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Identification of *Elizabethkingia meningoseptica* from American bullfrog (*Rana catesbeiana*) farmed in Sabah, Malaysia using PCR method and future management of outbreak

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ABSTRACT

Aims: High demand for frog meat in Malaysia especially the American bullfrog (*Rana catesbeiana*) has promoted intensive farming of the animal. However, the farming of American bullfrog is restricted by the occurrence of diseases. This study reports the first isolation of *Elizabethkingia meningoseptica* from specimens of American bullfrog that suffer from cataract and 'red-leg' syndrome.
Methodology and Result: The pathogen was isolated from eyes and internal organs (liver, kidney and spleen) of the diseased bullfrog specimens. All the bacterial isolates were subjected to phenotypic characterization and antibiotic susceptibility assay, and further identified by using the 16S rDNA sequencing analysis. We designed two pair of specific PCR primers (22-25 mers) which are complimentary to the β-lactamase gene in the reference strain of *E. meningoseptica* ATCC49470. The result showed all the bacterial isolates shared similar phenotypic characters and antibiotic susceptibility. BLAST analysis of the 16S rDNA sequences indicated that the bacterial isolates had very high sequence homology (100%) with *E. meningospetica* ATCC49470 and *E. meningoseptica* isolates from mosquito. The two PCR primers were very specific to *E. meningoseptica* isolates of this study.
Conclusion, significance and impact of study: This is the first isolation and characterization of bacterial pathogen, *E. meningoseptica* in cultured American bullfrog (*Rana catesbeina*) that suffered from eye cataract and 'red-leg' syndrome in Sabah, Malaysia. It is suspected that one of the possible transmission routes of the bacterial pathogen could be via mosquito bites. The findings suggest that there is urgent requirement for standard guideline of good farming practice to be adopted in frog farms throughout the country. Such a guideline can help in minimizing economic losses, preventing transmission of the zoonotic bacterial pathogen to farm workers, and sustaining the industry in Malaysia and upgrading frog meat quality for international market.

Keywords: American bullfrog, *Elizabethkingia meningoseptica,* cataract, red-leg syndrome, Malaysia

INTRODUCTION

American bullfrog, *Rana catesbeiana,* is not native to Malaysia but it was introduced in the country for aquaculture purposes. The farming of this anuran species has gained popularity because of its excellent attributes of adaptability to various tropical environmental conditions and relatively large size, with rich muscle mass. In Malaysia, the American bullfrog is farmed commercially to satisfy both local and international markets. Initially, there were only 12 bullfrog farms operating in Malaysia with the annual production of 80 tons of anuran meat (Kechick, 1995). After 14 years of introduction, the frog meat industry in Malaysia has undergone much development whereby the current meat production is estimated at 100 tons per month (Lee *et al.*, 2009). However, in the recent years the production of bullfrog in some farms in Malaysia is limited by the high mortality due to diseases. The most frequently occurring diseases are the 'red-leg' syndrome and cataract. These diseases affect mainly the adult frogs. The cataract is characterized by opaque eye lens, lethargic behaviour and loss of appetite. The frog with red-leg syndrome seemed to have limited hopping ability with no appetite for food. These two diseases can sometimes concurrently occur in the same individual frog. The diseases caused mortality within few days to weeks after the onset. Previous study on various tissues of bullfrog with redleg syndrome showed presence of variety bacterial pathogens which include *Aeromonas hydrophila, Elizabethkingia* (*Chryseobacterium*) *meningoseptica*,

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Streptococcus innie, *Edwardsiella tarda*, *Citrobacter frundii* and *Pseudomonas* spp. (Mauel *et al*., 2002). Contrary to the previous finding, this study reports the isolation of single bacterial pathogen, *E. meningoseptica* from bullfrog with redleg and cataract. In addition, this study also describes the specific PCR method for detection of the bacterial pathogen.

MATERIALS AND METHODS

Bacterial isolation and preservation

Bacteria were isolated from internal organs (liver, spleen and kidney) and eyes of the diseased frogs. Briefly, the frogs were aseptically dissected using sterile surgical tools to expose internal organs including spleen, kidney, heart and liver, and eyes. Sterile inoculating loop was aseptically swabbed on each organ, streaked on tryptic soy agar (TSA, Difco, USA) plates that were supplemented with 1.5% (w/v) sodium chloride and incubated at 28 °C for 48 h. Subsequently, the bacteria were serially sub-cultured on TSA plates to obtain single pure colony. Finally, 5 well characterized bacterial isolates were preserved at -86 °C according to the method described by Floodgate and Hayes (1961).

Phenotypic characterization

The 5 bacterial isolates were subjected to various biochemical tests according to the method described by Ransangan and Mustafa (2009). Tests included Gram staining, motility test, oxidative-fermentative test, catalase test, oxidase test, acid and gas production from sugars, citrate utilization, urease test, methyl-red reaction, Voges Proskauer, indole production, phenylalanine test, β-galactosidase test, lysine decarboxylase and arginine dehydrolase. The bacterial isolates were also grown at four different temperatures (10 °C, 28 °C, 37 °C and 40 °C) in four concentrations (0, 2, 4 and 6%) of NaCl (w/v).

Antibiotic susceptibility test

The bacterial isolates were then subjected to antibiotic susceptibility assay. They were first grown on TSA plates for 24 h at 28 °C. Each bacterial isolate was suspended in sterile phosphate buffered saline (PBS) (pH 7.2) and diluted to a turbidity equivalent to a MacFarland No. 0.5 standard solution. Then, 0.1 mL bacterial suspension was spread onto Mueller–Hinton agar (Difco) plate. Subsequently, antibiotic discs were aseptically placed onto the inoculated plates according to the method described by Dalsgaard *et al.* (1999). The antibiotic disks (Oxoid, Hampshire, England) used in this assay included ampicilin (10 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), sulphamethoxazole (100 µg), furazolidone (100 µg), kanamycin (30 µg), nalidixic acid (3 µg), neomycin (10 µg), nitrofurantoin (300 µg), novobiocin (5 µg), oxolinic acid (2 µg), oxytetracycline (30 µg), penicillin G (10 units), streptomycin (25 µg), sulphonamide (300 µg), tetracycline (10 µg) and vancomycin (30 µg). The plates were incubated at 28 °C for 48 h and inhibition zones were scored according to the method described by Barry *et al.*, (1979).

DNA isolation

Genomic DNA from the bacterial isolates was extracted using the CTAB-DTAB method as described by Phillips and Simon (1995). First, each bacterium was inoculated in 5 mL sterile tryptic soy broth (TSB, Difco) and incubated overnight at 28 °C overnight following the method described by Kim and Jeong (2001). Subsequently, 1.0 mL of the bacterial suspension was transferred into microtube and centrifuged at 7,500 *g* for 5 min at 4 °C. After centrifugation, the supernatant was discarded, the bacterial pellet was re-suspended in 600 µL DTAB solution [8% DTAB; 1.5 M NaCl; 100 mM Tris-HCl (pH 8.8) and 50 mM EDTA] and incubated at 75 °C for 5 min. The mixture was added with 700 µL of chloroform and vortex for 20-30 sec before centrifugation at 13,400 *g* for 5 min. Later on, 450 µL of the aqueous layer was transferred into the new sterile microtube, and added with 100 µL CTAB solution (5% CTAB; 0.4 M NaCl) and 900 µL sterile dH_2O. The mixture was incubated again at 75 °C for 5 min and centrifuged at 13,400 *g* for 10 min. Supernatant was discarded and DNA pellet dissolved in 150 µL dH_2O and incubated further at 75 °C for 5 min before centrifugation at 13,400 g for 5 min. Once again the clear solution was transferred into new microtube and added with equal volume of 95% ethanol. The microtube was finger flicked several times and centrifuged again at 13,400 *g* for 10 min. The DNA pellet was washed with 75% ethanol and centrifuged at similar speed as described above. Finally, the DNA pellet was dissolved in 50 µL 1X TE buffer and stored at -20 °C until use. The DNA concentration was determined using GeneQuant Pro RNA/DNA calculator (Pharmacia).

