Front. Agric. China 2010, 4(1): 116–120
DOI 10.1007/s11703-009-0085-z

RESEARCH ARTICLE

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# Antibiogram and heavy metal resistance of pathogenic bacteria isolated from moribund cage cultured silver catfish (*Pangasius sutchi*) and red hybrid tilapia (*Tilapia* sp.)



**Abstract** Antibiogram and heavy metal resistance patterns of pathogenic bacteria isolated from moribund cage cultured silver catfish (*Pangasius sutchi*) and red hybrid tilapia (*Tilapia* sp.) from Sungai Manir, Terengganu, Malaysia were studied and characterized. Sungai Manir is one of the famous rivers in Terengganu for its wide variety of cage cultured freshwater fish. However, to date, the baseline information of antibiogram and heavy metal resistance patterns of the pathogenic bacteria attacking the freshwater fish cultured in Sungai Manir is still lacking. Therefore, this study was carried out, which may be useful for fish farmers as a guideline for fish prophylactic and treatment purposes. Furthermore, present studies also provide information on the safety level of consuming freshwater fish produced from Sungai Manir. In the present study, bacteria were isolated from 100 fish of each moribund silver catfish and red hybrid tilapia using seven 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) and Baird Parker media. Identification of bacteria was carried out using conventional biochemical tests and confirmed by commercial bacterial identification kit. Antibiogram of the bacterial isolates against 18 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), tetracycline (30 μg), nitrofurantoin (50 μg) and spiramycin (100 μg) was carried out using disk diffusion method, whereas heavy metal resistance patterns ($Hg^{2+}$, $Cd^{2+}$, $Cr^{6+}$ and $Cu^{2+}$) of the bacterial isolates was determined through two-fold agar dilution method. The results showed that the percentage of sensitivity case of the 120 bacterial isolates to the tested antibiotics was 62.7%. This was followed by resistance (26.9%) and intermediary sensitive (10.4%) cases. In terms of the heavy metal resistance patterns, all bacterial isolates were resistant to $Hg^{2+}$ and $Cr^{6+}$. However, only 27.8% and 16.7% of the bacterial isolates were sensitive to $Cu^{2+}$ and $Cd^{2+}$, respectively. The multiple antibiotic resistance (MAR) indices indicated that the cage cultured silver catfish and red hybrid tilapia were under high exposure to the tested antibiotic. Overall, the results of the present studies showed that Sungai Manir may be polluted with heavy metal and antibiotic residues.

**Keywords** antibiogram, heavy metal resistance of bacteria, silver catfish (*Pangasius sutchi*), red hybrid tilapia (*Tilapia* sp.)

## 1 Introduction

The intensive and wide use of antibiotics for controlling pathogens in humans, animals and plants have contributed to the increase of antibiotic resistant bacteria populations in the environment (Hirsch et al., 1999; Mazel and Davis, 1999; Gordon et al., 2007). Furthermore, other wastes such as waste effluents from hospitals, pharmaceutical plants (Guardabassi et al., 1998; Goni-Urriza et al., 2000), agricultural sewage (Halling-Sorensen et al., 1998) and wastewater treatment plants (Tennstedt et al., 2003) are discharged into the environment, which is also a main factor leading to the increasing cases of antibiotic and heavy metal resistance among bacteria population in the environment. Up to the present, few scientific reports on

Received May 11, 2009; accepted June 22, 2009

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antibiotic and heavy metal resistance in the freshwater fish culturing environment have been published (Guardabassi et al., 2000; Schmidt et al., 2000). For instance, though freshwater fish aquaculture is widely practiced in Sungai Manir, Terengganu, Malaysia, there have been no studies carried out to evaluate fish pathogenic bacteria associated in these cultures as well as their antibiogram and heavy metal resistance patterns. Therefore, this study was carried out to determine the causative agent of the bacterial diseases infected in float-cage cultured silver catfish (*Pangasius sutchi*) and red hybrid tilapia (*Tilapia* sp.) as well as their antibiogram and heavy metal resistance patterns. The results of the present study can provide valuable information to fish farmers along Sungai Manir in terms of cultured fish health management as well as the level of antibiotic and heavy metal residues contamination in Sungai Manir.

