Thesis for the Degree of Master of Fisheries Science

Occurrence of Multiple Antibiotic Resistant Bacteria in Selected Aquaculture Farms of Korea

by

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Pukyong National University

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한국의 일부 양식장에서 항생제 다재내성 박테리아 존재

Advisor: Prof. Tae Jin Choi

by

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Abstract

Antibiotics have been used for the control of microbial growth in fish, humans and other animals globally. Due to the continuous application of antibiotics in aquaculture, the levels of antibiotic residues have drastically increased in the aquatic environment for years, which have increased the aquatic bacterial exposure leading to various challenges of antibiotics resistance among bacteria, antibiotic resistant gene transfer among pathogenic and non-pathogenic bacteria strains. Hence, in this study we aim to identify Multiple Antibiotic Resistance (MAR) gene in bacteria isolated from aquatic farms. We have also investigated possible transfer of genes between bacterial strains. We have collected 21 water samples from 4 aquaculture farms

in Korea. Bacterial strains in each samples were isolated and tested for antibiotic resistance on Mueller Hinton Agar (MHA) media containing Tetracycline, Kanamycin, Streptomycin, Ampicillin, Vancomycin, Amikacin, Impenem, Gentamicin, Aztreonam, Meropenem, Cefepime, Cefotaxime, Ertapenem and Ceftazidime. As a result, we have isolated 10 bacterial strains with ability to grow in relatively high antibiotic concentrations. Among isolated bacteria, *Halomonas alkaliphila* strains showed relatively higher Minimum Inhibitory Concentrations (MICs) greater than 32 mg/ml while other isolated bacteria, *Pseudomonas segitis, Pseudomonas species* and *Psychrobacter celer* showed MICs of 0.96 mg/ml, 0.24 mg/ml and 0.48 mg/ml, respectively. In this study, we have also investigated transfer of gene *aph-6-id* in conjugation assay with *E. coli.* The results clearly indicate that Multiple Antibiotic Resistance bacteria, carrying resistant genes on mobile genetic elements are present in the different aquaculture farms. The development and reserving of antibiotic resistance and the dissemination of the resistance genes among bacteria are likely to increase in aquatic environments due to the continuous application of antibiotics in fish farms.

Keywords: Multiple Antibiotic Resistance (MAR), Minimum Inhibitory Concentration (MIC), Aquaculture

INTRODUCTION

Aquaculture plays an important role in supplying constant aquatic protein as well as providing the source of livelihood for the fast growing human population. The increasing intensification of aquaculture has led to emergency of aquatic animal healthy challenges plus other environmental concerns (FAO, 2016). Antimicrobial agents have been widely used in fish farming for either therapeutic, prophylactic or other purposes (Chuah et al., 2016; Kathleen et al., 2016; Cabello et al., 2016; Vignash et al., 2011; Cabello et al., 2006). Antibiotics are also continuously released into various environments including aquatic ecosystem through waste water discharges and agricultural runoffs (Mohante and Sudha, 2014).The intensive use of antibiotics in aquaculture has led to a selection pressure creating reservoirs of drug resistant bacteria and transferable resistant genes in both pathogenic and non-pathogenic aquatic bacteria (Romero et al., 2012; Angulo, 2009)

Therapeutic methods play a vital role in aquatic disease control. Antibiotics, fungicides and parasiticides have been widely used in aquaculture. More other strategies of disease control such as vaccine use and application of immunostimulants are increasingly being used in aquatic

disease management (Indrani and Iddya, 1999). Bacteriophages are increasingly on use in aquaculture as a biological control method of fish pathogens (Akmal et al., 2020). Probiotics, prebiotics, synbiotics have been used to control bacterial diseases whereas medicinal plants have been recognized as promising feed supplements for the treatment of bacterial, viral and parasitic diseases of fin and shellfish (Hoseinifar et al., 2018). Chemotherapeutic disease control methods have drawn a lot of criticism due their environmental challenges. The wide spread use of antimicrobial agents to treat and control fish diseases has led to the emergency of antimicrobial resistance to single and multiple classes of antimicrobial agents in important fish pathogens (Miller and Harbottle, 2018). The selective processes leading to the emergency and maintenance of bacteria resistant to antibiotics are mainly brought about by the incorrect or abusive utilization of drugs (Kumar et al., 2011; Bondad et al., 2012). Thus, the use of antibiotics is the most important factor amplifying the level of resistance in a given reservoir (Matyar et al., 2004).

The wide spread distribution of Antibiotic Resistance Genes (ARGs) among environmental bacteria and commensal bacterial in humans and animals is an outcome of horizontal gene transfer (Capkin et al., 2015). Horizontal gene transfer may occur in the aquaculture environment, in the food chain or in the human intestinal tract (Angulo, 2009). Sousa et al (2011) has isolated antibiotic resistant genes of *blaTEM-52, bla SHV-12,* as well as *CMLA, tetA, aadA, Sul1, Sul2* and *Sul3* in *E. coli* isolates from gilthead seabream. The same resistant

genes have been isolated from different human pathogenic bacteria. The susceptible bacteria may become resistant to antibiotics through multiple and complex mechanisms such as the inactivation of the antibiotic (or modification); 2) altering the target site of the antibiotic; 3) the modification of effected metabolic pathways to mitigate the effect of the antibiotic; and 4) decreasing permeability or the use of efflux pumps to reduce the antibiotic accumulation intracellularly (Schmieder, 2012; Vignash et al., 2011). Studies showed that genetic elements and resistance determinants are shared between aquatic bacteria, fish pathogens, and human pathogens, and appear to have originated from aquatic bacteria (Cabello et al., 2013).Thus, these represent a potential hazard since they can mediate the transfer of antibiotic resistant genes to other bacteria in the fish and aquaculture environment which can enter the food chain (Budiata et al., 2013).

