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ISOLATION AND IDENTIFICATION OF *Elizabethkingia meningoseptica* FROM DISEASED AFRICAN CATFISH *Clarias gariepinus*

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ABSTRACT

Two isolates of *Elizabethkingia meningoseptica* were successfully isolated from kidney and skin tissue of diseased African Catfish (*Clarias gariepinus*) in Malaysia. The percentage similarity of both physical and biochemical characteristics for the isolates from kidney (K1) and skin samples (S1) as determined by BBL-Crystal and API 20E were 99 % and 95.4%, respectively. Furthermore, both isolates were identified *via* 16S rRNA gene sequences and showed more than 97% homology to sequences deposited in GenBank. The API ZYM results were analogous for both strains, with only minor quantitative variations. However, the isolates from kidney sample (K1) showed higher levels of enzymatic activity reaction towards esterase lipase and leucine arylamidase. Moreover, the enzymatic activity of α-galactosidase was detected at low level in kidney isolate and absence in skin isolate.

Keywords: *Elizabethkingia meningoseptica*, polymerase chain reaction, *Clarias gariepinus*, BBL-Crystal, Enzymatic activity

INTRODUCTION

Bacteria display diverse morphologies when they are grown on different culture media. Thus, bacterial species identification is determined using morphological, biochemical and physiological investigations. For instance, Gram staining, which is based on the amount of peptidoglycan in the bacteria cell wall, as well as several series of biochemical tests are classical method for bacterial characterization **(Pollack *et al.*, 2008)**. Nowadays, molecular methods are used to identify an organism **(Jacobs & Chenia *et al.*, 2011**).
Elizabethkingia meningoseptica has been recently isolated from diseased American bullfrog in Malaysia **(Ransangan *et al.*, 2013)**; however, never has it been isolated from *Clarias gariepinus* . *E. meningoseptica* is the etiological agent of disease in South African clawed frog (*Xenopus laevis*) and leopard frog (*Rana pipiens*) **(Xie *et al.*, 2009)**. This bacterium is also a significant human pathogen, where it has been found to cause pneumonia, meningitis, postoperative bacteraemia in adults, fatal necrotizing fasciitis in diabetic patients, and meningitis in premature neonates **(Bloch *et al., 1997;* Gupta *et al.*, 1997; Lee *et al.*, 2006; Lee *et al.*, 2008).**
Presently, *E. meningoseptica* infections in aquaculture are unknown **(Kirby *et al.*, 2004)**. Therefore, the present study was aimed to isolate and identify *E. meningoseptica* from diseased catfish (*Clarias gariepinus*). This is the first study to isolates *E. meningoseptica* from fish in Malaysia.

MATERIALS AND METHODS

Bacterial Isolation

Diseased *Clarias gariepinus* catfish were collected from a local fish farm located at Marang River Terengganu, Malaysia (05°12'N, 103°13'E). The fish weighed from 350 to 400 g. The fish were dissected according to **Wilson *et al* (2009)** which were performed using standard methods **(Buller *et al.*, 2004)**. Shieh agar supplemented with tobramycin (1 mg ml^{-1}), enriched Anacker and Ordal's agar (EAOA) supplemented with polymyxin B (10 U ml^{-1}) and neomycin (5 µg ml^{-1}) were prepared. Bacteriological swabs were aseptically taken from the skin lesions, liver and kidney of catfish. Incubation was done at 28°C for 48 h. The presence of flexirubin-type pigments in bacterial colony was determined using the KOH method **(Bernardet *et al.*, 2002)**. Shieh agar and Anacker and Ordal's agar (EAOA) were selected to isolate and maintain the bacteria. The pure cultures were kept in enriched Anacker and Ordal's broth (EAOB) supplemented with 20% glycerol at -80°C.

Bacterial Identification

The Gram reaction was performed as described by **Gerhardt *et al* (1994).**The bacterial colony morphology was determined after 48 h of incubation at 28°C. The bacterial isolates were characterized biochemically by standard biochemical tests (e.g. Indole Test, Methyl Red Test, Voges Proskauer Test, Urease Test, Catalase Test, Oxidase Test, Gelatin Hydrolysis, Oxidative fermentative utilization of glucose, Simmons citrate test, H_2S production, Casein hydrolysis, Acid and gas production from sugars, phenylalanine test, β-galactosidase test, lysine decarboxylase and arginine dehydrolase, and Starch Hydrolysis). Physiological temperature tolerance tests at 5°C, 28°C, 37°C, 42°C, and salt tolerance at different concentrations (0.5 %, 1.5 %, 3 %, 6 %, 8 %, 10 %) of NaCl (w/v) were also performed according to **Cappuccino & Sherman *et al* (1996)**; **Prescott *et al* (2005)**; **Pollack *et al* (2008)**. Flexirubin-type pigment was detected with 20 % KOH according to the method of **Fautz & Reichenbach *et al* (1980)**. The isolates were subjected to hemolysis test as described by **Pavlov *et al* (2004).** The isolates were further biochemically identified using BBL-Crystal™Enteric/Non Fermenter Identification System Kit (USA), API 20E, and API ZYM System (bioMerieux, France) according to the manufacturers' instructions.

