Antimicrobial Resistance Pattern and Characteristics of Integrons in *Escherichia Coli* Strains Isolated from Aquatic Retail Products in Zhejiang Province, China

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Abstract: The aquatic retail products are among the potential reservoirs of antimicrobial resistant microorganisms. The main objective of this study was to analyze the antimicrobial susceptibility patterns and integrons and their associated Gene Cassettes (GCs) of *Escherichia coli* isolates from aquatic retail products. *E. coli* isolates were obtained from 450 aquatic retail products samples at different markets in Zhejiang Province, China. Antimicrobial resistance among the selected isolates was studied against 19 different antibiotics by disk diffusion method and all isolates were detected for the presence of integrase genes and Gene Cassettes (GCs) using PCR assays. The results showed that a total of 181 (40.22%) *E. coli* were obtained and the resistance pattern in the isolates was highly variable and significant differences were observed between seven antibiotics (p<0.05). Furthermore, 65.19% (118/181) of the isolates exhibited Multi-Drug Resistance (MDR) and the multiple resistance patterns were highly diverse with 77 different ones. Additionally, multiple-antibiotic resistance index values of more than 0.2 were determined in 62.43% of the isolates, which were diversely distributed in the three aquatic products. Of the 181 *E. coli* isolates obtained, 56 (30.94%) were positive for class 1 integrons and five (2.76%) were positive for class 2 integrons. Among these, 31 GCs with nine different arrays were detected in class 1 integrons and five GCs with two different arrays were detected in class 2 integrons. This study suggests that aquatic products may act as a reservoir and facilitate the dissemination of MDR.

**Keywords:** Antimicrobial Resistance Patterns, Multi-Drug Resistance, Multiple-Antibiotic Resistance Index Values, Gene Cassette Arrays

Introduction

Antibiotics have been used routinely for the prophylaxis and therapy of bacterial infection as well as growth promotion in animals for several decades (Fang et al., 2019). However, with the large-scale and intensive development of the farming industry, animal diseases have increased and the increasing and inappropriate use of antibiotics has led to the emergence and spread of Antimicrobial-Resistant (AMR) bacteria (He et al., 2016; Kang et al., 2018). In particular, the overuse and misuse of antibiotics have accelerated the occurrence of Multi-Drug Resistant (MDR) bacteria and even “super bacteria,” which are emerging to harm the environment and endanger public and animal health (Park et al., 2018; Li et al., 2020).

MDR is mainly due to the Horizontal Gene Transfer (HGT) of Antimicrobial Resistance Genes (ARGs) by Mobile Genetic E (MGEs) (Top and Springael, 2003; Baharoglu et al., 2010). The HGT of bacteria through MGEs can confer the adaptive evolution of bacteria in a changing environment and promote the rapid proliferation of ARGs between different bacterial species, even in human pathogens (Martínez, 2008; Zhu et al., 2017; Beridze et al., 2020).
Integrons are one of bacterial MGEs that play an important role in the acquisition, expression, dissemination and distribution of ARGs between strains (Stalder et al., 2012; Fang et al., 2019; Trang et al., 2020). Integrons are composed of three elements: (a) The integrase gene (intI), which encodes an integrase protein IntI and mediates the integration and excision of the Gene Cassettes (GCs) through site-specific RecA-independent recombination; (b) a specific recombination site attI; and (c) a Pc promoter (Barraud and Ploy, 2015; Li et al., 2018; Dokić et al., 2020). Relative to the amino acid sequence of the IntI integrase protein, five general classes of integrons have been identified and distinguished (Theingi et al., 2019). Classes 1, 2 and 3 are detected in descending order and Classes 4 and 5 are rarely detected (Hochhut et al., 2001; Stalder et al., 2012; Læ et al., 2019).

Escherichia coli are ubiquitous commensal bacteria, some strains can be pathogenic to humans (Rehman et al., 2017; Ebrahimipour et al., 2017). Studies have found that AMR in the Enterobacteriaceae family has increased significantly in the past few years due to the abuse of antibiotics (Shaikh et al., 2015; Liu et al., 2017; Manikandan et al., 2020). Additionally, E. coli has a wide range of hosts and resistance genes can be easily obtained through HGT. Thus, E. coli is often used to monitor the occurrence of AMR (Huddleston, 2014; Paraoan et al., 2017).

Since 2002, China has become the world’s largest producer and consumer of aquatic products (FAO, 2002). However, in China, a high occurrence of AMR bacteria has already been detected in aquatic products and their related environments especially in coastal regions of southeastern China (Shao et al., 2018; Jiang et al., 2019). To our knowledge, little comprehensive research has been conducted on antibiotic resistance in E. coli isolates from both crustaceans, fish and shellfish in coastal region of southeastern China.

