

*Short Communication***Retrospective Identification of Bacterial Depository Revealed that *Streptococcus iniae* was Responsible for Some of the Streptococcosis Cases in Cultured Red Tilapia in Malaysia since 2006****Siti Hawa Mohamad Ali¹, Mohd Syafiq Mohammad Ridzuan^{2*}, Siti Zahrah Abdullah², Nur Nazifah Mansor³, Mohd Firdaus Nawi³, Mohd Zamri-Saad⁴ and Mohammad Noor Azmai Amal⁴**¹Sarawak and F.T. Labuan Division, Fisheries Research Institute (FRI) Bintawa, 93744 Kuching, Sarawak, Malaysia²National Fish Health Research Division (NaFisH), Fisheries Research Institute (FRI) Batu Maung, 11960 Batu Maung, Penang, Malaysia³Kulliyah of Science, International Islamic University Malaysia, 25200 Kuantan, Pahang, Malaysia⁴Laboratory of Marine Biotechnology, Institute of Bioscience, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia**ABSTRACT**

This paper reports the identification of *Streptococcus iniae* from a large collection of isolates previously identified as *Streptococcus* sp., *Lactococcus lactis* subsp. *lactis* or *Leuconostoc* sp. A total of 204 bacterial isolates recovered either from the brain, eye, or kidney of red tilapias in previous disease outbreaks and disease monitoring in Malaysia from 2006 to 2008 were used. PCR identification revealed that 34 (16.7%) of the isolates were confirmed as *S. iniae*. Our records showed that *S. iniae*-infected fish exhibited lethargy, exophthalmia, and erratic swimming patterns. Pathological lesions including generalised congestion of the internal organs, splenic infarction with soft and oedematous brain. Histopathological examination revealed multifocal encephalitis as one of the major findings. However, 44%

and 26.5% of the tilapias from which *S. iniae* was isolated did not manifest any clinical sign and pathological lesion, respectively. This study revealed that *S. iniae* was responsible for streptococcosis in cultured red tilapia in Malaysia since 2006.

Keywords: *Oreochromis* sp., red tilapia, streptococcosis, *Streptococcus iniae*

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INTRODUCTION

The intensification of tilapia culture in this country has led to disease outbreaks, especially due to *Streptococcus* spp., which cause high mortality and severe economic losses to the industry (Zamri-Saad et al., 2014). To date, *Streptococcus agalactiae*, *S. iniae*, and *S. dysgalactiae* were identified as the main aetiological agents of streptococcosis (Costa et al., 2014; El-Aamri et al., 2010; Rahmatullah et al., 2017). In Malaysia, infection by *S. agalactiae* has been previously reported affecting red tilapia (Syuhada et al., 2020) and golden pompano (Amal et al., 2012).

Streptococcus iniae has been associated with several disease outbreaks in both freshwater and marine cultured fishes, such as hybrid tilapia (Al-Harbi, 2011), Nile tilapia (Shoemaker et al., 2001), red porgy (El-Aamri et al., 2010), hybrid striped bass (Shoemaker et al., 2001), seabass (Colorni et al., 2002), and Japanese flounder (Nguyen et al., 2002). However, infection by *S. iniae* in cultured red hybrid tilapia in Malaysia was only reported in 2017 (Rahmatullah et al., 2017).

From 2006 to 2008, *Streptococcus* outbreaks and fish disease screening revealed the responsibility of *S. agalactiae* in infecting red tilapia in various aquaculture sites in Peninsular Malaysia (Amal et al., 2010), but no identification of *S. iniae* from the diseased fish was made. However, in this study, we revealed that *S. iniae* was also actually responsible for streptococcosis in cultured tilapia in Malaysia as early as

2006, where it was previously identified as *Streptococcus* sp. or other bacteria species.

MATERIALS AND METHODS

In our previous studies, between 2006 and 2008, samplings of cultured red tilapias were carried out for bacterial isolation following complaints of disease outbreaks from farmers and routine disease monitoring. The sampling sites covered three different types of water bodies including reservoir, river, and pond, which comprised of nine different locations in the north and east part of Peninsular Malaysia (Table 1). The fish clinical signs, external and internal abnormalities of the fish were observed and recorded before the bacterial isolation was made from the brain, eye, and kidney. Selected organs of the diseased fish such as skin, liver, spleen, brain, and kidney were also collected for histopathological analyses (Amal et al., 2010, 2015; Zamri-Saad et al., 2010).

