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ANTIBIOGRAM AND PLASMID PROFILING FROM *Edwardsiella tarda* ISOLATED FROM FRESHWATER FISH IN EAST COAST MALAYSIA

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Abstract: This study was carried out to investigate the antibiogram, plasmid profiling and Multiple Antibiotic Resistance (MAR) index of *Edwardsiella tarda* isolated from freshwater-fish cultures. To date, the information on antibiogram of local *E. tarda* isolates is still lacking. Therefore, this study was conducted to reveal the most suitable choice of antibiotic for aquaculture use among six types of commonly-used antibiotics (ampicillin, kanamycin, tetracycline, nalidixic acid, furazolidone and sulphamethoxazole). In the present study, antibiotic susceptibility test against local *E. tarda* was tested using disk diffusion and two-fold microdilution method was applied to determine its sensitivity and Minimum Inhibitory Concentration (MIC) values, respectively. The results showed that antibiotic sensitivity and resistance cases were reported as 63.0 % and 28.7 %, respectively. Intermediary sensitivity case was recorded as 8.3 %. The MIC value of the 6 antibiotics against the present isolates ranged from 1 mg/L to equal or more than 128 mg/l. 12 out of 18 isolates were found to carry plasmid where the sizes of plasmids were in the range of 54kb to 300 bp. All the isolates from cultured freshwater fish were found to carry plasmid except for Isolate T2. Only 4 (E1, G1, G2 and G3) out of 9 isolates from wild freshwater fish were found to carry plasmid, whereas Isolate E2, E3, E3, E4, G4 and G5 did not possess any plasmid. The total number of plasmid carried by the present isolates ranged from 1 to 8 plasmids. No correlation was found between the incidence of antibiotic resistance and plasmid carried by the present isolates. MAR index revealed that cultured freshwater fish in Terengganu received high-risk exposure to the tested antibiotics. On the other hand, wild freshwater fish were under the level of exposure to the antibiotics. Overall, ampicillin, kanamycin, tetracycline, nalidixic acid and furazolidone were successfully found to inhibit more than 50 % of the present bacterial isolates. On the other hand, more than 80 % of bacterial isolates were resistant to sulphamethoxazole. In terms of MIC values, ampicillin and nalidixic acid showed the lowest MIC value (1 mg/l) to control the growth of *E. tarda*. Therefore, we suggested that ampicillin and nalidixic acid can be used for combating Edwardsiellosis due to *E. tarda* in freshwater-fish cultures in Malaysia.

KEYWORDS: *Edwardsiella tarda*, antibiogram, plasmid profiling, MAR index

Introduction

Edwardsiella tarda is reported as a well-known pathogen for many species of animals including humans (Zheng *et al.,* 2004). It infects both freshwater and marine fish. Consequently, these bacteria pose a threat to fish farming. Recently, it was reported in turbot (Padros *et al.,* 2006), Japanese flounder (Zheng *et al.,* 2006), Nile tilapia (Kim *et al.,* 2003), *Clarias batrachus* and *Anabas testidineus* (Sahoo *et al.,* 2000). At present, the antibiogram for both environmental and clinical isolates of *Edwardsiella tarda*, one of the main bacterial diseases in freshwater fish in Malaysia, is lacking. This has led many local

