

Phenotyping, Genotyping and Whole Cell Protein Profiling of *Edwardsiella tarda* Isolated from Cultured and Natural Habitat Freshwater Fish

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Abstract: A total of 18 isolates of *Edwardsiella tarda* was successfully isolated from African Catfish (*Clarias gariepinus*), Red Hybrid Tilapia (*Tilapia* sp.), Asian Swamp Eel (*Monopterus albus*) and Snakeskin Gouramy (*Trichogaster pectoralis*). The percentage similarity of phenotypic, genotypic and proteotypic characteristics of the present isolates was ranged from 80.8 to 100%, 27.9 to 89.4% and 46.3 to 100%, respectively. 3 dendrograms were generated using NTSYSpc version 2.1 software based on phenotypic, genotypic and proteotypic characteristics of the present isolates. Numerical analysis of 59 unit characters of biochemical and physiological of the present isolates was failed to discriminate them as well as RAPD fingerprinting using 3 types of primers namely, (GTG)₅, M13 Universal and M13 Wild Type Phage. However, it was interesting to note that whole cell protein profiling of present isolates by using one dimensional SDS PAGE was able to discriminate the isolates. Generally, the isolates obtained from cultured African Catfish and Red Hybrid Tilapia were included into one group whereas isolates that isolated from wild caught Asian Swamp Eel and Snakeskin Gouramy were shared in one similar group.

Key word: *Edwardsiella tarda* % Phenotypic % Genotypic % Proteotypic % Freshwater fish

INTRODUCTION

Edwardsiella tarda is well known as a causative agent of edwardsiellosis that can devastate both freshwater and marine fish culture. At present, the information on phenotypic, genotypic and proteotypic characteristics of *E. tarda* from both environmental and clinical, are lacking. This may lead our fish farmer misidentified the actual pathogen which attacking their culture fish and misuse the appropriate treatment against edwardsiellosis outbreak. Subsequently, fish farmer was suffered the loss due to this fish disease. [1] reported that infected catfish with edwardsiellosis can cause noxious odours whenever the fish enter processing plant. Thus the processing plants had to stop production for disinfection and deodorization. This may result heavy financial losses due to only the presence of small infected fishes. However, catastrophic losses of catfish culture due to this disease have not been recorded. According to [2], *E. tarda* is distributed worldwide and edwardsiellosis is a problem principally in Africa, America and Asia and is an important disease of cultured eels in Japan and Taiwan.

The losses attributed to *E. tarda* infectious in cultured eel in Japan for the year 1984 are about 815 million yen, which corresponds to quart of the total losses. Due to that, edwardsiellosis has been known as a disease of primary importance and posed a big problem in eel culture. Although infectivity of *E. tarda* is not very high but the bacterium is thought to survive in eel ponds throughout the rearing period and causes disease when the physiological condition of the fish or the quality of the water deteriorates. It is difficult to eliminate the pathogen from the environment, as there is a variety of natural reservoirs including worms, reptiles and birds. Passive transmission by water, equipment, tools and food may occur. No vertical transmission of the disease is suspected. *E. tarda* possesses pilus on the cell surface and it is likely these are the means by which the bacterium initially attaches to its host and establishes infection. Thus, this study was conducted to reveal phenotypic, genotypic and proteotypic characteristics of *E. tarda* isolated from freshwater fishes. Therefore, the information is useful for bacterial fish disease diagnosis purpose especially for our fish farmer.

MATERIALS AND METHODS

Isolation and Identification of *Edwardsiella tarda*: Ten of each cage cultured African Catfish (*Clarias gariepinus*) and Red Hybrid Tilapia (*Tilapia* sp.) with weighing around 250 g were bought from fish farms located at Sungai Manir, Terengganu. Whereas, 10 of each wild caught Asian Swamp Eel (*Monopterus albus*) and Snakeskin Gourami (*Trichogaster pectoralis*) with weighing around 100 g were bought from Pasar Tani, Terengganu. All the fishes applied in this were having haemorrhages and necrosis lesions were externally. At the laboratory, the fish were killed under anesthesia at 100 ppm Tricaine Methanesulfonate (TMS) (MS-222) (Syndel, Canada). Kidney, liver and alimentary canal of the fish were taken out aseptically and homogenized separately in sterile physiological saline. Aliquots of 0.1 ml were inoculated on Xylose Lysine Dextrose (XLD) agar (Merck, Germany) plate by spread plate method followed by 48 hours incubation at room temperature. After 48 hours, clear colony with black at the centre and reddish peripheral ring in diameter 1 to 2 mm on the XLD agar plate was selected and stocked in deep tube Tryptic Soy Agar (TSA) (Merck, Germany) containing 0.5% NaCl. In the present study, 20 conventional biochemical tests, 8 physiological tests and a commercial enteric and non fermenter bacterial identification kit (BBL, USA) containing 30 biochemical tests were used to identify the isolated strains.

Numerical taxonomy of the present isolates: Firstly, a data matrix was prepared based on the biochemical and physiological properties of the present isolates. The positive and negative reactions from the tested biochemical and physiological tests of the present isolates will give scores of 1 and 0, respectively. The matrix data was then analysis by using Numerical Taxonomy and Multivariate Analysis System (NTSYSpc) version 2.1 [3] based on unweighted pair-group method with arithmetic means (UPGMA) [4]. The similarity of the phenotypic among the isolates was calculated according to [5] formulation as follow:

$$\text{Percentage of similarity, } F = \frac{2N_{xy} \times 100\%}{N_x + N_y}$$

Where,

N_{xy} = number of shared similar phenotypes

N_x = total number of positive reactions in lane X,

N_y = total number of positive reactions in lane Y

RAPD-PCR fingerprinting

Extraction of genomic DNA: DNA extraction of the present isolates was done using boiling technique. The bacterial isolates were cultured on TSA for 24 h. The bacterial colony was then collected and suspended in TE buffer in the micro centrifuge tube (Eppendoff, Germany). The sample was heated at 95°C using water bath for 5 min followed by immediate storage at the-20°C. The sample was then thawed at room temperature before it was centrifuged at 13,000 rpm for 10 min. The supernatant was collected and transferred into new micro centrifuge tube whereas the pelleted cells were discarded.