16S rRNA Identification

The DNA was extracted from the isolates using Wizard Genomic DNA Purification Kit (Promega, USA) according to the manufacturer's instruction. 16S rRNA gene PCR was carried out using published universal bacterial primers (5'-GAT TAG ATA CCC TGG TAG TCC AC-3' and 5'-CCC GGG AAC GTA TTC ACC G-3') to generate a fragment approximately 610 bp in length according to **Kim *et al* (2003)** and **Sun *et al* (2008)** using MasterCycler Personal (Eppendoft, Germany). The PCR was performed with minor modification. The reaction began with an initial denaturation step of 96°C for 2 min followed by 35 cycles of 96°C for 1 min, 60°C for 1 min, and 72°C for 1 min. The reaction was terminated by an extension step of 72°C for 10 min. Negative control with sterile distilled was included in PCR. The PCR products were analyzed using 1.5% agarose gel (molecular grade) electrophoresis, stained with ethidium bromide, and viewed under ultraviolet light. The PCR products were purified using Gene

MATRIX PCR/DNA Clean-Up DNA Purification Kit (EURx, Poland). The DNA sequences deduced were subjected to standard basic local alignment search tool (BLAST) analysis at National Center for Biotechnology Information **(NCBI, 2012)** to determine the identity of the isolates.

RESULTS

Bacterial Isolation

The diseased fish showed symptoms of increased respiration and lethargy, skin lesions such as white discoloration, shallow hemorrhagic ulcers and deep ulcers with exposed underlying muscle. Some fish showed marked haemorrhages on the base of the fins and vents. Others were dropsy, showed kidney congestion and enlargement, pale-coloured of liver and gills, and/or gall-bladder enlargement with the accumulation of yellowish fluid in the body cavity **(Figure 1).** Bacterial isolates were obtained from kidney (K1) and skin lesion (S1) that were grown on Shieh and enriched with Anacker and Ordal's agar (EAOA). Colonies were small (1 to 2 mm diameter), smooth surface, convex, circular, and shiny with complete edges on EAOA and Shieh agar. Colonies were yellowish and creamy in colour on EAOA agar Shieh agar, respectively. Bacteria were Gram negative, non-motile, straight, single short rods approx. 2 µm in length and 1 µm in width **(Figure 2)**. They were then subjected to identification using conventional, commercial kits, and 16S rRNA PCR.

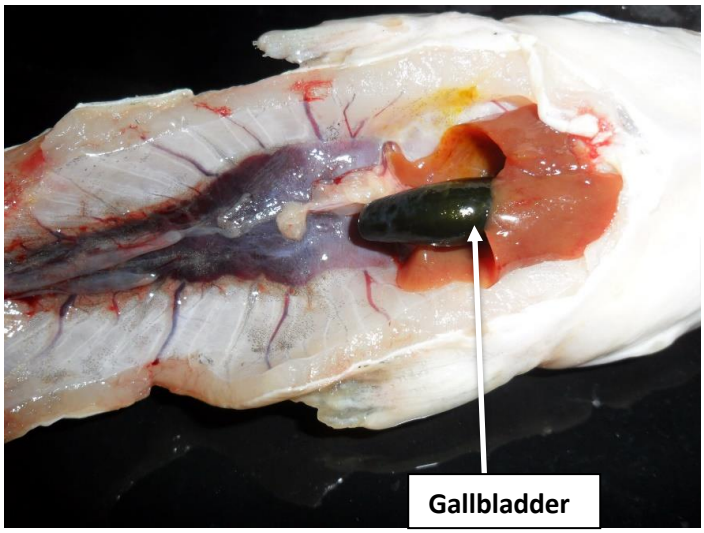

Figure 1 Catfish, *Clarias gariepinus*, infected with *Elizabethkingia meningoseptica* showing enlargement of the gallbladder