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fish farmers to use inappropriate treatments against Edwardsiellosis outbreak. As a result, fish farmers suffered economic loss due to this fish disease and cost of treatment. Meyer and Bullock (1973) reported that heavy financial losses of processing plants due to the presence of fish infected with Edwardsiellosis. These fish emit noxious odours causing immediate disinfection and deodorisation process of plant. However, catastrophic losses of catfish culture due to this disease have not been recorded. Due to that, Edwardsiellosis has been known as a disease of primary importance and it posed a big problem in eel culture. Economic losses of more than USD 70 million annually were estimated due to *E. tarda* infections in various types of cultured fish. Therefore, the baseline information of antibiogram towards *E. tarda* is needed. This is useful for our fish farmers in selecting the most suitable antibiotic during disease outbreak. In the present study, antibiogram of the 18 isolates of *Edwardsiella tarda* against 6 types of antibiotics: ampicillin, kanamycin, tetracycline, furazolidone, sulphamethoxazole and nalidixic acid, as well as the minimum inhibitory concentration (MIC) values of the antibiotic against the isolates, were determined. The plasmid profiling of the present isolates was also carried out to reveal the relationship between the incidence of antibiotic resistance of the tested antibiotics and the existence of plasmid in the isolates. MAR index (Multiple Antibiotic Resistance) was also determined to reveal the level of antibiotic exposure in cultured freshwater fish in Malaysia.

Materials and Methods

Bacterial Isolates

A total of 18 strains of *Edwardsiella tarda* isolated from diseased African catfish (*Clarias gariepinus*) (C1, C2, C3, C4, C5, C6 and C7), diseased Red Hybrid Tilapia (*Oreochromis* sp.) (T1 and T2), diseased Asian Swamp Eel (*Monopterus albus*) (E1, E2, E3, E4 and E5) and Snakeskin Gourami (*Trichogaster pectoralis*) (G1, G2, G3 and G4) at commercial farms in Terengganu, Malaysia were used in the experiment. Pheno-typic, genotypic and whole-cell protein profiles of these bacterial strains were previously described by Lee and Najiah (2008).

Antibiotic susceptibility test

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 14,500 rpm for 5 min by using minispin (Eppendorf, Germany). The concentration of the bacterial cells were adjusted into 10^9 Colony Forming Unit (CFU) by using physiological saline and monitored with a Biophotometer (Eppendorf, Germany) before being swabbed on the prepared Mueller Hinton agar (Oxoid, England). After 10 min, the tested antimicrobial disks were placed on the agar with a forcep. The plates were then placed invertly and incubated for 24 h at room temperature. Six antimicrobial agents were applied in the present study. They were ampicillin (10 µg/disk), kanamycin (30 µg/disk), tetracycline (30 µg/disk), nalidixic acid (30 µg/disk), furazolidone (15 µg/disk) and sulphamethoxazole (25 µg/disk) (Oxoid, England). Finally, antimicrobial susceptibility of the present isolates was determined according to National Committee for Clinical Laboratory Standards (NCCLS).

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

MAR index = X / (Y x Z)
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 that were seldom or never used for the animal in term of treatment, whereas a MAR index value higher than 0.2 it is considered that the animal has received high-risk exposure to those antibiotics.

Minimum Inhibitory Concentration (MIC) values determination

The values of Minimum Inhibitory Concentration (MIC) of the tested antibiotics against

Edwardsiella tarda isolates were determined through two-fold broth micro-dilution method. The concentrations of all the tested antibiotics ranged from 0.06 mg/l to ≤ 128 mg/l. The present isolates were cultured in TSB for 24 h at room temperature and the concentration of the cultures were adjusted into 10^6 CFU/ml by using saline and monitored with a Biophotometer (Eppendorf, Germany). The bacterial suspensions were then inoculated into a microtiter plate that contained a serial dilution of the tested antibiotics. The microplate was incubated at room temperature for 24 h. The MIC values of the tested antibiotic against the present isolates are defined as the lowest concentration of the tested antibiotics in the wells of the microtiter plate that shows no visible turbidity after 24 h incubation.

