Master's Thesis

Exploring Plasmid-Mediated Multidrug Resistance, Virulence, and Metal/Biocide Resistance in *Vibrio* Spp. Isolated from Influent and Effluent Water Samples of Fish Farms in Jeju, South Korea

ADEEL FAROOQ

Department of Biotechnology

GRADUATE SCHOOL

JEJU NATIONAL UNIVERSITY

June 2018

Exploring Plasmid-Mediated Multidrug Resistance, Virulence, and Metal/Biocide Resistance in *Vibrio* Spp. Isolated from Influent and Effluent Water Samples of Fish Farms in Jeju, South Korea

ADEEL FAROOQ

(Supervised by Professor Tatsuya Unno)

A thesis submitted in partial fulfillment of the requirement

for the degree of Master of Science

June 2018

This thesis has been examined and approved by

_____

Dong-Sun Lee, PhD., College of Applied Life Sciences, Jeju National University

_____

Soo-Je Park, PhD., College of Natural Life Sciences, Jeju National University

_____

Tatsuya Unno, PhD., College of Applied Life Sciences, Jeju National University

Department of Biotechnology

GRADUATE SCHOOL

JEJU NATIONAL UNIVERSITY

CONTENT

List of Figures

List of Tables

Abbreviations

MDR	Multidrug resistance
ARGs	Antibiotic resistance genes
CLSI	Clinical and Laboratory Standards Institute
EUCAST	European Committee on Antimicrobial Susceptibility Testing
CARD	Classification of antibiotic resistance database
BacMet	Antibacterial biocide and metal resistance genes
ORFs	Open reading frames
Prodigal	Prokaryotic Dynamic Programming Gene finding Algorithm
OTC	Oxytetracycline
AMX	Amoxicillin
CHL	Chloramphenicol
CIP	Ciprofloxacin
NAL	nalidixic acid
FFC	Florfenicol
N	Neomycin
SUL	Sulfamethoxazole
TET	Tetracycline
MLS	Macrolide-lincosamide-streptogramin
PH	Phenicol
PM	Polymyxin

FLQ	Fluoroquinolone
AMG	Aminoglycoside
SU	Sulfonamide
RIF	Rifampin
BET	Beta-lactam
GLY	Glycopeptides
PEP	Peptide

ABSTRACT

The objective of this study was to investigate the plasmid profiling, virulence genes, metal/biocide resistance genes and plasmid sequencing of multi-drug resistant (MDR) *Vibrio* in influent (inflow) and effluent (discharged) water samples of fish farms in Jeju, South Korea. MDR isolates identified through disc diffusion susceptibility tests were subjected to plasmid profiling. One hundred fifty *Vibrio* species isolates were obtained from each influent and effluent water sample. All MDR isolates were subjected for plasmid profiling. The greater number of bacteria were enumerated from effluents (61%) comparing to influents (39%). High incidence of neomycin, sulfamethoxazole, amoxicillin and oxytetracycline resistance was observed among the isolates, particularly more resistance rate in effluent *Vibrio*. In contrast, *Vibrio* isolates were more susceptible to florfenicol, chloramphenicol, ciprofloxacin, and nalidixic acid. Among 99 (influent 39 and effluent 60) MDR isolates, a total of 58 plasmid harboring isolates (influent 38 and effluent 20) were identified and showed Sixteen different resistance antibiograms. Influent MDR isolates showed six distinct plasmid profiles with size ranging from 2 kb to >10 kb, whereas effluent MDR showed four plasmid profiles with a molecular weight ranging from 1.7 kb to >10 kb. All three hundred isolates were also screened for the presence of *Vibrio* virulence genes, thermostable direct hemolysin (*tdh*), cholera toxin (*vct*), and cytotoxin-hemolysin (*vvh*). There were 17 (12 influent and 5 effluent) and 27 (8 influent and 19 effluent) *Vibrio* isolates found to harbor thermostable direct hemolysin gene (*tdh*) and cytotoxin-hemolysin gene (*vvh*), respectively. In addition, two isolates (02 influent) were found to harbor cholera toxin gene (*vct*). Detection of 19 *Vibrio vulnificus, vvh* genes, and 8 *Vibrio parahaemolyticus, the* genes in the fish farm discharged water may be responsible for their potential pathogenicity. Largely virulence genes were confined to chromosomes, only 9 (3 influent, 6 effluents) detected in plasmid bearing MDR *Vibrio.* Twenty-

four (12 influent, 12 effluent) plasmid bearing MDR *Vibrio* isolates selected for sequencing at Illumina Miseq platform predicted 14 influent putative plasmid sequences and 17 effluent putative plasmid sequences. Furthermore, 17 antibiotic resistance genes (ARGs) in influent plasmids and 21 ARGs in effluent plasmids were identified. Influent and effluent putative plasmid sequences encoding ARGs were resistant to 12 classes of antibiotics, among which beta-lactams (BET), glycopeptides (GLY) and peptides (PEP) were only confined to effluents whereas macrolide-lincosamide-streptogramin (MLS) and rifampin (RIF). Moreover, it was observed that chromosome-mediated antibiotic resistance contributed to the overall antibiotic resistance showed by plasmid bearing MDR *Vibrio* isolates. Two influent and three effluent putative MDR plasmids were carrying metal/biocide resistance genes. In addition, Illumina high throughput sequencing of metagenome plasmids from influents and effluents samples identified a higher abundance of ARGs encoding beta-lactams in effluents. Beta-lactam ARGs carrying bacterial genomes were studied to be involved in the foodborne diseases. Our results showed that more diverse plasmid profiles and antibiograms, higher abundance of plasmid carrying MDR *Vibrio*, virulence and metal/biocide resistance genes present in effluent samples, suggesting the accumulation of uneaten feed, fish excretion, residual antibiotics, metal/biocide contaminants and antibiotic resistance genes (ARGs) in fish farm tanks that may provide selective pressure and co-selection for acquisition and horizontal transfer of ARGs. The appearance of multidrug resistance plasmid carrying resistance to heavy metals is alarming. The presence of plasmid-bearing MDR *Vibrio* isolates in fish farm effluent water may contribute to the dissemination of MDR genes to the environments, which ultimately poses threat to human health.

1. INTRODUCTION

The *Vibrio* species are an important component of marine ecosystems[1] reported to cause mortalities and extensive economic losses in aquaculture production worldwide[2]. About one-third of *Vibrio* species are potential human pathogens involving in water and seafood-related outbreaks of gastrointestinal and wound infections in humans[3].

An excessive amount of antibiotics has been widely used to treat *Vibrio* infections in aquaculture, which has caused a high incidence of antibiotic resistance[4]. These antibiotic resistance genes (ARGs) can be easily disseminated through horizontal gene transfer[5]. Hence, antimicrobial resistant *Vibrio* not only disseminate ARGs between fish pathogens and other aquatic bacteria but also between other bacteria belonging to the different genera[6,7].

Some *Vibrio* isolates are known to cause diseases in fish and humans. The main pathogenic species are *V. cholera*, *V. parahaemolyticus*, and *V. vulnificus*[8]. There is a need to explore the antibiotic resistance patterns in fish farm pathogenic *Vibrio.*

Moreover, Metal salts and biocides are used for decontamination in fish farming. Copper and zinc are frequently used in aquaculture as antifouling whereas mercury used in fish-feed[9,10]. Fish farm bacteria may become resistant to metals, which may lead to co-selection of other antibiotics[11].

In South Korea, inland fish farms commonly apply ‘flow-through’ method, by which seawater near a fish farm is pumped in, passed through fish tanks and discharged untreated directly into the environment by a drainage system. This fish farm effluent is likely to repeatedly introduce uneaten feed, fish excretion, residual antibiotics and ARGs into coastal area, consequently affecting the human health[12].

In the present study, we investigated the abundance, antibiotic susceptibility pattern, plasmid profiling, virulence genes, MDR plasmid sequencing and metal/biocide resistance of *Vibrio* spp. isolated from influent and effluent water samples in coastal fish farms located in the northern part of Jeju island, South Korea to provide insights into the occurrence of plasmid harboring multi-drug resistant (MDR) *Vibrio* isolates. In addition, we explored the antibiotic resistance genes in metagenome plasmids of influents and effluents by high throughput sequencing (Hiseq).

STUDY OBJECTIVES

- Enumerate *Vibrio* from fish farm influent and effluent water samples
- Analyze antibiotic resistance pattern in influent and effluent *Vibrio* isolates
- Plasmid profiling of multidrug resistance (MDR) *Vibrio* isolates
- Antibiograms analysis to explore the relation between plasmid profiles and antibiotic resistance pattern
- Detect prevalence of potentially pathogenic *Vibrio*
- Identification of relation between potentially pathogenic and plasmid bearing MDR *Vibrio*
- Conduct Plasmid sequencing to identify putative plasmid sequences bearing ARGs
- Analyzing *in vitro* and *in silico* studied antibiotic resistance pattern in a plasmid carrying MDR *Vibrio*
- Detect metal/biocide resistance genes from putative plasmid sequences
- Comparative analysis of influent and effluent metagenome plasmids based on ARGs encoding antibiotics

2. MATERIALS AND METHODS

2.1 Sample collection

Influents and effluents of fish farms were sampled in the northern part (Haengwon Fish farms) of Jeju Island, South Korea, using four-liter sterilized sampling bottle in triplicates at the beginning of September 2017. Twelve liter of water samples (4L+4L+4L) were collected each from influent (33°33'35.1"N 126°48'58.5"E) and effluent (33°33'39.5"N 126°48'53.0"E) facilities (Figure 1). All the samples were transferred back to the laboratory within 2 hours after the collection.

Figure 1. Location of influent and effluent water sampling sites on Jeju island.

