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Research Article

High Occurrence of Multiple-Drug Resistance Mediated by Integron in *Aeromonas* Isolated from Fish-Livestock Integrated Farms

Yu Ting Deng,[1,2] **Yong Yong Feng**,[3] **Ai Ping Tan**,[1] **Fei Zhao**,[1] **Lan Jiang**,[1] **and Zhi Bin Huang**[1]

[1]*Key Laboratory of Fishery Drug Development of Ministry of Agriculture and Rural Affairs, Guangdong Provincial Key Laboratory of Aquatic Animal Immunology and Sustainable Aquaculture, Pearl River Fisheries Research Institute, Chinese Academy of Fishery Sciences, Guangzhou, China*
[2]*Key Laboratory of Control of Quality and Safety for Aquatic Products of Ministry of Agriculture and Rural Affairs, Chinese Academy of Fishery Sciences, Beijing, China*
[3]*College of Seed and Facility Agricultural Engineering, Weifang University, Weifang, China*

Correspondence should be addressed to Fei Zhao; zhaofei@prfri.ac.cn

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Fish-livestock integrated farming is a traditional practice in South China and Southeast Asia. The administration of antimicrobial agents in livestock may potentially facilitate the spread of antimicrobial resistance to aquaculture through the drainage of livestock manure. *Aeromonas* spp. are fish pathogens that are the predominant bacteria in water bodies. The aim of this study was to characterize multiple-drug-resistant*Aeromonas* spp. isolated from integrated and nonintegrated fish-livestock farms and to investigate the occurrence of class 1 integrons. Of the 481 *Aeromonas* strains examined in this study, isolates from pig manure, fish, and environmental samples from pig-fish or goose-fish integrated farms were significantly more resistant to various antimicrobial agents than those from nonintegrated farms ($P < 0.05$). High rates of resistance to nalidixic acid and sulfamonomethoxine were observed in the isolates from the integrated farm. Fifty *Aeromonas* isolates (10.4%) from integrated farms contained class 1 integrons. In addition, 96.0% (48/50) of the integron-positive strains displayed multiple-drug resistance. Ten types of gene cassette arrays were determined by sequencing, including *dfrA17*, *dfrA12-orfF-aadA2*, *catB8*, *dfrB4-catB3-aadA1*, *aac6(6′)-Ib-cr-arr-3*, *aac-II-bla*$_{\text{OXA-21}}$*-catB3*, *aar2-aacA4-drfA1-orfC*, *aac(6′)-Ib-cr*, *dfrA15,* and *dfrB4-catB3-bla*$_{\text{OXA-10}}$*-aadA1*. Notably, among the 50 integron-positive isolates, twenty isolates showed integrons located in plasmids, which may facilitate the transmission of resistance in integrated farms. Our investigation confirmed the high prevalence of multiple antimicrobial resistances mediated by class 1 integrons in *Aeromonas* isolates from integrated farms. Therefore, it is necessary to establish a risk assessment method for antimicrobial resistance in aquaculture.

1. Introduction

Fish-livestock integrated farming is a traditional practice in the countryside of South China and has been introduced into other Asian countries, such as Vietnam, Indonesia, Thailand, and India [1, 2]. Farmers often own these aquaculture systems. Fish are usually raised on farms with pigs, ducks, or geese that breed in sheds beside the fishponds. Untreated sewage from livestock urine, manure, and excess food was discharged directly into the ponds. Manure is often used as a source of organic matter for the growth of plankton and benthos and can be utilized by herbivorous and omnivorous fish [3]. The integrated fish-livestock farming system facilitates the input supply and marketing of products. However, the environmental impact of uncontrolled disposal of livestock waste is unacceptable. Within the integrated fish farming system, the accumulation of surplus antimicrobial agents and their residues from livestock may establish selective pressure that favours the selection and growth of antimicrobial-

resistant bacteria (ARB) [4]. ARB from livestock can enter ponds through animal manure and/or excess food [5]. A recent report found that an aquatic pathogen, *Aeromonas salmonicida* subsp. *salmonicida*, harbored two novel plasmid variants carrying multiple antibiotic resistance genes that were linked to a swine pathogen [6]. Previous studies on integrated farms in China and Southeast Asia have demonstrated that high levels of residual antimicrobial agents and antimicrobial resistance genes (ARGs) can be detected in polluted environments [2, 4, 5, 7]. Moreover, the potential transfer of ARB and ARGs from the aquaculture environment to humans may occur through direct or indirect consumption of fish and associated products harboring ARB [8, 9]. Faecal waste from livestock contains microbes and constituents that are potentially hazardous to pond ecology and food safety. To efficiently monitor antimicrobial resistance (AMR) in aquaculture systems, it is necessary to identify an effective general indicator bacterium. *Aeromonas* spp. are waterborne bacteria that are ubiquitous in aquatic animals and in the environment. *Aeromonas* are facultative pathogens that can infect various species of aquatic animals and may cause diseases in humans and warm-blooded animals through direct or indirect consumption of contaminated water or food [10]. *Aeromonas* spp. are the most convenient bacteria to isolate from freshwater aquatic animals and environments. Therefore, *Aeromonas* spp. are useful indicators for monitoring AMR in aquaculture systems [11].

The spread of AMR and ARGs among bacterial pathogens on integrated livestock farms is a major public health issue [2, 4, 5, 12]. Genetic elements, such as plasmids, transposons, and integrons, can be transferred horizontally between bacteria of different origins. Integrons are especially important among these genetic elements given their ability to capture and express resistance genes harbored by different gene cassettes [13]. Class 1 integrons, the most common integrons, have a complete structure containing a 5′-conserved segment, variable gene cassettes, and 3′-conserved segment. Integrons with gene cassettes have mostly been reported in Gram-negative bacteria, especially in Enterobacteriaceae [12, 14, 15]. Several studies have investigated AMR and mobile resistance elements in aquaculture systems [8, 16, 17]. Previous studies have found that class 1 integrons are the most predominant type of integrons detected in *Aeromonas* isolated from aquatic animals and the environment [18, 19]. It has also been shown that aminoglycoside resistance genes (*aadA1* and *aadA2*) and trimethoprim resistance genes (*dfrA1* and *dfrA12*) are the most prevalent resistance genes carried by class 1 integrons [19–21]. Integrated fish-livestock aquaculture systems seem to have a negative effect on ecosystems; however, studies on the genetics of AMR in integrated aquaculture systems are limited. This study aimed to determine the occurrence and characteristics of class 1 integrons in *Aeromonas* spp. isolated from integrated fish-livestock farms in Guangdong Province, China, and to provide information regarding the risk of AMR resistance in integrated farming systems.