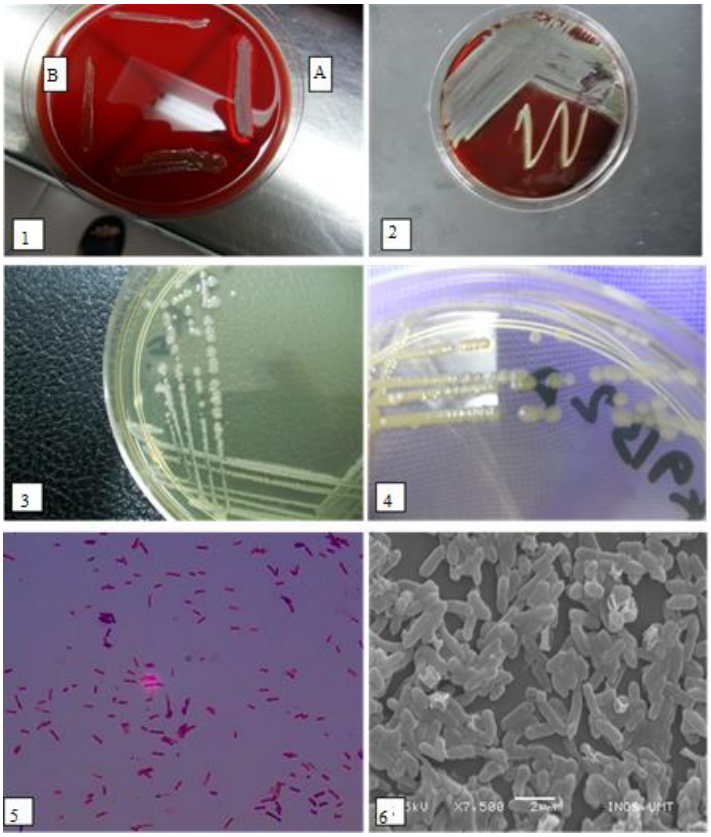

Figure 2 Morphology of *Elizabethkingia meningoseptica*. Blood agar showing haemolytic activity of *E. meningoseptica* (A): Kidney isolates showing beta hemolysis. (B): Skin isolates showing gamma hemolysis; **2**-*E. meningoseptica*, skin isolated growth on blood agar showing gamma haemolytic activity; **3**-(A) Colony morphologies displayed by *E. meningoseptica* isolate on Shieh agar; creamy-white, small shiny convex colonies with smooth edges; **4**-(B) Colony morphologies displayed by *E. meningoseptica* isolate on (EAOA); pale yellow, larger convex colonies with smooth edges; **5**- Light microscope image of Gram-negative staining reaction displayed by *E.meningoseptica* isolates (×1000 magnification); **6**- Scanning Electron Microscope (SEM) of *E.meningoseptica*

Bacterial Identification

Physical and biochemical characteristics of the isolates were carried out according to standard protocol. BBL-Crystal ™ Enteric/Non fermenter Identification System kit (USA), API 20E and API ZYM systems (bioMerieux, France) were used for the purpose. Phenotypic characteristics of kidney isolate and skin isolate are shown in Chyba! Nenašiel sa žiaden zdroj odkazov..

Table 1 Phenotypic characteristics of *E. meningoseptica* isolates

Characteristics	K1	S1
Gram Stain	-	-
Indole production	+	+
Methyl red	-	-
Voges-proskauer	-	-
H_2S production	-	+
Ornithine decarboxylase	-	-
Arginine dihydrolase	+	+
Motility	-	-
Oxidase	+	+
Catalase	+	+
Blood hemolysis	B	γ
Lipid hydrolysis	-	-
Gelatine hydrolysis	+	-
Starch hydrolysis	+	+
Casein hydrolysis	+	+
Utilization		
Glucose,acid	+	+
Lactose,acid	-	-
Maltose,acid	+	+
Raffinose,acid	-	-
Trehalose, acid	+	+
Xylose,acid	-	-

Characteristics	K1	S1
Mannose,acid	-	-
Sucrose,acid	-	-
Rhamnose,acid	-	-
Sorbitol,acid	-	-
Mannitol,acid	-	-
Inositol,acid	-	-
Melibiose	-	-
Arabinose	-	-
Adonitol	-	-
Galactose	-	-
p-nitrophenyl phosphate	+	+
p-nitrophenyl α-β-glucoside	+	+
p-nitrophenyl β-galactoside	-	+
Prolinenotroanilide	+	+
p-nitrophenylbis-phosphate	+	-
p-nitrophenylxyloside	-	-
p-nitrophenyl α-arabinoside	-	+
p-nitrophenylphosphorylcholine	+	-
p-nitrophenly β-glucuronide	-	-
p-nitrophenyl-N-acetyl glucosaminide	+	-
γ-L-glutamyl p-nitroanilide	+	+
Esculin	+	+
p-nitro-DL-phenylalanine	-	-
Urea	-	-
Glycine	+	+
Citrate	-	-
Malonate	-	-
Tetrazolium	+	+
Arginine	-	-
Lysine	+	-
Growth at NaCl		
0.5 %	+	+
1.5 %	+	+
3 %	-	-
6 %	-	-
8 %	-	-
10 %	-	-
Growth at		
5o C	-	-
28o C	+	+
37o C	+	+
42o C	-	-

***** K1:*E. meningoseptica* isolate from the kidney of catfish (*Clarias gariepinus*); S1:*E. meningoseptica* isolate from the skin of catfish (*Clarias gariepinus*)

The isolates produced indole, but scored negative in the Methyl red-Voges – proskauer and hydrogen sulfide tests. Additionally, *Elizabethkingia meningoseptica* strains isolated from kidney showed to be β-hemolytic on blood agar. *E. meningoseptica* strains isolated from skin showed to be γ- hemolytic activity **(**Chyba! Nenašiel sa žiaden zdroj odkazov.**)**.

