Random amplified polymorphic DNA (RAPD): a powerful method to differentiate *Streptococcus agalactiae* strains

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ABSTRACT

Streptococcus agalactiae from five different locations comprised of Pedu Lake (Kedah, Malaysia), Kenyir Lake (Terengganu, Malaysia), Pergau Lake (Kelantan, Malaysia), NTL Farm (Kelantan, Malaysia) and Wawasan Farm (Kelantan, Malaysia) were sampled and characterized by the API System. In order to achieve strain typing, all of the isolates were investigated by random amplified polymorphic DNA-PCR (RAPD-PCR). The RAPD-PCR technique was used to detect the genetic diversity of the five different sampling sites in Malaysia. Initially, 20 RAPD primers (Operon, USA) were used for screening before suitable primers that showed optimal level to be used later. Six 10-mer RAPD primers revealed significant bands ranging from 400-5000 bp. The primers were OPA3, OPA4, OPA5, OPA9, OPA11 and OPA20. The genetic identity and genetic distance were calculated to reveal the diversities among the five different locations of sampling sites by using RAPD Distance software before a phylogenetic tree was constructed. The results indicated that the genetic variation from different locations is relatively high. Four out of five places exhibited a very close relationship with each other but a relatively distant relationship with Pergau Lake isolates.

Keywords: *Streptococcus agalactiae,* random amplified polymorphic DNA (RAPD), phylogenetic tree, tilapia.

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INTRODUCTION

Streptococcal disease in fish was first reported in 1957, in cultured rainbow trout in Japan, (Hoshina *et al*., 1958). Since then, numerous other species of fish have been found susceptible to the infection, including tilapia (Plumb, 1997). In a study conducted by Ferguson *et al*. (1994), *Streptococcus agalactiae* was shown to be more aggressive than any other environmental bacterium, where mortality may reach 100%. This indicates a need for studies on streptococcal infection in tilapia in Malaysia, particularly on the characterization of *S. agalactiae* isolated from diseased tilapia. This may lead to a better understanding of the epidemiology and assist in formulating better management of this pathogenic bacterium especially in cage-cultured tilapia.

A surveillance study by the National Fish Health and Research Centre (NaFisH) Penang, Malaysia, found that *S. agalactiae* was frequently isolated from cases of high mortality of cage-cultured tilapia in Kenyir and Pergau in 2002 to 2003. This incidence was found to be related to seasonal changes that affect the water quality parameters leading to unbalance physiological conditions of the fish. Alpha and beta hemolytic types of *Streptococcus* spp. were isolated.

Streptococcus agalactiae is a Group B *Streptococcus* (GBS), typically Gram-positive, cocci in chains and displays beta-hemolysis when cultured on a blood agar plate. The polysaccharide anti-phagocytic capsule in this bacterium is the main virulence factor that causing a disease of *Streptococcus* septicemia in tilapia. Therefore, the objective of this study is to determine the molecular epidemiological pattern of streptococcal infection of tilapia in Malaysia using molecular characterization of randomly amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR). The PCR was used to amplify DNA samples of *S. agalactiae* with short oligonucleotide primers, which annealing process take place randomly throughout the genome of the isolates (Snowdon and Langsdorf, 1998).

MATERIALS AND METHODS

Bacterial isolates and growth conditions

Isolates of *S. agalactiae* were obtained from NaFisH, Penang. The isolates were collected from Kenyir Lake, Pergau Lake, Pedu Lake, NTL Farm and Wawasan Farm in Malaysia between 2003 and 2005. All isolates were cultured onto blood agar plates within 18 to 24 hr and incubated at 30°C. The bacteria were sub-cultured into brain heart infusion broth (BHIB) (MERCK, Germany) and incubated at 30°C on a shaker for 18 hr. The bacteria were harvested by centrifugation at 3,500 *g*, 4°C for 10 min.

DNA extraction

DNA from the isolates was extracted using Wizard Genomic DNA Purification Kit (Promega, USA) according to the manufacturer's instructions.