Plasmid Profiling

In the present study, plasmid profiling of the present isolates and *Escherichia coli* V517 (as a marker) was conducted using a commercial plasmid extraction kit (Genei, India). The commercial kit containing solution G1, G2, G3, RNase (Lyophilised), wash buffer I, wash buffer II, elution buffer, spin miniprep column and collection tube. All the isolates in the present study were cultured overnight in TSB (Oxoid, England) at room temperature before centrifuging at 10,000 rpm for 5 min. The pelleted bacterial cells were then suspended with 250 µl of solution G1 with RNase. 250 µl of solution G2 was added into the suspension, followed by gentle mixing. Solution G3 was added into the suspension by mixing invertly and centrifuged at 13,000 rpm for 10 min. Only supernatant was collected and transferred into spin miniprep column that placed on the collection tube. The sample was then centrifuged at 14,500 rpm for 1 min and the eluate was discarded. The sample was then washed twice using wash buffer 1 and 2. Finally, the sample was centrifuged for 3 min at 14,500 rpm and the spin miniprep column was transferred into a new 1.5 ml centrifuge tube and centrifuged at 14,500 rpm for 1 min after adding 50 µl of elution buffer. Separation of plasmid product was electrophoresed on 1% agarose gel (Mupid Ex, Japan). Electrophoresis was run at 110 V for 90 min. The gel was then stained with ethidium bromide at 5 µl/ml concentration. After that, plasmid profiles of the samples were visualised by using UV transilluminator (Bio Rad, USA).

Results

Table 1 shows the susceptibility of present isolates against 6 types of antibiotic: ampicillin, kanamycin, tetracycline, nalidixic acid, furazolidone and sulphamethoxazole, and the total plasmid carried by the present bacterial isolates as well. Generally, the percentage of antibiotic resistance in the present study was recorded as 28.7 %, whereas 63.0 % was reported as antibiotic sensitivity case. Another 8.3 % was intermediate sensitivity case. Figure 1 shows comparison of percentage of antibiotic resistance, intermediately sensitive and sensitive case between isolates from wild and cultured freshwater fish. Overall, the incidence of sensitive case among the isolates from wild freshwater fish against the tested antibiotics was 40.7 % compared to the isolates from cultured freshwater fish which was recorded as 22.2 %. The percentages of incidence of intermediately sensitive and resistance case among the isolates from cultured fish were 7.4 % and 21.3 %, respectively. Both values were higher than the isolates from wild freshwater fish which were recorded as 1.9 % and 6.5 % for intermediately sensitive and resistance case, respectively. All the present isolates were found sensitive to furazolidone except for Isolate C7 where it was resistant to furazolidone. On the other hand, all the isolates were resistant to sulphamethoxazole, excluding Isolate C1, E5 and G1. Isolates from cultured freshwater fish performed different patterns of antibiotic susceptibility result where majority of case were reported as sensitive (15 cases) and resistant (14 cases) to ampicillin, kanamycin, tetracycline and nalidixic acid. Only 7 cases were reported as intermediately sensitive to kanamycin and tetracycline among the isolates from cultured freshwater fish. Overall, all the isolates from wild freshwater fish were found to be sensitive to ampicillin, kanamycin, tetracycline and nalidixic acid except two cases

Table 1. The Susceptibility of Present Isolates to Antibiotics.

Isolate	AM	KM	TE	NA	FR	RL	Total of carried plasmid
C1	S	I	I	S	S	S	2
C2	S	S	R	R	S	R	2
C3	R	I	R	S	S	R	6
C4	S	S	I	R	S	R	8
C5	R	S	I	S	S	R	2
C6	S	S	I	R	S	R	7
C7	R	R	R	R	R	R	3
T1	R	R	I	S	S	R	6
T2	R	S	S	S	S	R	-
E1	S	S	S	S	S	R	1
E2	S	I	S	S	S	R	-
E3	S	S	S	S	S	R	-
E4	S	S	S	S	S	R	-
E5	S	S	S	S	S	S	2
G1	S	S	S	S	S	S	6
G2	S	S	S	R	S	R	8
G3	S	I	S	S	S	R	-
G4	S	S	S	S	S	R	-
Sensitive (%)	72.2	66.7	55.6	72.2	72.2	16.7	
Resistance (%)	27.8	11.1	16.7	27.8	27.8	83.3	
Intermediately sensitive (%)	0.0	22.2	27.8	0	0.0	0.0	

