Minimum inhibitory concentrations of antimicrobials against clinical *Vibrio* and *Streptococcus* isolated from aquaculture

HATHAIRAT MAISAK, NUTCHARNART TIPMONGKOLSILP and JANENUJ WONGTAVATCHAI

Department of Veterinary Medicine, Faculty of Veterinary Science Chulalongkorn University, Bangkok 10330, Thailand

ABSTRACT

Four antimicrobial agents: Amoxycillin, Oxytetracycline, Sulfadiazine/Trimethoprim (SXT) and Sulfadimethoxine/Ormetoprim (ORS) were tested for their *in vitro* antimicrobial activity against clinical *Streptococcus* and *Vibrio* isolates from clinical cases occurring between 2005 to 2006. The specimens or moribund animals from different culture areas in Thailand were collected for laboratory microbiological analysis. *Streptococcus* strains were isolated from the kidney of the diseased tilapia *Oreochromis niloticus* and *Vibrio* strains were obtained from the hepatopancreas of diseased black tiger shrimp *Penaeus monodon* or pacific white shrimp *Litopeneaus vannamei*. All clinical bacterial isolates were identified by conventional tube media or API system (Biomerieux, France). The agar dilution method, as described by the Clinical and Laboratory Standards Institute (CLSI), was used to determine Minimum Inhibitory Concentrations (MICs) of each chemical against the tested isolates. The effect of the components of seawater on the antimicrobial activity of ORS was also analysed by a comparison between MIC values tested on Mueller Hinton Agar (MHA) dissolved in distilled water with an added 1% NaCl and on agar dissolved in 10 ppt seawater. MIC values suggested that *Streptococcus* isolates obtained from the diseased tilapia were resistant to Oxytetracycline (MIC range 0.50-8.00 μg/ml) but susceptible to Amoxicillin (MIC range 0.031-0.250 μg/ml), STX (MIC range 0.285/0.015-9.50/0.50 μg/ml) and ORS (MIC range 0.152/0.008-4.75/0.25 μg/ml). While the test conducted against *Vibrio* isolates revealed broader MIC ranges; Amoxicillin 1.0-512 μg/ml, Oxytetracycline 1.0-512 μg/ml, STX 1.178/0.062- >152/8 μg/ml and ORS 0.152/0 008- >152/8 μg/ml, the activity of ORS was not substantially influenced by an addition of 10 ppt seawater to the tested system. .The study concluded that, in respect of MIC testing and the possible implications of a seawater effect, ORS is a functional antimicrobial for the tilapia *Streptococcus* and penaeid shrimp *Vibrio* pathogen.

Maisak, H., Tipmongkolsilp, N. and Wongtavatchai, J. 2011. Minimum inhibitory concentrations of antimicrobials against clinical *Vibrio* and *Streptococcus* isolated from aquaculture, pp. 309-316. *In* Bondad-Reantaso, M.G., Jones, J.B., Corsin, F. and Aoki, T. (eds.). Diseases in Asian Aquaculture VII. Fish Health Section, Asian Fisheries Society, Selangor, Malaysia. 385 pp.

Corresponding author: Janenuj Wongtavatchai, janenuj.w@chula.ac.th

Keywords: minimum inhibitory concentrations, antimicrobials, *Streptococcus spp.*, *Vibrio spp.*

INTRODUCTION

Bacterial diseases are considered to be the most important cause of mass mortality and economic loss when present in intensive aquaculture. Antimicrobial therapy is one of the strategies frequently applied to control bacterial diseases. As part of the prudent use of animal antimicrobials, antimicrobial susceptibility testing is strongly recommended prior to treatment. The aim of this study was to determine the Minimum Inhibitory Concentrations (MICs) for 4 antimicrobial agents: Amoxycillin, Oxytetracycline, Sulphadiazine/Trimethoprim (SXT) and Sulfadimethoxine/Ormetoprim (ORS) against *Vibrio* isolated from diseased Black Tiger shrimp (*Penaeus monodon*) and Pacific White shrimp (*Penaeus vannamei*), and *Streptococcus* isolated from diseased tilapia (*Oreochromis nilotica*) in Thailand. The MIC tests reveal the antimicrobial susceptibility of bacterial pathogens associated with the aquaculture industry of Thailand and hence, provide data to direct the prudent use of antimicrobials in aquaculture production.