Thus, in this study, we selected three popular and important aquatic products in China: Pacific whiteleg shrimps (P. vannamei), Pacific mackerel (Pseudotaxodorus japonicus) and Pacific oysters (Crassostrea gigas) to represent crustaceans, fish and shellfish, respectively. The samples were obtained from different markets in Zhejiang Province, a representative coastal region in southeastern China with high production and consumption of important aquatic products. The aim was to analyze the antimicrobial susceptibility profiles and integrons and the associated GCs of E. coli isolates. This information will help monitor the changes in AMR of E. coli isolated from aquatic products in China and will provide insight into the appropriate use of antibiotics and the safe consumption of aquatic products. The remainder of present paper is organized as follows. Section 2 briefly describes the method of sample collection, isolation of E. coli isolates, antimicrobial susceptibility testing of the isolates, the detection of integrons and statistical analysis of drug resistance in the E. coli isolates from different samples. The isolation rates of E. coli from aquatic product samples, the antibiotic resistance profiles of E. coli isolates for 19 antibiotics from nine classes and the prevalence of integrons in E. coli isolates will be presented in section 3. Section 4 provides the conclusion and prospect of this paper.

Materials

Sample Collection

A total of 450 samples from Pacific whiteleg shrimps (P. vannamei; n = 150), Pacific mackerel (P. japonicus; n = 150) and Pacific oysters (C. gigas; n = 150) were collected evenly from five different retail markets in Zhejiang Province, China between July and August 2019. Each sample was dispensed into a sterile sealed sampling bag and shipped back to the laboratory in an icebox within 2 h for bacterial isolation in a sterile environment.

Isolation and Identification of E. coli Strains

Meat (10 g) from each Pacific whiteleg shrimp, Pacific mackerel and Pacific oyster was used for E. coli isolation. Each sample was mixed with sterile Luria-Bertani (LB) broth (Hope Bio-Technology Co., Qingdao, China) (1:1, w/v) and homogenized for 2 min in a homogenizer (Scietz, Ningbo, China). Homogenates were incubated overnight (about 16-18 h) in a shaker at 37°C, 200 rpm for pre-enrichment. A 50 μL aliquot of the overnight cultivated broth used in primary isolation was transferred to Eosin Methylene Blue (EMB) agar plates (Hope Bio-Technology Co.) and incubated at 37°C for 24 h. Thereafter, colonies with a green metallic sheen on EMB plates were presumed to be E. coli and inoculated into LB broth at 37°C for 16-18 h and then kept at-20°C. Because the microbial cells from the top of the homogenates mentioned above were enriched with many of the same colonies, one E. coli isolate per sample was selected for further analysis, as done in previous studies (Cheng et al., 2019; Fang et al., 2019).

Polymerase Chain Reaction (PCR) was used to detect the uidA gene (Cheng et al., 2019). Genomic DNA templates of putative E. coli strains were isolated using a Bacteria Genomic DNA Kit (Kangwei Century Biotechnology Co. Ltd., Beijing, China). E. coli ATCC 25922 was used as a positive control. The design of primers was based on the uidA gene (Table 1) (Fang et al., 2019). Each PCR reaction mixture consisted 5 U of Ex-Taq DNA polymerase (Takara-Bio, Dalian, China), 200 mM of each deoxynucleotide triphosphate, 10× PCR buffer, 400 nM of each primer, 250 ng of DNA template...
and double-distilled water (ddH₂O) to a total volume of 25 μL. PCR amplification included pre-denaturation at 98°C for 1 min, followed by 35 cycles of denaturation at 98°C for 30 s, annealing at 56°C for 30 s and polymerization at 72°C for 30 s and a final extension at 72°C for 10 min. The PCR products were electrophoresed on a 1.2% agarose gel stained with GoldView II (Gentihold, Beijing, China) (Fig. 1).

**Antimicrobial Susceptibility Testing**

E. coli isolates (n = 181) were tested for antimicrobial susceptibility according to the Kirby-Bauer disk diffusion method recommended by the Clinical and Laboratory Standards Institute (CLSI, 2019). Nineteen antimicrobial agents of nine different classes: β-lactam (ampicillin, 10 μg; cefazolin, 30 μg; cefamandole, 30 μg; cefotaxime, 30 μg; cefepime, 30 μg; meropenem, 10 μg), polypeptide (polymyxin B, 300 IU), furan (furazolidone, 100 μg), tetracycline (tetracycline, 30 μg; doxycycline, 30 μg), sulfonamides (trimethoprim-sulfamethoxazole, 1.25/23.75 μg; sulfadiazine, 300 μg), macrolide (erythromycin, 15 μg), quinolone (enrofloxacin, 10 μg; lomefloxacin, 10 μg), chloramphenicol (florfenicol, 30 μg; chloramphenicol, 30 μg), aminoglycoside (spectinomycin, 100 μg; neomycin, 30 μg), were obtained from Hangzhou Microbiology Co. Ltd. (Hangzhou, China). All confirmed E. coli isolates were incubated at 37°C overnight and diluted to a 0.5 McFarl and standard with 0.9% (w/v) NaCl then transferred to Mueller-Hinton agar (Hope Bio-Technology Co.) plates with a sterile cotton swab and incubated at 37°C for 16-18 h. Interpretation of inhibition zones was performed according to the CLSI guidelines and classified as Resistant (R), Intermediate (I), or Susceptible (S). E. coli ATCC 25922 was used as a quality control strain. MDR was defined as E. coli strains exhibiting resistance to three or more classes of antimicrobials (Schwarz et al., 2010). Multiple-Antibiotic Resistance (MAR) index was calculated (MAR index = a/b, where a is the number of antibiotics with which the isolate was resistant and b is the total number of antibiotics tested) (Krumperman, 1983).