Quantification of DNA sample: The extracted DNA of the present isolates was screened for it's quality and quantity by using Bio photometer (Eppendoff, Germany) at absorbance of 260nm and 280nm. The ratio of $A_{260} : A_{280}$ of the DNA fell between 1.6 to 2.0 showed that the extracted DNA was suitable for PCR reaction. The quantity of the DNA of the isolates was then determined by using the following formulation:

$$\text{DNA quantity } (\mu\text{g/mL}) = \frac{\text{ABS}_{260} \times 50 \mu\text{g/mL} \times \text{total volume } (\mu\text{l})}{\text{Volume of sample } (\mu\text{l})}$$

RAPD-PCR assay: PCR reactions were performed in a 25 μl PCR 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). Three primers used were (GTG)₅ (5'-GTGGTGGTGGTGGT-3'), Universal M13 (5'-TTATGTAACGACGCGCCAGT-3') and Wild-type Phage M13 (5'-GAGGGTGCCGTTCT-3'). Amplifications for primers Universal M13 and Wild-type Phage were carried out by programming the thermal cycler (Eppendoff, Germany) to the following profile: 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 whereas amplification for primer (GTG)₅ was initially denatured at 95°C for 1 min followed by 30 cycles of at 94°C for 1 min, 40°C for 1 min and 65°C for 16 min. The RAPD-PCR products together with 1000bp and 100bp DNA markers (Fermentas, USA) were electrophoresed on 1% agarose gel containing ethidium bromide (5 $\mu\text{g}/\mu\text{L}$) submerged in 1 x TBE buffer.

Gel electrophoresis and RAPD fingerprint: The gels for electrophoresis used in this study were prepared by boiling 1% agarose powder in 1 X Tris borate EDTA

(TBE) buffer solution together with 2 µl of ethidium bromide and poured into a mold after cooled to 50°C. Before started electrophoresis by using electrophoresis machine (Mupid Ex, Japan), the gel was submerged in a TBE buffer which contains ethidium bromide at 5 µl/ml concentration. By using 10 µl micro pipette, RAPD PCR products which have already mixed with loading dye (Promega Madison, USA) were loaded into wells of the prepared gel. After that, electrophoresis was run at 110 V for 90 min. RAPD-PCR fingerprints of the samples were visualized by using UV transilluminator and the image was captured with digital camera (Nikon, Japan).

RAPD analysis and genetic relationship: A data matrix was generated based on the RAPD fingerprints of the isolates by giving scores of 0 and 1 for the absence or presence of bands, respectively, at each position for all isolates. The obtained matrix data was analysis by using Numerical Taxonomy and Multivariate Analysis System (NTSYSpc) version 2.1 [3] based on unweighted pair-group method with arithmetic means (UPGMA) [4]. The similarity of the genetic among the isolates was calculated according to [5] formulation as follow:

$$\text{Percentage of similarity, } F = \frac{2N_{xy} \times 100 \%}{N_x + N_y}$$

Where

N_{xy} = number of shared bands

N_x = total number of bands in lane X,

N_y = total number of bands in lane Y

SDS-PAGE (Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis)

Extraction of whole cell protein: Whole cell protein of the present isolates was extracted as described by [6]. The isolates of the present study were cultured using TSB (Tryptic Soy Broth) (Merck, Germany) for overnight at room temperature. The cultures were then adjusted into 109 cell using Bio photometer (Eppendoff, Germany) before harvested 1 ml from each the culture by centrifuged at 13,500 rpm for 5min. The supernatant was discarded and the bacterial cell was resuspended in 1 ml of chilled aqueous 0.5% (v/v) Triton X-100. The samples were then sonicated for three cycles with 20 s each were cooled. The sample was placed on ice between treatments. After that, the samples were centrifuged for 1 min at 13,500 rpm. Finally, Laemmli sample buffer containing 100 mM DTT was added to the samples followed by 2 min boiling.

SDS-PAGE assay and whole cell protein fingerprint:

Protean II xi electrophoresis unit (Bio-Rad, USA) was used in the present study. Firstly, 4% T of stacking gel (5.36 ml of 30% acrylamide, 10 ml of stacking gel buffer, 24 ml of H₂O, 0.4 ml of 10% SDS stock, 0.2ml of 10% ammonium persulfate and 0.04 ml of TEMED) and 12% T of resolving gel (40 ml of 30% acrylamide, 25 ml of resolving gel buffer, 33.2 ml of H₂O, 1 ml of 10% SDS, 0.75 ml of 10% ammonium persulfate and 0.05 ml of TEMED) were prepared. The extracted whole cell protein from the isolates together with higher and lower range of protein molecular weight marker were mixed with 2X SDS PAGE sample buffer in a 1: 1 ratio. The mixtures were then heated in a heater block for 2 min at 95°C. The samples were then cool at room temperature and insoluble materials were removed by centrifuged at 13,500 rpm for 10 min. 2 X SDS PAGE sample buffer was added into the pelleted sample followed by mixing with micro pipette. The samples were reheated in a heater block for 2 min at 95°C and left cool at room temperature. The insoluble materials were removed by centrifuged and the samples were loaded into sample well by using micro pipette followed by electrophoresis at 110 V until the bromophenol blue dye front reaches the bottom of the gel. After electrophoresis, the gel was stained overnight with Coomassie Blue R-250. The gel was then destained by soaking in distilled water for 15 min. Finally, the whole cell protein profiles of the samples were visualized and captured using Gel Compac (Bio Rad, USA).

Whole cell protein fingerprint analysis: Firstly, a data matrix was prepared based on the absence or presence of bands, respectively, at each position for all isolates whole cell protein profiles by giving scores of 0 and 1. The matrix data was then analysis by using Numerical Taxonomy and Multivariate Analysis System (NTSYSpc) version 2.1 [3] based on unweighted pair-group method with arithmetic means (UPGMA) [4]. The similarity of the whole cell protein profile among the isolates was calculated according to [5] formulation as follow:

$$\text{Percentage of similarity, } F = \frac{2N_{xy} \times 100 \%}{N_x + N_y}$$

Where,

N_{xy} = number of shared bands

N_x = total number of bands in lane X,

N_y = total number of bands in lane Y

RESULTS

Isolation, identification and numerical taxonomy of *Edwardsiella tarda*: In the present study, a total of 18 isolates of *Edwardsiella tarda* were successfully isolated by using xylose lysine dextrose (XLD) agar plate from African Catfish (*Clarias gariepinus*), Red Hybrid Tilapia (*Tilapia* sp.), Asian Swamp Eel (*Monopterus albus*) and Snakeskin Gouramy (*Trichogaster pectoralis*). Table 1 and 2 showed biochemical and physiological characteristics of the present isolates. 7 isolates namely, C1 to C7 were isolated from African Catfish (*Clarias gariepinus*) whereas two isolates; T1 and T2 were isolated from red hybrid tilapia. Five isolates; E1, E2, E3, E4 and E5 were isolated from Asian Swamp Eel and the rest of isolates (G1, G2, G3 and G4) were obtained from Snakeskin Gourami. Morphological appearance of the all isolates on XLD agar plate was small (1-2 mm diameter) and clear single colony with black at the centre and reddish peripheral ring.