MATERIALS AND METHODS

Bacterial Strains

Test strain: Fifty four *Streptococcus* spp. isolates were derived from tilapia from disease cases occurring between 2005 and 2006. Streptococcosis has been observed in most regions of tilapia farming; in the north-eastern, eastern and central parts of Thailand. Isolates were identified by conventional biochemical methods described in the API system (BioMerieux, Marcy l'Etoile, France). Of the fifty four *Streptococcus* strains isolated from the kidney of the diseased tilapias were 4 *S. dys. equisimilis*, 43 *S. agalactiae*, 6 *S. porcinus* and one *Streptococcus* sp. All bacterial strains were stored in a maintenance broth containing 40% glycerol and supplemented with 10% fetal bovine serum, at -70 ^{O}C. Before each experiment the stored bacteria strains were transferred to Tryptic Soy Agar (TSA, Difco Laboratory, USA) supplemented with 10% sheep blood. After incubation at 30 ^{O}C for 18-24 hr, the inocula were transferred to Tryptic Soy Broth and the cell density was adjusted to McFarland standard 0.5 or approximately 10^8 Colony Forming Unit (CFU)/ml. The inocula were then diluted ten-fold in sterile normal saline, giving a final cell density of approximately 10^7 CFU/ml.

Vibrio isolates derived from clinical cases of diseased penaeid shrimp were obtained from the culture collection of the Department of Medicine, Faculty of Veterinary Science, Chulalongkorn University, Thailand. The collection has culture specimens from disease cases occurring between 2004 and 2006, mainly from the western and southern parts of Thailand. Previously, the identification of the isolates had been performed using conventional biochemical methods described in the API system (BioMerieux, France). Of the fifty *Vibrio* strains isolated from the hepatopancreas of the diseased penaeid shrimp were

4 *V. alginolyticus*, 7 *V. cholerae*, 7 *V. damsela*, 14 *V. fluvialis*, 10 *V. parahaemolyticus* and 8 *V. vulnificus* strains. All bacteria strains were stored in a maintenance broth containing 40% glycerol, at -70 ^{O}C. The isolates were treated with similar procedures described for streptococcal isolates, but 1%NaCl was added to Tryptic Soy Agar.

Quality control strains: Additional bacterial organisms were obtained from the American Type Culture Collection for use as quality controls: *Esteriachia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923 and *V. parahaemolyticus* ATCC 17802.

Antibacterial Agents

The antimicrobials that were tested were Amoxicillin, Oxytetracycline, Sulfadiazine, Trimethoprim (Sigma Chemical Co., USA.), Sulfadimethoxine and Ormetoprim (PHARMAQ, St Louis, MO, Norway). A serial two-fold dilution of antimicrobials dissolved in solvent was processed with distilled water, except that Trimethoprim and Ormetoprim were dissolved in solvent only as suggested by CLSI, giving a series of tested concentrations.

Minimum Inhibitory Concentration (MIC)

The procedures described here are in accordance with the international recommendations provided by the Clinical and Laboratory Standards Institute (CLSI). Mueller Hinton Agar (MHA) plates, containing serial two dilutions with the four antimicrobial agents, were inoculated with a standardized inoculum of the test strain (10^7 CFU/ml). Using a standard multipoint inoculator, bacteria from mature cell cultures were allocated at approximately 10^4 CFU/spot on the surface of MHA. After 18-20 hr., incubation, the MIC was recorded as the lowest concentration of antimicrobials with no visible growth of bacteria. The minimum concentrations of antimicrobials required to inhibit 50% and 90% of the tested isolate were reported as MIC_{50} and MIC_{90}, respectively. Additional tests on Meuller Hinton Agar, dissolved in sea water (10 ppt salinity), were performed to evaluate any possible effects of components in the sea water that might affect the antivibrio activity of Sulfadimethoxine and Ormetoprim. Quality controls of the methods were regularly performed on each test. Inhibition of the quality control strains by antimicrobial agents are required to be comparable with the interpretive standards.

RESULTS AND DISCUSSION

The values of the MICs of Amoxicillin, Oxytetracycline, Sulfadiazine/Trimethoprim (SXT) and Sulfadiamethoxine/Ormetoprim (ORS) against 54 *Streptococcus* isolates associated with the diseased tilapia are presented in Table 1. The MIC_{50} and MIC_{90} were, respectively, the minimum concentrations of antimicrobials required to inhibit 50% and 90% of the tested isolates. The data showed that Amoxicillin, SXT and ORS were effective against the tested *Streptococcus* isolates and their MIC_{90} corresponded with the susceptible range of MIC interpretive standards against *S. pneumonia* (CLSI, 2002) (Table 2).

