Antibiogram and Heavy Metal Resistance Pattern of *Flavobacterium* spp. Isolated From Asian Seabass (*Lates Calcarifer*) Hatchery

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ABSTRACT

This paper described antibiogram and heavy metal resistance pattern of *Flavobacterium* spp. isolated from Asian Seabass (*Lates calcarifer*) commercial hatchery. *Flavobacterium* spp. was recognized as a causative agent of bacterial systemic disease in Asian seabass and may cause a huge loss to fish farmer. Therefore, this study was conducted to help fish farmer in selecting suitable antibiotics in controlling this bacterial. In the present study, a total of 150 *Flavobacterium* spp. was successfully isolated from diseased Asian Seabass fingerling and water sample from the fingerling tank by using cytophaga medium. The sensitivity of the bacteria against 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), nitrofuratoin (50 µg), florfenicol (30 µg), flumequine (30 µg), tetracycline (30 µg) and spiramycin (100 µg) and four heavy metals mercury (Hg^{2+}), cadmium (Cd^{2+}), chromium (Cr^{6+}) and copper (Cu^{2+}) were determined. In the present study, high percentage of antibiotic resistance case (71.4%) was observed compared to the intermediary sensitive (10.5%) and sensitive case (18.1%) among the present bacterial isolates. The occurrence of high percentage of antibiotic resistance case might due to over or misuse of the tested antibiotics in the hatchery.

Keywords: Antibiogram, heavy metal, *Flavobacterium* spp., Asian seabass, *Lates calcarifer*

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INTRODUCTION

Asian seabass, *Lates calcarifer* (Bloch) is a native fish species in Indo−Pacific region (Greenwood, 1976). It is an important species for aquaculture in Southeast Asia (Catacutan and Coloso, 1997). It becomes a popular fish species among Malaysian aquaculturist due to high value and huge demand from local and abroad seafood market. Tucker *et al.* (2002) reported that production of *L. calcarifer* was recorded 20,000 tons per year with the value more than 65 million dollars. However, pathogenic bacteria such as *Flavobacterium* spp. are considered one of the serious pathogen to Asian seabass culture. It was reported as the most second costly pathogen in channel catfish culture in the Unites States (Darwish *et al*., 2004). Furthermore, *Flavobacterium* spp. was also reported infected various types of fish species such as golden shiners, striped bass, largemouth bass and sunfishes (Darwish *et al*., 2004). Arias *et al*. (2004) is also highlighted that *Flavobacterium* spp. the causative

agent of columnaris, is an important bacterium in aquaculture especially in freshwater fish culture. This bacterium can be commonly found in worldwide aquatic environment, wild and cultured fish including ornamental fish (Austin and Austin, 1999). The external pathology of this disease exhibited lesion on fin, body surface and gills of the infected fish. Skin of the infected fish appeared yellowish mucoid material. This disease will result in mortality when the lesions appeared tremendously on the gills of the diseased fish. In some cases, no pathological signs were observed of the infected fish. The infected fish was found lingering on the surface water. In the present study, fish farmer claimed that this bacterial disease outbreak was occurred during the monsoon season where the temperature of the water source was not constant. Subsequently, the cultured fish may stress due to the dramatically change of temperature. However, only Asian seabass fingerling was found susceptible to this disease. So far, no mortality of Asian seabass brood stock was reported during the disease outbreak. Therefore, the aim of this study was to investigate antibiogram and heavy metal resistance pattern of *Flavobacterium* spp. isolated from Asian seabass. The information from the present study may be useful for fish farmer in selecting suitable antibiotics for controlling bacterial disease due to *Flavobacterium* spp.

MATERIALS AND METHODS

Bacterial Isolation and Identification

Asian seabass, *Lates calcarifer* fingerling with the size 8 to 12 cm and water samples of *L. calcarifer* nursery tank were collected from commercial Asian seabass hatchery. The water parameters of the sampling sites were measured using pH meter (YSI, USA). The temperature, dissolved oxygen, pH and salinity of the sampling sites were recorded as 25.6ºC, 6.47 mg/L, 6.89 and 15 ppt, respectively. Water samples were collected from *L. calcarifer* fingerling water reservoir tank in four replicates. One millimeter of water sample was serially diluted (tenfold dilution) in sterile physiological saline and plated on two medium; Tryptic Soy Agar (TSA) and cytophaga medium. 200 diseased *L. calcarifer* fingerlings were randomly sampled from nursery tank. Swab was aseptically taken from organs such as eyes, kidney, liver and abdominal fluid of the fish using sterile cotton bud and spread onto cytophaga medium. All the inoculated media were incubated at room temperature (25–28ºC) for 24 to 48 h. The bacterial colonies that grew on the selective media were further selected for the identification test. The bacterial isolates were identified using conventional biochemical tests (Holt *et al*., 1994) and confirmed with commercial identification kit, BBL Crystal ID System Enteric/ Nonfermenter (BBL, USA) (Lee *et al*., 2009a; Lee *et al*., 2009b).