## 2 Materials and methods

### 2.1 Fish and sampling sites

One hundred of moribund silver catfish (*Pangasius sutchi*) and red hybrid tilapia (*Tilapia* sp.) cultured in two separate cages were collected from Sungai Manir, Terengganu, Malaysia. The three sampling locations were N5°12.253′ E103°2.213′, N5°12.255′ E103°2.217′ and N5°12.261′ E103°2.223′ (Table 1). The water parameters of the sampling sites were measured using a pH meter (YSI, USA). The temperature, dissolved oxygen, pH and salinity of the sampling sites ranged from 29.65 to 30.72°C, 6.37 to 7.38 $mg \cdot L^{-1}$, 5.43 to 5.95 and 0.00 to $0.01 \times 10^{-12}$, respectively (Table 1).

### 2.2 Isolation and identification of bacterial isolates

Each species of fish was divided into 4 groups with 25 fish in each group. All internal organs (liver, kidney and intestine) of fish were homogenized in 100 mL of sterile physiological saline using sterile glass rods. One millimeter of sample was serially diluted in sterile physiological saline and plated on seven media: 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 (BP) (Merck, Germany).

All the inoculated media were incubated at room temperature for 24–48 h. The bacterial colonies that grew on the selective media were further selected for identification test. The obtained bacterial isolates were identified using conventional biochemical tests (Holt et al., 1994) and confirmed with commercial identification kit (BBL, USA).

### 2.3 Antibiotic susceptibility test and MAR (multiple antibiotic resistance) index

The present isolates were cultured in tryptic soy broth (TSB) (Oxoid, England) for 24 h at room temperature. The bacterial cells were then centrifuged at 14500 $r \cdot min^{-1}$ for 5 min by using minispin (Eppendorf, Germany). The concentration of bacterial cells were adjusted into $\times 10^6$ colony forming unit (CFU) by using saline and monitored with a 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). The tested antibiotics included oxolinic acid (2 μg), ampicillin (10 μg), erythromycin (15 μg), furazolidone (15 μg), lincomycin (15 μg), oleandomycin (15 μg), amoxicillin (25 μg), colistin sulphate (10 μg), sulphamethoxazole (25 μg), chloramphenicol (10 μg), doxycycline (30 μg), florfenicol (30 μg), flumequine (30 μg), kanamycin (30 μg), nalidixic acid (30 μg), tetracycline (30 μg), nitrofurantoin (50 μg) and spiramycin (100 μg) (Oxoid, England). The interpretation of results of sensitive (S), intermediary sensitive (I) and resistance (R) was made in accordance to the standard measurement of inhibitory zones in millimeter (mm).

MAR (multiple antibiotic resistance) index of the present isolates against the tested antibiotics was calculated based on the formula as follows (Sarter et al., 2007):

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

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 was defined as antibiotics that were seldom or never used for the animal in terms of the treatments whereas a MAR index value higher than 0.2 was considered as animals having high risk exposure to those antibiotics.

### 2.4 Heavy metal tolerance test

Heavy metal resistance test was carried out as described by Miranda and Castillo (1998). Bacterial tolerance to four

**Table 1** Water parameters and sampling location

| location | pH | temperature/°C | dissolved oxygen/($mg \cdot L^{-1}$) | salinity/$\times 10^{-12}$ |
|---|---|---|---|---|
| N5°12.253′ E103°2.213′ | 5.47 | 29.65 | 7.38 | 0.00 |
| N5°12.255′ E103°2.217′ | 5.95 | 29.72 | 7.05 | 0.01 |
| N5°12.261′ E103°2.223′ | 5.43 | 30.69 | 6.37 | 0.01 |

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 $HgCl_2$, $CdCl_2$, $K_2Cr_2O_7$ and $CuSO_4$ (Fluka, Spain). In terms of two-fold dilutions, the concentration of both $Cd^{2+}$ and $Cr^{6+}$ ranged from 100 to 400 $\mu g \cdot mL^{-1}$, while the concentration of $Hg^{2+}$ and $Cu^{2+}$ ranged from 25 to 400 $\mu g \cdot mL^{-1}$ and 2400 to 600 $\mu g \cdot mL^{-1}$, respectively. For the purpose of defining metal resistance, the isolates were considered to be resistant when growing at a 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 in our study was based on the positive bacterial growth when the concentration of heavy metals was above the stated concentration for resistance.

