Thesis for the Degree of Master of Science

Studies on re-identification, quantification, and control of *Vibrio scophthalmi* in olive flounder (*Paralichthys olivaceus*)

by

Ebrahim Osman

Department of Aquatic Life Medicine

Graduate School, College of Fisheries Science

Pukyong National University

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양식 넙치 (*Paralichthys olivaceus*) 를 감염시키는 *Vibrio scophthalmi* 의 재동정, 정량 및 제어에 관한 연구

Advisor: Prof. Do-Hyung Kim

by

Ebrahim Osman

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Studies on re-identification, quantification, and control of *Vibrio scophthalmi* in olive flounder (*Paralichthys olivaceus*)

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Approved by:

(Chairman) Prof. Hyun-Do Jeong

(Member) Prof. Jun-Ki Jeon

(Member) Prof. Do Hyung KIM

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Publications

1- Ebrahim Osman and Do-Hyung Kim (2016) Molecular approaches for detection and quantification of *Vibrio scophthalmi* using *recA* gene. Poster presentation at spring meeting of Korean society of fish pathology. 17 Jun 2016, Busan, Korea.

2- Ebrahim Osman and Do-Hyung Kim (2016) Re-identification, quantification, and antibiograms of *Vibrio scophthalmi.* Oral presentation at 2016 Korean federation of fisheries science and technology societies, international conference. 28 Oct 2016, Bexco, Busan, Korea.

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젊은과학자상
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소 속 : Pukyong National University

귀하는 한국수산과학총연합회 국제학술대회에서 발표한 아래의 논문이 우수하며, 젊은 과학자로써 발전 잠재력이 뛰어나므로 이에 젊은 과학자상을 수여합니다.

논문제목: Re-identification, quantification, and antibiograms of *Vibrio scophthalmi*

2016년 10월 28일

한국해양과학기술진흥원장 임 광 수

양식 넙치 (*Paralichthys olivaceus*) 를 감염시키는 *Vibrio scophthalmi* 의 재동정, 정량 및 제어에 관한 연구

이브라힘 오스만

부경대교 대학원 수산생명 의학과

요약

Vibrio scophthalmi 는 스페인의 건강한 터봇 *Scophthalmus maximus* 치어의 장에서 처음 분리되었다. 이 세균은 주로 넙치 및 터봇에서 기회 감염 병원체로 간주되고 있다. 한국에서 넙치 비브리오병의 주요 원인 종으로 보고되었으며 분리주에 따른 독성의 차이 및 본 세균의 감염 실험에서 75%에 달하는 누적폐사가 보고되었다. *V. scophthalmi* 는 다른 Vibrio 속 종들과 생화학적 성상 및 16S rRNA 유전자 염기서열의 유사성으로 인하여 이들 종을 구분하는 것이 매우 어렵다. 이로 인하여, 매우 오랜 시간동안 *V. scophthalmi* 는 *V. ichthyoenteri* 로 동정되었다. *rpoD, recA, mreB* 와 같은 유전자는 *V. scophthalmi* 와 *V. ichthyoenteri* 를 구별하기 위한 좋은 분자 마커로 보고되었다.

본 연구의 목적은 *V. scophthalmi* 의 검출과 정량을 위한 분자생물학적 방법을 개발하고, 이와 더불어 본 병원체로 인한 질병을 제어할 수 있는 방법들을 확인하기 위함이다. 본 연구에서는 *V. scophthalmi* 의 검출과 정량을 위하여 *recA* 유전자에 기초한 일반 PCR 및 Real time PCR 방법을 사용하였다. 또한 *V. scophthalmi* (n=62)에 대한 7 개의 항생제에 대한 최소 억제 농도(minimum inhibitory concentrations, MICs)를 확인하였으며 각 항생제에 대한 역학적내성기준값을 구하기 위하여 normalized resistance interpretation(NRI) 방법을 이용하였다.

일반 PCR 및 real time PCR 에서 병원체의 검출한계농도는 각각 1ng 과 100fg(≈19×104 and 19 copies)이었다. Vibrio 속의 세균들을 포함한 어류 세균성 병원체 30 종 이상을 검사한 결과 교차반응은 없었으며 이는 본 시험이 *V. scophthalmi* 에 매우 특이적임을 나타냈다.

본 연구는 recA 유전자가 *V. scophthalmi* 와 다른 유사 종들을 구분할 수 있는 좋은 분자 마커로 작용하는 것을 확인하였다. 본 연구에서 개발한 방법은 빠르고, 특이적이며, 민감한 분석법으로 본 병원체를 검출하기 위한 일상적인 진단 업무와 후속 연구에 매우 유용할 것으로 사료된다. 마지막으로 MICs 와 역학적내성기준값 결과는 7 개 중 3 개의 항생제(Ceftiofur, Florfenicol, Trimethoprim)가 본 질병에 좋은 치료제로 작용할 수 있는 가능성을 나타냈다.

Studies on re-identification, quantification, and control of *Vibrio scophthalmi* in olive flounder (*Paralichthys olivaceus)*

Ebrahim Osman

Department of Aquatic Life Medicine, The Graduate School,
Pukyong National University

Abstract

Vibrio scophthalmi was first isolated from the intestine of juvenile turbot *Scophthalmus maximus* in Spain. It is considered to be an opportunistic pathogen of flat fish, primarily infecting olive flounder and turbot. In Korea, it has been reported as common species associated with olive flounder vibriosis, with strain dependent virulence, and cumulative mortality reach to 75% in experimental challenge. It is difficult to distinguish *V. scophthalmi* from its closely related species due to their high similarity in biochemical characteristics and *16S* rRNA gene sequences. Therefore, for a long time *V. scophthalmi* had been identified as *V. ichthyoenteri*. The genes: *rpoD, recA*, and *mreB* were reported as good molecular markers to differentiate *V. scophthalmi* from *V. ichthyoenteri*.

The aims of this study were to develop molecular approaches for detection and quantification of *V. scophthalmi,* in addition to indicate some good ways for control. We used conventional and real time PCR methods based on recombinase A (*recA*) gene for detection and quantification of *V. scophthalmi*; in addition to that we determined the minimum inhibitory concentration (MIC) values of seven antibiotics against *V. scophthalmi* (n=62), which were used to generate epidemiological cut off (ECOFF) values using normalized resistance interpretation (NRI) analysis.

The detection limits of PCR assays were 1ng and 100 fg (≈19×10^4 and 19 copies) of genomic DNA per reaction, respectively, for conventional and real time PCR assay. There was no any cross-reactivity to more than thirty different species of other aquatic bacterial pathogens including *Vibrio* spp., indicating that our approaches are highly specific to *V. scophthalmi.* NRI analysis successfully estimate the ECOFF value for all examined antibiotics with a limit of 2 standard deviation above the NRI calculated mean value of *V. scophthalmi* isolates distribution.

Our study confirms that *recA* gene is a good molecular marker to distinguish between *V. scophthalmi* and the other closely related species. Our developed approaches are rapid, specific and sensitive which are helpful tools for the routine laboratory diagnosis and future studies. Finally, MIC and ECOFF values results showed three antibiotics (Ceftiofur, Florfenicol, and Trimethoprim) out of seven seems likely to be good choice for the treatment of this infection in Korea.

General Introduction

Olive flounder *Paralichthys olivaceus* is one of the most important commercial food fishes, and it is commonly cultured through flatfish aquaculture tanks in inland Korea. Culture systems for this species have expanded rapidly since 1980s with production reaching 22,174 metric tons in 2016, which consider over 50% of the total fish aquaculture production in Korea (Statistics Korea, 2016). Diseases emergence is an important constraint to the expansion of aquaculture causing severe financial losses through decrease production of farmed species or increase production costs (Murray, and Peeler, 2005). The annual economic loss in aquaculture industry through diseases - caused by bacteria, fungi, viruses, and parasites - is estimated to be billions of US dollars worldwide (Defoirdt et al., 2011). Given that bacteria can survive well in aquatic environment independently of their hosts, bacterial diseases have become major impediments to aquaculture, especially when water temperature is warm (Klesius and Pridgeon, 2011). Virulent bacteria are likely to cause disease in an unstressed, well-fed host in a clean environment, and opportunistic bacteria can cause mortality under adverse environmental conditions (Murray, and Peeler, 2005). The bacterium *Vibrio scophthalmi* has been reported to be one of the main bacterial species present in diseased olive flounder in Korea (Kang.2003; and Qiao et al., 2013).

Vibrio scophthalmi was first isolated from juvenile turbot *Scophthalmimus maximus* in Spain 1997, and considered as a species commonly found in the intestinal bacterial population. The phenotypic characteristics, DNA G+C content, 16S rRNA gene sequence, and DNA–DNA hybridization similarity of *V. scophthalmi* isolates formed a homogeneous group and failed to place them in any of the known *Vibrio* species at that time (Cerdà-Cuéllar et al., 1997). At first detection time, *V. scophthalmi* considered a possible probiotic as it has been isolated only from healthy turbot (Cerdà-Cuéllar et al., 1997; Farto et al., 1999; and Montes

et al. 2003). But later on it has been isolated from the intestine of different fish species include farmed and wild olive flounder *Paralichthys olivaceus* (Kim and Kim 2013), summer flounder *Paralichthys dentatus* (Gauger et al., 2006), puffer fish *Takifugu rubripes* (Li et al., 2015), skin and rearing water of turbot *Scophthalmimus maximus* (Montes et al., 1999), and other aquatic animal like clams (Hidalgo et al., 2008). As well it has been isolated as a pure culture or associated with other bacterial pathogens from different diseased fish species e.g., turbot (Wang et al., 2004), summer flounder (Gauger et al., 2006); and commen dentex *Dentex dentex* (Sitjà-Bobadilla et al., 2007). In Korea, *V. scophthalmi* reported as one of the common dominant bacteria associated with diseased olive flounder, which became more sensitive to infection under stressful conditions (Jo et al., 2006; Qiao et al., 2012a). The virulence factors and pathogenicity study of *V. scophthalmi* revealed strain dependent virulence reaching to 75% mortality in experimental challenge (Kim et al., 2013).

All above-mentioned reasons pulled the alarm bell for us to take a foreword step since rapid and accurate identification of pathogens warrant successful treatment and remains of critical importance for aquatic animal health management (Bondad-Reantaso et al.,2005). In this context, this study aims to develop an accurate molecular approaches for specific detection and quantification of *V. scophthalmi* using conventional and real time polymerase chain reaction (PCR) assays (chapter 1), in addition to estimate the Epidemiological Cut Off (ECOFF) values for seven antibiotics and indicate the antibiotics that likely would be effective as a way to control this infection (chapter 2).