Antibiotic Susceptibility Test

The present bacterial isolates (n = 150) were cultured in tryptic soy broth (TSB) (Oxoid, England) for 24 h at room temperature. The bacterial cells were then centrifuged at 14,500 rpm for 5 min by using minispin (Eppendorf, Germany). The concentration of the bacterial cells were adjusted into 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). Antibiotic susceptibility test was conducted according to Kirby–Bauer disk diffusion method using Mueller−Hinton agar (Oxoid, England) (Bauer *et al*., 1966; Lee *et al*., 2009b). Antibiotics tested including 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, fosfomycin (50 µg); FOS 50, nitrofuratoin (50 µg); F 50, florfenicol (30 µg); FFC 30, flumequine (30 µg); UB 30, tetracycline (30 µg); TE 30 and spiramycin (100 µg); SP 100 (Oxoid, England). Interpretation of the results namely sensitive (S), intermediary sensitive (I) and resistance (R) was made in accordance to the standard measurement of inhibitory zones in millimeter (mm).

Multiple Antibiotic Resistance (MAR) Index Determination

MAR index (multiple antibiotic resistance) of the present isolates against the tested antibiotics was calculated based on the formula as follows (Sarter *et al*., 2007; Lee *et al*., 2010a; Lee and Wendy, 2011; Lee and Wendy, 2012):

MAR index (multiple antibiotic resistance)
= X / (Y x Z)
X = total of antibiotic resistance case
Y = total of antibiotic used in the study
Z = total of isolates

A MAR index value of equal or less than 0.2 was defined as those antibiotics were seldom or never used for the animal in term of treatment whereas the MAR index value higher than 0.2 is indicating that fish highly expose to the tested antibiotics.

Heavy Metal Resistance Test

Heavy metal resistance test was carried out as described by Miranda and Castillo (1998); Lee *et al*. (2013). 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 agar dilution method. Overnight bacterial suspension was spread onto plates of TSA medium incorporated with different concentrations of $HgCl_2$, CdCl, $K_2Cr_2O_7$ and $CuSO_4$ (Fluka, Spain). By two−fold dilutions, concentration of both Cd^{2+} and Cr^{6+} were ranging from 25 to 400 µg/mL while concentration of Hg^{2+} and Cu^{2+} were ranging 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 as resistant if growth was obtained at concentration 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*., 2010b). The operational definition of tolerance as used in this study was based on the positive bacterial growth when concentration of heavy metals was above the stated concentration for resistance.

RESULTS AND DISCUSSION

RESULTS

The total plate count of *Flavobacterium* spp. from the water sample in the Asian seabass hatchery was 3.5 X 10^3 (CFU)/ml. In the present study, all the bacterial isolates were found resistant to colistin sulphate, fosfomycin, erythromycin and furazolidone (Figure 1). All the tested antibiotics were found not effective in controlling *Flavobacterium* spp. in the present study. Only 53.3% of the present bacterial isolates were found to be sensitive to nitrofuratoin and spiramycin. Whereas the percentage of bacterial isolates that showed sensitive to another 12 antibiotics were ranged from 0 to 33.3%. Overall, the percentage of resistance case was recorded as 71.4%. On the other hand, intermediary sensitive and sensitive case was recorded as 10.5% and 18.1%, respectively. The MAR value of the present study was 0.71. All the bacterial isolates were found resistant to the tested heavy metals. However, Hg^{2+} was found can inhibit the growth of 52.3% and 73.3% of the present bacterial isolates at concentration of 200 µg/mL and 400 µg/mL, respectively. Whereas, Cd^{2+} was found can be inhibited the growth of 33.3% and 73.3% of the present bacterial isolates at the concentration of 200 µg/mL and 400 µg/mL, respectively.