![Fig. 1: Research methods in this study](image)

**Table 1: Primers used in this study**

| Target          | Primer | Sequence (5’-3’) | Amplicon size (bp) | Reference                  |
|-----------------|--------|------------------|--------------------|---------------------------|
| uidA            | uidA-F | GTCCGTGAGAAAACCCCAACCGTGAA | 424                | Fang et al. (2019)        |
|                 | uidA-R | GGATAGCTGCTGAGTTGTGTCGT |                   |                           |
| intI            | intI-F | GGCTCTGATGCTGCTGCTT | 146                | Lao et al. (2010)         |
|                 | intI-R | CATTTCTGGCCTGTGTTCT |                   |                           |
| intI2           | intI2-F | CACGGATATGCCCAAAAAAGGT | 789                | Odumosu et al. (2013)    |
|                 | intI2-R | GTAGCAACCGAGTACAAATG |                   |                           |
| intI3           | intI3-F | GCCTCCGGACGACCTTCAG | 980                | White et al. (2001)       |
|                 | intI3-R | ACGGATCTGCAAAACCTGCT |                   |                           |
| Class 1 integron| hep58  | TCATGGGCTTTGTTAATGCTGT | Class 1 integron   | Class 1 integron          |
| variable region | hep59  | GTAGGGCTTTATATGACGC | variable region    | variable region           |
| Class 2 integron| hep51  | GATGCGATCGCAAGTAGCAGG | Class 2 integron   | Class 2 integron          |
| variable region | hep74  | CCGGATCCCCGGACGACGACATGCTGGA | variable region | variable region           |
Detection of Integrons and Relevant Gene Cassettes (GCs)

All isolates were detected for the presence of integrase genes (intI1, intI2 and intI3) and GCs using PCR assays. The primers used for these genes are listed in Table 1 (Luo et al., 2010; Odumosu et al., 2013). The genomic DNA extraction and PCR reaction mixture were as described previously (section 2.2). Amplification was initiated by incubating the reaction mixture at 94°C for 1 min followed by 35 cycles of 30 s at 98°C, annealing at 55°C (intI1 and intI2) or 60°C (intI3) for 30 s, an extension for 30 s (intI1) or 1 min (intI2 and intI3) at 72°C and a final extension at 72°C for 10 min.

Although the intI3 gene was not detected in any identified E. coli strains, the Variable Regions (VRs) of strains that were positive for the intI1 and/or intI2 genes were further analyzed by PCR. For intI1-positive E. coli isolates, PCR amplification annealed at 55°C for 30 s and extended at 72°C for 4 min with primers hep58-hep59 (Odumosu et al., 2013). For intI2-positive E. coli isolates, the procedure was as follows: Pre-denaturation (98°C, 1 min), followed by 12 cycles of denaturation (98°C, 30 s), annealing (68°C, 30 s; an initial temperature of 68°C decreasing by 1°C each cycle), extension (72°C, 1 min) and followed by 30 cycles of denaturation (98°C, 30 s), annealing (56°C, 30 s), extension (72°C, 4 min) and a final extension (72°C, 10 min) with primer pairs hep51-hep74 (White et al., 2001). The products were subsequently sequenced by Shanghai Sangon (Shanghai, China) and sequences were compared with the GenBank reference sequences using NCBI BLAST (www.ncbi.nlm.nih.gov/blast/).

Statistical Analysis

All experiments were performed in triplicate. Data analysis was performed by SPSS (version 20.0). Independent sample t-tests were used for the analysis of drug resistance in the E. coli isolates from different samples against different antibiotics and significant differences were assigned when p<0.05.

Results

E. coli Isolates

A total of 181 (40.22%) E. coli isolates were obtained from 450 aquatic product samples. Of these, 51.33% (77/150) were from Pacific whiteleg shrimps, 40.00% (60/150) were from Pacific mackerels and 29.33% (44/150) were from Pacific oysters.