Chapter 1

1. Development of molecular approaches for detection and quantification of *Vibrio scophthalmi*

1.1. Introduction

The genus *Vibrio* members in marine habitats are known by have multiple roles, from symbiotic luminescence to pathogenicity in different organisms. *Vibrio scophthalmi* is considered to be an opportunistic pathogen of flat fish, primarily infecting olive flounder and turbot (Qiao et al., 2012b). Based on biochemical characteristics, it is difficult to distinguish *V. scophthalmi* from other *Vibrio* species, including *Vibrio ichthyoenteri* (Tarazona et al., 2015). Cerdà-Cuéllar and Blanch (2004) reported that 28 different phenotypes were retrieved from 136 isolates obtained from eight various ages of sampled turbot larvae. This may indicate that the strains, which remain during a certain period, might have certain characteristics that allow them to colonize the intestine and maintain their population more easily. The possibility of *V. scophthalmi* to colonize the intestine - reflected by phenotypic diversity - and maintain their population will increase the hazard for fish to get infection under stress, as well as reduce the ability to use the biochemical test as a marker for identification. This justify the needs to develop an accurate rapid identification tools for *V. scophthalmi*.

Several studies (Cerdà-Cuéllar and Blanch, 2002) have already shown that 16S rRNA gene sequences cannot distinguish *V. scophthalmi* from *V. ichthyoenteri*. Recently, studies using multilocus sequence analysis (MLSA) based on different housekeeping genes were performed for the typing of multiple loci of bacterial pathogens, including *Vibrio* spp. (Sawabe et al., 2007, Thompson et al., 2005). Tarazona et al., 2015 demonstrated that recombinase A (*recA*) is an excellent marker to differentiate *V. scophthalmi* from *V. ichthyoenteri*, with 99-100% and 89% for intraspecies and interspecies variance, respectively.

In this chapter, we aimed to develop molecular approaches for detection and quantification of *V. scophthalmi* using conventional and real time PCR based on *recA* gene, which can play a great role in the specific rapid identification,

differentiate *V. scophthalmi* from other closely related species, as well as early sensitive quantification directly from tissue to help in control and future researches to reduce economic losses caused by this infection.

1.2. Materials and Methods

1.2.1. Bacterial strains

Sixty-four *Vibrio ichthyoenteri*-like isolates identified based on 16S rRNA sequences and biochemical characters, were collected and used in this study. They were isolated from different locations and farmed fish species in South Korea between 2004 and 2016 as shown in Table 1-1. Four reference strains; *V. scophthalmi CECT* (Colección Española de Cultivos Tipo, Spanish Type Culture Collection) *4638^T*, *V. scophthalmi CAIM* (Collection of Aquatic Important Micro-organisms) *1797*, *V. ichthyoenteri ATCC* (American Type Culture Collection) *700023^T* and *ATCC 700024* were purchased and used for comparison in this study. Ten strains of *V. scophthalmi* and thirty aquatic pathogens, including 22 *Vibrio* spp. were used for the specificity of PCR assay (Table 1-3). Reference strains were obtained from the following sources: ATCC, BCCM (Belgian Co-ordinated Collections of Micro-organisms), CAIM, CECT, KCCM (Korean Culture Center of Microorganisms), KCTC (Korean Collection for Type Culture) and NCIMB (National Collection of Industrial and Marine Bacteria) as indicated in Table 1-3. All bacterial strains used in this study were stored at -80°C until further use.

Table 1-1 Bacterial isolates and reference stains information (identified bacterial sp. Based on 16S rRNA)

Location	Year	Isolates Number	Origin
		V. ichthyoenteri (n = 61)	
Kijang	2015	19	Olive flounder adult
Jeju	2004 – 2011	18	Olive flounder adult (2), larva (12); Starry flounder larva (2), Large scale black fish, Scar breast tusk fish
-	2008 – 2011	11	Starry flounder (2), Chub mackerel, Damsel fish, Black spot hog fish, Rainbow trout , Red sea bream, Rock fish, Bamboo leaf wrasse, Belted beard grunt
Yeosu	2007	5	Starry flounder adult
Ulsan - Sosang	2004 – 2015	3	Olive flounder adult (1); Starry flounder adult (1)
Hanam	2004	2	Olive flounder adult
Sajon	2005	1	Olive flounder adult ; Grey mullet adult
Japan (Reference strains)	1986-1988	2	Olive flounder larva
		V. scophthalmi (n = 7)	
Jeju	2005	2	Olive flounder adult
Ulsan	2012	2	Olive flounder adult
Pohang	2005	1	Olive flounder adult
Spain (Reference strains)	1997 – 2005	2	Turbot; Commen dentex

1.2.2. DNA extraction, amplification and sequencing

All cultures were grown routinely in brain heart infusion agar (BHIA; BD Difco) containing 1% NaCl (w/v) overnight at 28°C. Genomic DNA was extracted from the bacterial samples using the AccuPrep® Genomic DNA Extraction Kit (Bioneer, Daejeon, Republic of Korea) according to the manufacturer's instructions. The separated DNA was quantified by the NanoVue system (GE healthcare, Piscataway, NJ, USA). The validity of the DNA was checked using *16S* rRNA universal primers (Table 1-2).

PCR amplification and sequencing were performed with a thermal cycler (Gene Amp® PCR System 2400; Applied Biosystems, USA) on sixty-four isolates and four reference strains listed in Table 1-1 using VrecA130F and VrecA720R (Table 1-2) to target approximately 600 bp of *recA*. Amplified DNA has been sequenced by High throughput DNA Analyzer (Cosmo genetech, Busan, Korea).

1.2.3. Primers and probe design

All obtained *recA* sequences of *V. scophthalmi* were aligned together using CLUSTALW software implemented in the software package MEGA 6.06 (Tamura et al., 2013) and then compared with *V. ichthyoenteri* sequences. The primer sets; (V.sco recA-F, V.sco recA-R) and (V.sco recA2-F, V.sco recA2-R) and the probe; V.sco recA-probe had been picked using the Primer3Plus program. The BLAST analysis of NCBI (National Center for Biotechnology Information) database showed that the primer sets and probe are complementary to the parts (222 to 240, 526 to 507 bp), (513 to 532, 621 to 602 bp) and 539 to 561 bp respectively of *recA* gene of *V. scophthalmi LMG 19158* (GenBank accession Number: HM771381.1) as indicated in Figure 1-1. The probe sequence labelled with the fluorescent reporter dye FAM (6-Carboxyfluorescein) at the 5'end and double fluorescent quencher dye

(ZEN in the middle and Iowa Black FQ at the 3’end). All primers and probe were purchased from m.biotech (Hanam, Korea).

Table 1-2 Primers and probe information used for study of *V. scophthalmi* by conventional and real-time PCR

Primers and probe	Sequence (5'-3')	Target gene	Amplicon size (bp)	Target organism	Reference
V.sco recA-F	GCACTTGCACGTTCAGGTG	Recombinase A	305	*Vibrio scophthalmi*	This study
V.sco recA-R	TTGATCGAACCCGTACGACG				
V.sco recA2-F	ACGGGTTCGATCAAAGATGG	Recombinase A	109	*Vibrio scophthalmi*	This study
V.sco recA2-R	AAAGAATCTGTGTTTCAGCT				
V.sco recA-probe	/56-FAM/ AGT GGT GGG /ZEN/ TAA CGA AAC ACG CA /3IABkFQ/				
VrecA130F	GTCTACCAATGGGTCGTATC	Recombinase A	600	Most of *Vibrios*	Sawabe et al. (2007)
VrecA720R	GCCATTGTAGCTGTACCAAG				
16S1	AGAGTTTGATCMTGGCTCAG	16S rRNA	1466	Universal	Kim et al. (2008)
16S2	TACGGYTACCTTGTTACGACTT				