The distribution of MIC values also correlated with the estimated values of MIC_{50}, MIC_{90} and the MIC range, showing that ORS had a better overall MIC range than did other antimicrobials (Fig. 1).

Table 1
Minimum Inhibitory Concentrations (MICs) of antimicrobials against 54 *Streptococcus* isolates associated with tilapia disease. MIC_{50} and MIC_{90} were the minimum concentrations of antimicrobials required to inhibit 50% and 90% of the tested isolates

Antimicrobial agent	MIC (µg/ml)		
	MIC_{50}	MIC_{90}	MIC range
Amoxicillin	0.062	0.125	0.031-0.250
Oxytetracycline	0.500	8.000	0.500-8.000
Sulfadiazine/Trimethoprim (19:1)	2.375/0.125	4.750/0.25	0.285/0.015 – 9.50/0.50
Sulfadimethoxine/Ormetoprim (19:1)	2.375/0.125	4.750/0.25	0.15/0.008 – 4.750/0.25

Table 2
MIC Interpretive Standards (µg/ml) for S. pneumoniae using the Agar Dilution Method (CLSI, 2002)

Antimicrobials	MIC Interpretive Standard (µg/ml)		
	Susceptible	Intermediate	Resistance
Amoxicillin	≤ 0.5	1	≥ 2
Tetracycline	≤ 2	4	≥ 8
Sulfamethoxazole/Trimethoprim	≤9.5/0.5	19/1-38/2	≥76/4

MIC testing for 50 *Vibrio* isolates associated with the diseased black tiger shrimp or Pacific white shrimp showed MIC_{50}, MIC_{90} and a MIC range in Table 3. MIC values of antimicrobials observed in the study, in comparison to MIC interpretive standards (Table 4), indicated that most of *Vibrio* isolates were susceptible to ORS and moderately susceptible to SXT and Oxytetracycline, while being resistant to Amoxicillin. The MIC distribution figures also showed that the frequency of MICs in the susceptible range were observed more in ORS than other compounds (Fig. 2).

Changes in MIC values due to the addition of seawater to the medium have been reported for several antimicrobial agents, resulting in implications for MIC testing (Lunestad and Samuelsen, 2001). In the present study, moderate differences in the MIC values of ORS were noticed between *Vibrio* strains tested on MHA dissolved in distilled water, with 1% NaCl added and MHA dissolved in seawater. The test on MHA dissolved in distilled water, with an added 1% NaCl, showed a MIC_{50} and MIC_{90} of 1.178/0.062 (Sulfadiamethodoxine/Ormetoprim) µg/ml while the MICs observed in a test on MHA dissolved in seawater was 1.178/0.062 (Sulfadiamethodoxine/Ormetoprim) µg/ml for MIC_{50}, and 4.750/0.250 (Sulfadiamethodoxine/Ormetoprim) µg/ml for MIC_{90} (Table 5).

