

Plasmid profiling of multi-drug resistant *Vibrio* sp. isolated from influent and effluent water samples of fish farms in Jeju, South Korea

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제주 양식장 유입수와 방출수에서 분리한 다제내성 *Vibrio* 균 플라스미드 프로파일링

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The objective of this study was to investigate the plasmid profiling 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* isolates were obtained from each influent and effluent water sample. All MDR isolates were subjected to plasmid profiling. 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, which was higher in effluent samples. 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 (influent 38 and effluent 20) were found to bear plasmids ranging from 1.7 kb to >10 kb and showed 19 different antibiograms according to the size of plasmids. MDR isolates showed six and four distinct plasmid profiles in influent and effluent, respectively. Effluent samples contained more plasmid-carrying MDR *Vibrio* isolates with more diverse plasmid profiles and antibiograms, suggesting that fish farm tanks may serve as a reservoir of antibiotic resistance genes. The presence of plasmid-carrying MDR *Vibrio* isolates in fish farm effluent water may contribute to the

dissemination of antibiotic resistance genes to the environments, which ultimately poses threat to human health.

Keywords: *Vibrio*, antibiotic resistance genes, fish farm effluent, plasmid profile

The *Vibrio* sp. are important component of marine ecosystems reported to cause mortalities and extensive economic losses in aquaculture production worldwide (Martinez-Urtaza *et al.*, 2012; Yang *et al.*, 2017). About one third of *Vibrio* sp. are potential human pathogens involved in water and seafood related outbreaks of gastrointestinal and wound infections in humans (Thompson *et al.*, 2005).

Excessive amount of antibiotics has been widely used to treat *Vibrio* infections in aquaculture, which has caused high incidence of antibiotic resistance (Malla *et al.*, 2014). These antibiotic resistance genes (ARGs) can be easily disseminated through horizontal gene transfer (Serrano, 2013). 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 (Vezzulli *et al.*, 2002; Chelossi *et al.*, 2003).

In South Korea, inland fish farms commonly apply 'flow-

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through' method, by which sea water 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 may contain uneaten feed, fish excretion, and residual antibiotics, providing an ideal place where ARGs can be disseminated. Therefore, fish farm effluent may repeatedly introduce ARGs into coastal area and consequently affect the human health (Pruden *et al.*, 2013).

In the present study, we investigated the abundance, antibiotic susceptibility patterns, and plasmid profiling 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.

Materials and Methods

Sample collection

Influents and effluents of fish farms were sampled in the northern part of Jeju Island, South Korea. Water samples were collected using a four-liter sterilized sampling bottle in triplicates in the beginning of September 2017. All the samples were transferred back to the laboratory within 2 h after the collection.

Enumeration of *Vibrio* spp.

Influent and effluent water samples (30, 10, and 3 ml) were directly filtered through a sterile mixed cellulose ester membrane filter with 0.2 µm pore size and a diameter of 47 mm (Hyundai Micro Co., Ltd.). The membranes were then transferred onto the surface of Thiosulfate Citrate Bile Salts Sucrose Agar (TCBS, DIFCO™ Becton & Dickenson) 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™ Becton & Dickenson), all the isolates were preserved at -80°C in 50% of glycerol in phosphate buffer saline for further experiments.

Analysis of antibiotic resistance pattern

The susceptibility of isolates was examined by the disc

diffusion method according to the guidelines of the National Committee on Clinical Laboratory Standards, NCCLS (Marie, 2005). Briefly, MH agar containing 1.5% NaCl and eight different antibiotic discs including oxytetracycline (OTC, 30 µg), amoxicillin (AMX, 30 µg), chloramphenicol (CHL, 10 µg), ciprofloxacin (CIP, 5 µg), nalidixic acid (NAL, 30 µg), florfenicol (FFC, 30 µg), neomycin (N, 10 µg) and sulfamethoxazole (SUL, 25 µg) representing different classes of antibiotics used in this study. 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-carrying *Vibrio* were tested for four additional antibiotics NAL, FFC, N, and SUL. All agar plates were incubated at 37°C for 18 h. The results were expressed as susceptible (S), intermediate (I), or resistant (R) following the guidelines of the CLSI (CLSI, 2015).

Plasmid profiling

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

Results and Discussion

Vibrio abundance

Greater number of *Vibrio* were present in effluent (61%) samples comparing to influent (39%) samples (data not shown). It has been previously reported that fish farm sediments receive a large amount of organic matter due to uneaten food, residual antibiotics and fecal materials, which could consequently increase the number of *Vibrio* in fish farms (Sandaa *et al.*, 1992; Vezzulli *et al.*, 2002; Igbino, 2016). Although we did not measure the amount of organic matter in the fish tank, accumulated organic matter in fish tanks may have served as a reservoir for ARGs.

