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Characterization of *Edwardsiella tarda* isolated from Asian Seabass, *Lates calcarifer*

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Abstract: This study described the antibiotic and heavy metal resistance pattern of 17 isolates of *Edwardsiella tarda* obtained from Asian seabass (*Lates calcarifer*). *E.tarda* isolates were resistant to oleandomycin, lincomycin, novobiocin and spiramycin. In contrast, most of the isolates showed high level of susceptibility to tetracycline, doxycycline, florfenicol, chloramplenicol, nitrofurantoin, fosfomycin, kanamycin, oxolinic acid and flumequine. MAR value was 0.35 which indicated that the cultured Asian seabass have received high exposure to those tested antibiotics. Besides, very high level of heavy metal resistance among these isolates was observed. Genotypic profile of DNA fingerprintings generated by RAPD-PCR using M13 universal primer and M13 wild type phage primer showed high degree of genetic diversity with percentages similarity and genetic distance among the isolates were ranging from 10.5% to 100% and 0 to 0.895, respectively. This result indicates that strains that belong to the same origin were not always closely related genetically.

Keywords: ***Edwardsiella tarda, Lates calcarifer,*** **antibiotic, heavy metal, genotypic profiling**

Introduction

Lates calcarifer, commonly known as giant sea perch, Asian seabass or barramundi can be found in the tropical and subtropical regions of Asia and the Pacific, and is considered as an important coastal, estuarine and freshwater food fish. Due to its high market price, it develops into small scale to large scale of aquaculture activities in many Asian countries (Kungvankij *et al*., 1985).

Edwardsiellosis is one of the most important bacterial disease occur in fish farming. Fish species normally infected by Edwardsiellosis including both freshwater and marine fish such as carp, tilapia, eel, catfish, salmon, trout and flounder (Mohanty and Sahoo, 2007). Edwarsiellosis is epizootic, which had affect the culture in South of USA, Southeast Asia, northwest of Pacific and many other of the tropical fish culture (Najiah *et al*., 2007). To date, the cases of edwardsiellosis infecting freshwater fish culture were reported particularly in channel catfish. However, the study of Edwardsiellosis on locally cultured seabass has not been accounted in Malaysia.

Recently, the incidence of antibiotics resistance among the bacterial pathogen is increasing due to the irresponsible use of antibiotic by fish farmers. On the other hand, most of bacteria have particular resistance gene to heavy metal elements such as Cd^{2+}, Hg^{2+}, Zn^{2+}, Cr^{6+}, Cu^{2+} and others (Silver, 1996). Bacteria with high resistance against heavy metals may pose risk to public health. Thus, the study of both antibiotic and heavy metal resistance among the pathogenic bacteria is important.

At present, there is lacking of database on antibiogram, heavy metal resistance and genotypic profile report of *E. tarda* in the Asian seabass (*L. calcarifer*) hatchery in Malaysia. This study was carried out to study the antibiogram, heavy metal resistance and genotyping profile of the *E. tarda* isolated from Asian seabass. Moreover, the information gathered would help the farmers in selecting the suitable antibiotic for fish health management.

Materials and Methods

Stock culture samples

The present study was conducted at fish disease laboratory in University Malaysia Terengganu during 2010-2011. Seventeen isolates of E. tarda were obtained from bacterial stocks and were cultured in Trypticase Soy Broth, TSB (Oxoid, England) for 24 h at room temperature.

Antibiotic susceptibility test

Antibiotics susceptibility test was conducted

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according to Kirby-Bauer disk diffusion method (Bauer *et al*., 1966) by using Muller-Hinton Agar (Merck, Germany). Antibiotics tested included oxolinic acid (2 µg per disc), ampicillin (10 µg per disc), erythromycin (15 µg per disc), lincomycin (15 µg per disc), oleandomycin (15 µg per disc), amoxicillin (25 µg per disc), chloramphenicol (30 µg per disc), doxycycline (30 µg per disc), florfenicol (30 µg per disc), flumequine (30 µg per disc), kanamycin (30 µg per disc), nalidixic acid (30 µg per disc), novobiocin (30 µg per disc), tetracycline (30 µg per disc), nitrofurantoin (50 µg per disc), fosfomycin (50 µg per disc) and spiramycin (100 µg per disc) (Oxoid, England). Briefly, the sample of *E. tarda* culture in TSB was swabbed onto Muller-Hinton Agar uniformly for a lawn of bacterial growth. Antibiotic discs were gently placed on the surface of the agar using forceps and were kept in incubator for 24 h at 25ºC. Interpretation of the resulted inhibition zones, namely sensitive (S), intermediary sensitive (I) and resistance (R), was done according to the standard measurement in millimeter (mm) following National Committee for Clinical Laboratory Standards (NCCLS, 1998). The Multiple Antibiotic Resistance (MAR) index of the isolates against the tested antibiotics was calculated following Sarter *et al.* (2007):