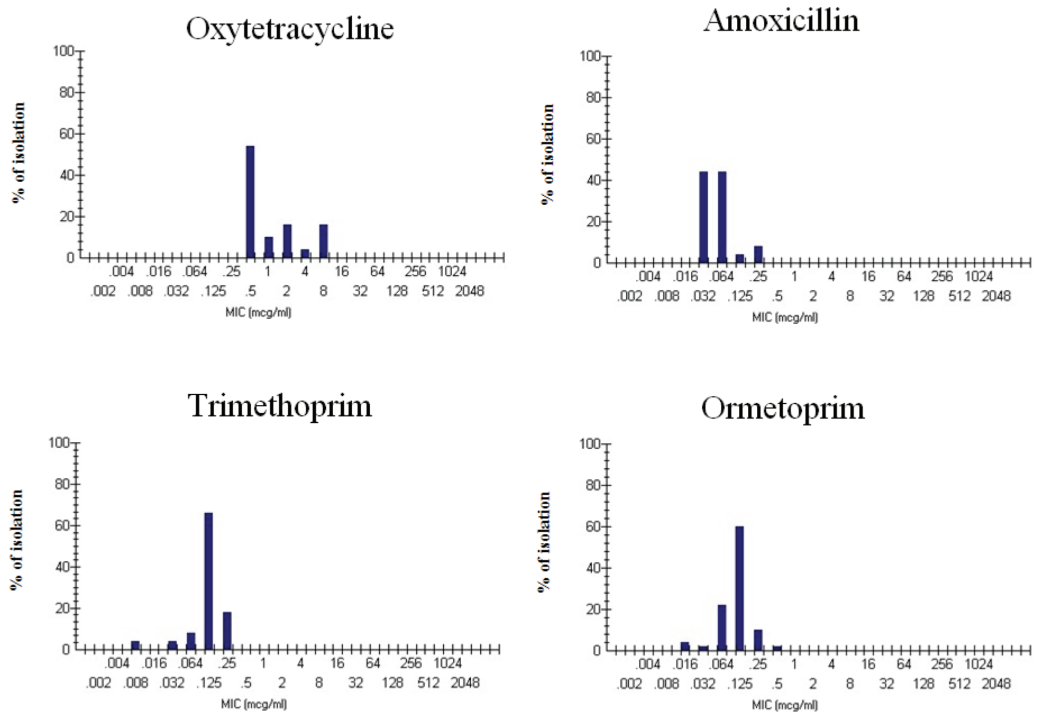

Figure 1. Frequencies of the Minimum Inhibitory Concentrations (MICs) observed for four Antimicrobials; Amoxicillin, Oxytetracycline, Sulfadiazine/Trimethoprim (SXT) (19:1) and Sulfadimethoxine/Ormetoprim (ORS) (19:1); against 54 *Streptococcus* isolates associated with tilapia disease.

Table 3
Minimum Inhibitory Concentrations (MICs) of antimicrobials against 50 *Vibrio* isolates associated with penaeid shrimp disease. MIC_{50} and MIC_{90} were the minimum concentrations of antimicrobials required to inhibit 50% and 90% of the tested isolates.

Antimicrobial agent	MIC (µg/ml)		
	MIC_{50}	**MIC_{90}**	**MIC range**
Amoxicillin	64	512	1-512
Oxytetracycline	2	64	1-512
Sulfadiazine/Trimethoprim (19:1)	9.500/0.500	>152/8	1.178/0.062 – >152/8
Sulfadimethoxine/Ormetoprim (19:1)	1.178/0.062	152/8	0.152/0.008 – >152/8

The frequencies of MICs observed for ORS indicated that the distribution of isolates with different MIC values was within the susceptible range and comparable in both test conditions, distilled water and seawater (Fig. 3). Our observation that ORS did not show a significant increase in MIC values when tested on a seawater based medium, compared to a 1% NaCl supplemented medium, is necessary for the optimal therapeutic application of the compound, particularly when the compound is used in a marine environment.

Table 4
MIC Interpretive Standards (µg/ml) for Vibrio cholerae using the Agar Dilution Method (CLSI, 2002).

Antimicrobials	MIC Interpretive Standard (µg/ml)		
	Susceptible	Intermediate	Resistance
Amoxicillin	≤ 8	16	≥ 32
Tetracycline	≤ 4	8	≥ 16
Sulfamethoxazole/Trimethoprim	≤ 38/2	-	≥ 76/4