## 3 Results

In the present study, a total of 60 bacterial isolates (*Aeromonas* spp. $n$ = 12, *Escherichia coli* $n$ = 10, *Edwardsiella* spp. $n$ = 12, *Salmonella* spp. $n$ = 11 and *Vibrio* spp. $n$ = 15) were successfully isolated from moribund cage cultured silver catfish. Whereas, 55 bacterial isolates (*Aeromonas* spp. $n$ = 9, *Escherichia coli* $n$ = 14, *Edwardsiella* spp. $n$ = 12, *Salmonella* spp. $n$ = 13 and *Vibrio* spp. $n$ = 7) were isolated from red hybrid tilapia. *Vibrio* spp. isolates exhibited the lowest percentage of antibiotic resistance (13.3%), followed by *E. coli* (22.0%), *Edwardsiella* spp. (24.0%) and *Aeromonas* spp. (29.0%). On the other hand, *Salmonella* spp. showed evidence of highest percentage of antibiotic resistance (54.0%). From the overall number of isolates tested by antibiotic sensitivity testing 62.7% was reported as sensitive, whereas 26.9% and 10.4% were reported as resistance and intermediary sensitivity, respectively (Fig. 1). More than 70% of the bacteria were sensitive to erythromycin, oxolinic acid, furazolizone, chloramphenicol, doxycycline, flumequine, kanamycin, nalidixic acid and tetracycline (Fig. 2). Conversely, the highest resistance rate of 72.2% was recorded against colistin sulphate. The multiple antibiotic resistance (MAR) values of the present study indicated that the sampled fish have high risk exposure to the tested antibiotics in which the MAR value recorded was 0.27, higher than the normal value of 0.2. In the present study, all the bacteria isolates were found resistant to mercury and chromium whereas 38.9% and 26.7% of the bacteria isolates were sensitive to cooper and cadmium, respectively.

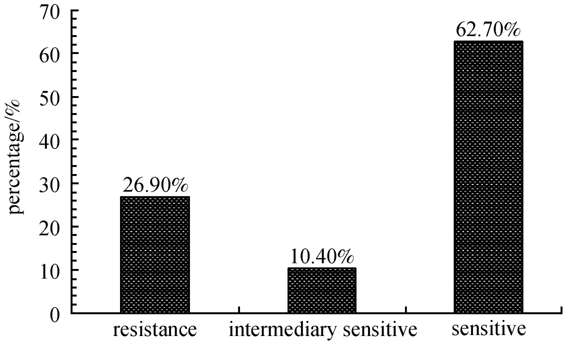


**Fig. 1** Total percentage of sensitivity of 18 antibiotics against bacteria isolated from moribund cage cultured silver catfish and red hybrid tilapia

## 4 Discussion

Both red hybrid tilapia and silver catfish in the present study were suspected to be infected with bacterial diseases due to *Aeromonas* spp. and *Edwardsiella* spp. in which *Escherichia coli*, *Salmonella* spp. and *Vibrio* spp. developed secondary infection. The clinical signs of the moribund fish indicated that the fish were primarily infected by *Aeromonas* spp. and *Edwardsiella* spp. Moribund fish demonstrated dark coloration, swollen, erratic swimming, lethargy and appetite loss. Internal examination of the fish showed that the gill was pale, liver was swollen and cherry red, while the spleen was grayish. According to the investigation among the local fish farmers, disease outbreak of the cultured fish usually occurs during the hot and dry season when the water temperature of Sungai Manir rises up to 30°C, which is optimum for most of the isolated bacterial species (mesophilic bacteria) to grow (Rheinheimer, 1985). Furthermore, many reports claim that bacteria population increases with the increase of water temperature (Chowdhury et al., 1989; Fernandes et al., 1997; Hossain et al., 1999; Al-Harbi and Uddin, 2004). Therefore, we suggested that aquaculture activity should be discouraged in Sungai Manir during hot and dry seasons (March to June) to avoid economic losses due to bacterial disease outbreak in fish cultures.