$$MAR\ index = X/(Y \times Z)$$

X=Total number of bacterial isolates resistant to antibiotics
Y=Total number of antibiotics used in the study
Z=Total number of isolates

A MAR index value equivalent or less than 0.2, indicate that those antibiotics were seldom or never used for the animals for the purpose of treatment whereas the MAR index value exceed than 0.2 is signify that animal have received high-risk exposure to those antibiotics.

Heavy metal resistant test

Heavy metal resistant test was carried out as described by Miranda and Castillo (1998). Four elements of heavy metal with different concentration was used: mercury (Hg^{2+}), cadmium (Cd^{2+}), chromium (Cr^{6+}) and copper (Cu^{2+}). Overnight bacterial suspension was spread onto Tryptic Soy Agar (TSA) (Merck, Germany) medium and integrated with different concentration of Mercury(II) chloride, $HgCl_2$; admium chloride, $CdCl_2$; Potassium dichromate, $K_2Cr_2O_7$ and Copper(II) sulfate, $CuSO_4$ (Fluka, USA). The concentration of $HgCl_2$ was 2.5µg/ml, 5 µg/ml, 10 µg/ml, 20 µg/ml, 40 µg/ml and 80 µg/ml. The concentration of $K_2Cr_2O_7$ and $CdCl_2$ was 25 µg/ml, 50 µg/ml, 100 µg/ml, 200 µg/ml, 400 µg/ml and 800 µg/ml (Calomiris *et al*., 1984). On the other hand, the concentration of CuSO4 was 150 µg/ml, 300 µg/ml, 600 µg/ml, 1200 µg/ml, 2400 µg/ml and 4800 µg/ml (Calomiris *et al*., 1984).

In terms of defining metal resistance, the bacterial strains were considered resistant if they grown at the concentration of 10 µg/ml for mercury (Hg^{2+}) and 100 µg/ml for both cadmium (Cd^{2+}) and chromium (Cr^{6+}) (Allen *et al*., 1997) and 600 µg/ml for copper (Cu^{2+}) (Miranda and Castillo, 1998).

DNA extraction

DNA extraction of the present isolates was done using boiling technique described by Sambrook and Russell (2001). Briefly, bacterial isolates were cultured on Tryptic Soy Agar (TSA) (Merck, Germany) for 24 h. The bacterial colony was collected and suspended in TE butter in the micro centrifuge tube (Eppendoff, Germany). The sample was then heated at 95°C using water bath for 5 min and then instantly stored at -20°C. Frozen sample was thawed at room temperature, followed by centrifugation at 13,000 rpm for 10 min. The quantity of the DNA was determined by using Bio photometer (Eppendoff, Germany) at absorbance of 260nm and 280nm, following the formula:

$$DNA\ quantity\ (\mu g\ ml^{-1}) = \frac{ABS260 \times 50\ \mu g\ ml^{-1} \times total\ volume\ (\mu l)}{Volume\ of\ sample\ (\mu l)}$$

RAPD-PCR assay

RAPD-PCR assay was performed in a total volume of 25µl mixture containing 10 mM Tris-HCl, 50 mM KCl, 0.1% Triton® X-100, 2.5 mM MgCl2 , 0.5 µM universal primers, 0.2 mM nucleotide mix and 1.25 U of *Taq* DNA polymerase (Genensis Biotech, Malaysia). Two primers used were Universal M13 (5’-TTATGTAAAACGACGGCCAGT-3’) and Wild-type Phage M13 (5’-GAGGGTGGCGGTTCT-3’). Amplication for Universal M13 and Wild-type Phage was performed by programming the thermal cycler (Eppendoff, Germany) to 1 cycle at 94°C for 5 min; 2 cycles at 94°C for 5 min, 40°C for 5 min and 72°C for 5 min; 35 cycles at 94°C for 1 min, 60°C for 1 min and 72°C for 1 min; and 1 cycle at 72°C for 5 min. The RAPD-PCR products were electrophoresed on 2% agarose gel containing ethidium bromide (5 µg µl-1) submerged in 1 x TBE buffer (Lee and Najiah, 2008).

