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Characterization of *Vibrio alginolyticus* isolated from white leg shrimp (*Litopenaeus vannamei*) with emphasis on its antibiogram and heavy metal resistance pattern

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ABSTRACT

This paper describes the antibiogram and heavy metal resistance pattern of *Vibrio alginolyticus* isolated from farmed white leg shrimp (*Litopenaeus vannamei*). *V. alginolyticus* has been recognized as a causative agent of vibriosis in shrimp culture and can devastate an entire shrimp farm. Therefore, this study was carried out to investigate the suitability of using antibiotics against *V. alginolyticus,* as well as its tolerance to heavy metals, to reveal the exposure level of cultured shrimp to heavy metals. In the present study, a total of 14 antibiotics; oxolinic acid (2 µg), ampicillin (10 µg), erythromycin (15 µg), furazolidone (15 µg), lincomycin (15 µg), colistin sulphate (25 µg), oleandomycin (15 µg), doxycycline (30 µg), fosfomycin (50 µg), florfenicol (30 µg), flumequine (30 µg), tetracycline (30 µg), fosfomycin (50 µg) and spiramycin (100 µg) as well as four heavy metals; mercury (Hg^{2+}), cadmium (Cd^{2+}), chromium (Cr^{6+}) and copper (Cu^{2+}) were applied. Based on the results for antibiotic sensitivity, it was shown that oxolinic acid is the most effective antibiotic in controlling *V. alginolyticus,* where 92.8% of the bacterial isolates present were sensitive to it. It was followed by furazolidone (85.6%), nitrofuratoin (79.8%), tetracycline (78.8%), doxycycline (72.2%) and florfenicol (71.2%). On the other hand, all the bacterial isolates present were resistant to lincomycin. In the heavy metal tolerance test, all the bacterial isolates present were resistant to Hg^{2+}, Cd^{2+} and Cr^{6+} whereas only 26.7% of them were resistant to Cu^{2+}.

Key words: antibiogram, heavy metal, *Vibrio alginolyticus*, white leg shrimp, *Litopenaeus vannamei*

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Introduction

White leg shrimp (*Litopenaeus vannamei*) is a shrimp species native to the Pacific region and an important species in aquaculture in the Asia-Pacific region. It has become a popular shrimp species among Malaysian aquaculturists, due to its high value and the huge demand for it in local and international seafood markets. However, bacterial diseases, especially vibriosis, have been recognized as a significant constraint to the development of shrimp culture worldwide. Vibriosis has been reported as a fatal disease in many marine fish and invertebrates such as shrimp (DE LA PENA et al., 1993), crab and lobster (BOWSER et al., 1981), mollusks (NOTTAGE et al., 1989) and fish (HUSTVEDT et al., 1992). At the moment, information on vibriosis due to *V. alginolyticus* is still scarce and insufficient for the prevention and control of the disease in aquaculture. Therefore, this study was carried out to provide valuable information for fish farmers in selecting the most suitable antibiotic or for treatment or prophylactic purposes.

Materials and methods

Sampling. A total of 220 moribund white leg shrimp (*L. vannamei*) 8 to 10 cm in length were sampled randomly from a farm at Selangor, Malaysia. Water samples from the *L. vannamei* grow-out pond were also been collected and water parameter were measured using a multiparameter meter (YSI, USA). The temperature, dissolved oxygen, pH and salinity of the sampling sites were 26.41 ºC, 7.66 mg/L, 8.73 and 25.11 ppt, respectively.

Bacterial isolation and identification. Swabs were aseptically taken from the shrimp hemolymph and hepatopancreas using sterile cotton buds and spread onto Thiosulphate Citrate Bile Salt (TCBS) (Merck, Germany) medium (LEE et al., 2009a). Next, 1 mL of water sample was serially diluted in sterile physiological saline and plated onto Tryptic Soy Agar (TSA) and TCBS medium (Merck, Germany). All the inoculated media were incubated at room temperature for 24 to 48 hours. The bacterial colonies grew on the culture media were further selected for identification using conventional biochemical tests (HOLT et al. 1994) and further confirmed by a commercial identification kit (BBL, USA).

Antibiotic sensitivity test. The isolates present (n = 320) were cultured in Tryptic Soy Broth (TSB) (Oxoid, England) for 24 hours at room temperature. The bacterial cells were then centrifuged at 14,500 rpm for 5 min using MiniSpin (Eppendorf, Germany). The concentration of bacterial cells was adjusted into a 10^6 colony forming unit (CFU) by using saline and monitored with BioPhotometer (Eppendorf, Germany), before being swabbed on the prepared Mueller Hinton agar (Oxoid, England). An antibiotic susceptibility test was conducted according to the Kirby-Bauer disk diffusion method, using Mueller-Hinton agar (BAUER et al., 1966). Antibiotics tested included oxolinic acid

(2 µg) OA 2; ampicillin (10 µg) AMP 10; erythromycin (15 µg) E 15; furazolidone (15 µg) FR 15; lincomycin (15 µg) MY 15; colistin sulphate (25 µg) CT 25; oleandomycin (15 µg) OL 15; doxycycline (30 µg) DO 30; florfenicol (30 µg) FFC 30; flumequine (30 µg) UB 30; tetracycline (30 µg) TE 30; fosfomycin (50 µg) FOS 50 and spiramycin (100 µg) SP 100 (Oxoid, England). Interpretation of the results was made in accordance with the standard measurement of inhibitory zones in millimetres (mm) as sensitive (S), intermediary sensitive (I) and resistant (R).