PCR amplification of 16S rRNA gene

PCR amplification of 16S rRNA gene was conducted against total genomic DNA extracted from all the 5 isolates using primers shown in Table 1. The forward and reverse primers were correspondent to nucleotide positions 3776045 to 3776026 and 3774654 to 3774678 of the 16S rRNA gene of *E. coli* ATCC 8739 (Figure 1), respectively. The PCR The PCR amplification was conducted in 25 µL total reaction which consisted of 2.5 µL of 10X i-Taq PCR buffer (iNtRON, Korea), 1.0 µL of each (10 µM) forward and reverse primers, 0.5 µL i-Taq Polymerase (iNtRON), 2.0 µL DNA template (0.307 µg/ µL l) and 18.0 µL nuclease-free water. The amplification was carried out one cycle at 95 °C for 3 min followed by 30 cycles at 95 °C for 1 min, at 58 °C for 1 min and at 72 °C for 1 min, and final extension at 72 °C for 5 min. The PCR products were analyzed on 1.5% agarose gel electrophoresis and visualized under UV using the Alphaimager® Imaging System (Alpha Innotech Corporation).

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Table 1: PCR primers used in this study.

Primer	Nucleotide Sequence (5'-3')	Target gene	Expected size (bp)
16SFJR	ATBNAGAGTTTGATCMTGGC	16S rRNA	1400
16SRJR	CAAGGCCCGGGAACGTATTCAC	16S rRNA	
JREMF1	ATATTACGTAGGAACCTATGATTTG	β-lactamase	612
JREMR1	ATGGAGATCGAACTGACTTGCAT	β-lactamase	
JREMF2	ATGATTTGGCTTCTTACCTTATTG	β-lactamase	644
JREMR2	TATCCATAAACAATTGCGGATT	β-lactamase	

Figure 1: Locations of PCR primers (16SFJR: nt3776045 to nt3776026; 16SRJR: nt3774654 to nt3774678) of 16S rRNA gene within the genome of *Escherichia coli* ATCC 8739 (CP000946).

DNA cloning and sequencing

PCR products (16S rDNA fargments) were purified using *AccuPrep*™. PCR purification Kit (Bioneer Corporation, Seoul, Korea) according to the procedures described in the manufacturer's instruction manual. Two microlites (2.0 µL) of the PCR product was cloned into pGEM®-T Easy (Promega, Madison, USA) cloning vector following the method described by the manufacturer. The plasmid was purified using PureLink™ Quick Plasmid Miniprep Kit (Invitogen™, USA) following manufacturer's instruction. The plasmids were restricted using EcoR1 (New England Biolabs, USA) and analyzed on 1.5% agarose gel electrophoresis. Finally, 20 µL of each purified plasmid harbouring correct fragment of the 16S rDNA was sequenced using M13 primers (Macrogen, DNA sequencing service, Seoul, Korea). Bacterial isolates were identified based on the result of BLAST analysis of the partial 16S rDNA sequences. The percentage identity of 16S rDNA sequences of the 5 bacterial isolates against 16S rDNA sequences downloaded from the genbank was computed using the ClustalW (DNASTAR, Madison, United States). The construction of phylogenetic tree was achieved using the MegAlign program (DNASTAR) and the TREECON for Windows (Van de peer and De Wachter, 1994).

Specific PCR detection of E. meningoseptica

Two PCR primer pairs were designed based on β-lactamase gene sequences downloaded from genbank (DQ004496, GU188445, EF394442, EF394444, EF394445 and EF394446). The first primers pair was correspondent to the nucleotide positions, 132 to 256 and 744 to 722 of *E. meningoseptica* GOB-18 gene (DQ004496) and second primers pair was correspondent

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to the nucleotide positions, 149 to 172 and 793 to 772 of *E. meningoseptica* GOB-18 gene (DQ004496) (Figure 2). The PCR amplification was carried out in 25 µL total reaction which consisted of 12.5 µL 10X i-Taq PCR buffer (iNtRON), 1.0 µL of each primer (10 µM), 0.3 µL i-Taq polymerase (iNtRON), 2.0 µL DNA (0.307 µg/ µL) and 8.2 µL sterile Milli-Q water. The optimum PCR condition when using primers (JREMF1 and JREMR1) was as follow: initial DNA denaturation at 95 °C for 3 min followed by 30 cycles at 95 °C for 30 sec, 58 °C for 30 sec and 72 °C for 30 sec, and final extension at 72 °C for 5 min. Whereas the optimum PCR condition when using primers (JREMF2 and JREMR2) was as described above except for the annealing temperature set at 55 °C.

The specificity of the primers was evaluated against DNA from *Vibrio alginolyticus* (ATCC 17749), *V. parahaemolyticus* (ATCC 17802), *V. harveyi* (ATCC 35084), *V. anguillarum* (ATCC 19264), *Aeromonas salmonicida* subsp*. salmonicida* (ATCC33658), *A. hydrophila* (ATCC 7965), *A. caviae* (ATCC 15468), *Edwardsiella tarda* (ATCC 15947), *Yersinia ruckeri* (ATCC 29473), *Pseudomonas fluorescens* (ATCC 13525), *Pseudomonas aeruginosa* (ATCC 27853), *Proteus mirabilis* (ATCC 29245), *Escherichia coli* (ATCC 25922) and *E. meningoseptica* (ATCC 13253). The PCR products were analyzed on 1.5% agarose gel electrophoresis and visualized under UV using using the Alphaimager® Imaging System.

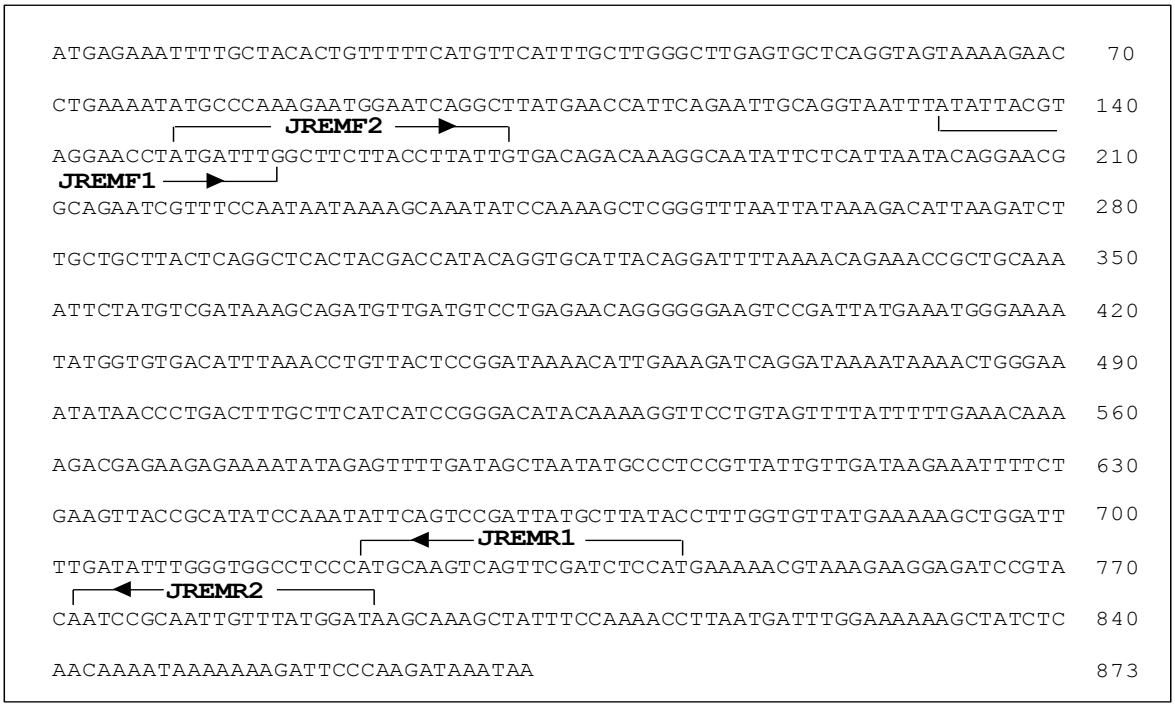

Figure 2: Locations of β-lactamase PCR primers (JREMF1: nt132 to nt156, JREMF2: nt149 to nt172, JREMR1: nt744 to nt722 and JREMR2: nt793 to nt772) within the complete coding sequence of *Elizabethkingia meningoseptica* class B carbapenemase COB-18 gene (DQ004496).