Gel electrophoresis and RAPD fingerprint

The gel for electrophoresis was prepared by boiling 2% agarose powder in 1 X Tris borate EDTA (TBE) buffer solution with 10 µl of ethidium bromide

and was poured into a mold after cooled to about 50°C. The electrophoresis was run at 110 V for about 90 minutes. RAPD-PCR fingerprints of the samples were visualized using UV transilluminator and the image was captured.

RAPD analysis and Genetic Relationship

A data matrix was created by giving scores of 0 and 1 for the absence or presence of bands, respectively, at each position for all isolates. Analysis for the obtained matrix data was done using Numerical Taxonomy and Multivariate Analysis System (NTSYSpc) version 2.1(Rohlf, 2000) based on unweighted pair-group method with arithmetic means (UPGMA) (Sneath and Sokal, 1973). The genetic distance and percentages of similarity among the isolates was calculated based on (Nei and Li, 1979) formulation as below:

$$\text{Percentage of similarity, } F = \frac{2Nxy \times 100\ \%}{Nx + Ny}$$

Where
Nxy = number of shared bands
Nx = total number of bands in lane X,
Ny = total number of bands in lane Y

Results and Discussion

The application of antibiotics in aquaculture has been the main purpose of managing and treating fish disease. These synthetic drugs effectively inhibit bacterial cell wall synthesis (penicillins, aminopenicillins), protein synthesis (tetracyclines, aminoglycosides, chloramphenicol, florfenicol, macrolides, lincosamides) and DNA function (nitrofurans, quinolones) (Harold, 1992). However, the irresponsible use of the antibiotics in fish farming has caused the development of antibiotics resistant in fish pathogen (Sarter *et al*., 2007). Some researchers claimed that the reservoirs of antibiotic resistance can interact between different ecological systems and the possibility transfer of resistant bacteria or resistant genes from animals to humans may occur through the food chain (Van den Bogaard and Stobberingh, 2000 ; Teuber, 2001).

Out of 17 isolated strains (Table 1), 100% of isolates were resistant to oleandomycin, lincomycin, novobiocin, followed by Spiramycin (94.12%), ampicilin (58.82%) and amoxicillin (58.82%). According to Stock and Wiedemann (2001), *Edwardsiella* species were naturally resistant to macrolides and lincosamides. Almost all the Enterobacteriaceae species is typically intrinsic resistance to these agents and it has been largely

Table 1. Percentages of sensitivity of bacterial isolates against 17 types of antibiotic

Antibiotic, µg	Resistance		Intermediate sensitive		Sensitive	
	no	%	no	%	no	%
Tetracycline	0	0.00	0	0.00	17	100.00
Doxycycline	0	0.00	0	0.00	17	100.00
Kanamycin	3	17.65	0	0.00	14	82.35
Ampicillins	10	58.82	3	17.65	4	23.53
Amoxicillins	10	58.82	4	23.53	3	17.65
Erythromycin	2	11.76	11	64.71	4	23.53
Oleandomycin	17	100.00	0	0.00	0	0.00
Spiramycin	16	94.12	1	5.88	0	0.00
Lincomycin	17	100.00	0	0.00	0	0.00
Oxolinic acid	3	17.65	0	0.00	14	82.35
Nalidixic acid	4	23.53	0	0.00	13	76.47
Flumequine	3	17.65	0	0.00	14	82.35
Florfenicol	0	0.00	0	0.00	17	100.00
Chloramphenicol	0	0.00	0	0.00	17	100.00
Novobiocin	17	100.00	0	0.00	0	0.00
Nitrofurantoin	0	0.00	0	0.00	17	100.00
Fosfomycin	0	0.00	0	0.00	17	100.00

attributed to the outer membranes of these bacteria (Stock and Wiedemann, 2001). Spizek and Rezanka (2004) stated that lincomycin is bacteriostatic, acts as inhibitor of protein synthesis in sensitive bacteria and may even be bactericidal at the higher concentrations. Analysis of lincomycin-resistant lines of *Chlamydomonas reinhardtii* (Harris *et al*., 1989), Nicotiana plubaginifolia (Cseplo et al., 1993), and Solanum nigrum (Kavanagh *et al*., 1994) found that resistance to lincomycin is confer by a mutation in the 26S rRNA gene in plastids.