Following the bacterial isolation and identification in the period of 2006 to 2008, besides *S. agalactiae*, a total of 204 Gram positive isolates were also identified either as *Streptococcus* sp., *Lactococcus lactis* subsp. *lactis*, and *Leuconostoc* sp. using API 20 Strep (bioMérieux, Marcy l'Etoile, France) (Table 1). In order to screen for *S. iniae*, all of the isolates were then retrieved from our collections, and subcultured onto tryptic soy agar (Merck, Darmstadt, Germany) with 5% goat's blood and incubated at 27°C for 24 h to 48 h. A single colony from each plate with pure bacterial growth was then selected and

inoculated into 10 mL brain heart infusion broth (Merck) before incubated in an orbital incubator at 27°C for 24 h at 150 rpm. DNA from the isolates was extracted using Wizard Genomic DNA Purification Kit (Promega, Madison, USA) as per manufacturer's protocol. The extracted DNA was optimized for concentrations ranging between 3.5 and 4.0 µg/µL prior to PCR amplification.

A published primers sequence for the targeted gene *lctO* of *S. iniae* was used (Mata et al., 2004). The nucleotide sequences for the forward and reverse direction were LOX-1 5'AAGGGGAAATCGCAAGTGCC3' and LOX-2 5'ATATCTGATTGGGCCGTCTAA3', respectively. The PCR assay was carried out based on the method described by Mata et al. (2004). For comparison and validation of the PCR technique, *S. iniae* strain ATCC® 29178™ (positive control), distilled water (negative control), and *S. agalactiae* strain ATCC® 27956™ (negative control) were included in each test.

RESULTS

A total of 16.7% (34) of the isolates were successfully identified as *S. iniae*, following amplification of the 870 bp band in PCR methods. The positive control of *S. iniae* ATCC® 29178™, negative control of distilled water, and negative control of *S. agalactiae* ATCC® 27956™ were used to validate the methods revealing their respective expected results (Figure 1). All *S. iniae* isolated in this study showed β-haemolysis on blood agar.

Streptococcus iniae was detected from cultured red tilapia at six (67%) of the nine sampling sites. Most isolates originated from Terengganu (31 isolates; 91.2%), while the remaining two (5.8%) and one (2.9%) were from Kedah and Perlis, respectively. They were isolated either from the brain, eye or kidney of the fish that between 19.7 to 31.5 cm in length and 175 to 967 g of body weight (Table 1). Interestingly, it was found that larger tilapias seemed to be more susceptible to streptococcosis. Our previous data showed that all 34 isolates of *S. iniae* were earlier identified as *L. lactis* subsp. *lactis* (28 isolates; 82.4%), *Streptococcus* spp. (3 isolates; 8.8%), and *Leuconostoc* spp. (3 isolates; 8.8%) when using the API 20 Strep kit.

Database showed that approximately 44.1% of red tilapias from which *S. iniae* was isolated did not show any gross lesion and clinical sign, while 55.9% either showed corneal opacity, unilateral or bilateral exophthalmia, inflammation along the base of the pectorals region, ventral region, operculum, and erratic swimming patterns. Following post-mortem examination, 26.5% of the affected tilapias appeared normal, while the remaining 73.5% either showed congestion of gill, liver, spleen, and kidney, with soft and oedematous brain. Histopathological examination confirmed the generalised congestion of internal organs and splenic infarcts, while the brain revealed multifocal encephalitis and oedema (Figures 2 and 3).

Table 1
Details of the identified *Streptococcus iniae* from cultured red tilapias in several sampling sites in Peninsular Malaysia