RESULTS

Phenotypic characterization

All the 5 bacterial isolates exhibited similarity in the phenotypic features such as Gram staining negative, non-motile, and positive for oxidase and catalase, and negative for Voges-proskauer reaction. Although the bacteria were not able to produce indole, they utilized citrate and gelatine. They did not produce arginine dihydrolyse, lysine decarboxylase and urease but produced β-galactosidase (ONPG). Acid production was only recorded from glucose and cellobiose but not from other sugars. Nevertheless, gas was not produced from all the sugars tested. The bacteria 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 inhibited at 6% (w/v). However, the 5 bacterial isolates differed from the ATCC strains of *E. menignoseptica* in the acid production from cellobiose, D-fructose, maltose, lactose and manitol. Details of the phenotypic characteristics of the bacterial isolates are shown in Table 2.

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Table 2: Phenotypic features of the *E. menigoseptica isolates* from American bullfrog, Rana catesbeiana farmed in Sabah, Malaysia.

	Bacterial Isolates					
Characteristics	EKMS1	EKML1	EKMK1	EKMLE1	EKMRE1	*E. meningoseptica*[a]
Gram staining Shape	- rod	- rod	- rod	- rod	- rod	- rod
Oxidase test	+	+	+	+	+	+
Catalase test	+	+	+	+	+	nd
Voges-Proskauer	-	-	-	-	-	nd
Indole production	-	-	-	-	-	+
Citrate utilization	+	+	+	+	+	+
Gelatine	+	+	+	+	+	nd
Arginine dihydrolase	-	-	-	-	-	nd
Lysine decarboxylase	-	-	-	-	-	nd
Phenylalanine agar	-	-	-	-	-	nd
*ONPG	+	+	+	+	+	nd
Methyl-Red	-	-	-	-	-	nd
Urease test	-	-	-	-	-	-
Growth at 10 °C	-	-	-	-	-	nd
28 °C	+	+	+	+	+	nd
37 °C	+	+	+	+	+	+
40 °C	-	-	-	-	-	nd
Growth at 0 % NaCl	+	+	+	+	+	nd
2% NaCl	+	+	+	+	+	nd
4% NaCl	+	+	+	+	+	nd
6% NaCl	-	-	-	-	-	nd
O/F glucose	O	O	O	O	O	nd
Gas (acid) from glucose	- (+)	- (+)	- (+)	- (+)	- (+)	nd(+)
D-fructose	- (-)	- (-)	- (-)	- (-)	- (-)	nd(+)
Cellobiose	- (+)	- (+)	- (+)	- (+)	- (+)	nd(-)
Mannose	- (-)	- (-)	- (-)	- (-)	- (-)	nd(nd)
Sorbitol	- (-)	- (-)	- (-)	- (-)	- (-)	nd(nd)
Arabinose	- (-)	- (-)	- (-)	- (-)	- (-)	nd(-)
Dextrose	- (-)	- (-)	- (-)	- (-)	- (-)	nd(nd)
Sucrose	- (-)	- (-)	- (-)	- (-)	- (-)	nd(nd)
Maltose	- (-)	- (-)	- (-)	- (-)	- (-)	nd(+)
Mannitol	- (-)	- (-)	- (-)	- (-)	- (-)	nd(+)
Lactose	- (-)	- (-)	- (-)	- (-)	- (-)	nd(+)
Salicin	- (-)	- (-)	- (-)	- (-)	- (-)	nd(nd)
Raffinose	- (-)	- (-)	- (-)	- (-)	- (-)	nd(nd)
Galactose	- (-)	- (-)	- (-)	- (-)	- (-)	nd(nd)
Rhamnose	- (-)	- (-)	- (-)	- (-)	- (-)	nd(nd)

Bacterial identification

The PCR primers designed in this study successfully amplified partial fragment of 16S rDNA from all the bacterial isolates. Based on the BLAST analysis, it was found that all the bacterial isolates had high nucleotide sequence homology (98-100%) to the 16S rDNA sequences belonging to *E. meningoseptica* strains (Table 3, Figure 3). On this basis, the 5 bacterial isolates described here are identified as those of *E. meningoseptica*. The partial 16S rDNA sequences of the 5 bacterial isolates were deposited in GenBank (http://www.ncbi.nih.gov) with the accession numbers as shown in Table 4.

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Table 3: Percentage similarity (above diagonal) of 16S rDNA sequences of *E. meningoseptica* in reference to nt75-1350 of the 16S rDNA sequence (X80724) of *Escherichia coli* ATCC25922