The emergency of antibiotic resistant bacteria may be very greater than detected since most studies have been focused on demonstrating resistance in culturable bacteria and this constitutes a small proportion of all the bacteria available in the aquatic environment (Biseet et al., 2005). The aim of this study was to investigate the occurrence of antibiotic resistant bacteria from four different aquaculture farms in Korea, presence of antibiotic resistant genes and to show the incidence of antibiotic resistant gene transfer among bacteria in laboratory environment.

MATERIALS AND METHODS

2.1. Sample collection

The study area included 4 sampling sites, 3 on Jeju Island and 1 in Busan as shown in Table 1. Two farms, one in Jeju and one in Busan, (Farm A and B respectively), were culturing olive flounder (*Paralichthys olivaceus*), 1 farm in Jeju was culturing abalone and sea cucumber (Farm C), and the third farm in Jeju was culturing different grouper fish species (Farm D). All the farms were using re circulating aquaculture system

Twenty-one water samples of approximately 35 ml were collected in 50 ml sterile centrifuge bottles from the surface and 15cm below the water surface. Samples were carried to laboratory and stored at 4 ºC until analysis. Five water samples were collected from each of the farms in Jeju and 6 water samples were picked from the farm in Busan.

Table 1. Summary of sample collection

Farm name	Type of fish cultured	Location	Number of samples collected
A	Abalone, Sea cucumber	Jeju	5
B	Olive flounder	Busan	6
C	Olive flounder	Jeju	5
D	Grouper species	Jeju	5

Fig. 1. A map of South Korea showing location of the sampling sites.

2.2. Isolation of antibiotic resistant bacteria

Bacterial isolation was done based on standard differential identification that included growth on selective media. Samples were serially diluted to 10^{-3} dilutions, plated on the respective selective agar media. One hundred microliter of each sample were directly plated onto LB-agar (Agar 10 .0 g/l, Sodium chloride 5.0 g/l, Tryptone 10.0 g/l, yeast extract 5.0 g/l) containing antibiotics of (Tetracycline, 30 µg/ml, Kanamycin,30 µg/ml, Ampicillin,10 µg/ml, Streptomycin,10 µg/ml, Doxycycline,30 µg/ml, Oxolinic acid, 10 µg/ml and Enrofloxacin, 5µg/ml). The plates were incubated at 30 ºC for 48 hours. Duplicate counting of bacteria was performed and number of CFU/ML were calculated. The same procedure was done for all samples on LB without antibiotics and the CFU/ML were determined for each sample. More combinations (double and triple) of antibiotics were prepared in LB agar and plated with 100 µl of sample and incubated at the 30 ºC for the same time. The number of colonies were counted. The antibiotics used were purchased from Sigma Aldrich.

2.3. PCR amplification and sequencing of Antibiotic resistant bacteria

2.3.1. DNA extraction

Total DNA of the antibiotic resistant strains was extracted using the Accuprep Genomic DNA extraction Kit, (BIONEER,Daejeon). 1 ml of an overnight bacterial cell culture (1x 10^9

CFU/ML) were harvested by centrifugation at 6000 X g for 5 minutes.DNA was extracted from the pellet as described in the manual. 16SrRNA gene in of each bacterium were amplified by PCR using primers 149R and 27F shown on Table 2. The 20 µl PCR mixture comprised of 2 µL of primer, 13 µL of distilled water , 1 µL of DNA and 4 µl of enzyme rTaq 5X master mix PCR mixture (ELPIS- BIOTECH, Daejeon), 10 pmol of forward and reverse primers. MiniAmp plus thermal cycler was used for thermal recycling, with the conditions as follows, initial denaturation at 95 ºC for 4 minutes, followed by 31 cycle of at 95 ºC for 15 seconds, annealing at 54 ºC for 15 seconds , extension at 72 ºC for 30 seconds and 72 ºC a for the final cycle of amplification for 7 minutes. The control experiment comprised of the mixture of PCR and no DNA template. After the cycle, 5 µl of the PCR reaction mixture were used for gel electrophoresis in 1 % agarose gel. The gel was stained and further run at 100V for 28 minutes in the tank. The stained gels were after viewed by UV irradiation.

2.3.2. Sequencing

DNA sequencing was done for the different PCR products from the antibiotic resistant bacteria. PCR products were purified with GeneAll® Expin™ PCR SV protocol. BIONICS Company Limited did DNA sequencing. The resulting sequences were aligned with GenBank database through the BLAST search (hhtp://www.ncbi.n/m.nih.gov/BLAST/)

2.4. Determination of Minimum Inhibitory Concentration (MIC)

MICs were determined in triplicate for every strain of bacteria using broth micro dilution method (Weigand et al., 2008). The MICs for the 10 isolates were determined for; Kanamycin, Tetracycline and Streptomycin. The MIC tests were conducted using 96 well sterile polypropylene microtiter plate from Thermofisher Scientific (Waltham, USA). The overall procedure was carried out using the above mentioned protocol, and samples were incubated at 30 ºC for 20 hours. The microplate reader was used to read growth in different wells at a wavelength of 600 nm. The MIC was recorded as less than or equal to the lowest concentration when no growth occurs at any of the concentrations tested.