2.2 Enumeration of *Vibrio* spp.

Influent and effluent water samples (30ml, 10ml, and 3ml) were directly filtered through a sterile mixed cellulose ester membrane filter with 0.2-um pore size and a diameter of 47 mm (Hyundai Micro co., LTD. Korea). The membranes were then transferred onto the surface of

Thiosulfate Citrate Bile Salts Sucrose Agar (TCBS, DIFCO^{TM} Becton & Dickenson, USA) agar plates for incubation. Distinctive 300 (150 each from influent and effluent) colonies on TCBS agar plates were streaked again for single colony isolation. After culturing individual colonies on Mueller Hinton II (MH) agar (BBL^{TM} Becton & Dickenson, USA), all the isolates were preserved at -80ºC in 50% of glycerol in phosphate buffer saline for further experiments.

2.3 Analysis of antibiotic resistance pattern

The susceptibility of isolates was examined by the disc diffusion method according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI). Briefly, MH agar containing 1.5% NaCl and eight different antibiotic discs including oxytetracycline (OTC), amoxicillin (AMX), chloramphenicol (CHL), ciprofloxacin (CIP), nalidixic acid (NAL), florfenicol (FFC), neomycin (N) and sulfamethoxazole (SUL) representing different classes of antibiotics used in this study (Table 1). Initially, three hundred isolates were screened based on resistance to AMX, OTC, CIP, and CHL. MDR isolates that are resistant to more than one antibiotics were screened for plasmid profiling. Plasmid-bearing *Vibrio* were tested for four additional antibiotics NAL, FFC, N, and SUL. All agar plates were incubated at 37°C for 18 hours. The results were expressed as susceptible (S), intermediate (I) or resistant (R) following the guidelines of the CLSI, except for FFC and N that were evaluated based on European Committee on Antimicrobial Susceptibility Testing (EUCAST) guideline[13].

Table 1. The classification of antibiotics used in this study.

Antibiotics (Class)	**Potency per disk (µg)**	**Bactericidal mechanisms**
Amoxicillin (Penicillin)	30 µg	Inhibits Cell wall synthesis
Chloramphenicol (Phenicols)	10 µg	Inhibits Protein synthesis
Oxytetracycline (Tetracyclines)	30 µg	Inhibits Protein synthesis
Ciprofloxacin (Fluoroquinolones)	5 µg	Inhibits DNA synthesis
Nalidixic acid (Fluoroquinolones)	30 µg	Inhibits DNA synthesis
Sulfamethoxazole (Sulfonamides)	25 µg	Inhibits DNA synthesis
Florfenicol (Phenicols)	30 µg	Inhibits protein synthesis
Neomycin (Aminoglycoside)	10 µg	Inhibits protein synthesis

2.4 Plasmid profiling

Plasmid DNA was extracted from an overnight culture of *Vibrio* isolates in MH broth using QIAprep spin miniprep kit (Valencia, CA, USA) according to the manufacturer's instructions. Isolated plasmid DNA samples were subjected to electrophoresis using 0.7% agarose gel for 3 hours at 50V.

2.5 Prevalence of *Vibrio* virulence genes

All three hundred isolates were screened for the detection of *Vibrio parahaemolyticus* thermostable direct hemolysin (*tdh)* gene, *Vibrio vulnificus* cytotoxin-hemolysin (*vvh*) gene and *Vibrio cholera* toxin (*vct)* gene. Genomic DNA was extracted from all the isolates using AccuPrep Genomic DNA Mini Extraction Kit (BIONEER Corporation, Republic of Korea) according to manufacturer's instructions. Detection of *the vvh* and *vct* genes was performed by PCR using three different sets of primers (Table 2).

Table 2. Primer sets used in the detection of *Vibrio* virulence genes

Virulence gene	**Product size (bp)**	**Cycling conditions**	**Primer sequence**
tdh	269	94 ˚C for 5 min, 30 cycles of 94 ˚C for 60 s, 58 ˚C for 1 min and 72 ˚C for 30 s	L-tdh: GTAAAGGTCTCTGACTTTTGGAC R-tdh: TGGAATAGAACCTTCATCTTCACC
vct	308	94 ˚C for 3 min, 30 cycles of 94 ˚C for 45 s, 55 ˚C for 30 s and 72 ˚C for 30 s	VCT-1: ACAGAGTGAGTACTTTGACC VCT-2: ATACCATCCATATATTTGGGAG
vvh	383	94 ˚C for 5 min, 30 cycles of 94 ˚C for 1 min, 60 ˚C for 30 s and 72 ˚C for 30 s	F-vvh73: CTC ACT GGG GCA GTG GCT R-vvh1113: CCA GCC GTT AAC CGA ACC A

Total Volume of each reaction mixture was 20 µl including 1 µl of each forward and reverse primer (10 pmol/µl), 16 µl of nuclease-free water and 2 µl of template DNA. Maxime PCR Premix kit (iNtRON Biotechnology, Republic of Korea) contains i-Taq DNA polymerase 2.5U, dNTPs 2.5mM each, reaction buffer 1X and gel loading buffer 1X was used for all reactions. PCR was conducted in a Bio-RAD T-100 Thermal Cycler (Bio-RAD, CA, USA) using specific programming conditions for each primer set (Table 2). PCR products were separated by gel electrophoresis using 2% agarose gel and ran for 40 minutes at 100V. The images were captured using a bench-top UV transilluminator, (Major science, CA, USA). *Vibrio parahaemolyticus* strain KCTC 2471, *Vibrio vulnificus* strain KCTC 2959^{T}, *Vibrio cholera* strain NCCP11179 were used as positive controls and nuclease-free water used as the negative control.

2.6 Plasmid DNA isolation and sequencing

Plasmid DNA was extracted from selected twenty-four plasmids bearing MDRs (12 influent and 12 effluent) using QIAprep spin miniprep kit (Valencia, CA, USA) according to the manufacturer's instructions. In addition, ATP dependent plasmid-safe DNase ((EPICENTRE® Biotechnologies, USA) was then added to remove any remaining chromosomal DNA. Plasmid DNA concentration was determined by Qubit Fluorometer (Life Technologies, Carlsbad, USA). Purified plasmid DNA samples (>5 ng/ul) were sent to Macrogen (Macrogen Inc. South Korea) for Illumina Miseq sequencing.

2.7 Plasmid sequencing analysis

Illumina MiSeq Sequencer was used for paired-end (2×300 bp) sequencing of 24 plasmids. Paired-end Miseq reads were filtered by quality using sickle (https://github.com/najoshi/sickle.git). All clean reads were de novo assembled using SPAdes[14], with the options "–sc –only-assembler "and

contigs larger than 1000 bp were kept for further analysis. PlasFlow[15] was used to extract putative plasmid and chromosome sequences from the contigs (contiguous sequences). Open reading frames (ORFs) from putative plasmid and chromosome contigs were detected by Prodigal (Prokaryotic Dynamic Programming Gene finding Algorithm)[16]. ORFs with size more than 100bp were kept for further analysis. Blastp was used to align translated gene sequences to protein sequences in the classification of antibiotic resistance database (CARD)[17], which includes 3996 ontology terms, 2506 reference sequences, and 2536 AMR detection models. The threshold was set to an alignment length ≥25 amino acids, an E-value cutoff of 10−5, and an identity ≥75 %. Relevant antibiotic classes for the identified antibiotic resistance genes (ARGs) were also obtained from CARD. The prevalence of metal resistance genes was identified by blasting ORFs extracted from putative plasmid contigs against BacMet against the BacMet (antibacterial biocide and metal resistance genes database)[18] by using Blastp. The BLAST hits were filtered with an identity ≥60% and alignment lengths of more than 25 amino acids. ARG-miner[19] (http://bench.cs.vt.edu/argminer) was used to obtain evidence about ARGs being carried by pathogenic bacterial genomes involved in diseases.

2.8. Metagenome plasmid DNA extraction and sequencing

Twelve liters of each of the influent and effluent water samples were directly filtered through a sterile mixed cellulose ester membrane filter with 0.2-um pore size and a diameter of 47 mm (Hyundai Micro co., LTD. Korea). Membranes were wrapped in the aluminum foil and kept at -

20ºC. Total community DNA from the membranes was extracted using DNeasy Power Water Kit (Qiagen: USA) as per manufacturer's instructions. The concentration of extracted DNA was measured with Qubit fluorometer (Life Technologies, Carlsbad, USA). The chromosomal DNA fragments were removed by treating with Plasmid-Safe ATP-Dependent DNase (EPICENTRE® Biotechnologies, USA) for 16 h at 37°C by doubling the recommended ATP and enzyme amount. The presence of chromosomal DNA contamination in the plasmid DNA was monitored by detecting Eubacterial 16S rRNA gene by polymerase chain reaction (PCR) using primers Eub338F and Eub518R[20]. Purified plasmid DNA (18.7 ng/ul Influent and 22.6 ng/ul Effluent) were subjected to High-throughput sequencing conducted at Macrogen (Macrogen Inc. South Korea) using Illumina Hiseq 3000 platform with paired-end sequencing,150-bp reads strategy. Approximately 4.87 and 5.07 GB of data were generated for influents and effluents respectively.

2.9 Metagenome plasmid DNA sequencing data analysis

Raw reads were filtered by Sickle to remove the low-quality reads. Clean reads were assembled into contigs using SPAdes assembler with default parameters. All the assembled contigs were further subjected for plasmid detection by PlasFlow using default parameters and excluding contigs smaller than 1000 bp that classify contigs into plasmid, chromosome, and unclassified sequences. Prodigal was used for ORF prediction from plasmid contigs. All the ORFs were searched against CARD database using Blastp with an E-value of ≤10-5. The relative abundance of ARGs was described using "ppm" namely one read per million reads

3. RESULTS AND DISCUSSION

3.1 *Vibrio* abundance

The greater number of *Vibrio* were present in effluent (61%) samples comparing to influent (39%) samples. It has been previously reported that fish farm sediments receive a large amount of organic matter due to uneaten food, antibiotic remains, and fecal materials, which consequently increases the number of *Vibrio* in fish farm[7,21]. Although we did not measure the amount of organic matter in the fish tank, accumulated organic matter in fish tanks may have increased the abundance of *Vibrio*.