RAPD-PCR

RAPD-PCR was performed to detect the genetic diversity of five different sampling sites of *S. agalactiae* in Malaysia. Six 10-mer RAPD primers (Operon, USA) were screened out of 20 (OPA1 - OPA20). The primers are as in Table 1.

Table 1
Primers use in RAPD-PCR of *S. agalactiae*

No.	Primers	Primer Sequence
1.	OPA 3	5'- AGT CAG CCA C-3'
2.	OPA 4	5'- AAT CGG GCT G -3'
3.	OPA 5	5'- AGG GGT CTT G -3'
4.	OPA 9	5'- GGG TAA CGC C -3'
5.	OPA 11	5'- CAA TCG CCG T -3'
6.	OPA 20	5'- GTT GCG ATC C -3'

Polymerase chain reaction

All PCR's were carried out in a thermal cycler (Eppendorf, Germany) in 25 µl reaction volumes. The 0.2 ml thin-walled tubes were used to amplify the DNA of each reaction mixtures. Each reaction mixture contained 1.0 µg genomic DNA, 0.5 µl of 10 mM dNTPs mix, 2.0 µl of 25 mM $MgCl_2$, 2.5 µl of 10× PCR buffer (100 mM Tris-HCl (pH 8.8 at 25°C); 500 mM KCl; 0.8% Nonidet P40), 0.5 µl of *Taq* DNA polymerase (MBI Fermentas, USA) and 25 mM of each primers (First Base Laboratory, Malaysia). Standard amplification conditions were 94°C for 4 min followed by 44 cycles of 94°C for 1 min, 36°C for 1 min, 72°C for 1 min and the reaction was completed by a final extension step of 72°C for 10 min.

Gel electrophoresis

A gel consisted of 1% agarose in 1× TBE running buffer was prepared. Following that, the PCR product solution was mixed with 3 µl of 6× loading dye solution (Fermentas, Lithuania) and electrophoresed at 100 V for 45 min in a MT-108 Wide Mini Horizontal Gel Electrophoresis System. The gels were stained with 1 µg/ml ethidium bromide (Sigma, USA) for 30 min and visualized with an ultraviolet transilluminator.

Data analysis

Target DNA amplicon were scored (1 for band visible, 0 for no band visible) for each suitable marker (bp). Faint bands were considered. Results were then entered in NTedit 1.2a software according to the markers selected. Construction of a phylogenetic tree was done using NTSYSpc 2.10j (Numerical Taxonomy and Multivariate Analysis System version 2.1j) software.

RESULTS

By using six significant primers (OPA3, OPA4, OPA5, OPA9, OPA11 and OPA20), results revealed that the bands ranged from 4000 bp (OPA20) to ~ 300 bp (OPA5). OPA5 produced the most DNA fragments (7 fragments). Fig. 1 showed RAPD amplification of *S. agalactiae*

using OPA1 - OPA20 primers, which highlighted that different primers synthesize different molecular weight of bands. Primers that gave the most number of synthesized bands and the highest density of fragment bands (referring to the bright color on the agarose gels) were chose to use on the rest of the isolates. Figs. 2 - 7 showed RAPD amplification of *S. agalactiae* using OPA3, OPA4, OPA5, OPA9, OPA11 and OPA20. As referred to Figs. 2 - 7, some isolates looks similar but the faint band is the key that differentiates each isolate tested.