2.5. Antibiotic susceptibility test

Antibiotic susceptibility tests were performed by agar disc diffusion method, using 6 mm diameter discs on Mueller Hinton Agar (MHA), following the Clinical and Laboratory Standards Institute (CLSI, 2020) guidelines. Single bacterial colonies from each strain were transferred into sterile LB broth in different tubes (corresponding to 1.5×10^8 CFU/ML). Ten different isolates were tested for susceptibility to 14 antimicrobials agents. One hundred microliter of each bacterial suspension was directly inoculated on to MHA plates and spread evenly using a sterile swab. Antimicrobial susceptibility test discs were placed with the aid of

a sterile pair of forceps to the inoculated media. The plates were incubated at 30 ºC for 20 hours. The zone sizes were measured to the nearest millimeter using a ruler. The zones were described as resistant or susceptible based on CSLI guidelines 2020.The antimicrobial agents included; Ampicillin (AM,10 µg), Kanamycin (KAN, 30 µg/ml), Tetracycline (T, 30 µg/ml), Streptomycin (S, 10 µg/ml), Gentamycin (GM, 30 µg/ml), Cefepime (FEP, 30 µg/ml), Ertapenem (ETP, 10 µgml) Amikacin (AN, 30 µg/ml), Meropenem (MEM, 10 µg/ml), Aztreonam (ATM,30 µg/ml), Ceftazidime (CAZ, 30 µg/ml), Vancomycin (30 µg/ml), Imipenem (IPM, 10 µg/ml),and Cefotaxime (CTX, 30 µg/ml).

2.6. Plasmid isolation and conjugation

Strain S3TSV5 was selected as the donor strain, while *E. coli* J53 was used a recipient strain for conjugation assay. The conjugation was carried out following a conjugation manual (Alexander and Tonya, 2019). Streptomycin and Sodium azide were used as test antibiotics. The DNA was extracted and PCR was done using primers STR-F and STR-R. The set PCR cycling conditions were, initial denaturation at 95 ºC for 4 minutes, followed by 31 cycle of at 95 ºC for 15 seconds, annealing at 68 ºC for 15 seconds , extension at 72 ºC for 30 seconds and 72 ºC a for the final cycle of amplification for 7 minutes. PCR prep and sequencing was done as mentioned above and results were then blast searched.

Table 2. Primers used for the different PCR assays

Primer name	Sequence	Annealing temperature (Tm)
27F	AGA GTT TGA TCC TGG CTC AG	58.7º C
149R	CGG TTA CCT TGT TAC GAC TT	61.5 ºC
STR-F	TTC ATT GCC AGA CGG GAC TC	68 ºC
STR-R	GAC CGC GTT GCT CCT CTT CT	68 ºC

RESULTS

3.1. Isolation of antibiotic resistant bacteria

Normal LB- agar plates exhibited more bacteria growth counts compared to the antibiotic treated plates as shown in Table 3. Samples 2, 3 and 4 had bacteria resistant to more than 3 different antibiotics (Kanamycin, Tetracycline, Vancomycin and Streptomycin). Fourteen out of 21 samples had bacteria resistant to Kanamycin, followed by Tetracycline (13/21), Ampicillin (10/21), Vancomycin (6/21), Streptomycin (5/21), Doxycycline (2/21), Enrofloxacin (1/21) and Oxolinic acid (0/21). Samples 2, 3 and 4 were further subjected a combination of antibiotics. Sample 3 had 18 colonies showing resistance to a combination of 2 different antibiotics (Table 4), with T+S and T+V having 6 colonies each. The single isolated colonies from Table 4 on further antibiotic testing showed resistance to 3 antibiotic combinations (Table 5). High resistance to a combination of T+S+V was observed followed by K+V+T. No bacteria was resistant to T+S+K antibiotic combination. Nine out of 10 resistant strains were from farm B and one resistant strain was from farm A as shown in Table 6.

Table 3. Bacterial counts of different samples on different single antibiotics.

Bacteria counts on agar plates containing different antibiotic agents									
Sample No.	Control	Single antibiotics							
	LB	A	K	T	V	S	D	O	E
1	14	0	10	8	1	0	0	0	0
2	14	0	5	8	6	3	2	0	0
3	53	0	43	11	23	4	0	0	0
4	19	0	4	11	19	7	0	0	3
5	23	0	0	0	0	0	3	0	0
6	$3.8x10^{-3}$	23	1	46	0	5	0	0	0
7	210	0	0	2	0	0	0	0	0
8	3	0	0	0	20	0	0	0	0
9	11	0	0	0	0	3	0	0	0
10	$5x\ 10^{-4}$	$2.3x10^{-4}$	11	0	3	0	0	0	0
11	$3.3x10^{-3}$	35	0	0	0	0	0	0	0
12	$7x10^{-2}$	48	32	$1x10^{-2}$	0	0	0	0	0
13	$1\ x10^{-2}$	30	40	14	0	0	0	0	0
14	$1.8x10^{-3}$	$8x10^{-3}$	76	5	0	0	0	0	3
15	$1x\ 10^{-2}$	27	68	1	0	0	0	0	0
16	$6.3x\ 10^{-1}$	15	1	2	0	0	0	0	0
17	$6x10^{-2}$	30	31	3	0	0	0	0	0
18	$2.2x10^{-1}$	0	0	0	0	0	0	0	0

19	$6.9x\ 10^{-1}$	0	22	0	0	0	0	0	0
20	$2x10^{-1}$	0	0	0	0	0	0	0	0
21	$7x10^{-3}$	6	257	$4x10^{-2}$	0	0	0	0	0

A-Ampicillin, K-Kanamycin, T-Tetracycline, V-Vancomycin, S-Streptomycin, D-Doxycycline, O-Oxolinic acid, E-Enrofloxacin

Table 4. Bacteria counts on double antibiotic combinations.

