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BRIEF NOTE

Seong Wei LEE, Musa NAJIAH, Wee WENDY

Bacteria associated with golden pompano (*Trachinotus blochii*) broodstock from commercial hatchery in Malaysia with emphasis on their antibiotic and heavy metal resistances

Abstract This paper was the first report on pathogenic bacteria isolated from golden pompano (*Trachinotus blochii*) with the antibiogram, as well as the heavy metal resistance pattern. Golden pompano becomes popular among Malaysian fish farmers due to its high value and demand from local and oversea markets. However, the baseline information on antibiogram of pathogenic bacteria associated with golden pompano is not well established. Therefore, the information from this study may be useful to fish farmers in selecting appropriate antibiotic for treatment and prophylactive purpose in golden pompano culture. Isolation of bacterial isolates was carried out using 5% of Horse Blood agar, Tryptic Soy Agar (TSA), Mac Conkey, Thiosulphate Citrate Bile Salt (TCBS), Eosin Methylene Blue (EMB), Glutamate Starch Pseudomonas (GSP), Xylose Lysine Deoxycholate (XLD), and Baird Parker media. The bacterial isolates were then identified using conventional biochemical tests and confirmed by commercial bacterial identification kit. Antibiotic susceptibility test of bacterial isolates against 21 antibiotics (oxolinic acid 2 μg, ampicillin 10 μg, erythromycin 15 μg, furazolidone 15 μg, lincomycin 15 μg, oleandomycin 15 μg, amoxicillin 25 μg, colistin sulphate 25 μg, sulphamethoxazole 25 μg, chloramphenicol 30 μg, doxycycline 30 μg, florfenicol 30 μg, flumequine 30 μg, kanamycin 30 μg, nalidixic acid 30 μg, novobiocin 30 μg, oxytetracycline 30 μg, tetracycline 30 μg, nitrofurantoin 50 μg, fosfomycin 50 μg, and spiramycin 100 μg) was determined using disk diffusion method, whereas the heavy metal resistance pattern (mercury Hg^{2+}, cadmium Cd^{2+}, chromium Cr^{6+}, and copper Cu^{2+}) of the bacterial isolates was characterized using two-fold agar dilution method. Five bacterial species were successfully found from 50 diseased golden pompano. They were *Streptococcus* spp. (n = 12), *Escherichia coli* (n = 30), *Salmonella* spp. (n = 20), *Pseudomonas* spp. (n = 36), and *Vibrio* spp. (n = 50). More than 80% of the bacterial isolates were found sensitive to 11 out of 21 antibiotics (tetracycline, nitrofurantoin, florfenicol, chloramphenicol, oxytetracycline, nalidixic acid, doxycycline, furazolidone, flumequine, fosfomycin, and oxolinic acid. However, all bacterial isolates were found resistant to all tested heavy metals except for copper and cadmium. The multiple antibiotic resistance (MAR) index of the present study indicated that the fish samples were under high risk exposure to the tested antibiotics. Florfenicol is suggested to be used as antimicrobial agent for golden pompano culture since all bacterial isolates were sensitive to it.

Keywords bacteria, golden pompano, *Trachinotus blochii*, antibiotic, heavy metal

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Seong Wei LEE (✉), Musa NAJIAH, Wee WENDY
Faculty of Agro Industry and Natural Resources, University Malaysia Kelantan, Kota Bharu 16100, Malaysia
E-mail: leeseongwei@yahoo.com

1 Introduction

Marine aquaculture production is increasing rapidly with the annual world production rate at 8.8% (Garza-Gil et al., 2009). More new species were introduced in this sector for human consumption as well as supply for other related business activity (Garza-Gil et al., 2009). One example of marine fish species in mass production in aquaculture is Atlantic salmon (*Salmo salar*), where Norway is the largest world producer (Toranzo et al., 2005). Other main producers are Chile, United Kingdom, Canada, and

Ireland. On the other hand, Greece, Italy, France, Spain, and Portugal lead in gilthead seabream (*Sparus aurata*), seabass (*Dicentrarchus labrax*), and turbot (*Scophthalmus maximus*) production (Toranzo et al., 2005). In Asia, Japan has been the primary producer for yellowtail (*Seriola quinqueradiata*), ayu (*Plecoglossus altivelis*), flounder (*Paralichthys olivaceus*), and seabream (*Pagrus major*) (Toranzo et al., 2005). However, bacterial diseases are recognized as the main factor, which limited the success of worldwide and accounted for economic losses (Toranzo et al., 2005).