RAPD analysis

The band migration distances for each lane were determined and the molecular size of each fragment was calculated from migration distances. By using NTedit 1.2a, all data were entered as shown in Tables 2 - 7. The phylogenetic tree for the 5 different sampling

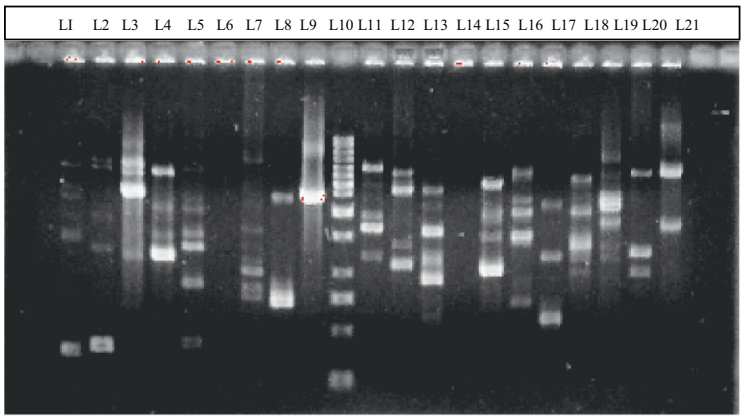

Figure 1. RAPD amplification of *S. agalactiae* using OPA1-OPA20 primers (L10: 1kb DNA markers, Fermentas) by using RT isolate. This is the preliminary RAPD-PCR to ensure which of the primer gave a significant band. L1-L9 represents OPA1-OPA9. L11-L21 represents OPA11-OPA20.

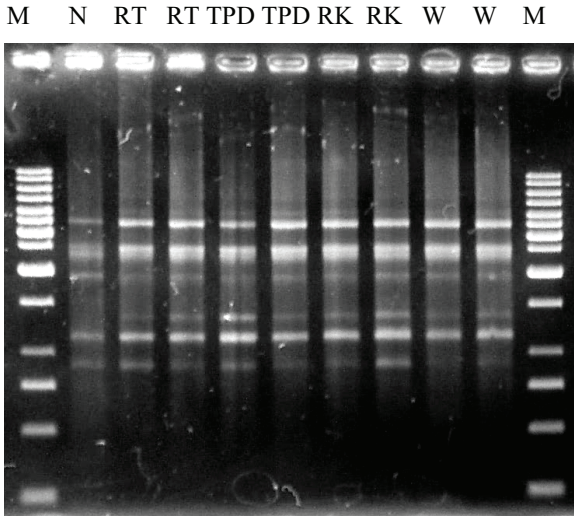

Figure 2 RAPD fingerprints of *S. agalactiae* using OPA3 primer. Differences between isolates can be seen in the faint band observed on the 1% agarose gel.

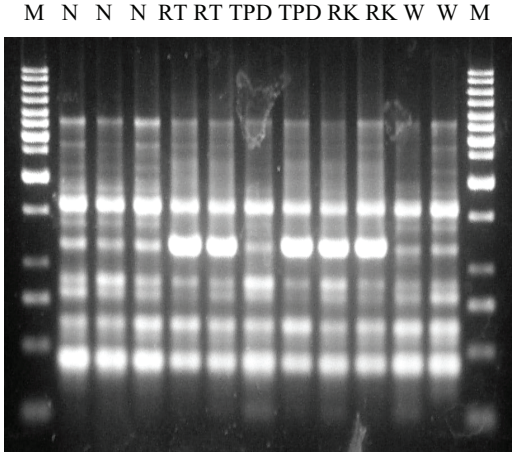

Figure 3. RAPD fingerprints of *S. agalactiae* using OPA4 primer. Differences between isolates can be seen in the faint band observed on the 1% agarose gel.

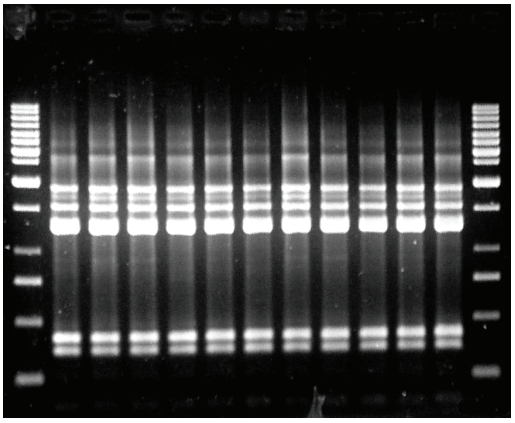

Figure 4. RAPD fingerprints of *S. agalactiae* using OPA5 primer. Differences between isolates can be seen in the faint band observed on the 1% agarose gel.