The present isolates were identified using commercial enteric identification kit (BBL, Crystal, USA) with a combination of 20 conventional biochemical tests such as: gram stain, catalase test, oxidase test, indole production test, starch hydrolysis test, casein hydrolysis test, lipid hydrolysis test, gelatine hydrolysis test, blood hemolysis test, hydrogen sulphide production test, motility test, methyl red-voges proskauer (MRVP) tests, arginine dihydrolase test, ornithine decarboxylase test, utilization of glucose test, utilization of lactose test, utilization of maltose test, utilization trehalose test and utilization of xylose test. Physiological tests for temperature tolerance at 4, 28, 37 and 55°C and salt tolerance at 0, 1, 6 and 8% were also carried out. Under a light microscope (Leica, USA) with magnification 1000 X showed that all isolates were Gram negative short rods. All the isolates were able to produce indole, positive in methyl red test and failed in voges-proskauer test. The present isolates were showed positive in hydrogen sulphide production test but failed in arginine dihydrolase test. All the isolates were positive in ornithine decarboxylase test, motility test, catalase test, lipid hydrolysis test, starch hydrolysis test but negative in oxidase test and gelatine hydrolysis test. 66.7 and 27.8% of the present isolates showed β -hemolysis and α -hemolysis against red blood cell of horse, respectively. 5.5% or only one isolates showed γ -hemolysis against red blood cell of horse. All the isolates in the present study were able to ferment

Table 1: Phenotypic characteristics of isolate C1, C2, C3, C4, C5, C6, C7, T1, T2 and E1

Characteristics	C1	C2	C3	C4	C5	C6	C7	T1	T2
Gram stain	-	-	-	-	-	-	-	-	-
Indole production	+	+	+	+	+	+	+	+	+
Methyl red	+	+	+	+	+	+	+	+	+
Voges-Proskauer	-	-	-	-	-	-	-	-	-
H2S production	+	+	+	+	+	+	+	+	+
Arginine dihydrolase	-	-	-	-	-	-	-	-	-
Ornithine decarboxylase	+	+	+	+	+	+	+	+	+
Motility	+	+	+	+	+	+	+	+	+
Oxidase	-	-	-	-	-	-	-	-	-
Catalase	+	+	+	+	+	+	+	+	+
Blood hemolysis	β	β	a	β	a	a	β	a	?
Lipid hydrolysis	+	+	+	+	+	+	+	+	+
Gelatin hydrolysis	+	+	+	+	+	+	+	+	+
Starch hydrolysis	+	+	+	+	+	+	+	+	+
Utilization									
Glucose, acid	+	+	+	+	+	+	+	+	+
L-arabinose	-	-	-	-	-	-	-	-	-
D-Galactose	-	-	-	-	-	-	-	-	+
Inositol, acid	-	-	-	-	-	-	-	-	-
Lactose, acid	-	-	-	-	-	-	-	-	-
Maltose, acid	+	+	+	+	+	+	+	+	+
Mannitol, acid	-	-	-	-	-	-	-	-	+
Mannose, acid	+	+	+	+	+	+	-	+	+
Raffinose, acid	+	-	+	-	-	+	+	+	+
Rhamnose, acid	-	-	-	-	-	-	-	-	-
Sorbitol, acid	-	-	-	-	-	-	-	-	-
Sucrose, acid	-	-	-	-	-	-	-	-	-
Trehalose, acid	-	-	-	+	-	-	-	-	+
Xylose, acid	-	-	-	-	-	-	-	-	-
Melibiose	-	-	-	-	-	-	-	-	-
Adonitol	-	-	-	-	-	-	-	-	-
p-n-p-phosphate	+	+	+	-	+	+	+	+	+
p-n-p a- β -glucoside	-	-	-	+	-	-	-	-	-
p-n-p- β -galactoside	-	-	-	-	-	-	-	-	+
Proline nitroanilide	+	+	+	+	+	+	+	+	+
p-n-p bis-phosphate	-	-	-	-	-	-	-	-	-
p-n-p-xyloside	-	-	-	-	-	-	-	-	-
p-n-p-a-arabinoside	-	-	-	-	-	-	-	-	+
p-n-p-phosphorylcholine	-	-	-	-	-	-	-	-	-
p-n-p- β -glucuronide	-	-	-	-	-	-	-	-	-
p-n-p-N-acetyl glucosaminide	+	+	+	+	+	+	+	+	+
G-L-glutamyl p-nitroanilide	-	-	-	-	-	-	-	+	-
Esculin	-	-	-	-	-	-	-	-	-
p-nitro-DL-phenylalanine	-	-	-	-	-	-	-	-	-
Urea	-	-	-	-	-	-	-	-	-
Glycine	-	-	-	-	-	-	-	-	-
Citrate	+	+	+	+	+	+	+	+	+
Malonic acid	-	-	-	-	-	-	-	-	-
Triphenyl Tetrazolium chloride	+	+	+	+	+	+	+	+	+
Arginine	+	+	+	+	+	+	+	+	+
Lysine	+	+	+	+	+	+	+	+	+
Growth at NaCl									
0%	+	+	+	+	+	+	+	+	+
1%	+	+	+	+	+	+	+	+	+
6%	-	-	-	-	-	-	-	-	-
8%	-	-	-	-	-	-	-	-	-
Growth at									
40C	-	-	-	-	-	-	-	-	-
280C	+	+	+	+	+	+	+	+	+
370C	+	+	+	+	+	+	+	+	+
550C	-	-	-	-	-	-	-	-	-
Identification	ET	ET	ET	ET	ET	ET	ET	ET	ET

