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Antibiogram and heavy metal resistance pattern of *Aeromonas hydrophila* isolated from Guppy, *Poecilla reticulata*, ornamental fish from aquarium shop

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Abstract: This study was carried out to investigate the mass mortality in ornamental fish Guppy, *Poecilia reticulata*, infected Motile Aeromonad Septicemia (MAS) due to *Aeromonas hydrophila* from ornamental fish aquarium shop in Tanah Merah, Kelantan, Malaysia with emphasis the bacterial isolates antibiogram and heavy metal resistance pattern. MAS due to *A. hydrophila* is an important disease to both human and aquatic animals. Mass mortality of MAS infection among ornamental fish lead to significant economic lose. Hence, there is a must to find out solution to overcome the arise problem. In the present study, a total of 100 bacterial isolates was successfully isolated and identified from diseased Guppy fish by using Glutamate Starch Pseudomonad (GSP) agar media. The bacterial isolates were then subjected to antimicrobial agent sensitivity test and heavy metal resistance assay by using disc diffusion and two fold dilution method, respectively. The findings of the present study showed Oxolinic acid was found can be effectively control the present bacterial isolates. High heavy metal resistance pattern and MAR index revealed that the sampled fish were highly exposed to the tested antimicrobial agent and heavy metals.

INTRODUCTION

World ornamental fish industry is recorded billions USD value where more than 125 countries involved in ornamental fish trading annually (Karthick et al., 2019). Guppy and zebra danio alone contributed about 14% of the total world ornamental fish industry value (Karthick et al., 2019). United States is the largest ornamental fish importer whereas European is the largest ornamental fish market in the world (Casey, 2016). In Asia, Singapore is remain largest ornamental fish exporter mainly Koi, Guppy and Goldfish where ornamental fish products of Singapore is mainly derivate from Malaysia. In Malaysia there are 259 companies involved in exporting ornamental fish throughout the world and has significant economic contribution (Casey, 2016). Total value of Malaysia ornamental fish industry was recorded about RM 340 million with the main ornamental fish families were Cyprinids and Poecilids (Department of Fisheries Malaysia, 2015). One of the famous ornamental fish under Poecilids family in Malaysia is Guppy fish. Guppy fish is not Malaysia native species and it was imported to this country to control mosquito population in natural and artificial waterbodies. The Guppy was found can control mosquito population by eating mosquito larvae subsequently can reduce transmission of diseases such as dengue

fever and malaria (Misha et al., 2017). Beside Guppy was used as fish to control mosquito population, it was also accepted as ornamental fish in worldwide and through selective breeding program in ornamental fish generating new varieties of ornamental fish including Guppy (Asem et al., 2010). However, Guppy is reported prone to be infected by bacterial diseases and one of the diseases is Motile Aeromonas Septicemia (MAS) due to *Aeromonas hydrophila*.

Motile *Aeromonas Septicemia* (MAS) due to *Aeromonas hydrophila* was reported as a disease that may lead to significant economically lose (Austin and Austin, 2016). This bacterium was also reported is a zoonotic pathogen to human (Li and Saghaian, 2011). Hence, *A. hydrophila* is an important pathogen to human and aquatic animals. *A. hydrophila* was reported infected various of aquatic animals. For example, *A. hydrophila* was successfully isolated from diseased Guppy (Cigdem, 2020), Malaysian Giant Freshwater Prawn (*Macrobrachium rosenbergii*) (Lee et al., 2009), red hybrid tilapia (*Oreochromis* spp.) (Lee and Wendy, 2017), silver catfish (*Pangasius sutchi*) (Lee et al., 2010) and freshwater Asian sea bass (*Lates calcarifer*) (Lee et al., 2010). In the present study, antibiogram and heavy metal resistance pattern of *A. hydrophila* were characterised in order to reveal the effectiveness of tested antibiotics in controlling the bacteria at the mean time to determine the exposure level of tested heavy metals against the isolated bacteria.

MATERIALS AND METHODS

Fish sampling

A total of 50 diseased Guppy, *Poecilla reticulata*, was sampled from ornamental fish shop located in Tanah Merah, Kelantan, Malaysia. The sampled fish appeared skin lesions, swollen abdomen, swollen eye and eroded fin. The sampled fish were brought back to laboratory in Universiti Malaysia Kelantan Jeli Campus for microbiology analysis.