2. Materials and Methods

2.1. Sample Collection. Eight ponds belonging to three types of freshwater farms in Zhaoqing, Foshan City, and Guangdong Province, South China, were selected. Three pig fishponds (PF1, PF2, and PF3), two goose-fish ponds (GF1 and GF2), and three nonintegrated farms (F1, F2, and F3) were sampled between September and October 2014. The fishes reared in these ponds were tilapia (*Oreochromis aureus*), grass carp (*Ctenopharyngodon idella*), and mud carp (*Cirrhinus mrigala*). For all of these farming systems, the Xijiang River was the main water source. All the investigated integrated farms applied animal waste directly to the fishponds. Furthermore, the sediments were seldom removed or left untreated.

Ten fish of the same type were collected by the farmers from each pond. Manure of pigs or geese was obtained from pig or goose farms near the fishponds. Five representative sites in the pond, including locations for animal waste discharge and fish-feeding hacks, were selected as sampling points. At each sampling point, a water sample of approximately 2 L was collected from 1 m below the water surface. Approximately 100 g of the sediment sample was collected from the 10 cm water-sediment interface. In total, 72 fish samples (20 grass carp, 20 tilapias, and 32 mud carp), 35 pig manure, 33 samples of goose manure, 35 samples of pond water samples, and 35 sediment samples were collected from both integrated and nonintegrated farms. All the samples were placed in separate plastic bags, stored in a cooler, and transported to the laboratory within 12 h of collection.

2.2. Bacterial Isolation and Genetic Identification. Fish were euthanized by immersion in MS222 solution (Sigma, Beijing, China) (250 mg/L; 25–30°C) that was assumed to cause rapid unconsciousness according to the American Veterinary Medical Association (AVMA) guidelines [22]. Pretreatment of fish and environmental samples was conducted as described previously, with some modifications [23, 24]. In brief, approximately 25 g of the gills and intestines were aseptically removed using sterile scissors and forceps, cut into small pieces, and placed in a sterile blender bag containing 250 mL of alkaline water and peptone medium (Oxoid, UK). For solid samples, 5 g of sediment or manure was suspended in a 100 mL alkaline water peptone medium. All sample suspensions were shaken on a horizontal shaker for 30 min at 200 r/min and left to stand for 1 h at room temperature, after which bacterial suspensions were obtained. All applicable international, national, and institutional guidelines for the care and use of animals were followed.

Ten-fold serial dilutions of each sample, including pond water and sample suspensions, were prepared in a sterile saline solution (0.85% NaCl). Each dilution (0.1 mL) was then spread on Rimler–Shotts agar (Oxoid, UK) and incubated at 28 ± 2°C for 18–24 h. Individual yellow colonies were subsequently identified using an oxidation test [25]. The oxidase-positive isolates were further identified by PCR amplification of the 16S rRNA and *gyrB* genes, as previously

described [26, 27]. Nucleotide sequences were analyzed using BLAST in GenBank (https://blast.ncbi.nlm.nih.gov/). No more than 3 *Aeromonas* strains were selected from each sample.

2.3. Antimicrobial Susceptibility Tests. Antimicrobial susceptibility tests (ASTs) of *Aeromonas* isolates were performed according to the broth microdilution method described in the Clinical and Laboratory Standards Institute (CLSI) guideline VET03 [28]. The antimicrobial agents tested included ampicillin (AMP), cefotaxime (CTX), chloramphenicol (CHL), florfenicol (FFC), thiamphenicol (THI), neomycin (NEO), gentamicin (GEN), amikacin (AMK), nalidixic acid (NAL), norfloxacin (NOR), enrofloxacin (ENR), ciprofloxacin (CIP), sulfamonomethoxine (SMM), sulfadiazine/trimethoprim (SD/TMP), sulfamethoxazole/trimethoprim (SXT), tetracycline (TET), doxycycline (DOX), and nitrofurantoin (NIT). Antimicrobial agents were double diluted using Mueller–Hinton broth (Oxoid, UK) in 96-well plates at the following ranges: AMP (2–128 mg/mL), CTX (0.03–64 mg/mL), CHL (2–64 mg/mL), FFC (1–32 mg/mL), THI (2–32 mg/mL), NEO (1–64 mg/mL), GEN (0.25–16 mg/mL), AMK (0.5–64 mg/mL), NAL (0.5–128 mg/mL), NOR (0.03–16 mg/mL), ENR (0.008–8 mg/mL), CIP (0.004–8 mg/mL), SMM (16–512 mg/mL), SD/TMP (2.5/0.5–20/4 mg/mL), SXT (9.5/0.5–76/4 mg/mL), TET (0.5–64 mg/mL), DOX (0.5–32 mg/mL), and NIT (4–64 mg/mL).

Aeromonas spp. isolates were inoculated on Rimler–Shotts agar (Oxoid, UK), and incubated at $28 \pm 2^{\circ}$C for 18–24 h. Growth was resuspended in 2 mL of sterile PBS, adjusting the concentration to 1.5×10^8 CFU/mL in accordance with the 0.5 McFarland standard. The turbidity was adjusted using a turbidimeter (BioMérieux, USA). The bacterial suspension was delivered to the wells at a final concentration of approximately 5×10^5 CFU/mL. Inoculated plates were incubated at $28 \pm 2^{\circ}$C for 24–28 h. *Escherichia coli* ATCC 25922 was used as a quality control strain.

Currently, no criteria have been established for interpreting AST results for aquatic pathogens, except for *Aeromonas salmonicida* subsp. *salmonicida*. The minimal inhibitory concentration (MIC) results were interpreted, and the isolates were divided into resistant, intermediate, and susceptible groups according to the CLSI guideline VET01 (5th ed.) for animal isolates [29]. Multiple-drug resistance (MDR) was considered when isolates were resistant to three or more different antimicrobial classes. The multiple antibiotic resistance (MAR) index was determined using the procedure described by Krumperman [30]. The MAR index over 0.20 was presumed to be affected by high-risk environments [30].

2.4. Polymerase Chain Reaction (PCR) Assays for Detection of Integrons and Gene Cassettes. Genomic DNA was extracted using a whole-cell boiling lysate protocol [31]. PCR amplification of *intI1*, *intI2*, and *intI3* was performed using template DNA from *Aeromonas* isolates. All *intI1*-positive strains were analyzed for *sulI* and *qacE*△*1* fragments by PCR using the previously described primers [32–34].

2.5. Enterobacterial Repetitive Intergenic Consensus (ERIC)-PCR Fingerprinting. Integron-positive strains were used in the ERIC-PCR DNA fingerprinting assay. The PCR reaction mixture (TaKaRa, Dalian, China) contained 100 ng of DNA, 0.2 mmol/L of each dNTP, 2.5 mmol/L $MgCl_2$, $10 \times$ PCR buffer, 1.5 U of Taq DNA polymerase, and 2 μmol/L of each ERIC primer (ERIC-F: 5′-ATG TAA GCT CCT GGG GAT TCA C-3′; ERIC-R: 5′-AAG TAA GTG ACT GGG GTGA GCG-3′) [35]. The PCR conditions were as described by Versalovic et al. [35]. Isolates with ERIC-PCR fingerprint patterns with an index greater than 0.80 were considered clonally related [36].