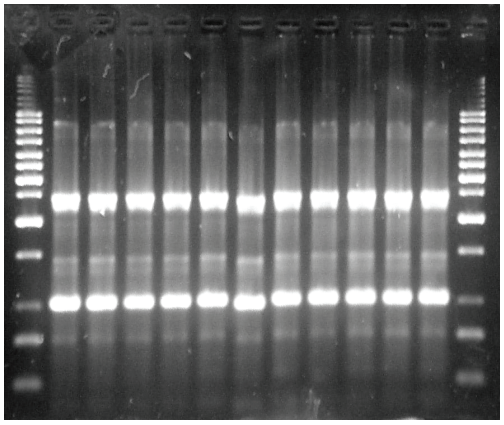

Figure 5 RAPD fingerprints of *S. agalactiae* using OPA9 primer. Differences between isolates can be seen in the faint band observed on the 1% agarose gel.

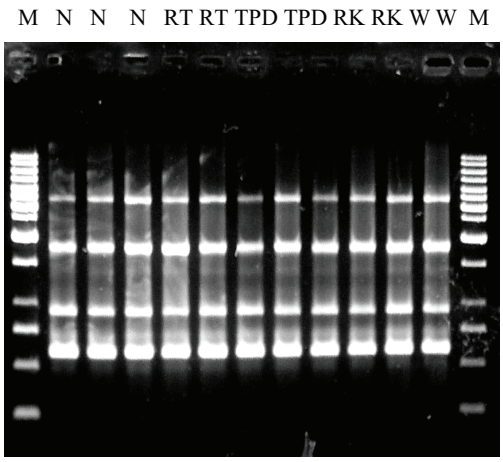

Figure 6 RAPD fingerprints of *S. agalactiae* using OPA11 primer. Differences between isolates can be seen in the faint band observed on the 1% agarose gel.

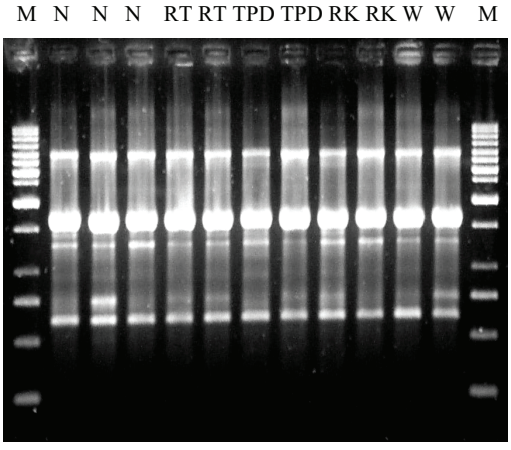

Figure 7 RAPD fingerprints of *S. agalactiae* using OPA20 primer. Differences between isolates can be seen in the faint band observed on the 1% agarose gel.

sites locations, Pedu Lake (TPD), Kenyir Lake (RT), Pergau Lake (RK), NTL Farm (N) and Wawasan Farm (W) was constructed. Cluster analysis generated two arbitrary groups. The phylogenetic tree (Fig. 8) revealed two major clusters. The first cluster showed two subclusters, which contained *S. agalactiae* isolated from four different sampling sites, while the second cluster showed that RK was totally different compared to the other *S. agalactiae* from the other sampling sites.

DISCUSSION

As shown in Fig. 8, the phylogenetic tree demonstrated that *S. agalactiae* isolated from RK are genetically different compared to the other *S. agalactiae* isolates. *S. agalactiae* isolated from RT, TPD, W and N were genetically similar as they are in the same cluster. However, *S. agalactiae* isolated from N were slightly different from the other even though they were in the same cluster. DNA sequencing is required to detect any slight mutation that may be present.

