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Antibiotics Resistance of *Vibrio* spp. Isolated from Diseased Seabass and Tilapia in Cage Culture

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ABSTRACT

Vibriosis has become one of the most important bacterial diseases in marine cultured organisms in recent years. This study was focusing on isolation and identification of *Vibrio* spp. isolated from diseased seabass (*Lates calcarifer*), tilapia (*Oreochromis niloticus*) and seawater from Sri Tujuh lagoon in East Coast of Malaysia; also determination of antibiotic resistance patterns among *Vibrio* spp. *Vibrio* species isolated from diseased seabass in Pantai Sri Tujuh, Tumpat, Kelantan were screened for their antibiotic sensitivity patterns by Kirby-Bauer method. A total of 47 isolates belonging to three different species were identified which are *V. parahaemolyticus*, *V. vulnificus*, and *V. alginolyticus*. In this study, high incidence of erythromycin, chloramphenicol and sulfamethoxazole resistance was observed among the *Vibrio* isolates, whereas all isolates were susceptible to oxytetracycline. *Vibrio* isolates were 96% resistant to one or more different classes of antibiotic, and 17 different resistance patterns were identified. The MAR index of 0.4 indicating the *Vibrio* spp. in these farmed fish might have been indiscriminately and continuously exposed to those antibiotics during culturing stages of the fish. This study showed that multidrug-resistant *Vibrio* spp. were common in diseased seabass and tilapia cultured at Sri Tujuh lagoon. These essential findings suggested involvement of seafood in transmission of these pathogen to human. In addition, oxytetracycline can be used as a treatment to combat vibriosis in diseased seabass and tilapia.

Key Words:*Vibrio* spp., *Lates calcarifer*, *Oreochromis niloticus*, Antibiotic Resistance

INTRODUCTION

Vibrios are ubiquitous in aquatic environments, depending on their salt requirement for optimum growth. However, clinical disease outbreaks only occur when a stressed fish get exposed to infectious agent (Abdel-Aziz et al. 2013). Vibriosis affects aquaculture of many commercially important species elsewhere, which include penaeid shrimp (Ransangan et al. 2013), gilthead sea bream (Abdel-Aziz et al. 2013), European seabass (Abdel-Aziz et al. 2013), tiger puffer (Mohi et al. 2013), groupers (Anand et al. 2008; Mienda 2012), flounder (Kim et al. 2014) and Asian seabass (Ransangan et al. 2011). You et al. (2016) stated that genus *Vibrio* encompases more than 63 species, whereas one third are potential human pathogens and have been implicated in water- and seafood-related outbreaks of gastrointestinal and wound infections in humans. *Vibrio vulnificus*, *V. haryei*, *V. parahaemolyticus*, *V. alginolyticus*, and *V. mimicus* are the common aetiology of vibriosis in marine aquaculture industry that have been reported to cause mortality and severe economic losses (You et al. 2016). Bondad-Reantaso et al. (2005) stated that Malaysia losses US$ 7.4 million to vibriosis in 1990. Multiple antibiotic resistance (MAR) was defined as resistance to two or more antibiotics (Shaw et al. 2014). Antibiotics have been widely used to treat vibriosis in humans and aquaculture livestock. The use of antibiotics in various clinical applications and aquaculture resulted in emerging of

antibiotic-resistant bacteria. Hence, reduces the effectiveness of antibiotic to combat both human and animal infections (Malla et al. 2014). In this study, evaluation of antibiotic resistance patterns of *Vibrio* spp. was done which were isolated from diseased seabass and tilapia; and water systems at Sri Tujuh lagoon, Tumpat, Kelantan, Malaysia

MATERIAL AND METHODS

Bacterial isolation and identification

Diseased seabass and tilapia showed clinical signs of exophthalmia, emaciation, skin darkening and body ulceration were collected from Sri Tujuh Lagoon. Loopful of kidney, spleen and external lesion of the fish were streaked separately onto thiosulfatecitrate-bile salts-sucrose (TCBS, Oxoid, England). For seawater, 0.1 ml of sample were spread plated onto TCBS. The inoculated plates were incubated at 30°C overnight. The selected colonies were identified using Gram staining, oxidase, catalase and API 20E (Biomerieux, France).

Antibiotic sensitivity tests

The Kirby-Bauer method was used to determine the antibiotic sensitivity patterns of isolates. The resistance of all strains to different antimicrobial agents was determined by the disc diffusion method. The pellet was inoculated onto 0.85% saline and compared to 0.5 McFarland Equivalence Turbidity Standard (Remel USA). The entire Mueller Hinton (MH, Oxoid, England) agar surface was streaked horizontally, vertically and around the outer edge of the plate by sterile swab. An antibiotic loaded paper disc was put onto MH agar. Antibiotics used were ampicillin (10 µg), erythromycin (15 µg), gentamicin (10 µg), oxytetracycline (30 µg), sulfamethoxazole (25 µg), and chloramphenicol (30 µg) (Oxoid, England). All cultures plates were incubated for 24 h at 30°C. The results of inhibition zones were interpreted as sensitive (S), intermediary sensitive (I), and resistance (R) according to the reference to the standard provided by the National Committee for Clinical Laboratory Standards (CLSI 2015).