Sample No.	Combination of antibiotics					
	T+K	T+V	T+S	K+V	K+S	V+S
2	0	0	0	0	0	0
3	3	6	6	2	0	0
4	0	0	1	0	0	0

Table 5. Bacteria counts on triple antibiotic combinations

Sample No.	Bacterial strains	Combination of antibiotics		
		T+S+K	T+S+V	K+V+T
4	S4TS1	0	0	0
3	S3TSV1	0	0	0
	S3TSV2	0	0	0
	S3TSV3	0	$2.86X\ 10^{-5}$	0
	S3TSV4	0	2.21X10-5	0
	S3TSV5	0	$1.66X10^{-6}$	0
	S3TSV6	0	$1.80X10^{-6}$	0
	S3TSK1	0	0	0
	S3TSK2	0	0	0
	S3TSK3	0	0	0
	S3TSK4	0	0	0
	S3TSK5	0	0	0
	S3TSK6	0	0	0
	S3TVS1	0	0	0
	S3TVS2	0	0	0
	S3TVS3	0	0	0
	S3TVS4	0	0	0
	S3TVS5	0	$4.12X\ 10^{-6}$	0
	S3TVS6	0	$4X10^{-1}$	0

	S3TKS1	0	0	0
	S3TKS2	0	0	0
	S3TKV1	0	0	0
	S3TKV2	0	0	$1.64X10^{-6}$
	S3TVK1	0	0	0
	S3TVK2	0	0	0
	S3TVK3	0	0	0
	S3TVK4	0	0	0
	S3TVK5	0	0	0
	S3TVK6	0	0	$1.47X10^{-6}$
	S3KVT1	0	0	$7.10X10^{-6}$
	S3KVT2	0	0	0
	S3KVS1	0	0	0
	S3KVS2	0	0	0

A-Ampicillin, K-Kanamycin, T-Tetracycline, V-Vancomycin, S-Streptomycin, D-Doxycycline, 0-Oxolinic acid, E-Enrofloxacin

Table 6. Antibiotic test results for the different bacterial isolates on single and combined antibiotic

Bacterial strains	Sample source	Resistance to antibiotics																
		A	K	T	V	S	D	O	E	T + K	T + V	T + S	K + V	K + S	V + S	T+ S+ V	T+ S+ K	K+ V+ T
S4TS1	Farm A	-	+	+	+	+	–	–	+	-	-	+	–	–	–	-	–	–
S3TSV3	Farm B	–	+	+	+	+	–	–	–	+	+	+	+	–	–	+	–	–
S3TVK6	Farm B	–	+	+	+	+	–	–	–	+	+	+	+	–	–	–	–	+
S3TKV2	Farm B	–	+	+	+	+	–	–	–	+	+	+	+	–	–	–	–	+
S3KVT1	Farm B	–	+	+	+	+	–	–	–	+	+	+	+	–	–	–	–	+
S3TSV4	Farm B	–	+	+	+	+	–	–	–	+	+	+	+	–	–	+	–	–
S3TSV5	Farm B	–	+	+	+	+	–	–	–	+	+	+	+	–	–	+	–	–
S3TSV6	Farm B	–	+	+	+	+	–	–	–	+	+	+	+	–	–	+	–	–
S3TVS6	Farm B	–	+	+	+	+	–	–	–	+	+	+	+	–	–	+	–	–
S3TVS5	Farm B	–	+	+	+	+	–	–	–	+	+	+	+	–	–	+	–	–

A-Ampicillin, K-Kanamycin, T- Tetracycline, V- Vancomycin, S-Streptomycin, D-Doxycycline, O-

Oxolinic acid, E- Enrofloxacin,

3.2. Identification of Antibiotic resistant bacterial strains

The isolated Multiple Antibiotic Resistant bacteria strains were identified based on the sequencing of 16SrRNA gene sequencing; 1 strain of *Pseudomonas species* (S3KVT1), 1 strain of *Pseudomonas segitis* (S3TVK6), 1 strain *Psychrobacter celer* (S4TS1) and 7 strains of *Halomonas alkaliphila* were identified as shown on Table 7.

Table 7. Identification of antibiotic resistant bacteria

Strain ID	Species name
S4TS1	*Psychrobacter celer* (Isolate 1)
S3KVT1	*Pseudomonas species* (Isolate 2)
S3TVK6	*Pseudomonas segitis* (Isolate 3)
S3TKV2	*Halomonas alkaliphila* (Isolate 4)
S3TSV4	*Halomonas alkaliphila* (Isolate 5)
S3TSV5	*Halomonas alkaliphila* (Isolate 6)
S3TSV6	*Halomonas alkaliphila* (Isolate 7)
S3TVS6	*Halomonas alkaliphila* (Isolate 8)
S3TVS5	*Halomonas alkaliphila* (Isolate 9)
S3TSV3	*Halomonas alkaliphila* (Isolate 10)

3.3. Minimum Inhibitory Concentrations (MICs)

Generally all isolates had slightly high MICs .*Halomonas alkaliphila* isolates had extremely higher MICs on Tetracycline. *Halomonas alkaliphila* (S3TVS5) exhibited the highest MIC value of 38.4 mg/ml on Tetracycline while *Halomonas alkaliphila* (S3TSV4) had the lowest MIC value of 0.00117 mg/ml on Streptomycin as shown on Table 8.