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	
***	96.0	96.0	95.9	94.1	94.2	94.2	94.1	94.0	94.4	94.1	94.5	94.4	94.3	94.7	94.3	94.2	94.0	94.4	94.2	94.3	94.4	94.4	94.4	94.3	94.3	94.4	94.2	94.2	94.3	94.4	94.1	94.2	94.2	93.7	93.7	94.8	94.4	94.4	94.4	94.4	94.4	93.4	94.7	94.2	94.7	94.7	93.7	70.1	*Epillthonimonas lactis* (EF204460)
4.2	***	100	99.9	94.0	94.0	94.1	94.3	94.2	94.8	94.3	94.7	94.4	94.4	95.2	94.1	94.1	94.2	94.4	94.1	94.3	94.4	94.4	94.4	94.4	94.4	94.4	94.3	94.4	94.4	94.4	94.3	94.2	94.2	93.7	93.7	95.1	94.4	94.4	94.4	94.4	94.4	93.7	95.2	94.2	95.1	95.2	93.9	69.3	*C. hominis* (AM423083)
4.2	0.0	***	99.9	94.0	94.0	94.1	94.3	94.2	94.8	94.3	94.7	94.4	94.4	95.2	94.1	94.1	94.2	94.4	94.1	94.3	94.4	94.4	94.4	94.4	94.4	94.4	94.3	94.4	94.4	94.4	94.3	94.2	94.2	93.7	93.7	95.1	94.4	94.4	94.4	94.4	94.4	93.7	95.2	94.2	95.1	95.2	93.9	69.3	*C. hominis* (AM423083)
4.2	0.1	0.1	***	94.1	94.1	94.2	94.4	94.3	94.7	94.4	94.8	94.4	94.5	95.1	94.2	94.2	94.3	94.5	94.2	94.4	94.4	94.5	94.5	94.4	94.4	94.5	94.4	94.5	94.4	94.5	94.4	94.3	94.3	93.8	93.8	95.0	94.5	94.5	94.5	94.5	94.5	93.7	95.1	94.3	95.0	95.1	94.0	69.3	*C. isbiliense* (AM159194)
6.2	6.2	6.2	6.1	***	99.8	99.7	98.4	98.2	98.4	98.4	98.1	99.5	99.6	98.5	99.8	99.7	98.2	99.5	99.7	98.4	99.5	99.5	99.5	99.5	99.5	99.5	99.4	99.5	99.6	99.5	98.2	98.3	98.3	99.0	99.0	98.3	99.5	99.5	99.5	99.5	99.5	96.9	98.5	98.5	98.2	98.5	98.0	71.6	*C. meningosepticum* (AF207070)
6.1	6.2	6.2	6.1	0.2	***	99.8	98.4	98.2	98.2	98.4	98.1	99.5	99.6	98.5	99.9	99.8	98.2	99.5	99.8	98.4	99.5	99.5	99.5	99.5	99.5	99.5	99.4	99.5	99.6	99.5	98.2	98.3	98.3	99.0	99.0	98.3	99.5	99.5	99.5	99.5	99.5	96.9	98.5	98.5	98.2	98.5	98.0	71.6	*C. meningosepticum* (AF207071)
6.1	6.1	6.1	6.0	0.3	0.2	***	98.4	98.2	98.1	98.4	97.9	99.5	99.6	98.5	99.9	100	98.2	99.7	100	98.4	99.6	99.7	99.7	99.6	99.6	99.7	99.5	99.7	99.6	99.7	98.2	98.3	98.3	99.1	99.1	98.3	99.7	99.7	99.7	99.7	99.7	96.8	98.5	98.5	98.2	98.5	98.0	71.6	*C. meningosepticum* (AF207072)
6.2	5.9	5.9	5.8	1.7	1.7	1.7	***	99.8	98.3	99.9	99.2	98.7	98.6	98.6	98.5	98.4	98.8	98.7	98.4	99.8	98.6	98.7	98.7	98.6	98.6	98.7	98.5	98.7	98.6	98.7	99.8	99.8	99.8	97.8	97.8	98.5	98.7	98.7	98.7	98.7	98.7	97.1	98.6	99.9	98.5	98.6	99.6	71.3	*C. meningosepticum* (AF207073)
6.3	6.0	6.0	5.9	1.8	1.8	1.8	0.2	***	98.1	99.8	99.1	98.5	98.5	98.5	98.3	98.2	100	98.5	98.2	99.8	98.5	98.5	98.5	98.5	98.5	98.5	98.4	98.5	98.5	98.5	99.8	99.7	99.7	97.8	97.8	98.4	98.5	98.5	98.5	98.5	98.5	97.0	98.5	99.8	98.3	98.5	99.6	71.3	*C. meningosepticum* (AF207074)
5.8	5.3	5.3	5.4	1.7	1.8	2.0	1.8	1.9	***	98.3	98.8	98.5	98.6	99.6	98.1	98.1	98.1	98.4	98.1	98.1	98.3	98.4	98.4	98.3	98.3	98.4	98.2	98.4	98.3	98.4	98.1	98.1	98.1	97.5	97.5	99.6	98.4	98.4	98.4	98.4	98.4	98.3	99.6	98.2	99.7	99.6	97.9	71.2	*C. meningosepticum* (AF207075)
6.2	5.9	5.8	5.8	1.7	1.7	1.7	0.1	0.2	1.8	***	99.2	98.7	98.6	98.6	98.5	98.4	98.8	98.7	98.4	99.8	98.6	98.7	98.7	98.6	98.6	98.7	98.5	98.7	98.6	98.7	99.8	99.7	99.7	97.8	97.8	98.5	98.7	98.7	98.7	98.7	98.7	97.1	98.6	99.8	98.5	98.6	99.5	71.4	*C. meningosepticum* (AF207076)
5.8	5.4	5.4	5.3	2.0	2.0	2.2	0.8	0.8	1.3	0.8	***	98.2	98.3	98.8	98.0	97.9	99.1	98.2	97.9	99.2	98.1	98.2	98.2	98.1	98.1	98.2	98.1	98.2	98.1	98.2	99.2	99.1	99.1	97.5	97.5	99.0	98.2	98.2	98.2	98.2	98.2	97.5	98.8	99.1	98.8	98.8	98.8	71.1	*C. meningosepticum* (AF207077)
5.9	5.8	5.8	5.8	0.5	0.5	0.6	1.4	1.5	1.5	1.4	1.8	***	99.8	98.8	99.6	99.5	98.5	99.8	99.5	98.7	99.8	99.8	99.8	99.8	99.8	99.8	99.7	99.8	99.8	99.8	98.5	98.6	98.6	99.0	99.0	98.6	99.8	99.8	99.8	99.8	99.8	97.2	98.8	98.8	98.5	98.8	98.3	71.5	*C. meningosepticum* (AF207078)
6.0	5.8	5.8	5.7	0.4	0.4	0.6	1.4	1.6	1.4	1.4	1.8	0.2	***	98.8	99.5	99.5	98.5	99.8	99.5	98.6	99.7	99.8	99.8	99.7	99.7	99.8	99.6	99.8	99.7	99.8	98.5	98.5	98.5	98.9	98.9	98.5	99.8	99.8	99.8	99.8	99.8	97.3	98.8	98.7	98.6	98.8	98.2	71.6	*C. meningosepticum* (AF207079)
5.5	4.9	4.9	5.0	1.6	1.6	1.6	1.4	1.6	0.4	1.4	1.2	1.3	1.2	***	98.5	98.5	98.5	99.8	98.5	98.6	99.7	98.8	98.8	99.7	99.7	98.8	98.6	98.8	99.7	98.8	98.5	98.4	98.4	97.9	97.9	99.7	98.8	98.8	98.8	98.8	98.8	98.4	100	98.5	99.8	100	98.2	71.3	*E. meningoseptica* ATCC 13254^T (AJ704540)
6.0	6.1	6.1	6.0	0.2	0.1	0.1	1.6	1.8	1.9	1.6	2.1	0.4	0.5	1.5	***	99.9	98.3	99.6	99.9	98.5	99.5	99.6	99.6	99.5	99.5	99.6	99.5	99.6	99.5	99.6	98.3	98.4	98.4	99.1	99.1	98.4	99.6	99.6	99.6	99.6	99.6	97.0	98.5	98.5	98.3	98.5	98.1	71.6	*E. meningoseptica* ATCC 13254 (AJ704541)
6.1	6.1	6.1	6.0	0.3	0.3	0.0	1.7	1,8	2.0	1.7	2.2	0.5	0.6	1.6	0.1	***	98.2	99.7	100	98.4	99.6	99.7	99.7	99.6	99.6	99.7	99.5	99.7	99.6	99.7	98.2	98.3	98.3	99.1	99.1	98.3	99.7	99.7	99.7	99.7	99.7	96.9	98.5	98.5	98.2	98.5	98.0	71.5	*E. meningoseptica* ATCC 13255 (AJ704542)
6.3	6.0	6.0	5.9	1.8	1.8	1.8	0.2	0.0	1.9	0.2	0.9	1.5	1.6	1.6	1.8	1.8	***	98.5	98.2	98.8	98.5	98.5	98.5	98.5	98.5	98.5	98.4	98.5	98.5	98.5	99.8	99.7	99.7	97.8	97.8	98.4	98.5	98.5	98.5	98.5	98.5	97.0	98.5	99.8	98.3	98.5	99.5	71.3	*E. miricola* ATCC 333958 (AJ704543)
5.9	5.8	5.8	5.7	0.5	0.5	0.3	1.4	1.5	1.7	1.4	1.8	0.2	0.2	1.3	0.4	0.3	1.5	***	98.7	98.7	99.9	100	100	99.9	99.9	100	99.8	100	99.9	100	98.5	98.6	98.6	99.1	99.1	98.6	100	100	100	100	100	97.2	98.8	98.8	98.5	98.8	98.3	71.5	*E. meningoseptica* ATCC 49470 (AJ704544)
6.1	6.1	6.1	6.0	0.3	0.2	0.0	1.7	1.8	2.0	1.7	2.2	0.5	0.6	1.6	0.1	0.0	1.8	0.3	***	98.4	99.6	99.7	99.7	99.6	99.6	99.7	99.5	99.7	99.6	99.7	98.2	98.3	98.3	99.1	99.1	98.3	99.7	99.7	99.7	99.7	99.7	96.9	98.5	98.5	98.2	98.5	98.0	71.5	*E. meningoseptica* ATCC 51720 (AJ704545)
6.0	5.9	5.9	5.8	1.7	1.7	1.7	0.2	0.2	1.9	0.2	0.8	1.4	1.4	1.6	1.6	1.7	0.2	1.4	1.7	***	98.6	98.7	98.7	98.6	98.6	98.7	99.5	98.7	98.6	98.7	99.8	99.9	99.9	98.0	98.0	98.4	98.7	98.7	98.7	98.7	98.7	97.1	98.5	99.9	98.3	98.5	99.5	71.3	*C. meningosepticum* (AY468477)
6.0	5.8	5.8	5.8	0.6	0.6	0.4	1.4	1.6	1.8	1.4	1.9	0.2	0.3	1.4	0.5	0.4	1.6	0.1	0.4	1.4	***	99.9	99.9	99.8	99.8	99.9	99.8	99.9	99.8	99.9	98.5	98.5	98.5	99.1	99.1	98.5	99.9	99.9	99.9	99.9	99.9	97.1	98.7	98.7	98.5	98.7	98.2	71.5	*E. meningoseptica* (EF426425)
5.9	5.8	5.8	5.7	0.5	0.5	0.3	1.4	1.5	1.7	1.4	1.8	0.2	0.2	1.3	0.4	0.3	1.5	0.0	0.3	1.4	0.1	***	100	99.9	99.9	100	99.8	100	99.9	100	98.5	98.6	98.6	99.1	99.1	98.6	100	100	100	100	100	97.2	98.8	98.8	98.5	98.8	98.3	71.5	*E. meningoseptica* (EF426426)