Bacterial isolation and identification

A total of 100 bacterial isolates were successfully isolated from diseased Guppy. Sterile cotton bud was swab on swollen eye, skin lesion area, kidney and liver and streaked directly onto Glutamate Starch Pseudomonad (GSP) agar media (Merck, Germany). The media plates were then incubated for 24 to 48 h at room temperature. Bacterial colonies appeared yellow in color on medium plate with surrounded with yellow color were selected for bacterial identification. Selected bacterial isolates were screened with conventional biochemical test such as Gram staining, oxidase and catalase test. Only Gram-negative bacterial isolates performed positive in both oxidase and catalase tests were further identified by using commercial bacterial identification kit (BBL Crystal, Singapore).

Antimicrobial sensitivity test

Antimicrobial sensitivity test was carried out by using Kirby Bauer disk diffusion method as described in the study of Lee and Wendy (2017). The isolated bacteria were cultured in Tryptic Soy Broth (TSB) (Merck, Germany) with supplemented with 0.85% of NaCl (R&M Chemical, Malaysia) and incubated for 24 to 48 h at room temperature. The bacterial cells was collected by using MiniSpin (Eppendorf, Germany) at 14,500 rpm for 10 min. The bacterial cells suspension was prepared by using physiological saline and the concentration of the bacterial cells was adjusted to 10^9 colony forming unit (CFU) by monitored using Biophotometer (Eppendorf, Germany). The bacterial suspension was then swabbed entirely on the surface area of Tryptic Soy Agar (TSA) (Merck, Germany) by using sterile cotton bud. After 10 mins swabbing bacterial cells on the agar surface, 16 antimicrobial agents were placed on the agar media. The inoculated agar media were then incubated at room temperature for 24 h. The results of inhibition zone were measured by using ruler and the size of the inhibition zone was analysed by referred to CLSI to determine the sensitivity of the antibiotic against the bacterial isolates (CLSI, 2015). The antimicrobial agents (n = 16) were used in the present study were Compound sulphonamides (300 µg/disk), Nalidixic acid (30 µg/disk), Ampicillin (10 µg/disk), Doxycycline (30 µg/disk), Novobiocin (30 µg/disk), Oxytetracycline (30 µg/disk), Chloramphenicol (30 µg/disk), Erythromycin (15 µg/disk), Sulphamethoxazole (25 µg/disk), Flumequine (30 µg/disk), Kanamycin (30 µg/disk), Oxolinic acid (2 µg/disk), Spiramycin (100 µg/disk), Fosfomycin (50 µg/disk), Amoxycillin (25 µg/disk) and Tetracycline (30 µg/disk).

Multiple Antibiotic Resistance (MAR) index determination

Multiple Antibiotic Resistance (MAR) index determination of the present study was calculated as described in the study of Lee et al. (2013) as follow:

Multiple antibiotic resistance (MAR) index = X/(Y × Z)

Where,

X = Total number of antibiotic resistance cases;

Y = Total number of antibiotics applied;

Z = Total number of bacteria isolates.

MAR index equal to or <0.2 indicated that the sampled Guppy fish were seldom or never exposed to the tested antibiotics; on the other hand, the sampled Guppy fish may have a high risk of exposure to the tested antibiotics if the MAR index is more than 0.2 (Lee et al., 2013).

Characterization of bacterial isolates heavy metal resistance pattern

Characterization of bacterial isolates heavy metal resistance pattern was carried out by using two-fold dilution method as described in the study of Lee et al. (2009) and Lee and Wendy (2017). Heavy metals (mercury Hg^{2+}, chromium Cr^{6+}, zinc Zn^{2+}, and copper Cu^{2+}) were supplemented in Tryptic Soy Agar (TSA) (Merck, Germany) at five different concentrations each by using mercury II chloride, potassium dichromate, zinc sulphate, and copper II sulphate (Merck, Germany). 5 concentrations of Cr^{6+} and Zn^{2+}were same from 25 to 400 µg/mL whereas Hg^{2+} and Cu^{2+} were ranging from 2.5 to 40 µg/mL and 150 to 2400 µg/mL, respectively. Bacterial suspension was prepared as mentioned in the antimicrobial sensitivity test. The bacterial suspension was swabbed onto prepared heavy metal media agar surface followed by 24 h incubation period. After incubation period, the growth of the bacterial isolates on TSA supplemented with heavy metals was recorded. Any bacterial isolate was able to grow on TSA supplemented with 10 µg/mL of Hg^{2+}, 100 µg/mL of Zn^{2+} and Cr^{6+}, and 600 µg/mL of Cu^{2+} was categorised as resistant to the tested heavy metals (Allen et al., 1977; Lee et al., 2013).