Recently, golden pompano (*Trachinotus carolinus*) was introduced to Malaysia by Taiwanese investors to produce *T. carolinus* fry for both local and overseas demand. Several attributes including tolerance to a wide range of salinity (Sampaio et al., 2003), good growth rate and high survival rate in captivity (Lazo et al., 1998) created popularity of this fish species among marine fish farmers, with high demand and return. Nevertheless, one of the commercial *T. carolinus* hatchery in Pahang, Malaysia, was hit by the bacterial disease outbreak. Therefore, efforts were made to study the causative agents of the bacterial disease infected the *T. carolinus* broodstock as well as their antibiogram and heavy metal resistance pattern.

2 Materials and methods

2.1 Bacterial isolation, identification, and storage

A total of 50 diseased golden pompano broodstock weighing 5 to 10 kg were sampled from a commercial hatchery in Pahang, situated adjacent to South China Sea. The water parameters of the sampling sites were measured using a pH meter (YSI, USA). Temperature, dissolved oxygen, pH, and salinity of the sampling sites were recorded at 25°C, 7.3 mg·L^{-1}, and 7.8 and 28 ppt, respectively.

Approximately 10 g wet weight of *T. carolinus* liver and kidney was homogenized in 10-mL sterile physiological saline using sterile glass rods. One millimeter of each sample was serially diluted in sterile physiological saline and plated on eight types of media including Tryptic Soy Agar (TSA), Mac Conkey, Thiosulphate Citrate Bile Salt (TCBS), Eosin Methylene Blue (EMB), Glutamate Starch Pseudomonas (GSP), Xylose Lysine Deoxycholate (XLD), 5% of Horse Blood agar, and Baird Parker medium (Merck, Germany).

All the inoculated media were incubated at room temperature for 24 to 48 h. The bacterial colonies grown on the selective media were randomly selected for the identification test using conventional biochemical tests (Holt et al., 1994) and confirmed with a commercial identification kit (BBL, USA). The selected bacterial isolates were then stored with 20% glycerol and kept at −80°C as stock cultures.

2.2 Antibiotics susceptibilities tests (disk diffusion method)

The present isolates were incubated in tryptic soy broth (TSB) (Oxoid, England) for 24 h at room temperature. The bacterial cells were then centrifuged at 14500 r·min^{-1} for 5 min by using minispin (Eppendorf, Germany). The concentration of the bacterial cells were adjusted into $\times 10^6$ colony forming unit (CFU) using saline and monitored by Biophotometer (Eppendorf, Germany) prior to testing. Antibiotic susceptibility test was conducted according to Kirby-Bauer disk diffusion method using Mueller-Hinton agar (Oxoid, England) (Bauer et al., 1966). Antibiotics tested including oxolinic acid (2 μg), ampicillin (10 μg), erythromycin (15 μg), furazolidone (15 μg), lincomycin (15 μg), oleandomycin (15 μg), amoxicillin (25 μg), colistin sulphate (25 μg), sulphamethoxazole (25 μg), chloramphenicol (30 μg), doxycycline (30 μg), florfenicol (30 μg), flumequine (30 μg), kanamycin (30 μg), nalidixic acid (30 μg), novobiocin (30 μg), oxytetracycline (30 μg), tetracycline (30 μg), nitrofurantoin (50 μg), fosfomycin (50 μg), and spiramycin (100 μg) (Oxoid, England). Interpretation of the resulted inhibition zones of sensitivity (S), intermediary sensitivity (I), and resistance (R) was made in accordance with the standards provided by manufacturers. Finally, antimicrobial susceptibility of the present isolates was determined according to Clinical and Laboratory Standards Institute (CLSI, 2006).

2.3 Multiple antibiotic resistance (MAR) index

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):

$$\text{MAR index(multiple antibiotic resistance)} = X/(Y \times Z),$$

where X = total of antibiotic resistance case; Y = total of antibiotic used in the study; and Z = total of isolates.