5.9	5.8	5.8	5.7	0.5	0.5	0.4	1.4	1.5	1.7	1.4	1.8	0.2	0.2	1.3	0.4	0.3	1.5	0.0	0.3	1.4	0.1	0.0	***	99.9	99.9	100	99.8	100	99.9	100	98.5	98.6	98.6	99.1	99.1	98.6	100	100	100	100	100	97.2	98.8	98.8	98.5	98.8	98.3	71.5	*E. meningoseptica* (EF426427)
6.0	5.8	5.8	5.8	0.6	0.6	0.4	1.4	1.6	1.8	1.4	1.9	0.2	0.3	1.4	0.5	0.4	1.6	0.1	0.4	1.4	0.2	0.1	0.1	***	99.8	99.9	99.8	99.9	99.8	99.9	98.5	98.5	98.5	99.1	99.1	98.5	99.9	99.9	99.9	99.9	99.9	97.1	98.7	98.7	98.5	98.7	98.2	71.4	*E. meningoseptica* (EF426428)
6.0	5.8	5.8	5.8	0.6	0.6	0.4	1.4	1.6	1.8	1.4	1.9	0.2	0.3	1.4	0.5	0.4	1.6	0.1	0.4	1.4	0.2	0.1	0.1	0.2	***	99.9	99.8	99.9	99.8	99.9	98.5	98.5	98.5	99.1	99.1	98.5	99.9	99.9	99.9	99.9	99.9	97.1	98.7	98.7	98.5	98.7	98.2	71.6	*E. meningoseptica* (EF426429)
5.9	5.8	5.8	5.7	0.5	0.5	0.3	1.4	1.5	1.7	1.4	1.8	0.2	0.2	1.3	0.4	0.3	1.5	0.0	0.3	1.4	0.1	0.0	0.0	0.1	0.1	***	99.8	100	99.9	100	98.5	98.6	98.6	99.1	99.1	98.6	100	100	100	100	100	97.2	98.8	98.8	98.5	98.8	98.3	71.5	*E. meningoseptica* (EF426430)
6.1	5.9	5.9	5.8	0.6	0.6	0.5	1.5	1.7	1.7	1.5	2.0	0.3	0.4	1.4	0.6	0.5	1.7	0.2	0.5	1.5	0.2	0.2	0.2	0.2	0.2	0.2	***	99.8	99.8	99.8	98.4	98.5	98.5	99.0	99.0	98.5	99.8	99.8	99.8	99.8	99.8	97.1	98.6	98.6	98.4	98.6	98.1	71.3	*E. meningoseptica* (EF426431)
5.9	5.8	5.8	5.7	0.5	0.5	0.5	1.4	1.5	1.8	1.4	1.8	0.2	0.2	1.3	0.4	0.3	1.5	0.0	0.3	1.4	0.1	0.0	0.0	0.1	0.1	0.0	0.2	***	99.9	100	98.5	98.6	98.6	99.1	99.1	98.6	100	100	100	100	100	97.2	98.8	98.8	98.5	98.8	98.3	71.5	*E. meningoseptica* (EF426432)
6.0	5.8	5.8	5.8	0.6	0.6	0.4	1.4	1.6	1.7	1.4	1.9	0.2	0.3	1.4	0.5	0.4	1.6	0.1	0.4	1.4	0.2	0.1	0.1	0.2	0.1	0.1	0.2	0.1	***	99.9	98.5	98.5	98.5	99.1	99.1	98.5	99.9	99.9	99.9	99.9	99.9	97.1	98.7	98.7	98.5	98.7	98.2	71.5	*E. meningoseptica* (EF426433)
5.9	5.8	5.8	5.7	0.5	0.5	0.5	1.4	1.5	1.7	1.4	1.8	0.2	0.2	1.3	0.4	0.3	1.5	0.0	0.3	1.4	0.1	0.0	0.0	0.1	0.1	0.0	0.2	0.0	0.1	***	98.5	98.6	98.6	99.1	99.1	98.6	100	100	100	100	100	97.2	98.8	98.8	98.5	98.8	98.3	71.5	*E. meningoseptica* (EF426434)
6.1	5.8	5.8	5.8	1.8	1.8	1.8	0.1	0.2	1.8	0.2	0.7	1.4	1.5	1.5	1.7	1.8	0.2	1.4	1.8	0.1	1.5	1.4	1.4	1.5	1.5	1.4	1.6	1.4	1.5	1.4	***	99.8	99.8	97.8	97.8	98.4	98.5	98.5	98.5	98.5	98.5	97.1	98.5	99.8	98.3	98.5	99.5	71.3	*E. meningoseptica* (EU128742)
6.1	6.0	6.0	5.9	1.8	1.8	1.8	0.2	0.3	2.0	0.3	0.9	1.4	1.5	1.7	1.7	1.8	0.3	1.4	1.8	0.1	1.5	1.4	1.4	1.5	1.5	1.4	1.6	1.4	1.5	1.4	0.2	***	100	97.9	97.9	98.3	98.6	98.6	98.6	98.6	98.6	97.0	98.4	99.8	98.2	98.4	99.4	71.3	*E. meningoseptica* (EU128743)
6.1	6.0	6.0	5.9	1.8	1.8	1.8	0.2	0.3	2.0	0.3	0.9	1.4	1.5	1.7	1.7	1.8	0.3	1.4	1.8	0.1	1.5	1.4	1.4	1.5	1.5	1.4	1.6	1.4	1.5	1.4	0.2	0.0	***	97.9	97.9	98.3	98.6	98.6	98.6	98.6	98.6	97.0	98.4	99.8	98.2	98.4	99.4	71.3	*E. meningoseptica* (EU128744)
6.7	6.5	6.5	6.4	1.0	1.0	0.9	2.2	2.3	2.6	2.2	2.6	1.0	1.1	2.2	1.0	0.9	2.3	0.9	0.9	2.1	1.0	0.9	0.9	1.0	1.0	0.9	1.0	0.9	1.0	0.9	2.2	2.2	2.2	***	100	97.8	99.1	99.1	99.1	99.1	99.1	96.5	97.9	97.9	97.7	98.4	99.4	71.3	*E. meningoseptica* (FJ816020)
6.7	6.5	6.5	6.4	1.0	1.0	0.9	2.2	2.3	2.6	2.2	2.6	1.0	1.1	2.2	1.0	0.9	2.3	0.9	0.9	2.1	1.0	0.9	0.9	1.0	1.0	0.9	1.0	0.9	1.0	0.9	2.2	2.2	2.2	0.0	***	97.8	99.1	99.1	99.1	99.1	99.1	96.5	97.9	97.9	97.7	97.9	97.4	71.5	*E. meningoseptica* (FJ816028)
5.4	5.1	5.1	5.2	1.8	1.8	1.8	1.5	1.7	0.4	1.5	1.0	1.4	1.5	0.3	1.7	1.8	1.7	1.4	1.8	1.7	1.5	1.4	1.4	1.5	1.5	1.4	1.6	1.4	1.5	1.4	1.6	1.8	1.8	2.3	2.3	***	98.6	98.6	98.6	98.6	98.6	98.4	99.7	98.5	99.8	97.9	97.4	71.5	*E. meningoseptica* (FJ839441)
5.9	5.8	5.8	5.7	0.5	0.5	0.3	1.4	1.5	1.7	1.4	1.8	0.2	0.2	1.3	0.4	0.3	1.5	0.0	0.3	1.4	0.1	0.0	0.0	0.1	0.1	0.0	0.2	0.0	0.1	0.0	1.4	1.4	1.4	0.9	0.9	1.4	***	100	100	100	100	97.2	98.8	98.8	98.5	99.7	98.1	71.2	*E. meningoseptica* (GU180602)
5.9	5.8	5.8	5.7	0.5	0.5	0.3	1.4	1.5	1.7	1.4	1.8	0.2	0.2	1.3	0.4	0.3	1.5	0.0	0.3	1.4	0.1	0.0	0.0	0.1	0.1	0.0	0.2	0.0	0.1	0.0	1.4	1.4	1.4	0.9	0.9	1.4	0.0	***	100	100	100	97.2	98.8	98.8	98.5	98.8	98.3	71.5	*E. meningoseptica* (GU180603)
5.9	5.8	5.8	5.7	0.5	0.5	0.3	1.4	1.5	1.7	1.4	1.8	0.2	0.2	1.3	0.4	0.3	1.5	0.0	0.3	1.4	0.1	0.0	0.0	0.1	0.1	0.0	0.2	0.0	0.1	0.0	1.4	1.4	1.4	0.9	0.9	1.4	0.0	0.0	***	100	100	97.2	98.8	98.8	98.5	98.8	98.3	71.5	*E. meningoseptica* (GU180604)
5.9	5.8	5.8	5.7	0.5	0.5	0.3	1.4	1.5	1.7	1.4	1.8	0.2	0.2	1.3	0.4	0.3	1.5	0.0	0.3	1.4	0.1	0.0	0.0	0.1	0.1	0.0	0.2	0.0	0.1	0.0	1.4	1.4	1.4	0.9	0.9	1.4	0.0	0.0	0.0	***	100	97.2	98.8	98.8	98.5	98.8	98.3	71.5	*E. meningoseptica* (GU180605)
5.9	5.8	5.8	5.7	0.5	0.5	0.3	1.4	1.5	1.7	1.4	1.8	0.2	0.2	1.3	0.4	0.3	1.5	0.0	0.3	1.4	0.1	0.0	0.0	0.1	0.1	0.0	0.2	0.0	0.1	0.0	1.4	1.4	1.4	0.9	0.9	1.4	0.0	0.0	0.0	0.0	***	97.2	98.8	98.8	98.5	98.8	98.3	71.5	*E. meningoseptica* (GU180606)
5.8	5.2	5.2	5.3	2.0	2.0	2.0	1.8	1.9	0.6	1.8	1.4	1.7	1.6	0.6	1.9	2.0	1.9	1.7	2.0	1.8	1.8	1.7	1.7	1.8	1.8	1.7	1.8	1.7	1.8	1.7	1.8	1.9	1.9	2.4	2.4	0.6	1.7	1.7	1.7	1.7	1.7	***	98.4	97.0	98.5	98.8	96.7	70.6	*E. meningoseptica* (HM480364)
5.5	4.9	4.9	5.0	1.6	1.6	1.6	1.4	1.6	0.4	1.4	1.2	1.3	1.2	0.0	1.5	1.6	1.6	1.3	1.6	1.6	1.4	1.3	1.3	1.4	1.4	1.3	1.4	1.3	1.4	1.3	1.4	1.7	1.7	2.2	2.2	0.3	1.3	1.3	1.3	1.3	1.3	0.5	***	98.5	99.8	100	98.2	71.3	*E. meningoseptica* (HM748801)
6.1	6.0	6.0	5.9	1.6	1.6	1.6	0.1	0.2	1.8	0.2	0.9	1.3	1.4	1.5	1.5	1.6	0.2	1.3	1.6	0.1	1.4	1.3	1.3	1.4	1.4	1.3	1.4	1.3	1.4	1.3	1.4	0.2	0.2	2.2	2.2	1.6	1.3	1.3	1.3	1.3	1.3	1.9	1.5	***	98.4	98.5	99.5	71.3	*E. meningoseptica* (HQ154560)
5.6	5.1	5.1	5.9	1.8	1.8	1.8	1.6	1.8	0.3	1.6	1.3	1.5	1.4	0.2	1.8	1.8	1.8	1.5	1.8	1.8	1.6	1.5	1.5	1.6	1.6	1.5	1.6	1.5	1.7	1.5	1.6	1.8	1.8	2.4	2.4	9.2	1.5	1.5	1.5	1.5	1.5	0.4	0.2	1.7	***	99.8	98.1	71.3	*E. meningoseptica* (JN201943)
5.5	4.9	4.9	5.0	1.6	1.6	1.6	1.4	1.6	0.4	1.4	1.2	1.3	1.2	0.0	1.5	1.6	1.6	1.3	1.6	1.6	1.4	1.3	1.3	1.4	1.4	1.3	1.4	1.3	1.4	1.3	1.4	1.7	1.7	2.2	2.2	0.3	1.3	1.3	1.3	1.3	1.3	0.5	0.0	1.5	0.2	***	98.2	71.3	*E. meningoseptica* (NR_042267)
6.4	6.1	6.1	6.0	1.8	1.8	1.8	0.2	0.3	1.9	0.2	1.0	1.5	1.6	1.6	1.8	1.8	0.3	1.5	1.8	0.3	1.6	1.5	1.5	1.6	1.6	1.5	1.7	1.5	1.6	1.5	0.2	0.4	0.4	2.4	2.4	1.7	1.5	1.5	1.5	1.5	1.5	2.0	1.6	0.2	1.8	1.6	***	70.9	*E. miricola* (NR_036862)
35.9	37.1	37.1	37.1	33.4	33.4	33.5	33.8	33.8	34.0	33.6	34.2	33.5	33.2	33.8	33.4	33.5	33.8	33.5	33.5	33.8	33.5	33.5	33.5	33.6	33.4	33.5	33.8	33.5	33.5	33.5	33.8	33.9	33.9	33.5	33.5	34.0	33.5	33.5	33.5	33.5	33.5	34.1	33.8	33.8	33.8	33.8	34.1	***	*E. coli* ATCC 25922 (X80724)

