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Determination of epidemiological tetracycline MIC cut-off value for *Vibrio ichthyoenteri*

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Normalized resistance interpretation (NRI) analysis for tetracycline was applied to generate information on the epidemiological cut-off value for *Vibrio ichthyoenteri* isolated from diseased olive flounder (*Paralichthys olivaceus*) larvae. Thus, 42 strains of *V. ichthyoenteri* were used to determine minimum inhibitory concentration (MIC) values of tetracycline using Etest. Also, 11 tetracycline resistance related genes were investigated by PCR method. Most tetracycline-resistant strains harbored both *tetB* and *tetM* with a few exceptions. NRI-derived mean and 2 SD above the mean of theoretical normal distributions of susceptible isolates were 0.33 mg/L and 1.66 mg/L, respectively. The epidemiological cut-off value for *V. ichthyoenteri* from the calculations could be set to $S \leq 2$ mg/L. Of the 42 strains, 15 were classified as non-wild type (NWT), and MIC values of the NWT strains vary regardless of *tetB* and *tetM* detection, suggesting that there may be other mechanisms involved in tetracycline resistance in this *Vibrio* species.

Key words : *Vibrio ichthyoenteri*, Olive flounder, Tetracycline, Normalized resistance interpretation, Epidemiological cut-off value

Bacterial enteritis in olive flounder caused by *Vibrio ichthyoenteri* is a serious issue in Korean and Japanese hatcheries (Muroga *et al.*, 1990; Kim *et al.*, 2004). This disease is often characterized by opaque intestine (intestinal necrosis) and high larval susceptibility (Muroga *et al.*, 1990; Kim *et al.*, 2004). For prevention and treatment of bacterial diseases, tetracyclines are one of the most frequently used antibiotics in Korean olive flounder hatcheries. However, data on the use of tetracyclines against and the susceptibility phenotypes of *V. ichthyoenteri* are scant. In addition, it is unknown that what tetracycline resistance genes (*tet* genes) are carried by this pathogen.

Currently, over 40 different *tet* genes have been characterized (Roberts 2005; Whittle *et al.*, 2003). *tet* genes are often carried on plasmid and conjugative transposons, which contribute to their dissemination via horizontal gene transfer (Roberts, 2005).

Due to lack of interpretive criteria (clinical breakpoint or epidemiological cut-off value) for bacteria isolated from aquatic animals, it is very difficult to classify resistant and sensitive strains. In this study, therefore, we used minimum inhibitory concentration (MIC) of tetracycline using the Etest methodology (AB biodisk), which is based on an agar diffusion MIC method using a thin plastic

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strip coated with continuous antimicrobial gradient, to provide the epidemiological cut-off value for *V. ichthyoenteri* isolated from olive flounder (Table 1). Also, 11 tetracycline resistance related genes of forty-two strains of *V. ichthyoenteri* were investigated using PCR method.

Materials and Methods

Bacterial isolation

Strains of *V. ichthyoenteri* were isolated from the gastrointestinal tracts of 9-30 dph olive flounder larvae with symptoms of bacterial enteritis in Jeju hatchery from 2007 to 2009. To acquire more isolates of *V. ichthyoenteri*,

Table 1. Primers and conditions used for gene-specific PCRs

Target gene	Forword primer	Reverse primer	Annealing temp.	Extenstion time	Amplicon size (bp)	Reference(s) for primers and PCR conditions
tetA	GCGCTNTATGCGTTGATGCA	ACAGCCCGTCAGGAAATT	55	30sec	387	Jun *et al.* (2004)
tetB		TGAAAGCAAACGGCCTAA	55	30sec	171	
tetC		CGTGCAAGATTCCGAATA	50	45sec	631	
tetD		CCAGAGGTTTAAGCAGTGT	50	30sec	484	
tetE		ATGTGTCCTGGATTCCT	50	30sec	246	
tetG		ATGCCAACACCCCCGGCG	59	1min	803	
tetM	GTTAAATAGTGTTCTTGGAG	CTAAGATATGGCTCTAACAA	56	1min	659	Kim *et al.* (2004)
tetO	AACTTAGGCATTCTGGCTCAC	TCCCACTGTTCCATATCGTCA	54	30sec	515	Ng *et al.* (2001)
tetS	ATGTTTTTGGAACGCCAGAG	CATAGACAAGCGTTGACC	54	45sec	667	Villedieu *et al.* (2003)
tetK	TCGATAGGAACAGCAGTA	CAGCAGATCCTACTCCTT	54	30sec	169	Macovei and Zurek (2006)
tetZ	CCTTCTCGACCAGGTCGG	ACCCACAGCGTGTCCGTC	54	30sec	204	Aminov *et al.* (2002)
tet39	CTCCTTCTCTATTGTGGCTA	CACTAATACCTCTGGACATCA	58	1min	701	Agerso and Petersen (2007)
16S rRNA	CAGGCCTAACACATGCAAGTC	ACGGGCGGTGTGTRC	55	1min 30sec	1,343	