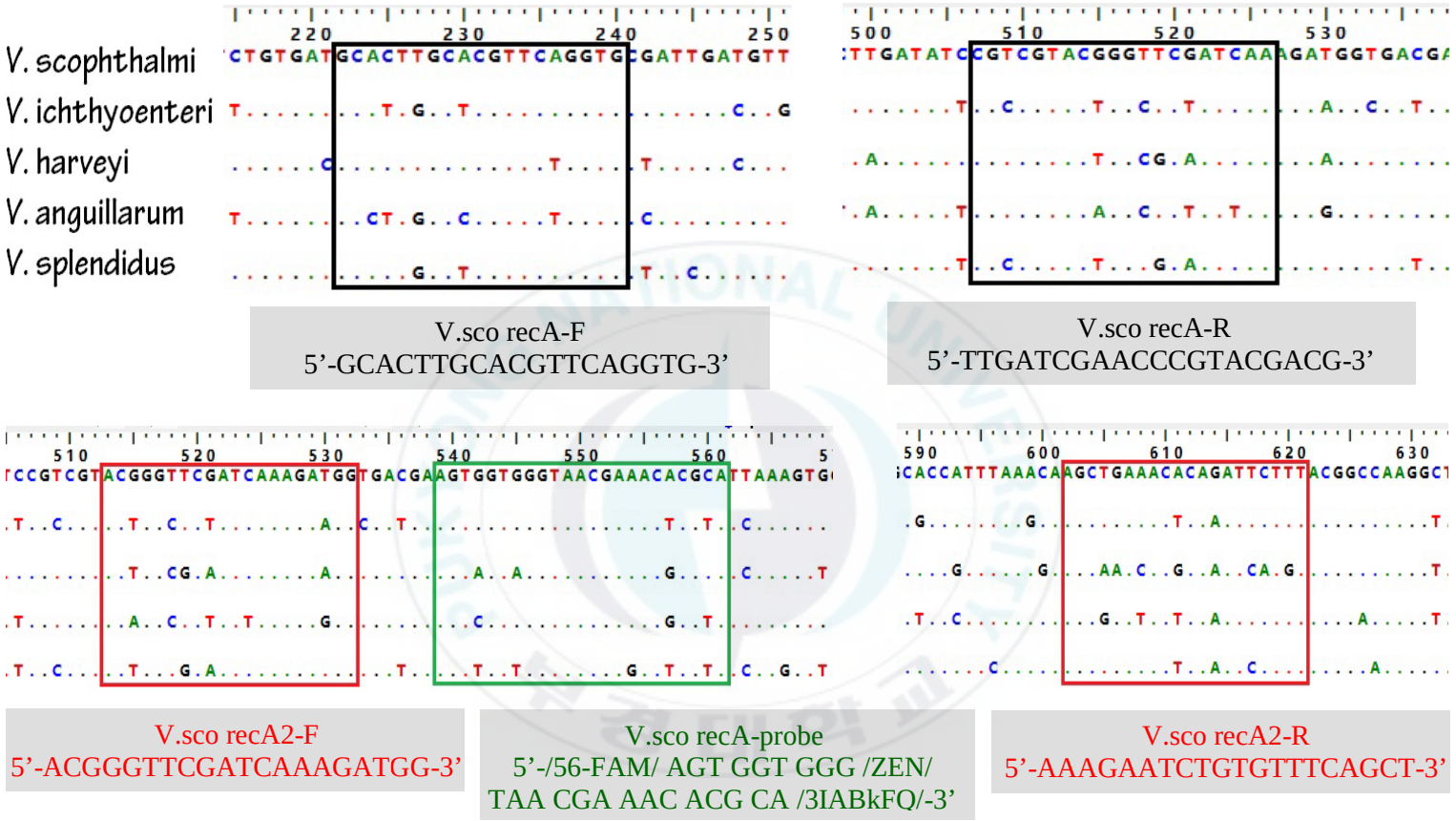


Figure 1-1. Multiple sequence alignments of *V. scophthalmi and other close vibrios recA* gene sequence. Boxes with different colors are indicating *recA* primers and probe binding sites corresponding to *V. scophthalmi LMG 19158 recA* gene sequence (GenBank accession Number: HM771381.1). Nucleotides identical to those of *V. scophthalmi* are indicated with dots.

1.2.4. Conventional PCR assay

PCR assay of *V. scophthalmi* strains to get *recA* sequences (≈600 bp) were performed with *recA* primers (VrecA130F and VrecA720R) according to Sawabe et al. (2007). 10 pmole of primer pair, diluted DNA 1 μl, and sterile distilled water up to total volume 20 μl were added to AccuPower® PCR PreMix (Bioneer, Daejeon, Korea).

Gradient PCR was carried out in order to select an appropriate annealing temperature without non-specific reactions for detection of *V. scophthalmi* with *recA* primers (V.sco recA-F and V.sco recA-R). Then the PCR condition was set as follows: initial denaturation at 94˚ C for 5 min; 30 cycles of amplification (denaturation at 94˚ C for 30 s, annealing at 69˚ C for 18 s, extension at 72˚ C for 30 s); and final extension of the incompletely synthesized DNA at 72˚ C for 7 min.

Colony PCR for *V. scophthalmi* strains appointed in Table 1-3 (n=10) has been run according to the indirect reaction described by Fukui and Sawabe (2007). A small amount (≈ 1 mm^3) of a single colony was picked using autoclaved 10 μl micropipette tip and suspended in 10 μl autoclaved distilled water and vortexed for 20 second, and then added to the PCR tubes as DNA template following the same PCR condition mentioned previously. Amplified products were displayed by electrophoresis on ethidium bromide-stained 1.5% agarose gels (Bio-Rad).

1.2.5. Real time PCR assay

Quantification of the gene *recA* was performed with an Exicycler^{TM} 96 Real-Time Quantitative Thermal Block in 0.2ml White 8-strip qPCR tube (Bioneer). Each reaction tube of 25 μl reaction mixture (rxn) containing 12.5 μL qPCR probe 2X mastermix (m.biotech, Gyeonggi Hanam, Korea), 1 μl of each 10 μM primer (V.sco recA2-F and V.sco recA2-R), 1 μl of 10 μM probe and 5 μl of DNA template (2ng μl^{-1}). Adhesive optical sealing film from Bioneer was used to seal the qPCR tube.

The amplification protocol set as follows: denaturation at 95°C for 10 min, followed by 45 cycles of 15 s of denaturation at 95°C and 13 s of annealing/extension at 64°C. Standard curves for quantification constructed from 10-fold serial dilutions ($10 – 10^{-5}$ ng rxn $^{-1}$) of DNA template extracted from *V. scophthalmi CECT 4638^T* in triplicate using cycle threshold (Ct) values. The results of real-time PCR were confirmed by electrophoresis on ethidium bromide-stained 1.5% agarose gels (Bio-Rad) and were displayed as absolute amounts of genomic DNA. All analysis used the Exicycler3 Analysis Program (ver. 3.53 from Bioneer) and Microsoft Excel 2013.

1.2.6. Specificity and sensitivity

The species specificity of the *recA* gene primers were assessed by testing purified genomic DNA derived from *V. scophthalmi* strains and a wide range of other aquatic bacterial pathogens (non-*V.scophthalmi*) listed in Table 1-3. A concentration of > 30 ng and 10 ng of purified genomic DNA has been used, respectively, for conventional and real-time PCR assay.

The sensitivity of detection has been checked using genomic DNA purified from *V. scophthalmi CECT 4638^T* which tenfold serially diluted in distilled water in a triplicate sets and subjected to PCR amplification using V.sco recA primers (Table 1-2). The dilution range for conventional PCR assay was $10^3 – 10^{-3}$ ng amplified in 30 cycles, while $10 –10^{-5}$ ng amplified in 45 cycles for real-time PCR assay.

1.2.7. Repeatability and reproducibility of the real time PCR assay

The intra-assay variance (repeatability) and inter-assay variance (reproducibility) of the reaction were estimated by amplifying six consecutive 10-fold serial dilutions ($10 – 10^{-4}$ ng rxn^{-1}) of purified DNA from *V. scophthalmi CECT 4638^T* in triplicate and repeated in three separate reactions. A standard curve was constructed for each reaction based on DNA serial dilutions, on which Ct values were plotted against the

log values of the target DNA amount. The coefficient of variation (CV) for copy number variance, which is defined as percentage of standard deviation of the mean calculated copy number for each of different DNA dilutions, was used to evaluate the repeatability and reproducibility of the reaction according to Kuhar et al. (2013). Copy number was calculated from the Ct values of DNA dilutions using regression equations obtained from the standard curves.

1.2.8. Detection of *V. scophthalmi* from inoculated tissue homogenates

Kidney tissue and Gut (chyme) samples were harvested from healthy olive flounder fish (n=2), which were obtained from a commercial fish farm in Korea. The samples were thoroughly homogenized with a suspension of overnight cultivated *V. scophthalmi CECT 4638*^{T} diluted in PBS to a final concentration of 1x10^9 and 1x10^7 CFU. Same concentrations were also prepared in PBS (without tissue) to check the effect of fish tissues existence on real time PCR result. DNA was totally extracted according to the instructions of the manufacturer using the AccuPrep® Genomic DNA Extraction Kit (Bioneer, Daejeon, Republic of Korea). Real-time PCR was used to enumerate the number of *V. scophthalmi*, and the results were compared with standard plate counting method values in brain heart infusion agar (BHIA; BD Difco) containing 1% NaCl (w/v). The statistical analysis was conducted with Student's t-test using.

1.2.9. Determining the copy number of *V. scophthalmi* genomic DNA

Based on *V. scophthalmi CECT 4638*^{T} (Gen-Bank accession no. GCA_000222585.2) genome size 4,854,288 bp, The DNA copy number was calculated with a formula available online (Staroscik 2004).

$$\text{Number of copies} = \frac{6.022 \times 10^{23}\ (\text{copies mol}^{-1}) \times \text{DNA amount (ng)}}{\text{DNA length (bp)} * 1 \times 10^{9} * 650\ (\text{g mol}^{-1}\ \text{bp}^{-1})}$$

1.3. Results

1.3.1. Analytical specificity of detection

The validity of the DNA was checked using 16S rRNA universal primer which yielded a band of 1466 bp appeared from all 23 *Vibrio* and 10 non-*Vibrio* bacterial species.

All *V. Scophthalmi* strains were successfully amplified in both conventional and real time PCR assays with a product size 305 and 109 bp, respectively (Table 1-3). Moreover, Colony PCR results showed a clear band for all *V. scophthalmi* strains appointed in Table 1-3. No amplification band/signal was obtained from any of non-*V. scophthalmi* samples, including olive flounder tissues, and negative control. All results indicated that this assay is highly specific to *V. scophthalmi*.

1.3.2. Analytical sensitivity of detection

The minimum level of detection using V.sco recA primers was 1 ng for conventional PCR assay and 10^{-4} ng for real-time PCR assay (Figure 1-2). A standard curve was generated from Ct values against the common logarithm log of quantities DNA, Linear dynamic range 10 to 10^{-4} ng rxn^{-1}, and correlation coefficient $R^2 = 0.9976$ (Figure 1-2.B). Using the formula mentioned before and Based on the *V. scophthalmi CECT 4638^T* (Gen-Bank accession no. GCA_000222585.2), The DNA copy number corresponding to minimum level of detection was $\approx 19\times10^4$ and 19 copies of genomic DNA per reaction, for conventional and real time assay, respectively.

Table 1-3 Specificity of conventional and real time PCR assay.