2.6. Plasmid Analysis. Plasmid isolation from integron-positive strains was performed using the QIAGEN Plasmid Midi Kit (QIAGEN, Germany) according to the manufacturer's protocol, with several modifications, such as on-ice incubation, centrifugation, using elution buffers at room temperature and 65°C, sequential elution, and isopropanol storage (−20°C). The yield, purity, and integrity were evaluated using a spectrophotometer (Thermo Fisher Scientific, NanoDrop-1000, USA). Plasmid profiles were determined by 0.8% agarose gel electrophoresis.

2.7. Statistical Analysis. The ERIC-PCR fingerprints were compared and analyzed using Tocan Gel analysis software (Shanghai, China). Cluster analysis was performed using NTSYS-pc software V2.2, according to the method reported by Dos Anjos Borges et al. [36]. Based on Dice's similarity coefficient (1% position tolerance) and the unweighted pair group method, arithmetic averages (UPGMAs) were used and similarity values greater than 80% were classified as the same genotype, representing the same clonal type. The variables were compared using Fisher's exact test. Statistical significance was considered at $P \leq 0.05$, highly significant at $P \leq 0.01$, and extremely significant at $P \leq 0.001$.

3. Results

3.1. Isolation of Aeromonas spp. from Farm Samples. A total of 481 presumed *Aeromonas* strains were isolated from the integrated and nonintegrated farms. Species identification of the 481 *Aeromonas* strains was performed using PCR amplification of the 16S rRNA and *gyrB* genes. The dominant *Aeromonas* species was *A. veronii* (51.4%), while other *Aeromonas* species included *A. jandaei* (22.7%), *A. caviae* (10.8%), *A. sobria* (8.5%), *A. hydrophila* (6.0%), *A. simiae* (0.4%), and *A. schubertii* (0.2%) (Table 1).

3.2. Antimicrobial Susceptibility of Aeromonas. The frequencies of antimicrobial resistance of *Aeromonas* from integrated and nonintegrated farms are shown in Table 2. *Aeromonas* isolates were resistant in varying degrees to 16 of the 18 tested antimicrobial agents. Resistance was most

Table 1: Distribution of *Aeromonas* spp. in different sources from farm samples.

Aeromonas spp. (no., %)	Pig manure	Goose manure	Fish	Pond water	Pond sediment
A. veronii (247, 51.4%)	2	0	139	69	37
A. jandaei (109, 22.7%)	1	0	33	36	39
A. caviae (52, 10.8%)	12	0	13	7	20
A. sobria (41, 8.5%)	0	0	23	14	4
A. hydrophila (29, 6.0%)	1	0	7	9	12
A. simiae (2, 0.4%)	2	0	0	0	0
A. schubertii (1, 0.2%)	0	0	1	0	0
Total (481)	18	0	216	135	112

prevalent to ampicillin (97.9%) (intrinsic resistance), sulfamonomethoxine (52.6%), and nalidixic acid (45.1%) (Table 2). Most isolates (>85%) were sensitive to fluoroquinolones, tetracyclines, amphenicol, aminoglycosides, and β-lactams. All the isolates were sensitive to amikacin and nitrofurantoin.

The diversity of antimicrobial resistance was compared among different sources (pig manure, fish, pond water, and pond sediment) from integrated and nonintegrated farms (Table 2). High resistance rates of 94.4% and 72.1% for nalidixic acid and 66.7% and 68.0% for sulfamonomethoxine, respectively, were observed in isolates from pig slurry and fish samples. The frequencies of nalidixic acid, sulfadiazine/trimethoprim, sulfamethoxazole/trimethoprim, florfenicol, thiamphenicol, and chloramphenicol resistance in *Aeromonas* isolates from pig slurry and fish were significantly higher than those in pond water and sediment ($P < 0.05$). The MDR ratios for pig slurry and fish (38.9% and 20.5%, respectively) were significantly higher than those for pond water and sediment samples from integrated farms (12.0% and 5.3%, respectively) ($P < 0.05$) (Table 2). MDR was identified in 47 isolates (9.8%) from integrated farms, with an MAR index ranging from 0.06 to 0.72 from integrated farms, whereas no multiple-drug-resistant isolates were identified in nonintegrated farms (Table 2) ($P < 0.001$).

3.3. Detection and Characterization of Integrons and Gene Cassettes. Among the 481 *Aeromonas* strains, 50 (10.4%) were positive for *intI1*, whereas *intI2* and *intI3* were not detected in any isolate. All integron-positive isolates also contained *sul1* and *qacE*$\triangle$*1,* which indicated that these isolates contained structurally complete integrons. All 50 integron-positive strains were isolated from integrated farms, whereas no integrons were detected in the nonintegrated farms. Of the 50 *intI1*-positive isolates, 36 (72%) harbored gene cassettes. Ten types of gene cassette arrays were determined by sequencing, including *dfrA17*, *dfrA12-orfF-aadA2*, *dfrB4-catB3-aadA1*, *catB8*, *aac6(6′)-Ib-cr-arr-3*, *aac-II-bla*$_{\text{OXA-21}}$*-catB3*, *aar2-aacA4-drfA1-orfC*, *aac(6′)-Ib-cr*, *dfrA15,* and *dfrB4-catB3-bla*$_{\text{OXA-10}}$*-aadA1* (Table 3). Among them, *dfrA17* was the most predominant type of gene cassette array associated with seven isolates, followed by *dfrA12-orfF-aadA2* and *dfrB4-catB3-aadA1*, each of which was associated with six isolates (Table 3). The characteristics of 50 integron-positive isolates are listed in Table 4.

The comparison of the resistance profiles of integron-positive and integron-negative strains to 18 antimicrobial agents showed significantly higher resistance rates to 13 drugs in integron-positive strains ($P < 0.001$) (Figure 1). In addition, there are 96.0% (48/50) of the integron-positive strains that displayed multiple-drug resistance with a MAR index from 0.11 to 0.67 (Table 4). The resistance ratios of the 18 antimicrobial agents in integron-positive and integron-negative strains are listed in Table 5. Among the carriers of the gene cassettes, strains harboring *aac(6′)-cr-aar3* and *aac(6′)-cr* displayed resistance to 13 and 11 drugs, respectively. Other gene cassette carriers displayed resistance to only three to six types of drugs. Strains that did not carry gene cassettes (empty integrons) were found to be resistant to sulfamonomethoxine. More details on the resistance phenotypes of different gene cassette carriers are shown in Figure 1 and Table 5.

