ANNALES
UNIVERSITATIS MARIAE CURIE-SKŁODOWSKA
LUBLIN – POLONIA

VOL. LXIV (2) SECTIO DD 2009

[*]Faculty Agro Industry and Natural Resources, Universiti Malaysia Kelantan,
Karung Berkunci 36, Pengkalan Chepa, 16100, Kota Bahru, Kelantan, Malaysia,
e-mail: leeseongwei@yahoo.com

SEONG WEI LEE[*], MUSA NAJIAH, WEE WENDY

Antibiogram and heavy metal resistance pattern of *Aeromonas* spp. isolated from Asian seabass (*Lates calcarifer*) hatchery

Antybiogram oraz model odporności na metale ciężkie *Aeromonas* spp. izolowanego z wylęgarni *Lates calcarifer*

Summary. The paper was described antibiogram and heavy metal tolerance of *Aeromonas* spp. isolated from Asian seabass (*Lates calcarifer*) hatchery in Malaysia. *Aeromonas* spp. was recognized as causative agent of motile aeromonad septicemia (MAS) which attack in Asian seabass and caused tremendous loss to fish farmer. Therefore this study was carried out to help fish farmer in selecting the suitable antibiotic in controlling MAS due to *Aeromonas* spp. diseased Asian seabass fingerling and water sample from the fingerling tank were collected from a commercial farm. *Aeromonas* spp. was isolated using Glutamate Starch Pseudomonas (GSP). The isolated bacterial isolates were identified using conventional biochemical tests and confirmed with commercial identification kit. 150 bacterial isolates of *Aeromonas* spp. were randomly selected from antibiotic susceptibility and heavy metal tolerance test. All the bacterial isolates were found to be sensitive to nitrofuratoin, furazolidone, tetracycline, doxycycline, flumequine, oxolinic acid and florfenicol. Therefore, fish farmer may apply those antibiotics as prophylactive or treatment to against MAS due to *Aeromonas* spp. in Asian seabass culture.

Key words: antibiotic, heavy metal, *Aeromonas* spp., Asian seabass, *Lates calcarifer*

INTRODUCTION

Asian seabass, *Lates calcarifer* (Bloch) is a native fish species in Indo-Pacific region [Greenwood 1976]. It becomes a popular fish species among Malaysian aquaculturist due to high value and huge demand from local and abroad seafood market. The production of *L. calcarifer* was recorded 20,000 tons per year with the value more than 65 million dollars [Tucker *et al.* 2002]. However, bacterial diseases were recognized as a significant constraint of the development of Asian seabass culture in worldwide. Whereas, *Aeromonas* spp. is a causative agent of motile aeromonad septicemia (MAS) which is

well known bacterial disease in aquaculture that can devastate whole fish farm. For instance, *A. salmonicida* was responsible to the significantly loss nearly USD 18 millions from 2002 to 2004 in salmon farming in Atlantic Canada [McIntosh *et al.* 2008]. Till present, there is lacking of database of *Aeromonas* spp. in Asian seabass especially antibiotic susceptibility information of this species of bacterial. Therefore, this study was carried out to characterize antibiogram of *Aeromonas* spp. To our knowledge, this is first report on antibiogram and heavy metal resistance pattern of *Aeromonas* spp. isolated from Asian seabass in Malaysia. The information of the present study may be useful for fish farmer in selecting suitable antibiotic for prophylactive and treatment purpose.

MATERIALS AND METHODS

Asian seabass, *Lates calcarifer* fingerling with the size 10 to 15 cm and water samples of *L. calcarifer* nursery tank were collected from commercial Asian seabass hatchery located at Setiu, Terengganu, Malaysia. 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 30.4°C, 6.66 mg/l, 8.73 and 30.31 ppt, respectively.

Water samples were collected from *L. calcarifer* fingerling water reservoir tank in four replicates. One millimeter of water sample was serially diluted in sterile physiological saline and plated on two medium: Tryptic Soy Agar (TSA) and Glutamate Starch Pseudomonas (GSP) (Merck, Germany).

10 diseased *L. calcarifer* fingerling 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 GSP medium.

All the inoculated media were incubated at room temperature 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, USA).

The present 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]. 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, florfenicol (30 μg); FFC 30, flumequine (30 μg); UB 30 and fosfomycin (50 μg); FOS 50 (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).

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]:

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

were: 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 considered that animal have received high risk exposure to those antibiotics.