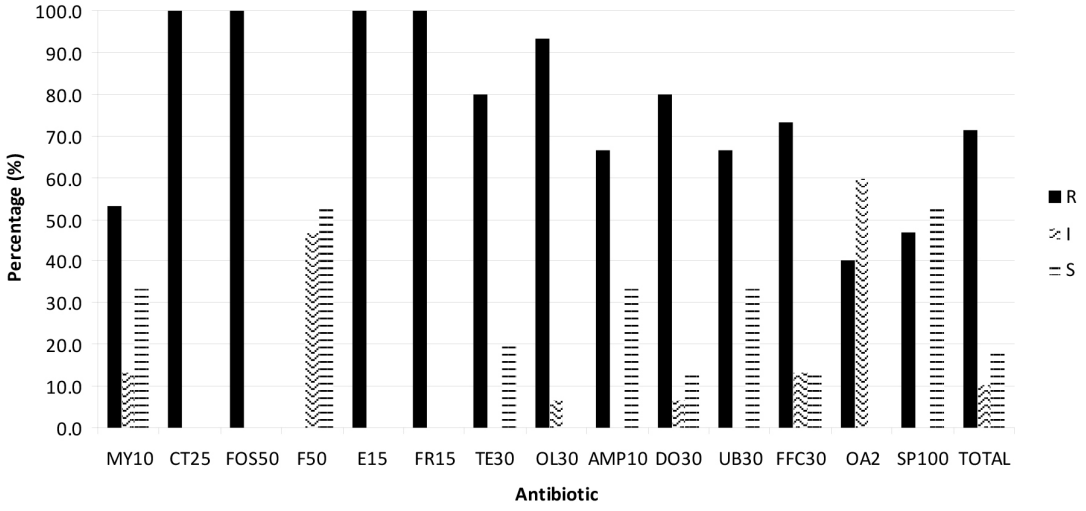

Figure 1 Antibiotic sensitivity of *Flavobacterium* spp. isolated Asian seabass hatchery; R = Resistance; I = Intermediate Resistance; S = Sensitive

DISCUSSION

Flavobacterium spp. was recognized as a pathogenic bacterial in aquaculture which caused damage on skin and gills of the infected fish (Kubilay *et al*., 2008). Subsequently, this bacterial disease can result huge mortality to the cultured fish (Noga, 2000). Till present, *Flavobacterium* spp. was reported successfully isolated from various types of ornamental fish (Najiah *et al*., 2008) and American bullfrog (Lee *et al*., 2009) in Malaysia. However, *Flavobacterium* spp. in Asian seabass in Malaysia was not well documented. To our knowledge, this is first report on *Flavobacterium* spp. in Asian seabass culture in Malaysia.

High antibiotic resistance rate was observed in this study where may be due to overuse or misuse of antibiotic among fish farmer in the hatchery. None of the tested antibiotics was found effective in controlling isolated *Flavobacterium* spp. However, nitrofuratoin and spiramycin were found can inhibit the growth more than 50% present bacterial isolates. Thus, fish farmer may try to apply these two antibiotics to control *Flavobacterium* spp. in Asian seabass culture. But, further study on antibiogram of *Flavobacterium* spp. should be carried out to find the effective antibiotic to combat *Flavobacterium* spp. in Asian seabass culture. For instance, Bruun *et al*. (2003) reported that oxytetracycline was found effective in controlling *F. psychrophilum* in rainbow trout culture. Another study of Kubilay *et al*. (2008) reported that *Flavobacterium* spp. isolated from rainbow trout in Turkey was found sensitive to oxytetracycline (30 µg), chloramphenicol (30 µg), furazolidone (100 µg), nitrofurantoin (300 µg), erythromycin (15 µg) and streptomycine (10 µg). However, Malaysian government had banned several antibiotics such as oxytetracycline, chloramphenicol, furazolidone and nitrofurantoin. The results of antibiotic test of the present study gave us insight information on the level exposure of antibiotic residues in the sampling area. Instead of using antibiotics in fish health management, farmer may find alternative ways in controlling disease problem at aquaculture sites such as application of probiotic and natural antimicrobial agent.

In the present study, all bacterial isolates were found resistant to the tested heavy metals. There is very little information on heavy metal resistance pattern of bacterial from aquaculture sites (Lee and Wendy, 2011). Therefore it is quite difficult to make comparison the heavy metal pattern of the present bacteria isolates to other study. High heavy metal resistance cases were observed among present bacterial isolates could be resulted from the water source of the hatchery contaminated with fertilizer which contains heavy metal residues since the hatchery in our study was surrounded in agricultural activities (Lee and Wendy, 2012). Thus, this factor may contribute to the incidence of heavy metal resistance among the isolated bacteria.

CONCLUSION

The findings of the present study are indicating that the sampled fish was over exposure to the tested antibiotics and heavy metals. In spite of the fact, farmer should alerted to these findings by having a good filtration system in order to ensure the water source is free from antibiotic and heavy metal residues which may trigger contamination to the fish.

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