Antibiotic Resistance Profiles of E. coli Isolates

The antibiotic resistance profiles of 181 E. coli isolates for 19 antibiotics from nine classes are displayed in Table 2. Overall, E. coli strains showed relatively higher resistance to enrofloxacin (59.12%, 107/181), erythromycin (47.51%, 86/181) and tetracycline (40.33%, 73/181). In addition, these isolates exhibited moderate resistance rates for ampicillin (37.02%, 67/181), cefazolin (32.60%, 59/181), doxycycline (32.04%, 58/181), furazolidone (29.83%, 54/181), polymyxin B (27.62%, 50/181), sulfadiazine (25.97%, 47/181), florfenicol (21.55%, 39/181), trimethoprim-sulfamethoxazole (20.44%, 37/181), chloramphenicol (17.68%, 32/181) and lomefloxacin (10.50%, 19/181). Low levels of resistance were observed for spectinomycin (4.97%, 9/181), cefamandole (4.42%, 8/181), cefepime (4.42%, 8/181), meropenem (3.87%, 7/181), ceftizoxime (3.31%, 6/181) and neomycin (2.21%, 4/181). Individually, in Pacific whiteleg shrimp samples, E. coli isolates showed the highest resistance to enrofloxacin (50.65%, 39/77) and no resistance to ceftizoxime, cefepime and meropenem; in Pacific mackerel samples, E. coli isolates showed the highest resistance to erythromycin (61.67%, 37/60) and no resistance to neomycin; and in Pacific oyster samples, E. coli isolates showed the highest resistance to enrofloxacin (72.73%, 32/44) and no resistance to cefamandole, cefepime and meropenem. Furthermore, independent sample t-tests revealed that resistant rates of strains to ampicillin, cefazolin, doxycycline, lomefloxacin, enrofloxacin, florfenicol and chloramphenicol were significantly different among the three different species (p<0.05), while resistant rates to spectinomycin and neomycin were not significantly different among the three species (p>0.05). It seems that E. coli isolates exhibited high rates of resistance to several older antibiotics, including enrofloxacin, erythromycin and tetracycline, that have commonly been used in animal husbandry in China for many years (Gong et al., 2013).

Additionally, results showed that 65.19% (118/181) of E. coli isolates were classified as MDR (Pacific whiteleg shrimps, 51.95%, 40/77; Pacific mackerels, 71.67%, 43/60; and Pacific oysters, 79.55%, 35/44). The multiple resistance patterns were highly diverse with 77 different patterns and the most common pattern was the combination of furazolidone/erythromycin/enrofloxacin observed in three E. coli isolates: One from Pacific whiteleg shrimp samples and two from Pacific oyster samples. Furthermore, MAR index values were between 0 and 0.68 with an abundance of 92 different resistance patterns (data not shown). The MAR index values of most isolates were 0.21, 0.26 and 0.37 (each 14 isolates), but was as high as 0.68 in two isolates that were resistant to 13 antibiotics (Table 3). In aquaculture, antibiotics are used to control bacterial infections and promote growth (Tan et al., 2017). It is a common practice to add antibiotics to feed and water bodies. In addition, the
widespread use of antibiotics in clinics, agriculture and livestock production has also promoted the spread of drug-resistant bacteria, which also explains the multiple MDR patterns found in our research (Tan et al., 2017; Yassin et al., 2017). Although the prevalence of MDR was higher than that reported in our previous researches, it was still lower than that reported for animal husbandry in China (Cheng et al., 2019; Zhang et al., 2017).

Table 2: Antibiotic susceptibility profiles of 181 E. coli isolates

| Antibiotics | Breakpoints (CLSI, 2019) | Resistants isolates (%) | Resistant isolates (%) | Total (%) |
|-------------|-------------------------|-------------------------|------------------------|-----------|
|             | R, I, S (mm)            | Pacific mackerels (n = 77) | Pacific oysters (n = 44) | Total (n = 181) |
| β-Lactams   |                         |                         |                        |           |
| AM          | ≤13|14-16|≥17             | 20.78                  | 43.33      | 36.82      | 56.82      | 37.02      |
| CZ          | ≤19|20-22|≥23             | 31.17                  | 50.00      | 11.36      | 13.66      | 32.60      |
| CM          | ≤14|15-17|≥18             | 2.60                   | 10.00      | 0.00       | 0.00       | 4.42       |
| ZOX         | ≤21|22-24|≥25             | 0.00                   | 6.67       | 4.55       | 3.31       | 4.42       |
| FEP         | ≤18|19-24|≥25             | 0.00                   | 13.33      | 0.00       | 0.00       | 4.42       |
| MEM         | ≤19|20-22|≥23             | 0.00                   | 11.67      | 0.00       | 0.00       | 3.87       |
| Polymyxins  |                         |                         |                        |           |
| PB          | ≤12|13-19|≥20             | 20.78                  | 43.33      | 18.18      | 27.62      |           |
| Furan       |                         |                         |                        |           |
| FZ          | ≤14|15-16|≥17             | 23.38                  | 48.33      | 15.91      | 29.83      |           |
| Tetracyclines|                       |                         |                        |           |
| TET         | ≤11|12-14|≥15             | 32.47                  | 60.00      | 27.27      | 40.33      |           |
| DOX         | ≤10|11-13|≥14             | 15.58                  | 53.33      | 31.82      | 32.04      |           |
| Sulfonamides|                         |                         |                        |           |
| TMP-SMZ     | ≤10|11-15|≥16             | 11.69                  | 13.33      | 45.45      | 20.44      |           |
| SZE         | ≤10|11-15|≥16             | 18.18                  | 21.67      | 45.45      | 25.97      |           |
| Macrolides  |                         |                         |                        |           |
| ERY         | ≤12|12-23|≥24             | 38.96                  | 61.67      | 43.18      | 47.51      |           |
| Quinolones  |                         |                         |                        |           |
| LOM         | ≤18|19-21|≥22             | 9.09                   | 3.33       | 22.73      | 10.50      |           |
| ENR         | ≤27|28-36|≥37             | 50.65                  | 60.00      | 72.73      | 59.12      |           |
| Amphenicols |                         |                         |                        |           |
| FLO         | ≤11|12-18|≥19             | 11.69                  | 16.67      | 45.45      | 21.55      |           |
| CHL         | ≤12|13-17|≥18             | 9.09                   | 18.33      | 31.82      | 17.68      |           |
| Aminoglycosides|                   |                         |                        |           |
| SPT         | ≤14|15-17|≥18             | 3.90                   | 5.00       | 6.82       | 4.97       |           |
| NEO         | ≤11|12-14|≥15             | 2.60                   | 0.00       | 4.55       | 2.21       |           |
| MDR         |             |                         |                        |           |