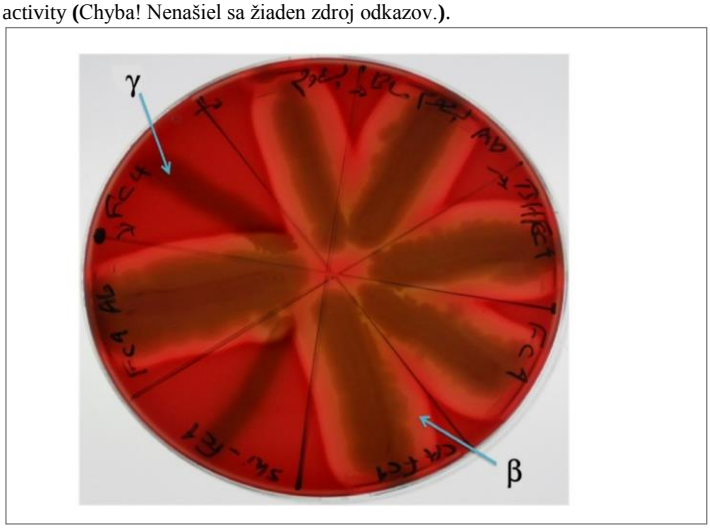

Figure 3 Isolate from kidney showing beta haemolytic activity (β) while Isolate from skin showing gamma haemolytic activity (γ)

In physiological test, both isolates grew at 28˚C and 37˚C. In the salt tolerance test, both isolates grew on media supplemented with 0.5 % and 1.5 % NaCl media. BBL results showed that isolates K1 and S1 were 99 % similar to *E. meningoseptica* **(**
). On the other hand, API 20E identified isolates K1 and S1 as *E. meningoseptica* with 95.4% similarity **(**Chyba! Nenašiel sa žiaden zdroj odkazov.**)**.

Table 2 Biochemical characteristics of *Elizabethkingia meningoseptica* based on BBL-Crystal system

Test	Isolate	
	K1	S1
Oxidase	+	+
Indole	+	+
Arabinose	-	-
Mannose	-	-
Sucrose	-	-
Melibiose	-	-
Rhamnose	-	-
Sorbitol	-	-
Mannitol	-	-
Adonitol	-	-
Galactose	-	-
Inositol	-	-
p-nitrophenyl phosphate	+	+
p-nitrophenyl α-β-glucoside	+	+
p-nitrophenyl β-galactoside	-	+
Prolinenitroanilide	+	+
p-nitrophenylbis-phosphate	+	-
p-nitrophenylxyloside	-	-
p-nitrophenyl α-arabinoside	-	+
p-nitrophenylphosphorylcholine	+	-
p-nitrophenly β-glucuronide	-	-
p-nitrophenyl-N-acetyl glucosaminide	+	-
γ-L-glutamyl p-nitroanilide	+	+
Esculin	+	+
p-nitro-DL-phenylalanine	-	-
Urea	-	-
Glycine	+	+
Citrate	-	-
Malonate	-	-
Tetrazolium	+	+
Arginine	-	-
Lysine	+	-

***** K1: kidney; S1: skin; +: positive - : negative

Table 3 Identification of *Elizabethkingia meningoseptica* based on API20E

Tests	Substrate	Stain Reaction	
		K1	S1
ONPG	ONPG	+	+
ADH	Arginine	-	-
LDC	Lysine	-	-
ODC	Ornithine	-	-
CIT	Citrate	+	+
H2S	Na thiosulfate	-	-
URE	Urea	+	+
TDA	Tryptophan	-	-
IND	Tryptophan	+	+
VP	Na pyruvate	-	-
GEL	charcoal gelatin	+	-
GLU	glucose	-	-
MAN	mannitol	-	-
INO	inositol	-	-
SOR	sorbitol	-	-
RHA	rhamnose	-	-
SAC	sucrose	-	-
MEL	melibiose	-	+
AMY	amygdalin	+	-
ARA	arabinose	-	-
OX	Oxidase	+	+

***** K1: kidney; S1: skin; +: positive - : negative

The characteristics of each isolate tested on the API ZYM system are listed in Chyba! Nenašiel sa žiaden zdroj odkazov. and

Table 5 Hydrolytic enzymes and their substrates assayed based on API ZYM test. The API ZYM results were very similar for both strains, with only minor qualitative variations. Both isolates showed a low level of reactions to the enzymes; Esterase (C4), Lipase (C14), Cystinearylamidase, Trypsin, α-chymotrypsin, β-galactosidase, α-glucosidase, β-glucosidase and N-acetyl-β-glucosaminidase. They were negative for β-glucuronidase and α-mannosidase, but there were higher levels of enzymatic activities such as alkaline phosphatase, valinearylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase. However, isolate K1 showed higher levels of enzymatic activity reaction towards esterase lipase and leucine arylamidase. Furthermore, the enzymatic activity of α-galactosidase was detected at low level in kidney isolate and absence in skin isolate.