AM = Ampicillin 10 μg/disk,
K = Kanamycin 30 μg/disk,
TE = Tetracycline 30 μg/disk,
NA = Nalidixic Acid 30 μg/disk,
FR = Furazolidone 15 μg/disk,
RL = Sulphamethoxazole 25 μg/disk,
R = Resistant, I = Intermediately sensitive, S = Sensitive.
C1 – C7: bacterial isolates from cultured African catfish
T1 – T2: bacterial isolates from cultured red hybrid tilapia
E1 – E5: bacterial isolates from wild Asian swamp eel
G1 – G4: bacterial isolates from snakeskin gourami

that were reported intermediately sensitive to kanamycin and one case that was resistant to nalidixic acid. Figure 2 shows plasmid profiling of the present isolates. Overall, in the present study, 12 out of 18 isolates were found to carry plasmid where the sizes of plasmids were in the range of 54kb to 300 bp. All the isolates from cultured freshwater fish were found to carry plasmid except for Isolate T2. Only 4 (E1, G1, G2 and G3) out of 9 isolates from wild freshwater fish were found to carry plasmid whereas Isolate E2, E3, E3, E4, G4 and G5 did not possess any plasmid. The total number of plasmid carried by the present isolates ranged from 1 to 8 plasmids. Table 2 shows MAR index value of the present study. Isolates from wild freshwater fish showed lower MAR index value than 0.2. On the other hand, the MAR index value of isolates from cultured freshwater fish was 0.43, where it was much higher than 0.2. Overall, the MAR index value of all present isolates was 0.29. Table 3 shows MIC values of the tested antibiotics against the present isolates. The MIC values of the tested antibiotics against the present isolates were in the range of 1 to ≥128 mg/l. Tetracycline at concentration of 64 mg/l and more or equal

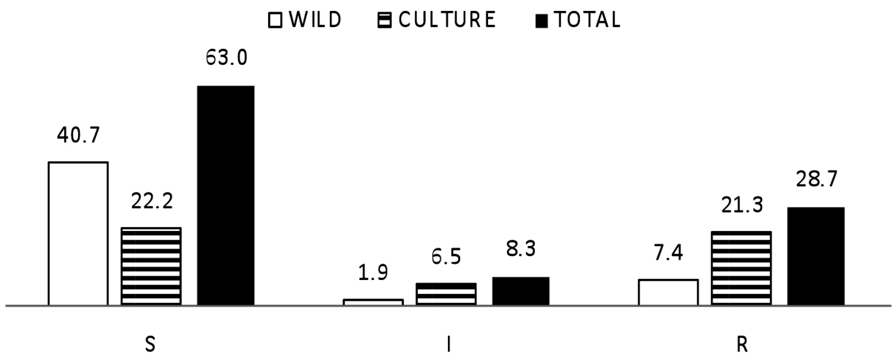
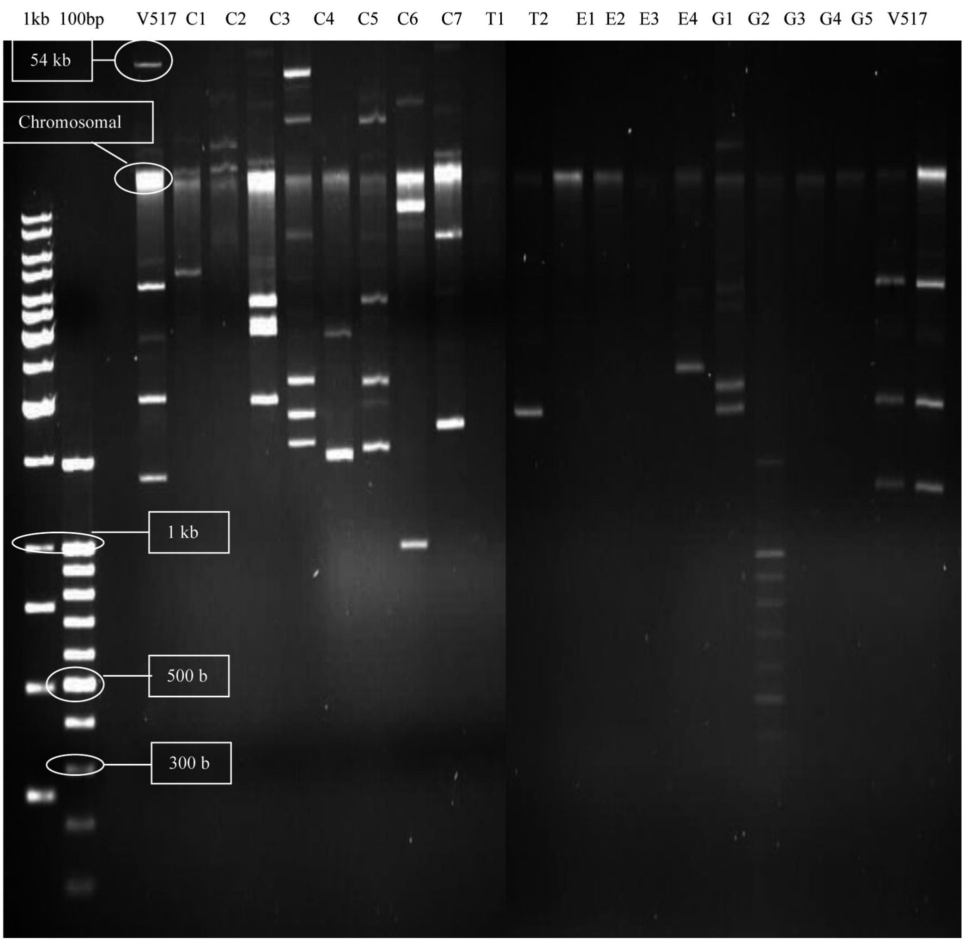