N = Number of Investigated E. coli Isolates; R = Resistance; I = Intermediate Resistance; S = Susceptible; AM, Ampicillin; CZ, Cefazolin; CM, Cefamandole; ZOX, Cefizoxime; FEP, Cefepime; MEM, Meropenem; PB, Polymyxin B; FZ, Furazolidone; TET, Tetracycline; DOX, Doxycycline; TMP-SMZ, Trimethoprim-Sulfamethoxazole; SZE, Sulfadiazine; ERY, Erythromycin; LOM, Lomefloxacin; ENR, Enrofloxacin; FLO, Florfenicol; CHL, Chloramphenicol; SPT, Spectinomycin; NEO, Neomycin

Table 3: MAR index values of E. coli isolates

| MAR index | No. of isolates | Origin (n) |
|-----------|-----------------|------------|
| 0         | 19              | Pacific whiteleg shrimps (15); Pacific mackerels (3); Pacific oysters (1) |
| 0.05      | 19              | Pacific whiteleg shrimps (7); Pacific mackerels (9); Pacific oysters (3) |
| 0.11      | 19              | Pacific whiteleg shrimps (6); Pacific mackerels (10); Pacific oysters (3) |
| 0.16      | 11              | Pacific whiteleg shrimps (5); Pacific mackerels (2); Pacific oysters (4) |
| 0.21      | 20              | Pacific whiteleg shrimps (4); Pacific mackerels (9); Pacific oysters (7) |
| 0.26      | 20              | Pacific whiteleg shrimps (7); Pacific mackerels (9); Pacific oysters (4) |
| 0.32      | 18              | Pacific whiteleg shrimps (2); Pacific mackerels (13); Pacific oysters (3) |
| 0.37      | 20              | Pacific whiteleg shrimps (4); Pacific mackerels (9); Pacific oysters (7) |
| 0.42      | 19              | Pacific whiteleg shrimps (3); Pacific mackerels (12); Pacific oysters (4) |
| 0.47      | 7               | Pacific whiteleg shrimps (2); Pacific mackerels (5) |
| 0.53      | 6               | Pacific whiteleg shrimps (3); Pacific mackerels (3) |
| 0.63      | 1               | Pacific mackerels (1) |
| 0.68      | 2               | Pacific mackerels (2) |
Table 4: Integrons and cassette arrays identified in 181 E. coli isolates