Key: ET = *Edwardsiella tarda*, - = negative reaction, + = positive reaction

Table 2: Phenotypic characteristics of isolate E2, E3, E4, E5, G1, G2, G3 and G4

Characteristics	E1	E2	E3	E4	E5	G1	G2	G3	G4
Gram stain	-	-	-	-	-	-	-	-	-
Indole production	+	+	+	+	+	+	+	+	+
Methyl red	+	+	+	+	+	+	+	+	+
Voges-Proskauer	-	-	-	-	-	-	-	-	-
H ₂ S production	+	+	+	+	+	+	+	+	+
Arginine dihydrolase	-	-	-	-	-	-	-	-	-
Ornithine decarboxylase	+	+	+	+	+	+	+	+	+
Motility	+	+	+	+	+	+	+	+	+
Oxidase	-	-	-	-	-	-	-	-	-
Catalase	+	+	+	+	+	+	+	+	+
Blood hemolysis	a	β	β	β	β	β	β	β	β
Gelatin hydrolysis	+	-	-	-	-	-	-	-	-
Lipid hydrolysis	+	+	+	+	+	+	+	+	+
Starch hydrolysis	+	+	+	+	+	+	+	+	+
Utilization									
Glucose, acid	+	+	+	+	+	+	+	+	+
L-arabinose	-	-	-	-	-	-	-	-	-
D-Galactose	-	-	-	-	-	-	-	-	-
Myo-Inositol, acid	-	-	-	-	-	-	-	-	-
Lactose, acid	-	-	-	-	-	-	-	-	-
Maltose, acid	+	+	+	+	+	+	+	+	+
D-Mannitol, acid	-	-	-	-	-	-	-	-	-
D-Mannose, acid	-	+	+	+	+	+	+	+	+
Raffinose, acid	-	-	-	-	-	-	-	-	-
Rhamnose, acid	-	-	-	+	-	-	-	-	-
D-Sorbitol, acid	-	-	-	-	-	-	-	-	-
Sucrose, acid	-	-	-	-	-	-	-	-	-
Trehalose, acid	-	+	+	-	+	-	-	-	-
D-Xylose, acid	-	-	-	-	-	-	-	-	-
Melibiose	-	-	-	-	-	-	-	-	-
Adonitol	-	-	-	-	-	-	-	-	-
p-n-p-phosphate	+	-	+	+	+	+	+	+	+
p-n-p a-β-glucoside	-	-	-	-	-	-	-	-	-
p-n-p-β-galactoside	-	-	-	-	-	-	-	-	-
Proline notroanilide	+	+	+	+	+	+	+	+	+
p-n-p bis-phosphate	-	-	-	-	-	-	+	-	-
p-n-p-xyloside	-	-	-	-	-	-	-	-	-
p-n-p-a-arabinoside	-	-	-	-	-	-	-	-	-
p-n-p-phosphorylcholine	-	-	-	-	-	-	-	-	-
p-n-p-β-glucuronide	-	-	-	-	-	-	+	+	-
p-n-p-N-acetyl glucosaminide	+	+	+	+	+	+	+	+	+
G-L-glutamyl p-notroanilide	-	-	-	-	-	-	-	-	-
Esculin	-	-	-	-	-	-	-	-	-
p-nitro-DL-phenylalanine	-	-	-	-	-	-	-	-	-
Urea	-	-	-	-	-	-	-	-	+
Glycine	-	-	-	-	-	-	-	-	-
Citrate	+	+	+	+	+	+	+	+	+
Malonic acid	+	+	+	+	+	+	+	+	+
Triphenyl Tetrazolium chloride	+	+	+	+	+	-	+	-	+
Arginine	+	+	+	+	+	+	+	+	+
Lysine	+	+	+	+	+	+	+	+	+
Growth at NaCl									
0%	+	+	+	+	+	+	+	+	+
1%	+	+	+	+	+	+	+	+	+
6%	-	-	-	-	-	-	-	-	-
8%	-	-	-	-	-	-	-	-	-
Growth at									
40C	-	-	-	-	-	-	-	-	-
280C	+	+	+	+	+	+	+	+	+
370C	+	+	+	+	+	+	+	+	+
550C	-	-	-	-	-	-	-	-	-
Identification	ET	ET	ET	ET	ET	ET	ET	ET	ET

Key: ET = *Edwardsiella tarda*, + = positive reaction, - = negative reaction

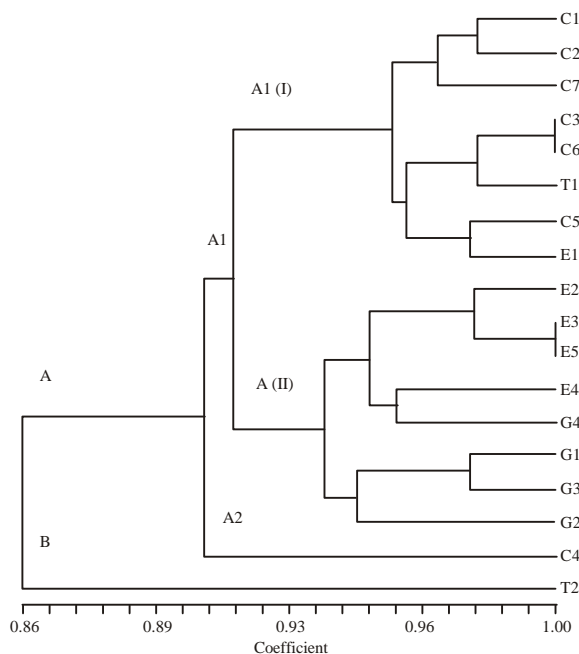


Fig. 1: Dendrogram generated based on phenotypic characteristics of present isolates

glucose but failed to ferment L-arabinose, inositol, lactose, rhamnose, sorbitol, sucrose, xylose, melibiose, adonitol, p-n-p bis-phosphate, p-n-p-xyloside, p-n-p-phosphorylcholine, p-n-p-β-glucuronide, Esculin, p-nitro-DL-phenylalanine, glycine, malonic acid and lysine. In the present study, only one or 5.5% of isolate was found to ferment D-galactose, D-mannitol, L-Rhamnose, p-n-p bis-phosphate, p-n-p-β-glucoside, p-n-p-a-arabinoside, L-glutamyl p-notroanilide, p-nitro-DL-phenylalanine and urea. 88.9% of the present isolates were able to ferment mannose and triphenyl tetrazolium chloride. 77.8, 22.2 and 11.1% of the present isolates were able to ferment raffinose, trehalose and p-n-p-β-glucuronide, respectively. Furthermore, only two or 11.1% of the present isolates were found to be failed in p-n-p-phosphate. In the physiological test, all the isolates of the present study can grow well at 28 and 37°C. However, they were failed to grow at 0 and 55°C. In the NaCl tolerance test, all isolates were found to grow on medium that supplemented with 1% and without NaCl and but failed to grow in medium supplemented with 6 and 8% NaCl.