Multiple antibiotic resistance index. The multiple antibiotic resistance (MAR) index of the bacterial isolates was calculated based on the following formula (SARTER et al., 2007; LEE et al., 2009a; LEE et al., 2010):

MAR index = X / (Y × Z)

X = total antibiotic resistance case

Y = total antibiotic used in the study

Z = total isolates

A MAR index equals to or less than 0.2 indicates that antibiotics seldom or never been used on animals for treatment purposes, whereas a MAR index higher than 0.2 indicated that animals were in high risk exposure to these antibiotics.

Heavy metal resistance test. A heavy metal resistance test was carried out as described by MIRANDA and CASTILLO (1998). Bacterial tolerance to four elements of heavy metal, i.e., mercury (Hg^{2+}), cadmium (Cd^{2+}), chromium (Cr^{6+}) and copper (Cu^{2+}) was determined by the agar dilution method (LEE et al., 2009c). Overnight bacterial suspension was spread on plates of TSA medium with different concentrations of $HgCl_2$, CdCl, $K_2Cr_2O_7$ and $CuSO_4$ (Fluka, Spain). By two-fold dilutions, concentrations of both Cd^{2+} and Cr^{6+} ranged from 25 to 400 µg/mL while concentrations of Hg^{2+} and Cu^{2+} ranged from 2.5 to 40 µg/mL and 150 to 2400 µg/mL, respectively. For the purpose of defining metal resistance, the isolates were considered resistant if growth was obtained at concentrations of 10 µg/mL (Hg^{2+}), 100 µg/mL (Cd^{2+} and Cr^{6+}) and 600 µg/mL (Cu^{2+}) (ALLEN et al. 1977; LEE et al., 2009c; LEE et al., 2010). The operational definition of tolerance as used in this study was based on positive bacterial growth when the concentration of heavy metals was above the stated concentration for resistance.

Results

The total plate count of *V. alginolyticus* from the water sample from the white leg shrimp farm was 1.3 ×10^4 colony forming unit (CFU)/mL. In the present study, the majority (more than 70%) of the bacterial isolates present were found to be sensitive to nitrofuratoin, furazolidone, tetracycline, doxycycline, florfenicol and oxolinic acid (Fig 1). However, the percentage of bacterial isolates present which were sensitive to colistin sulphate, oleandomycin, fosfomycin, erythromycin, lincomycin, ampicillin, flumequine, and spiramycin ranged from 0% to 60%. All the bacterial isolates present were found to be resistant to lincomycin. Overall, the sensitive category accounted for 50.5%, whereas 40% and 9.5% were reported as resistant and intermediary sensitive categories. The MAR value of the present study was 0.40. All the bacterial isolates present were found to be resistant to Hg^{2+}, Cd^{2+} and Cr^{6+}. However, Cd^{2+} was found capable of inhibiting the growth of the bacterial isolates by 80% at a concentration of 400 µg/mL. On the other hand, 73.3% of bacterial isolates present were found to be sensitive to Cu^{2+}.

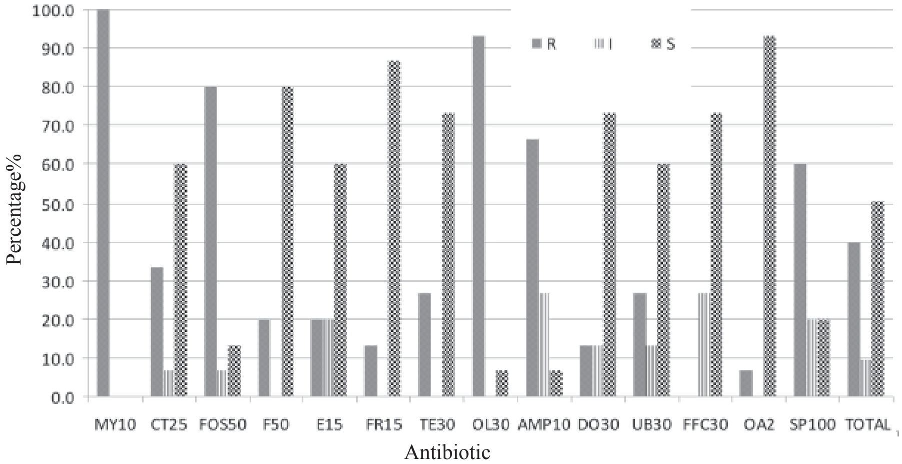

Fig. 1. Antibiotic susceptibility of *Vibrio alginolyticus* isolated from Asian seabass.
R - Resistant, I - Intermediary sensitive, S - Sensitive