3.4. ERIC-PCR Analysis of Integron-Positive Isolates. ERIC-PCR analysis was performed to analyze the genetic similarity of the 50 *Aeromonas* isolates carrying class 1 integrons (Figure 2(a)). These isolates were assigned to 16 major clusters based on 80% similarity and were designated as A to P (Table 3, Figure 2(b)). Each cluster was further subdivided into different ERIC types at the individual strain level, based on more than 95% similarity between the strains (Table 4). Strains showing differences in one or more bands were considered to belong to the different ERIC types. Thus, 42 ERIC types were obtained from 16 ERIC clusters among the 50 integron-positive strains (Table 4, Figure 2(b)). The results of the cluster analysis revealed that some integron-positive isolates were clonally related. Two fish isolates (7F9-1 and 7F9-2) showed indistinguishable ERIC patterns. Both isolates 7F9-1 and 7F9-2 were "empty" with no gene cassettes inserted between the conserved segments of the integron. One strain (isolate 5S33) from the pond sediment and one strain (isolate 5W13) from the pond water also showed indistinguishable ERIC-PCR patterns. Isolates 5S33 and 5W13 carry the same gene cassette (*catB8*). More details on the ERIC-PCR clusters of the integron-positive strains are presented in Table 4.

3.5. Plasmid Analysis. Plasmid analysis showed that single and/or multiple plasmids, ranging from 1.5 to 23 kb, were detected in 40% (20/50) of integron-positive strains (Table 3 and Supplementary Figure 1). Plasmids were not restricted

Table 2: Comparison of *Aeromonas* spp. in different sources from integrated farms and nonintegrated farm resistant to 18 antimicrobial agents.

Antimicrobial agents	All of the isolates no. (%) ($n = 481$)	Pig manure no. (%)	Fish no. (%)		Pond water no. (%)		Pond sediment no. (%)		Total no. (%)	
		Integrated farm ($n = 18$)	Integrated farm ($n = 122$)	Nonintegrated farm ($n = 94$)	Integrated farm ($n = 92$)	Nonintegrated farm ($n = 43$)	Integrated farm ($n = 75$)	Nonintegrated farm ($n = 37$)	Integrated farm ($n = 307$)	Nonintegrated farm ($n = 174$)
AMP	471 (97.9%)	16(88.9%)	118 (96.7%)	94 (100%)	91 (98.9%)	43 (100%)	72 (96.0%)	37 (100%)	297 (96.7%)*	174 (100%)
NAL	217 (45.1%)	17 (94.4%)^{a}	88 (72.1%)***^{b}	29 (30.9%)	44 (47.8%)**^{c}	9(20.9%)	19 (25.3%)^{d}	11 (29.7%)	168 (54.7%)***	49 (28.2%)
SMM	253 (52.6%)	12 (66.7%)	83 (68.0%)***	37 (39.4%)	53 (57.6%)**	10 (23.3%)	44 (58.7%)*	14 (37.8%)	192 (62.5%)***	61 (35.1%)
SD/TMP	59 (12.3%)	8 (44.4%)^{a}	30 (24.6%)***^{ab}	1 (1.1%)	14 (15.2%)**^{bc}	0	5 (6.7%)^{c}	1 (2.7%)	57 (18.6%)***	2 (1.1%)
SXT	59 (12.3%)	8 (44.4%)^{a}	30 (24.6%)***^{ab}	1 (1.1%)	14 (15.2%)**^{bc}	0	5 (6.7%)^{c}	1 (2.7%)	57 (18.6%)***	2 (1.1%)
THI	58 (12.1%)	7 (38.9%)^{a}	29 (23.8%)***^{ab}	1 (1.1%)	14 (15.2%)*^{bc}	1 (2.3%)	5 (6.7%)^{c}	1 (2.7%)	55 (17.9%)***	3 (1.7%)
FFC	42 (8.7%)	6 (33.3%)^{a}	26 (21.3%)***^{a}	0	12 (13.0%)**^{ab}	0	4 (5.3%)^{b}	0	48 (15.6%)***	0
CHL	25 (5.2%)	5 (27.8%)^{a}	16 (13.1%)***^{a}	0	3 (3.3%)^{bc}	0	1 (1.3%)^{c}	0	25 (8.1%)***	0
DOX	16 (3.3%)	2 (11.1%)	9 (7.4%)**	0	2 (2.2%)	1 (2.3%)	2 (2.7%)	0	15 (4.9%)**	1 (0.6%)
CTX	13 (2.7%)	0	9 (7.4%)**	0	2 (2.2%)	0	2 (2.7%)	0	13 (4.2%)**	0
CIP	14 (2.9%)	1 (5.6%)	9 (7.4%)**	0	3 (3.3%)	0	1 (1.3%)	0	14 (4.6%)**	0
NOR	11 (2.3%)	1 (5.6%)	8 (6.6%)*	0	2 (2.2%)	0	0	0	11 (3.6%)**	0
ENR	3 (0.6%)	0	0	0	3 (3.3%)	0	0	0	3 (1.0%)	0
TET	9 (1.9%)	0	8 (6.6%)**	0	1 (1.1%)	0	0	0	9 (2.9%)*	0
NEO	4 (0.8%)	0	4 (3.3%)	0	0	0	0	0	4 (1.3%)	0
GEN	1 (0.2%)	0	1 (0.8%)	0	0	0	0	0	1 (0.3%)	0
NIT	0	0	0	0	0	0	0	0	0	0
AMK	0	0	0	0	0	0	0	0	0	0
Multiple resistance	47 (9.8%)	7 (38.9%)^{a}	25 (20.5%)***^{a}	0	11 (12.0%)*^{b}	0	4 (5.3%)^{bc}	0	47 (15.3%)***	0
MAR index	73 (15.2%)	9 (50.0%)^{a}	35 (28.7%)***^{a}	2 (2.1%)	18 (19.6%)*^{b}	1 (2.3%)	7 (9.3%)^{bc}	1 (2.7%)	69 (22.5%)***	4 (2.3%)

Note. *P value <0.05, **$P < 0.01$, and ***$P < 0.01$. Superscript letters indicate $P < 0.05$.

Table 3: Characteristics of integron-positive strains carrying different gene cassettes.

Types of gene cassette arrays	GenBank accession no.	No. of strains	ERIC clusters	No. of strains carrying plasmid	Size of plasmid (kb)
*dfrA*17	KR067581.1	7	G, H, M, P	2	1.5–23
*dfrA*12-*orfF*-*aadA*2	KR067578.1	6	A, C, G, M, J	2	2–7.5
*dfrB*4-*catB*3-*aadA*1	KR067582.1	6	G, I, L	4	3–15
*catB*8	KR067580.1	5	K	0	
aac(6′)-Ib-cr-arr-3	KR868994.1	5	E, F	4	6.5–15
aac6-II-bla$_{OXA-21}$-*catB*3	KR067583.1	3	E, J	2	2.5–15
*aar2-aacA*4-*drfA*1-*orfC*	KR067585.1	1	H	0	
aac(6′)-Ib-cr	KR868995.1	1	F	1	2–3
*dfrA*15	KR868993.1	1	L	1	15
*dfrB*4-*catB*3-*bla*$_{OXA-10}$-*aadA*1	KR067584.1	1	G	1	15
Empty integron		14	B, C, D, J, N, O	4	1.8–15

to a specific gene cassette array of isolates but appeared to be present in isolates carrying the *aac(6′)-Ib-cr* gene (83.3%, 5/6), or *dfrB4-catB3-aadA1* (71.4%, 5/7). In addition, four isolates (7F2-3, 7F5-2, 7F6-1, and 7F6-2) carrying the *aac(6′)-Ib-cr* gene and two isolates (7F4-2 and 7W4-6) carrying the *dfrA12-orfF-aadA2* appeared to share similar plasmid profiles (Table 4).