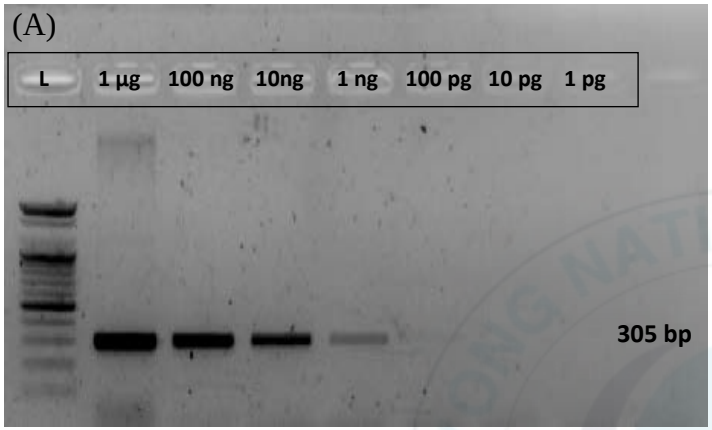
Name	Code	Conventional PCR	Real time PCR
1. *Vibrio scophthalmi*	*CECT 4638 ^{T}, CAIM 1797,* 8 isolates (*A19010, A19008, ViOF-1, Vi9-7, ViSF-1, CM1, 24-a, ViRF-2*)	+	+
2. *Vibrio ichthyoenteri*	*ATCC 700023^T, ATCC 700024,* 1 isolate (*12-b-K*)	—	—
3. *Vibrio ponticus*	1 isolate (*8-a-S-MA*)	—	—
4. *Vibrio parahaemolyticus*	*KCCM 11965 (ATCC 17802^T), ATCC 33844*	—	—
5. *Vibrio campbellii*	*KCCM 41986*	—	—
6. *Vibrio alginolyticus*	*KCTC 2928,* 1 isolate (*Vi9-3*)	—	—
7. *Vibrio vulnificus*	*ATCC 27562^T, LMG 16868, LMG 16852*	—	—
8. *Vibrio anguillarum*	*ATCC 19264^T*	—	—
9. *Vibrio harveyi*	*ATCC 14126^T, ATCC 35084, 2 isolates* (*FR2, FF10*)	—	—
10. *Aliivibrio logei*	*KCTC 12281^T*	—	—
11. *Vibrio pomeroyi*	1 isolate (*F2-1aL*)	—	—
12. *Vibrio fischeri*	1 isolate (*Vibrio fischeri 1*)	—	—
13. *Vibrio fortis*	2 isolate (*8-1-a, Q4-2S*)	—	—
14. *Vibrio tubiashi*	2 isolate (*S.R bK, 12/22*)	—	—
15. *Aliivibrio finisterrensis*	1 isolate (*6EP2-1*)	—	—
16. *Vibrio tasmaniensis*	1 isolate (*6EP6-1*)	—	—
17. *Vibrio hepatarius*	1 isolate (*11W6 FAP 2-1*)	—	—
18. *Enterovibrio nigricans*	1 isolate (*AT3*)	—	—
19. *Vibrio splendidus*	*KCTC 12679^T*	—	—

Table 1-3 (continued) Specificity of conventional and real time PCR assay.

Name	Code	Conventional PCR	Real time PCR
20. Vibrio diazotrophicus	*KCCM 41666*	—	—
21. Vibrio fluvialis	*KCCM 40827*^{T}	—	—
22. Vibrio mimicus	*ATCC 33653*^{T}	—	—
23. Vibrio sp. CECT 8220	1 isolate (*N1-3aK*)	—	—
24. Streptococcus parauberis	LMG 12174^T	—	—
25. Streptococcus iniae	*ATCC 29178*^{T}	—	—
26. Lactococcus garvieae	*ATCC 43921*^{T}	—	—
27. Aeromonas hydrophila subsp. hydrophila	*ATCC 7966*^{T}	—	—
28. Aeromonas salmonicida subsp. salmonicida	*NCIMB 833*	—	—
29. Aeromonas salmonicida subsp. Masoucida	*KCCM 40239*^{T}, 1 isolate (*RFAS1*)	—	—
30. Edwardsiella tarda	*ATCC 15947*^{T}, 1 isolate (*KE-1*)	—	—
31. Tenacibaculum maritimum	1 isolate (*TmOF-1*)	—	—
32. Photobacterium damselae subsp. damselae	*ATCC 33539T*, 2 isolate (*FP2260, Fp2261*)	—	—
33. Photobacterium damselae subsp. pisicida	1 isolate (*PdOF-1*)	—	—
34. NTC		—	—

Abbreviations: ATCC, American Type Culture Collection; BCCM/LMG, Belgian Co-ordinated Collections of Micro-organisms; CAIM, Collection of Aquatic Important Micro-organisms; CECT, Colección Española de Cultivos Tipo, Spanish Type Culture Collection; KCCM, Korean Culture Center of Microorganisms; KCTC, Korean Collection for Type Culture; and NCIMB National Collection of Industrial and Marine Bacteria.

NTC, No template control; +, positive detection (only 1 amplification product); —, no amplification.



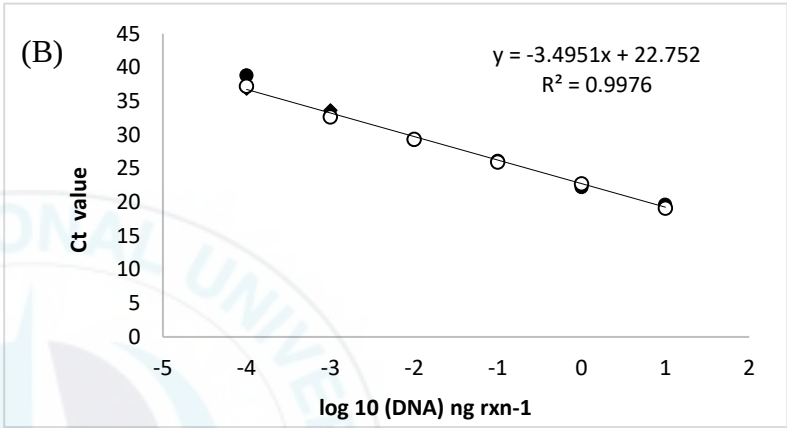
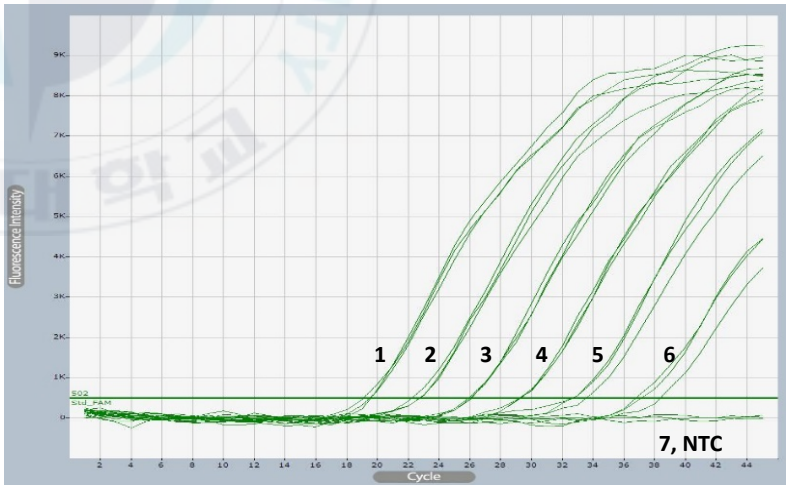


Figure 1-2. The standard curve and detection sensitivity of conventional and real-time PCR assay. (A) Sensitivity of conventional PCR for detection of serial dilutions of *V. Scophthalmi* DNA (10^{3} – 10^{-3}ng) after 30 cycles; (B) The standard curve generated from the mean of Ct values against log10 of tenfold serial dilutions of *V. Scophthalmi CECT 4638* ^{T} genomic DNA; (C) Sensitivities of real-time PCR for detection of tenfold serial dilutions of *V. Scophthalmi* DNA (10^{-5}–10ng) after 45 cycles. Curves 1–7: 10^{1}, 10^{0}, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} ng µL $^{-1}$ *V. Scophthalmi CECT 4638* ^{T} genomic DNA; The curve NTC: negative control. Each concentration performed in triplicate (represented by ♦, ● and ○, respectively).

(C)



1.3.3. Repeatability and reproducibility of the real time PCR assay

The serial dilutions that used to evaluate repeatability and reproducibility of the reaction showed a wide dynamic range of Ct values: 19.41–37.60 and 19.45 – 37.39 for intra-assay and inter-assay, respectively, of reliable amplification linearity. Intra-assay CV for all dilutions ranged from 0.41% to 37.33% with the most variation observed in the highest dilution, while the Inter-assay CV values ranged from 0.23% to 8.33% for the lowest and highest dilutions, respectively (Table 1-4).

1.3.4. Detection of *Vibrio scophthalmi* in inoculated fish tissue homogenate

Regression equation obtained from the standard curve was used to calculate the log copy number from the Ct values of DNA isolated from the PBS suspension and inoculated tissue homogenates. The comparison of bacterial concentrations, as determined by plate counts and real-time PCR results, showed a good Pearson's correlation coefficient (R^2=0.922; $P < 0.05$) between log copy number of purified DNA and V. *scophthalmi* log of CFUs determined by plate counting method (Figure 1-3; Table 1-5).

Table 1-4 Repeatability and reproducibility of the real time PCR assay

Dilution of DNA (ng rxn^{-1})	Intra-assay performance				Inter-assay performance			
	Mean Ct	SD	average log copy number[a]	CV[b] (%)	Mean Ct	SD	average log copy number[a]	CV[b] (%)
10^{1}	19.41	0.25	6.17 ± 0.10	1.58	19.43	0.04	6.16 ± 0.01	0.23
10^{0}	22.57	0.29	5.29 ± 0.12	2.20	22.43	0.24	5.33 ± 0.10	1.80
10^{-1}	26.03	0.08	4.32 ± 0.03	0.74	26.01	0.03	4.34 ± 0.01	0.31
10^{-2}	29.27	0.04	3.42 ± 0.01	0.41	29.46	0.33	3.37 ± 0.13	3.83
10^{-3}	32.99	0.53	2.38 ± 0.21	8.72	33.22	0.39	2.33 ± 0.16	6.65
10^{-4}	37.60	1.04	1.10 ± 0.41	37.33	37.46	0.24	1.15 ± 0.10	8.33

[a], Calculation of copy number was based on average Ct values.

[b], Coefficient of variation.

Table 1-5 Detection of *V. scophthalmi* in PBS suspension and inoculated tissue homogenates

	Inoculum (CFU)	Mean of Ct	real time assay (log of copy) [a]	plate count (log of CFU)
PBS-1	10^9	15.49 ± 1.04	8.56 ± 0.30	9.5 ± 0.02
PBS-2	10^7	20.73 ± 0.90	7.06 ± 0.26	7.46 ± 0.10
KID-1	10^9	16.45 ± 0.23	8.29 ± 0.07	10.01 ± 0.01
KID-2	10^7	21.24 ± 0.71	6.92 ± 0.20	7.99 ± 0.02
GUT-1	10^9	16.12 ± 0.50	8.38 ± 0.14	ND
GUT-2	10^7	21.86 ± 0.49	6.74 ± 0.14	ND
NTC	-	NA	NA	-

[a], Calculation of copy number was based on average Ct values.

PBS: bacterial suspension in PBS; KID: bacterial homogenate with kidney tissue; GUT: bacterial homogenate with gut tissue; NA: No amplification; ND: Not done.