3.2 Analysis of antibiotic resistance pattern

Initial antimicrobial susceptibility tests for 300 isolates against four antibiotics identified 99 MDR *Vibrio* isolates (Table 3). Briefly, AMX resistance was 39% and 70%, OTC resistance was 21% and 31%, CIP resistance was 23% and 25%, CHL resistance was 6% and 8%, among influent and effluent isolates respectively. The greater number of effluent isolates showed resistance against AMX and OTC.

Table 3. Percentages of *Vibrio* isolates resistant to antibiotics

	Influents (n=150)			**Effluent (n=150)**		
Antibiotics	**R**	**I**	**S**	**R**	**I**	**S**
OTC	21.59	26.32	52.09	30.78	48.55	20.67
AMX	38.74	48.69	12.57	70.11	26.49	3.4
CIP	23.48	55.28	21.24	25.20	65.69	9.12
CHL	6.78	11.61	81.61	8.32	28.15	63.53

In this study, we found 99 MDR isolates that were resistant to more than two antibiotics tested. Of the 99 MDR isolates, 58 were found to harbor plasmids. To investigate antibiograms

according to the size of plasmids, antibiotics susceptibility testing with 4 additional antibiotics were conducted (Table 4). Among the 58 isolates, high resistance was observed among effluent *Vibrio*.

Table 4. Multidrug resistance profile of 58 plasmids harboring *Vibrio* isolates. R, I, and S indicate resistant, intermediate, and susceptible, respectively.

Antibiotics	Influent samples (n=20)			Effluent samples (n=38)		
	R	I	S	R	I	S
OTC	10	5	5	17	19	2
AMX	14	4	2	26	12	-
CIP	8	8	4	15	19	4
CHL	4	6	10	12	16	10
NAL	7	10	3	21	11	6
N	16	3	1	34	2	2
FFC	5	1	14	9	3	26
SUL	13	5	2	33	3	2

More than 68% of the influent and effluent isolates were resistant to N, SUL, and AMX. While a portion of antibiotic-resistant plasmid-bearing isolates was similar between influent and effluent, a greater number of an influent plasmid bearing isolates were shown for OTC resistance (influent 65% and effluent 45%). Tetracycline has long been the most commonly used antimicrobial in Korean fisheries, particularly for the treatment of infection by *Vibrio* species[22]. In the present study, high influents resistance against OTC may be due to the presence of tetracycline resistance genes in the Seawater. Tetracycline resistance genes tet (M) and tet (S) genes which encode ribosomal protection proteins and tet (A)-(E), tet (G) and tet (Y) encoding active efflux pumps already been reported in *Vibrio* from sea water in previous studies[23,24].

Whereas, less than half the influent and effluent isolates were resistant to FFC, CHL, CIP, NAL, with exception of effluents which were resistant against NAL (above 50%). Effluents showed intermediate resistance against NAL which is in agreement with the previous study where half of the *Vibrio* isolates were resistant to quinolones[25]. Quinolones were effective against *Vibrio*[26]. This increasing resistance in Enterobacteriaceae against quinolones is most probably owing to the presence of plasmid-mediated quinolone resistance (PMQR)[27].

Similarly, the result that most of the *Vibrio* isolates in the present study were resistant to amoxicillin agrees with the previous report that showed the resistance of *Vibrio* isolates against AMX isolated from farmed fish and shellfish in Korean coastal areas[26]. Sulfonamides have a wide spectrum of antimicrobial activity, thus widely used all over the world including agriculture, livestock, aquaculture and human therapy. In the present study, 85% of influents and 86.8% of the effluents *Vibrio* isolates were resistant to sulfamethoxazole. In other studies, sulfonamide resistance is already reported in aquacultures[26,28,29]. This is suggestive of widespread of the sulfa resistance gene in the sea water and fish farm discharged water. Around 85% of the influent and effluent *Vibrio* isolates were resistant to neomycin. These results are in consensus with the previous study that showed the high resistance of *Vibrio* isolates against neomycin and other aminoglycosides[30,31].

It was analyzed that most of the *Vibrio* isolates were susceptible to chloramphenicol and florfenicol. Our results are in agreement with the previous studies showing susceptibility of *Vibrio* to phenicols[27,32 ,25].

3.3 Plasmid profiling

Of the 99 isolates analyzed, 58 had one or more plasmids with four and six distinct plasmid profiles in influent and effluent *Vibrio* isolates, respectively (Figure 2).

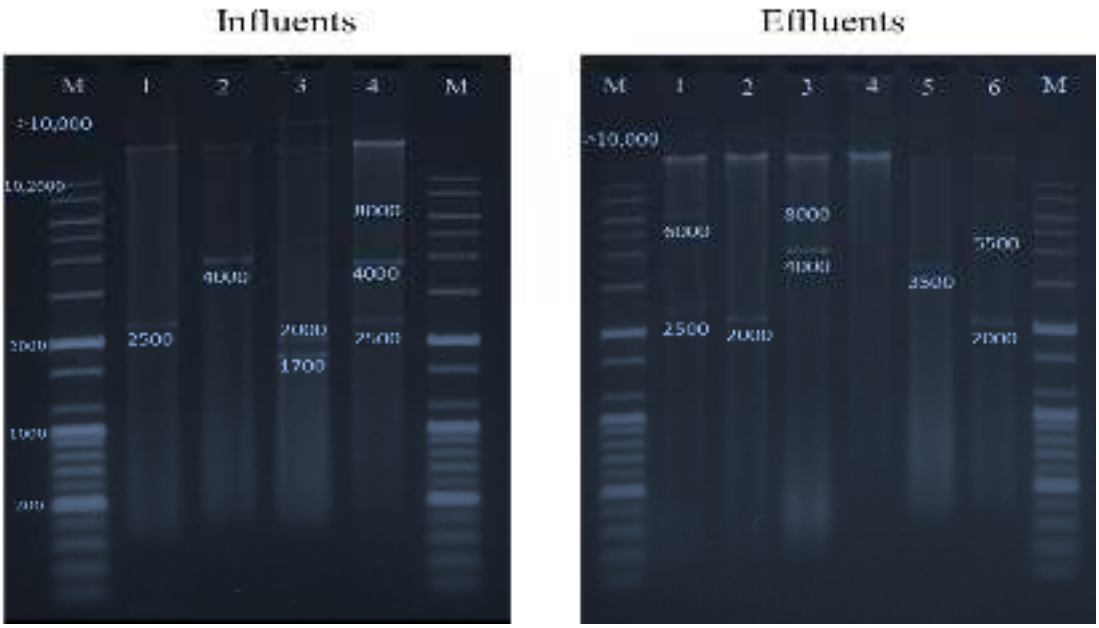

Figure 2. Plasmid profiling of influent and effluent isolates.

Influent *Vibrio* isolates were found to have four distinct molecular weight plasmids ranging from 1,700 bp to >10,000 bp whereas effluent isolates had six different molecular weight plasmids ranging from 2,000 bp to >10,000bp. Plasmids with molecular weights 2,000bp, 2,500bp, 4,000bp, 8,000bp and >10,000 bp were common among *Vibrio* isolates obtained from influent and effluent water samples. Plasmids with size 3,500bp, 5,500bp, 6,000bp were only present among effluents and only one plasmid molecular weight 1,700 bp was present in influent isolates.

Detection of *Vibrio* isolates harboring plasmids ranging from 1.7 kb to >10 kb is consistent with a previous study where 50% of the *Vibrio* spp. having plasmids from 1.5 kb to 26 kb were isolated from coastal water[33].

Total twenty-two antibiograms were encountered, out of which, ten were only present among effluents and six in influents whereas rest were shared (Table 5).

Table 5. The relationship between plasmid profiles and resistance patterns of 20 influent and 38 effluent isolates.

Plasmid Size (bp)	Antibiograms	Frequency	
		Influents	Effluents
1700	OTC, AMX, NAL, N	03	-
2000	AMX, CHL, NAL, N, SUL	-	05
	OTC, AMX, NAL, N	03	02
	CIP, N, NAL, SUL	-	02
	CIP, N, NAL, FFC, SUL	-	03
2500	OTC, AMX, CIP, N, SUL	4	-
	AMX, CHL, N, SUL	1	-
	CIP, N, FFC, SUL	05	-
	OTC, AMX, N, SUL	-	02
3500	AMX, CHL, N, SUL	-	04
	AMX, CIP, CHL, SUL	-	02
	NAL, N, FFC	-	01
4000	AMX, NAL, SUL	04	-
	OTC, AMX, CHL, N	03	-
	OTC, AMX, CIP, N, SUL	04	03
5500	AMX, CHL, N, NAL, SUL	-	05
6000	OTC, AMX, N, SUL	-	02
8000	OTC, AMX, CIP, N, SUL	04	03
>10,000	OTC, NAL, N, SUL	-	06
	CHL, NAL, N, SUL	-	01
	AMX, CIP, N, SUL	-	05
	OTC, AMX, NAL, N	03	03

Fourteen antibiograms observed having resistance against four antibiotics showing frequency of occurrence in effluents and influents as 29 and 18, respectively.

Likewise, six antibiograms showed resistance against five antibiotics with the frequency of 19 and 12 in effluents and influents respectively. Whereas two antibiograms showing resistance against three antibiotics were found among effluents and influents with the frequency of 04 and 01, respectively. Results showed the presence of diverse antibiograms particularly among MDR effluent *Vibrio* which indicates the plasmid-mediated resistance[34].

Furthermore, multiple plasmids were found in two and three isolates obtained from influent and effluent samples, respectively (Table 6).

Table 6. *Vibrio* isolates carrying more than one plasmid. Plasmid IDs starting with IN and EF indicate influent and effluent, respectively.