Table 4 Enzymatic profiles for *Elizabethkingia meningoseptica* based on API ZYM

Enzymes/Isolates	Substrate	K1	S1
Control		-	-
Alkaline phosphatase	2-naphtylophosphate	+/s	+/s
Esterase (C4)	2-naphtylbutyrate	+/w	+/w
Esterase Lipase (C8)	2-naphtylcapylate	+/s	+/w
Lipase (C14)	2-naphtylmyristate	+/w	+/w
Leucinearylamidase	L-leucyl-2-naphthylamide	+/s	+/w
Valinearylamidase	L-leucyl-2-naphtylamide	+/s	+/s
Cystinearylamidase	L-cystyl-2-naphthylamide	+/w	+/w
Trypsin	N-benzoyl-DL-arrginine-2-naphthylamide	+/w	+/w
α-chymotrypsin	N-glutaryl-phenylalanine-2-naphthylamide	+/w	+/w
Acid phosphatase	2-naphthylphosphate	+/s	+/s
Naphthol-AS-BIphosphohydrolase	Naphthyl-AS-BI-phosphate	+/s	+/s
α-galactosidase	6-Br2-naphthyl- D-galactopyranoside	+/w	-
β-galactosidase	2-naphthyl- D-galactopyranoside	+/w	+/w
β-glucuronidase	Naphthol-AS-BI- D-glucuronide	-	-
α-glucosidase	2-naphthylyl- D-glucopyranoside	+/w	+/w
β-glucosidase	6-Br-2-naphthyl- D-glukopyranoside	+/w	+/w
N-acetyl-β-glucosaminidase	1-naphthyl-N-acetylo- D glucosaminide	+/w	+/w
α-mannosidase	6-Br-2-naphthyl- D-mannopyranoside	-	-
α-fucosidase	2-naphthyl- -L-fukopiranoza	-	-

***** K1: kidney; S1: skin; +: positive; - : negative; W: weak; S: strong

Table 5 Hydrolytic enzymes and their substrates assayed based on API ZYM test

Enzymes/Isolates	K1	S1	Sum
Control	0	0	0
Alkaline phosphatase	5	4	9
Esterase (C4)	3	3	6
Esterase Lipase (C8)	5	3	8
Lipase (C14)	2	2	4
Leucinearylamidase	5	2	7
Valinearylamidase	4	4	8
Cystinearylamidase	3	2	5
Trypsin	1	2	3
α-chymotrypsin	1	1	2
Acid phosphatase	5	5	10
Naphthol-AS-BIphosphohydrolase	4	5	9
α-galactosidase	2	0	2
β-galactosidase	1	1	2
β-glucuronidase	0	0	0
α-glucosidase	1	1	2
β-glucosidase	1	1	2
N-acetyl- β-glucosaminidase	2	3	5
α-mannosidase	0	0	0
α-fucosidase	0	0	0
Sum	45	39	84

* Numbers indicate the relative expression of enzymes which is estimated from the color intensity as strong (4-5), weak (1-3), or negative (0). Mean values were rounded up.

Both isolates had a similar enzymatic profile but kidney isolate had a weak positive reaction for α-galactosidase as compared to skin **(Table 5)**. Both isolates were identified using 16S rRNA gene sequences analysis. PCR amplification of 16S rRNA gene sequences of isolates is presented in **Figure 4**. All 16S rRNA gene sequences showed more than 97% homology to sequences deposited in GenBank. The isolates were identified as *E. meningoseptica*,
16S rRNA sequence alignments of *Elizabethkingia meningoseptica* bacteria isolated from kidney and skin isolates of diseased catfish (*Clarias gariepinus*) are shown in Appendix A and Appendix D, respectively. Genbank accession 16S rRNA S1 (skin) strain and 16S rRNA K1 (kidney) strain of *Elizabethkingia meningoseptica*are highlighted in Appendix H and Appendix G, respectively.

