Antibiotic and Heavy Metal Resistance of *Aeromonas* spp. Isolated from diseased Red Hybrid Tilapia (*Oreochromis* sp.)

Lee Seong Wei[1*], Mustakim MT[1], Noor Azlina I[1]., Zulhisyam A.K.[1], An'amt MN[2], Wendy Wee[3], Huang NM[4]

[1]Faculty of Agro Based Industry, Universiti Malaysia Kelantan Jeli Campus, 17600, Jeli, Kelantan, Malaysia

[2]Faculty of Earth Science, Universiti Malaysia Kelantan Jeli Campus, 17600, Jeli, Kelantan, Malaysia

[3]School of Fisheries and Aquaculture Sciences, Universiti Malaysia Terengganu, Kuala Terengganu, 21030, Terengganu, Malaysia.

[4]Department of Physics, Faculty of Science Building, University of Malaya, 50603, Kuala Lumpur, Malaysia.

[*]Email of corresponding author: leeseongwei@yahoo.com

Abstract

A total of 300 bacterial isolates of *Aeromonas* spp. were isolated from diseased Red Hybrid Tilapia, *Oreochromis* sp.. All the bacterial isolates were tested for antibiotic susceptibility against 16 types of antibiotics by using disk diffusion method. The antibiotics tested in this study were nalidixic acid (30 µg/disk), oxolinic acid (2 µg/disk), compound sulphonamides (300 µg/disk), doxycycline (30 µg/disk), tetracycline (30 µg/disk), novobiocin (30 µg/disk), chloramphenicol (30 µg/disk), kanamycin (30 µg/disk), sulphamethoxazole (25 µg/disk), flumequine (30 µg/disk), erythromycin (15 µg/disk), ampicillin (10 µg/disk), spiramycin (100 µg/disk), oxytetracycline (30 µg/disk), amoxycillin (25 µg/disk) and fosfomycin (50 µg/disk). Heavy metal resistance pattern of the present bacterial isolates against mercury (Hg^{2+}) chromium (Cr^{6+}), copper (Cu^{2+}) and Zinc (Zn^{2+}) was also determined by using two fold agar dilution method. The results of the present study showed the percentage of antibiotic sensitivity of the present bacterial isolates ranged from 22.5 % to 100 % in which most of the present bacteria isolates were not sensitive to ampicillin on the other hand all the bacteria isolates were sensitive to nalidixic acid, flumequine and oxytetracycline. Overall, the total of sensitive case was reported as 75.5% whereas antibiotic resistance and intermediate sensitive case was recorded as 19.0% and 5.5 %, respectively. Multiple Antibiotic Resistance (MAR) value was recorded 0.25. The MAR value indicating that the sampled fish were highly exposed to the test antibiotics. Low resistance activity of the present bacterial isolates to the tested heavy metals (Cr^{6+}: 35.8 %, Zn^{2+}: 5.5 %, Cu^{2+}: 25 % and Hg^{2+}: 30 %) was observed revealed that low exposure of the tested heavy metals to the sampled fish.

Key words: *Aeromonas* spp., Red Hybrid Tilapia; *Oreochromis* sp.**;** antibiotic, heavy metal, MAR index

INTRODUCTION

Red Hybrid Tilapia, *Oreochromis* sp., is a popular aquaculture species in worldwide and known as chicken aquatic due to its faster growth characteristic. At present the market price of Red Hybrid Tilapia in Malaysia ranges RM 10 – 15/kg. Due to the high demand of tilapia in the market the production of tilapia is increasing rapidly. However, the expansion tilapia production is constrained by diseases problem. One of the diseases is Motile Aeromonad Septicemia (MAS) due to *Aeromonas hydrophila*.

Based on the literature survey, many reports on the disease outbreak in tilapia farm due to *A. hydrophila*. For instance, Yambot (1998) reported that disease outbreak occurred in tilapia farm in Philippines from 1994 to 1996. Other report on *A. hydrophila* infection in the aquatic animals was Lee et al. (2009a) claimed that this bacterium was found in Malaysian Freshwater Giant Prawn, *Macrobrachium rosenbergii*. *A. hydrophila* was also detected in Silver Catfish, *Pangasius sutchi* (Lee et al, 2010a), America bullfrog, *Rana catesbeina* (Lee et al., 2009c), Asian seabass (*Lates calcarifer*) (Lee et al, 2010b), Asian clam (*Corbicula fluminea*) and many more. Thus, *A. hydrophila* was reported can be found in most of the aquatic animals that may posed a threat to aquatic animal health. Hence, this present study is carried out to reveal *A. hydrophila* colonized in Red Hybrid Tilapia with emphasis their antibiogram and heavy metal resistance pattern.