| Gene cassette array | Source | No. of isolates | Size (bp) | Antibiotic resistance profile (n) |
|---------------------|--------|-----------------|-----------|----------------------------------|
| Class 1 Integrons   |        |                 |           |                                  |
| aadA1               | Pacific whiteleg shrimps | 3              | 750       | AM-CZ-PB-FZ-DOX-TMP-SMZ-ERY-ENR (2); CZ-PB-FZ-TET-ERY-LOM (1) |
|                     | Pacific mackerels          | 3              |           | AM-CZ-MEM-FZ-TET-DOX-SZE-ERY-LOM-ENR-FLO-CHL (1); FZ-TET-ERY-ENR (2) |
|                     | Pacific oysters            | 2              |           | FZ-ERY-ENR (2) |
| aadA2               | Pacific whiteleg shrimps   | 2              | 791       | AM-PB-FZ-TET-ERY-LOM (1); CM-TMP-SMZ-ERY-ENR (1) |
|                     | Pacific mackerels          | 3              |           | CZ-PB-FZ-TET-DOX-ERY-FLO (1); PB-FZ-TET-DOX-ERY-ENR (2) |
|                     | Pacific oysters            | 2              |           | AM-CZ-PB-TMP-SMZ-SZE-ERY-ENR-FLO-NEO (1); AM-TET-TMP-SMZ-SZE-ERY-ENR-FLO-CHL (1) |
| dfrA17-aadA5        | Pacific whiteleg shrimps   | 1              | 1391      | FZ-TET-DOX-TMP-SMZ-ERY-ENR-FLO (1) |
|                     | Pacific mackerels          | 2              |           | AM-TET-DOX-SZE-ERY-ENR-FLO-CHL (2) |
| dfra1-aadA1         | Pacific whiteleg shrimps   | 1              | 1704      | AM-CZ-CM-PB-FZ-TET-DOX-TMP-SZE-ERY-ENR-FLO-CHL (1); MEM-PB-TET-ENR (1) |
| dfra1-aadA1         | Pacific mackerels          | 1              | 1357      | AM-CM-TET-ENR (1) |
|                     | Pacific oysters            | 2              | 2903      | TET-SMZ-ERY-ENR-FLO-CHL (2) |
| aadB-aadA1-cmlA6    | Pacific whiteleg shrimps   | 2              | 2903      | AM-CZ-SZE-CHL (2) |
| arr3-aac(6')-Ib     | Pacific whiteleg shrimps   | 2              | 1148      | AM-PB-TET-ERY-ENR (2) |
| dfra12-orf-aadA2    | Pacific mackerels          | 1              | 1696      | AM-CZ-CM-MEM-FZ-TET-DOX-TMP-SMZ-SZE-ERY-ENR (1) |
| orf                 | Pacific oysters            | 1              | 290       | AM-PB-TET-ERY-ENR (1) |
| Class 2 Integrons   |        |                 |           |                                  |
| dfra1-sat2-aadA1    | Pacific whiteleg shrimps   | 1              | 1938      | CZ-PB-FZ-TET-ERY-ENR-CHL (1); AM-TMP-SMZ-ERY-ENR-FLO-CHL (1) |
|                     | Pacific mackerels          | 2              |           | AM-TMP-SMZ-ERY-ENR-FLO-CHL (1); CZ-FZ-TET-ERY-ENR (1) |
| dfrA1-catB2-sat2-aadA1 | Pacific whiteleg shrimps | 1              | 2697      | CZ-DOX-ERY-LOM-ENR (1) |

AM, Ampicillin; CZ, Cefazolin; CM, Cefamandole; MEM, Meropenem; PB, Polymyxin B; FZ, Furazolidone; TET, Tetracycline; DOX, Doxycycline; TMP-SMZ,Trimethoprim-Sulfamethoxazole; SZE, Sulfadiazine; ERY, Erythromycin; LOM, Lomefloxacin; ENR, Enrofloxacin; FLO, Florfenicol; CHL, Chloramphenicol; SPT, Spectinomycin; NEO, Neomycin

Prevalence of Integrons in E. coli Isolates and Associated GCs

Of the 181 E. coli isolates identified, 56 (30.94%) were positive for intI1 and five (2.76%) were positive for intI2. All intI2-positive strains were also carrying intI1. However, no intI3-positive isolates were detected in our study. The prevalence of class 1 and class 2 integrons in E. coli isolates and their associated GCs (where possible) from different samples are shown in Table 4.

Of the 56 intI1-positive isolates identified, 31 (55.36%) were positive for GCs and nine distinct types of GCs arrays were identified by VR fragment sequencing. In addition, of the 5 intI2-positive isolates, 5 (100%) were positive for GCs and two different types of GCs arrays were identified. Third, no E. coli isolates contained both GCs of class 1 integrons and GCs of class 2 integrons. Table 4 summarizes the sizes and the antibiotic resistance profiles associated with the different GCs. In total, four streptomycin and spectinomycin resistance genes (aadB, aadA1, aadA2 and aadA5), three trimethoprim resistance genes (dfrA1, dfrA12 and dfrA17), one chloramphenicol resistance gene (cmlA6), one rifampin resistance gene (arr3), one amikacin resistance gene (aac(6')-Ib), one streptothricin resistance gene (sat2), one amphenicol resistance gene (catB2) and one open reading frame of unknown function (orf) were detected. It was noted that all E. coli isolates carrying GCs were MDR isolates. Similar to previous studies, intI1 is most present in the environment and may be related to its structure, while intI3 is rarely detected in the environment. In addition, it has been reported that dfrA and aadA resistance genes are highly stable and low cost structures which are commonly found in class 1 and class 2 integrons (Paraoan et al., 2017; Dib et al., 2018).

Discussion

Many studies have reported that a large amount of antibiotics has already been discharged into aquatic environments.
environments (Cabello et al., 2013; Shimizu et al., 2013; Zhang et al., 2015). Under this selective pressure, the occurrence of AMR bacteria and ARGs and their transmission between the same or different bacteria in aquatic species have developed into a global concern (Shao et al., 2018). At present, a high prevalence of AMR E. coli isolates has already been detected in many aquatic products worldwide (Lin et al., 2016; Jiang et al., 2019). In our study, we characterized antimicrobial resistance profiles and integrons and their associated GCs of E. coli strains isolated from three species of aquatic products sold at different markets in Zhejiang Province, China.