4. Discussion

4.1. AMR in Fish-Livestock Integrated Farms. Integrated farming systems in the countryside are a major source of increase in farmers' incomes. However, they also represent ecological impacts and potential food safety hazards [23, 37–39]. Studies from China have reported high frequencies of antimicrobial resistance to multiple drugs in bacteria isolated from integrated farms [12, 40]. In this study, isolates from pig manure and fish were more resistant to the tested antimicrobial agents than those from the aquaculture environment. The frequencies of nalidixic acid, sulfadiazine/trimethoprim, sulfamethoxazole/trimethoprim, florfenicol, thiamphenicol, and chloramphenicol resistance in *Aeromonas* isolates from pig manure and fish were significantly higher than those from pond water and sediment ($P < 0.05$). In the pig-fish integrated farm, pig manure water was flushed into the ditch and then discharged into the pond without treatment. Pig-manure samples were collected from the ditch, and only 18 *Aeromonas* strains were isolated. *Aeromonas* was not dominant in pigs and had lower isolation rates than other sources; however, higher resistance rates of *Aeromonas* from pig manure were determined in this study. This is presumably due to the extensive use of antimicrobials in livestock husbandry. In addition, we found that the risk of the emergence of AMR from livestock husbandry is significantly higher in integrated farms as compared to that from nonintegrated farms ($P < 0.05$). Similarly, no *Aeromonas* strains were isolated from goose manure, but isolates from fish and environmental samples from two goose-fish farms showed high multiple-drug resistance, twenty-one of which carried integrons. In goose-fish integrated farms, the increase in water geese stocking density has also led to bacterial pollution. Excessive use of antimicrobial agents in feed has been reported to reduce bacterial infections in geese and has a negative effect on antimicrobial resistance [41, 42]. These data imply that antimicrobial resistance may spread from livestock to the aquatic environment. A study by Petersen and Dalsgaard [43] showed that a significantly higher level of resistant *Enterococci* spp. was found in the intestines of fish from ponds where chicken manure was discharged compared to the level of resistance present in fish from control ponds. We also found that 15.3% of isolates were resistant to multiple antimicrobial agents in samples from integrated farms, whereas no multiple-drug-resistant isolates were observed in nonintegrated farms. This implies that livestock manure might be a source of ARB in aquaculture systems. In our previous study, multiple-drug resistance was detected in *Aeromonas* spp. isolated from cultured freshwater animals [31]. We also found multiple resistant phenotypes in *Aeromonas* strains from fish and pig slurries, indicating that bacterial resistance originates from animals. The frequencies of resistance to certain extended-spectrum antimicrobial agents in *Aeromonas* were moderately high compared with those in *Enterobacteriaceae* isolates reported in previous studies [23, 40]. Therefore, the reduction in the sensitivity of *Aeromonas* to certain drugs warrants further investigation.

4.2. Integrons in Fish-Livestock Integrated Farms. Class 1 integrons are also found extensively in *Aeromonas* spp. isolated from aquatic animals as well as from the environment and are associated with a variety of gene cassettes [44]. The prevalence of integrons was also assessed in the present study, wherein class 1 integrons were detected in 10.4% of isolates. The association of *sul1* and *qacEΔ1* indicated that integrons contained the typical 3′-conserved region in these 50 *intI1*-positive strains. This prevalence is comparable to that reported for fish and aquatic environments in other geographical locations [19, 45]. Ten types of gene cassette arrays were identified in this study, and 36 integron-positive strains (72%) carried gene cassettes. The amplicon sequences revealed gene cassettes encoding dihydrofolate reductases (*drfA1*, *dfrA12*, *dfrA17*, *dfrA15*, and *dfrB4*), aminoglycoside adenylyl transferases (*aadA1* and *aadA2*), aminoglycoside acetyl transferase (*aacA4* and

Table 4: Characterization of 50 integron-positive*Aeromonas* isolates from integrated-farms.