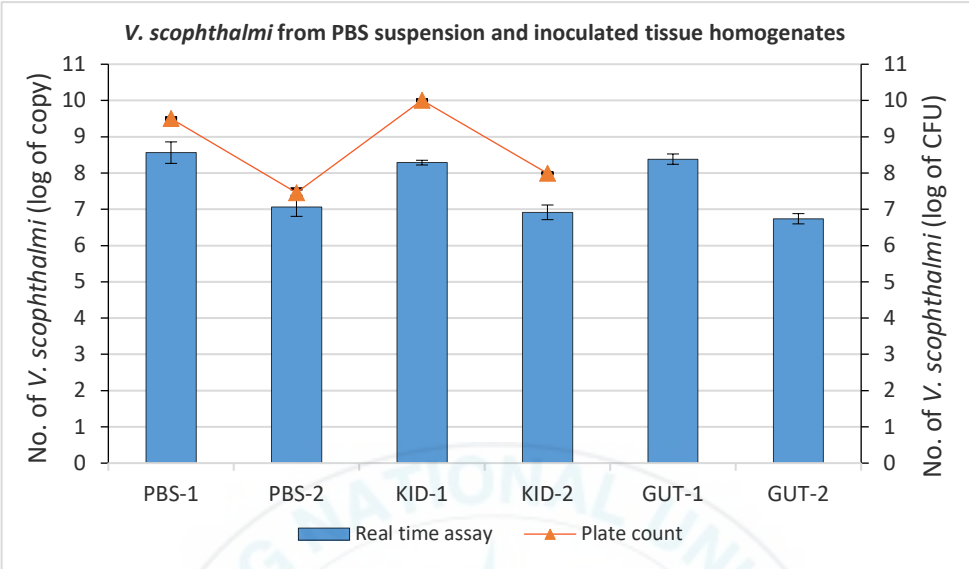


Figure 1-3. Detection of *V. scophthalmi* in PBS suspension and inoculated kidney and gut tissue homogenates by real time PCR assay and plate count method, with a strong pearson's correlation coefficient between the two method (R^2=0.922; $P < 0.05$).

1.4. Discussion

We demonstrate that molecular approaches based on *recA* gene developed in this study successfully detected and quantified only *V. scophthalmi* with high sensitivity and specificity. Although several conventional and real time PCR assays have been developed for pathogenic Vibrios (Cano-Gomez et al.,2015, Haldar et al.,2010, Kim et al., 2008), to our best knowledge, there is no PCR assays for *V. scophthalmi*.

Most of *Vibrio* species cannot be delineated using only 16S rRNA sequence, and other house-keeping genes that are less conserved are required (Tarazona et al., 2015). A previous study (Sawabe et al., 2007) revealed that 58 vibrio taxa were divided into 14 monophyletic clades with a significant bootstrap value on the basis of MLSA using nine gene sequences (*ftsZ*, *gapA*, *gyrB*, *mreB*, *pyrH*, *recA*, *rpoA*, *topA* and the16S rRNA gene). A recent study (Sawabe et al., 2014) using MLSA with the same house-keeping genes except for the16S rRNA gene showed that the clade Scophthalmi consists of *V. ichthyoenteri*, *V. scophthalmi*, and *V. ponticus*, indicating they are very tightly related, although a study conducted by Tarazona et al. (2015) showed the latter was always external to the Scophthalmi clade. In this recent study (Tarazona et al., 2015), both individual gene trees and the tree obtained from concatenated sequences of five genes (*rpoD, mreB, recA, ftsZ, and gyrB*) were able to consistently differentiate four subclades, including *V. scophthalmi* and *V. ichthyoenteri*, within the Scophthalmi clade. They concluded that *rpoD*, *recA* and *mreB* were the best genes to differentiate the clade. *mreB* gene was the one provided the maximum differentiation between the members, but it showed wide range of intra-specific identity for *V. scophthalmi* 95-100%, while average of 99.4% for the other 2 gene. *recA* gene had showed more power than *rpoD* to distinguish *V. scophthalmi* with inter-specific identity 89.2 %.

The gene *recA* is well known housekeeping gene frequently concluded in MLSA and phylogenetic studies for several bacterial spp. (Thompson et al., 2008, Menna et al., 2009, Ramos et al., 2011, Suharjo et al., 2014). As shown by Tarazona et al. (2015), *recA* gene was proved to be a good marker in this study with 100% and 99% for intra-species similarity of *V. ichthyoenteri* (6 isolates) and *V. scophthalmi* (40 isolates), respectively, and 89% for inter-species similarity.

Indeed, *dnaJ* gene has also been used for identification of *Vibrio* species (Nhung et al., 2007), although previous studies using MLSA have not included the gene. Qiao et al. (2012a) identified successfully five bacterial isolates obtained from diseased olive flounder as *V. scophthalmi*. In our study, although inter-species similarity between *V. ichthyoenteri* and *V. scophthalmi* was approximately 92%, intra-species similarity showed comparatively a wide range of 97% and 97.8 ± 2.5% for the species, respectively, suggesting that discriminating power of recA gene is higher than *dnaJ*. However, sequences of *dnaJ* genes obtained in this study were only 1 sequence of *V. ichthyoenteri* and 4 sequences of *V. scophthalmi*, which are not enough. Also, unavailability of publicly-accessible *dnaJ* gene sequences of *V. scophthalmi* and other vibrios was constraint for extensive analysis. Further study may be needed for this gene. Another house-keeping gene, *rpoB*, was used to develop multiplex primer sets to detect a few *Vibrio* species, including *V. ichthyoenteri* (Kim et al., 2014). However, they did not use any *V. scophthalmi* strains, and we found that *rpoB* gene of *V. scophthalmi* has complementary sequences for the probes when primer-BLAST was used. To avoid this problem, we tried to be sure that the designed primers are highly conserved to *V. scophthalmi* and we investigated their specificity with wide range of aquatic vibrios to keep away from any cross-reactivity.

Our real time assay using TaqMan probe successfully quantified not only nucleic acids of *V. scophthalmi* but those in artificially inoculated fish tissue sample (Figure 1-3, Table 1-5), without any cross-reactivity with healthy un-inoculated tissue (data not shown). The detection limit of our quantitative PCR assay (100 fg = ~19 copies) was approximately 100 times sensitive than conventional method (1 ng = ~19×10^4 copies) developed in this study. TaqMan probe used in study was tagged by one reporter dye (FAM) to the 5′ end, and two quenchers (ZEN and 3IABkFQ), allowing the reduction of background and increases of the sensitivity and precision of the assay. Our real time assay standard curve showed linear dynamic range with high correlation (R^2= 0.9976) and good efficiency (E= 0.932) of the reaction. According to Bustin et al. (2009), the precision of qPCR has been determined by expressing the variance in copy numbers (coefficient variations). Our real time PCR assay showed good level of accuracy as the vast majority of dilutions in intra-assay and inter-assay performance gave relatively low percentage of CVs (except the highest dilution in intra-assay), indicating a good level of repeatability and reproducibility (Table 1-4). In the comparison of conventional plate counting method with real time PCR assay, we considered that one colony contain one chromosome since the CFU number was highly correlated to copy number (R^2=0.922; $P < 0.05$) with no significant difference. As well there was no significant difference in qPCR results for the inoculated fish tissues (kidney and gut) when it compared with suspension in PBS (Figure 1-3). Thus, potentiate the accuracy and show the benefits of our qPCR assay to detect and quantify *V. scophthalmi* copy number directly from tissues and gut chyme.

Our study confirms that *recA* gene is a good molecular marker to distinguish between *V. scophthalmi* and the other closely related species. These developed approaches are rapid, specific and sensitive; they can be used for routine laboratory diagnosis, as well as it will be helpful for the future

researches like bacterial replication, host – pathogen interaction, and investigating the in-vivo effect of any factor on bacteria inside the host body.

Chapter 2

2. Generation of antibiograms for *Vibrio scophthalmi* using international standardized broth dilution method

2.1. Introduction

Controlling diseases caused by various pathogens is considered the most important factor to maintain healthy stocks and to increase production. The basic strategies are either by disease control with chemo- and non-chemotherapeutic agents, and by host control with vaccines and immune stimulants (Park, 2009). Antibacterial chemotherapy has been the cornerstone upon which the aquaculture industry has been built (Inglis, 2000). Despite the adverse effects of antimicrobial agents, it remains the effective remedial method available for several bacterial diseases in aquaculture industry (Lee et al., 2012; Valdés et al., 2009; and Rodger, 2016).

In recent years, the World Animal Health Organization (OIE) stated that antibiotic susceptibility testing plays essential roles in the responsible use of therapeutic agents in aquaculture. To avoid the difficulties in comparisons of antibiotic susceptibility levels, one uniform standard for the whole world and a unified basis for interpretations has been suggested by the introduction of so called 'bacteriological breakpoints' separating the normal, wild type susceptible population from strains with any kind of resistance mechanism (Williams, 1978; and Williams, 1990). Later on, another terminology 'epidemiological cut off values' were proposed by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (2011). Minimum inhibitory concentration (MIC) distributions can be used to create epidemiological cut off values to distinguish susceptible from resistant populations of pathogens.

In this chapter, all *V. scophthalmi* isolates (n=62) that were re-identified in this study were used to determine their MIC values of seven antimicrobials by means of standardized broth micro dilution protocol recommended by clinical and laboratory standards institute (CLSI 2014a) document VET 04-A2 for group 1 non-fastidious bacteria. Then the MIC

distributions were used to generate epidemiological cut off values of the antimicrobials for all strains using normalized resistance interpretation (NRI) analysis which previously proposed by Kronvall (2010) and Kronvall et al. (2006). The application of NRI analysis and species-specific epidemiological cut-off values to our data allowed determining whether strains should be categorized as either fully susceptible, wild type (WT) members of their species or, because they manifest reduced susceptibility, as non-wild-type (NWT) members.

2.2. Material and Methods

2.2.1. Bacterial Strains and culture

Sixty Korean *Vibrio scophthalmi* isolates (identified by *recA*) in addition to two reference strain: *V. scophthalmi CECT 4638*^{T} and *V. scophthalmi CAIM* 1797 have been used for antimicrobial susceptibility testing. They were isolated from different locations and farmed fish species in South Korea between 2004 and 2016 as shown in Table 2-1. All isolates had been specifically identified by conventional polymerase chain reaction (PCR) method that described in chapter 1 using V.sco *recA* primer set (Table 1-2) as well confirmed by blasting *recA* gene sequences to NCBI database with identity 99-100 %.