55-dph olive flounder larvae obtained from the Pohang hatchery in 2008 were also used for bacterial isolation.

Gastrointestinal tracts of the larvae were aseptically removed and homogenized in 10 mL of sterile 0.85% (w/v) saline. A volume (0.1 mL) of 10-fold dilutions of the homogenate was spread onto tryptic soy agar (Difco, USA) containing 1% (w/v) sodium chloride. The plates were incubated at 25°C for 48 hrs, after which representative colonies, normally in pure culture were selected and streaked for isolation. Forty-two strains of *V. ichthyoenteri* were isolated for 3 years in this study (Table 1).

Minimal inhibitory concentration (MIC) testing

Bacterial strains were suspended in sterile saline (0.85% w/v) to match the density of a McFarland No. 5 Standard and then inoculated onto Mueller Hinton agar supplemented with 1% NaCl (MHA). Etest inoculum preparation and plating, strip application and MIC determinations were performed according to guidelines and illustrations provided by the manufacturer. Each strain was grown on MHA plates for 24 h at 22°C. As recommended in the CLSI document M42-A (CLSI, 2006), *Aeromonas salmonicida* subsp. *salmonicida* ATCC 33658 and *Escherichia coli* ATCC 25922 obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) were used as the reference strains for quality control in every test run and were incubated on MHA plates at 22°C for 24 h.

Normalized resistance interpretation (NRI)

Normalized resistance interpretation (NRI) was performed according to the method previously described by Kronvall (2003) and Kronvall *et al.* (2003; 2006). The normal distribution of ideal population of susceptible strains were calculated using a plot of probit values against zone diameters, into which MIC values derived in this study were converted using the procedure as described earlier (Kronvall *et al.*, 2006). In this study the epidemiological cut-off value was set at two standard deviations (SD) above the mean, since Kronvall (2010) suggested 2.0 SD limit was better than others for MIC distributions.

Detection and sequence analysis of *tet* genes

Bacterial strains were grown in TSB supplemented with 1% (W/V) NaCl at 25°C for 18 h. After culture, cells were harvested by centrifugation at 8000 × g for 10 min. Bacterial genomic DNA was isolated using High Pure PCR template preparation kit (Roche). The genes *tetA*, *tetB*, *tetC*, *tetD*, *tetE*, *tetM*, *tetO*, *tetS*, *tetK*, *tetZ* and *tet39* were amplified using specific primers and PCR conditions indicated in Table 2. PCR products (5 μl) were analyzed by electrophoresis on 1.5% agarose gels stained with ethidium bromide. Sequencing of the amplified DNA fragment was performed using an automatic sequencer (Applied biosystems 3730xl DNA Analyzer) and the nucleotide sequences obtained were used for a BLAST searches.

Table 2. Susceptibility testing and MIC values for tetracycline against *Vibrio ichthyoenteri* isolates used in this study