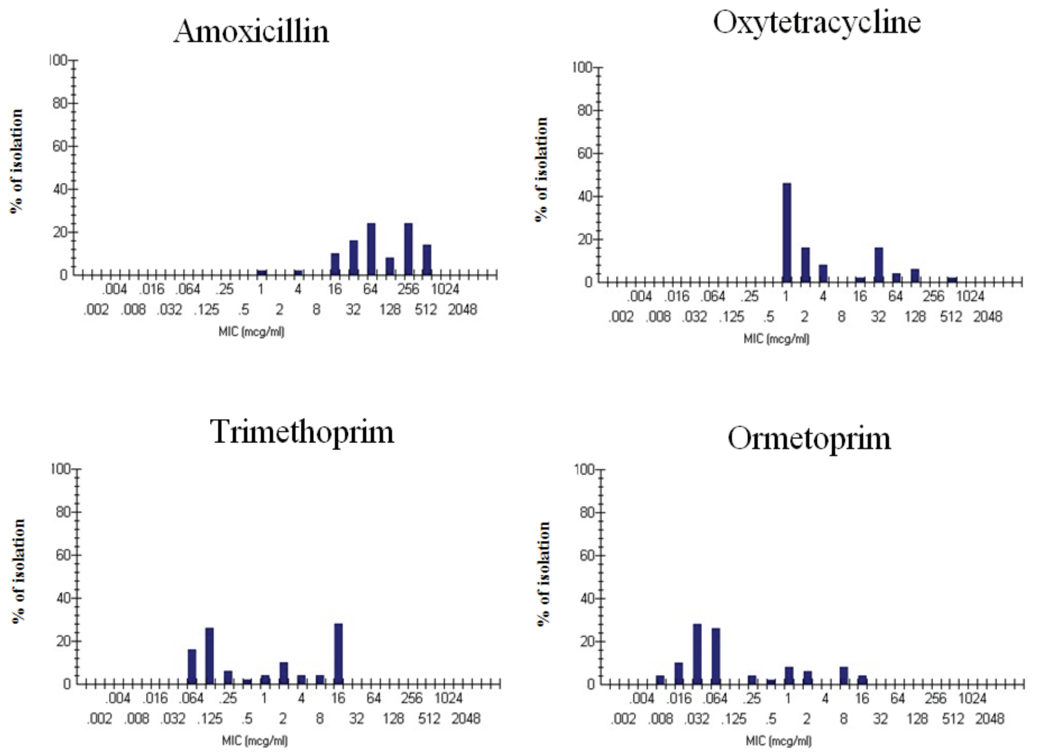

Figure 2. Frequencies of Minimum Inhibitory Concentration (MICs) observed for four antimicrobials; Amoxicillin, Oxytetracycline, Sulfadiazine/Trimethoprim (SXT) (19:1) and Sulfadimethoxine/Ormetoprim (ORS) (19:1); against 50 *Vibrio* isolates associated with penaeid shrimp disease.

Additional tests were conducted for SXT and ORS to compare the MIC testing system using different ratios of Sulfadiazine/Trimethoprim and Sulfadiamethoxine/Ormetoprim. The agar dilution method described by CLSI indicates the ratio to be used in the MIC testing of Sulfadiazine to Trimethoprim is 19:1, however, the ratio commonly used in veterinary formulae of these compounds is 5:1. Therefore, the present study compares MIC values of SXT and ORS of both ratios. The antimicrobial activity of SXT or ORS against 22 *Streptococcus* isolates, considering the MIC of Trimethoprim or Ormetoprim, were the same for both ratios (19:1 and 5:1) (Fig. 4). Corresponding results were also observed in MICs

Table 5
Minimum Inhibitory Concentrations (MICs) observed for Sulfadimethoxine/ Ormetoprim (19:1) that was tested on Meuller Hinton Agar dissolved in distilled water (DW) and in 10 ppt seawater (SW), against 22 *Vibrio* isolates associated with penaeid shrimp disease. MIC_{50} and MIC_{90} were the minimum concentrations of antimicrobials required to inhibit 50% and 90% of the tested isolates.

Antimicrobial agent	MIC (µg/ml)		
	MIC_{50}	MIC_{90}	MIC range
	Sulfadimethoxine/Ormetoprim		
DW	1.178/0.062	1.178/0.062	0.152/0.008 – 19/1
SW	1.178/0.062	4.750/0.250	0.589/0.031 – 19/1

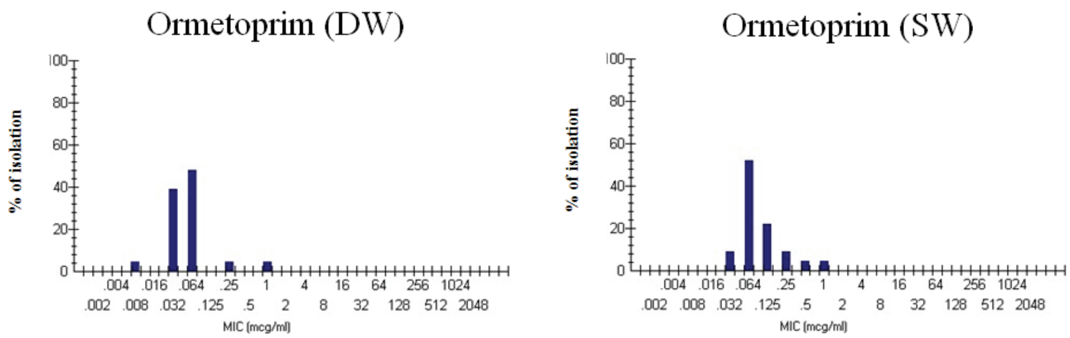

Figure 3. Frequencies of Minimum Inhibitory Concentrations (MICs) observed for Sulfadimethoxine/Ormetoprim (ORS) (19:1) that was tested on Meuller Hinton Agar dissolved in distilled water (DW) and in 10 ppt seawater (SW).