Strains[a]	Sources	Farms[a]	Molecular identification	Gene cassette arrays	ERIC clusters	Plasmid	Resistance phenotypes
2W05	Pond water	GF2	*A. veronii*	*dfrA17*	G1	−	FFC/THI/SMM/NAL/AMP/SXT
2F4-7	Fish	GF2	*A. veronii*	*dfrA17*	G2	−	THI/SMM/NAL/AMP/SXT
2F4-6	Fish	GF2	*A. veronii*	*dfrA17*	H1	+	THI/SMM/NAL/AMP/SXT
2W13	Pond water	GF2	*A. veronii*	*dfrA17*	H2	−	THI/SMM/NAL/AMP/SXT
2F4-5	Fish	GF2	*A. veronii*	*dfrA17*	M1	−	FFC/THI/NAL/AMP/SXT
2W09	Pond water	GF2	*A. veronii*	*dfrA17*	M2	+	CHL/FFC/THI/SMM/NAL/AMP/SXT
2W19	Pond water	GF2	*A. sobria*	*dfrA17*	P1	+	SMM/DOX/NAL/AMP/SXT
2F2-4	Fish	GF2	*A. veronii*	*dfrA12-orfF-aadA2*	A1	−	CHL/FFC/THI/SMM/NAL/AMP/SXT
5S13	Sediment	PF1	*A. caviae*	*dfrA12-orfF-aadA2*	C1	−	CHL/FFC/THI/SMM/NAL/AMP/SXT
2F7-2	Fish	GF2	*A. veronii*	*dfrA12-orfF-aadA2*	G3	−	SMM/NAL/AMP/SXT
2W11	Pond water	GF2	*A. veronii*	*dfrA12-orfF-aadA2*	M3	−	CHL/FFC/THI/SMM/NAL
7F4-2	Fish	PF3	*A. veronii*	*dfrA12-orfF-aadA2*	J1	+	FFC/THI/SMM/NAL/CIP/ENR/AMP/SXT
7W4-6	Pond water	PF3	*A. veronii*	*dfrA12-orfF-aadA2*	J2	+	CHL/FFC/THI/SMM/NAL/CIP/NOR/AMP/CTX/SXT
2F2-2	Fish	GF2	*A. veronii*	*dfrB4-catB3-aadA1*	G4	−	SMM/NAL/AMP/SXT
2F5-5	Fish	GF2	*A. veronii*	*dfrB4-catB3-aadA1*	I1a	+	THI/SMM/NAL/AMP/SXT
2F7-1	Fish	GF2	*A. veronii*	*dfrB4-catB3-aadA1*	I1b	+	FFC/THI/SMM/NAL/AMP/CTX/SXT
2W15	Pond water	GF2	*A. veronii*	*dfrB4-catB3-aadA1*	I2	−	SMM/NAL/AMP/SXT
2W18	Pond water	GF2	*A. veronii*	*dfrB4-catB3-aadA1*	I3	+	CHL/FFC/THI/SMM/NAL/AMP/SXT
2F8-1	Fish	GF2	*A. sobria*	*dfrB4-catB3-aadA1*	L1	+	FFC/THI/SMM/NAL/AMP/SXT
2F1-2	Fish	GF2	*A. veronii*	*catB8*	K1a	−	THI/SMM/NAL/AMP/SXT
2F5-3	Fish	GF2	*A. veronii*	*catB8*	K1b	−	THI/SMM/NAL/AMP/SXT
5S33	Sediment	PF1	*A. veronii*	*catB8*	K2a	−	SMM/SXT
5W13	Pond water	PF1	*A. veronii*	*catB8*	K2a	−	FFC/THI/SMM/NAL/AMP/SXT
5W14	Pond water	PF1	*A. veronii*	*catB8*	K2b	−	CHL/FFC/THI/SMM/NAL/NEO/AMP/SXT
7F2-3	Fish	PF3	*A. caviae*	*aac6(6′)-Ib-cr-arr-3*	E3	+	CHL/FFC/THI/SMM/NAL/CIP/ENR/AMP/SXT
7F2-2	Fish	PF3	*A. caviae*	*aac6(6′)-Ib-cr-arr-3*	F1	−	SMM/NAL/NOR/ENR/AMP/SXT
7F5-2	Fish	PF3	*A. caviae*	*aac6(6′)-Ib-cr-arr-3*	F2a	+	CHL/FFC/THI/SMM/DOX/TET/NAL/CIP/NOR/AMP/CTX/SXT
7F6-1	Fish	PF3	*A. caviae*	*aac6(6′)-Ib-cr-arr-3*	F2b	+	CHL/FFC/THI/SMM/DOX/TET/NAL/CIP/NOR/AMP/CTX/SXT
7F6-2	Fish	PF3	*A. caviae*	*aac6(6′)-Ib-cr-arr-3*	F2c	+	CHL/FFC/THI/SMM/DOX/TET/NAL/CIP/NOR/AMP/CTX/SXT
5P3-3	Pig manure	PF1	*A. caviae*	*aac6-II-bla*$_{OXA-21}$*-catB3*	E1	−	CHL/FFC/THI/SMM/NAL/NEO/AMP/SXT
6S1-3	Sediment	PF2	*A. caviae*	*aac6-II-bla*$_{OXA-21}$*-catB3*	E2	−	CHL/FFC/THI/SMM/NAL/AMP/SXT
5P2-3	Pig manure	PF1	*A. caviae*	*aac6-II-bla*$_{OXA-21}$*-catB3*	J4	+	SMM/AMP/SXT
6S2-2	Sediment	PF2	*A. hydrophila*	*aar2-aacA4-drfA1-orfC*	H3	−	FFC/THI/SMM/NAL/CIP/AMP/SXT
7F8-3	Fish	PF3	*A. caviae*	*aac(6′)-Ib-cr*	F5	+	CHL/FFC/THI/SMM/DOX/TET/NAL/CIP/NOR/AMP/CTX/SXT
2F6-4	Fish	GF2	*A. veronii*	*dfrA15*	L2	+	CHL/FFC/THI/SMM/NAL/AMP/SXT
5P2-1	Pig manure	PF1	*A. caviae*	*dfrB4-catB3-bla*$_{OXA-10}$*-aadA1*	G4	+	THI/SMM/AMP
2F3-7	Fish	GF2	*A. veronii*	−	B1	−	SMM/NAL/AMP/SXT
2W14	Pond water	GF2	*A. veronii*	−	B2	−	CHL/FFC/THI/SMM/NAL/AMP/SXT
5S16	Sediment	PF1	*A. veronii*	−	B3	−	SMM/NAL/CIP/AMP/SXT
7F9-1	Fish	PF3	*A. caviae*	−	B4	+	CHL/FFC/THI/SMM/DOX/TET/NAL/CIP/NOR/AMP/CTX/SXT
7F9-2	Fish	PF3	*A. caviae*	−	B4	−	CHL/FFC/THI/SMM/TET/NAL/CIP/NOR/CN/AMP/CTX/SXT
7F10-3	Fish	PF3	*A. caviae*	−	B5	+	CHL/FFC/THI/SMM/DOX/TET/NAL/CIP/NOR/AMP/CTX/SXT
7F6-3	Fish	PF3	*A. caviae*	−	C2	+	CHL/FFC/THI/SMM/DOX/TET/NAL/CIP/NOR/AMP/CTX/SXT
2W12	Pond water	GF2	*A. caviae*	−	D1	−	SMM/NAL/AMP/SXT
5P3-2	Pig manure	PF1	*A. caviae*	−	D2	−	THI/SMM/AMP/SXT
6S2-3	Sediment	PF2	*A. sobria*	−	J3	−	CHL/FFC/THI/SMM/DOX/NAL/AMP/SXT/CTX
5P3-4	Pig manure	PF1	*A. simiae*	−	N1	−	FFC/SMM/NAL/NEO/AMP/SXT
6F6-2	Fish	PF2	*A. veronii*	−	N2a	−	FFC/THI/SMM/NAL/AMP/SXT
6F10-3	Fish	PF2	*A. veronii*	−	N2b	−	CHL/FFC/THI/SMM/NAL/NEO/AMP/CTX/SXT
5P1-5	Pig manure	PF1	*A. caviae*	−	O1	−	SMM/NAL/AMP/SXT

Note. P, pig manure; F, fish; W, pond water; S, sediment. GF, goose-fish integrated farm; PF, pig-fish integrated farm; AMP, ampicillin; CTX, cefotaxime; CHL, chloramphenicol; FFC, florfenicol; THI, thiamphenicol; NEO, neomycin; NAL, nalidixic acid; NOR, norfloxacin; ENR, enrofloxacin; CIP, ciprofloxacin; SMM, sulfamonomethoxine; SD/TMP, sulfadiazine/trimethoprim; SXT, sulfamethoxazole/trimethoprim; TET, tetracycline; DOX, doxycycline.