Table 2
Band Scoring Table for isolates using OPA3 primer

Band (kb)	RT	RK	TPD	W	N
4.00	0	0	0	0	0
3.50	1	1	1	1	1
2.50	1	1	1	1	1
2.25	0	0	0	0	0
2.00	0	0	0	0	1
1.80	0	0	0	0	0
1.70	0	0	0	0	0
1.40	0	0	0	0	0
1.50	0	0	0	0	0
1.25	1	1	1	1	1
1.00	0	0	0	0	0
0.80	0	1	0	0	0
0.75	0	0	0	0	0
0.60	0	0	0	0	0
0.40	0	0	0	0	0
0.30	0	0	0	0	0

Table 3
Band Scoring Table for isolates using OPA4 primer

Band (kb)	**RT**	**RK**	**TPD**	**W**	**N**
4.00	0	0	0	0	0
3.50	1	1	1	1	1
2.50	0	0	0	0	0
2.25	0	0	0	0	0
2.00	0	0	0	0	0
1.80	0	0	0	0	0
1.70	0	0	0	0	0
1.40	0	0	0	0	0
1.50	1	1	1	1	1
1.25	1	1	1	1	1
1.00	0	0	0	0	0
0.80	0	0	0	0	0
0.75	1	1	1	1	1
0.60	1	1	1	1	1
0.40	1	1	1	1	1
0.30	0	0	0	0	0

Table 4
Band Scoring Table for isolates using OPA5 primer

Band (kb)	**RT**	**RK**	**TPD**	**W**	**N**
4.00	0	0	0	0	0
3.50	0	0	0	0	0
2.50	1	0	1	1	1
2.25	0	0	0	0	0
2.00	0	0	0	0	0
1.80	1	1	1	1	1
1.70	1	1	1	1	1
1.40	0	0	0	0	0
1.50	1	1	1	1	1
1.25	1	1	1	1	1
1.00	0	0	0	0	0
0.80	0	0	0	0	0
0.75	0	0	0	0	0
0.60	0	0	0	0	0
0.40	1	1	1	1	1
0.30	1	1	1	1	1

Table 5
Band Scoring Table for isolates using OPA9 primer

Band (kb)	**RT**	**RK**	**TPD**	**W**	**N**
4.00	0	0	0	0	0
3.50	0	0	0	0	0
2.50	1	1	1	1	1
2.25	0	0	0	0	0
2.00	0	0	0	0	0
1.80	0	0	0	0	0
1.70	0	0	0	0	0
1.40	0	0	0	0	0
1.50	0	0	0	0	0
1.25	1	1	1	1	1
1.00	1	1	1	1	1
0.80	0	0	0	0	0
0.75	0	0	0	0	0
0.60	0	0	0	0	0
0.40	0	0	0	0	0
0.30	0	0	0	0	0

Table 6
Band Scoring Table for isolates using OPA11 primer

Band (kb)	RT	RK	TPD	W	N
4.00	0	0	0	0	0
3.50	1	1	1	1	1
2.50	0	0	0	0	0
2.25	0	0	0	0	0
2.00	0	0	0	0	0
1.80	0	0	0	0	0
1.70	1	1	1	1	1
1.40	0	0	0	0	0
1.50	0	0	0	0	0
1.25	0	0	0	0	0
1.00	0	0	0	0	0
0.80	1	1	1	1	1
0.75	0	0	0	0	0
0.60	1	1	1	1	1
0.40	0	0	0	0	0
0.30	0	0	0	0	0

Table 7
Band Scoring Table for isolates using OPA20 primer

Band (kb)	RT	RK	TPD	W	N
4.00	1	1	1	1	1
3.50	0	0	0	0	0
2.50	0	0	0	0	0
2.25	0	0	0	0	0
2.00	0	0	0	0	0
1.80	0	0	0	0	0
1.70	0	0	0	0	0
1.40	0	0	0	0	0
1.50	1	1	1	1	1
1.25	1	1	1	1	1
1.00	0	0	0	0	0
0.80	0	0	0	0	0
0.75	0	1	0	0	1
0.60	1	1	1	1	1
0.40	0	0	0	0	0
0.30	0	0	0	0	0