Water body	Sampling site	State	Year of isolation	No. of tested isolates	No. of positive isolates (%)	Organ	Infected fish			API20 Strep identification (%)
							Fish length Mean \pm SD (cm)	Fish weight Mean \pm SD (g)		
Reservoir	Pedu Lake	Kedah	2006	55	2 (3.6)	E, K	19.75 \pm 2.47	175.00 \pm 63.64		<i>Streptococcus</i> sp. (100)
	Kenyir Lake	Terengganu	2008	4	2 (50.0)	E	28.25 \pm 0.35	443.50 \pm 20.51		<i>Lactococcus lactis</i> subsp. <i>lactis</i> (100)
River	Kuala Kejir	Terengganu	2008	3	0 (0.0)	-	-	-	-	-
	Pantai Ali	Terengganu	2007-2008	15	2 (13.3)	B, E	16.00 \pm 6.36	125.50 \pm 132.23		<i>Lactococcus lactis</i> subsp. <i>lactis</i> (100)
	Beladau Selat	Terengganu	2006-2007	64	24 (37.5)	B, E, K	20.69 \pm 3.99	224.71 \pm 133.32		<i>Lactococcus lactis</i> subsp. <i>lactis</i> (95.83) and <i>Leuconostoc</i> spp. (4.17)
	Beladau Kepong	Terengganu	2006-2008	23	3 (13.0)	E	20.50 \pm 4.09	176.67 \pm 82.02		<i>Lactococcus lactis</i> subsp. <i>lactis</i> (33.3) and <i>Leuconostoc</i> spp. (66.6)
Pond	Arau	Perlis	2006	30	1 (3.3)	B	31.50	967.00		<i>Streptococcus</i> sp. (100)
	Kodiang	Kedah	2008	5	0 (0.0)	-	-	-	-	-
	Jitra	Kedah	2006	5	0 (0.0)	-	-	-	-	-
Total				204	34 (16.7)					

Note. B = brain; E = eye; K = kidney

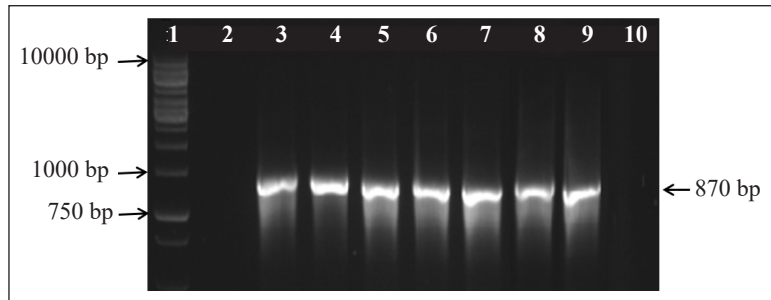


Figure 1. Agarose gel electrophoresis showing PCR amplification products generated by the LOX-1/LOX-2 primers at 870 bp. Lane 1: 10000 bp molecular size ladder; Lane 2: distilled water (negative control); Lane 3: *Streptococcus iniae* ATCC® 29178™ (positive control); Lane 4: TP3 (isolate from Pedu Lake); Lane 5: TCT1044 (isolate from Kenyir Lake); Lane 6: TSP299 (isolate from Pantai Ali); Lane 7: TSB274 (isolate from Beladau Selat); Lane 8: TSK682 (isolate from Beladau Kepong); Lane 9: TA127 (isolate from Arau); Lane 10: *Streptococcus agalactiae* ATCC® 27956™ (negative control)

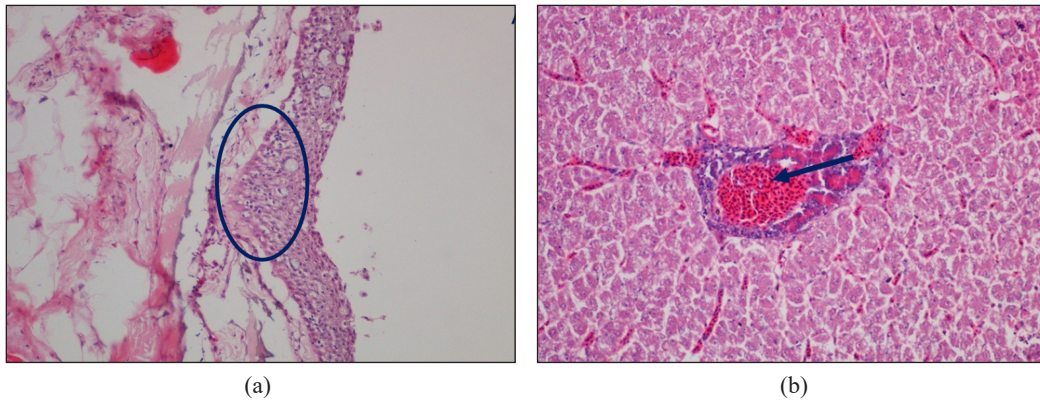


Figure 2. (a) Skin section showing inflamed epithelial layer with infiltration of inflammatory cells (oval). H.E., obj. 100x; (b) Liver section showing congested portal vein (arrow). H.E., obj. 400x