Plasmid ID	**Size of plasmids (bp)**	**No. of antibiograms**
Influent	1700, 2000, >10,000	OTC, AMX, NAL, N
Influent	2500, 4000, 8000	OTC, AMX, CIP, N, SUL
Effluent	2500, 6000	OTC, AMX, N, SUL
Effluent	4000, 8000	OTC, AMX, CIP, N, SUL
Effluent	2000, 5500	AMX, CHL, N, NAL, SUL

Usually, plasmids with sizes ≥30kb are thought to be R plasmid but plasmids isolated in this study in spite of the small size likely be involved in the horizontal transfer of MDR genes[32]. Moreover, total 41 out of 99 MDR isolates detected in this study were without the presence of plasmids, suggesting that MDR-related genes in this *Vibrio* spp. can potentially be obtained

through transposition, chromosomal-mediated conjugation among *Vibrio* spp. and other bacterial species contribute to antibiotic resistance in *Vibrio*[32,35]. Chromosomally mediated tetracycline resistance in *Vibrio* has already been studied which occurred through mutation[36]. It is also known that management of chromosomal mediated resistance is difficult comparing to plasmid-mediated resistance as resistant genes reside in the chromosomes[37].

3.4. Prevalence of *Vibrio* virulence genes

Among the 300 *Vibrio* isolates, there are 17 (12 influent and 5 effluent) and 27 (8 influent and 19 effluent) *Vibrio* isolates found to harbor thermostable direct hemolysin gene (*tdh*) and cytotoxin-hemolysin gene (*vvh*), respectively. In addition, two isolates (02 influent) were found to harbor cholera toxin gene (*vct*) (Fig 4,5,6).

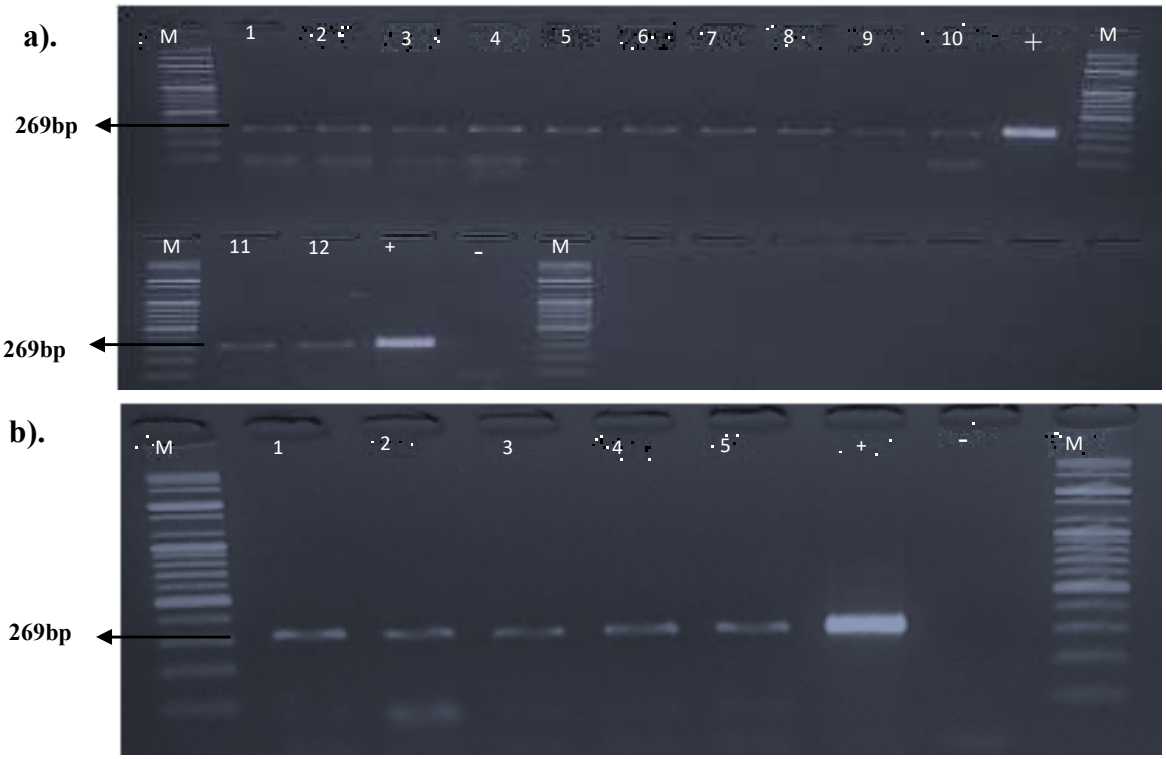

Figure 4. Gel showing the 269 bp PCR amplified *Vibrio parahaemolyticus* virulence gene *tdh.* **a)** lanes 1-10 & 12-13: influent positive isolates, M: molecular marker (100bp plus), lanes 11 & 14:

positive controls, lane 15: negative control. **b)** lanes 1-5: effluent positive isolates, M: molecular marker (1000p plus), lane 6: positive control, lane7: negative control

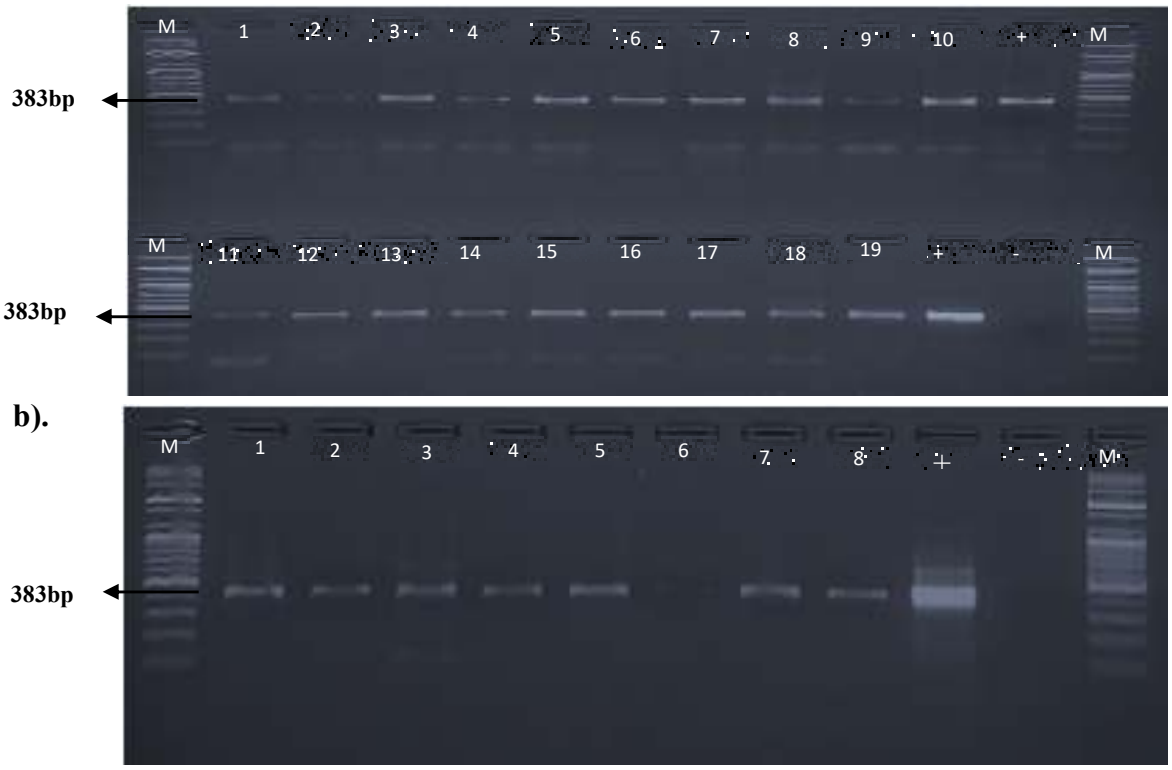

Figure 5. Gel showing the 383 bp PCR amplified *Vibrio vulnificus* gene, *vvh* **a).** lanes 1-10 & 12-20: effluent positive isolates, M: molecular marker (100bp plus), lanes 11& 21: positive controls, lane 22: negative control. **b).** lanes 1-8: influent positive isolates, M: molecular marker) lane 9: positive control, lane 10: negative control

Figure 6. Gel representation of 308 bp PCR amplified *Vibrio cholera, vct* gene. lanes 1-2: influent positive isolates, M: molecular marker, lane3: positive control, lane4: negative control.

3.4.1 The relationship between virulence genes harboring *Vibrio* isolates and their antibiotic resistance

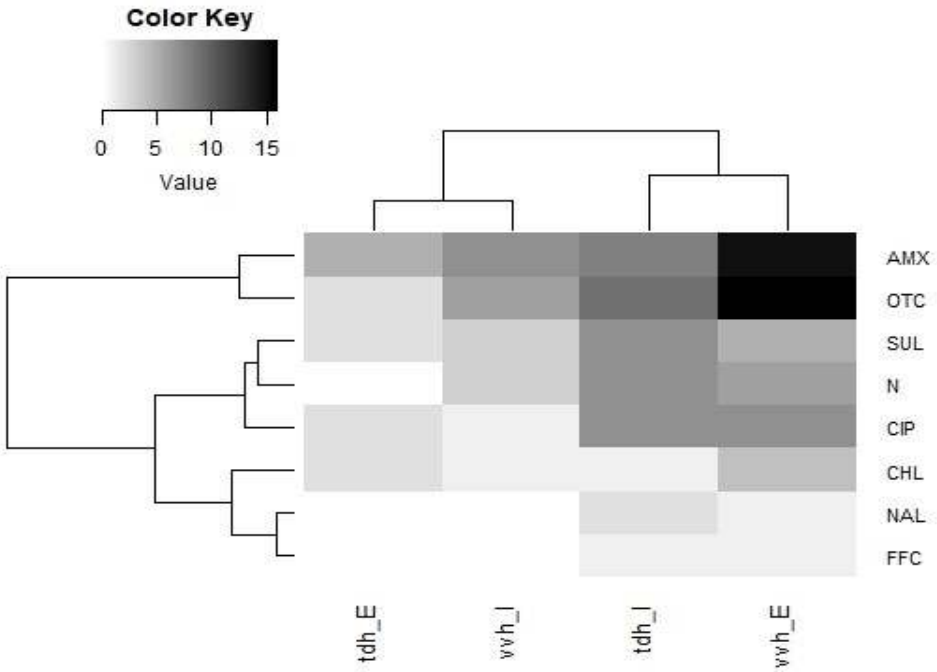

Figure 7. Heat map showing the relation between virulence genes carrying *Vibrio* isolates and antibiotic resistance.