Table 8. MIC results for the resistant strains

Strain ID	Species name	MIC values (mg/ml)			
		T	S	K	V
S4TS1	*Psychrobacter celer*	0.3	0.48	0	0
S3KVT1	*Pseudomonas species*	0.075	0.06	0.24	0.24
S3TVK6	*Pseudomonas segitis*	0.15	0.015	0.24	0.96
S3TKV2	*Halomonas alkaliphila*	0.0375	0.00117	0.12	1.92
S3TSV4	*Halomonas alkaliphila*	2.4	2.5	0.96	15.36
S3TSV5	*Halomonas alkaliphila*	2.4	20	15.36	0.24
S3TSV6	*Halomonas alkaliphila*	2.4	1.25	3.84	15.36
S3TVS6	*Halomonas alkaliphila*	2.4	2.5	0.24	0.96
S3TVS5	*Halomonas alkaliphila*	38.4	3.84	1.92	0.48
S3TSV3	*Halomonas alkaliphila*	0.0375	0.3125	0.48	0.48

T: Tetracycline, S; Streptomycin, K: Kanamycin, V: Vancomycin

3.4. Antibiotic susceptibility test

The results in Tables 9 and 10 showed that both glucose fermenting and no-fermenting isolates were resistant to 5 antibiotic agents; Ampicillin (AM, 10 µg/ml), Tetracycline (T, 30mg/ml), Streptomycin (S, 10 µg/ml), Vancomycin (VAN, 30 µg/ml), Kanamycin (KAN, 30 µg/ml). All isolates had varying susceptibilities to 9 antibiotic of; Gentamycin (GM, 30 µg/ml), Cefepime (FEP, 30 µg/ml), Ertapenem (ETP, 10 µg/ml) Amikacin (AN, 30 µg/ml), Meropenem (MEM, 10 µg/ml), Aztreonam (ATM, 30 µg/ml), Ceftazidime (CAZ, 30 µg/ml), Imipenem (IPM, 10 µg/ml), and Cefotaxime (CTX, 30 µg/ml). All susceptible isolates had slightly different inhibition zones for all antibiotics. The diameter of the inhibition zones for the resistant strains were almost similar for the respective resistant antibiotics on (Table 9 and 10, Fig. 2).

Table 9. Disk diameters and susceptibility test results for glucose non-fermenting bacteria

Non-fermenting bacteria		
Antibiotics	Bacteria and its diameter interpretation (mm)	
	S3KVT1	S3TVK6
Impenem	32 (S)	38 (S)
Gentamicin	17 (S)	17 (S)
Aztreonam	33 (S)	30 (S)
Amikacin	24 (S)	23 (S)
Cefepime	26 (S)	21 (S)
Ceftazidime	32 (S)	28 (S)
Meropenem	36 (S)	36 (S)
Tetracycline	8 (R)	8 (R)
Kanamycin	9 (R)	10 (R)
Streptomycin	20 (R)	19 (R)
Vancomycin	8 (R)	8 (R)

R: Resistant, S: Susceptible, S3KVT1-*Pseudomionas species*, S3TVK6- *Pseudomonas species*

Table 10. Disk diameters and susceptibility test results for glucose fermenting bacteria

Fermenting bacteria								
Antibiotics	Bacterial isolates and their diameter interpretation (mm)							
	S4TS1	S3TKV2	S3TSV4	S3TSV5	S3TSV6	S3TVS6	S3TVS5	S3TVS3
Cefotaxime	41 (S)	29 (S)	34 (S)	32 (S)	33 (S)	30 (S)	37 (S)	33 (S)
Ertapenem	45 (S)	36 (S)	39 (S)	41 (S)	43 (S)	41 (S)	44 (S)	37 (S)
Ampicillin	6 (R)	6 (R)	6 (R)	10 (R)	9 (R)	12 (R)	13 (R)	7 (R)
Aztreonam	26 (S)	30 (S)	26 (S)	21 (S)	21 (S)	24 (S)	23 (S)	28 (S)
Gentamicin	31 (S)	16 (S)	26 (S)	27 (S)	27 (S)	26 (S)	27 (S)	28 (S)
Impenem	50 (S)	34 (S)	41 (S)	43 (S)	40 (S)	40 (S)	41 (S)	41 (S)
Amikacin	35 (S)	26 (S)	29 (S)	30 (S)	30 (S)	29 (S)	31 (S)	33 (S)
Cefepime	31 (S)	40 (S)	36 (S)	37 (S)	39 (S)	35 (S)	37 (S)	36 (S)
Ceftazidime	38 (S)	36 (S)	37 (S)	35 (S)	36 (S)	34 (S)	39 (S)	34 (S)
Tetracycline	8 (R)	8 (R)	8 (R)	8 (R)	8 (R)	9 (R)	11 (R)	9 (R)
Kanamycin	37 (S)	9 (R)	9 (R)	8 (R)	13 (R)	10 (R)	9 (R)	10 (R)
Streptomycin	8 (R)	20 (R)	11 (R)	8 (R)	8 (R)	13 (R)	11 (R)	13 (R)
Vancomycin	31 (S)	8 (R)	8 (R)	8 (R)	8 (R)	8 (R)	8 (R)	9 (R)