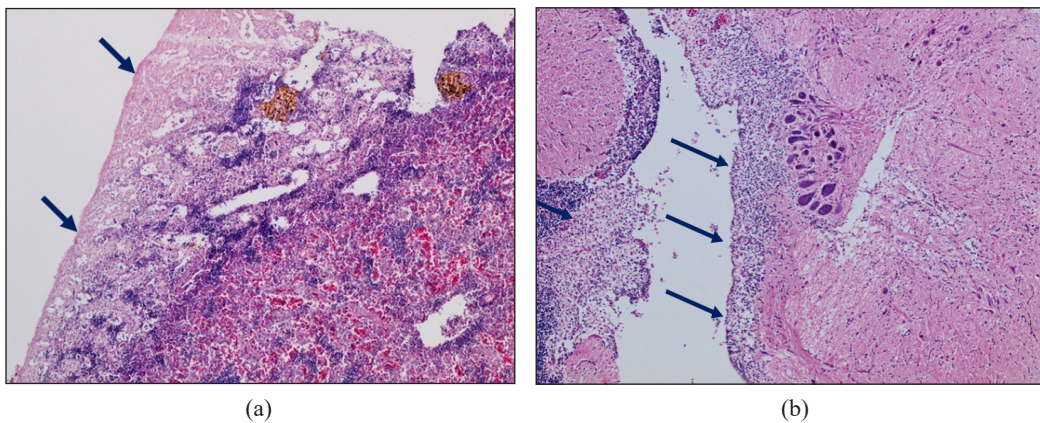


Figure 3. (a) Infarct in spleen (arrow). H.E., obj. 200x; (b) Brain section showing extensive encephalitis (arrow). H.E., obj. 100x

DISCUSSION

The inability of API 20 Strep to correctly identify *S. iniae* has been previously described (Al-Harbi, 2011; El-Aamri et al., 2010; Roach et al., 2006). Previous studies reported that biochemical analyses using API 20 Strep misidentified *S. iniae* as *S. dysgalactiae* subsp. *equisimilis* (El-Aamri et al., 2010; Lau et al., 2003; Suanyuk et al., 2010). However, in this study, *S. iniae* were previously identified as *Streptococcus* spp., *L. lactis* subsp. *lactis*, and *Leuconostoc* spp., which we believed to be due to misinterpretation on the reading of the biochemical tests based on the visual interpretation, as raised by Gomes et al. (2007). Other commercial test kits, such as BioMérieux Vitek, MicroScan WalkAway System (Facklam et al., 2005), and ATB Expression System (Lau et al., 2003) were also unable to identify *S. iniae*. Due to this limitation, specific PCR primer sequences have been developed as a useful alternative approach for the rapid and accurate identification of *S. iniae*, such as the 16S rRNA gene, the 16S–23S rRNA gene intergenic spacer region, the chaperonin HSP60, and the *lctO* gene (Berridge et al., 1998; Goh et al., 1998; Mata et al., 2004; Roach et al., 2006).

In this study, we successfully identified *S. iniae* from cultured red tilapia in Malaysia by PCR based on the *lctO* gene. Utilization of this primer sets has also successfully identified *S. iniae* from several species of fish in different regions (Al-Harbi, 2011; Lee & Park, 2014; Suanyuk et al., 2010).

Affected tilapia in this study showed similar clinical signs as previous reports (Chen et al., 2007; Rahmatullah et al., 2017). However, there were evidences of asymptomatic carriers, as observed in striped piggy and variegated lizardfish (Colorni et al., 2002). They showed neither clinical signs nor pathological changes, and could be a source of infection as observed in *S. agalactiae* (Amal & Zamri-Saad, 2011).

CONCLUSION

Future bacterial identification should include the molecular and sequencing analysis for better accuracy of the results. Moreover, this study revealed that *S. iniae* was actually responsible for streptococcosis in cultured red tilapia in Malaysia since 2006.

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