Analysis of antibiotic resistance pattern

Initial antimicrobial susceptibility tests for 300 isolates

Table 1. Percentages of *Vibrio* isolates resistant to antibiotics. R, I, and S indicate resistant, intermediate, and susceptible, respectively.

Antibiotics*	Influent (n = 150)			Effluent (n = 150)		
	R	I	S	R	I	S
OTC ^b	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

*OTC, AMX, CIP, and CHL indicate oxytetracycline, amoxicillin, ciprofloxacin, and chloramphenicol, respectively.

against four antibiotics identified 99 MDR *Vibrio* isolates (Table 1). 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. Greater number of effluent isolates showed resistance against AMX and OTC. In this study we found 99 MDR isolates that were resistant to more than two tested antibiotics, of which, 58 (influent 20, effluent 38) isolates were found to harbor plasmids.

Antibiograms of the 58 MDR plasmid-carrying *Vibrio* isolates

To investigate antibiograms, antibiotic susceptibility testing with four additional antibiotics was conducted for the 58 plasmid-carrying isolates (Table 2). More than 68% of them were resistant to N, SUL, and AMX, whereas less than 50% were resistant to NAL, OTC, CIP, CHL, and FFC. In particular, 86% of them (influent 16, effluent 34) were resistant to neomycin, which is in line with the previous studies that showed high resistance of *Vibrio* isolates against neomycin and other aminoglycosides (Hörmansdorfer *et al.*, 2000; Manjusha and Sarita, 2011). In addition, 79% of the *Vibrio* isolates (influent 13, effluent 33) were resistant to SUL, resistance to which has been widely spread in the sea and fish farm discharged water (Yoo *et al.*, 2003; Suzuki *et al.*, 2015; You *et al.*, 2016). Similarly, 40 of them (influent 14, effluent 26) were resistant to AMX which is in agreement with the previous report where more than half of the *Vibrio* isolates in Korean coastal areas were resistant to AMX (Yoo *et al.*, 2003). There were 27 MDR isolates (influent 10, effluent 17) resistant to OTC, the most commonly used antimicrobial in Korean fisheries, particularly for the treatment of infection by *Vibrio* sp. (Bien *et*

Table 2. Multi-drug resistance profile of 58 plasmid 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

*OTC, AMX, CIP, CHL, NAL, N, FFC, and SUL indicate oxytetracycline, amoxicillin, ciprofloxacin, chloramphenicol, nalidixic acid, neomycin, florfenicol, and sulfamethoxazole, respectively.

et al., 2015). Fewer MDR isolates resistant to NAL and CIP were found (influent 7, effluent 21, and influent 8, effluent 15, respectively). Resistance of *Vibrio* isolates against quinolones has already been reported (Kang *et al.*, 2017), although quinolones had been considered effective against *Vibrio* infections (Yoo *et al.*, 2003). It has been suggested that quinolone resistant Enterobacteriaceae gradually increase, probably owing to the presence of plasmid mediated quinolone resistance (PMQR) (Palmer *et al.*, 1992). Conversely, most of the MDR *Vibrio* isolates were susceptible to CHL and FFC, which is in agreement with the previous studies showing susceptibility of *Vibrio* to phenicols in aquaculture (Palmer *et al.*, 1992; Norman *et al.*, 2009; Kang *et al.*, 2017).

Plasmid profiling of the 58 MDR plasmid-carrying *Vibrio* isolates

Of the 58 isolates analyzed, four and six distinct plasmid profiles were observed for influent and effluent samples, respectively (Fig. 1). Plasmid sizes ranged from 1,700 bp to >10,000 bp, which is consistent with a previous study where 50% of the *Vibrio* isolates were found to have plasmids with sizes ranging from 1.5 kb to 26 kb in coastal water (Zanetti *et al.*, 2001). Among them, 2,000 bp, 2,500 bp, 4,000 bp, 8,000 bp and > 10,000 bp were common in influent and effluent *Vibrio* isolates. Conversely, plasmids with size 3,500 bp, 5,500 bp, 6,000 bp were only present among effluent isolates and only one plasmid having size 1,700 bp was present among influent