R: Resistant, S: Susceptible, S4TS1- *Psychrobacter celer,* S3TKV2- *Halomonas alkaliphila,* S3TSV4- *Halomonas alkaliphila,*S3TSV5- *Halomonas alkaliphila,* S3TSV6- *Halomonas alkaliphila* , S3TSV6- *Halomonas alkaliphila,* S3TVS5- *Halomonas alkaliphila,* and S3TSV3- *Halomoinas alkaliphila*

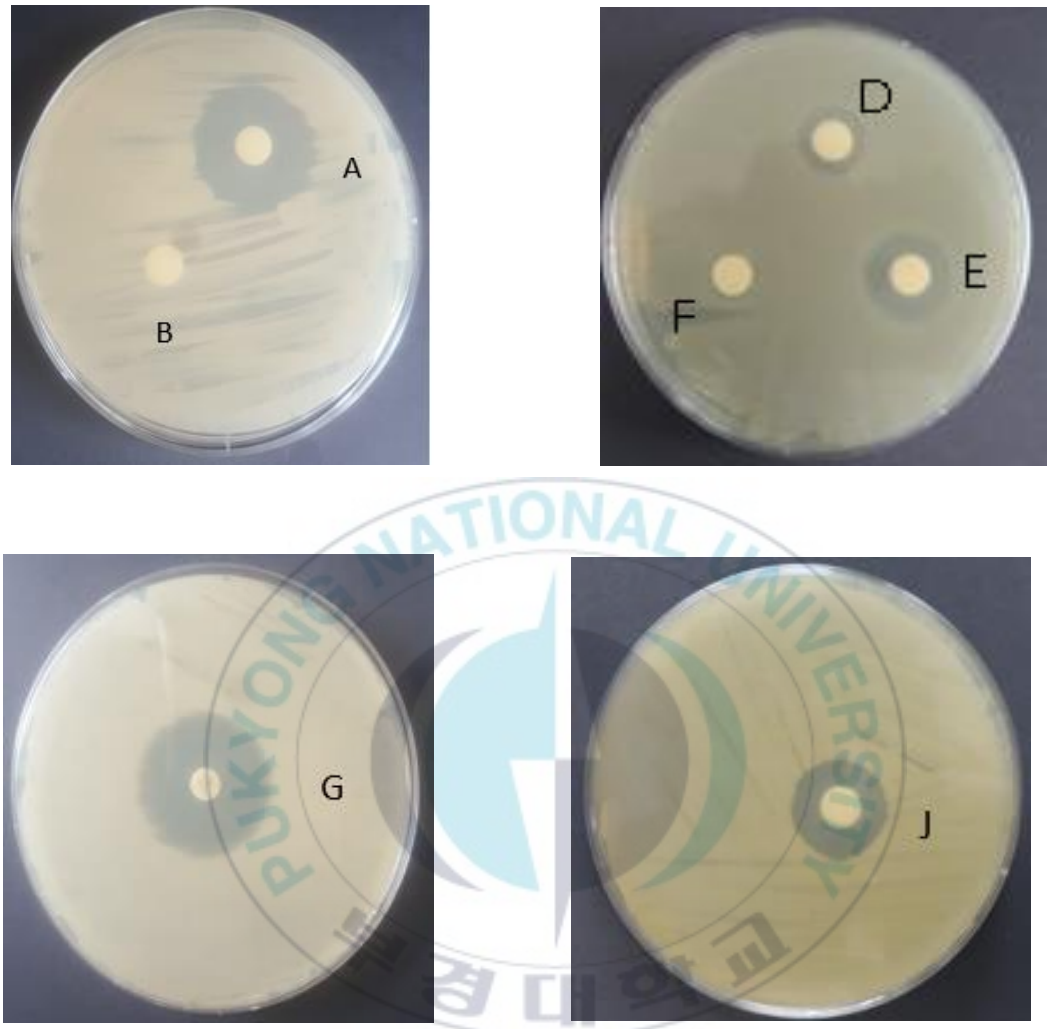

Fig. 2. Inhibition zones of selected bacteria strains and antibiotics. A and B are Aztreonam and Tetracycline respectively on S3TSV5, D, E and F are Streptomycin, Kanamycin and Tetracycline on respectively on S3TSV4, G-Amikacin on S3TSV5 , J-Gentamicin on S3TVS5.

3.5. Plasmid isolation and conjugation

Bacteria strain S3TSV5 was assessed for plasmid by conjugation experiments with *E.coli* J-53 (Fig.3).The donor strain S3TSV5 was able to grow on media containing Streptomycin. The recipient strain could not grow on media containing Streptomycin but could grow on Sodium azide. After conjugation, the recipient could grow on media containing both Sodium azide and Streptomycin. The PCR assays conducted on the trans conjugants to determine plasmid mediated resistance ARG, further produced a gel image showing the bands for trans conjugant, donor and recipient strain (Fig.3). The sequencing result of the PCR product indicated that the transferred gene was *aph -6-id* .The results show that the gene was carried on a plasmid ECPF5 plasmid p1 with a band size of 700 bp as shown in Fig 4.