All bacterial strains used in this study were taken from stored stocks at −80˚C and cultivated for 24 hours at 28°C in brain heart infusion agar (BHIA; BD Difco) containing 1% NaCl (w/v).

Table 2-1 *Vibrio scophthalmi* isolates (n=60) and reference stains (n=2) information.

Location	Year	Isolates Number	Origin
Gijang	2015 – 2016	19	Olive flounder adult
Jeju	2004 – 2011	19	Olive flounder adult (4), larva (12); Starry flounder larva (2), Scar breast tusk fish
-	2008 – 2011	8	Starry flounder, Chub mackerel, Damsel fish, Black spot hog fish, Rainbow trout, Red sea bream, Rock fish.
Yeosu	2007	5	Starry flounder adult
Ulsan	2012 – 2015	4	Olive flounder adult (3); Starry flounder adult (1)
Hanam	2004	2	Olive flounder adult
Sosang	2005	1	Olive flounder adult ; Grey mullet adult
Sajon	2004	1	Olive flounder adult
Pohang	2005	1	Olive flounder adult
Spain (Reference strains)	1997 – 2005	2	Turbot; Commen dentex

- Identification Based on *recA*.
- larva & adult Olive flounder (n = 42); larva & adult starry flounder (n = 10); Other fish sp. (n = 10

2.2.2. Determination of minimum inhibitory concentrations (MICs)

Minimum inhibitory concentrations for all isolates were determined following the broth micro dilution protocol recommended by clinical and laboratory standards institute (CLSI 2014a) document VET 04-A2 for group 1 non-fastidious bacteria. The test was performed using 96 well cell culture plates - U type (SPL Life science, Korea). These plates contained the following antimicrobials: Ceftiofur hydrochloride, Florfenicol, Enrofloxacin, Flumequine, Oxolinic acid, Tetracycline hydrochloride, and Trimethoprim; All were obtained from Sigma–Aldrich. All antimicrobials were prepared and ordered in a 2-fold serial dilution of concentration 256 to 29×10^{-4} mg L^{-1} in cation-adjusted Mueller Hinton II Broth (CAMHB, BD, BBL^{TM}) with 1% NaCl to total volume 100 µl per well. Each 24 hours old *V. scophthalmi* isolate was suspended in PBS and inoculated at a cell density equivalent to 5×10^{4} CFU per well, each plate included a growth control well (without antibiotics) and a negative (un-inoculated) broth well. All plates were wrapped by plastic cover and then incubated for 24 to 28 hours at 28°C in an ambient air incubator.

2.2.3. Quality control

Following the recommendation of clinical and laboratory standards institute (CLSI. 2014b, document VET 03/ VET 04-S2), the reference strains *Escherichia coli* (*E. coli*) *ATCC 25922* was included on each test day for quality control, and were tested on CAMHB (BD, BBL^{TM}) without NaCl supplementation at 35 ± 2°C for 16 - 20 h.

Quality control ranges for broth dilution testing that approved by CLSI. (2014b) were used for comparison.

2.2.4. Statistical analysis

Epidemiological cut off values (ECOFF) of seven antimicrobial agents against *V. scophthalmi* were estimated using normalized resistance interpretation (NRI) analysis according to the method previously described by Kronvall et al. (2006) and Kronvall (2010). The NRI analysis was performed using the MS Excel spreadsheet available on-line at http://www.bioscand.se/nri/, which adapted to be applied to data sets obtained using two overlapping doubling dilution series.

The NRI method was used with permission from the patent holder, Bioscand AB, TÄBY, Sweden (European Patent No. 1383913, US Patent No. 7,465,559). Epidemiological cut-off values were calculated based on 2 standard deviations (SD) above the NRI calculated mean value (Kronvall, 2010).

2.3. Results

2.3.1. MICs and application of NRI analysis

The minimum inhibitory concentration for seven antimicrobials against *V. scophthalmi* (n=62) were shown in Table 2-1, and its distribution in histograms were shown in Figures 2-1, and 2-2. On all test days the MIC values of the reference strain (*E. coli ATCC 25922)* used in this study were all within the quality control ranges provided by the CLSI (2014b) guideline. Using a 2 SD limit above the NRI calculated mean values, the wild type cut off values (COwt) of *V. scophthalmi* when treated with ceftiofur, florfenicol, enrofloxacin, flumequine, oxolinic acid, tetracycline, and trimethoprim were ≤ 0.375, 2, 0.094, 0.375, 0.25, 0.75 and 0.5 mg/L, respectively (Table 2-3).

Table 2-2

MIC values for seven antimicrobials against *V. scophthalmi* (n=62).

Isolate Code	**Class/antimicrobial agent code**						
	Cephalosporin	Phenicol	Quinolones			Tetracycline	Folic Acid Antagonist
	CEF	FLO	ENR	FLU	OXO	TC	TMP
ViOF-1	0.0938	1.5	0.0313	0.1875	0.0625	0.0313	0.0625
ViOF-2	0.0313	1.5	0.0156	0.0938	0.0313	**96**	0.5
ViOF-3	**≥256**	0.5	0.0156	0.0938	0.0313	0.0313	**0.75**
ViOF-4	0.0234	0.75	0.0234	0.0938	0.0469	**96**	**≥256**
ViOF-6	0.0469	0.75	**2**	**12**	**12**	0.75	0.1875
ViOF-9	0.0234	**3**	0.0313	**0.75**	0.0938	**128**	**0.75**
A19010	0.0234	0.75	0.0313	0.25	0.125	0.0625	**≥256**
A19006	**≥256**	1	0.0313	0.125	0.0625	0.0625	0.5
A19008	**≥256**	0.5	**1**	**12**	**12**	0.75	0.1875
ViOF-12	**≥256**	1	0.0156	0.0938	0.0313	0.0625	0.5
Vi07-11-2	0.1875	1	**6**	**24**	**24**	**64**	0.125
Vi07-11-3	0.0938	1	**4**	**16**	**12**	**1**	0.5
Vi07-14-1	0.375	**96**	**2**	**6**	**6**	**48**	0.0938
Vi07-17-1	0.0234	**24**	**1**	**4**	**4**	**48**	0.0469
Vi08-30-2	0.0469	**24**	0.0313	0.1875	0.0938	**96**	0.0938
Vi08-30-4	0.0469	**32**	0.0234	0.0938	0.0625	**64**	0.0234
Vi08-30-1	0.0469	**24**	0.0313	0.1875	**≥256**	**48**	0.0938
Vi08-30-6	0.0469	**24**	0.0313	0.25	0.0625	**32**	0.125
Vi09-14-8	0.375	1.5	0.0625	0.25	0.125	0.125	0.125
Vi09-21-4	0.375	1.5	0.0625	0.25	**0.5**	0.0625	0.0938
Vi09-21-6	0.1875	1.5	0.0625	0.25	0.125	**48**	0.0938
Vi09-21-3	**0.75**	1.5	**1**	**3**	**3**	0.0625	0.25
A28003	**0.75**	1.5	**1**	**4**	**8**	0.0469	0.375

Table 2-2 (continued)

Isolate Code	**Class/antimicrobial agent code**						
	Cephalosporin	Phenicol	Quinolones			Tetracycline	Folic Acid Antagonist
	CEF	FLO	ENR	FLU	OXO	TC	TMP
A28004	0.0938	1.5	**1**	**3**	**4**	0.0234	0.25
Vi2	0.0938	2	**1**	**4**	**2**	0.375	0.125
Vi3	0.0938	**6**	**2**	**16**	**16**	0.375	0.125
Vi4	0.1875	1.5	**3**	**8**	**16**	**96**	0.125
Vi5	0.1875	1.5	**2**	**8**	**6**	0.375	0.1875
Vi6	0.0938	1.5	**2**	**8**	**6**	0.5	0.1875
Vi9-7	**0.75**	1.5	**0.25**	**0.75**	0.25	0.375	0.375
Vi9-9	**0.5**	2	**0.25**	**0.75**	**0.5**	0.5	0.25
ViSF 3-3	0.1875	1.5	0.0938	0.25	0.0469	**96**	0.1875
ViSF 3-4	0.0469	1.5	0.0156	0.1875	0.0938	**96**	0.125
ViSF-1	0.0938	**≥256**	**2**	**6**	**6**	**96**	0.1875
ViGM-17	0.0625	1	0.0313	0.125	0.0938	0.0625	0.125
CM1	0.0938	1.5	0.0313	0.125	0.0625	0.0234	**0.75**
2-2-b	0.0469	1.5	0.0313	0.125	0.0469	0.1875	0.125
12-2-a	0.1875	1.5	0.0313	0.1875	0.0938	0.0938	0.1875
24-a	0.0469	1	0.0078	0.0625	0.0313	**48**	**≥256**
F1-1bS	**0.5**	1.5	0.0625	0.1875	0.0625	0.1875	0.125
L1-2bS	0.1875	1.5	0.0469	0.125	0.0469	**12**	0.25
ViRF-2	0.0625	0.75	0.0313	0.1875	0.0938	**128**	0.125
V1 MAF	0.1875	**192**	**1**	**6**	**4**	**16**	0.125
V2 MAF	**0.75**	**≥256**	**1**	**4**	**4**	**96**	**0.75**
V4 MAF	**1.5**	**≥256**	**1.5**	**4**	**3**	**128**	**1**
V7 MAF	0.0625	1.5	**2**	**6**	**6**	0.0625	0.1875
Vs OFK1	**0.75**	**≥256**	**1**	**3**	**4**	**96**	**0.75**
Vs OFK2	0.1875	1.5	**1.5**	**6**	**4**	0.0625	0.0625

Table 2-2 (continued)

Isolate Code	**Class /antimicrobial agent code**						
	Cephalosporin	Phenicol	Quinolones			Tetracycline	Folic Acid Antagonist
	CEF	FLO	ENR	FLU	OXO	TC	TMP
Vs OFK3	**0.75**	**96**	**1.5**	**6**	**6**	**128**	0.5
Vs OFK4	0.0625	**192**	**1**	**4**	**4**	**96**	0.125
Vs OFK5	0.0234	**96**	**1.5**	**12**	**8**	0.0234	0.125
VS C1	0.0938	1.5	0.0313	0.0938	0.0938	0.0156	**0.75**
VS B1	0.0938	1.5	**1.5**	**6**	**6**	0.0625	0.0938
VS B2	0.1875	1.5	0.0625	0.1875	0.1875	0.0625	**0.75**
VS C3	0.1875	1.5	0.0625	0.125	0.0938	0.125	**≥256**
V10 G	0.0625	0.75	0.0313	0.0938	0.0625	**96**	0.1875
V12 G	**0.75**	**3**	**2**	**8**	**4**	**96**	0.1875
V17 G	**0.75**	**≥256**	**1**	**4**	**4**	**128**	**0.75**
V19 G	0.0234	0.75	0.0313	0.0938	0.0625	**96**	0.125
V23 G	0.0469	**3**	**2**	**8**	**6**	**128**	0.5
CECT4638	0.0625	1	**1.5**	**6**	**≥256**	0.0938	**≥256**
CAIM 1797	0.0625	0.75	**1**	**4**	**3**	0.0625	0.125
QC Range	0.25-1	2-8	0.008-0.03	0.25-1	0.06-0.25	0.5-2	0.5-2
QC result	0.75	8	0.0117	0.25	0.125	0.75	0.75

Bold numbers indicate NWT isolates.