A dendrogram (Fig. 1) was successfully generated based on the biochemical and physiological characteristics of the present isolates. The dendrogram was consisted of 2 clusters, namely Cluster A and B. Cluster A possess 2 subclusters (Subcluster A1 and

Table 3: Percentage similarity of phenotypic characteristics of present isolates

	C1	C2	C3	C4	C5	C6	C7	T1	T2	E1	E2	E3	E4	E5	G1	G2	G3	G4	
C1	100																		
C2	98.0	100																	
C3	98.0	95.8	100																
C4	92.0	93.9	89.8	100															
C5	95.8	97.9	97.9	91.7	100														
C6	98.0	95.8	100.0	89.8	97.9	100													
C7	98.0	95.8	95.8	89.8	93.6	95.8	100												
T1	96.0	93.9	98.0	88.0	95.8	98.0	93.9	100											
T2	88.9	86.8	90.6	85.2	88.5	90.6	86.8	88.9	100										
E1	93.6	95.7	95.7	89.4	97.8	95.7	95.7	93.6	86.3	100									
E2	91.7	93.6	89.4	95.8	91.3	89.4	89.4	87.5	84.6	88.9	100								
E3	93.9	95.8	91.7	93.9	93.6	91.7	91.7	89.8	86.8	91.3	97.9	100							
E4	93.9	95.8	91.7	89.8	93.6	91.7	91.7	89.8	83.0	91.3	93.6	95.8	100						
E5	93.9	95.8	91.7	93.9	93.6	91.7	91.7	89.8	86.8	91.3	97.9	100.0	95.8	100					
G1	93.6	95.7	91.3	89.4	93.3	91.3	91.3	89.4	82.4	90.9	93.3	95.7	95.7	95.7	100				
G2	92.0	93.9	89.8	88.0	91.7	89.8	89.8	88.0	81.5	89.4	91.7	93.9	93.9	93.9	93.6	100			
G3	91.7	93.6	89.4	87.5	91.3	89.4	89.4	87.5	80.8	88.9	91.3	93.6	93.6	93.6	97.8	95.8	100		
G4	93.9	95.8	91.7	89.8	93.6	91.7	91.7	89.8	83.0	91.3	93.6	95.8	95.8	95.8	95.7	93.9	93.6	100	

A2). Subcluster A1 included 2 groups, namely A1 (I) and A1 (II). Group A1 (I) contained 2 subgroups. The first subgroup included C1, C2 and C7 whereas the second subgroup consisted of C3, C5, C6, T1 and E1; which C3 and C6 shared similar phenotype characteristics. Group A1 (II) was also possessing 2 subgroups. The first subgroup included isolate E2, E3, E4, E5 and G4 which E3 and E5 shared similar phenotype characteristics. The second subgroup contained 3 isolates (G1, G2 and G3). Only isolate (C4) was found in the Subcluster A2 as well as Cluster B contained only isolate T2 alone in its own cluster.

The percentage of similarity of the biochemical and physiological characteristics of the present isolates was ranged from 80.8 to 100% as showed in Table 3. The percentage similarity of the isolates from African Catfish was ranged from 89.8 to 100%. 2 isolates from Red Hybrid Tilapia shared 88.9% of similarity of biochemical and physiological characteristic. The percentage similarity of the isolates from Asian Swamp Eel and Snakeskin Gouramy were ranged from 88.9 to 100% and 93.6% to 97.8%, respectively. Comparison percentage of similarity among the isolates from African catfish to red hybrid tilapia, Asian Swamp Eel and Snakeskin Gouramy was ranged from 85.2 to 98.0%, 89.4 to 95.8% and 87.5 to 95.8%, respectively. The percentage similarity of the isolates from Red Hybrid Tilapia compare to the isolates from Asian Swamp Eel and Snakeskin Gouramy was ranged from 83.0 to 93.6% and 80.8 to 89.8%, respectively. Comparison the percentage

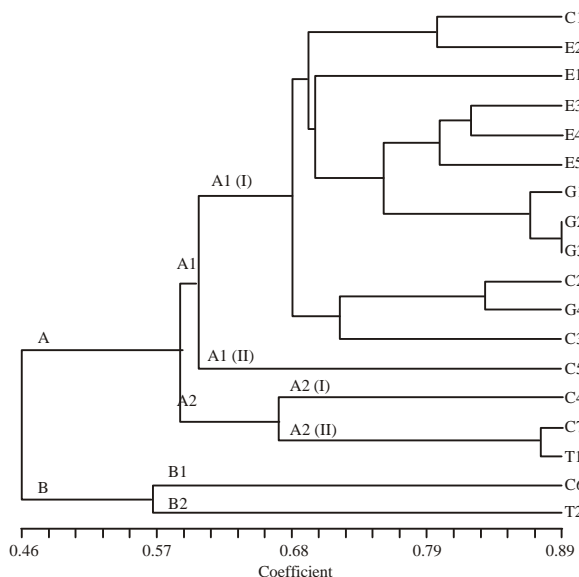


Fig. 2: Dendrogram generated based on RAPD-PCR profile of the present isolates

similarity of the isolates from Asian Swamp Eel to the isolates from Snakeskin Gouramy was ranged from 88.9% to 95.8%.

