas-

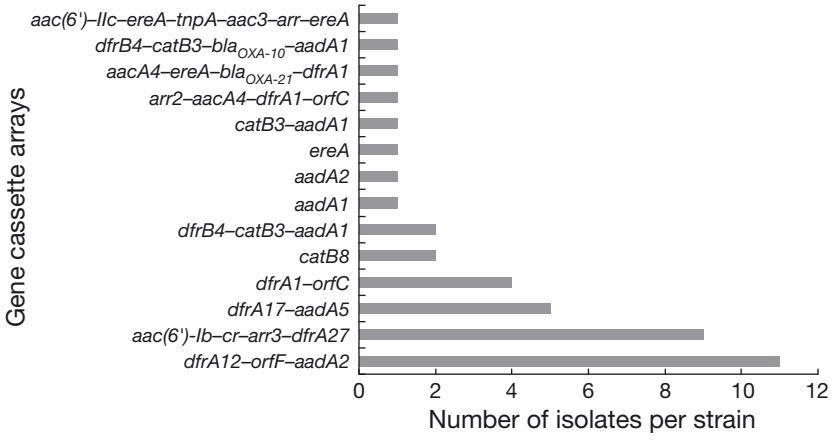

Fig. 3. Abundance of different class I integron gene cassette arrays in antibiotic-resistant bacteria isolated from eels *Anguilla rostrata* and aquaculture ponds

sette array *dfrA12–orfF–aadA2* was the most widely distributed, being present in 11 of the class I integron-positive isolates. Those isolates included *Aeromonas* (2), *Citrobacter* (4), and *Shewanella* (5). We found that 71.43% (5/7) of the *Shewanella* isolates contained the *dfrA12–orfF–aadA2* array. The *aac(6′)-Ib–cr–arr3–dfrA27* array was carried by 9 strains. Other gene cassettes included *dfrA17–aadA5*, *dfrA1–orfC*, *catB8*, *dfrB4–catB3–aadA1*, *arr2–aacA4–dfrA1–orfC*, *aacA4–ereA–bla$_{OXA-21}$–dfrA1*, *aadA1*, *aadA2*, *dfrB4–catB3–bla$_{OXA-10}$–aadA1*, *aac(6′)-IIc–ereA–tnpA–aac3–arr–ereA*, *catB3–aadA1*, and *ereA*. Among these gene cassette arrays, 82.93% contained aminoglycoside-resistance genes, and trimethoprim-resistance genes were also found in 82.93% of the arrays, with approximately 75% of the arrays containing both aminoglycoside- and trimethoprim-resistance genes in the same array. In summary, 23 different gene cassettes encoding resistance to 6 classes of antibiotics were identified in the class I integrons, including the following: genes encoding for resistance to aminoglycosides (*aac(6′)-IIc*, *aac3*, *aacA4*, *aadA1*, *aadA2*, *aadA5*), trimethoprim (*dfrA1*, *dfrA12*, *dfrA17*, *dfrA27*, *dfrB4*), β-lactams (*bla$_{OXA-10}$*, *bla$_{OXA-21}$*), rifampicins (*arr*, *arr2*, *arr3*), amphenicols (*catB3*, *catB8*), quinolones (*aac(6′)-Ib–cr*), and macrolides (*ereA*). We detected 2 open reading frames (*orfC* and *orfF*) of unknown function as well as 1 transposase gene (*tnpA*).

In this study, class II integrons were identified by PCR in all 5 strains of *Proteus*. Other species that contained class II integrons were *Aeromonas* (2 strains), *Staphylococcus* (1 strain), *Citrobacter* (1 strain), and *Shewanella* (1 strain). All 10 of these strains had the same gene cassette array, viz. *dfrA1–catB2–sat2–aadA1.* The trimethoprim (*dfrA1*), amphenicol (*catB2*), and aminoglycoside (*aadA1*)-resistance genes were also found as part of the gene cassettes in class II integrons. In addition, the *sat2* gene cassette encodes a streptothricin acetyltransferase, which gives rise to streptothricin resistance.