A MAR index value of equal or less than 0.2 indicates that those antibiotics are seldom or never used for animals in terms of treatment, whereas the MAR index value higher than 0.2 is considered that animal have received high risk exposure to those antibiotics.

2.4 Heavy metal resistance test

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 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, the concentration of both Cd^{2+} and Cr^{6+} ranged from 25 to 400 μg·mL^{-1}, while that of Hg^{2+} and Cu^{2+} ranged from 2.5 to 40 μg·mL^{-1} and 150 to 2400 μg·mL^{-1},

respectively. For the purpose of defining metal resistance, the isolates were considered to be resistant if growth was obtained at the concentration of 100 $\mu g \cdot mL^{-1}$ Cd^{2+} and Cr^{6+} and 600 $\mu g \cdot mL^{-1}$ Cu^{2+} (Allen et al., 1977). The operational definition of tolerance as used in this study was based on the positive bacterial growth when the concentration of heavy metals was above the stated concentration for resistance.

3 Results

Total colony forming unit (CFU) of five types bacterial species in the fish liver sample ranged from 1×10^6 to 1.6×10^{16} $CFU \cdot g^{-1}$ in which *Escherichia coli* showed the lowest total CFU, and *Streptococcus* spp. was the highest total CFU (Table 1). On the other hand, *Vibrio* spp. showed the lowest total CFU 1.1×10^{16} $CFU \cdot g^{-1}$, and *Pseudomonas* spp. was the highest total CFU (9.1×10^{16} $CFU \cdot g^{-1}$) bacterial species associated in fish kidney samples. A total of 148 bacterial isolates (*Streptococcus* spp. $n = 12$, *Escherichia coli* $n = 30$, *Salmonella* spp. $n = 20$, *Pseudomonas* spp. $n = 36$, and *Vibrio* spp. $n = 50$) were successfully isolated in the present study. More than 80% of the bacterial isolates were found sensitive to 11 out of 21 antibiotics (tetracycline, nitrofurantoin, florfenicol, chloramphenicol, oxytetracycline, nalidixic acid, doxycycline, furazolidone, flumequine, fosfomycin, and oxolinic acid) with all bacterial isolates sensitive to florfenicol (Fig. 1). The multiple antibiotic resistance (MAR) index of the present study indicated that the fish samples were under high risk exposure to the tested antibiotics, in which all the MAR values were more than 0.2 (Table 2). All bacterial isolates were found resistant to all tested heavy metals except for copper and cadmium in which only 6.8% of bacteria were resistant to both heavy metals (Table 3).

4 Discussion

Based on the total plate count result, all bacterial species (*Vibrio* spp., *Salmonella* spp., *Escherichia coli*, *Edwardsiella* spp., *Pseudomonas* spp., and *Streptococcus* spp.) except *Aeromonas* spp. were found dominant bacterial species in the diseased liver and kidney of *T. carolinus* broodstock. Various types of bacterial diseases have been reported to attack marine fish culture, including edwardsiellosis due to *Edwardsiella tarda* in yellowtail (*Seriola quinqueradiata*), Japanese flounder (*Paralichthys olivaceus*) and red sea bream (*Pagrus major*) (Matsuyama et al., 2005); *Vibrio* spp., the causative agent of vibriosis, that was reported to infect salmonids, turbot, seabass, striped bass, eel, ayu, cod, red seabream, Atlantic salmon and cod; furunculosis in salmonids and turbot with *Aeromonas* spp. as causative agent; *Pseudomonas anguilliseptica* responsible for pseudomonadiasis in seabream, eel, turbot, ayu and *Streptococcus* spp., the causative agent of streptococcosis in yellowtail, flounder, seabass, barramundi, turbot, Atlantic salmon (Toranzo et al., 2005). In this case, bacterial infections in *T. carolinus* broodstock were mixed of edwardsiellosis, vibriosis, pseudomonadiasis, and streptococcosis. Although there was no literature reviewed on *Salmonella* spp. and *E. coli* infecting marine fish, the high concentration of these bacteria were observed in the liver and kidney of diseased *T. carolinus* broodstock in the present study. Thus, we can conclude that these bacteria may be secondary pathogens of the diseased fish.