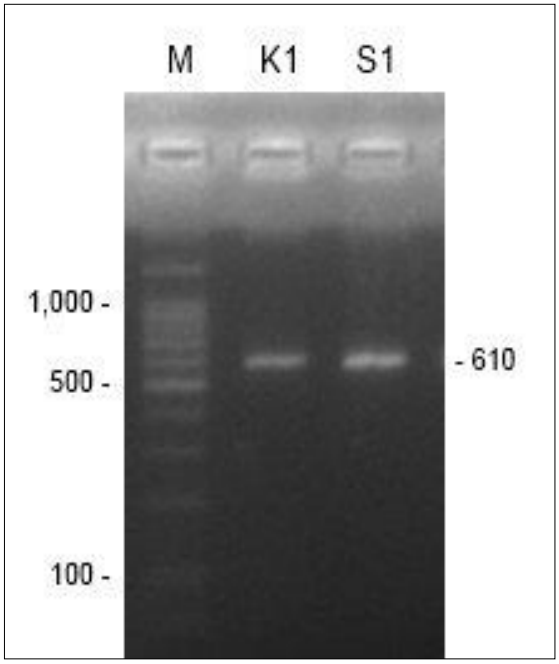

Figure 4 Electrophoresis on 1.5% agarose gel of universal PCR products of 16S rRNA gene of *Elizabethkingia meningoseptica* isolated from kidney (Lane K1) and *Elizabethkingia meningoseptica* isolated from skin (Lane S1) amplified at 610 bp with standard molecular weight marker (M 1 kb)
***** Lane M: 100 bp marker; Lane 2: K1 isolate; Lane 3: S1 isolate

All 16S rRNA gene sequences of the 2 bacterial isolates showed more than 97% homology to sequences were deposited in GenBank with the accession number for kidney isolate BankIt 1604905 KC757123 and for skin isolate BankIt 1611391KC757124. The isolates were identified as *E. meningoseptica*. Blast result and electropherogram are shown in respective appendices (Appendix A, B, C & D).

DISCUSSION

Recently, Gram-negative, non-fermentative bacteria are increasingly implicated in human disease **(Gautam *et al*., 2011)**. In 1959, American bacteriologist Elizabeth O. King was examining unclassified bacteria associated with meningitis in infants when she isolated an organism that she named *Flavobacterium meningosepticum*. In 1994, *Flavobacterium meningosepticum* was reclassified in the genus *Chryseobacterium* and was named *Chryseobacteriummeningosepticum*. In 2005, a 16S rRNA phylogenetic tree illustrated that *C. meningosepticum* and *C. miricola* were related; however, dissimilar to the rest of the Chryseobacteria family. They were then placed in a new genus: *Elizabethkingia* **(Kim *et al*., 2005).**

Elizabethkingia meningoseptica are opportunistic pathogens in both veterinary and human infections. Their pathogenicity and association with food spoilage has caused some economic losses in the aquaculture and food industries. *E. meningoseptica* causes equipment-associated infections in immuno-compromised humans **(Jacobs & Chenia *et al*., 2011**; **Kirby *et al*., 2004; Michel *et al*., 2005)**. *E. meningoseptica* was formerly reported as the etiological agent of disease in South African clawed frogs (*Xenopus laevis*) and leopard frogs (*Rana pipiens*) **(Xie *et al*.,2009)**. In the present research, an appropriate approach was used to analyse bacterial isolates from diseased catfish. Moreover, the present study is the first report of *Elizabethkingia meningoseptica* isolated from catfish in Malaysia. All isolates were identified as *E. meningoseptica* by biochemical and physiological tests, commercial identification kit, and were conformed based on their 16S rRNA gene sequences. Most of the biochemical characteristics of these strains were consistent with *E. meningoseptica* **(Decostere *et al*., 1998**; **Kim *et al*., 2005)**, with the exception of acid being produced from xylose from a few isolates especially those isolated from skin. Based on previous research, the study was carried out to isolate the bacteria from fish. Primary isolates were placed on Shieh agar enriched with a 1 µg ml^{-1}tobramycine **(Decostere *et al*., 1998)**, and the isolates were later enriched on Enriched Anacker and Ordal's agar (EAOA) supplemented with polymyxin B (10 U ml^{-1}) and neomycin (5 µg ml^{-1}) (**Plumb *et al*., 1999)** . In the study of **Bernardet *et al* (2005),** it was reported that Shieh agar and enriched Anacker and Ordal's agar (EAOA) performed well for growing bacteria. On the other hand, these isolates also grew on trypticase soy agar (TSA) at 28˚C for 48 h. The colonies were pale yellow or creamy-white in these entire medium. In the present study, both isolates showed no growth at 5˚C or 42˚C, these finding are in accordance to **Kim *et al* (2005)**, in that good growth was observed on trypticase soy agar (TSA) and nutrient agar at 28˚C to 37˚C. In order to purify the colonies, 2 or 3 replications were performed. For long-term storage, the cultures were kept in Enriched Anacker and Ordal's broth (EAOB) supplemented with 20% glycerol and stored at -80^0 C **(Jacobs & Chenia *et al*., 2011)**. According to **Fijan *et al* (1969)**, addition of polymyxin B (10 U ml^{-1}) and neomycin (5 µg ml^{-1}) to Cytophaga agar could increase the number of *Chondrococcus columnaris* colonies and suppress the growth of other bacteria. Moreover, **Flint *et al* (1985)** showed that inclusion of 10 µg ml-1 kanamycin in nutrient agar could increase the percentage of yellow pigmented colonies from 15% to 55%. In another study, **Decostere *et al* (1998)** revealed that *Flavobacterium columnare* was inhibited by 1 µg ml^{-1} of tobramycin than other fish-associated bacteria.