In the current study, the most effective antibiotics for controlling the growth of E. tarda were tetracycline, doxycycline, florfenicol, chloramplenicol, nitrofurantoin and fosfomycin. Other antibiotics that were also effective were kanamycin, oxolinic acid and flumequine. However, chloramplenicol and nitrofurantoin has been banned for aquaculture use in Malaysia (Lee *et al.*, 2009) due to its severe toxicity and its importance in the treatment of typhoid fever. Meanwhile, fluorinated derivatives, florfenicol was available and applied in aquaculture activities since it did not give any inconvenience as chloramphenicol and had no application in human medicine (Michel *et al*., 2003).

The MAR index value of the present isolates was 0.35, which indicated that the cultured Asian seabass have received high exposure to those tested antibiotics. In the study done by Najiah *et al.* (2009), 30 isolates of *E.tarda* have been successfully isolated from African catfish, (*Clarias gariepinus*) and the

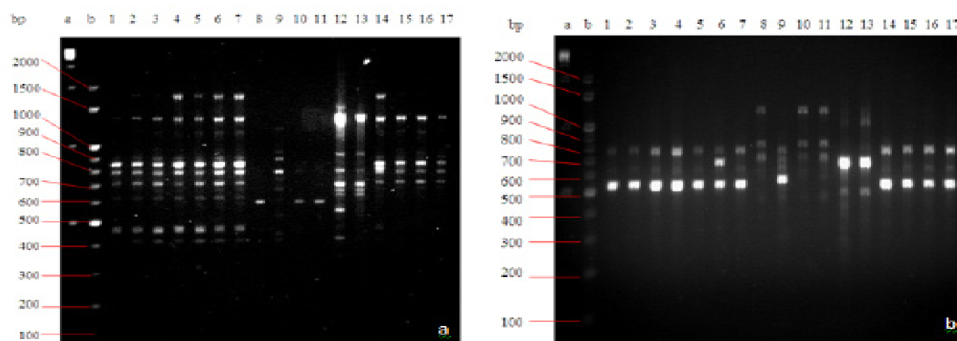

Figure 1. RAPD pattern obtained using a) Wild-type Phage (WTP) primer.; b) M13 universal primer

Lane a: 1kb DNA ladder; lane b: 100bp DNA ladder; lane 1: isolate 1; lane 2: isolate 2, lane 3: isolate 3; lane 4: isolate 4; lane 5: isolate 5; lane 6: isolate 6; lane 7: isolate 7; lane 8: isolate 8; lane 9: isolate 9; lane 10: isolate 10; lane 11: isolate 11, lane 12: isolate 12, lane 13: isolate13; lane 14: isolate 14; lane 15: isolate 15, lane 16: isolate 16, lane 17: isolate 17

MAR value was 0.40. High multiresistance may be a sign of the presence of fluctuating pressure which produces bacterial strains with multiple mechanisms or a single mechanism of resistance to survive under the variable environmental conditions (Baquero *et al*., 1998).

All bacterial isolates in the present study were found resistant to all the tested heavy metals (mercury, cadmium, chromium and copper). Heavy metal resistance can be occurring due to enzyme mediated resistance mechanism (Cloete, 2003). Currently, there has been very few information on the heavy metal resistance pattern of *E. tarda* from aquaculture activity. Hence, present finding may be able provide data for further detailed investigation.