Florfenicol has been found effective for treatment and prophylactic purposes in *T. carolinus* hatchery and in the farm since all present bacterial isolates were sensitive to it. In other literatures, the application of florfenicol has been recommended for bacterial diseases in aquaculture, e.g., vibriosis in cod (*Gadus morhua*) (Samuelsen and Bergh, 2004), enteric septicemia in pond-reared channel catfish fingerlings (Gaunt et al., 2006), furunculosis and vibriosis in Atlantic salmon (Nordmo et al., 1998), and furunculosis in Atlantic salmon (Inglis et al., 1991; Samuelsen et al., 1998). Though florfenicol was closely related to chloramphenicol, it has been proven to be effective in combating chloramphenicol-resistant bacteria species (Yanong et al., 2005). In addition, florfenicol is currently being registered for use in food fish in the United States due to its lack of the functional group associated with human toxicity (Yanong et al., 2005).

The multiple antibiotic resistance (MAR) index of the present study indicated that the fish samples were under

Table 1 Total plate count of bacterial isolated from liver, kidney, and water sample of (*Trachinotus carolinus*) broodstock from commercial hatchery in Malaysia

medium	liver/($CFU \cdot g^{-1}$)	kidney/($CFU \cdot g^{-1}$)
Vibrio spp.	4×10^{15}	1.1×10^{16}
Salmonella spp.	4×10^{15}	5×10^{16}
Escherichia coli	1×10^{6}	8.9×10^{16}
Pseudomonas spp.	7.3×10^{15}	9.1×10^{16}
Streptococcus spp.	1.6×10^{16}	5.1×10^{16}
total plate count on TSA medium	9.8×10^{16}	4.2×10^{16}