The results of this study demonstrated that *E. meningoseptica* colonies exhibit the following features: pale yellow to creamy color, round colonies 1 to 2 mm in diameter, this finding is in agreement with previous report by **Kim *et al* (2005)** where *E. meningoseptica* colonies are smooth and fairly large, white yellowish, colonies diameter of 1.0 to 1.5 mm grown on Shieh and Enriched Anacker and Ordal's agar (EAOA). In our study, *E. meningoseptica* was Gram negative, straight rods, non-motile; catalase, and oxidase positive comparable to reports by **Sarma *et al* (2011)**. In addition, *E. meningoseptica* could not ferment the carbohydrate (sugar) as: mannose, sucrose, rhamnose, sorbital, melibiose, arabinose and adonitol as a carbon source as well as nitrate and nitrite were not reduced as an electron acceptor, which is similar to **Kim *et al* (2005)**. The present result revealed *E. meningoseptica* was negative for H_2S production. It was unable to reduce sulfur-containing compounds to sulfides during the process of metabolism. It was unable to hydrolyze*o*-nitrophenyl-B-D-galactopyranoside (ONPG) and esculin. Furthermore, *E. meningoseptica* was able to hydrolysis starch, as a source of carbon and energy for growth. An enzyme called alpha-amylase accomplishes use of starch. These findings are in conformity to the previous research performed by **Kim *et al* (2005)**.Both isolates of *E. meningoseptica* were also tested for gelatinase test with isolates from kidney showed positive result. Our findings are in agreement with **Connell *et al* (2011)** who documented *E. meningoseptica* as being able to produce gelatinases, which contributed to their virulence. Nevertheless, the production of certain enzymes promotes the degradation of macromolecules in the medium, there is a clearing in the areas of the medium where bacteria grow **(Pilarski *et al*., 2008)**. According to the results obtained from study by **Mottar *et al* (1989)**, *E. meningoseptica* produces phospholipase C on lecithin agar, enhancing the lipolysis of fat and causing rancidity.

The result of this study demonstrated that *E. meningoseptica* strains were positive for the following conventional tests: production of catalase and oxidase production; growth in 0.5% and 1.5% NaCl nutrient broth; growth at 28˚C and growth on brain–heart infusion agar, trypticase soy agar, blood agar, and nutrient agars. This results is partially agreed with the results of **Ransangan *et al* (2013)** who reported that *E. meningoseptica* strains isolated from American bullfrog (*Ranac atesbeiana*) were Gram staining negative, non-motile, positive for oxidase and catalase, grew at 28˚C and 37˚C but not at 10˚C and 40˚C, respectively. The bacteria were tolerant to NaCl concentrations up to 4% (w/v) but were inhibited at 6% (w/v).In this study both isolates were able to ferment glucose and this result corroborates with the works of **Kim *et al* (2005)**. Additionally, flexirubin-type pigments were not produced from the isolates. The absence of flexirubin pigments is a unique feature differentiating *E.meningoseptica* from all other *Chryseobacterium* species except *C. hominis* **(Vaneechoutte *et al*., 2007)**. Haemolysis is one of the indicators for the virulent of isolates **(Chang *et al*.,2000)**. In this study, isolate from skin exhibited gamma haemolytic activity, and isolate from kidney exhibited beta haemolytic activity. According to **Williams & Lawrence *et al* (2005),** haemolysis is a vital virulence factor for many species of bacteria. Previous research by **Hirono *et al* (1997)** revealed that the haemolytic activity of *Edwardsiella tarda* encoded by gene which are responsible for haemolysis of red blood cells. *E. meningoseptica* was capable of growth at 1.5 % NaCl but unable to grow at 3 %, 6 %, 8 % or 10 % NaCl, indicating that NaCl concentrations above 1.5 % had an inhibitory effect. Previous study by **Bernardet *et al* (2002)** reported that all *Chryseobacterium* species are able to grow on marine agar. Therefore, the salinity range should be determined when NaCl is sufficient for growth. In addition, environmental studies of **Bloch *et al* (1997)** revealed that *Chryseobacterium meningosepticum* can survive in chlorine-treated municipal water supplies, and has become a potential reservoir for infections in the hospital environment.