Based on the antibiotic test result, we suggested that fish farmers can apply flumequine for prophylactic and treatment purposes since 94.4% of the present bacterial isolates in this study were sensitive to this antibiotic. Flumequine is commonly used for red mouth, vibriosis and furunculosis in trout as well as pasteurella in sea-bass among Italian fish farmers (Lalumera et al., 2004). However, this antibiotic residue may persistently remain in sediment and the surroundings after application. Thus, other antibiotics such as erythromycin, kanamycin, doxycycline and nalidixic acid can be used alternately to avoid constant use of flumequine. Seventy percent of the bacterial isolates were found sensitive to oxolinic acid, furazolizone, chloramphenicol and tetracycline but application of these types of antibiotics in aquacultures have been banned.

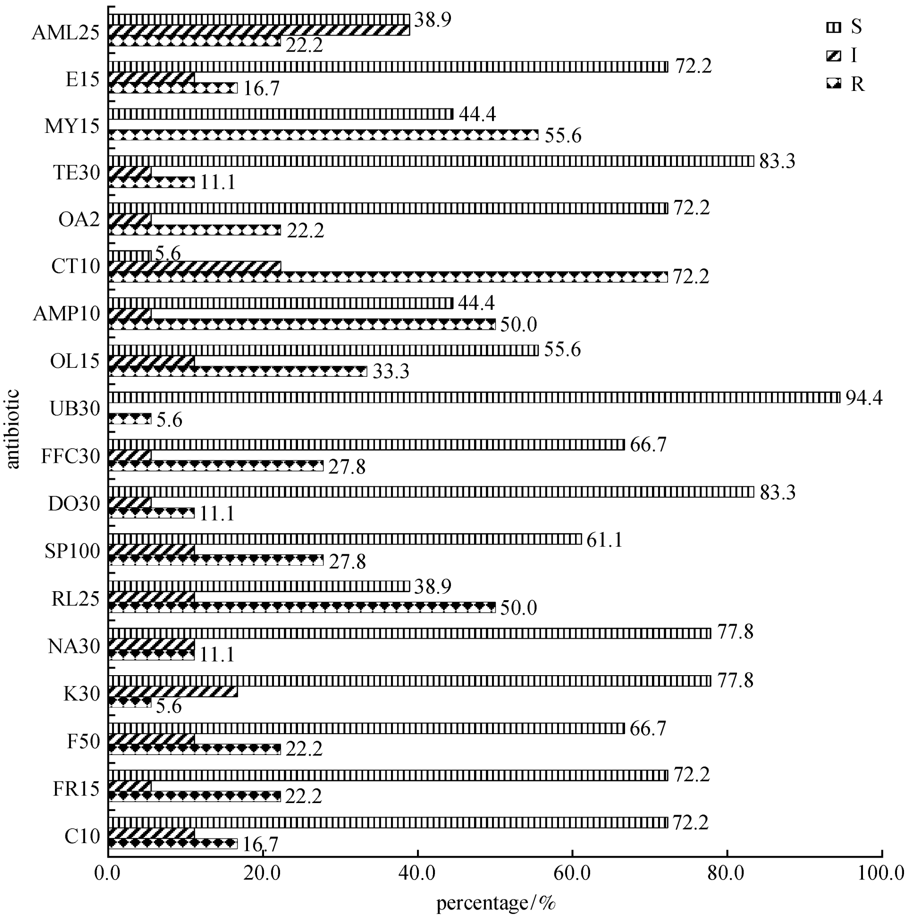


**Fig. 2** Percentage of sensitivity of 18 antibiotics against bacteria isolated from moribund cage cultured silver catfish and red hybrid tilapia

Overall, the multiple antibiotic resistance (MAR) values of the present study indicated that the sampled fish received high risk exposure to the tested antibiotics. Therefore, we may conclude that Sungai Manir is contaminated with antibiotic residues, specifically with sulphate-based antibiotics. This may be due to overuse and misuse of antibiotics among fish farmers. Furthermore, the results of bacterial tolerance to heavy metals indicate that Sungai Manir contains high residues of heavy metals due to the drainage of industrial wastes and domestic sewage (Wright and Welbourn, 1994) and agricultural activities (Akinbowale et al., 2007).

**Acknowledgements** This project was funded by Universiti Malaysia Terengganu.

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