Most of the potentially pathogenic *Vibrio* isolates are resistant to amoxicillin and oxytetracycline, whereas they are more susceptible to florfenicol, nalidixic acid, and chloramphenicol. More effluent isolates containing *vvh* were found resistant to AMX and OTC, compared to those from influents. In contrast, influent *tdh* carrying isolates seem to be resistant to various antibiotics compared to those from the effluent (Fig 7).

3.4.1 Incidence of antibiotic-resistant potentially pathogenic *Vibrio* isolates bearing plasmids

Plasmid-bearing potentially pathogenic isolates showing almost similar resistance pattern to plasmid bearing pathogenic and non-pathogenic isolates (Table 7). Most of the effluent plasmid bearing pathogenic isolates (with different virulence genes) are resistant to oxytetracycline, amoxicillin, sulfamethoxazole, and neomycin.

Table 7. The incidence of antibiotic-resistant potentially pathogenic *Vibrio* isolates bearing plasmids.

Isolates	Virulence genes	Plasmid (bp)	Antibiotic resistance							
			OTC	AMX	CIP	CHL	NAL	N	FFC	SUL
Influents	*Tdh*	1700	2	2	0	0	2	2	0	0
	Vvh	2500	2	2	2	0	0	2	0	2
	Tdh	2500	0	1	0	1	0	1	0	1
Effluents	*Vvh*	2000	0	1	1	1	2	2	1	2
	Vvh	2500	1	1	0	0	0	1	0	1
	Tdh	3500	0	1	1	1	0	0	0	1
	Vvh	3500	0	2	0	2	0	2	0	2
	Tdh	>10,000	0	1	1	0	0	1	0	1
	Vvh	>10,000	1	0	0	0	1	1	0	1

4.1. Plasmid sequencing data analysis

Illumina Miseq platform generated a total of 5.8 GB data for plasmid DNA samples. Raw reads were produced with a median of 2175581 reads/effluent sample, and 2105261 reads/influent

sample (Table). After *de novo* assembly with SPAdes and filtering (>1kb) a median of 303 contigs/influent sample and 638 contigs/effluent sample were generated. Plasmid and chromosomal contigs were isolated by using PlasFlow. Plasmid and chromosome sequences obtained from PlasFlow were annotated against NCBI nucleotide database to select best hits based on the query coverage, percentage identity, alignment length and expected size of the plasmid for plasmid bearing isolates. Total of 31(14 influent and 17 effluent) putative plasmid sequences were collected for further study. ORFs were extracted from plasmid and chromosomal sequences by using Prodigal. ORFs with size >100bp were used for further analysis. After aligning putative plasmid ORFs through Blastp against CARD, we obtained 17 influent and 21 effluent sequences carrying ARGs. In the same way, chromosomal sequences carrying ARGs were identified by blasting chromosomal ORFs against CARD.

4.2 Identification of plasmid-mediated ARGs encoding antibiotics

Twelve influent plasmids sequencing data were used to identify 17 different ARGs carrying plasmids conferring resistance to 9 classes of antibiotics, including 5 resistance genes for multi drugs (MDR), 3 for tetracycline (TET), 2 for macrolide-lincosamide-streptogramin (MLS), 2 for phenicol (PH) and 1 each for polymyxin (PM), fluoroquinolone (FLQ), aminoglycoside (AMG), sulfonamide (SU) and rifampin (RIF). On the other hand, effluent sequencing data predicted 21 different ARGs dispensing resistance to 10 antibiotic classes including 6 resistance genes for multidrug (MDR), 5 for beta-lactam (BET), 2 each for tetracycline (TET), and aminoglycoside (AMG), 1 each for polymyxin (PM), sulfonamide (SU), glycopeptides (GLY), peptide (PEP), fluoroquinolone (FLQ) and phenicol (PH).

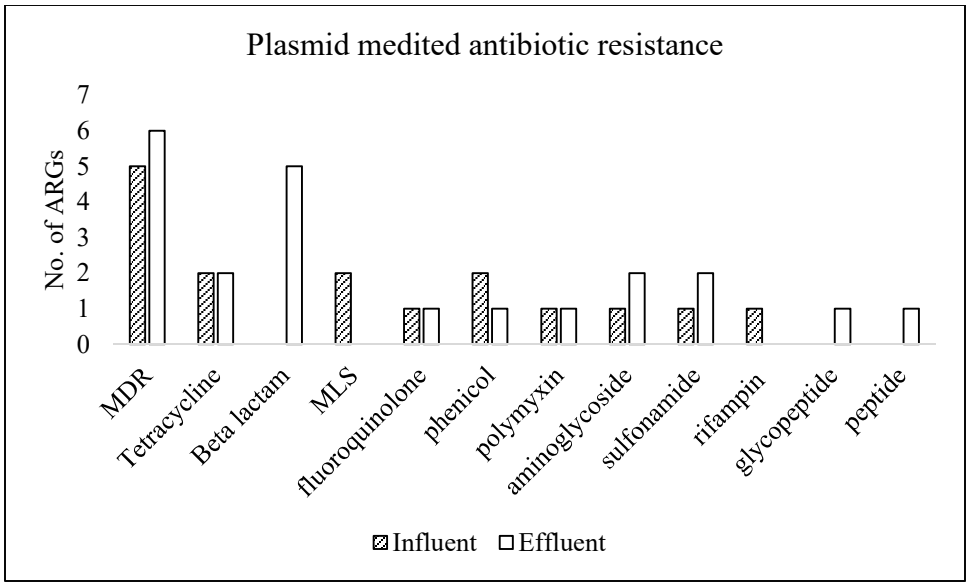

Figure 8. Graph showing plasmid-mediated antibiotic resistance in influent and effluent putative plasmids.

Our results suggested that a high number of effluent putative plasmid sequences (897) carrying ARGs (22) were detected comparing to influent putative plasmid sequences (1084) with ARGs (16). These results were quite consistent with the *in vitro* plasmid profiling and susceptibility. Furthermore, a similar trend of resistance against MDR, TET, PH, SU, PM, FLQ, and AMG, in both influent and effluent putative plasmids was observed. Resistance against BET, GLY and PEP were only confined to effluent putative plasmids. On the other hand, resistance against MLS and RIF was just present in influent putative plasmids (Fig 8).

In a previous study, 18 ARGs (3 FLQ, 1 AMG, 3 MLS, 2 TET, and 9 BET resistance genes) were detected from the marine environment. Influent putative plasmid-mediated antibiotic resistance results showed by the present study following the same trend except for the absence of BET genes. The absence of beta-lactam (BET) genes in the influent water may be because of the dilution factor present in the oceans[38].

Identification of effluent putative plasmid carrying ARGs is in consensus with a previous study conducted on fish farm bacteria have shown plasmid-mediated MDR, TET, SU, BET, FLQ, and PH[39]. Plasmid-mediated MDR, TET, FLQ is well established[40].

Detection of BET genes from putative effluent plasmids may be owing to the excessive usage of BET antibiotics in the fish farms as in a previous study, plasmids carried ARGs related to BET, AMG, SU, TET, and MLS were isolated from an aquaculture where penicillin, BET, and aminoglycoside were used for prophylactic purposes[39].

4.3 Identification of chromosome-mediated ARGs encoding antibiotics

Influent chromosomal sequences carried 43 different types of antibiotic resistance genes encoding 4 classes of antibiotics including 12 resistance genes for MDR, 11 for BET, 12 for TET and 8 for PM. Effluent chromosomal sequences identified with 38 different ARGs conferring resistance to 4 antibiotic classes including 11 resistance genes for MDR, 11 for TET, 10 for the PM and 6 for BET (Fig 9).

Results suggested that chromosome-mediated ARGs are conferring resistance to only 4 classes of antibiotics whereas plasmid-mediated ARGs responsible for more variety of antibiotic classes.

Intrinsic chromosome-mediated antibiotic resistance through naturally occurring genes to BET and MDR has been reported (levy and marshal). In previous studies, it was observed that mutations in the bacterial chromosomes are responsible for antibiotic resistance against FLQ, RIM, SU, TET, AMG, MLS, BET[41,42].

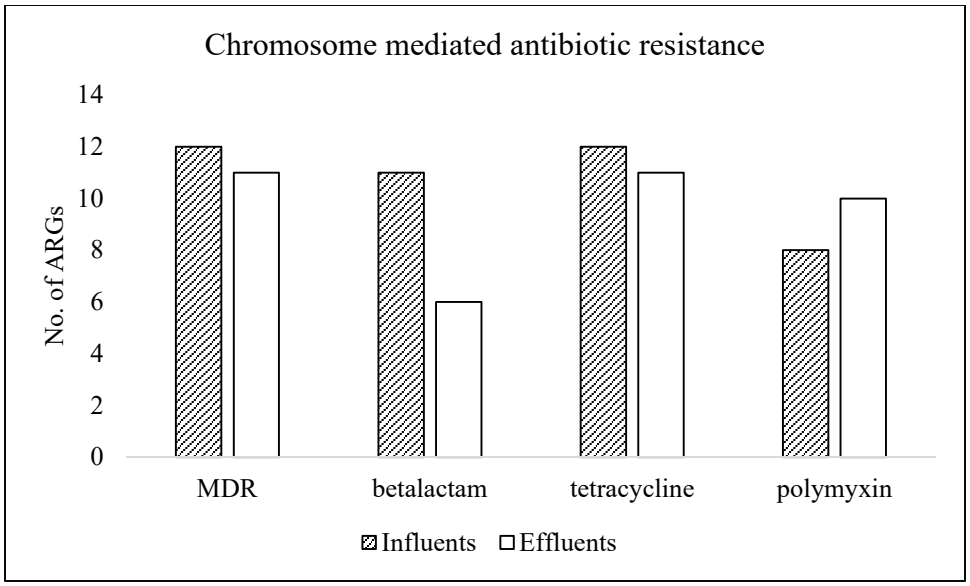

Figure 9. Graph showing percentage of chromosome-mediated antibiotic resistance in influent and effluent putative plasmids.