MATERIALS AND METHODS

Bacterial isolation and identification

100 pieces of diseased Red Hybrid Tilapia, *Oreochromis* sp. with body weight approximately 520 to 725 g were collected from commercial fish farm located at Pasir Puteh, Kelantan, Malaysia. Fluid from external and internal part of fish was collected by using sterile cotton bud and spread plated on Glutamate Starch Pseudomonas (GSP) (Merck, Germany) of agar plate medium. All the inoculated media were incubated at room temperature of 25^{o}C for 24 to 48 h (s) to incubation in inverted position. The bacterial colonies that growth on the selective media will further selected for the identification test. The bacterial isolates were identified using conventional biochemical tests (Lee et al., 2009b; Lee et al., 2010a) and were confirmed with commercial identification kit (BBL, USA) (Lee et al., 2009d; Lee et al., 2010b).

Antibiotic Susceptibility Test

Antibiotic susceptibility test were performed according to Kirby-Bauer disk diffusion method (Lee et al., 2009d; Lee et al., 2010a). Bacterial isolates were randomly selected from the plates were cultured in Tryptic Soy Broth (TSB) for 18 to 24 h (s) at room temperature, 25^{o}C. The bacterial cells were then centrifuged at 14500 rpm for 5 min (s) using minispin (Eppendorf, Germany). The concentration of the bacterial cells were adjusted to 10^{9} colony forming unit (CFU) by using saline and monitored with Biophotometer (Eppendorf, Germany). The bacterial inoculums were swab on Trypticase

Soy Agar (TSA) with zig zag pattern using sterile cotton swabs. Antibiotics were tested including Nalidixic acid (30µg), Oxolinic acid (2µg), Compound sulphonamides (300µg), Doxycycline (30µg), Tetracycline (30µg), Novobiocin (30µg), Chloramphenicol (30µg), Kanamycin (30µg), Sulphamethoxazole (25µg), Flumequine (30µg), Erythromycin (15µg), Ampicillin (10µg), Spiramycin (100µg), Oxytetracycline (30µg), Amoxycillin (25µg) and Fosfomycin (50µg) (Oxoid, England). Sterile forceps were used to place the antibiotic disc from antibiotic disc dispenser onto the agar surface. After incubation, the diameter (in mm) of inhibit zone that produced from each antibiotic disk were measured using ruler. Interpretation of the results namely 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, 2006) and was made in accordance to the standard measurement of inhibitory zones in millimeter (mm).

Multiple Antibiotic Resistance (MAR) Index Determination

Multiple Antibiotic Resistance (MAR) Index of all the present isolates against the 16 tested antibiotics were calculated based on the formula as follows (Lee et al., 2009a; Lee et al., 2010b):

$$\text{Multiple Antibiotic Resistance (MAR) Index} = X / (Y \times Z)$$

Where,

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 were 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 (Lee et al., 2013).

Heavy Metal Resistance Test

The heavy metal resistance test was conducted by agar dilutions method with bacterial tolerance to four elements of heavy metal as follows: mercury (Hg^{2+}) chromium (Cr^{6+}), copper (Cu^{2+}) and Zinc (Zn^{2+}). Bacteria suspension was spread onto the Trypticase Soy Agar (TSA) medium of Kalium Dichromate ($K_2Cr_2O_7$), Zinc Sulphate ($ZnSO_4$), Copper II Sulphate ($CuSO_4$) and Mercury II Chloride ($HgCl_2$) (Merck) for overnight in five different concentrations. The concentration for Zn^{2+} and Cr^{6+} was ranging from 25 to 400 µg mL^{-1} while the concentration of Hg^{2+} and Cu^{2+} was ranging from 2.5 to 40 µg mL^{-1} and 150 to 2400 µg mL^{-1}, respectively. The bacterial were considered resistant if they were able to grow at concentrations of 10 µg mL^{-1} Hg^{2+}, 100 µg mL^{-1} Cr^{6+} and 600 µg $mL^{-1}Cu^{2+}$ (Lee et al., 2009a; Lee et al., 2010b).