The frequency of antibiotic resistance was higher in the integron-positive strains than in the integron-negative strains (Fig. 4). Several integron-positive strains were resistant to streptomycin, norfloxacin, and ofloxacin.

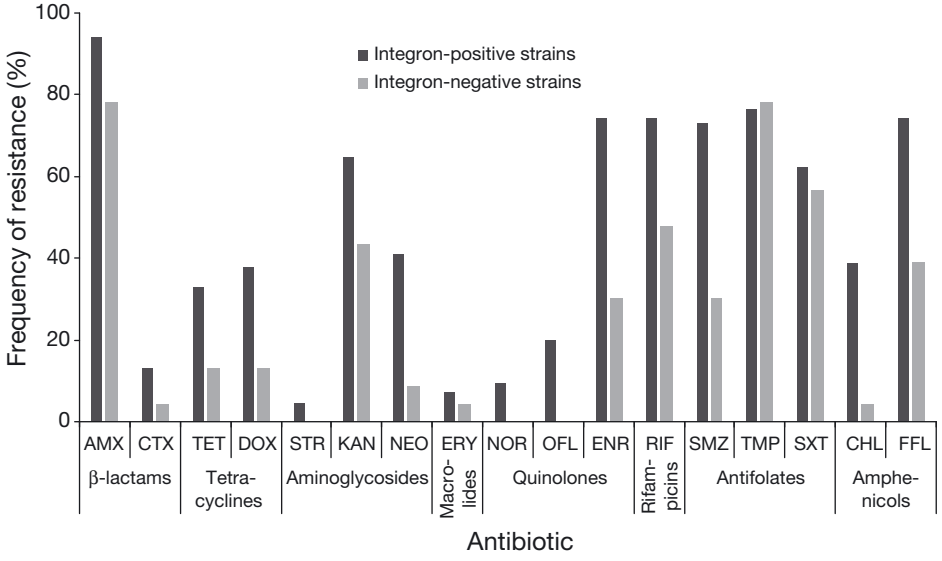

Fig. 4. Frequency of resistance to 17 antimicrobials among the integron-positive and integron-negative bacterial strains isolated from eels *Anguilla rostrata* and aquaculture ponds. AMX: amoxicillin; CTX: cefotaxime; TET: tetracycline; DOX: doxycycline; STR: streptomycin; KAN: kanamycin; NEO: neomycin; ERY: erythromycin; NOR: norfloxacin; OFL: ofloxacin; ENR: enrofloxacin; RIF: rifampicin; SMZ: sulfamethoxazole; TMP: trimethoprim; SXT: sulfamethoxazole/trimethoprim; CHL: chloramphenicol; FFL: florfenicol

DISCUSSION

In this study, we have reported the distribution of different classes of antibiotic-resistance genes in antibiotic-resistant bacteria isolated from eels and farming water in China. We detected specific resistance genes based on primers designed during previous studies (Table S1). Molecular characterization showed great diversity in the antimicrobial-resistance genes (Fig. 1). Many previous studies have focused on 1 or 2 bacterial genera and/or strains to identify antimicrobial-resistance genes that correspond to antibiotic-resistant bacteria. Dolejska et al. (2007) carried out an assessment of the occurrence of antimicrobial-resistance genes in *Escherichia coli* isolated from black-headed gulls. The bla_{TEM} gene was detected in 29 of 30 beta-lactam resistant isolates, while bla_{SHV} and bla_{OXA} genes were not found. Other resistance genes, such as *cat*, *strA*, *aadA*, *tetA*, *tetB*, *sul1*, and *sul2* were also detected in the resistant strains (Dolejska et al. 2007). These results are similar to our study, where bla_{TEM} was detected in all 108 antibiotic-resistant isolates, while few bla_{SHV}, bla_{OXA}, *DHA*, and *EBC* genes were found, and the *MOX* gene was not detected. The bla_{TEM} gene is the most prevalent gene detected in many antibiotic-resistant isolates from humans and animals (Bakour et al. 2013, Ahmed et al. 2014). Tetracycline-resistance genes encoding active efflux pumps occur frequently among bacteria isolated from different environments. The most predominant gene is *tetC*, which was detected in all strains of *Aeromonas* spp. from rainbow trout farms in Australia (Ndi & Barton 2011). A study on *E. coli* from catfish showed that *tetB* was present in 75% of all isolates tested, which was the highest percentage of the *tet* genes tested (Nawaz et al. 2009). Nawaz et al. (2006) also studied *A. veronii* from catfish; among these isolates, *tetE* was the most predominant antibiotic-resistance gene, occurring in 73/81 (90.0%) strains. Furthermore, in our study, the largest proportion of isolates tested was positive for *tetC*, followed by *tetD* and *tetA*. Therefore, it appears that it is not difficult to find different tetracycline-resistance genes in various ecosystems, and the distribution of these genes differs with environment.