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, 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]. 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

The total plate count of *Aeromonas* spp. from the water sample in the Asian seabass hatchery was 3.8 × 10 colony forming unit (CFU)/ml. In the present study, the percentage of sensitive case was 61.5% whereas intermediary sensitive and resistance case was 11.8% and 26.7%, respectively (Fig. 1). Nitrofuratoin, furazolidone, tetracycline, doxycycline,

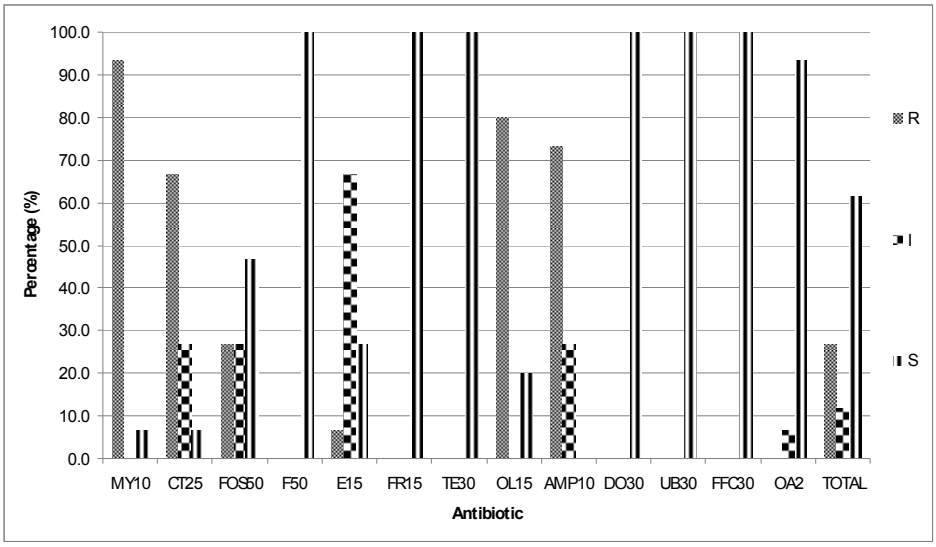

Fig. 1. Antibiotic sensitivity of *Aeromonas hydrophila* isolated from Asian seabass

flumequine, oxolinic acid and florfenicol were found effective in controlling present bacterial isolates in which all the bacterial isolates were sensitive to the antibiotics. On the other hand, more than 60% present bacterial isolates were resistant to lincomycin, colistin sulphate, oleodomycin and ampicillin. Most of the bacterial isolates (66.7%) were intermediary sensitive to erythromycin whereas only 6.7% bacterial isolates was resistant to erythromycin. The MAR value of the present study was 0.27. All bacterial isolates in the present study were found resistant to all tested heavy metal.

DISCUSSION

Aeromonas spp. was recognized as a pathogenic bacterial in worldwide aquaculture. It was successfully isolated from seabass in Thailand [Ruangpan 1988], seabass in Aegean sea [Doukas *et al.* 1998], wild freshwater fish in Croatia [Topic *et al.* 2000] and wild *Piaractus mesopotamicus* and *Oreochromis niloticus* in Brazil [Belem-Costa and Cyrino 2006]. In Malaysia, it was reported infected in various types of ornamental fish [Najiah *et al.* 2008], American bullfrog and giant freshwater prawn [Lee *et al.* 2009]. Therefore, this study was conducted to give us information on the existence of *Aeromonas* spp. in Asian seabass hatchery in Malaysia and alerted to the fish farmer.

In the study of Doukas *et al.* [1998] found that oxytetracycline was effective in controlling *A. hydrophila* in seabass. On the other hand, *A. hydrophila* strains isolated from wild *P. mesopotamicus* and *O. niloticus* in Brazil were found highly resistant to amoxicillin, ampicillin, lincomycin, novobiocin, oxacillin, penicillin and trimetoprim + sulfametoxazole [Belem-Costa and Cyrino 2006]. The authors claimed that the incidence occurred was due to overuse or widely use of antibiotics among Brazil fish farmer for a long period. However, low antibiotic resistance incidence was observed in the present study. The present bacterial isolates were found to be sensitive to nitrofuratoin, furazolidone, tetracycline, doxycycline, flumequine, oxolinic acid and florfenicol. Therefore, Malaysian fish farmer may use the mentioned antibiotics for prophylactive and treatment purpose in seabass culture to against *Aeromonas* spp. The present bacterial isolates were found resistant to all tested heavy metals. This phenomenon occurred may be due to residues of agricultural activities which consisted of various types of heavy metals were contaminated the water source of hatchery for a long period. The continuous exposure to these heavy metal residues may lead to the development of heavy metals resistance strain of the bacteria. At present, there have been very few data base on heavy metal resistance pattern of bacteria from aquaculture sites in the literature. Therefore, comparison between the finding of heavy metal resistance pattern of the present bacterial isolates and other studies can not be make.

REFERENCE

Allen D.A., Austin B., Colwell R.R., 1977. Antibiotic resistance patterns of metal tolerant bacteria isolated from an estuary. Antimicrob. Agents Chemotherap., 545–547.

Bauer A.W., Kirby W.M.M, Sherris J.C., Turck M., 1966. Antibiotic susceptibility testing by a standardized single disk method. Amer. J. Clin. Pathol., 45, 493–496.