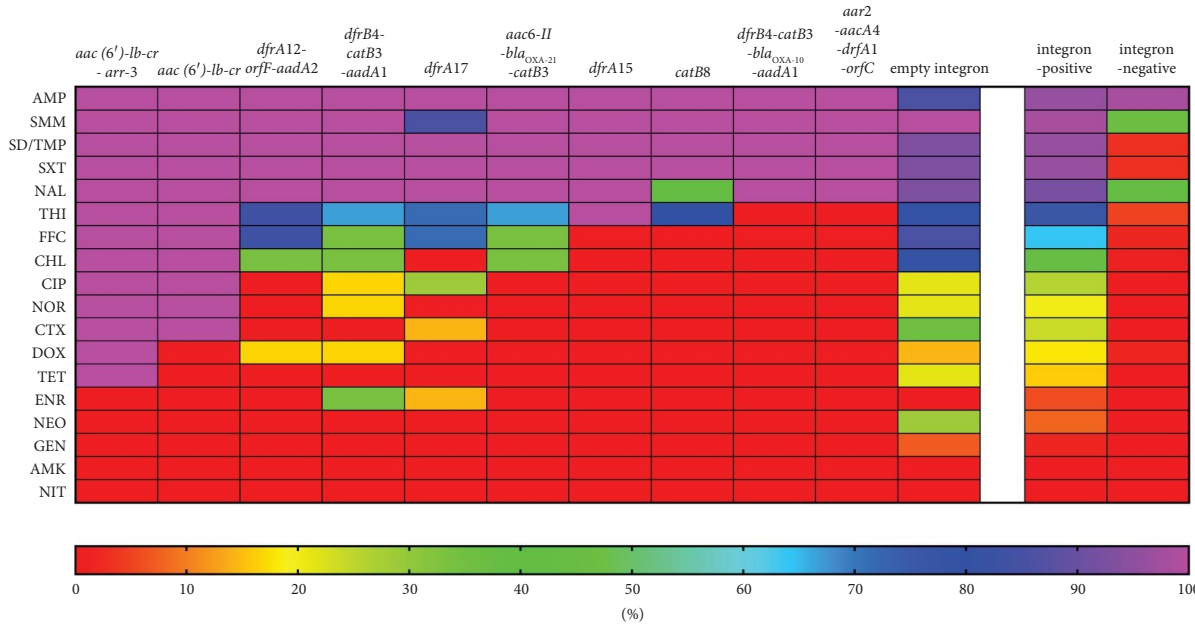


Figure 1: Antimicrobial resistance phenotypes of *Aeromonas* strains carrying different gene cassette arrays.

aac(6′)-II), chloramphenicol acetyltransferase (*catB3* and *catB8*), β-lactamase (bla_{OXA-21}), and plasmid-mediated quinolone resistance (*aac(6′)-Ib-cr*), and ribosyl transferase (*aar-3*).

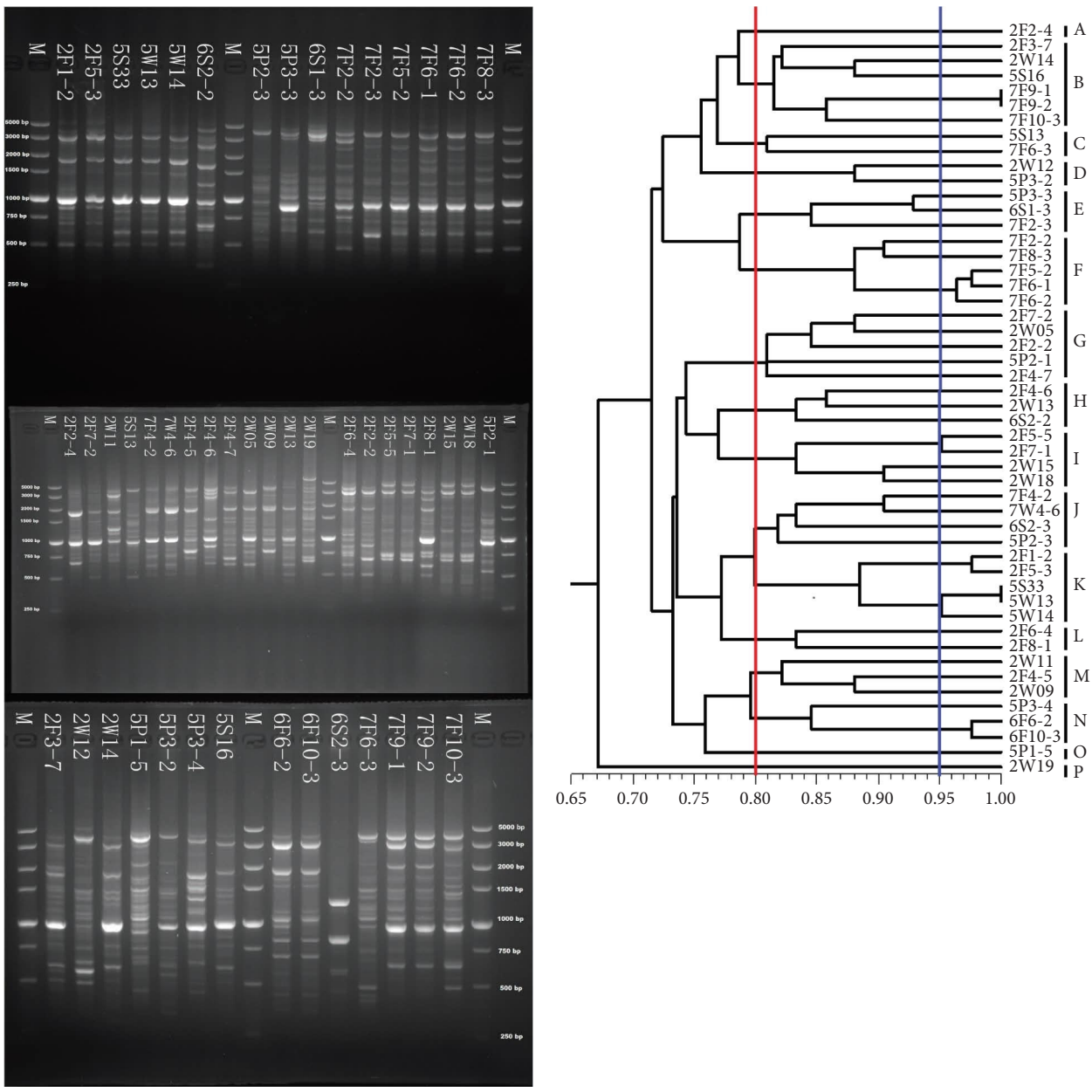
Bacteria acquire resistance to antimicrobial agents mainly because of genetic resistance determinants. In this study, 44% (22/50) of integron-positive*Aeromonas* strains carried various dihydrofolate reductase genes and displayed resistance to sulfamonomethoxine and sulfamethoxazole/trimethoprim. Chloramphenicol acetyltransferase genes were carried by 30% (15/50) of the strains, and 66% (10/15) of the strains were resistant to florfenicol or thiamphenicol. The *aac (6′)-Ib-cr* gene was identified in six isolates and displayed resistance to ciprofloxacin and enrofloxacin. Previous studies reported that the most prevalent resistance genes, *addA* and *dfr,* confer resistance to streptomycin and sulfamethoxazole/trimethoprim [17, 46]. The *aac(6′)-Ib-cr*, *arr3*, and *catB3* genes are responsible for resistance to fluoroquinolone, rifampin, and chloramphenicol, respectively [17, 19]. In these studies, the strains harboring *aac(6′)-Ib-cr* genes displayed multiple-drug resistance compared to strains harboring other gene cassettes. The *aac(6′)-Ib-cr* gene is a plasmid-mediated quinolone resistance gene detected in *Aeromonas* isolated from different aquatic animals and environments [19, 31]. This aminoglycoside resistance gene modified with two amino acids is generally located in a large plasmid, which also harbors resistance genes of various antimicrobial groups and contributes to multiple-drug resistance [47].