Abbreviations, CEF: Ceftiofur; FLO: Florfenicol; ENR: Enrofloxacin; FLU: Flumequine; OXO: Oxolinc acid; TC: Tetracycline; TMP: Trimethoprim; QC: Quality control (*E. coli ATCC 25922)*. QC Range: quality control ranges provided by CLSI. (2014b).

2.3.2. Ceftiofur, Florfenicol, and Trimethoprim

Based on NRI calculation, COwt values for these three antimicrobials (trimethoprim, ceftiofur, and florfenicol) categorized a relatively higher number of wild type (WT) isolates, respectively, 48, 47, and 43 isolates (Table 2-3), therefore, lower number of Non-wild type (NWT) isolates among the seven antimicrobials (Figure 2-1). In these three antimicrobials the modal classes of WT and NWT isolates, was overlapped and difficult to categorize by visual examination.

2.3.3. Enrofloxacin, Flumequine, Oxolinic Acid, and Tetracycline

COwt values for these four antimicrobials (tetracycline, enrofloxacin, flumequine, and oxolinic acid) categorized lower number of wild type isolates among the seven antimicrobials, respectively, 32, 30, 29, and 29 isolates (Table 2-3), therefore, higher number of Non-wild type isolates (Figure 2-2). In these four antimicrobials, the MIC distribution was bimodal with a clear separation between the modal classes.

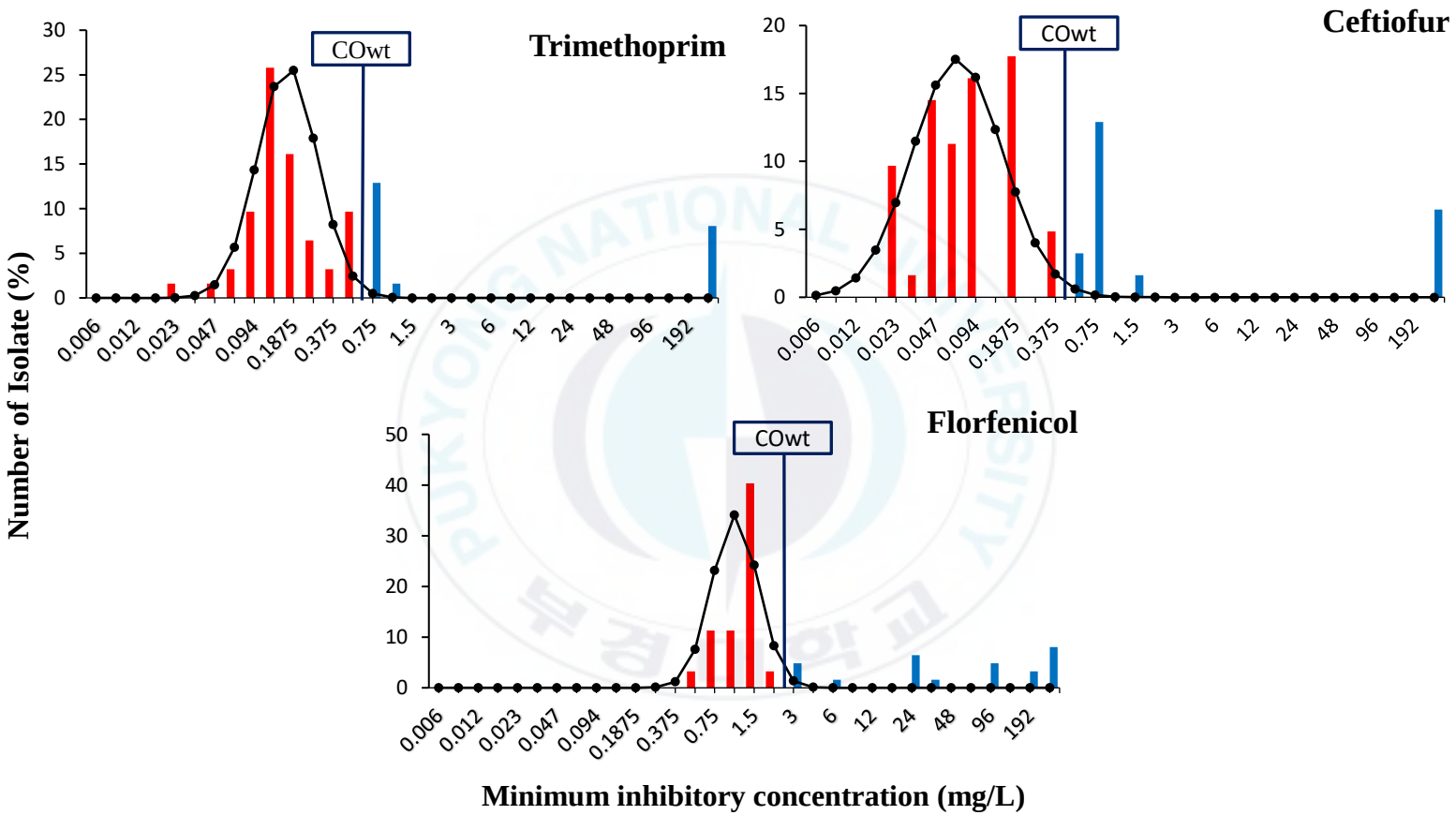


Figure 2-1 MIC values histogram for antimicrobials (ceftiofur, florfenicol, and trimethoprim) against *V. scophthalmi* (n=62). Red bars indicate WT isolates, blue bars indicate NWT isolates. COwt indicates wild type cut off breakpoint.

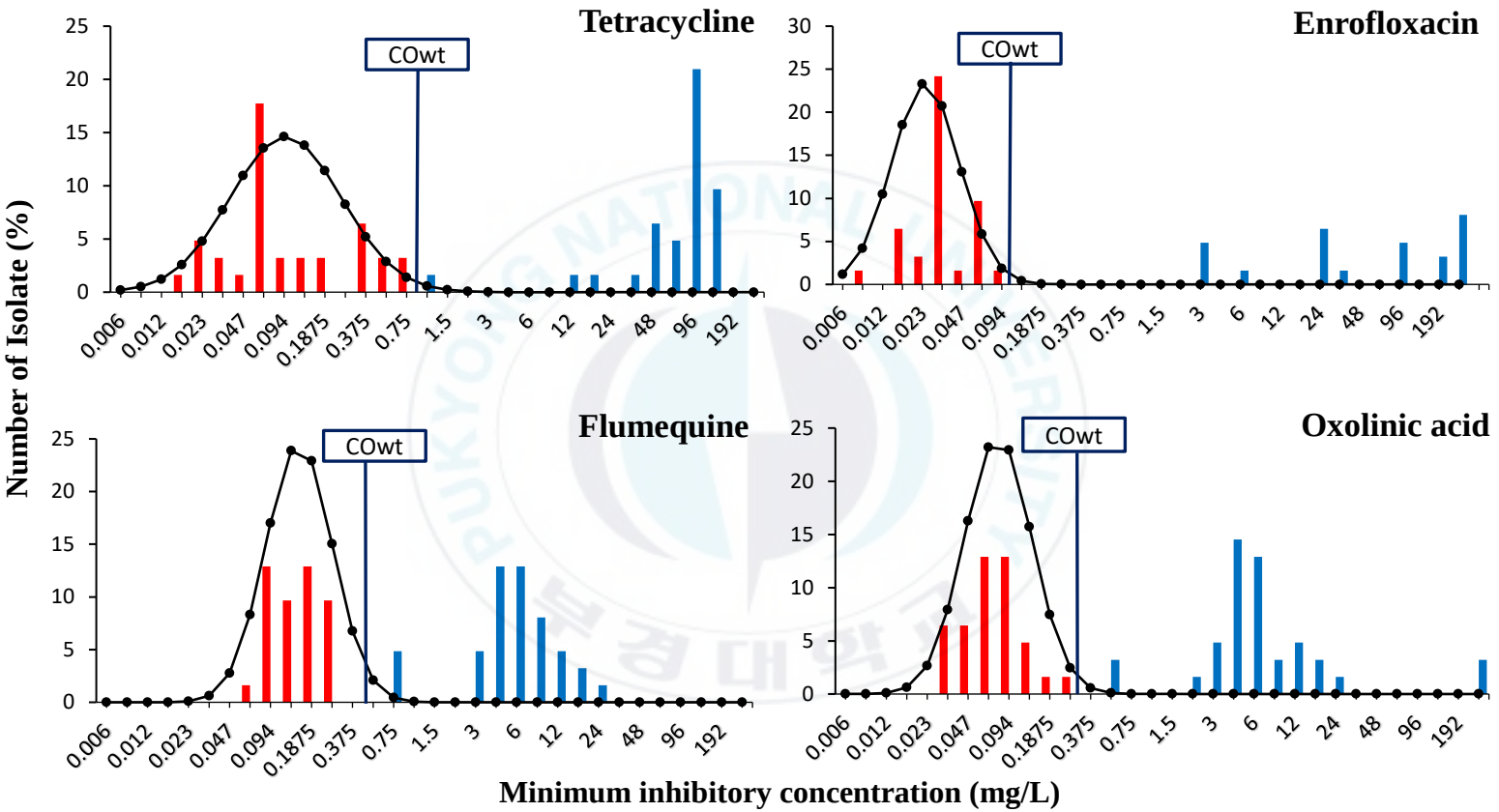


Figure 2-2 MIC histograms for antimicrobials (Enrofloxacin, Flumequine, Oxolinic acid, and Tetracycline) against *V. scophthalmi*. Red bars indicate WT isolates, blue bars indicate NWT isolates. COwt indicates wild type cut off breakpoint.