4.4 Comparison of *in vitro* and *in silico* analysis of antibiotic resistance

Illumina Miseq sequencing results demonstrated that antibiotic resistance dispensed by both putative plasmid and chromosome sequences is responsible for the overall antibiotic resistance presented by the plasmid bearing MDR Vibrio isolates (Table 8,9).

Although chromosome-mediated resistance is important, the real concern is plasmid-mediated resistance as it may involve in the dissemination of ARGs through horizontal gene transfer to other environments[43].

Table 8. Comparison of antibiotic resistance in influent plasmid bearing MDR *Vibrio* obtained through susceptibility testing (*in vitro*) and predicted from sequencing results (*in silico*).

***in vitro* analysis**		***in silico* analysis**	
Plasmid-bearing MDR *Vibrio*	**Susceptibility test**	**Sequencing data analysis**	
	Antibiotic Class	**Plasmid-mediated ARGs encoding antibiotics**	**Chromosome-mediated ARGs encoding antibiotics**
IN_1	TET, PEN, FLQ, AMG	TET	BET, TET, PM, MDR
IN_2	PEN, PH, AMG, SU	PH, MLS	BET, TET MDR, PM
IN_3	TET, PEN, PH, AMG	PM, PH, AMG, SU	MDR, TET BET
IN_4	PEN, PH, AMG, SU	MDR	BET, TET, MDR
IN_5	TET, PEN, FLQ, AMG, SU	TET	TET, BET, MDR
IN_6	TET, PEN, FLQ, AMG	RIF	BET, TET, PM, MDR
IN_7	PEN, FLQ, SU	MDR	BET, TET, PM, MDR
IN_8	TET, PEN, FLQ, AMG, SU	MDR	BET, TET, PM, MDR
IN_9	FLQ, AMG, PH, SU	FLQ, TET	BET, TET, MDR
IN_10	TET, PEN, FLQ, AMG, SU	MLS	TET, BET, PM, MDR
IN_11	PEN, FLQ, SU	MDR	TET, PM, MDR, BET
IN_12	TET, PEN, FLQ, AMG	MDR	TET, PM, MDR

(TET= tetracycline, PEN= penicillin, AMG= aminoglycoside, PH= phenicol, SU= sulfonamide, FLQ= fluoroquinolone, MLS= macrolide-lincosamide-streptogramin, PM= polymyxin, MDR= multidrug resistance, RIF=rifampin, BET=beta-lactam)

Table 9. Comparison of antibiotic resistance in effluent plasmid bearing MDR *Vibrio* obtained through susceptibility testing (*in vitro*) and predicted from sequencing results (*in silico*)

***in vitro* analysis**		***in silico* analysis**	
Plasmid-bearing MDR *Vibrio*	**Susceptibility Test**	**Sequencing data analysis**	
	Antibiotic class	**Plasmid-mediated ARGs encoding antibiotics**	**Chromosome-mediated ARGs encoding antibiotics**
EF_1	PEN, PH, FLQ, AMG, SU	MDR, GLY	PM, TET, MDR
EF_2	PEP, PH, AMG, SU	MDR	PM, TET, MDR, BET
EF_3	PEN, PH, FLQ, AMG, SU	BET	PM, TET, MDR
EF_4	TET, PEN, FLQ, AMG, SU	MDR	-
EF_5	TET, PEN, AMG, SU	PPT	BET, TET, PM, MDR
EF_6	PEN, FLQ, PH, SU	MDR	TET, BET, PM, MDR
EF_7	PEN, FLQ, PH, SU	MDR, SU, PH, FLQ, PM, BET	PM, TET, MDR
EF_8	TET, FLQ, AMG, SU	TET, BET	TET, PM, MDR
EF_9	PEP, PH, AMG, SU	MDR, TET	MDR, TET
EF_10	PEP, PH, AMG, FLQ, SU	BET	PM, TET, MDR, BET
EF_11	TET, PEN, FLQ, AMG	AMG	BET, TET, PM, MDR
EF_12	AMG, FLQ, SU	BET, AMG	TET, BET, PM, MDR

(TET= tetracycline, PEN= penicillin, AMG= aminoglycoside, PH= phenicol, SU= sulfonamide, FLQ= fluoroquinolone, GLY= glycopeptides, PM= polymyxin, MDR= multidrug resistance, RIF=rifampin, BET=beta-lactam, PPT= peptide)

Furthermore, evidence about beta-lactam genes carried by an effluent putative plasmid, being carried by pathogenic bacterial genomes involved in foodborne diseases was obtained from ARG-miner.

4.4 Detection of plasmid-borne Metal and Biocide resistance

Two influent putative plasmids were having resistance genes for arsenic, antimony, and sodium dodecyl sulfate. On the other hand, three effluent putative plasmids were found to contain genes conferring resistance to hydrochloric acid, hydrogen peroxide, silver, mercury, cadmium, and cyclohexane (Table 10).

Table 10. Prevalence of Biocide and Metal resistance genes in the putative plasmids sequences extracted from influent and effluent plasmid sequenced data (Miseq).

Plasmid	**Gene**	**Metal/biocide**	**Plasmid-mediated Antibiotic resistance**
Influent_1	*pgpA/ltpgpA*	Arsenic (As), Antimony (Sb)	MDR
Influent_2	*vmeT*	Sodium Dodecyl Sulfate,	MDR
Effluent_1	*eefA*	Hydrochloric acid (HCl)	MDR
Effluent_2	*cpxA*	Hydrogen Peroxide (H2O2)	MDR
Effluent_3	*robA*	Silver (Ag), Mercury (Hg), Cadmium (Cd), Cyclohexane	MDR, TET

Co-occurrence of metal/biocide and multidrug resistance was observed in the putative plasmid sequences (Table 7). This may be because metal contamination is responsible for the propagation of antibiotic-resistant bacteria through co-selection. As metals are not subjected to degradation and may remain as agents of selective pressure over long periods[44,45].

One influent putative plasmid sequence was found carrying Arsenic and antimony resistance genes. Arsenic and antimony compounds are widely distributed as pollutants in soil and aquatic

environments because of their massive usage in agriculture as herbicides, insecticides, fungicides, pesticides and treating parasitic infections in farm animals. Resistance to these metals in Gram-positive and Gram-negative pathogens and carried on MDR plasmids is already reported[46].

One effluent putative plasmid sequence was identified bearing *robA* gene responsible for silver, mercury, cadmium and cyclohexane resistance. Our results are in line with a previous study demonstrated plasmid carrying *robA* gene conferred resistance not only to Ag^{+}, Hg^{2+}, and Cd^{2+}, but also increase in resistance to several antibiotics[47].

Detection of metal resistance genes might be due to the usage of metals as feed ingredients, antifouling agents, and decontaminants. These metals are released in the form of uneaten feed or feces and settle on sediment. Thus, the metal resistance of bacteria can be triggered and disseminated. The linkage between metal and antibiotic resistances on plasmids is now well established, and, despite a reduction in the use of antimicrobial metals in medicine and agriculture, antimicrobial metal resistances are still often found on the same plasmid as an antibiotic[48].

Furthermore, two effluent putative sequences showed resistance against biocides including hydrochloric acid and hydrogen peroxide. Biocides are widely used as disinfectants in fish farming facilities. In a study including gram-negative bacteria, several genes for resistance to biocide were detected[49]. It was investigated that biocides may also co-select strains resistant to antibiotics[50,51,52].

5.1 Metagenome plasmid DNA sequencing analysis

Illumina Hiseq sequencer produced 4.87GB and 5.07GB of data for influent and effluent metagenomic plasmids, respectively. Total of 13356528 reads for influent metagenome plasmid and 13912764 reads for effluent metagenome plasmid was generated. After assembly and filtering (>1kb), we obtained 25054 influent and 24800 effluent contigs. PlasFlow isolated 7350 influent and 7357 effluent plasmid sequences. Prodigal produced 21100 ORFs from influent plasmid

sequences and 18790 ORFs from effluent metagenome sequences. Putative plasmid sequences were aligned with CARD, produced 594 influent sequences and 410 effluent sequences carrying ARGs encoding different antibiotics.

5.2 Presence of ARGs encoding antibiotics in the plasmid metagenome

Influent plasmid metagenome was found having 10058 antibiotic resistance genes encoding 19 antibiotic classes. On the other hand, effluent plasmid metagenome identified 7487 antibiotic resistance genes encoding 18 antibiotic classes. Relative abundance of influent plasmid metagenome ARGs encoding antibiotic classes was like MDR (29%), MLS (21%), TET (13%), and equal or less than 10% for pleuromutilin, BET, PEP, FLQ, aminocoumarins, AMG, PH, triclosan, fusidic acid, rifampicin, SU, trimethoprim, bicyclomycin, fosfomycin, and others. Relative abundance of effluent plasmid metagenome ARGs encoding antibiotic classes was like BET (24%), MDR (20%), MLS (17%), TET (14%), and equal or less than 10% for GLY, pleuromutilin, aminocoumarins, AMG, FLQ, PEP, PH, triclosan, trimethoprim, fosfomycin, fusidic acid, rifampicin, bicyclomycin and others (Fig 10).