In our findings, the strains had highly variable resistance patterns and significant differences were observed in the resistant rates of seven antibiotics, belonging to β-Lactams, tetracyclines, quinolones and amphenicols classes, between isolates from Pacific whiteleg shrimp, Pacific mackerel and Pacific oyster samples (p<0.05). Resistance patterns were highly diverse as can be seen in Table 2 and only resistant rates to aminoglycosides displayed no significant difference in strains from three aquatic products (p>0.05). Overall, in E. coli isolates the highest resistance rate was observed for enrofloxacin (59.12%), followed by erythromycin (47.51%), tetracycline (40.33%) and ampicillin (37.02%). These resistance rates were quite different when compared to other studies as ampicillin was always the highest (Lin et al., 2016; Dib et al., 2018; Cheng et al., 2019). The discrepancy may be attributed to the test methodology or the geographic variation of samples (Yu et al., 2016). In addition, E. coli isolates have high resistance to first-generation cephalosporins (cefazolin, 32.60%), but lower resistance to the second, third and fourth-generation cephalosporins (cefamandole, 4.42%; cefitizoxime, 3.31%; cefepime, 4.42%). This suggests that first-generation cephalosporins might have been widely misused in the environment in past decades thus reducing susceptibility and efficiency in the treatment of bacterial infections (Sudha et al., 2014). However, the accumulation of other generations of cephalosporins resulting in increased drug-resistant bacteria in the environment might take a longer time (Yu et al., 2016). Furthermore, the third- and fourth-generation cephalosporins, meropenem and polymyxin B tested in our study are regarded as critically important antimicrobial agents in human medicine by the (World Health Organization, 2019). Furthermore, polymyxin B was originally found in the Gram-positive bacterium Paenibacillus polymyxa as the last line of defense against serious infections with Gram-negative pathogens (Sun et al., 2018a). Meropenem belongs to the carbapenem class of β-lactams and has a broad-spectrum antibacterial activity (Cheng et al., 2019). Unfortunately, E. coli isolates from all three aquatic products exhibited high rates of resistance to polymyxin B (overall 27.62%) and Pacific mackerel samples exhibited a resistance rate of 11.67% to meropenem. In China, antibiotics including doxycycline, sulfadiazine, florfenicol and neomycin are permitted in aquaculture (PRC, 2019). Except for neomycin (overall resistance 2.21%), however, more than 20% of isolates showed resistance to these antibiotics, which may result in reducing the efficiency of such antibiotics. Furthermore, we observed some resistance to furazolidone (overall 29.83%) and chloramphenicol (overall 17.68%), which have already been banned in aquaculture in China since 2002 and lomefloxacin (overall 10.50%), which has been banned in aquaculture in China since 2015 (PRC, 2019). Thus, although certain antibiotics have been banned, the residues of resistance in bacteria can last for a long time. Finally, E. coli isolates exhibited resistance to older antibiotics trimethoprim-sulfamethoxazole (overall 20.44%) and spectinomycin (overall 4.97%), that have been used commonly in animal husbandry for many years (Gong et al., 2013).

Among the 181 E. coli isolates, 65.19% (118/181) exhibited MDR and the MDR rates of three species of aquatic samples were all higher than 50%. This is higher than the findings in our previous report (Cheng et al., 2019). A national surveillance study showed high levels of MDR E. coli isolates in pigs (90.00%, 6,806/7,562) and in chickens (89.20%, 6,751/7,568) in China from 2008-2015 (Zhang et al., 2017). Although E. coli isolates of aquatic products in the present study had a relatively lower level of MDR compared to the very high levels found in the pig and the chicken isolates in China mentioned above, there is growing evidence that the problem with MDR bacteria is becoming serious in China and guidelines and regulations are urgently needed to limit and rationalize antimicrobial use (Yassin et al., 2017). Furthermore, we determined MAR index values of more than 0.2 in 62.43% of the isolates, which were distributed diversely in the three aquatic products. An MAR index value higher than 0.2 indicates that the aquatic products originated from a source of high-risk antimicrobial contamination. The MAR index is used to evaluate the extent of environmental contamination by antimicrobials (Krumperman, 1983). Moreover, the MAR index value of two isolates sourced from Pacific mackerels was 0.68, which exhibited resistance to 13 antibiotics. These all implied that most aquatic products were extensively exposed to antimicrobials.

According to past studies, integrons are as one of the MGEs that play a vital role in the development and dissemination of ARGs and MDR bacteria (Stalder et al., 2012). In our study, class 1 integrons were the most common type, followed by class 2 integrons and no class 3 integrons were detected, which was consistent with some other studies showing that the predominance of...
class 1 integrons was in animal-derived *E. coli* and human-associated Gram-negative isolates (Paraaoan et al., 2017; Rehman et al., 2017). Meanwhile, all integron-positive strains are MDR in our study.