Resistance genes to antifolates include the genes encoding dihydrofolate reductase (*dfrA1*, *dfrA5*, *dfrA7*, *dfrA12*, *dfrB4*) and dihydropteroate synthase (*sulI*). The overall frequency of genes for sulfonamide and trimethoprim resistance in this study was *sulI* > *dfrA12* > *dfrA5* > *dfrA1* > *dfrA7*. Several studies have focused on *dfr* and *sul* genes together. Hu et al. (2011) showed that of 102 *Stenotrophomonas maltophilia* isolates analyzed, 50.99% contained the *sulI* gene and 15.69% carried *dfr* genes.

Among the aminoglycoside-resistance genes, the *aadA* gene had the highest prevalence (74.07%) and the *aphA1* gene was present in 44.44% of the isolates. This observation was consistent with Glenn et al. (2011), where the most prevalent aminoglycoside-resistance genes among all isolates were *aadA*, *aac*(*3*), *strA*, *strB*, and *aphA1*. Among the other classes of antimicrobial-resistance genes (quinolones, amphenicols, rifampicins, and macrolides), the most prevalent genes in the current study were *qnrB*, *floR*, *arr2/3*, and *ereA*, respectively. The distribution of antimicrobial-resistance genes depended on the source of the isolates (animal, water, sediment, etc.), the detection method, and the genes that were detected. Zhang et al. (2013) studied resistance genes of aquaculture systems in southern China, and their results revealed that the most prevalent resistance genes from 3 different gene classes were *tetA*, *sul2*, and bla_{TEM}. Other resistance genes were detected to different degrees in these resistant isolates, including *tetW*, *tetB*, *sul3*, *sul1*, bla_{OXA}, and bla_{CTX}; however, *tetE*, *tetX*, and bla_{SHV} were not detected in any isolates (Zhang et al. 2013). In the present study, we found 1 strain of the 108 antibiotic-resistant isolates that carried 18 resistance genes, and all strains in this study contained at least 2 antibiotic-resistance genes.

The occurrence of the 3 integrons in different ecosystems has been reported in many studies, particularly on class I integrons and their gene cassette arrays. Ndi & Barton (2011) detected class I integrons in 31% of *Aeromonas* spp. isolated from trout farms in Australia, while they failed to detect any class II or class III integrons. In addition, they also found that class I integrons were present in 23% (30/129) of *Pseudomonas* spp., and again, class II and class III integrons were not found in any of the strains (Ndi & Barton 2012). Similarly, class I integrons have been reported from approximately 33% of *Pseudomonas* spp. and 28% of *Aeromonas* spp. isolates from catfish, and the main gene cassette arrays were *dfrA12–orfF–aadA2*, *aadA1*, *catB8–aadA1*, *dfrA1–orfX*, and *dfrA21* (Nguyen et al. 2014). All isolates were negative for class II integrons. Due to the structure of the *intI*II gene, which contains an internal stop codon that renders *intI*II inactive, the occurrence of class II integrons is much lower than class I integrons. The most common gene cassette array found among class II integron-positive isolates is *dfrA1–sat2–aadA1* (Zhu et al. 2011). Class III integrons have only