Multiple antibiotic resistance (MAR) index

Multiple antibiotic resistance (MAR) index of present isolates against the tested antibiotics was calculated based on the following formula (Krumperman 1983):

$$\text{MAR index} = X/(Y \times Z)$$

Where, X = Total cases of antibiotic resistance; Y = Total number of antibiotic used in the study; Z = Total number of isolates. A MAR index value of equal or less than 0.2 was defined as antibiotics that were seldom or never used.

RESULTS AND DISCUSSION

In the present study, we found that *V. parahaemolyticus* and *V. alginolyticus* were the predominant isolates from diseased seabass and tilapia (Table 1). You et al. (2016) stated that both species are commonly detected in tropical marine regions. Those species also are among the most important disease causing agents of many commercially farmed marine invertebrate and vertebrate species in many Asian countries. Moreover, *V. vulnificus* also was isolated from seawater in Sri Tujuh lagoon. This pathogenic species in coastal, estuary, and river waters may pose a significant health hazard to local individuals who

have direct contact with water through recreational activities or via seafood consumption (You et al. 2016). Table 2 showed biochemical tests results for *V. parahaemolyticus*, *V. alginolyticus* and *V. vulnificus* isolated from diseased seabass, tilapia and seawater. The results obtained in this study was in accordance with several reports which using API 20E kit (Biomerieux) as identification tool to characterize the isolates of *V. parahaemolyticus*, *V. alginolyticus* and *V. vulnificus* (Biosca et al. 1996; Martinez-Urtaza et al. 2006; Martins et al. 2010).

Table 1. *Vibrio* spp. isolated from diseased seabass and tilapia

Strain ID	Spesies	Source
VP1	*V. parahaemolyticus*	Seawater
VP2, VP3, VP4, VP5, VP6, VP7, VP8, VP9, VP10, VP11, VP12, VP13, VP14, VP15, VP16, VP17, VP18, VP19, VP20, VP21, VP22, VP23, VP24, VP25, VP26, VP27, VP28, VP29, VP30	*V. parahaemolyticus*	Kidney of seabass
VA1, VA3, VA4, VA5, VA6, VA7, VA10, VA12, VA13, VA14, VA16,	*V. alginolyticus*	Ulser of tilapia
VA2, VA8, VA9, VA11, VA15	*V. alginolyticus*	Kidney of tilapia
VV1	*V. vulnificus*	Seawater

The frequencies of resistance were observed against erythromycin (85%), chloramphenicol (58%), sulfamethoxazole (49%) ampicillin (32%), and gentamycin (11%). You et al. (2016) stated that the resistance of Gram-negative bacteria to erythromycin is expected due to their intrinsic resistance. Ampicillin has been widely used since 1960 and ampicillin resistance is also commonly reported (Laganà et al. 2011, You et al. 2016). Common drugs used in agriculture, livestock operations, aquaculture, and human therapy are Sulfonamides (sulfa drugs) (Suzuki et al. 2013). This class of antibiotics are widely use all over the world especially in developing Asian countries as a result of their inexpensiveness and wide spectrum antimicrobial activity (Luo et al. 2011; Suzuki & Hoa 2012). Resistance to sulfonamides has been reported in clinical, aquaculture, and aquatic environments from different geographical regions (Hoa et al. 2008, 2011; You et al. 2012; Suzuki et al. 2013; Das et al. 2014). Pantai Sri Tujuh lagoon is adjacent to irrigation, a site where there have been numerous wastewater treatment overflows. Consequently, inputs from domestic sewage might be a cause of notable frequency of sulfonamide-resistant bacteria detected in diseased seabass and tilapia. In other hand, all the *Vibrio* isolates were found to be susceptible to oxytetracycline. This findings were in agreement with study done by You et al. (2016) which showed the sensitivity of oxytetracycline against *Vibrio* spp. isolated from waters in Peninsular Malaysia.

The resistance patterns of all the 47 *Vibrio* isolates are shown in Table 3. Multiple antibiotic resistance was observed and 17 different resistance patterns were identified among the isolates. In this study, 96% of *Vibrio* isolates were resistant to one or more different classes of antibiotic. None of the isolates susceptible to all antibiotics tested, indicating widespread occurrence of MAR *Vibrio* spp. in diseased seabass and tilapia. One *V. vulnificus* strain isolated from seawater found to be resistant to five different classes of

antibiotic. Our results showed that *Vibrio* isolates showed high frequency of MAR (80%). The MAR index of 0.4 indicating the *Vibrio* spp. in these farmed fish might have been indiscriminately and continuously exposed to those antibiotics during culturing stages of the fish. There were no clear species-specific antibiotic resistance patterns in this study. The current results are in agreement with others studies showing multiresistance incidence in *Vibrio* spp. from aquatic environments and seafood samples (Baker-Austin et al. 2009; Zulkifli et al. 2009; Lesley et al. 2011; Noorlis et al. 2011; You et al. 2016.).