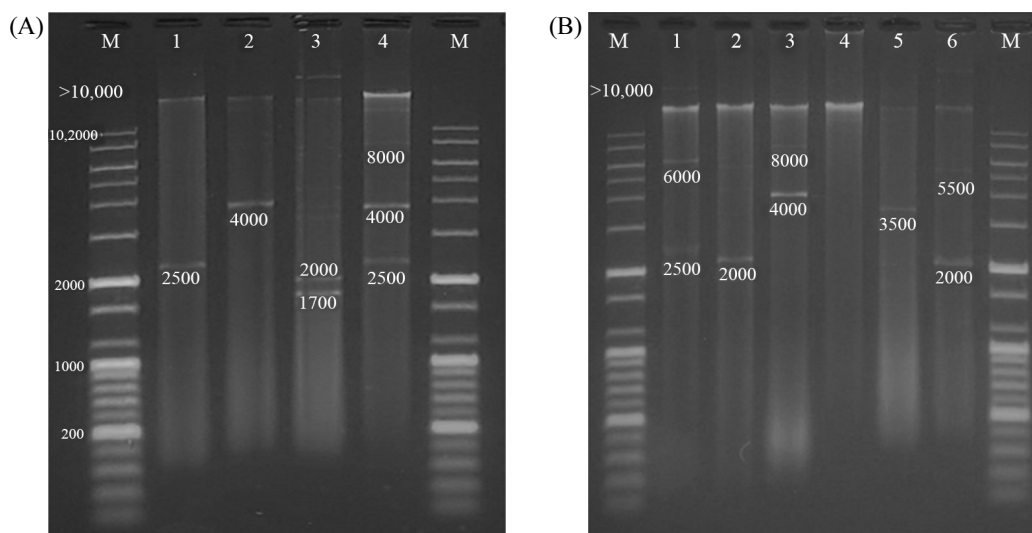


Fig. 1. Four plasmid profiles among influents (A) and six in effluents (B). Numbers (1-6) and the letter 'M' indicate different isolates and DNA ladder, respectively.

isolates.

Nine different sizes of plasmids were observed in this study. In each size of plasmid, 1 to 4 antibiograms were observed (total 19), out of which, 9 were distinctly present among effluent and 4 among influent isolates (Table 3). SUL, N, AMX, and OTC are found in most of the antibiograms, while CHL and FFC were only found in effluent antibiograms. There was low number of plasmid-carrying *Vibrio* resistant to FFC and CHL. The 4 distinct antibiograms in influent are mostly consisted of N, SUL, AMX and OTC, whereas the 9 distinctive antibiograms include the resistance to CHL and FFC in addition to the four antibiotics. While the number of antibiotics included in the influent antibiogram ranged from 4 to 5, effluent antibiogram showed 3 to 6 antibiotics. Although number of isolates analyzed in this study is not high, our results suggest antibiotic resistant *Vibrio* isolates carry different types of plasmids with different antibiograms. Further study may clarify what causes this difference between influent and effluent.

We observed 6 influent and 7 effluent MDR *Vibrio* isolates carrying multiple plasmids and some of them show different antibiograms (Table 4). It has been studied that the presence of multiple plasmid harboring *Vibrio* isolates is a common phenomenon in marine environment. Similar plasmid profiles were noticed in previous reports, although within the same plasmid profiles there exist different antibiograms that suggest these plasmids may encode different types of ARGs (Manjusha and

Sarita, 2013; Silvester *et al.*, 2017).

Norman *et al.* (2009) reported that plasmids equal or more than 30 kb are likely to be R-plasmids where multi-antibiotic resistance genes are coded. Although most of the plasmids we identified in this study are less than 10 kb, multi-antibiotic resistance genes may be involved in these plasmids. On the other hand, 41 out of 99 MDR isolates detected in this study were without the presence of plasmids, suggesting that MDR-related genes in these *Vibrio* can potentially be obtained through non-plasmid mediated horizontal gene transfers such as integron-mediated. García-Aljaro *et al.* (2017) reported that membrane vesicles can contain genetic elements and disseminate ARGs to other organisms especially in marine ecosystem. Chromosomal mediated ampicillin and tetracycline resistance in *Vibrio* has already been studied where all the ampicillin resistant isolates (176/200) and tetracycline resistant isolates (26/200) remain resistant to the respective antibiotics after plasmid curing (Letchumanan *et al.*, 2015). Nevertheless our results showed effluent water from fish farms have more plasmid-carrying MDR *Vibrio* comparing to that of influent water samples, suggesting fish farms may serve as a source of dissemination of antibiotic resistant genes to the other environments (Luo *et al.*, 2014). It has been reported that effluent water from flow through fish farms may look clean but could act as a potential pollutant containing antibiotic residues and ARGs (Mirzoyan *et al.*, 2010). The presence of subsequent dissociation of

uneaten feed, metabolic byproducts and antibiotic residues in fish farm tanks may suite the growth of fish pathogens including *Vibrio* (Buschmann *et al.*, 2012; Schumann *et al.*, 2017). In Mexico it was studied that about 70% of the *Vibrio* isolated from aquaculture settings were multidrug resistance (Molina-Aja

et al., 2006). It was further investigated that fish farms may serve as reservoir for pathogenic *Vibrio* strains, which can withstand environmental conditions established inside the tanks and would be able to cause infections (Ben-Haim *et al.*, 2003).