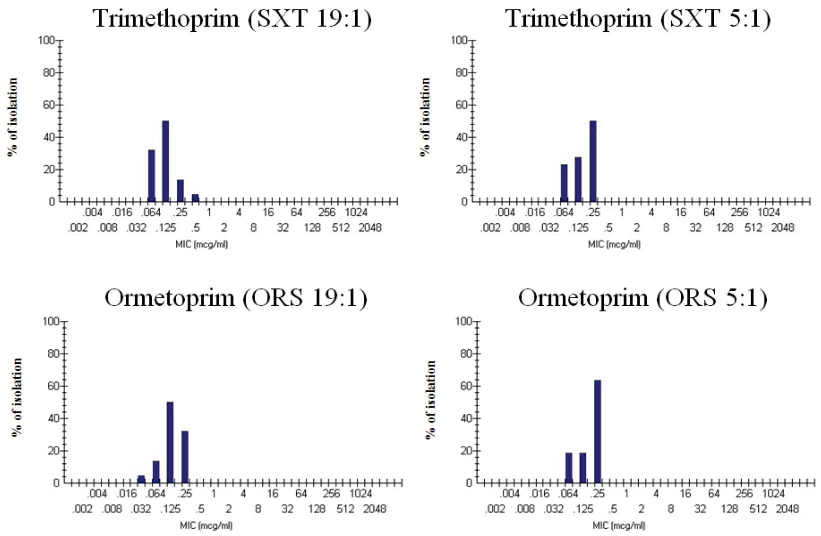

Figure 4. Frequencies of Minimum Inhibitory Concentrations (MICs) observed for Trimethoprim in Sulfadiazine/Trimethoprim (SXT) 19:1 and 5:1, Ormetoprim in Sulfadimethoxine/Ormetoprim (ORS) 19:1 and 5:1 against 22 *Streptococcus* isolates associated with tilapia disease.

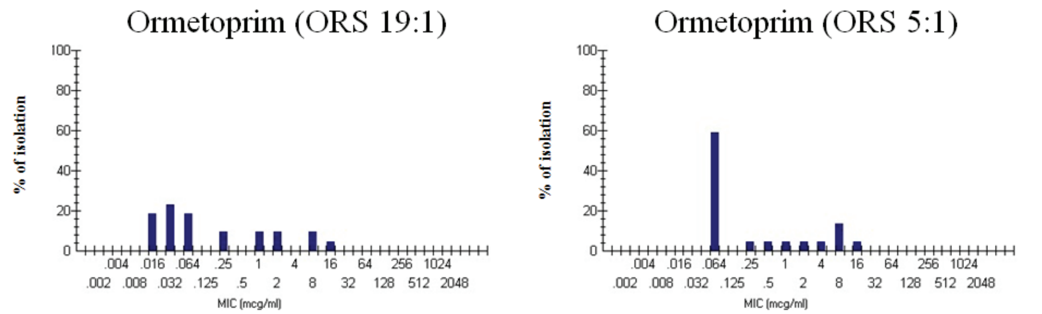

Figure 5. Frequencies of Minimum Inhibitory Concentrations (MICs) observed for Trimethoprim in Sulfadiazine/Trimethoprim (SXT) 19:1 and 5:1, Ormetoprim in Sulfadimethoxine/Ormetoprim (ORS) 19:1 and 5:1 against 22 vibrio isolates associated with penaeid shrimp disease.

tested against 22 *Vibrio* isolates (Fig. 5). Consequently, the MIC values of SXT and ORS acquired from the CLSI described ratio of 19:1 were used for the study's interpretation.

In conclusion, MIC data obtained in this study suggests that *Streptococcus* spp. isolated from diseased tilapia and *Vibrio* spp. from diseased penaeid shrimp are susceptible to ORS and SXT. Seawater has a minimal influence on the antimicrobial activity of ORS and this compound is approved for use in food fish species of many countries, making it the preferred therapeutic for aquaculture.

ACKNOWLEDGEMENTS

This study was supported by PHARMAQ (Norway) and the Royal Golden Jubilee Ph.D Program (RGJ) the Thailand Research fund.

REFERENCES

Lunestad, B.T. and Samuelsen, O.B. 2001. Effects of sea water on the activity of antimicrobial agents used in aquaculture, implications for MIC testing. *Aquaculture* 193: 319-323.

CLSI (formerly NCCLS). 2002. Performance standards for antimicrobial susceptibility tests. Ninth Informational Supplement-Sixth Edition. 19(1): 91-93.

WHO. 2000. WHONET5 Laboratory Database Software. Geneva, Switzerland.