In this study, similar ERIC-PCR fingerprints, resistance phenotypes, and resistance gene cassettes were observed in strains isolated from sediment, pond water, and different fishes. This implies the clonal transmission of multiple-drug-resistant*Aeromonas* strains of different origins. In contrast, some of the isolates were phylogenetically distant. This implies that clonal and horizontal transmission of resistance occurred simultaneously between bacteria. In this study, we confirmed that mobile genetic elements might play an important role in mediating multiple-drug resistance. To define the location of the class 1 integron in the chromosome or plasmid of the isolates, plasmids were extracted and used as templates for the amplification of *intI1* genes. Results showed that 40% (20/50) of the isolates contained integrons located in the plasmids. This implies that integrons may be present in plasmids or chromosomes. Plasmids may play an important role in integrons that mediate gene transfer and facilitate the transmission of resistance among bacteria. Most of the empty integrons without gene cassettes were found in the plasmids. These may have lost gene cassettes in the absence of antimicrobial selective pressure or on the other hand, never acquired the resistance gene insert [48]. However, they can acquire new resistance genes and facilitate the dissemination of resistance in bacteria [13].

In conclusion, our study demonstrated the widespread dissemination of class 1 integrons among *Aeromonas* strains isolated from integrated farms. These results confirmed that the selective pressure enforced by the use of antimicrobial agents in livestock husbandry increases the prevalence of integrons carrying resistance genes. A large number of antimicrobial agents and ARB contaminate fish-farming systems. It is necessary to establish a risk assessment for antimicrobial resistance in aquaculture to promote the healthy development of Chinese aquaculture.

Table 5: Comparison of *Aeromonas* spp. carrying different gene cassettes arrays resistant to 18 antimicrobial agents.

Drugs	Empty integron ($n = 14$)	*dfrA17* ($n = 7$) (%)	*dfrA12-orfF-aadA2* ($n = 6$) (%)	*dfrB4-catB3-aadA1* ($n = 6$) (%)	*aac6(6′)-Ib-cr-arr-3* ($n = 5$) (%)	*catB8* ($n = 5$) (%)	*aac6-II-bla*$_{OXA-21}$*-catB3* ($n = 3$) (%)	*aac6(6′)-Ib-cr* ($n = 1$) (%)	*dfrB4-catB3-bla*$_{OXA-10}$*-aadA1* ($n = 1$) (%)	*aar2-aacA4-drfA1-orfC* ($n = 1$) (%)	*dfrA15-* ($n = 1$) (%)	Integron-positive ($n = 50$) no. (%)	Integron-negative ($n = 431$) no. (%)	*P* value
SMM	100.0	85.7	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	49 (98.0%)	204 (47.3%)	≤0.001
SD/TMP	92.9	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	48 (96.0%)	11 (2.6%)	≤0.001
SXT	92.9	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	48 (96.0%)	11 (2.6%)	0.2790
AMP	85.7	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	48 (96.0%)	423 (98.1%)	≤0.001
NAL	92.9	100.0	100.0	100.0	100.0	40.0	100.0	100.0	100.0	100.0	100.0	46 (92.0%)	171 (39.7%)	≤0.001
THI	78.6	71.4	83.3	66.7	100.0	80.0	66.7	100.0	0.0	0.0	100.0	38 (76.0%)	20 (4.6%)	≤0.001
FFC	85.7	71.4	83.3	33.3	100.0	0.0	33.3	100.0	0.0	0.0	0.0	32 (64.0%)	10 (2.3%)	≤0.001
CHL	78.6	0.0	33.3	33.3	100.0	0.0	33.3	100.0	0.0	0.0	0.0	22 (44.0%)	3 (0.7%)	≤0.001
CIP	21.4	28.6	0.0	16.7	100.0	0.0	0.0	100.0	0.0	0.0	0.0	13 (26.0%)	1 (0.2%)	≤0.001
CTX	35.7	14.3	0.0	0.0	100.0	0.0	0.0	100.0	0.0	0.0	0.0	12 (24.0%)	1 (0.2%)	≤0.001
NOR	21.4	0.0	0.0	16.7	100.0	0.0	0.0	100.0	0.0	0.0	0.0	10 (20.0%)	1 (0.2%)	≤0.001
DOX	14.3	0.0	16.7	16.7	100.0	0.0	0.0	0.0	0.0	0.0	0.0	9 (18.0%)	7 (1.6%)	≤0.001
TET	21.4	0.0	0.0	0.0	100.0	0.0	0.0	0.0	0.0	0.0	0.0	8 (16.0%)	1 (0.2%)	≤0.001
NEO	28.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	4 (8.0%)	0	≤0.001
ENR	0.0	14.3	0.0	33.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3 (6.0%)	0	≤0.001
GEN	7.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1 (2.0%)	0	0.1040
AMK	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0	0	-
NIT	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0	0	-



(a) (b)

Figure 2: ERIC-PCR profiles of 50 integron-positive isolates. (a) Electrophoresis of ERIC-PCR fingerprints of 50 integron-positive isolates. M denotes the DL 5000 DNA marker (Takara, Dalian, China). (b) Dendrogram of ERIC-PCR fingerprint patterns of 50 integron-positive isolates. The bands were analyzed by applying the dice coefficient and the matrix was clustered using the UPGMA method. Isolates with >80% similarity represent the same clonal type.

Data Availability

The datasets generated and/or analyzed in this study are available from the National Center for Biotechnology Information (NCBI): KR067581.1, KR067578.1, KR067582.1, KR067580.1, KR868994.1, KR067583.1, KR067585.1, KR868995.1, KR868993.1, and KR067584.1.

Ethical Approval

All animal procedures were approved by the Committee on Ethics of Animal Experiments of the Pearl River Fisheries Research Institute, China Academy of Fishery Sciences. The study was conducted in accordance with the Chinese government principles for the utilization and care of vertebrate

animals used in testing, research, and training (https://www.gov.cn/gongbao/content/2017/content_5219148.htm).

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

FZ and LJ participated in the study design and discussion. YD, YF, and AT carried out the major experiments. YD and FZ analyzed the data. YD wrote the manuscript. FZ and ZH revised the manuscript. All the authors have read and approved the final version of the manuscript.

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Supplementary Materials

Supplementary Figure 1: Plasmid profiles of 50 integron-positive isolates. M1 denotes λ-Hind III digest marker (Takara, Dalian, China), and M2 denotes the DL 15000 DNA marker (Takara, Dalian, China). (*Supplementary Materials*)

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