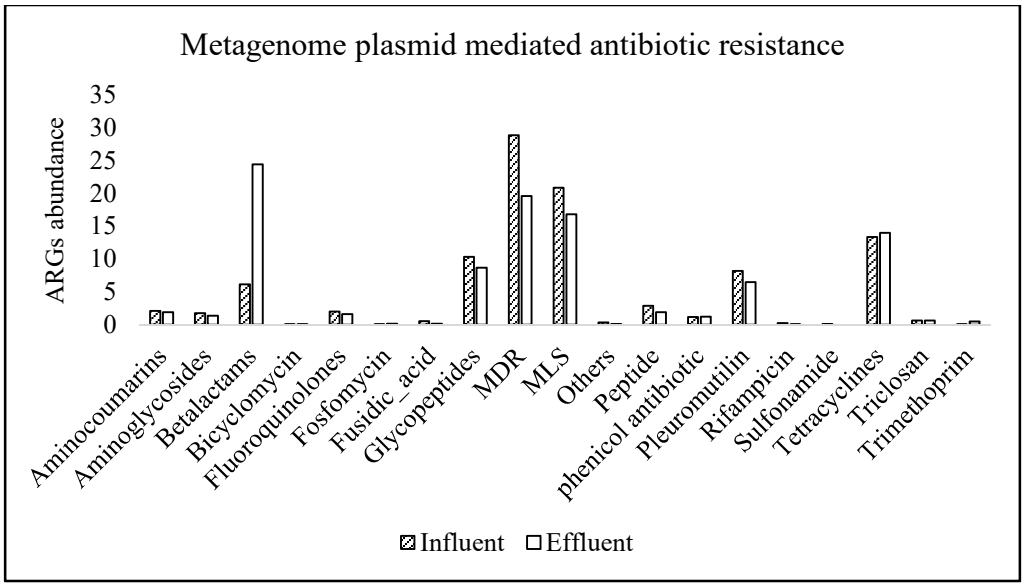

Figure 10. Graph showing metagenome plasmid-mediated antibiotic resistance in influent and effluent metagenomes.

Illumina high throughput sequencing results revealed that effluent plasmid metagenome having high resistance against BET (24%), comparing to influent BET (>10%). This result is in consensus with the resistance shown by the sequencing of culturable isolates. High prevalence of BET may be attributed to long exposure of β-lactam antibiotics in fish farming for treatment of Gram-negative infections[53]. Prevalence of high resistance in influent plasmid metagenome against MDR (29%) and MLS (21%) was identified, comparing to effluents MDR (20%) and MLS (17%). MDR resistance pattern was almost the same as predicted in culturable MDR *Vibrio* plasmids sequencing while MLS resistance was only confined to influent plasmid genomes. MDR is often related with natural processes[54] and widespread anthropogenic activity[55,56]. Horizontal gene transfer (HGT) in the environment, via natural transformation, transduction, or conjugation is the main driving force in propagating MDR[57]. High level of resistance in influents could be attributed by the association of majority of MLS with mobile elements and thus have the capacity to spread through the bacterial ecosystem[58,59]. The mobile elements associated with MLS resistance genes are often linked to

genes which confer resistance to other classes of antibiotics, heavy metal, and detergents as well[44]. Tetracycline resistance was almost similar in influent and effluent plasmid metagenomes which were in consensus with the sequencing results of culturable isolates. High prevalence of tetracycline resistance is due to its extensive usage in the aquaculture[59]. Excessive use of antibiotics generates a strong selective pressure that has resulted in the transfer of resistance genes associated with plasmids or transposons among bacterial species[60]. In gram-negative bacteria, plasmid-mediated genes coding for tetracycline efflux proteins are widely distributed and these are normally associated with large and conjugative plasmids[61,62].

These results showed that antibiotic resistance is encoded in some *Vibrio* isolates in plasmids and in others in the chromosomes. Secondly, discharged water from fish farms having more plasmid harboring MDR *Vibrio*, a higher number of virulence and biocide/metal resistance genes, comparing to influent water, suggesting its role in the dissemination of antibiotic-resistant genes to the other environments. It can be implied that plasmid bearing MDR *Vibrio* may ultimately cause serious health concerns to humans. The high abundance of beta-lactam resistance genes in effluents is suggestive of their extensive usage in fish farms. The co-occurrence of metal-resistance and antimicrobial-resistance genes can facilitate their persistence, co-selection, and dissemination[63].

CONCLUSION

This study revealed that the presence of greater number of *Vibrio*, more plasmid bearing MDR isolates, diverse range of antibiograms, large number of plasmid profiles, and higher virulence and metal/biocide resistance genes among the effluents (discharged water), suggestive of deposition of organic matter deriving from fish metabolism, uneaten feed, metal contaminants, and antibiotic residues in the fish farm tanks in Jeju, South Korea. This accumulation of organic matter and presence of ARGs in fish tank *Vibrio* may be the major reason for piling up MDR *Vibrio* in the fish farms which is not only harmful to the fish industry but a great risk to public health. It was also observed that *Vibrio* species were resistant to most of the commonly used antibiotics like SUL, N, Amx, and OTC. More *tdh* and *vvh* genes were found in effluent water advocating the existence of potentially pathogenic *Vibrio*. Plasmid-harboring MDR *Vibrio* may contribute in acerbating the propagation of ARGs in different environments. The appearance of multidrug resistance plasmids carrying resistance to heavy metals is alarming and requires additional supervision. Surveillance and monitoring of antibiotic resistance and the resulting pollution levels of antibiotics in aquatic environments should be encouraged to curtail improper usage of antibiotics. Also, proper treatment of coastal aquaculture effluent should be performed to control the dissemination of ARGs in the marine ecosystem.

ACKNOWLEDGMENT

This study was supported by the Korea Institute of Marine Science & Technology Promotion funded by the Ministry of Oceans and Fisheries, Korea (KIMST-20150307).

Thankful to my Lab mates Jo hyejun, Lee soo young & Shahbaz raza for helping me in this project.

Immensely grateful to “Professor Tatsuya Unno” for providing me this opportunity to work under his leadership.

REFERENCES

1. Martinez-Urtaza, J. *et al.* Ecological determinants of the occurrence and dynamics of Vibrio parahaemolyticus in offshore areas. *ISME J.* **6,** 994–1006 (2012).

2. Yang, J. H. *et al.* Distribution and antimicrobial susceptibility of Vibrio species associated with zooplankton in coastal area of Korea. *Mar. Pollut. Bull.* 1–6 (2017). doi:10.1016/j.marpolbul.2017.07.054

3. Thompson, F. L. *et al.* Phylogeny and Molecular Identification of Vibrios on the Basis of Multilocus Sequence Analysis Phylogeny and Molecular Identification of Vibrios on the Basis of Multilocus Sequence Analysis. *Appl. Environ. Microbiol.* **71,** 5107–5115 (2005).

4. Malla, S. *et al.* The challenges and successes of implementing a sustainable antimicrobial resistance surveillance programme in Nepal. *BMC Public Health* **14,** 269 (2014).

5. Serrano, P. H. *Responsible use of antibiotics in aquaculture*. *Journal of Chemical Information and Modeling* **53,** (2013).

6. Chelossi, E. *et al.* Antibiotic resistance of benthic bacteria in fish-farm and control sediments of the Western Mediterranean. *Aquaculture* **219,** 83–97 (2003).

7. Vezzulli, L., Chelossi, E., Riccardi, G. & Fabiano, M. Bacterial community structure and activity in fish farm sediments of the Ligurian sea (Western Mediterranean). *Aquac. Int.* **10,** 123–141 (2002).

8. Thompson, F. L., Iida, T. & Swings, J. Biodiversity of vibrios. *Microbiol. Mol. Biol. Rev.* **68,** 403–31, table of contents (2004).

9. Yebra, D. M., Kiil, S. & Dam-Johansen, K. Antifouling technology - Past, present and future steps towards efficient and environmentally friendly antifouling coatings. *Prog. Org. Coatings* **50,** 75–104 (2004).

10. Watermann, B. T. *et al.* Bioassays and selected chemical analysis of biocide-free antifouling coatings. *Chemosphere* **60,** 1530–1541 (2005).

11. Guardiola, F. A., Cuesta, A., Meseguer, J. & Esteban, M. A. Risks of using antifouling biocides in aquaculture. *Int. J. Mol. Sci.* **13,** 1541–1560 (2012).

12. Pruden, A. *et al.* Management options for reducing the release of antibiotics and antibiotic resistance genes to the environment. *Environ. Health Perspect.* **121,** 878–885 (2013).

13. Committee, T. E., Testing, A. S. & Routine, G. N. European Subcommittee on Antifungal Susceptibility Testing (EUCAST AFST) Routine and extended internal quality control for. 1–9 (2015).

14. Bankevich, A. *et al.* SPAdes: A New Genome Assembly Algorithm and Its Applications to Single-Cell Sequencing. *J. Comput. Biol.* **19,** 455–477 (2012).

15. Krawczyk, P. S., Lipinski, L. & Dziembowski, A. PlasFlow: predicting plasmid sequences in metagenomic data using genome signatures. *Nucleic Acids Res.* **46,** 1–14 (2018).

16. Hyatt, D. *et al.* Prodigal: Prokaryotic gene recognition and translation initiation site identification. *BMC Bioinformatics* **11,** (2010).

17. Jia, B. *et al.* CARD 2017: Expansion and model-centric curation of the comprehensive antibiotic resistance database. *Nucleic Acids Res.* **45,** D566–D573 (2017).

18. Pal, C., Bengtsson-Palme, J., Rensing, C., Kristiansson, E. & Larsson, D. G. J. BacMet: Antibacterial biocide and metal resistance genes database. *Nucleic Acids Res.* **42,** 737–743 (2014).

19. Argoty, G. A. A. *et al.* ARG-miner: A web platform for crowdsourcing-based curation of antibiotic resistance genes. *bioRxiv* 274282 (2018). doi:10.1101/274282

20. Fierer, N. & Jackson, J. Assessment of soil microbial community structure by use of taxon-specific quantitative PCR assays. *Appl. Environ. Microbiol.* **71,** 4117 (2005).