Belem-Costa A., Cyrino J.E.P., 2006. Antibiotic resistence of *Aeromonas hydrophila* isolated from *Piaractus mesopotamicus* [Holmberg, [1887]] and *Oreochromis niloticus* (Linnaeus, 1758). Sci. Agric. (Piracicaba, Braz.), 63 (3), 281–284.

McIntosh D., Cunningham M., Ji B., Fekete F.A., Parry E.M., Clark S.E., Zalinger Z.B., Gilg I.C., Danner G.R., Johnson K.A., Beattie M., Ritchie R., 2008. Transferable, multiple antibiotic and mercury resistance in Atlantic Canadian isolates of *Aeromonas salmonicida* subsp. salmonicida is associated with carriage of an IncA/C plasmid similar to *Salmonella enterica* plasmid pSN254. J. Antimicrob. Chemotherap., doi:10.1093/jac/dkn123

Doukas V., Athanassopoulou F., Karagouni E., Dotsika E., 1998. *Aeromonas hydrophila* infection in cultured seabass, *Dicentarchus labrax* L., and *Puntazzo puntazzo* Cuvier from the Aegean sea. J. Fish Dis., 21 (4), 273–280.

Greenwood P.H. 1976. A review of the family centropomidae (*Pisces perciformes*). Bull. Br. Mus. Nat. Hist. (Zoology) 29, 1–81.

Holt J.G., Krieg N.R., Sneath P.H.A., Staley J.T., Williams S.T., 1994. Bergey's manual of determinative bacteriology-9th edition. William & Wilkins, Maryland, USA.

Lee S.W., Najiah M., Wendy W., Nadirah M., Faizah S-H., 2009. Occurrence of heavy metals and antibiotic resistance in bacteria from organs of American bullfrog (*rana catesbeiana*) cultured in Malaysia. J. Venom. Anim. Toxin. Trop. Dis., 15 (2), 353–358.

Lee S.W., Najiah M., Wendy W., Zarol A., Nadirah M., 2009. Multiple antibiotic resistance and heavy metal resistance profile of bacteria isolated from giant freshwater prawn (*Macrobrachium rosenbergii*) hatchery. Agri. Sci. China 8(6), 740–745.

Miranda C.D., Castillo G., 1998. Resistance to antibiotic and heavy metals of motile aeromonads from Chilean freshwater. Sci. Tot. Environ., 224, 167–176.

Najiah M., Lee S.W., Faizah S., Wendy W., 2008. Surveillance of bacteria species in diseased freshwater ornamental fish from aquarium shop. J. World Appl. Sci. 3 (6), 903–905.

Ruangpan L., 1988. Seabass (*Lates calcarifer*) Culture in Thailand. Training Course on Seabass Culture, 1–22 August 1988, Satul, Thailand. FAO Regional Seafarming Project.

Sarter S., Nguyen H.N.K., Hung L.T., Lazard J., Montet D., 2007. Antibiotic resistance in Gram-negative bacteria isolated from farmed catfish. Food Control 18, 1391–1396.

Topic N., Popovic E., Teskered I., Strunjak-Perovic I., Rakovac R., 2000. *Aeromonas hydrophila* isolated from wild freshwater fish in Croatia. Vet. Res. Comm. 24 (6), 371–377.

Tucker J.W., Russell D.J., Rimmer M.A., 2002. Barramundi culture: a success story for aquaculture in Asia and Australia. World Aquac. 33, 53–59

Streszczenie. Niniejsza praca opisuje antybiogram oraz tolerancję na metale ciężkie *Aeromonas* spp. izolowanego z wylęgarni *Lates calcarifer* w Malezji. *Aeromonas* spp. uznano za przyczynę MAS, która atakuje *Lates calcarifer,* powodując ogromne straty ryb. Badanie przeprowadzono, by pomóc hodowcom ryb w wybraniu właściwego antybiotyku przeciw chorobie MAS, spowodowanej przez *Aeromonas* spp. Próbki chorych małych osobników *Lates calcarifer* oraz wody ze zbiornika zebrano z hodowli komercyjnej. *Aeromonas* spp. wyizolowano przy użyciu GSP. Izolaty bakterii zidentyfikowano konwencjonalnymi testami biochemicznymi i potwierdzono za pomocą komercyjnego zestawu identyfikacyjnego. 150 izolatów bakterii *Aeromonas* spp. wybrano losowo na podstawie testu wrażliwości i tolerancji na metale ciężkie. Wszystkie izolaty bakterii okazały się wrażliwe na nitrofurantoinę, furazolidon, tetracyklinę, diksycyklinę, flumekinę, kwas oksolinowy oraz florfenikol. Hodowca ryb może zatem stosować te antybiotyki profilaktycznie lub leczyć nimi chorobę MAS, spowodowaną *Aeromonas* spp. w hodowli *Lates calcarifer*.

Słowa kluczowe: antybiotyk, metal ciężki, *Aeromonas* spp., *Lates calcarifer*