Isolate code	Sampling time & location	16S rRNA sequence based identification (id %)*	Tetracycline MIC (mg/L)	Tetracycline Susceptibility*	Tetracycline resistance genes	
					tetB	*tetM*
Vi079-1	Nov., 2007, Jeju	*V. ichthyoenteri* (100%)	1.5	S	-	-
Vi079-2		***V. ichthyoenteri*** (100%)	1.5	S	-	-
Vi0711-1		***V. ichthyoenteri*** (100%)	1	S	-	-
Vi0711-2		***V. ichthyoenteri*** (100%)	48	**R**	+	+
Vi0711-3		***V. ichthyoenteri*** (100%)	1	S	-	-
Vi0711-4		***V. ichthyoenteri*** (100%)	0.75	S	-	-
Vi0714-1		***V. ichthyoenteri*** (100%)	32	**R**	+	+
Vi0717-1		***V. ichthyoenteri*** (100%)	32	**R**	+	+
Vi0717-2		***V. ichthyoenteri*** (100%)	32	**R**	+	+
Vi0717-3		***V. ichthyoenteri*** (100%)	32	**R**	+	+
Vi0717-4		***V. ichthyoenteri*** (100%)	1.5	S	-	-
Vi0717-5		***V. ichthyoenteri*** (100%)	48	**R**	+	+
Vi0721-1		V. ichthyoenteri (100%)	96	**R**	+	+
Vi0814-1	Nov., 2008, Jeju	***V. ichthyoenteri*** (100%)	96	**R**	+	+
Vi0830-1		***V. ichthyoenteri*** (100%)	96	**R**	+	+
Vi0830-2		***V. ichthyoenteri*** (100%)	96	**R**	+	+
Vi0830-3		***V. ichthyoenteri*** (100%)	48	**R**	+	+
Vi0830-4		***V. ichthyoenteri*** (100%)	24	**R**	+	+
Vi0830-5		***V. ichthyoenteri*** (100%)	0.25	S	-	-
Vi0830-6		***V. ichthyoenteri*** (100%)	48	**R**	+	+
Vi0830-7		***V. ichthyoenteri*** (100%)	0.25	S	-	-
Vi0855-1	Nov., 2008, Pohang	***V. ichthyoenteri*** (99.7%)	1.5	S	-	-
Vi0855-2		***V. ichthyoenteri*** (100%)	4	**R**	-	-
Vi0855-3		***V. ichthyoenteri*** (100%)	0.38	S	-	-
Vi0855-4		***V. ichthyoenteri*** (100%)	0.5	S	-	-
Vi0855-5		***V. ichthyoenteri*** (100%)	1	S	-	-
Vi099-7	Jul.-Aug. 2009, Jeju	***V. ichthyoenteri*** (100%)	0.19	S	-	-
Vi099-8		***V. ichthyoenteri*** (100%)	0.5	S	-	-
Vi099-9		***V. ichthyoenteri*** (100%)	0.25	S	-	-
Vi099-11		***V. ichthyoenteri*** (100%)	2	S	-	-
Vi0914-1		***V. ichthyoenteri*** (100%)	0.38	S	-	-
Vi0914-3		***V. ichthyoenteri*** (100%)	0.38	S	-	-
Vi0914-8		***V. ichthyoenteri*** (100%)	0.19	S	-	-

Vi0917-1	Jul.-Aug. 2009, Jeju	***V. ichthyoenteri*** (100%)	0.19	S	-	-
Vi0917-2		***V. ichthyoenteri*** (100%)	0.19	S	-	-
Vi0921-3		***V. ichthyoenteri*** (100%)	0.5	S	-	-
Vi0921-4		***V. ichthyoenteri*** (100%)	0.25	S	-	-
Vi0921-6		***V. ichthyoenteri*** (100%)	24	**R**	+	+
Vi0921-11		***V. ichthyoenteri*** (100%)	0.25	S	-	-
Vi0921-12		***V. ichthyoenteri*** (100%)	0.25	S	-	-
Vi0921-15		***V. ichthyoenteri*** (100%)	0.19	S	-	-
Vi0921-16		***V. ichthyoenteri*** (100%)	0.25	S	-	-

* The isolates were classified as susceptible (S) or resistant (**R**) according to the epidemiological cut-off value.

Results and Discussion

Many laboratories associated with fish diseases were less than reasonably confident in the interpretive criteria they were using (Smith *et al.*, 2009). Thus, there have been several attempts to generate epidemiological cut-off values for fish pathogens, allowing characterization of isolates as WT or NWT based on their relative susceptibility to a specific agent (e.g., Smith and Christofilogiannis, 2007; Smith *et al.*, 2007; Smith *et al.*, 2009; Avendaño-Herrera *et al.*, 2011). Those studies all used the NRI analysis that can reduce errors in interpreting susceptibility test data derived from aquatic bacterial pathogens. Furthermore, Smith *et al.* (2007) have already demonstrated that the NRI method generated laboratory-specific epidemiological cut-off values, as two laboratories in their study showed complete agreement in the classification of WT and NWT for all strains when NRI analysis was applied to their own data.