RAPD-PCR fingerprinting: In the present study, 3 different types of universal primers namely, (GTG)₅, Wild Type Phage (WTP) and M13 universal can generate multibanded RAPD PCR profile. Based on the generated RAPD PCR profile of the isolates in the present study a dendrogram (Figure 2) was generated by using NTSYSpc

Table 4: Genetic distance (below diagonal) and percentage of similarity,%F (above diagonal) between isolates

	C1	C2	C3	C4	C5	C6	C7	T1	T2	E1	E2	E3	E4	E5	G1	G2	G3	G4
C1	-	0.267	0.375	0.333	0.429	0.334	0.27	0.281	0.544	0.29	0.206	0.238	0.3	0.357	0.311	0.357	0.311	0.379
C2	73.3	-	0.222	0.391	0.391	0.5	0.472	0.407	0.574	0.346	0.321	0.321	0.283	0.304	0.255	0.261	0.294	0.167
C3	62.5	77.8	-	0.44	0.44	0.433	0.509	0.449	0.647	0.393	0.263	0.298	0.263	0.32	0.236	0.36	0.309	0.346
C4	66.7	60.9	56	-	0.333	0.577	0.306	0.36	0.581	0.458	0.429	0.429	0.49	0.476	0.49	0.476	0.49	0.409
C5	57.1	60.9	56	66.7	-	0.538	0.469	0.44	0.628	0.5	0.469	0.347	0.347	0.381	0.362	0.381	0.404	0.318
C6	66.6	50	56.7	42.3	46.2	-	0.492	0.433	0.434	0.414	0.492	0.424	0.492	0.462	0.333	0.462	0.404	0.519
C7	73	52.8	49.1	69.4	53.1	50.8	-	0.123	0.6	0.382	0.429	0.357	0.429	0.388	0.37	0.429	0.333	0.529
T1	71.9	59.3	55.1	64	56	56.7	87.7	-	0.569	0.393	0.404	0.333	0.404	0.36	0.309	0.4	0.382	0.462
T2	45.6	42.6	35.3	41.9	37.2	56.6	40	43.1	-	0.469	0.68	0.64	0.64	0.721	0.625	0.628	0.625	0.689
E1	71	65.4	60.7	54.2	50	58.6	61.8	60.7	53.1	-	0.345	0.309	0.273	0.333	0.245	0.333	0.321	0.44
E2	79.4	67.9	73.7	57.1	53.1	50.8	57.1	59.6	32	65.5	-	0.25	0.286	0.306	0.296	0.347	0.333	0.451
E3	76.2	67.9	70.2	57.1	65.3	57.6	64.3	66.7	36	69.1	75	-	0.179	0.224	0.222	0.265	0.259	0.412
E4	70	71.7	73.7	51	65.3	50.8	57.1	59.6	36	72.7	71.4	82.1	-	0.184	0.272	0.265	0.259	0.333
E5	64.3	69.6	68	52.4	61.9	53.8	61.2	64	27.9	66.7	69.4	77.6	81.6	-	0.234	0.238	0.277	0.318
G1	68.9	74.5	76.4	51	63.8	66.7	63	69.1	37.5	75.5	70.4	77.8	72.8	76.6	-	0.149	0.115	0.265
G2	64.3	73.9	64	52.4	61.9	53.8	57.1	60	37.2	66.7	65.3	73.5	73.5	76.2	85.1	-	0.106	0.273
G3	68.9	70.6	69.1	51	59.6	59.6	66.7	61.8	37.5	67.9	66.7	74.1	74.1	72.3	88.5	89.4	-	0.347
G4	62.1	83.3	65.4	59.1	68.2	48.1	47.1	53.8	31.1	56	54.9	58.8	66.7	68.2	73.5	72.7	65.3	-

program and successfully separated the present isolates into two distinct clusters namely, Cluster A and B. Cluster A contained of 2 subclusters (A1 and A2). Each of the subcluster contained of 2 groups. Under Subcluster A1 consists of Group A1 (I) and Group A1 (II). Group A1 (I) composed of 2 subgroups. The first subgroup consists of C1, E1, E2, E3, E4, E5, G1, G2 and G3; which G2 and G3 shared similar pattern of RAPD-PCR profile. The second subgroup comprised of C2, C3 and G4. Group A1 (II) included only C5. Subcluster A2 was subdivided into 2 group; A2 (I) and A2 (II). The Group A2 (I) was only consisted of C4 alone in its own cluster whereas C7 and T1 were included under Group A2 (II). Cluster B contained only isolate C6 and T2 which each under Subcluster B1 and B2, respectively.

Table 4 showed genetic distance [7] and percentage of similarity of RAPD-PCR profile among 18 isolates of the present study. The value for both genetic distance and percentage of similarity were invertly correlated. The percentage similarity and genetic distance among the present isolates based on the RAPD PCR profile were ranged from 27.9% to 89.4% and 0.106 to 0.721, respectively. The isolates from African Catfish showed the highest ranging of percentage of similarity and genetic distance recorded from 42.3% to 77.8% and 0.222 to 0.577, respectively. The percentage similarity and genetic distance among isolates obtained from Red Hybrid Tilapia were 43.1% and 0.569, respectively. The percentage similarity and genetic distance isolates isolated from Asian Swamp Eel were ranged 65.5 to 81.6% and 0.179 to 0.345, respectively. Whilst, the percentage of similarity and genetic distance of isolates from Snakeskin Gouramy

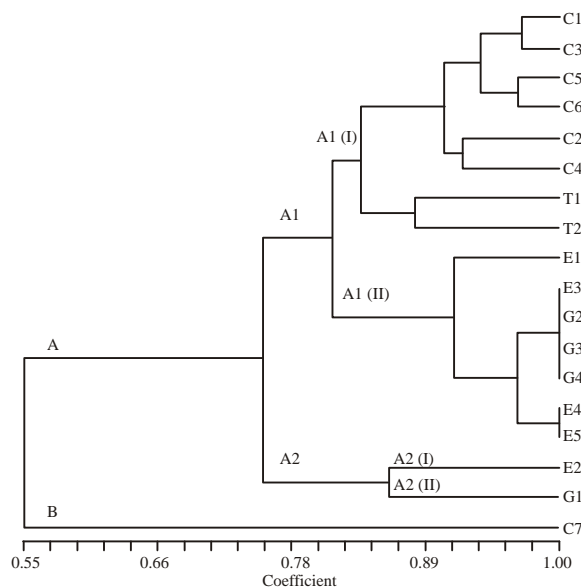


Fig. 3: Dendrogram generated based on whole cell protein profile of the present isolates

were ranged 65.3 to 89.4% and 0.106 to 0.347, respectively. The percentage similarity of isolates from African Catfish compared to isolates from Red Hybrid Tilapia, Snakeskin Gouramy and Asian Swamp Eel was ranged from 35.3 to 87.7%, 47.1 to 83.3% and 50 to 79.4%, respectively. Comparison percentage similarity of isolates from Red Hybrid Tilapia to Snakeskin Gouramy and Asian Swamp Eel was ranged from 27.9 to 66.7% and 31.1 to 69.1%, respectively. The percentage similarity between isolates from Snakeskin gouramy and Asian swamp eel was ranged from 54.9 to 76.6%.