RESULTS

In the present study, a total of 300 bacterial isolates of *Aeromonas* spp. were successfully isolated from Red Hybrid Tilapia, *Oreochromis* sp.. The present bacterial isolates showed positive in indole, catalase and motility tests. However, they were failed to utilize urea, inositol, raffinose and sorbitol. All present bacterial isolates possess blood hemolysis activity against horse blood cells. All the present study bacterial isolates were found sensitive to nalidixic acid, flumequine and oxytetracycline. More than 80 % of the bacterial isolates were found sensitive to oxolinic acid, compound sulphonamides, doxycycline, tetracycline, chloramphenicol and kanamycin. This present study was also documented a total of 70 % and 75 % of bacterial isolates were sensitive to erythromycin and fosfomycin, respectively. The percentage of sensitivity of present bacterial isolates to novobiocin, sulphamethoxazole, ampicillin, spiramycin and amoxycillin was ranged from 10.7 to 55.8 % (Table 1). Overall, the total of sensitive case (75.5%) was reported higher than the resistance case (19%) and followed by intermediate sensitive case (5.5%) (Table 1). The Multiple Antibiotic Resistance (MAR) values was 0.28. The MAR value indicated that the sampled fish in the present study was highly exposed to the tested antibiotics. Heavy metal test results showed the total percentage of bacteria resistant to Cr^{6+} was 35.8% whereas the percentage of present study bacterial isolates resistant to Zn^{2+} was 5.5%. The percentage of the present study bacterial isolates resistant to Cu^{2+} and Hg^{2+} was 25 % and 30 %, respectively.

DISCUSSION

The intensification of aquaculture activity in order to generate more production to fulfill the demand from market meet several constraints that restraint the expansion of aquaculture industry in worldwide. One of the major constraint is bacterial disease. Motile Aeromonad Septicemia (MAS) due to *A. hydrophila* was recognized as main problem in freshwater fish farming including tilapia. Many studies have been the existence of this bacterium in various aquatic animals.

In the present study, a total of 300 bacterial isolates of *Aeromonas* spp. was successfully isolated and identified from diseased tilapia from commercial. The biochemical reactions of the present bacterial isolates such as utilization activities of urea, inositol, raffinose and sorbitol were same as claimed in the study of Lee et al. (2009a) where the bacterial isolates of *A. hydrophila* were isolated from Malaysia Giant Prawn, *Macrobrachium rosenbergii*. The present bacterial isolates were also shared similar biochemical reaction in indole, catalase and motility activities with the bacterial isolates of *A. hydrophila* isolated from various types of tropical ornamental fish (Najiah et al., 2008). All the bacterial isolates were found pathogenic due to their blood hemolysis activity.

It is very important to reveal causative agent that responsible to the disease infection of the diseased fish in the present study. However, further study should be carried out to characterize antibiogram of the present bacteria isolates with the purpose to identify the most effective antibiotics for treatment uses. In the present study, nalidixic acid,

flumequine and oxytetracycline were found effective in controlling all the bacterial isolates in the present study. Therefore, we suggest that these antibiotics can be used as treatment in commercial tilapia farm except for oxytetracycline as this antibiotic is banned by Malaysian government to be used in aquaculture activity. As a result, tilapia farmers may use nalidixic acid and flumequine as antimicrobial agent in their farm. However, flumequine may remain in the sediment and the surroundings after application and will effect directly on the external bacteria population within the aquatic ecosystem (Lalumera et al., 2004). Hence, tilapia farmers can use nalidixic acid alternately instead of using flumequine continuously.