Figure 1. Percentage (%) of Antibiotic Resistance (R), Intermediately Sensitive (I) & Sensitive (S) between Isolates from Wild and Cultured Freshwater Fish.



Key: 1 kb= DNA 1 kilo bases ladder, 100 bp = DNA 100 bases,
V517= *Escherichia coli* strain V517 marker

Figure 2. Plasmid profiling of the present isolates

to 128 mg/l was found to inhibit 33.3 and 66.7 % of the present isolates, respectively. 11.1 and 5.6% of the present isolates failed to grow at 16 and 32 mg/l of sulphamethoxazole, respectively, whereas a concentration of more or equal to 128 mg/l sulphamethoxazole was able to inhibit the growth of majority of the present isolates (83.8 %). At 32 and 64 mg/l concentrations, furazolidone was able to inhibit the growth of 33.3 and 61.1 % of the present isolates, respectively. Only 5.6 % of the isolates failed to grow at concentration of more or equal to 128 mg/l of furazolidone. A total of 11.1 % of the present isolates failed to grow at 64 mg/l and more or equal to 128 mg/l of kanamycin. Majority of the isolates (72.2 %) failed to grow at concentration of 32 mg/l of kanamycin. Kanamycin at 16 mg/l was able to inhibit the growth of 5.6 % of the present isolates. Nalidixic acid and ampicillin could inhibit the growth of the present isolates at the highest concentration ranging from 1 mg/l to more or equal to 128 mg/l.

Discussion

Antimicrobial agents can be used as a tool to maintain the health and disease prevention of cultured animals (Bischoff *et al*., 2005). However, overuse of antimicrobial agents can potentially result in antibiotic resistance incidences in pathogenic bacteria; subsequently making them less responsive to antibiotic. Therefore, this study was carried out to reveal antibiogram of *E. tarda*, an important bacterial disease in freshwater fish, and the actual concentration of selected antibiotic to combat this bacterium. Plasmid profiling was carried out on the present *E. tarda* isolates to investigate the relationship between plasmid carried by the present isolates and the incidence of antibiotic resistance of the tested antibiotics.