RAPD pattern generated using Wild-type Phage (WTP) primer and M13 universal primer are shown in Figure 1(a) and 1(b). Genetic distance and percentage of similarity of RAPD-PCR profile among 17 isolates are as shown in Table 2. The value for both genetic distance and genetic similarity were inversely correlated. The percentages similarity and genetic distance among the isolates based on RAPD PCR profile were ranging from 10.5% to 100% and 0 to 0.895, respectively. Highest percentage of similarity was between isolates 4 and 5, 4 and 7, 5 and 7, 8 and 10, 8 and 11, 10 and 11, 15 and 16. Our results thus, clearly show that strains that belong to the same origin were not always closely related genetically. Study by Nucci *et al.* (2002) found that *E. tarda* strains from different countries, fish and human, were distinguished into two clustered related to the source of isolation. Besides, the intraspecific genetic diversity of *E. tarda* strains isolated from turbot using RAPD analysis (Castro *et al*., 2006) showed that the oligonucleotides P3 and P6 compiled the isolates into a unique group whereas primers P4 and P5 generate a pattern where two clone lineages was obtained. Another study of the genotyping profile on *E.tarda* isolated from cultured and natural habitat fish was done by Lee and Najiah (2008). Their results of RAPD-PCR fingerprinting also exhibited high degree

Table 2. Genetic distance (above diagonal) and percentage of similarity (below diagonal) among the 17 isolates of *Edwardsiella tarda*

Isolate no.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1	_	0.091	0.167	0.167	0.167	0.200	0.167	0.867	0.579	0.867	0.867	0.619	0.579	0.200	0.222	0.222	0.111
2	90.9	_	0.077	0.077	0.077	0.111	0.077	0.882	0.524	0.882	0.882	0.565	0.619	0.182	0.300	0.300	0.200
3	83.3	92.3	_	0.037	0.037	0.071	0.037	0.889	0.455	0.889	0.889	0.500	0.545	0.217	0.238	0.238	0.238
4	83.3	92.3	96.3	_	0.000	0.034	0.000	0.895	0.478	0.895	0.895	0.520	0.565	0.167	0.273	0.273	0.273
5	83.3	92.3	96.3	100.0	_	0.034	0.000	0.895	0.478	0.895	0.895	0.520	0.565	0.167	0.273	0.273	0.273
6	80.0	88.9	92.9	96.6	96.6	_	0.034	0.800	0.417	0.800	0.800	0.462	0.500	0.200	0.304	0.304	0.304
7	83.3	92.3	96.3	100.0	100.0	96.6	_	0.895	0.478	0.895	0.895	0.520	0.565	0.167	0.273	0.273	0.273
8	13.3	11.8	11.1	10.5	10.5	20.0	10.5	_	0.714	0.000	0.000	0.875	0.857	0.867	0.846	0.846	0.846
9	42.1	47.6	54.5	52.2	52.2	58.3	52.2	28.6	_	0.714	0.714	0.500	0.556	0.579	0.647	0.647	0.529
10	13.3	11.8	11.1	10.5	10.5	20.0	10.5	100.0	28.6	_	0.000	0.875	0.857	0.867	0.846	0.846	0.846
11	13.3	11.8	11.1	10.5	10.5	20.0	10.5	100.0	28.6	100.0	_	0.875	0.857	0.867	0.846	0.846	0.846
12	38.1	43.5	50.0	48.0	48.0	53.8	48.0	12.5	50.0	12.5	12.5	_	0.100	0.714	0.684	0.684	0.684
13	42.1	38.1	45.5	43.5	43.5	50.0	43.5	14.3	44.4	14.3	14.3	90.0	_	0.684	0.647	0.647	0.647
14	80.0	81.8	78.3	83.3	83.3	80.0	83.3	13.3	42.1	13.3	13.3	28.6	31.6	_	0.111	0.111	0.111
15	77.8	70.0	76.2	72.7	72.7	69.6	72.7	15.4	35.3	15.4	15.4	31.6	35.3	88.9	_	0.000	0.125
16	77.8	70.0	76.2	72.7	72.7	69.6	72.7	15.4	35.3	15.4	15.4	31.6	35.3	88.9	100.0	_	0.125
17	88.9	80.0	76.2	72.7	72.7	69.6	72.7	15.4	47.1	15.4	15.4	31.6	35.3	88.9	87.5	87.5	_

of genetic diversity among the isolates.

Conclusion

Asian seabass locally cultured have been exposed to tested antibiotics and heavy metals. Hence, reduction of the use of antibiotic and heavy metal was suggested. In addition, the strains that belong to the same origin were not always closely related genetically. A deeper understanding of *E. tarda* through further study on the plasmid profiling and proteotypic characteristics are warranted.

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