Table 3. Relationship between plasmid profiles and resistance patterns of 20 influent and 38 effluent isolates.

Plasmid size (bp)	Antibiograms*	Frequency	
		Influents	Effluents
1,700	OTC, CIP, N, SUL	4	0
	OTC, AMX, N, FFC, SUL	1	5
2,000	OTC, CIP, N, SUL	2	3
	OTC, AMX, CIP, N, FFC, SUL	0	2
	AMX, CHL, N, SUL	1	2
2,500	AMX, CIP, SUL	0	2
	OTC, AMX, N, SUL	7	5
	OTC, AMX, CHL, NAL, FFC, SUL	0	1
3,500	OTC, AMX, CIP, N, SUL	0	3
4,000	AMX, CIP, N, SUL	0	5
	OTC, AMX, N, SUL	2	0
5,500	OTC, AMX, CIP, N, SUL	0	1
6,000	OTC, AMX, N, SUL	0	2
	AMX, CIP, N, SUL	1	3
8,000	OTC, AMX, N, SUL	3	2
	OTC, AMX, NAL, N	0	5
> 10,000	AMX, CIP, CHL, SUL	0	1
	OTC, AMX, N, SUL	2	0
	OTC, AMX, NAL, N, SUL	2	0

*OTC, AMX, CIP, CHL, NAL, N, FFC, and SUL indicate oxytetracycline, amoxicillin, ciprofloxacin, chloramphenicol, nalidixic acid, neomycin, florfenicol, and sulfamethoxazole, respectively.

Table 4. *Vibrio* isolates carrying more than one plasmid

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

*OTC, AMX, CIP, CHL, NAL, N, FFC, and SUL indicate oxytetracycline, amoxicillin, ciprofloxacin, chloramphenicol, nalidixic acid, neomycin, florfenicol, and sulfamethoxazole, respectively.

Conclusion

This study revealed that fish farm effluent water contains greater number of *Vibrio*, more plasmid-carrying MDR isolates, diverse range of antibiograms and diverse plasmid profiles than those of influents. Although we did not investigate the cause, it is likely that deposition of organic matter deriving from fish metabolism, uneaten feed, and antibiotic residues in the fish farm tanks contributed to the dissemination of ARGs in effluent. MDR *Vibrio* may not only cause economical damage to fish industry, but also cause a great risk to public health. It was also observed that fish farm *Vibrio* were resistant to most of the commonly used antibiotics such as SUL, N, AMX, and OTC. Plasmid-carrying MDR *Vibrio* may contribute to the dissemination of ARGs in different environments. Surveillance and monitoring of antibiotic resistance and the resulting pollution levels of antibiotics in aquaculture 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 marine ecosystem.

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적 요

이 실험은 제주도내 양식장으로부터 유입수와 방출수의 다제내성(MDR)을 가진 *Vibrio* 균의 plasmid profiling을 위해 진행하였다. Plasmid profiling을 위해 사용한 다제내성을 가진 균주는 디스크 확산법을 통해 확인하였고, 유입수와 방출수으로부터 각각 150개의 *Vibrio* 균주를 분리하였다.

모든 다제내성 균주를 대상으로 plasmid profiling을 실시하였으며, 유입수와 비교하여 방출수에서 많은 *Vibrio* 균이 열거되었다(유입수 39%, 방출수 61%). 방출수에서는 neomycin, sulfamethoxazole, amoxicillin 및 oxytetracycline의 내성을 가진 균주가 유의적으로 많은 것으로 확인되었고, 대조적으로 *Vibrio* 균주는 florfenicol, chloramphenicol, ciprofloxacin 및

nalidixic acid에 더 민감한 것으로 확인이 되었다. 99종의 다제내성 균주(유입수 39종과 방출수 60종) 중에서 총 58종(유입수 38종과 방출수 20종)이 1.7 kb에서 10 kb 이상의 플라스미드를 가지고 있는 것을 확인하였으며 플라스미드 크기마다 19가지의 다른 다제내성 패턴을 보였다. 6종의 유입수와 4종의 방출수에서 다제내성 균주는 특이적인 plasmid profile이 확인되었다.

방출수 샘플은 보다 많은 플라스미드를 가진 다제내성 *Vibrio* 균주와 다양한 plasmid profile들과 다제내성 패턴을 가지고 있었고 이는 양식장의 저장탱크가 항생제내성 유전자의 저장소 역할을 할 수 있다는 점을 시사한다. 양식장의 방출수에서 plasmid를 가진 다제내성 *Vibrio* 균주의 존재는 항생제내성 유전자의 전파에 기여할 수 있으며 이로 인해 인간의 건강을 위협할 수 있다.

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