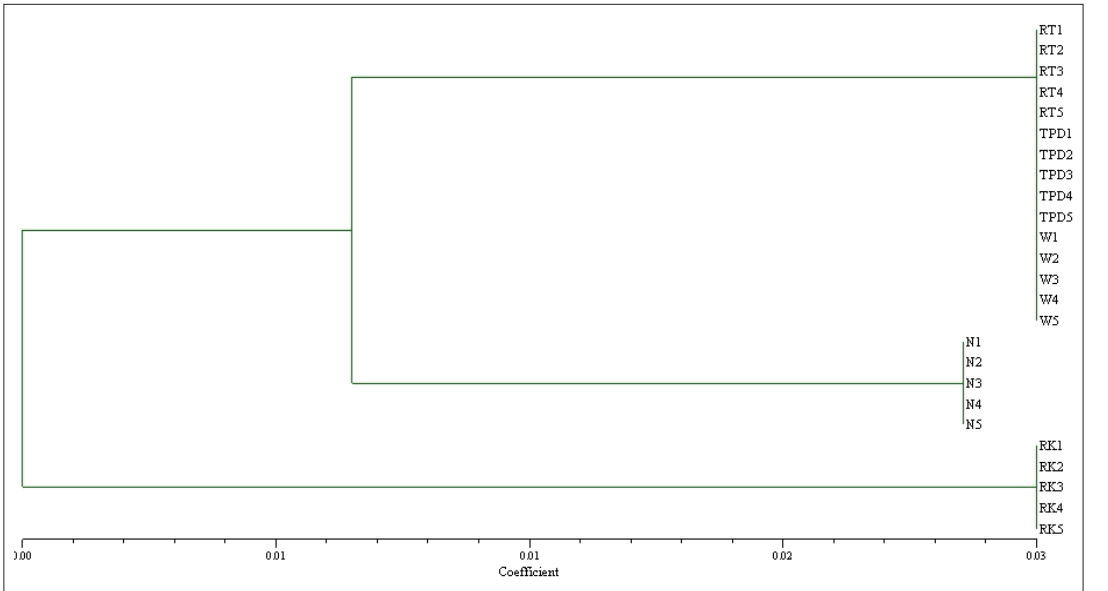

Figure 8 A simplified phylogenetic tree showing the degree of similarity of RAPD pattern of all the strains investigated using primer OPA3, OPA4, OPA5, OPA9, OPA11 and OPA20.

From the phylogenetic tree, geographical relations were not shown to be a factor because RT, RK, W and N are located at the west of Malaysia, while TPD is located at the east of Malaysia. So, the main factor may be the source of the fish obtained or perhaps it was the results of transposable elements, insertions or deletions in the DNA that need further sequencing for confirmation.

RAPD is one of the most powerful methods to differentiate *S. agalactiae* strains. Six of the twenty primers tested showed significant bands, which enabled construction of a phylogenetic tree. Interestingly, RAPD can also differentiate each *S. agalactiae* isolates within the species due to polymorphisms. Generally, if more isolates of *S. agalactiae* from various part of Malaysia were studied, the phylogenetic tree constructed will represent more of *S. agalactiae* strains in Malaysia.

In conclusion, RAPD is a useful method in analyzing complexes of species (Moschetti *et al.,* 1997; Martinez *et al.,* 1999). It has several advantages such as requiring very little genomic DNA, simple methodology, it is useful when there is no prior knowledge of sequencing data and it does not require the use of hazardous materials. In a lactobacillus study, the RAPD techniques have been extensively used in the strain and intraspecies typing of lactic acid bacteria (Sylviya *et al.,* 2008). On the other hand, Pulsed Field Gel Electrophoresis (PFGE) and Amplified Fragment Length Polymorphism (AFLP) may be used in order to discriminate better between the isolates.

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