In the present study, both resistant incidence rates of *E. tarda* to ampicillin and nalidixic acid shared similar values which were 27.8 %. The rate was low compared to isolates obtained from 3 catfish farms in Vietnam in the study of Sarter *et al.* (2007) where both resistant incidences of

Table 2. MAR (multiple antibiotic resistance) index

Source of Isolate	MAR (multiple antibiotic resistance) index
Wild Freshwater Fish	0.15
Cultured Freshwater Fish	0.43
Wild and Cultured Freshwater Fish	0.29

Table 3. Minimum Concentration Inhibitory (MIC) Values of Antibiotics against Present Isolates.

Antibiotic	Percentage of Isolate (%) MIC mg/l											
	0.06	0.13	0.25	0.5	1	2	4	8	16	32	64	≥128
TE											33.3	66.7
RL									11.1	5.6		83.3
FR										33.3	61.1	5.6
NA					44.4	5.6	5.6		16.7			27.7
KM									5.6	72.2	11.1	11.1
AM					5.6	11.1		11.1	22.2	16.7	5.6	27.7

AM = Ampicillin,
K = Kanamycin,
TE = Tetracycline,
NA = Nalidixic Acid,
FR = Furazolidone,
RL = Sulphamethoxazole

ampicillin and nalidixic acid rates were 69.6 % and 51.6 %, respectively. Another study of Hatha *et al*. (2005) reported that the incidence of ampicillin resistance of *Aeromonas* spp. isolated from aquaculture sites was higher than the incidence of antibiotic resistance to nalidixic acid in which both of the values was 100 % and 4 %, respectively. However, the MIC value for ampicillin in the present study was higher than other studies. In the present study, the MIC value of ampicillin ranged from 1 to 128 mg/l whilst Clark *et al.* (1991) reported that MIC value of ampicillin for 22 isolates of *E. tarda* ranged from 0.12 to 1 mg/l. MIC value of ampicillin of 103 isolates of *E. tarda*, *E. hoshinae* and *E. ictaluri* in the study of Stock and Wiedemann (2001) ranged from ≤ 0.03 to 4 mg/l. The incidence of antibiotic resistance to sulphamethoxazole was widely spread among the present *E. tarda* (83.3 %). However, the incidence of antibiotic resistance to sulphamethoxazole among 129 isolates of *Pseudomonas* spp. and 90 isolates of *Aeromonas* spp. isolated from 9 rainbow trout (*Oncorhynchus mykiss*) farms in Australia was less common (Akinbowale et al. 2007). According to the Akinbowale et al. (2007), only one isolate of *Pseudomonas* spp. from rainbow trout was resistant to sulphamethoxazole whilst 14.3 % and 18.8 % were reported for incidence of resistance to sulphamethoxazole among *Aeromonas* spp. isolated from sediment and fish, respectively. Perhaps fish farmers in Terengganu have widely used this antibiotic in terms of prophylactic and treatment measures to control fish disease due to the highest rate of antibiotic resistance cases to sulphamethoxazole for both isolates from aquaculture sites and the natural environment. Furthermore, Defoirdt *et al.* (2007) reported that sulfa drug has been widely used for aquaculture in South East Asia against bacterial disease. Similar to the study of Stock and Wiedemann (2001), they found that the MIC value of sulphamethoxazole of 103 isolates of *Edwardsiella* spp. ranged 16 to ≥ 128 mg/l whereas the MIC value of sulphamethoxazole against the present isolates ranged from 16 to ≥ 128 mg/l. Thus, it shows that the MIC value of sulphamethoxazole for most of *Edwardsiella* spp. isolates fall into this MIC range. The case of present *E. tarda* isolates resistant to tetracycline was less common which was only 16.7 % and all isolates involved were from cultured freshwater fish. However, *E. tarda* isolates from wild freshwater fish were found to be susceptible to tetracycline. The percentage of antibiotic resistance incidence to tetracycline of the present *E. tarda* isolates was found lower than the isolates in the study of Jacob and Chenia (2007) where 78.3 % of *Aeromonas* spp. isolated from aquaculture sites in South Africa were found to be resistant to tetracycline. This may be due to less application of this drug by fish farmers at sampling sites in the present study. This is supported by the finding that shows no case of incidence of resistance to tetracycline among the present isolates obtained from wild freshwater fish. So far, the information on antibiotic resistance of kanamycin and furazolidone among the isolates from aquaculture sites is lacking. However, the present study shows that the incidence of antibiotic resistance to both kanamycin (11.1 %) and furazolidone (27.8 %) was less common, but the MIC value of both antibiotic against the present isolates was high. The MIC value of kanamycin of 103 isolates of *Edwardsiella* spp. in the study of Stock and Wiedemann (2001) ranged from 0.25 to 4 mg/l, whereas the MIC value among the present isolates was 16 to ≥ 128 mg/l. Overall, the incidence of antibiotic resistance of ampicillin, kanamycin, tetracycline, furazolidone and nalidixic acid was found less common. However, case of antibiotic resistance of sulphamethoxazole was widely spread among the present isolates.