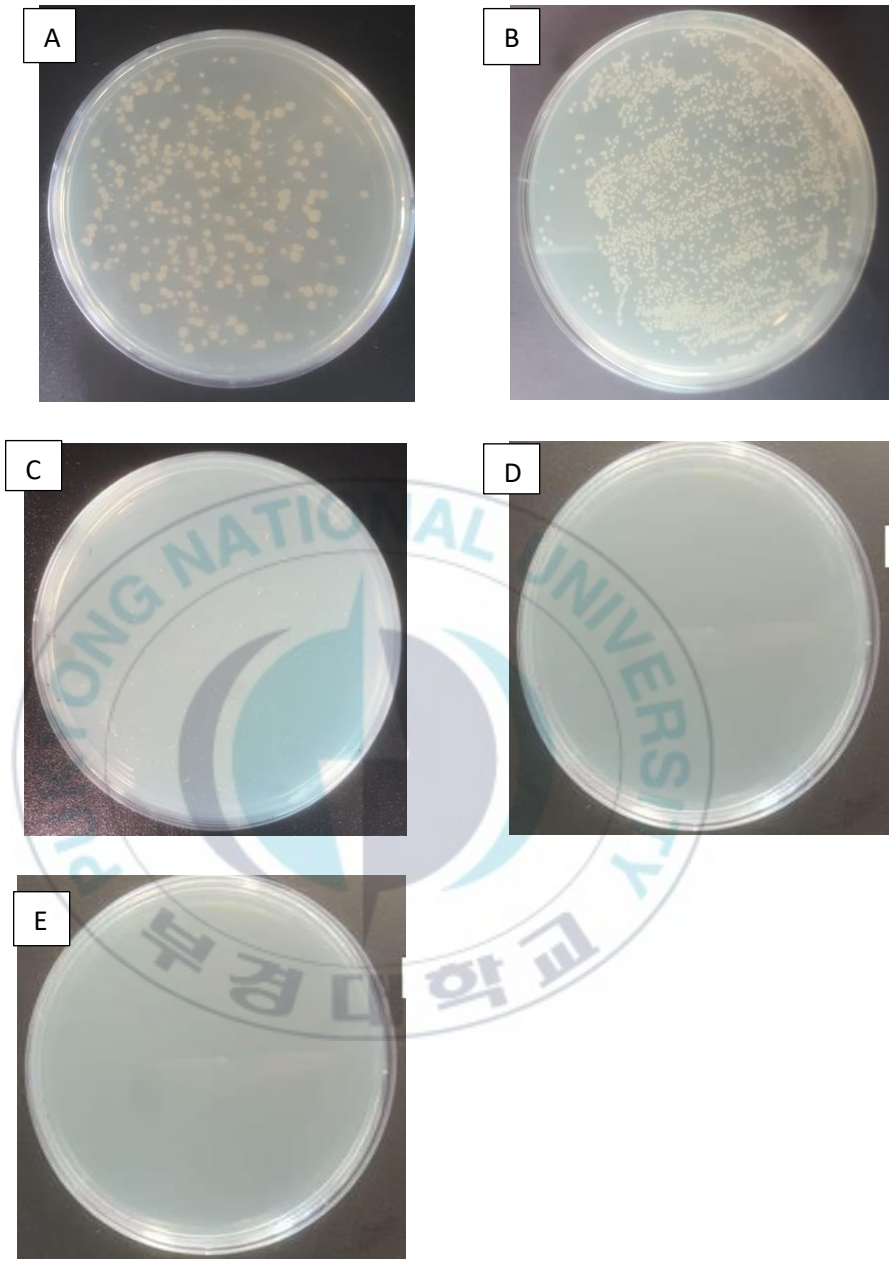
A
B
C
D
E

Fig. 3. Conjugation of antibiotics resistant S3TSV5 and *E.coli* J53. A- donor strain on streptomycin, B- recipient strain on sodium azide, C- trans conjugants on (Streptomycin+ Sodium azide), and D-donor strain on Sodium azide and E- recipient strain on Streptomycin

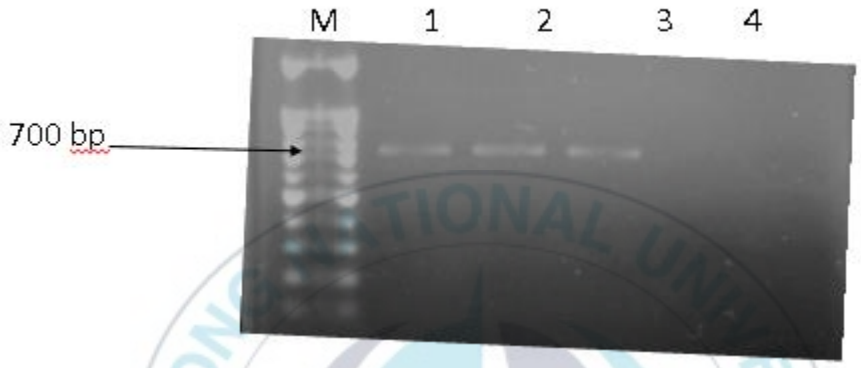

Fig. 4. Confirmation of conjugation mediated plasmid transfer between antibiotics resistant donor to susceptible receptor. Lanes 1, 2; PCR product from the donor; lane 3: PCR product from the trans conjugant; lane 4: PCR with recipient before conjugation; lane M: 1Kb DNA size marker

DISCUSSION

Antibiotics are generally used for therapeutic purposes in both humans and aquatic animals including fish. The study of antimicrobial resistant aquatic bacteria plays an important role in understanding the mechanisms of resistance exhibited by the bacteria, the resistance genes present and the potential for transfer of the resistance genes within the environment. The resistance is evident in both pathogenic and non-pathogenic bacteria. The antibiotic gene transfers has almost occurred in all natural environments. Thus, in this study, resistant bacteria from aquatic environments was associated with mobile genetic elements (plasmids) carrying the resistant genes. Horizontal gene transfer is still an important mechanism of resistant gene dissemination in most bacteria genera.

