American Journal of Biochemistry and Biotechnology

Original Research Paper

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

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Article history
Received: 12-10-2020
Revised: 14-12-2020
Accepted: 15-12-2020

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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 Springaely, 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; Lae *et al*., 2019).

Escherichia coli are ubiquitous commensal bacteria, some strains can be pathogenic to humans (Rehman *et al*., 2017; Ebrahimipour *et al*., 2020). 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 AM R 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 mackerels (*Pneumatophorus 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 mackerels (*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 (Scieatz, 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 *uid*A 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 *uid*A 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 (ddH_2O) 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; ceftizoxime, 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).

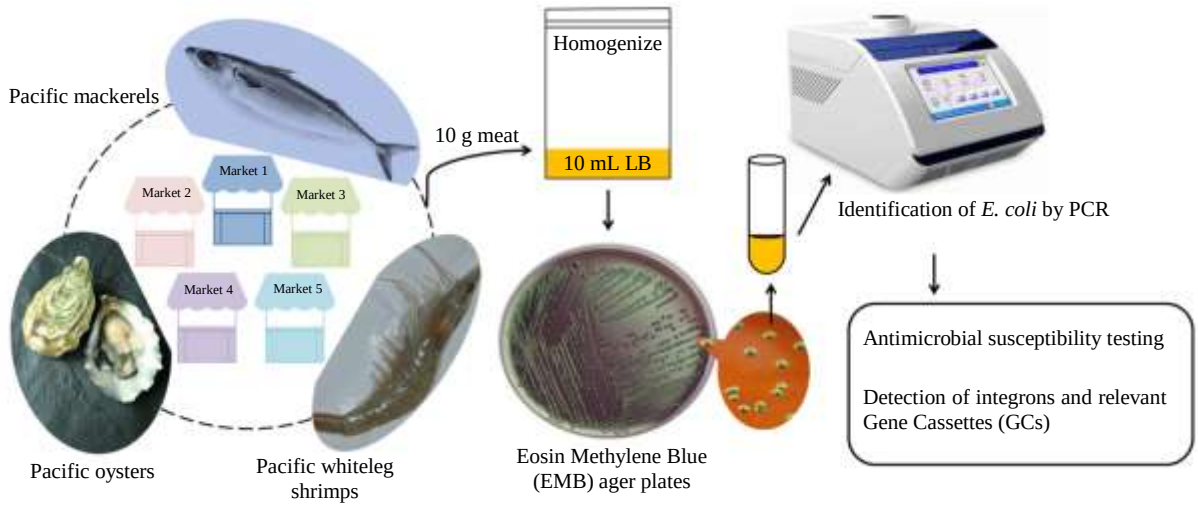

Fig. 1: Research methods in this study

Table 1: Primers used in this study

Target	Primer	Sequence (5' -3')	Amplicon size (bp)	Reference
*uid*A	*uid*A-F	GTCCTGTAGAAACCCCAACCCGTGAA		
	*uid*A-R	GGGATAGTCTGCCAGTTCAGTTCGT	424	Fang *et al.* (2019)
intI1	*intI1*-F	GGCTTCGTGATGCCTGCTT		
	intI1-R	CATTCCTGGCCGTGGTTCT	146	Luo *et al.* (2010)
intI2	*intI2*-F	CACGGATATGCGACAAAAAGGT		
	intI2-R	GTAGCAAACGAGTGACGAAATG	789	Odumosu *et al.* (2013)
intI3	*intI3*-F	GCCTCCGGCAGCGACTTTCAG		
	intI3-R	ACGGATCTGCCAAACCTGACT	980	White *et al.* (2001)
Class 1 integron variable region	hep58	TCATGGCTTGTTATGACTGT	Class 1 integron variable region	Odumosu *et al.* (2013)
	hep59	GTAGGGCTTATTATGCACGC		
Class 2 integron variable region	hep51	GATGCCATCGCAAGTACGAG	Class 2 integron variable region	White *et al.* (2001)
	hep74	CGGGATCCCGGACGGCATGCACGATTTGTA		

Detection of Integrons and Relevant Gene Cassettes (GCs)

All isolates were detected for the presence of integrase genes (*intI1*, *intI*2 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 are 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 30s (*intI1*) or 1 min (*intI2* and *intI3*) at 72°C and a final extension at 72°C for 10 min.

Although the *intI*3 gene was not detected in any identified *E. coli* strains, the Variable Regions (VRs) of strains that were positive for the *intI*1 and/or *intI*2 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 (72ºC, 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) R, I, S (mm)	Resistant isolates (%)			
		(*n* = 77)	Pacific mackerels (*n* = 60)	Pacific oysters (*n* = 44)	Total (*n* = 181)
β-Lactams					
AM	≤13|14-16|≥17	20.78	43.33	56.82	37.02
CZ	≤19|20-22|≥23	31.17	50.00	11.36	32.60
CM	≤14|15-17|≥18	2.60	10.00	0.00	4.42
ZOX	≤21|22-24|≥25	0.00	6.67	4.55	3.31
FEP	≤18|19-24|≥25	0.00	13.33	0.00	4.42
MEM	≤19|20-22|≥23	0.00	11.67	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		51.95	71.67	79.55	65.19

N = Number of Investigated *E. coli* Isolates; R = Resistance; I = Intermediate Resistance; S = Susceptible; AM, Ampicillin; CZ, Cefazolin; CM, Cefamandole; ZOX, Ceftizoxime; 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-ENR-FLO (1); PB-FZ-TET-DOX-ERY-ENR (2)
	Pacific oysters	2		AM-CZ-PB-TMP-SMZ-SZE-ENR-FLO-NEO (1); AM-TET-TMP-SMZ-SZE -ENR-FLO-CHL (1)
dfrA17-aadA5	Pacific whiteleg shrimps	1	1391	FZ-TET-DOX-TMP-SMZ-SZE-ENR-FLO (1)
	Pacific mackerels	2		AM-TET-DOX-SZE-ERY-ENR-FLO-CHL (2)
dfrA12-aadA2	Pacific whiteleg shrimps	1	1704	PB-ERY-ENR (1)
	Pacific mackerels	2		AM-CZ-CM-PB-FZ-TET-DOX-TMP-SMZ-SZE-ERY-ENR-FLO-CHL (1); MEM-PB-TET-ENR (1)
dfrA1-aadA1	Pacific whiteleg shrimps	1	1357	AM-CM-TET-ENR (1)
	Pacific mackerels	1		TET-TMP-SMZ-SZE-ERY (1)
	Pacific oysters	2		TET-DOX-TMP-SMZ-SZE -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-DOX-ENR (1)
Class 2 Integrons				
*dfr*A1-*sat*2-*aad*A1	Pacific whiteleg shrimps	1	1938	CZ-PB-FZ-TET-ERY-ENR-SPT (1)
	Pacific mackerels	2		AM-TMP-SMZ-SZE -ENR-CHL (1); CZ-FZ-TET-DOX-ERY-ENR (1)
	Pacific oysters	1		DOX-TMP-SMZ-SZE -ENR-FLO-CHL (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 *drfA* 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 (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%; ceftizoxime, 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 the aquaculture industry (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 in China 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%, 6,806/7562) 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 (Paraoan *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 adenylyl 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 (Paraoan *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 *sat*2 gene cassette encodes streptomycin acetyltransferase, which results in streptomycin resistance (Xia *et al*., 2013; Mostafa *et al*., 2015; Paraoan *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. LQ18C200004 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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