RESULTS

A total of 100 bacterial isolates, *Aeromonas hydrophila* was successfully isolated and identified from disease Guppy fish sampled from an aquarium shop located in Tanah Merah, Kelantan. Antibiogram of the bacterial isolates showed in Table 1 whereas their heavy metal resistance pattern showed in Table 2. Overall of antimicrobial sensitive case and intermediary sensitive case were 64.3% and 4.6 %, respectively. On the other hand, antimicrobial resistance case was recorded as 31.3 %. All bacterial isolates were found resistant to ampicillin and sulphamethoxazole on the other hand oxolinic acid was able to inhibit the growth of all the present bacterial isolates. High antimicrobial sensitive case (more than 90%) was observed among antimicrobial agents such as spiramycin, fosfomycin, kanamycin, flumequine, doxycycline and nalidixic acid. Moderate antimicrobial resistance case (more than 60%) was recorded for amoxycillin, novobiocin and compound sulphonamides. Besides that, tetracycline, erythromycin, chloramphenicol and oxytetracycline were found can control present bacterial isolates moderately. MAR index calculation recorded as 0.311. Heavy metal resistance test revealed the present bacterial isolates showed highly resistant to Cu^{2+}(90%). This was followed by Cr^{6+} (72 %), Zn^{2+} (36 %) and Hg^{2+}(11 %), respectively.

Table 1. Antibiogram of *Aeromonas hydrophila* isolated from Guppy, *Poecilla reticulata*, ornamental fish from aquarium shop

Antibiotic (µg/disk)	Resistance case (%)	Intermediary sensitive case (%)	Sensitive case (%)
Compound sulphonamides (300)	66	5	29
Nalidixic acid (30)	0	7	93
Ampicillin (10)	100	0	0
Doxycycline (30)	0	2	98
Novobiocin (30)	75	5	20
Oxytetracycline (30)	24	2	74
Chloramphenicol (30)	31	11	58
Erythromycin (15)	12	10	78
Sulphamethoxazole (25)	100	0	0
Flumequine (30)	0	2	98
Kanamycin (30)	3	2	95
Oxolinic acid (2)	0	0	100
Spiramycin (100)	0	3	97
Fosfomycin (50)	0	4	96
Amoxycillin (25)	73	16	11
Tetracycline (30)	14	4	82
Overall	31.1 %	4.6 %	64.3 %
Total Incidence cases	498	73	1029

Table 2. Susceptibility of bacterial isolates to four types of heavy metal

Heavy metals	Sensitive (%) (Total cases)	Resistance (%) (Total cases)
Hg^{2+}	89 (89)	11 (11)
Zn^{2+}	64 (64)	36 (36)
Cr^{6+}	28 (28)	72 (72)
Cu^{2+}	10 (10)	90 (90)

DISCUSSION

The findings of the present study revealed that the mass mortality of sampled Guppy fish from aquarium shop was infected by MAS due to *A. hydrophila*. *A. hydrophila* was responsible to many cases of ornamental fish in world wide. For example, Raja et al. (2018) claimed that *A. hydrophila* was identified as causative agent of mass mortality of gold fish, *Carassuis auratus*, from four ornamental fish farms in Kerala, India. Park et al. (2009) was reported that *A. hydrophila* was responsible to mass mortality of adult koi carp, *Cyprinus carpio* in the early winter in Samchunpo city, Korea. Other than ornamental fish, *A. hydrophila* was also reported responsible to mass mortality to other freshwater fish. For instance, Abadi et al. (2020) was reported that *A. hydrophila* infected in Nile tilapia (*Oreochromis niloticus*) and led to mass mortality in Sorong District, West Papua, Indonesia. Hence we may conclude that *A. hydrophila* is a bacterium can lead to mass mortality of fish and there is a must to find suitable antimicrobial agent to overcome MAS due to *A. hydrophila*.