In this study, multiple ARGs were observed in bacteria isolated from water samples taken from half of the sampled aquatic farms. Similar studies have isolated multiple antibiotic resistant *Pseudomonas species,* from salmon farms in Chile (Claudio and Zeleman, 2002; Spanggaarddet et al., 1993; Maha and Samy, 2011) and in *Pseudomonas segitis* from sea cucumber farm in China (Zheng et al., 2012). Other studies have isolated *Psychrobacter celer* from sea food (Palaparni et al., 2020). Romano et al., (2006) isolated antibiotic resistant

Halomonas alkaliphila from Pozzo de sale (salt well) in Italy. Similar studies by Dagha et al., (2020) have isolated *Halomonas alkaliphila* from Ratnagiri coast in India. The possible cause of resistance among these bacteria would be the antibiotic treated feeds or antibiotics applied in aquaculture for prophylactic purposes and those applied direct to the fish for therapeutic purposes leaving residues of antibiotics within the environment. This long term exposure can lead to emergency of resistance within the different environmental aquatic bacteria. Miranda et al., (2013) has also reported resistance of both *Pseudomonas segitis* and *Pseudomonas species* to Kanamycin and Streptomycin. The susceptibility results of both strains to the 9 antibiotics (Table 9) still agree with those of the same authors above.

In aquaculture, the use of antimicrobials influences the frequency and spread of antibiotic resistant bacteria in the aquatic environment (Aminov, 2009; Martinez, 2009). Selective pressure within the aquaculture systems as a result of antimicrobial use is the main cause of increase and persistence of wide spread antimicrobial resistant bacteria (Huer et al., 2009; Miranda, 2012). Thus, this could be one of the main reason for the resistance of *Pseudomonas species* and *Pseudomonas segitis* in the aquaculture system.

The 7 isolates of *H. alkaliphila* were resistant to Ampicillin, Kanamycin, Streptomycin, Vancomycin and Tetracycline. These findings agree with other authors (Romano et al., 2006) who reported similar antibiotic resistance by the same bacteria isolated from a salt pool in Italy. In the same line, the results agree that the strain was susceptible to Gentamicin. Alkaliphilic

bacteria have been investigated for their ability to produce novel antibiotics and can produce a unique number of antimicrobial compounds (Edwards, 2015). The resistance to Tetracycline is reported to occur in any aquatic environment with the absence of the antimicrobial agent itself (Vaughan et al., 1996; Kapetanaki et al., 1995). The resistance of Streptomycin and Tetracycline could further be explained with the fact that both antibiotics are among the most commonly used veterinary antimicrobial agents in the world (Pizella and Ricca, 2003). Thus the high resistance could be as a result of long term enrichment of the aquatic environment with the same antibiotics. This increases exposure of the aquatic bacteria to the antibiotic agents thus accelerating increase in resistance within the aquatic environment. The MICs exhibited were relatively higher for all the tested antibiotics. The strains are believed to have expressed several mechanisms of resistance including active efflux. According to Isabella et al., (2015), the variations in MICs may be due to intrinsic factors specific to each microorganism. The activity of antimicrobials depends on their classes. The presence of two different efflux pumps or multi component efflux pump could have significantly caused the variations in MIC in the different strains. Blake et al., (2003) and Kohansi et al., (2010), further suggested that the use of sub lethal levels of bactericidal antibiotics induce mutagenesis resulting in heterogeneous increases in the Minimum Inhibitory Concentrations of antibiotics.

The haphazard use antimicrobials has led to emergency of a selective pressure among different aquatic bacteria. Roser and Younger, (1999) have identified and characterized different

antibiotic resistance determinants linked with mobile genetic elements in the aquatic environment. In the present study, a *Halomonas alkaliphila* isolate 9 was able to transfer its plasmid carrying the Streptomycin resistant gene *aph-6–id*. These results agree with Overbeek et al., (2002) who also identified several *aph 6-id* determinant genes from the European habitats. Seveno et al., (2002) reported that the high cases of Streptomycin resistant bacteria in the environment have most likely been caused due the continuous use of the antibiotics. However, the mechanisms of distribution of Streptomycin resistant genes in intact habitats are less known. Thus bacteria populations of different genera have been able to exchange broad host range DNA through conjugation (Seveno et al., 2002; De la Cruz and Davies, 2000). Enzymatic modifications using the enzyme O phosphotransferases APH-6-id is the main mechanism of Streptomycin resistance used by the resistant bacteria. The gene is carried on the plasmid *Escherichia coli* PF5 plasmid 1. The reaction is catalyzed by the enzyme APH -6 -id resulting into high level resistance of the bacteria to streptomycin.

The continuous use of antimicrobials in aquaculture has resulted into serious health and environmental hazards. Increasing cases of antibiotic resistance are reported in both clinical and other environmental isolates. Therefore, with no stringent measures put in place, the aquatic environment may soon become a pool and reservoir of environmental antibiotic resistant bacteria, as well as genes. More studies should be conducted to further understand the

mechanisms of antibiotic resistance in different bacteria and modes of gene transfer between bacteria of different genera.

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