Discussion

Vibriosis due to *V. alginolyticus* has been recognized as a major problem in aquaculture. It has been reported as attacking various aquatic animals, such as tiger shrimp, *Penaeus monodon* (LEE et al., 1996), the larvae of the catarina scallop, *Argopecten ventricosus* (SAINZ et al., 1998), gilt-head sea bream (*Sparus aurata* L.) (BALEBONA et al., 1998), shrimp (GEORGE et al., 2005) and giant freshwater prawn (KHUNTIA et al., 2008). Up to now, there have been few reports of *V. alginolyticus* in Malaysian aquaculture. For instance, IDRIS et al. (2009) reported the virulence of *V. alginolyticus* in Asian seabass. *V. alginolyticus* was also reported in other aquatic animals in Malaysia. Other studies reported infections of *V. alginolyticus* in aquatic animals such as mantis shrimp (NAJIAH and LEE, 2008b), diseased seaweed, *Gracilaria changii* (NAJIAH and LEE, 2008a) and oyster, *Crassostrea iredalei* (NAJIAH et al., 2008a). To our knowledge, this is the second report of *V. alginolyticus* isolated from white leg shrimp in Malaysia, following the first report by LEE et al. (2009a).

In the present study, nitrofuratoin, furazolidone, tetracycline, doxycycline, florfenicol and oxolinic acid were found to be effective in controlling *V. alginolyticus* in white leg shrimp. Thus, it is recommended that shrimp farmers may use these antibiotics for prophylactic and treatment purposes in shrimp culture. However, these antibiotics cannot kill all strains of *V. alginolyticus*. The results of the present study showed that oxolinic acid could control almost 90% of all bacterial strains. Therefore, further study should be carried out to find which antibiotics can inhibit the growth of all strains of *V. alginolyticus*. For example, in the study by THAKUR et al. (2003), it was reported that all the bacteria strains of *Vibrio* spp. including *V. alginolyticus* isolated from moribund shrimp, were sensitive to erythromycin, streptomycin and chloramphenicol. Another study by NAJIAH and LEE (2008a) revealed that all strains of *V. alginolyticus* isolated from diseased mantis shrimp were sensitive to chloramphenicol, oxytetracycline and furazolidone. Instead of looking for other effective antibiotics, we suggested that fish farmers could use antimicrobial agents derived from natural resources, as described in the study by LEE and NAJIAH (2009), in which they found that *C. microcarpa* could inhibit the growth of *V. alginolyticu,s* whereas another study by LEE et al. (2009b) claimed that essential oil of cloves displayed inhibitory activity against *V. alginolyticus*.

In addition to antibiotic tests, a high percentage of heavy metal resistance to the heavy metals tested was observed among isolated *V. alginolyticus*. This would probably associated to the study site, which was surrounded by agricultural activities. Subsequently, agricultural waste such as fertilizer, consisting of heavy metal residues, may have seeped into the water source of the white leg shrimp farm. Therefore, bacteria may have developed heavy metal resistance genes after being exposed to heavy metal residues for an undefined period. Up to now, little information on the heavy metal resistance pattern of bacteria isolated from aquaculture sites has been available in the literature. Therefore, a comparison of the heavy metal resistance patterns of bacterial isolates in present study to those in other studies cannot be made.

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SAŽETAK

U radu je opisan antibiogram bakterije *Vibrio alginolyticus* izdvojene iz farmski uzgajane atlantske bijele kozice (*Litopenaeus vannamei*) i njezina otpornost na teške metale. Kao uzročnik vibrioze *V. alginolyticus* može dovesti do propadanja cijele farme. Stoga je poduzeto istraživanje s ciljem da se pronađe djelotvoran antibiotik protiv bakterije *V. alginolyticus* te da se ustanovi razina njezine podnošljivosti na teške metale kako bi se ustanovila razina izloženosti uzgajanih kozica tim metalima. Istražena je osjetljivost na 14 antimikrobnih tvari: oksolinsku kiselinu (2 μg), ampicilin (10 μg), eritromicin (15 μg), furazolidon (15 μg), linkomicin (15 μg), kolistin sulfat (25 μg), oleandomicin (15 μg), doksiciklin (30 μg), fosfomicin (50 μg), florfenikol (30 μg), flumekvin (30 μg), tetraciklin (30 μg) i spiramicin (100 μg), te na četiri teška metala: živu (Hg^{2+}), kadmij (Cd^{2+}), krom (Cr^{6+}) i bakar (Cu^{2+}). Najučinkovitijom se pokazala oksolinska kiselina na koju je bilo osjetljivo 92,8% izolata *V. alginolyticus*. Na furazolidon je bilo osjetljivo 85,6%, na nitrofuratoin 79,8%, na tetracillin 78,8%, na doksiciklin 72,2% i na florfenikol 71,2% izolata. S druge strane, svi su izolati bili otporni na linkomicin. U testu podnošljivosti na teške metale svi su bakterijski izolati bili otporni na ione žive, kadmija i kroma, dok je samo 26,7% bilo otporno na ione bakra.

Ključne riječi: antibiogram, teški metali, *Vibrio alginolyticus*, bijela kozica, *Litopenaeus vannamei*