Based on the result in the present study showed oxolinic acid is the most effective antimicrobial agent in controlling *A. hydrophila*. Many studies revealed the potential of oxolinic acid in fish disease treatment. For instance, Katharios et al. (2015) mentioned oxolinic acid was found effective in the treatment of edwardsiellosis infection in sharpsnout sea bream, *Diplodus puntazzo,* in Mediterranean. Beside oxolinic acid, flumequine is also can be used as antimicrobial agent to against MAS due to *A. hydrophila* in the present study. Both antimicrobial agents are under quinolone antimicrobial agent group. These quinolone antimicrobial agents were reported no lead to antimicrobial agent resistant case among bacteria in the environment of aquaculture system although high dose of the antimicrobial agent was used (Giraud et al., 2006). Furthermore, oxolinic acid and flumequine were found can be degraded faster

when exposed to sun light (Lai and Lin, 2009). Therefore, we may suggest that these antimicrobial agents can be used in the treatment of MAS due to *A. hydrophila* infected in Guppy fish. However, in the recent year, quinolone antimicrobial agents (oxolinic acid dan flumequine) were not allowed to use in Chilean salmon industry (Claudio et al., 2018). Therefore, Chilean salmon industry player mainly using florfenicol and oxytetracycline in managing salmon health. In spite Chilean government restrict salmon farmer from using quinolone antimicrobial agent but this group of antimicrobial agent was reported widely used in worldwide (Done et al., 2015; Quesada et al., 2013) and in Mediterranean aquaculture (Rigos and Troisi, 2005).

Beside of using oxolinic acid and flumequine, fish farmer can consider antimicrobial agents such as nalidixic acid, doxycycline, kanamycin, spiramycin, fosfomycin and tetracycline in the treatment of MAS due to *A. hydrophila* in Guppy. However, nalidixic acid and tetracycline were banned to applied in Malaysia aquaculture (Thiang et al., 2021). Application of doxycycline was also reported used in disease treatment of striped catfish, *Pangasianodon hypohthalmus* in Vietnam (Phu et al., 2015). Kanamycin and spiramycin was seldom or none reported use in aquaculture however fosfomycin was found effectively used in controlling streptococcosis in Nile tilapia (*Oreochromis niloticus*) (Hussien and Hassan, 2011). Instead of using commercial antimicrobial agent in controlling MAS due to *A. hydrophila*, farmer may have other options like using plant-based treatment. Many studies was conducted showed plant based treatment was promising against MAS. For instance, Riauwaty et al. (2020) revealed that turmeric can be used in the treatment of MAS infection due to *A. hydrophila* in *Pangasius hypothalmus*. High MAR index was observed in the present study indicating the sampled Guppy fish were highly exposed to the tested antimicrobial agent. Therefore scientist may explored new plant based antimicrobial agent to alternate the current commercial antimicrobial agent for environment betterment.

High resistant to chromium and copper was observed among the present bacterial isolates. These trace elements are important in animal immune system but will tend to persist in environment hence contribute to heavy metal resistance case among bacteria (Resende et al., 2012). Furthermore, bacterial may adapt with the presence of heavy metals because it have genetic to resistant the heavy metals (Yu et al., 2017). Although the finding in the present study showed low resistance case of zinc and mercury case but if bacteria were continuously expose to these heavy metals slowly the bacteria will resistant to the heavy metals. Low resistance case was found among the present bacterial isolates against mercury. This finding was agreed with the study of Hassen et al. (1998) where claimed that mercury is the most toxic against bacteria if compared to chromium, copper and zinc. The main source of heavy metals contamination into environment is from agricultural activities where fertilizers contain heavy metal elements were widely used allowed these heavy metals seeping into aquaculture system through soil and water. However, further study need to be carry out before we can come to a conclusion.

CONCLUSION

Based on the present study findings showed that oxolinic acid can be used in controlling MAS due to *A. hydrophila* in Guppy fish. However, further study can be used before it can be used. High MAR index and heavy metal resistance pattern copper and chromium indicating the sampled Guppy fish were highly exposed to the tested antimicrobial agent and heavy metals.

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