The MAR index in the present study indicates that cultured freshwater fish, namely African Catfish and Red Hybrid Tilapia, in Terengganu are under high-risk exposed-antibiotic sources. Sarter *et al.,* (2007) reported that 3 catfish farms located at Mekong Delta, Vietnam which used antibiotics to treat bacteria disease were also under exposed-antibiotic sources. However, the MAR values in the present study revealed that wild freshwater fish (Siamese Gouramy and Asian Swamp Eel) were below the level of high-risk exposed-antibiotic sources. McPhearson *et* al., (1991) reported that a river located at South-Eastern United States was also below the level of high-risk exposed-antibiotic sources. However, the MAR index of the catfish aquaculture pond situated near to the river where antibiotics were commonly used for treatment can be as high as 0.76.

To date, there is little study of plasmid profiling on *E. tarda*. However, plasmid profiles of the present *E. tarda* isolates have revealed that there is no correlation between the occurrence of antibiotic resistance of 6 tested antibiotics and the number and size of plasmid that harbored in the present *E. tarda* isolates. However, it is interesting to note that all the isolates from cultured freshwater fish possessed plasmid except for Isolate T2, on the other hand, only 4 out of 5 isolates from wild freshwater fish carried plasmid. The study of Smith and Bidochka (1998) described the loss of plasmid among the bacteria is due to starvation. In addition, the limitation of glucose and salt in the bacteria culture medium could cause starvation (Smith and Bidochka, 1998). However, in the present study, plasmid extraction was conducted from fresh cultured isolates. Therefore, cases of plasmid loss in the present *E. tarda* isolates due to starvation may not occur. The results of the present study showed that the occurrence of plasmid may not be necessary for resistance, might be important for virulence since some may enhance the resistance to antibiotics. For example, although the present bacterial isolates strain G2 and G3 which carried 6 and 8 plasmids, respectively, they did not exhibit high level of resistance to the tested antibiotics.

Overall, ampicillin, kanamycin, tetracycline, nalidixic acid and furazolidone were successfully found to inhibit more than 50 % of the present bacterial isolates. On the other hand, more than 80 % bacterial isolates were resistant to sulphamethoxazole. In terms of MIC values, ampicillin and nalidixic acid showed the lowest MIC value (1 mg/L) to control the growth of *E. tarda*. Therefore, it is suggested that ampicillin and nalidixic acid be the antibiotics of choice in combating Edwardsiellosis due to *E. tarda* in freshwater-fish culture in Malaysia.

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