More than 50 % of the present bacterial isolates were found resistant to novobiocin, sulphamethoxazole, ampicillin, spiramycin and amoxicillin. The incidence of bacteria resistant to these antibiotics may be due to the increasing input of the antibiotic into tilapia culture system or probably as a consequence of the disposal of untreated sewage, industrial wastes and agricultural activities. These human activities may also contribute to the incidence of heavy metal resistance pattern of the bacterial isolates in the present study. The MAR index indicated that the sampling site is under antibiotic residues contamination in which the farmed tilapia were found highly exposed to the tested antibiotics. Hence, the finding of the present study create awareness about the contamination level in the environment. However, further study should be carried out before we can come to a conclusion. Alternatively, fish farmer may use natural product in fish health management. Several studies in literature claimed that many plant extracts showed positive in controlling bacterial diseases in fish. For example, Lee et al. (2008) claimed that aqueous and methanolic extracts of edible plant were found effective in controlling *A. hydrophila*. Lee et al. (2011a) was also found that *Peperomia pellucida* extract can inhibit the growth of *A. hydrophila*. Other study of Lee and Wendy (2013) claimed that essential oil of *Cymbopogon nardus* showed antimicrobial activity against various types of systemic bacteria isolated from aquatic animals including *A. hydrophila*. Other plant that found can control *A. hydrophila* was *Ficus deltoidea* Jack leaf as described Lee et al. (2011b). The application of natural product in controlling fish diseases is consider popular among fish farmer because is cheap and environmental friendly.

In terms of heavy metal resistance pattern of bacterial isolates in the present study revealed that no more than 40 % of the present bacterial isolates were resistant to the tested heavy metals. Low incidence of heavy metal resistance case clearly showed that the water source of the sampling sites is suitable to be used for aquaculture activity. Based on the literature survey, there are studies claimed that high incidence of heavy metal resistance case was observed among isolated bacterial isolates. For instance, high antibiotic resistance case was found in the study of Lee and Wendy (2012) where the study is about to *Vibrio algino*lyticus, isolated from white leg shrimp, *Litopenaeus vannamei*. Other studies Lee and Wendy (2011) and Lee et al. (2010b) were also found high heavy metal resistance activity among bacteria isolated from wild Asian seabass and Asian seabass fingerling. Heavy metal resistance cases were observed among the present bacterial isolates could be the result of heavy metal contamination with fertilizer which contains heavy metal residues in which can contribute to heavy metal resistance cases

among the bacteria in the present study. In additional, alternatives to antibiotics should be explored for aquaculture industry development to ensure its sustainability and food safety. Besides that, further studies should be carried out in the near future to reveal the relationship between antibiotic and heavy metal resistance pattern and incidence of antibiotics and heavy metal resistance among opportunistic bacteria concerning the possible risks to tilapia and public health.

Acknowledgements

This project was funded by Malaysia Fundamental Research Grant Scheme (FRGS) vot no: R/FRGS/A0.700/00387A/005/2013/00107 and Malaysia Niche Research Grant Scheme (NRGS) vot no: R/NRGS/A07.00/00387A/006/2014/000152

References

Abraham, T.J., Manely, R., Palaniappan, R., Devendaran, K. Pathogenicity and antibiotic sensitivity of luminous *Vibrio harveyi* isolated from diseased penaeid shrimp. J. Aquat. Trop. 1997; 12 (1), 1 – 8.

Allen DA, Austin B, Colwell RR. Antibiotic resistance patterns of metal tolerant bacteria isolated from an estuary. Antimicrob Agents Chemother. 1997; 12:545-547.

CLSI (Clinical and Laboratory Standards Institute) Performance standards for antimicrobial susceptibility testing; sixteenth informational supplement. CLSI document 2006. M100-S16 Vol. 26 No.3. USA: Clinical and Laboratory Standards Institute.

Karunasagar, I., Pai, R., Malathi, G.R., Karunasagar, I. Mass mortality of *Penaeus monodon* larvae due to antibiotic resistant *Vibrio harveyi* infection. Aquaculture. 1994; 128: 203–209.

Lalumera, G. M., D. Calaman, P. Galli, S. Castiglioni, G. Crosa, R. Fanelli. Preliminary investigation on the environmental occurrence and effects of antibiotics used in aquaculture in Italy. Chemosphere. 2004; 54, 661-668

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

Lee SW, Najiah M, Chuah TS, Wendy W, Noor Azhar MS. Antimicrobial properties of tropical plants against 12 pathogenic bacteria isolated from aquatic organisms. African J Biotechnol. 2008; 7 (13): 234 – 238.

Lee SW, Najiah M, Wendy W, Zahrol A, Nadirah M. Multiple antibiotic resistance and heavy metal resistance profile of bacteria isolated from giant freshwater prawn

(*Macrobrachium rosenbergii*) hatchery. Agricultural Sciences in China. 2009a; 8 (6): 740-745.