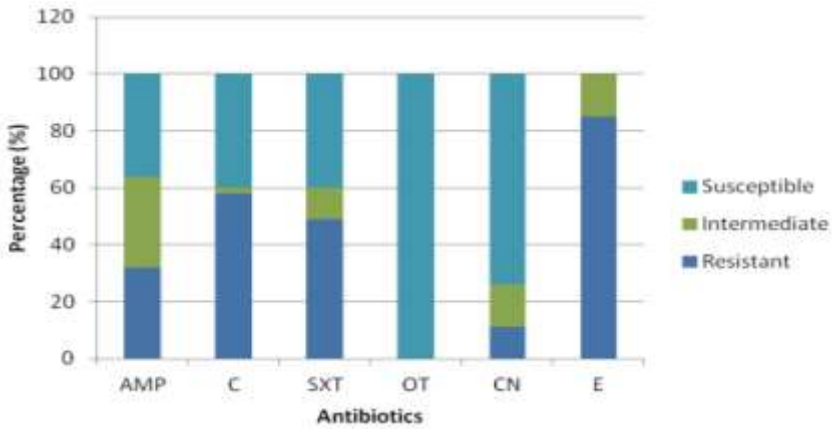
Table 2. Biochemical tests results for *V. parahaemolyticus*, *V. alginolyticus* and *V. vulnificus* isolated from diseased seabass, tilapia and seawater

Biochemical tests	*V. parahaemolyticus*	*V. alginolyticus*	*V. vulnificus*
ONPG	-	-	+
ADH	-	-	-
LDC	+	+	+
ODC	+	+	+
CIT	-	+	-
H_2S	-	-	-
URE	-	-	-
TDA	-	+	-
IND	+	+	+
VP	-	-	-
GEL	-	+	+
GLU	+	+	+
MAN	+	+	-
INO	-	-	-
SOR	-	-	-
RHA	-	-	-
SAC	-	-	-
AMY	V	+	+
ARA	-	-	-
OX	+	+	+
CAT	+	+	+

+: Positive reaction; -: Negative reaction; V: Variable; ONPG: β-galactosidase; ADH: Arginine dihydrolase; LDC: Lysine decarboxylase; ODC: Ornithine decarboxylase; CIT: Citrate; H_2S: Hydrogen sulfide; URE: Enzyme urease; TDA: Tryptophane deaminase; VP: Voges-proskauer; IND: Indole; GEL: Gelatine; GLU: Glucose; MAN: Mannitol; INO: Inositol; SOR: Sorbitol; RHA: Rhamnose; SAC: Saccharose; AMY: Amygdalin; ARA: Arabinose; OX: Oxidase; CAT: Catalase

Table 3. The antibiotic resistance profile patterns of *Vibrio* spp. from seabass, tilapia and seawater

Pattern	Strain number	Antibiotic profiles
I	VP1	CN, E
II	VA1, VP6, VP13, VP21, VP28	AMP, C, SXT, E
III	VP3, VP9, VP10, VP11, VP14, VP16, VA9, VP18, VP23, VP27, VA16	C, SXT, E
IV	VP5, VA7	AMP, C, E
V	VA3, VP20	SXT, E
VI	VA5	C, SXT, CN, E
VII	VA8	AMP, SXT, E
VIII	VV1	AMP, C, SXT, CN, E
IX	VA12, VA14	AMP, E
X	VP26	AMP, C
XI	VA13	AMP, C, CN, E
XII	VA15	AMP, SXT
XIII	VP19, VA10, VP24, VP25	C, E
XIV	VP2, VP4, VP8, VP12, VP15, VP17, VP22, VP29, VP30	E
XV	VA2	AMP
XVI	VA6	SXT
XVII	VA11	C



AMP: Ampicillin; C: Chloramphenicol; SXT: Sulfamethoxazole; OT: Oxytetracycline; CN: Gentamicin; E: Erythromycin

Figure 1. Antibiotic susceptibility of *Vibrio* spp. isolated from seabass, tilapia and seawater

CONCLUSION

This study revealed that the antibiotic-resistant and multidrug-resistant *Vibrio* spp. were common in seabass, tilapia and seawater of Sri Tujuh lagoon, East Coast Malaysia. The findings implying a great risk to public health. Constant surveillance and monitoring of antibiotic resistance and pollution levels of antibiotics should be done in order to compile more information on antibiotic sensitivity of *Vibrio* spp. and other known aquatic bacteria species in order to avoid the development of antibiotic superbug. Likewise, the increase of antibiotics resistant *Vibrio* spp. will lead to treatment challenge in vibriosis.

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