In this study, API ZYM test detects specific enzymatic activity in bacterial isolates. This method offers a simple way to identify strains based on their enzymatic activities. Therefore, many researchers used API ZYM test to distinguish phenotypic characteristics of bacteria **(Hesami *et al*., 2010; Joh *et al*., 2010; Ugur *et al*.,2012)**.In another study, **Liew *et al* (2012)** enzymatic profiling using the API ZYM system has been useful in the identification of many fermentative and non-fermentative bacteria. Previous study revealed that bacterial enzyme profiles can be used for taxonomy and bacterial typing **(Humble *et al*., 1977)**. Moreover, isolate identifications can be only performed at the genus but not the species level **(Laughon *et al*., 1982).**

The result of this study elucidated that isolates exhibited low level of enzymatic activity, but the reactions were reproducible and reliable for taxonomy identification, in agreement with **Ugur *et al* (2012)**. The API ZYM profiles of kidney isolates were similar to those of skin isolates except for the low level activities of enzymes. Esterase, lipase and leucinearyl amidase detection in skin isolates might be related to the variations in morphology and biochemistry, we hypothesized that the kidney and skin isolates are closely related species of same genus. On the other hand, leucine Arylamidase (LAP) or leucineaminop eptidase is a proteolytic enzyme that catalyzes the hydrolysis of peptides containing leucine.

The kidney isolates were β-hemolytic and might be more virulent than skin isolates. The hemolytic activity of kidney isolates as β-hemolysis may be used as an indicator of enterotoxicity **(Rahim *et al*., 1984).** However, it is not clear to what extent these enzymes are involved in biological events *in vivo* **(Liew *et al*., 2012)**.

Bacterial alkaline phosphatase (APs) is comparatively resistant to inactivation, denaturation, and degradation, and also has a higher rate of activity. Important role in metabolism of different phosphorus containing organic compounds Alkaline phosphatases (APs) occur in a broad diversity of microorganisms and are important in the utilization of phosphoesters, as well as play a role in virulence **(Pandey & Parveen *et al*., 2011)**. Both isolates kidney and skin had higher level activity of enzymeNaphthol-AS-BI phosphohydrolase , which is necessary for bacteria survival. The function of arylamidase enzyme is catalyzing the hydrolysis of N-terminal amino acid from peptides,which contributes to the virulent factor.

The API ZYM test revealed that the kidney and skin isolates shared similar patterns. One distinctive activity was seen in the positive α-glucosidase test, indicating the bacteria's ability to ferment maltose. Kidney and skin isolates were both positive for β-galactosidase and lactose fermentation **Slots *et al*.,1981)**. Esterase activity was seen in all isolates, which indicates that they produced acids and alcohol. Additional β-glucosidase activity proved that the isolates fermented cellobiose **(Hofstad *et al*., 1980).** Both isolates lacked α-mannosidase and α-fucosidase activities **(Laughon *et al*.,1982)**.

In general, there were no obvious correlations between the kidney and the skin isolates. Thus, these isolates were probably different strains, within the same species. Previous studies by **Colding *et al* (1994)** showed that *Chryseobacterium meningosepticum* are divided into subgroups depended on the difference in genetic structure for each other as shows through the difference in pathogenicity. In another study, **Lin *et al* (2004)** indicated that the species *C. meningosepticum*is highly heterogeneous and composed of many subgroups, which may be classified as separate species. This fact confirmed by **Kim *et al* (2005)** separated new genera of *Elizabethkingias pp* from *Chryseobacterium.* In this present study, to determine whether the isolates are closely related, distinguishing their specific enzymatic activity and patterns is necessary. The phenotypic, physiological and biochemical characterizations allow limited discrimination of *Elizabethkingia* species (**Bernardet *et al*., 2005; Hugo *et al*., 2003)**. Further, these methods are useful for preliminary genus identifications **(Bernardet *et al*., 2005; Hugo *et al*., 2003)**. The actual values of these results may be unreliable; therefore, confirmation by another method such as PCR is required **(Hesami *et al*., 2010)**. The BLAST analysis revealed that the 16S rRNA gene sequences of both isolates were more than 97% homology to *Elizabethkingia meningoseptica*.

This result is in agreement with previous study of **Xie *et al* (2009)** performed on frogs with typical clinical signs of cataract disease, identified six *E. meningoseptica* isolates by their 16S rRNA gene sequences and biochemical characteristics. Results of **Kim *et al* (2005)** using 16S rDNA sequences of five strains showing 98.2 to 100 % similarities to *E. meningoseptica.*

CONCLUSION

The results of the present study concluded that *Elizabethkingia meningoseptica* could be detected and identified in fish infected tissue using the universal PCR method accompanied with biochemical and enzymatic profiles. Further work on *E. meningoseptica* pathogenicity on virulent gene expression is under way.

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