Lee SW, Najiah M, Wendy W, Nadirah M. Comparative study on antibiogram of *Vibrio* spp. isolated from diseased postlarval and marketable-sized white leg shrimp (*Litopenaeus vannamei*). Front. Agric. China. 2009b; 3 (4): 446-451.

Lee S.W., Najiah M., Wendy W., Nadirah M., Faizah S-H. 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. 2009c. 15 (2), 353–358.

Lee SW, Najiah M, Wendy W. Antibiogram and heavy metal resistance pattern of *Aeromonas* spp. isolated from Asian seabass (*Lates calcarifer*) hatchery. Annales Universitatis Mariae Culrie-Sklodowska Lublin-Polonia. 2009d; 2: 9-13.

Lee SW, Najiah M, Wendy W, Nadirah M. Antibiogram and heavy metal resistance of pathogenic bacteria isolated from moribund cage cultured silver catfish (*Pangasius sutchi*) and red hybrid tilapia (*Tilapia* sp.). Front. Agric China. 2010a; 4 (1): 116-120.

Lee SW, Najiah M, Wendy W. Bacterial flora from a healthy freshwater Asian sea bass (*Lates calcarifer*) fingerling hatchery with emphasis on their antimicrobial and heavy metal resistance pattern. Vet Arhiv. 2010b; 80 (3): 411-420.

Lee SW, Wendy W, Julius Yong FS, Desy FS. Characterization of anticancer, antimicrobial, antioxidant properties and chemical compositions of *Peperomia pellucida* leaf extract. Act. Med. Iranica 2011a; 49 (10): 670-674.

Lee SW, Wendy W, Julius Yong FS, Desy FS. Characterization of anticancer, antimicrobial, antioxidant properties and chemical compositions of *Ficus deltoidea* Jack leaf extract. J Bio Activ Prod Nat. 2011b; 1 (1): 1-6.

Lee SW, Wendy W. Antibiogram and heavy metal resistance pattern of *Salmonella* spp. isolated from wild Asian seabass (*Lates calcarifer*) from Tok Bali, Kelantan, Malaysia. J Bio Sci. 2011; 4 (3): 125-128.

Lee SW, Wendy W. Characterization of *Vibrio alginolyticus* isolated from white leg shrimp (*Litopenaeus vannamei*) with emphasis on its antibiogram and heavy metal resistance pattern. Vet Arhiv. 2012; 82 (2), 221-227.

Lee SW, Wendy W. Chemical composition and antimicrobial activity of *Cymbopogon nardus* citronella essential oil against systemic bacteria of aquatic animals. Iranian J Microbiol. 2013; 5 (2) 147-150.

Sahul Hameed AS, Balasubramanian G. Antibiotic resistance in bacteria isolated from *Artemia nauplii* and efficacy of formaldehyde to control bacterial load. Aquaculture. 2000; 183: 195– 205.

Yambot AV. Isolation of *Aeromonas hydrophila* from *Oreochromis niloticus* during fish disease outbreaks in the Philippines. Asian Fisheries Soc. 1998; 10 (4): 347-354.

Table 1 Percentage of sensitivity of present bacterial isolates against 16 types of antibiotics

Antibiotic (µg/disk)	Resistance (%)	Intermediary sensitive (%)	Sensitive (%)
Nalidixic acid (30)	0.0	0.0	100.0
Oxolinic acid (2)	9.3	5.1	85.6
Compound sulphonamides (300)	10.7	0.0	89.3
Doxycycline (30)	5.1	5.1	89.8
Tetracycline (30)	6.1	0.0	93.9
Novobiocin (30)	44.0	10.5	45.5
Chloramphenicol (30)	17.5	0.0	82.5
Kanamycin (30)	0.0	3.1	96.9
Sulphamethoxazole (25)	58.5	17.9	23.6
Flumequine (30)	0.0	0.0	100.0
Erythromycin (15)	12.2	17.8	70.0
Ampicillin (10)	56.7	20.8	22.5
Spiramycin (100)	24.9	25.1	50.0
Oxytetracycline (30)	0.0	0.0	100.0
Amoxycillin (25)	36.8	19.7	43.5
Fosfomycin (50)	25.1	0.0	74.9
Overall	19	5.5	75.5