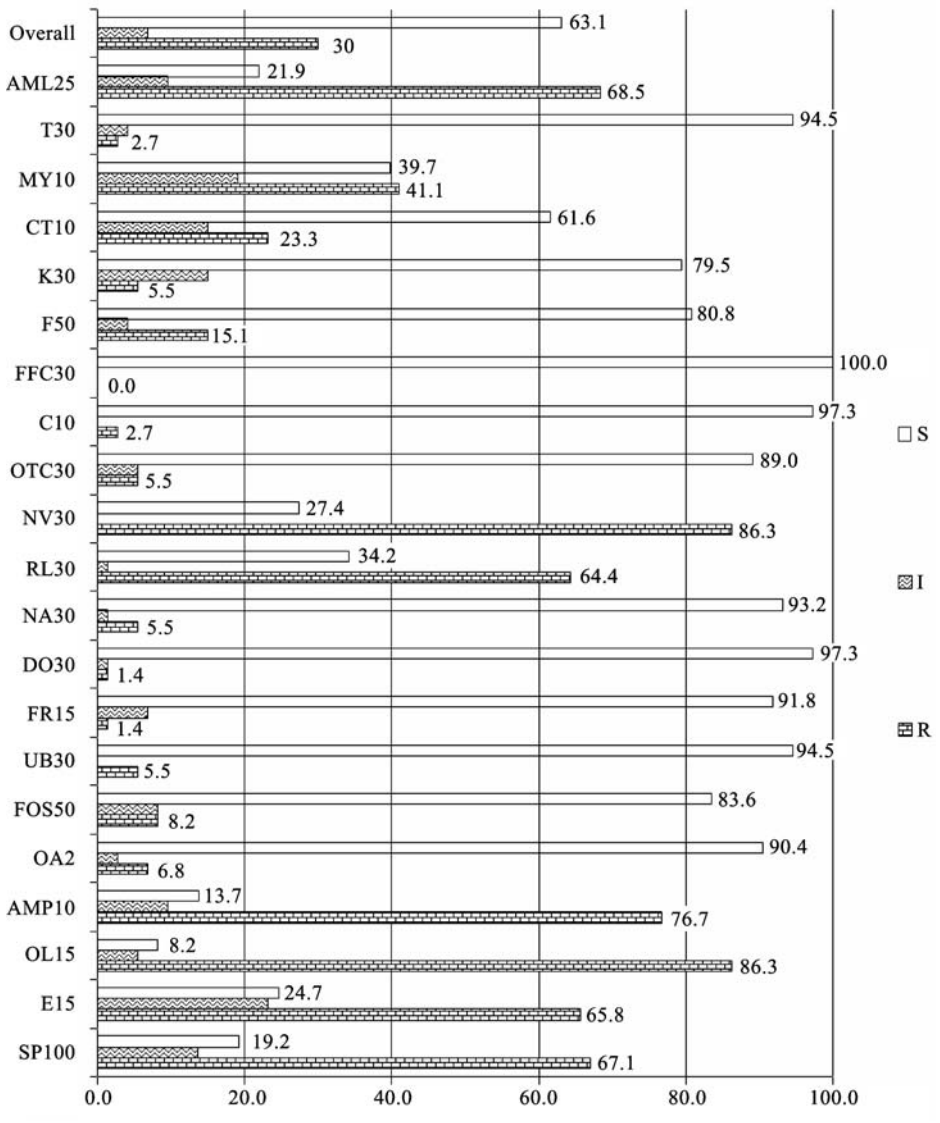

Fig. 1 Total percentage (%) sensitivity of 21 antibiotics against bacterial isolates from golden pompano

Table 2 Multiple antibiotic resistance (MAR) value of bacterial isolates from golden pompano

bacterial isolate	MAR value
total bacterial isolate	0.30
Streptococcus spp.	0.36
Escherichia coli	0.31
Salmonella spp.	0.37
Pseudomonas spp.	0.25
Vibrio spp.	0.28

high risk exposure to the tested antibiotics. These antibiotic residues were most probably introduced through the water source from South China Sea. This statement was supported by the findings of Miranda and Zemelman (2001), wherein they found bacterial isolates from the family of Enterobacteriaceae and Vibrionaceae isolated from demersal and pelagic fish in Concepcion Bay, Chile, which were resistant up to 10 types of antibiotics.

In addition to antibiotic resistance, all present bacterial isolates were found resistant to all tested heavy metals except for copper and cadmium, in which only 6.8% of bacteria were resistant to both heavy metals. There were difficulties in making comparison for the heavy metal pattern among the present bacteria isolates since the information from other aquaculture sites was limited. High heavy metal resistance cases observed among present bacterial isolates could result from fertilizers containing heavy metal residues since the hatchery in this study was surrounded by agricultural areas. In addition, the application of copper sulfate in algae and parasite controls practiced in the hatchery could be associated to the incidence of resistance. Therefore, the usage of chemicals

Table 3 The percentage present bacterial isolates liver and kidney of *Trachinotus carolinus* broodstock from commercial hatchery in Malaysia that growth on different concentration of Hg^{2+}, Cr^{6+}, Cu^{2+}, and Cd^{2+}

heavy metal concentration/(μg·mL^{-1})		*Vibrio* spp.	*Salmonella* spp.	*Streptococcus* spp.	*Escherichia coli*	*Pseudomonas* spp.	*Edwardsiella* spp.	total bacterial
Hg^{2+}	⩽2.5	–	–	–	–	–	–	–
	5	–	–	–	–	–	–	–
	10	–	–	–	–	–	–	–
	20	–	–	–	–	–	–	–
	⩾40	100	100	100	100	100	–	100
Cr^{6+}	⩽25	–	–	–	–	–	–	–
	50	–	–	–	–	–	–	–
	100	–	–	–	–	–	–	–
	200	–	–	–	–	–	–	–
	⩾400	100	100	100	100	100	–	100
Cu^{2+}	⩽150	–	–	66.7	–	–	–	5.5
	300	–	–	–	–	–	–	–
	600	–	–	–	–	17.6	–	6.8
	1200	48.0	20.0	33.3	30.0	17.6	–	34.2
	⩾2400	52.0	80.0		70.0	64.8		53.5
Cd^{2+}	⩽25	–	–	–	–	–	–	–
	50	–	–	–	–	–	–	–
	100	–	–	–	20.0	17.6	–	6.8
	200	4.0	–	–	4.0		–	2.7
	⩾400	96.0	100	100	76.0	82.4	–	90.5

containing heavy metal compounds within the culture site must be handled in proper way to limit the residue effect in aquatic environment.

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