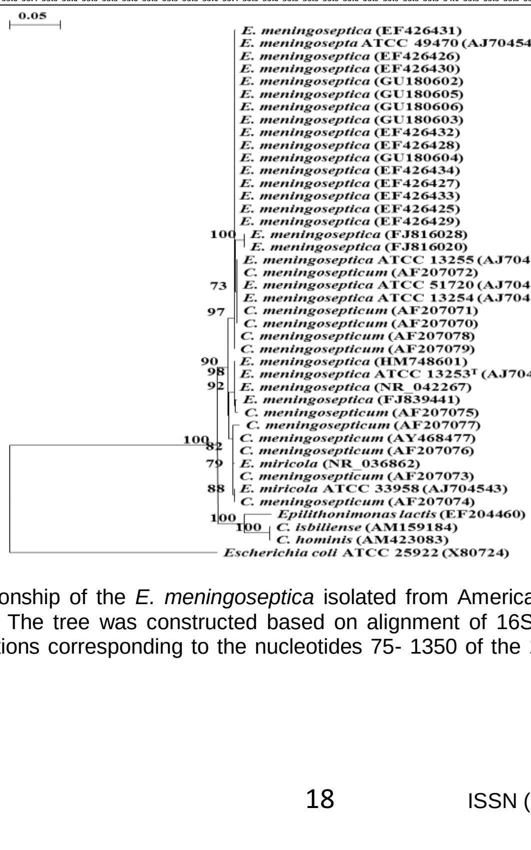

Figure 3: Phylogenetic relationship of the *E. meningoseptica* isolated from American bullfrog farmed in Sabah, Malaysia with other closely related bacteria. The tree was constructed based on alignment of 16S rDNA sequences using ClustalW method (DNASTAR Ver. 5.05) at positions corresponding to the nucleotides 75- 1350 of the 16S rDNA of *Escherichia coli* ATCC 25922 (X80724).