Table 5: Percentage of similarity based on proteotypic characteristics of isolates

	C1	C2	C3	C4	C5	C6	C7	T1	T2	E1	E2	E3	E4	E5	G1	G2	G3	G4
C1	100																	
C2	94.4	100																
C3	97	91.4	100															
C4	91.4	91.9	94.1	100														
C5	93.8	88.2	96.8	90.9	100													
C6	90.3	84.9	93.3	87.5	96.6	100												
C7	50	46.2	52.2	48	54.6	57.1	100											
T1	86.7	81.3	89.7	83.9	92.9	88.9	60	100										
T2	75.9	77.4	78.6	80	81.5	84.6	63.2	88	100									
E1	82.8	77.4	78.6	73.3	74.1	69.2	52.6	72	66.7	100								
E2	82.8	77.4	78.6	73.3	81.5	76.9	52.6	80	66.7	83.3	100							
E3	90.3	84.9	86.7	81.3	82.8	78.6	57.1	81.5	76.9	92.3	84.6	100						
E4	87.5	82.4	83.9	84.9	80	75.9	54.6	78.6	74.1	88.9	81.5	96.6	100					
E5	87.5	82.4	83.9	84.9	80	75.9	54.6	78.6	74.1	88.9	81.5	96.6	100	100				
G1	69.2	64.3	64	59.3	66.7	60.9	62.5	63.6	57.1	85.7	85.7	78.3	75	78.3	100			
G2	90.2	84.9	86.7	81.3	82.8	78.6	57.1	81.5	76.9	92.3	84.6	100	96.6	78.3	78.3	100		
G3	90.2	84.9	86.7	81.3	82.8	78.6	57.1	81.5	76.9	92.3	84.6	100	96.6	78.3	78.3	100	100	
G4	90.2	84.9	86.7	81.3	82.8	78.6	57.1	81.5	76.9	92.3	84.6	100	96.6	96.6	78.3	100	100	100

Whole cell protein profiling: In present study, SDS PAGE assay was successfully generated whole cell protein profile of the present isolates. A dendrogram (Figure 3) was also generated based on the whole cell protein profile of the isolates by using NTSYSpc programme. The dendrogram consisted of 2 clusters namely Cluster A and B. Cluster A was divided into 2 subclusters (A1 and A2). Subcluster A1 was subdivided into Group A1 (I) and A (II). Group A1 was contained 2 subgroups. The first subgroup comprised of C1, C2, C3, C4, C5 and C6 whereas the second subgroup included T1 and T2. The Group A2 was subdivided into 2 subgroups. The first subgroup included only E1 whereas the second subgroup possess 6 isolates namely E3, E4, E5, G2, G3 and G4. E3, G2, G3 and G4 were shared similar whole cell protein profile. Whilst, E4 and E5 shared similar whole cell protein profile. Subcluster A2 was contained 2 groups. Each Group A2 (I) and A2 (II) only contained one isolate; E2 and G1, respectively. The Cluster B was only consisted of C7 alone in its own cluster.

Table 5 showed percentage similarity and whole cell protein distance among the isolates in the present study. The values for both percentage similarity was calculated based on formulation [5]. The percentage similarity of the present isolates was ranged from 46.2 to 100%. The isolates from African Catfish showed the highest ranging of percentage similarity (46.2 to 97%). The percentage similarity between the 2 isolates from Red Hybrid Tilapia was 88%. The percentage similarity among the isolates from Asian Swamp Eel was ranged from 81.5 to 100%.

Among the isolate from Snakeskin Gouramy of percentage similarity was ranged from 78.3 to 100%. The comparison percentage similarity of the isolates from African Catfish to Red Hybrid Tilapia, Asian Swamp Eel and Snakeskin Gouramy was ranged from 60 to 92.9%, 52.6 to 90.3% and 57.1 to 90.2%, respectively. The percentage similarity of isolates from Red Hybrid Tilapia compared to isolates from Asian Swamp Eel and Snakeskin Gouramy was ranged from 66.7 to 81.5% and 51.7 to 81.5%, respectively. The percentage of similarity between the isolates from Asian Swamp Eel and Snakeskin Gouramy was ranged from 75 to 100%.

DISCUSSION

Currently, the dangerous and threatening of *Edwardsiella tarda* to several of hosts has been widely reported. Its hosts were ranged cases from amphibian, reptiles and fish as well as human beings. *E. tarda* was well known as causative agent of gastroenteritis, diarrheal, bacteremia, wound infection as well as death to human [8]. So far, there were reports on *E. tarda* infection freshwater fish cultures especially in channel catfish. However, the study of *E. tarda* infection in African Catfish, Red Hybrid Tilapia, Snakeskin Gouramy and Asian Swamp Eel has not been reported especially in Malaysia scenario. Furthermore, the information of phenotypic and genotypic as well as proteotypic characteristics of *E. tarda* especially from aquaculture sites is still lacking. This was supported by

[9] stated that study of phenotypic and genotypic characteristics of *E. tarda* from freshwater fish culture system was rarely conducted. Therefore, this study was carried out to reveal phenotypic, genotypic and proteotypic characteristics of *E. tarda* isolated from freshwater fish cultured in Malaysia as well as the suitable tool to discriminate them.