been detected in rare instances. In the present study, integrons were more widespread, with class I integrons being found in 79.63% of strains and class II integrons found in 9.26% of the strains. Furthermore, 47.67% of the class I integron-positive strains harbored gene cassette arrays. The most frequently found array was *dfrA12–orfF–aadA2,* as has also been seen in previous studies. The second most commonly observed array was *aac*(*6′*)*-Ib–cr–arr–3–dfrA27*, which was relatively uncommon in previous studies. Reasons for this might be that the sequence of the array was difficult to amplify and does not occur widely in most typical ecosystems. The *qacEΔ1/sul1* failed to amplify from several class I integron-positive isolates, which may be because they lack the non-essential structure 3′-CS. Nevertheless, the gene cassette arrays of the class II integrons all contained the array *dfrA1–catB2–sat2–aadA1*, which has only been reported in a few studies. However, the gene cassettes *dfrA1*, *catB2*, *sat2*, and *aadA1* were frequently detected in class II integrons. Three strains (1 *Shewanella* and 2 *Aeromonas* strains) carried class I as well as class II integrons, and the class I integron gene cassette arrays of the 3 strains were all *dfrA12–orfF–aadA2*. Thus, we found that the distribution of integrons was connected with different gene cassettes and/or bacterial genera.

Many studies have investigated integrons in different bacterial genera that have been isolated from humans, such as *Klebsiella* and *Acinetobacter*. Research on bacterial integrons isolated from aquatic animals is comparatively small in number, and most of these studies focused on the pathogens *Aeromonas* and *Pseudomonas*. Ndi & Barton (2011, 2012) studied resistance genes and integrons in *Aeromonas* and *Pseudomonas* isolated from rainbow trout farms, and they found that the frequency of resistance genes and integrons was higher in *Aeromonas* than in *Pseudomonas*. In our study, we analyzed the high-frequency strains from the genera *Aeromonas*, *Citrobacter,* and *Acinetobacter*. We found that 89.5% of *Citrobacter* isolates contained integrons, and 58.8% integron-positive *Citrobacter* strains carried gene cassettes. The gene cassette arrays were the following 3 arrays: *dfrA12–orfF–aadA2*, *aac*(*6′*)*-Ib–cr–arr3–dfrA27*, and *dfrA17–aadA5*. *Citrobacter* isolates had high multiple antibiotic-resistance indices, whereas those of *Acinetobacter* isolates were relatively lower, and the tendency towards antibiotic-resistance indices of *Citrobacter* and *Acinetobacter* was more obvious among the integron-positive isolates carrying gene cassettes. Of the *Aeromonas* strains, 65.4% carried integrons, and 70.6% of these were detected as gene cassettes, which included 6 types of arrays. Only 1 strain (7.7%) carried a gene cassette array in the 72.2% of *Acinetobacter* strains that were positive for integrons. Overall, the frequency of *Acinetobacter* strains was far below average. These data indicated that integrons were closely associated with antibiotic resistance across multiple bacterial genera.

Several earlier studies found that some antimicrobial-resistance genes were closely associated with integrons. Ndi & Barton (2012) suggested that the presence of the *aadA* gene was commonly associated with integrons. The *aadA1* gene was the most widely carried gene in strains isolated from 7 countries (L'Abée-Lund & Sørum 2001). The association of integrons with various resistance genes, such as *sul* and *dfr* genes, has been well documented (Petersen et al. 2000, Ndi & Barton 2011, Shah et al. 2014). The gene cassettes found in the present study demonstrate that the dominant cassettes belong to aminoglycoside (*aac* and *aad*) and trimethoprim (*dfr*) resistance genes, similar to other studies. The *qacEΔ1/sul1* genes were found in 73.26% of class I integron-positive isolates, and were also found in 18.18% of class I integron-negative isolates. From the data generated in this study, it is clear that there is a strong relationship between antimicrobial-resistance genes and integrons. The genes identified with high prevalence in this study were bla_{TEM}, *tetC*, *sulI*, *aadA*, *floR*, and *qnrB*, among others. Among these genes, *sulI* and *aadA* were found in a high percentage of class I integrons. However, bla_{TEM}, *tetC*, *floR*, and *qnrB* were not often found within integrons. This may be related to specific conditions associated with horizontal gene transfer and the characteristics of different genes and integrons.