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Antibiotic susceptibility assay

The 5 bacterial isolates exhibited strong susceptibility to ciprofloxacin, nalidixic acid, compound sulphoniamides and trimethoprim. However, they were resistant to nitrofurantion, chloramphenicol, ampicilin, oxytetracycline, tetracycline, streptomycin and kanamycin. The inhibition zones exhibited by individual antibiotics against the bacterial isolates are given in Table 5.

Specific PCR for detection of E. meningoseptica

The primers against the β-lactamase gene of *E. meningoseptica* were specific to the bacteria. All the bacterial isolates were successfully amplified using both pairs of the PCR primers with expected sizes. In contrast, the primer pairs did not amplify any of the ATCC bacterial strains tested in this study. The results of PCR amplification using the primer pair 1 and pair 2 are shown in Figure 4 and Figure 5, respectively.

Table 4: List of 16S rDNA sequences used in study.

Bacterial Strain	Accession No.	Reference
E. meningoseptica	EF426431	Lindh *et al.* 2008
E. meningoseptica ATCC 49470	AJ704544	Kim *et al.* 2005
E. meningoseptica	EF426426	Lindh *et al*. 2008
E. meningoseptica	EF426430	Lindh *et al.* 2008
E. meningoseptica	EF426432	Lindh *et al.* 2008
E. meningoseptica	EF426428	Lindh *et al.* 2008
E. meningoseptica	EF426434	Lindh *et al.* 2008
E. meningoseptica	EF426427	Lindh *et al.* 2008
E. meningoseptica	EF426433	Lindh *et al.* 2008
E. meningoseptica	EF426425	Lindh *et al.* 2008
E. meningoseptica	EF426429	Lindh *et al.* 2008
E. meningoseptica	FJ816028	Kajla *et al.* 2010
E. meningoseptica	FJ816020	Kajla *et al.* 2010
E. meningoseptica ATCC 13255	AJ704542	Kim *et al.* 2005
C. meningosepticum	AF207072	Bellais *et al.* 2000
E. meningoseptica ATCC 51720	AJ704545	Kim *et al.* 2005
E. meningoseptica ATCC 13254	AJ704541	Kim *et al.* 2005
C. meningosepticum	AF207071	Bellais *et al.* 2000
C. meningosepticum	AF207070	Bellais *et al.* 2000
C. meningosepticum	AF207078	Bellais *et al.* 2000
C. meningosepticum	AF207079	Bellais *et al.* 2000
E. meningoseptica	HM748601	Kim *et al.* 2011
E. meningoseptica ATCC 13253^T	AJ704540	Kim *et al.* 2005
E. meningoseptica	NR_042267	Kim *et al.* 2005
E. meningoseptica	FJ839441	Su and Ming, 2010
C. meningosepticum	AF207075	Bellais *et al.* 2000
C. meningosepticum	AF207077	Bellais *et al.* 2000
C. meningosepticum	AY468477	Bernardet *et al*. 2005
C. meningosepticum	AF207076	Bellais *et al.* 2000
E. miricola	NR_036862	Kim *et al.* 2005
C. meningosepticum	AF207073	Bellais *et al.* 2000
E. miricola ATCC 33958	AJ704543	Kim *et al.* 2005
C. meningosepticum	AF207074	Bellais *et al.* 2000
E. meningoseptica	EF204460	Shakěd *et al.* 2010
C. isbiliense	AM159184	unpublished
C. hominis	AM423083	Vaneechoutte *et al.* 2007
Echerichia coli ATCC 25922	X80724	Cilia *et al*. 1996
E. meningoseptica	GU180602	This study
E. meningoseptica	GU180603	This study
E. meningoseptica	GU180604	This study
E. meningoseptica	GU180605	This study
E. meningoseptica	GU180606	This study

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Table 5: Inhibition zone (cm) recorded on different antibiotics against different isolates of *E. meningoseptica*

Bacteria	F	C	CIP	AMP	OA	OT	TE	S	NA	K	S3	W
EKME1	0	0.9	2.0	0	0.8	1.2	0	0	2.1	0	2.7	1.8
EKME2	0	1.2	2.1	0	1.2	1.2	0	0	2.0	0	2.7	1.7
EKML1	0	1.1	2.2	0	1.0	1.1	0	0	2.0	0	2.8	1.5
EKMK1	0	1.1	2.1	0	1.0	1.0	0	0	2.0	0	2.9	2.0
EKMS1	0	1.4	2.5	0	1.4	1.2	0	0	2.5	0	2.5	1.5

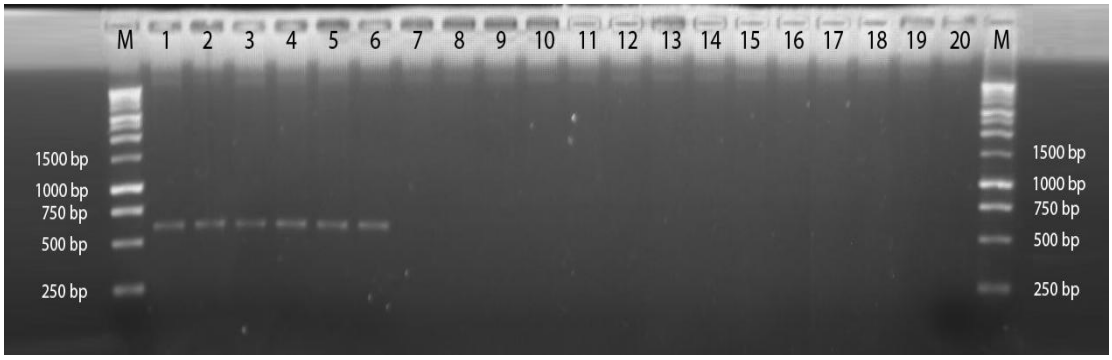

Figure 4: PCR amplification (612bp) using primers (JREMF1 and JREMR1) designed specifically for *E. meningoseptica*. Lanes M: 1 kb DNA Ladder (Promega); lane 1: *E. meningoseptica* ATCC 13253, lane 2: *E. meningoseptica* isolate EKMK1; lane 3: *E. meningoseptica* isolate EKML1; lane 4: *E. meningoseptica* isolate EKMLE1; lane 5: *E. meningoseptica* isolate EKMRE1; lane 6: *E. meningoseptica* isolate EKMS1; lane 7: *A. caviae* ATCC 15468; lane 8: *A. hydrophila* ATCC 7965; lane 9: *A. salmonicida* subsp. s*almonicida* ATCC 33658; lane 10: *Ed. tarda* ATCC 15947; lane 11: *Escherichia coli* ATCC 25922; lane 12: *Pr. mirabilis* ATCC 29245; lane 13: *P. aeruginosa* ATCC 27853; lane 14: *P. fluorescens* ATCC 13525; lane 15: *V. alginolyticus* ATCC17749; lane 16: *V. anguillarum* ATCC 19264; lane 17: *V. harveyi* ATCC 35084; lane 18: *V. parahaemolyticus* ATCC 17802; lane 19: *Y. ruckeri* ATCC 29473 and lane 20: Sterile double distilled water.