Of the 56 class 1 integron-positive isolates, 31 had GCs and nine different GC arrays were detected. The most prevalent genes detected in the VRs of integrons were those encoding adenyllyl transferases (*aadA1, aadA2, aadA5* and *aadB*) and dihydrofolate reductase (*dfrA1, dfrA12* and *dfrA17*), which are responsible for streptomycin-spectinomycin and trimethoprim resistance, respectively (Rehman et al., 2017). The *aadA* and *dfrA* genes occurred alone or in combination with other resistance genes in 33 integron-positive isolates in our study and are reported highly stable in integrons, even in the absence of selective pressures, because of the low fitness cost of their structures (Paraaoan et al., 2017; Dib et al., 2018; Fang et al., 2019). Additionally, two isolates from Pacific whiteleg shrimp samples carried the GC arr-3-aac (6′)-Ib, which has been reported as one of the most common GC arrays in China and the reason for this requires further study (Acosta-Pérez et al., 2015; Hu et al., 2016; Sun et al., 2018b). Moreover, the *cmlA* gene cassette was found in two isolates from Pacific whiteleg shrimp samples. This cassette encodes a chloramphenicol efflux pump located in both the plasmids and chromosomes and may be responsible for acquired or intrinsic resistance to chloramphenicol (Acosta-Pérez et al., 2015). Meanwhile, 44.64% (25/56) of class 1 integron-positive isolates lacked GCs despite harboring an *intI1* gene and exhibiting MDR. Some researchers believe that such bacteria have the potential to rapidly capture antibiotic resistance genes and acquire antibiotic resistance (Kotlarska et al., 2015; Park et al., 2018).

Of the five class 2 integron-positive isolates in our study, two different GC arrays were detected. The GCs *dfrA1-sat2-aadA1* were found most frequently associated with Class 2 integrons and the *sat2* gene cassette encodes streptomycin acetyltransferase, which results in streptomycin resistance (Xia et al., 2013; Mostafa et al., 2015; Paraaoan et al., 2017). Additionally, one isolate was found to carry the GC array *dfrA1-catB2-sat2-aadA1*, which has been reported by a few studies, such as in *Morganella morganii* from clinical specimens, in *Providencia* spp., *Proteus* spp. and *Proteus vulgaris* from wastewater environments and in *Proteus, Aeromonas, Staphylococcus, Citrobacter* and *Shewanella* from eels and aquaculture ponds (Lin et al., 2016; Cao et al., 2017; Moreira et al., 2019). The *intI2* gene contains an internal stop codon (TAA) at position 179 yielding and inactive 178-amino acid polypeptide, which might be the reason that occurrence of class 2 integrons is much lower than class 1 integrons (Hansson et al., 2002; Lin et al., 2016). In our study, all *intI2*-positive strains also carried *intI1*, which suggests that class 2 integrons can be complemented in trans by *intI1* (Hansson et al., 2002; Xia et al., 2013). In addition, we did not detect class 3 integrons. However, class 3 integrons were mostly detected in clinical and environmental samples such as in *Enterobacter cloacae* and *Delftia* spp. from hospital effluent and environmental sources, respectively (Xu et al., 2007; Barraud et al., 2013).

**Conclusion**

In conclusion, we analyzed the antimicrobial resistance profiles and integrons and their associated GCs of *E. coli* strains isolated from three species of aquatic products sold at different markets in Zhejiang Province, China. It showed that a total of 181 (40.22%) *E. coli* isolates were obtained from the 150 Pacific whiteleg shrimps, 150 Pacific mackerels and 150 Pacific oysters samples. Overall, the highest resistance rate observed in the isolates was to enrofloxacin (59.12%) and the resistance pattern in the isolates was highly variable and significant differences were observed between seven antibiotics (*p*<0.05). Furthermore, 65.19% (118/181) of the isolates exhibited Multi-Drug Resistance (MDR) and the multiple resistance patterns were highly diverse with 77 different ones. Additionally, multiple-antibiotic resistance index values of more than 0.2 were determined in 62.43% of the isolates, which were diversely distributed in the three aquatic products. Of the 181 *E. coli* isolates obtained, 56 (30.94%) were positive for class 1 integrons and five (2.76%) were positive for class 2 integrons. Among these, 31 GCs with nine different arrays were detected in class 1 integrons and five GCs with two different arrays were detected in class 2 integrons. This study suggests that aquatic products may act as a reservoir for MDR bacteria and facilitate the dissemination of ARGs. Continuous surveillance targeting AMR bacteria from aquatic products is necessary to ensure safe consumption. Furthermore, strict preventive measures should be taken to avoid the spread of ARGs and MDR bacteria in aquatic products in China, as well as worldwide.

**Acknowledgment**

This research was supported by the National Natural Science Foundation of China under Grant Nos. 31901792 and 31801655 and the Zhejiang Provincial Natural Science Foundation of China under Grant No. LZ18C200004 and 2019 University Students of Zhejiang Science and Technology Innovation Activity Fund (New Talent Program) No. 2019R409059.

**Author Contributions**

Ting Yu and Han Jiang: Completed the analysis of gene cassettes and all data analysis and prepared the manuscript.
Hui Cheng and Yingwen Chen: Completed antibiotic resistance testing and integron class and gene cassette detection.

Yiru Xuan, Xiang Lv, Yihao Chen and Jingyi Gu: Completed sample collection and E. coli isolation and identification.

Jiehong Fang and Cheng Zhu: Designed the project, completed the data analysis and revised the manuscript.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and no ethical issues involved.

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