Several antimicrobials have been banned for use in aquatic animals, but antibiotic resistance to these compounds still exists in several isolates. In earlier research, we identified the antimicrobial content in the same aquaculture ponds from which we took samples for the present study. Trace amounts of chloramphenicol and florfenicol were detected, and sulfamethoxazole, trimethoprim, and erythromycin were detected in the water samples. The index of multiple-drug resistance was high. The results showed that high concentrations of antimicrobials might lead to large numbers of antibiotic-resistant isolates. In contrast, the antibiotic-resistance level of the isolates did not always correlate with the low concentration of antimicrobials in farming water. Horizontal transfer of antibiotic-resistance genes might place emphasis on

molecular mechanisms to explain the frequency of antibiotic-resistance genes. Thus, research on the genes related to antimicrobial resistance is more specific, as we can control the antibiotic-resistance of isolates. In addition, isolates with inconsistent antibiotic-resistance phenotype and genotype were mainly related to the rifampicins, quinolones, and aminoglycosides. The strains resistant to quinolones were the most obvious in terms of the inconsistent antibiotic-resistance phenotype and genotype. The discordance for quinolones might be due to the fact that few *qnr* genes were tested that usually give low levels of resistance unless they are accompanied by other chromosomally encoded resistance mechanisms. The inconsistencies might be connected with a lack of expression, repression of the drug-resistance genes, or genes which were not detected in our study. Bacteria may also have entered dormancy to escape the antimicrobial agents.

Antibiotic-resistance genes and integrons are important contributors to the development of antibiotic resistance. Our study revealed that the frequency of antibiotic resistance was higher in the integron-positive strains than in the integron-negative strains (Fig. 3). Only several integron-positive strains were resistant to streptomycin, norfloxacin, and ofloxacin. Streptomycin is an aminoglycoside drug to which resistance is conferred by aminoglycoside-resistance genes, e.g. *aac* and *aad* genes. Thus, streptomycin resistance is usually associated with aminoglycoside-resistance genes and integrons. Also, we found strains that were resistant to antifolate agents that were not associated with integrons. The genes may confer resistance to antifolates through other mechanisms. The data show that the higher the frequency of antibiotic resistance of strains, the more resistance genes were detected. While some strains were found to be resistant to one class of antibiotics, we failed to detect a corresponding resistance gene. For example, we found that 31.48% of strains were resistant to erythromycin, while only 7.41% strains contained the *ere* resistance gene. This means that the erythromycin-resistant strains contain other genes encoding resistance to erythromycin. In addition, some strains contained resistance genes, but did not display the corresponding drug resistance. The bla_{TEM} gene was detected in all strains, but not all strains were resistant to β-lactamase drugs (amoxicillin and cefotaxime). This suggests that in some strains, the genes were not expressed and did not play a role in resistance.

CONCLUSION

From this study, we conclude that eels and farming water in China contain a large number of antibiotic-resistant bacteria that carry a wide range of antimicrobial-resistance genes and integrons. These results show that these bacteria are potential threats to the eel farming industry, other animals, and human health. Thus, we should constantly monitor the resistance genes of bacteria in aquaculture.

Acknowledgements. This study was financially supported by the Special Fund for Agro-scientific Research in the Public Interest (no. 201203085), National Natural Science Foundation of China (nos. 31202030 and 31272669), and Natural Science Foundation of Fujian Province (no. 2014J01131).

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Editorial responsibility: Alicia Toranzo, Santiago de Compostela, Spain

Submitted: November 20, 2015; Accepted: May 2, 2016
Proofs received from author(s): June 13, 2016