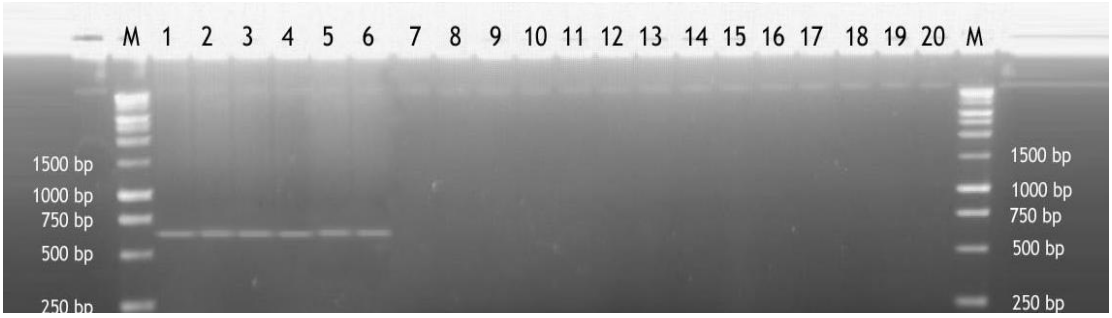

Figure 5: PCR amplification (644bp) using primers ((JREMF2 and JREMR2) designed specifically for *E. meningoseptica*. Lanes M: 1 kb DNA Ladder (Promega); lane 1: *E. meningoseptica* ATCC 13253, lane 2: *E. meningoseptica* isolate EKMK1; lane 3: *E. meningoseptica* isolate EKML1; lane 4: *E. meningoseptica* isolate EKMLE1; lane 5: *E. meningoseptica* isolate EKMRE1; lane 6: *E. meningoseptica* isolate EKMS1; lane 7: *A. caviae* ATCC 15468; lane 8: *A. hydrophila* ATCC 7965; lane 9: *A. salmonicida* subsp. s*almonicida* ATCC 33658; lane 10: *Ed. tarda* ATCC 15947; lane 11: *Escherichia coli* ATCC 25922; lane 12: *Pr. mirabilis* ATCC 29245; lane 13: *P. aeruginosa* ATCC 27853; lane 14: *P. fluorescens* ATCC 13525; lane 15: *V. alginolyticus* ATCC17749; lane 16: *V. anguillarum* ATCC 19264; lane 17: *V. harveyi* ATCC 35084; lane 18: *V. parahaemolyticus* ATCC 17802; lane 19: *Y. ruckeri* ATCC 29473 and lane 20: Sterile double distilled water.

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DISCUSSION

E. meningoseptica was previously known as *Flavobacterium meningosepticum* or *Chryseobacterium meningosepticum* (Kim *et al.,* 2005). It is a Gram-negative and non-fermenting bacterium which is widely distributed in nature. It constitutes common bacterial flora in freshwater (Vancanneyt *et al.,* 1994). However, the bacterium has also been isolated from diseased turtles, frogs and fish (Green *et al.,* 1999; Bernardet, 2006; Mauel *et al.,* 2003; Bernardet *et al.,* 2005). The bacterium was also recognized as an occasional but serious opportunistic pathogen to human, giving rise to meningitis, pneumonia, septic arthritis, endocarditis and conjunctivitis (Bernardet *et al.,* 2006).

Cataract is the most prevalent disease in farmed anurans and it quickly spreads within a relatively short period of time (Xie *et al.,* 2010). It is characterized by opaque eye lens, ascites in peritoneal cavity, lethargy and torticollis (Xie *et al.,* 2010). In the present study, we also observed eye opacity, sluggish behavior and ascites in peritoneal cavity of frogs with cataract and red-leg syndrome. In addition, the infected frogs had limited hopping ability and they were observed to suffer mortality from a few days to weeks after the onset of the disease. The bacterial isolation and 16S rDNA sequencing analysis revealed that all the bacterial isolates from eyes and internal organs of frogs belonged to *E. meningoseptica*. Although this bacterium has previously been isolated from farmed tiger frog (*R. tigerina rugulosa*) in China with cataract (Xie *et al.,* 2010) and in African clawed frog *Xenopus laevis* (Bernardet, 2006), this is the first report from Malaysia.

The farming of bullfrog in Malaysia is conducted either in earthen ponds or in concrete tanks. Broodstocks were first imported from Taiwan (Lee *et al.,* 2009). Ever since, they are propagated and maintained by the Fisheries Department of Sabah. Currently, the department maintains about 200-250 frog brooders in one of its aquaculture stations in Penampang. Frog larvae from this station are distributed to small-scale farms throughout Sabah as part of the government subsidy program. The cataract and red-leg syndrome occurred in frogs maintained in this station as well as in several private farms throughout Penampang district. The diseases were observed to affect adult frogs. It was found that the rapid spread of the disease among frogs could have been contributed by poor farming practices in most farms. This was apparent since diseased frogs are not isolated from clinically healthy animals. Furthermore, water quality in the culture tank was poor as indicated by smell of decaying organic matter. Uneaten foods are not removed but let to decay in the culture tanks. Similarly, several decaying dead frogs were also observed in the tanks. The workers who maintain the farms are not protected since they are handling the frogs by their bare hands. This could particularly be hazardous since *E. meningoseptica* has been reported as an opportunistic but serious human pathogen (Bernardet, 2006) especially those with respiratory problem (Weaver *et al.,* 2010).

E. meningoseptica can be contracted by the frogs from several sources including soils (Ahmad *et al.,* 2009), water (Vandamme *et al.,* 1994) and even mosquitoes (Lindh *et al.,* 2008; Rani et al. 2009). In the prevailing situation in Sabah, we understand that the bacterium may have been transmitted through mosquitos. This explains the 100% nucleotide sequence homology of the five *E. meningoseptica* isolated in this study to 16S rRNA gene sequences of *E. meningoseptica* isolates (EF426426, EF426427, EF426430, EF426432 and EF426434) from mosquitos, *Anopheles gambiae* (Lindh *et al.,* 2008). Although no bacterial isolation was done from mosquito specimens, the poorly maintained rearing water in the farms can be a perfect place for mosquito breeding. This is supported by the observation of many mosquito larvae in the rearing tanks in the farms where the disease outbreaks occurred.

The bacterium seemed to have developed resistance towards β-lactam antibiotics which include penicillin, nitrofurantion, ampicillin, tetracycline, streptomycin and kanamycin. Hence, the choice of antibiotics for treatmen of cataract and red-leg syndrome is limited except for ciprofloxacin and nalidixic acid. However, it has been shown that the use of ciprofloxacin in African clawed frog (*X. laevis*) showed that there was an increase of the antibiotic concentration in the habitat several hours after the administration (Howard *et al.,* 2010).

The PCR primers targeting β-lactamase gene described in the present study can be potentially be developed as a DNA-based diagnostic kit for *E. meningoseptica*. However, verification and validation of the technique are still required before such a diagnostic kit can be developed. Despite the availability of detection kit, we strongly believe that good farming practices should be carried all the time in order to effectively prevent disease outbreak from occurring in the farms. These include improving of quality of rearing water, avoiding of stagnation, preventing exposure to mosquitoes, regular tank cleaning, and removal of any uneaten food and dead animals from the culture tanks. Need for effective protection should also be explained to farm workers because of the zoonotic potential of *E. meningoseptica*. With these programs in place, disease outbreaks can be prevented or at least significantly minimized to curtail economic losses and to sustain the frog meat industry in the country

CONCLUSION

The first isolation of *E. meningoseptica* from frog farms in Sabah may form the basis for an extensive epidemiological study of the pathogen to be carried out throughout frog farms in Malaysia. We strongly believe that there is urgent requirement of standard guideline for good farming practice to be adopted in frog farms throughout the country. Such a guideline can help in minimizing economic losses, preventing transmission of the zoonotic bacterial pathogens to farm workers, and

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sustaining the industry in Malaysia as well as upgrading of frog meat quality for international market.

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