In the present study, 20 conventional biochemical and physiological tests together with used of bacterial commercial identification kit was successfully identify the 18 present isolates as *E. tarda*. Most of the phenotypic characteristics of the isolates were similar as claimed in [10]. The present isolates showed no variation was found in most of the biochemical and physiological tests except for red blood cell hemolysis, galactose, mannitol, mannose, raffinose, rhamnose, trehalose, p-n-p-phosphate, Triphenyl Tetrazolium chloride, p-n-p bisphosphate, (-L-glutamyl p-nitroanilide, p-n-p-\$-glucuronide, p-nitro-DL-phenylalanine, p-n-p "- β -glucoside, p-n-p bis-phosphate p-n-p-\$-galactoside and urea. Only one isolate, T2, in the present study was showed no hemolysis of red blood cell activity as well as 15 of the 16 isolates from study of [11] that obtained them from snakeheads (*Ophiocephalus punctatus*). According to [12], hemolysin is an important virulence factor of many species of bacteria including *E. tarda*. All isolates obtained from wild freshwater fish were performed beta hemolysis activity except for isolate E1 which showed alfa hemolysis activity. On the other hand, only 4 out of 9 isolates from cultured freshwater fish showed beta hemolysis activity whereas other 4 isolates were showed " hemolytic activity. Only one isolate failed to hemolysis red blood cell of horse. Here, we can concluded that *E. tarda* from wild freshwater fish were more virulent compared to the strains from cultured freshwater fish. Furthermore, [13] described the ability of *E. tarda* to perform hemolysis activity against red blood cell because it's possessed 2 types of gene, namely Eth A and Eth B. All the present isolates were failed in galactose utilization test except for the isolate, T2, was only found to be positive as well as 2 isolates of *E. tarda* in the study of [14]. In the present study, only one isolate was found able to utilize mannitol as well as the isolate from the study of [11]. However, all the 43 isolates in the study of [15] were unable to utilize mannitol. Only two of present isolates were found failed to utilize mannose whereas the rest isolates were able to ferment mannose. On the other hand, all the isolates in the studies of [15] and [16] were failed to utilize mannose. However, all the isolates obtained from diseased striped bass in the study of [14]

were also found able to utilize mannose. None of 44 isolates of *E. tarda* in the study of [15] were able to utilize rhamnose as well as the isolates in the studies of [14], [16] and [17]. Nevertheless, only one isolate of the present study was able to ferment rhamnose. 72% of the present isolates were not able to utilize trehalose as well as all the isolates from the study of [15]. Phenotypic characteristics of the 24 isolates of *E. tarda* in the study of [9] showed variation only in citrate utilization, one out of 13 biochemical tests conducted. The isolates of *E. tarda* in the study of [11] also exhibited variation in citrate utilization where 2 only out of 16 isolates were failed in citrate utilization. However, in the present study, no variation was found in terms of citrate utilization test where all isolates of *E. tarda* showed positive in citrate utilization test. On contrast, all the isolates in the studies of [14] and [17] failed in citrate utilization test. In the present study, only one isolate from Snakeskin Gouramy namely G5 was found able to utilize urea whereas the rest isolates were failed in urea test. However, [10] claimed that more than 90% isolates of *E. tarda* were failed to utilize urea as well as the isolates from the studies of [11] and [9] as well as [18]. [9] suggested that the occurrence of variation in phenotypic characteristics among *E. tarda* may due to the presence and absence of plasmid that control the metabolic of the phenotypic characteristics of the *E. tarda*.

RAPD-PCR fingerprinting revealed high degree of genetic diversity of present isolates with primers (GTG)₅, M13 universal and M13 wild type phage. This molecular tool showed the highest ranging of percentage similarity as well as genetic distance among the isolates compared to numerical taxonomy and whole cell protein profiling of the present isolates. Many studies has been used primer (GTG)₅ to discriminate bacteria especially for *Vibrio* species. The studies of [19] and [20] claimed the usefulness of primer (GTG)₅ in terms of discriminating *Vibrio* species whereas primer M13 Universal was also able to discriminate 40 strains of *Francisella tularensis* [21]. Primer M13 wild type phage in the study of [22] was found to discriminate *Cryptococcus neoformans* accordingly to their geographical area. However, application these primers in the present study were failed to discriminate the isolates based on their sources and hosts. Many studies showed that RAPD-PCR was failed to discriminate *E. tarda* according the sources or hosts where they were isolated. For instance, in the study of [9], RAPD-PCR was failed to discriminate 24 isolates of *E. tarda* according to their sources where they were obtained. In the study of [23], genotyping of 53 isolates

of *E. tarda* was also failed to group the isolates according to sources and hosts properly. Hence, we could assume that *E. tarda* possess high degree of genetic diversity may be due to the environmental adaptation [24].

Whole cell protein profiling of the present isolates analysis by SDS PAGE was successfully grouping the isolates according to their sources where they were obtained. Generally, the isolates from cultured freshwater fish (African Catfish and Red Hybrid Tilapia) were included in the same group as well as the isolates from wild freshwater fish were grouped in the similar group. Actually, whole cell protein profiling of bacteria analysis by SDS PAGE has been widely used for discriminating bacteria. The study of [25] characterized SDS PAGE profiles of whole cell proteins of 68 strains and successfully discriminating them. Furthermore, [26] claimed that whole cell protein analysis by SDS PAGE is a useful method and able to differentiate various strains of *Xylella fastidiosa*, a plant pathogen. A recent study of [27] showed that whole cell protein analysis by SDS PAGE of 9 and 18 isolates of *E. tarda* and *E. ictaluri*, respectively, was successfully discriminating and clustered the isolates according to their species properly. Therefore, whole cell protein analysis by SDS PAGE may provide a rapid, low cost, simple and powerful tool to discriminate and differentiate various strains of bacteria.

In the present study, biochemical and physiological tests were successfully identify a total of 18 strains of *E. tarda* but failed to discriminate the present isolates as well as genotyping by using primers of (GTG)₅, M13 universal primer and M13 wild type phage primer. However, it was interesting to note that proteomic typing by using one dimension SDS PAGE was able to discriminate the present isolates. Generally, the isolates that obtained from fish that sampled from freshwater culture system were gathered into one group as well as the isolates from fish that caught from natural environment. In other words, based on the data of whole cell protein profiling obtained from this study, we can hypothesize that cross infection of edwardsiellosis between cultured and wild freshwater fish might not occur. Since whole cell protein profiling of the present isolates using one dimensional SDS PAGE can discriminate the isolates, we concluded that this molecular tool may be helpful in establishing the source of infection of cases of *E. tarda* in both cultured and wild freshwater fish. This study is the first report on edwardsiellosis in African Catfish, Red Hybrid Tilapia, Asian Swamp Eel and Snakeskin Gouramy in Malaysia. This information should alert fish farmers as well as processing plants to monitor the presence of *E. tarda* from both cultured and wild freshwater fish in Malaysia.

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