21. Sandaa, R.-A., Torsvik, V. L. & Goksøyr, J. Transferable drug resistance in bacteria from fish-farm sediments. *Can. J. Microbiol.* **38,** 1061–1065 (1992).

22. Bien, T. L. T., Sato-Takabe, Y., Ogo, M., Usui, M. & Suzuki, S. Persistence of Multi-Drug Resistance Plasmids in Sterile Water under Very Low Concentrations of Tetracycline. *Microbes Environ.* **30,** 339–343 (2015).

23. Kim, S. R., Nonaka, L. & Suzuki, S. Occurrence of tetracycline resistance genes tet(M) and tet(S) in bacteria from marine aquaculture sites. *FEMS Microbiol. Lett.* **237,** 147–156 (2004).

24. Akinbowale, O. L., Peng, H. & Barton, M. D. Diversity of tetracycline resistance genes in bacteria from aquaculture sources in Australia. *J. Appl. Microbiol.* **103,** 2016–2025 (2007).

25. Kang, C. H. *et al.* Characterization of Vibrio parahaemolyticus isolated from oysters in Korea: Resistance to various antibiotics and prevalence of virulence genes. *Mar. Pollut. Bull.* **118,** 261–266 (2017).

26. Yoo, M. H., Huh, M. Do, Kim, E. H., Lee, H. H. & Jeong, H. Do. Characterization of chloramphenicol acetyltransferase gene by multiplex polymerase chain reaction in multidrug-resistant strains isolated from aquatic environments. *Aquaculture* **217,** 11–21 (2003).

27. Palmer, R., Kawai, K. & Kusuda, R. In vitro activity of quinolone antibacterials against selected fish pathogens. *Fish Pathol.* **27,** 131–142 (1992).

28. Suzuki, S., Ogo, M., Koike, T., Takada, H. & Newman, B. Sulfonamide and tetracycline resistance genes in total- and culturable-bacterial assemblages in south african aquatic environments. *Front. Microbiol.* **6,** 1–8 (2015).

29. You, K. G., Bong, C. W. & Lee, C. W. Antibiotic resistance and plasmid profiling of Vibrio spp. in tropical waters of Peninsular Malaysia. *Environ. Monit. Assess.* **188,** 1–15 (2016).

30. Manjusha, S. & Sarita, G. B. Plasmid associated antibiotic resistance in Vibrios isolated from coastal waters of Kerala. *Int. Food Res. J.* **18,** 1171–1181 (2011).

31. Hörmansdorfer, S., Wentges, H., Neugebaur-Büchler, K. & Bauer, J. Isolation of Vibrio alginolyticus from seawater aquaria. *Int. J. Hyg. Environ. Health* **203,** 169–175 (2000).

32. Norman, A., Hansen, L. H. & Sorensen, S. J. Conjugative plasmids: vessels of the communal gene pool. *Philos. Trans. R. Soc. B Biol. Sci.* **364,** 2275–2289 (2009).

33. Zanetti, S. *et al.* In vitro susceptibility of Vibrio spp. isolated from the environment. *Int J Antimicrob Agents* **17,** 407–409 (2001).

34. Aoki, T., Kitao, T., Watanabe, S. & Takeshita, S. Drug resistance and R plasmids in

Vibrio anguillarum isolated in cultured ayu (Plecoglossus altivelis). *Microbiol. Immunol.* **28,** 1–9 (1984).

35. Manjusha, S. & Sarita, G. B. Characterization of plasmids from multiple antibiotic resistant Vibrios isolated from molluscan and crustacean of Kerala. *Int. Food Res. J.* **20,** 77–86 (2013).

36. Letchumanan, V., Chan, K. G. & Lee, L. H. An insight of traditional plasmid curing in Vibrio species. *Front. Microbiol.* **6,** (2015).

37. Letchumanan, V. *et al.* Occurrence and antibiotic resistance of Vibrio parahaemolyticus from Shellfish in Selangor, Malaysia. *Front. Microbiol.* **6,** 1–11 (2015).

38. Mirzoyan, N., Tal, Y. & Gross, A. Anaerobic digestion of sludge from intensive recirculating aquaculture systems: Review. *Aquaculture* **306,** 1–6 (2010).

39. Kim, Y. B. *et al.* Use of a filtering process to remove solid waste and antibiotic resistance genes from effluent of a flow-through fish farm. *Sci. Total Environ.* **615,** 289–296 (2018).

40. Zhao, Z. *et al.* Nutrients, heavy metals and microbial communities co-driven distribution of antibiotic resistance genes in adjacent environment of mariculture. *Environ. Pollut.* **220,** 909–918 (2017).

41. Andersson, D. I. Persistence of antibiotic resistant bacteria. *Curr. Opin. Microbiol.* **6,** 452–456 (2003).

42. Machuca, J. *et al.* Interplay between plasmid-mediated and chromosomal-mediated fluoroquinolone resistance and bacterial fitness in Escherichia coli. *J. Antimicrob. Chemother.* **69,** 3203–3215 (2014).

43. Capkin, E., Ozdemir, S., Ozturk, R. C. & Altinok, I. Determination and transferability of plasmid-mediated antibiotic resistance genes of the bacteria isolated from rainbow trout. *Aquac. Res.* **48,** 5561–5575 (2017).

44. Baker-Austin, C., Wright, M. S., Stepanauskas, R. & McArthur, J. V. Co-selection of antibiotic and metal resistance. *Trends Microbiol.* **14,** 176–182 (2006).

45. Stepanauskas, R. *et al.* Coselection for microbial resistance to metals and antibiotics in freshwater microcosms. *Environ. Microbiol.* **8,** 1510–1514 (2006).

46. Hughes, M. F., Beck, B. D., Chen, Y., Lewis, A. S. & Thomas, D. J. Arsenic exposure and toxicology: A historical perspective. *Toxicol. Sci.* **123,** 305–332 (2011).

47. Nakajima, H., Kobayashi, K., Kobayashi, M., Asako, H. & Aono, R. Overexpression of the robA gene increases organic solvent tolerance and multiple antibiotic and heavy metal ion resistance in Escherichia coli. *Appl. Environ. Microbiol.* **61,** 2302–2307 (1995).

48. Pal, C., Bengtsson-Palme, J., Kristiansson, E. & Larsson, D. G. J. Co-occurrence of resistance genes to antibiotics, biocides and metals reveals novel insights into their co-selection potential. *BMC Genomics* **16,** 1–14 (2015).

49. Kücken, D. Association of *qacE* and *qacE*Δ1 with multiple resistance to antibiotics and antiseptics in clinical isolates of Gram-negative bacteria. *FEMS Microbiol. Lett.* **183,** 95–98 (2000).

50. Chuanchuen, R. *et al.* Cross-Resistance between Triclosan and Antibiotics in. *Society* **45,** 428–432 (2001).

51. Braoudaki, M. & Hilton, A. C. Adaptive Resistance to Biocides in Salmonella enterica

and Escherichia coli O157 and Cross-Resistance to Antimicrobial Agents Adaptive Resistance to Biocides in Salmonella enterica and Escherichia coli O157 and Cross-Resistance to Antimicrobial Agents. *J. Clin. Microbiol.* **42,** 73–78 (2004).

52. Ortega Morente, E. *et al.* Biocide tolerance in bacteria. *Int. J. Food Microbiol.* **162,** 13–25 (2013).

53. Zaniani, F. R. *et al.* The Prevalence of TEM and SHV Genes among Extended-Spectrum Beta- Lactamases Producing Escherichia Coli and Klebsiella Pneumoniae. *Iran. J. Basic Med. Sci.* **15,** 654–60 (2012).

54. Dcosta, V. M. *et al.* Antibiotic resistance is ancient. *Nature* **477,** 457–461 (2011).

55. Levy, S. B. & Bonnie, M. Antibacterial resistance worldwide: Causes, challenges and responses. *Nat. Med.* **10,** S122–S129 (2004).

56. Davies, J. & Davies, D. Origins and Evolution of Antibiotic Resistance. *Microbiol. Mol. Biol. Rev.* **74,** 417–433 (2010).

57. Aminov, R. I. Horizontal gene exchange in environmental microbiota. *Front. Microbiol.* **2,** 1–19 (2011).

58. Zhao, Y. *et al.* Metagenomic analysis revealed the prevalence of antibiotic resistance genes in the gut and living environment of freshwater shrimp. *J. Hazard. Mater.* **350,** 10–18 (2018).

59. Roberts, M. C. Environmental macrolide-lincosamide-streptogramin and tetracycline resistant bacteria. *Front. Microbiol.* **2,** 1–8 (2011).

60. Han, J. E., Mohney, L. L., Tang, K. F. J., Pantoja, C. R. & Lightner, D. V. Plasmid mediated tetracycline resistance of Vibrio parahaemolyticus associated with acute hepatopancreatic necrosis disease (AHPND) in shrimps. *Aquac. Reports* **2,** 17–21 (2015).

61. Chopra, I. & Roberts, M. Tetracycline Antibiotics : Mode of Action , Applications , Molecular Biology , and Epidemiology of Bacterial Resistance Tetracycline Antibiotics : Mode of Action , Applications , Molecular Biology , and Epidemiology of Bacterial Resistance. *Microbiol. Mol. Biol. Rev.* **65,** 232–260 (2001).

62. Schmidt, A. S., Bruun, M. S., Dalsgaard, I. & Larsen, L. Incidence , Distribution , and Spread of Tetracycline Resistance Determinants and Integron-Associated Antibiotic Resistance Genes among Motile Aeromonads from a Fish Farming Environment Article a prendre comme exemple et comportant les amorces pour les in. *Appl. Environ. Microbiol.* **67,** 5675–5682 (2001).

63. Wales, A. & Davies, R. Co-Selection of Resistance to Antibiotics, Biocides and Heavy Metals, and Its Relevance to Foodborne Pathogens. *Antibiotics* **4,** 567–604 (2015).