Table 2-3

Results of NRI calculations for MIC distribution of seven antimicrobials against *V. scophthalmi* (n=62).

	NRI calculated Mean value (mg/L)	SD [a] (log_2 mg/L)	COwt [b] (mg/L)	WT [c]
TMP	0.132751713	0.75133	≤ 0.5	48
CEF	0.054277827	1.128332	≤ 0.375	47
FLO	0.85002626	0.567044	≤ 2	43
TC	0.076362884	1.355703	≤ 0.75	32
ENR	0.019651911	0.839936	≤ 0.0938	30
FLU	0.120393566	0.798522	≤ 0.375	29
OXO	0.061804196	0.813139	≤ 0.25	29

[a] Standard deviation of the normalized distribution of WT

[b] Wild type cut off values (COwt) Using a 2 SD limit, above the NRI calculated Mean value.

[c] Number of wild type isolates categorized by NRI application of relevant COwt value.

Table 2-4

Distribution of *V. scophthalmi* isolates (n = 62) based on MICs values.

mg/L	CEF	FLO	ENR	FLU	OXO	TC	TMP
256	4	5			2		5
192		2					
128						6	
96		3				13	
64						3	
48						4	
32		1				1	
24		4		1	1		
16				2	2	1	
12				3	3	1	
8				5	2		
6		1	1	8	8		
4			1	8	9		
3		3	1	3	3		
2		**2**	9		1		
1.5	1	**25**	6				
1		**7**	12			1	1
0.75	8	**7**		3		**2**	8
0.5	2	**2**			2	**2**	**6**
0.375	**3**					**4**	**2**
0.25			2	**6**	**1**		**4**
0.1875	**11**			**8**	**1**	**2**	**10**
0.125				**6**	**3**	**2**	**16**
0.09375	**10**		**1**	**8**	**8**	**2**	**6**
0.0625	**7**		**6**	**1**	**8**	**11**	**2**
0.046875	**9**		**1**		**4**	**1**	**1**
0.03125	**1**		**15**		**4**	**2**	
0.023438	**6**		**2**			**3**	**1**
0.015625			**4**			**1**	
0.011719							
0.007813			**1**				
0.00293							

- Antimicrobial agents abbreviated as Table 2-2.
- Shaded area determine the ECOFF breakpoints and wild type (WT) isolates.

2.3.4. Estimation of Treatability

Based on the percentage of NWT isolates for each antimicrobial agent, we tried to predict the estimation of treatability. Then we ordered antimicrobial agents vertically from the lowest percentage on NWT isolates (relatively highest treatability) to the highest percentage on NWT isolates (low treatability) as indicated in Table 2-5.

Table 2-5
Estimation of treatability against *V. scophthalmi* isolates in Korea.

Antibiotics	**Non-wild type percentage (%)**				**Estimation of Treatability**
	Olive flounder (n=42)	Starry flounder (n=10)	Other Species (n=10)	Total (n=62)	
Trimethoprim	26	0	30	**23**	Relatively high
Ceftiofur	29	20	10	**24**	
Florfenicol	40	20	0	**31**	
Tetracycline	55	40	30	48	
Enrofloxacin	52	80	20	52	
Flumequine	55	80	20	53	
Oxolinic acid	57	70	20	53	low

2.3.5. Multiple Drug Resistance

Among *V. scophthalmi* isolates (n=62) approximately 64% showed multiple drug resistance (MRD) against two to seven antimicrobial agents. Those isolates come from different fish species, origin, and year of isolation (Table 2-6). More analysis revealed that, most isolates that have MRD to five antimicrobial agents and more come from olive flounder fish, as well isolated recently 2015-2016 (Table 2-7).

Table 2-6

MDR patterns	Isolates Code	Year	Origin
1. CEF, FLO, ENR, FLU, OXO, TC, TMP	V2 MAF, V4 MAF, Vs OFK1 , V17 G	2016	kijang
2. CEF, FLO, ENR, FLU, OXO, TC	Vs OFK3 , V12G	2007, 2016	yeosu, kijang
3. FLO, ENR, FLU, OXO, TC	Vi07-14-1, Vi07-17-1, ViSF-1, V23 G, V1 MAF, Vs OFK4	2007(n=2), 2015, 2016 (n=3)	Jeju,ulsan, Kijang
4. CEF, ENR, FLU, OXO	A19008, Vi09-21-3, A28003	2005, 2009, 2012	Jeju, Ulsan
5. FLO, ENR, FLU, OXO	Vi3, Vs OFK5	2007, 2016	yeosu, kijang
6. FLO, FLU, TC, TMP	ViOF-9	2010	Sosang
7. ENR, FLU, OXO, TC	Vi07-11-2, Vi07-11-3, Vi4	2007	Jeju, Yeosu
8. ENR, FLU, OXO, TMP	CECT4638	1997	Spain
9. CEF, ENR, FLU	Vi9-9, Vi9-7	2009	Jeju
10. FLO, ENR, TC	Vi08-30-1	2008	Jeju
11. ENR, FLU, OXO	ViOF-6, CAIM 1797, V7 MAF, VS B1, Vi2, Vi5, Vi6	2004 , 2005, 2007 (n=3),2012, 2016 (n=3)	Jeju, Yeosu, Spain, Ulsan, Kijang
12. CEF, TMP	ViOF-3	2004	Hanam
13. FLO, TC	Vi08-30-2, Vi08-30-4, Vi08-30-6	2008	Jeju
14. TC, TMP	ViOF-4, 24-a	2004, 2010	Hanam, open sea

- Multiple Drug Resistance (MDR) patterns of *V. scophthalmi* isolates
- Antibiotic agents abbreviated as in Table 2-1.

Table 2-7

Percentage of MDR of *V. scophthalmi* isolates based on fish species and year of isolation

Number of Antibiotics	Percentage of multiple drug resistance %					
	olive flounder (n=42)	starry flounder (n=10)	Other species (n=10)	1997-2009 (n=33)	2010-2016 (n=29)	total (n=62)
0	5% (2)	0	30% (3)	6% (2)	10% (3)	8%
1	26% (11)	20% (2)	40% (4)	24% (8)	31% (9)	27%
2	12% (5)	0	10% (1)	15% (5)	3% (1)	10%
3	14% (6)	50% (5)	10% (1)	24% (8)	14% (4)	19%
4	17% (7)	20% (2)	10% (1)	24% (8)	7% (2)	16%
5	12% (5)	10% (1)	0	6% (2)	14% (4)	10%
6	5% (2)	0	0	0	7% (2)	2%
7	10% (4)	0	0	0	14% (4)	6%

2.4. Discussion

The present chapter study aimed to define laboratory-specific MIC ECOFF values for Korean isolates of *V. scophthalmi* in our laboratory using standardized procedures flowing broth micro dilution protocol recommended by CLSI (2014a) for group 1 non-fastidious bacteria. Previously, NRI analysis has been applied successfully to species populations with antimicrobial susceptibility results in the form of inhibition zone diameter values (Joneberg et al., 2003; Kronvall et al., 2003; and Petersen 1999). Kronvall et al. (2006) and Kronvall (2010) reported that NRI analysis provides an objective method to analyze MICs distribution data and estimate epidemiological cut off values. We used the same principals to analyze our data by using the MS Excel spreadsheet available on-line at http://www.bioscand.se/nri/, which adapted to be applied to data sets obtained using two overlapping doubling dilution series.

It was very helpful when some data sets be proximal to or overlap with the measures for strains that are fully susceptible (Figure 2-1). Kronvall (2010) revealed that the best agreement between NRI generated cutoff values and ECOFF values (published by EUCAST) were with NRI cutoff values set at +2.0 SD. We successfully performed the NRI calculations on the MIC distributions of all the seven examined agents with two SD limit above the NRI calculated mean values as presented in Table 2-3.

The estimated ECOFF values separated our isolates into WT and NWT, and it gave a number of WT above 30 for all antibiotics except for oxolinic acid and flumequine, which were both 29 isolates (Tables 2-3, and 2-4). In respect to CLSI recommendations (2011), our calculated cut-off values are statistically valid as it approximated to or exceeded 30 WT. For oxolinic acid and tetracycline however, the calculated number of WT was not that far from 30. This suggest that it still can be accepted since Smith et al.

(2009) demonstrated that a good performance by the NRI can manifested even when determined cut-off values from only 10 strains in a data set.

Smith et al. (2012) argued that the standard deviation (SD) of the normalized distributions of the WT observations can provide a measure of the precision of the WT disc diffusion data distributions. Smith (personal communication, December 2016) had set the limits of SD values for 133 MIC data sets. He argued that the mean + 2 standard deviations of the SD calculated for the 133 data sets would make reasonable estimate of the acceptable level of precision that should be required to set a valid CO_{WT}. These arguments would suggest any CO_{WT} value of data sets where the SD is >1.19 should be viewed with some caution. With respect to our MIC data of *V. scophthalmi*, the SD values for 5 agents here are completely acceptable. For ceftiofur, the SD is close to this value, while for tetracycline the SD value is > 1.09 (Table 2-3). Therefore, the cut-off values calculated for those two antimicrobials must be treated with caution, and at best, they (especially for tetracycline) should be considered as only provisional approximations.

Categorizing WT and NWT in a data set by using epidemiological cut-off values can predict a probable clinical significance through estimating the treatability of an antimicrobial agent. For example, trimethoprim, ceftiofur, and florfenicol showed relatively high treatability estimation comparing to other examined antimicrobials, as most isolates were classified as wild type. It can be inferred that these agents would the best of choice for treatment of *V. scophthalmi* infection in Korea (Table 2-5). In contrast, more than 50% of *V. scophthalmi* were categorized as non-wild type for antibiotics belonging to quinolone, suggesting that it would be reasonable not to select those for the treatment. Cabello et al. (2013) demonstrate that several genetic elements and resistance determinants for antibiotics including quinolones are shared between aquatic bacteria, fish pathogens, and human pathogens. In addition, 65% of our isolates appear to be multiple drug resistant as they were classified

as NWT for two antibiotics and more (Table 2-6). Moreover, more than three-quarters of the isolates that has MDR ≥ 5 antimicrobials obtained in recent two years (Table 2-7), suggesting recent increase of use and types of antibiotics in fish farms. Therefore, the excessive use of antimicrobials in aquaculture can thus potentially negatively impact animal and human health as well as the aquatic environment and should be better assessed and regulated.

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