The CLSI guideline M42-A (CLSI, 2006) and M45-A2 (CLSI, 2010) provide standard testing protocols and clinical breakpoints for *Vibrio* spp. However, the tests were performed at only 35°C for 16-20 h incubation, and they do not contain interpretive criteria for the genus *Vibrio* tested at 22°C, which will need to be developed. In such a situation, the best method is to establish an epidemiological cut-off value for data derived under the protocols suggested in M42-A (CLSI, 2006). MIC values of the *A. salmonicida* subsp. *salmonicida* and *E. coli* were 0.064 and 0.5 mg/L, respectively, all of which were within acceptable quality control ranges indicated in M45-A2 (CLSI, 2010). The tetracycline MIC and NRI-generated distributions of *V. ichthyoenteri* are shown in Figure 1. NRI-derived mean and 2 SD above the mean of theoretical normal distributions of susceptible isolates were 0.33 mg/L and 1.66 mg/L, respectively, and its co-efficient variation was 7.1%. The epidemiological cut-off value for *V. ichthyoenteri* from the calculations could be set to $S \leq 2$ mg/L (Fig 1). This epidemiological cut-off value suggest that 15 strains should be treated as non wild-type (NWT) and 27 as wild type (Table 1). About 46% (n=12/26) of strains of 2007 and 2008 were classified as NWT, while all of 2009 except to one strain as WT.

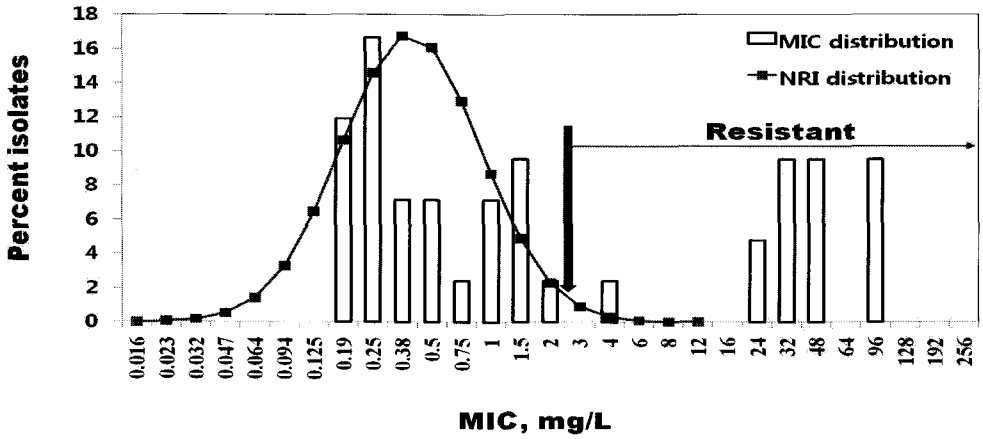

Fig. 1. *Vibrio ichthyoenteri* MIC distributions for tetracycline. Diagram bars show the percent *Vibrio ichthyoenteri* isolates. The NRI calculated susceptible peak is shown as line graph with a mean for the population of 0.33 mg/L and two standard deviations above the mean of 1.66 mg/L, which is indicated by a vertical arrow.

Approximately 93% (n=14/15) of tetracycline-resistant strains harbored *tetB* and *tetM* genes, but the other 9 resistance related genes were not detected by PCR method. *tetM* sequence of all *V. ichthyoenteri* strains (except to Vi0721-1) deposited in Genbank (accession no. AB625449), showed 100% identities with that of previously sequenced *Vibrio* species (Genbank accession no. DQ886586). The sequences of *tetB* were most closely related to that of the same *Vibrio* species (Identities= 98-99%). Our *tetB* sequences were divided into 2 groups (Genbank accession no. AB625447 and AB625448), presenting only one nucleotide discrepancy between them. There was no correlation between nucleotide difference and MIC values. As an early study (Kim *et al.*, 2007) found that almost all tetracycline-resistant *Vibrio* spp. isolated from Korean marine environments contained both *tetB* and *tetM*, our most resistant strains also had the two genes. However, the genes were not detected in one NWT strains, Vi0855-2.

In conclusion, wild-type MIC distribution of tetracycline was determined, including the epidemiological cut-off value ($S \leq 2$ mg/L). Of the 42 strains, 15 (approximately 37%) comprised seven, seven and only one strain for 2007, 2008 and 2009, respectively, were classified as NWT. In addition, MIC values of 15 NWT strains vary regardless of *tetB* and *tetM* detection. These results indicate that there may be some other genes or mechanisms involved in tetracycline resistance in this *vibrio* species.

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