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Original Article

Multiplex PCR for simultaneous detection of *Streptococcus agalactiae*, *Streptococcus iniae* and *Lactococcus garvieae*: a case of *S. agalactiae* infection in cultured Nile tilapia (*Oreochromis niloticus*) and red tilapia (*Oreochromis niloticus* x *Oreochromis mossambicus*)

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Abstract

A multiplex PCR (m-PCR) technique was developed for simultaneous detection of the causative agents responsible for streptococcosis of cultured fish in Thailand i.e., *Streptococcus agalactiae*, *Streptococcus iniae,* and *Lactococcus garvieae*. The study on the sensitivity of the technique indicated that the minimum detected DNA concentration was 9.76, 39.06, and 19.53 pg for *S. agalactiae, S. iniae* and *L. garvieae,* respectively. Detection of streptococcosis in healthy and diseased Nile tilapia (*Oreochromis niloticus*) and red tilapia (*Oreochromis niloticus* x *Oreochromis mossambicus*) cultured in Paphayom and Bangkaew District, Phatthalung Province and Sichon and Hua Sai District, Nakhon Si Thammarat Province, Thailand, by m-PCR technique showed positive results for *S. agalactiae* from tilapia cultured in Bangkaew and Hua Sai and negative results for samples from Paphayom and Sichon. The m-PCR results were in accordance with microbiological culture techniques, which detected *S. agalactiae* from tilapia cultured in Bangkaew and Hua Sai indicating that our m-PCR assay is a sensitive and specific diagnostic tool for simultaneous detection of streptococcosis caused by *S. agalactiae*, *S. iniae* and *L. garvieae* in cultured fish in Thailand.

Keywords: multiplex PCR, Streptococcosis, *Streptococcus agalactiae, Streptococcus iniae, Lactococcus garvieae,* Tilapia

1. Introduction

Streptococcosis in fish is a generic term used to designate diseases caused by at least six different species of Gram-positive cocci including *Streptococcus*, *Lactococcus,* and *Vagococcus*. The main pathogenic species responsible for these infections are *S. parauberis*, *S. iniae*, *S. difficilis*, *L. garvieae*, *L. piscium,* and *V. salmoninarum* (Mata *et al.*, 2004b). Outbreaks of streptococcosis have been described in many countries in different species of fish, including Nile tilapia (*O. niloticus*) and the hybrid (*O. niloticus* x *O. aureus*) in the USA and Saudi Arabia (Al-Harbi, 1994; Perera *et al.*, 1994; Shoemaker *et al.*, 2000), turbot (*Scophthalmus maximus*) in Spain (Doménech *et al.*, 1996), barramundi (*Lates calcarifer*) in Australia (Bromage *et al.*, 1999), yellowtail (*Seriola quinqueradiata*), red drum (*Sciaenops ocellatus*) and Japanese flounder (*Paralichthys olivaceus*) in Japan (Matsuyama *et al.*, 1992; Eldar *et al.*, 1999; Nguyen *et al.*, 2002), sea bream (*Sparus auratus*) and wild mullet (*Liza klunzingeri*) in Kuwait (Evans *et al.*, 2002) and aquarium fish (*Brachydanio rerio* and *B. albolineatus*) in Canada (Ferguson *et al.*, 1994). They also infected other aquatic species, such as frogs (Teska and Shotts, 1994) and eel (*Anguilla japonica*) (Kusuda *et al.*, 1978). Clinical signs of

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streptococcosis in fishes vary among species. However, the most common signs include loss of equilibrium, unilateral or bilateral exophthalmia, eye opacity, haemorrhages at the base of fins, and darkening of skin (Evans *et al.*, 2002; Duremdez *et al.*, 2004).

Our previous studies reported the outbreaks of *S. agalactiae*, *S. iniae* and *L. garvieae* in tilapia and Asian sea bass cultured in Thailand (Suanyuk, 2009; Suanyuk *et al.*, 2008, 2010). The studies used traditional microbiological methods for diagnosis of bacterial infection as well as PCR assays, which required several days to achieve the results. The present study aims to develop the m-PCR technique for simultaneous detection of *S. agalactiae*, *S. iniae* and *L. garvieae*, the causative agents responsible for streptococcosis of cultured fish in Thailand.

2. Materials and Methods

2.1 Bacterial isolates and culture conditions

S. agalactiae from the Culture Collection for Medical Microorganisms, Department of Medical Sciences, Thailand (DMST)17129, and *S. iniae* and *L. garvieae*, which were kindly provided by Dr. T. Itami, Department of Biological Production and Environmental Science, University of Miyazaki, Japan, were used in the study. The bacterial isolates were cultured on tryptic soy agar (TSA: Merck, Germany) or tryptic soy broth (TSB: Merck, Germany) at 30ºC for 24-48 hrs.

2.2 M-PCR technique

2.2.1 Nucleic acid extraction

Total nucleic acid was extracted from pure cultures of each bacterial isolate using the phenol-chloroform method described by Ausubel *et al.* (1999). Purified DNA was dissolved in distilled water and then stored at -20ºC until use.

2.2.2 Target genes, primers and optimization of m-PCR

The target genes and oligonucleotide primer sets used for the m-PCR assay are shown in Table 1. Three sets of oligonucleotide primers (Operon Biotechnologies, Germany), capable of detecting specific sequences of the 16S rRNA gene of *S. agalactiae* (Martinez *et al.*, 2001), the lactate oxidase-encoding gene (*lctO*) of *S. iniae* (Mata *et al.*, 2004a) and the 16S rDNA gene of *L. garvieae* (Zlotkin *et al.*, 1998) were used. Optimal conditions for the m-PCR were empirically determined by varying the annealing temperature (55 to 60ºC) and the $MgCl_2$ concentrations (1.0 to 3.0 mM). The optimal m-PCR condition was based on good intensity of the PCR amplicons for each target DNA and the absence of a non-specific band. Amplification of target DNA was performed using a PTC-100™ thermal cycler (MJ Research Inc., USA). A negative control (no template DNA) was included in the m-PCR. The m-PCR amplified samples were subjected to electrophoresis (30 min, 110V; Mupid®-exu, Japan) in 1.5% agarose gel with TBE buffer (90 mM Tris, 90 mM Borate and 2 mM ethylenediamine tetraacetic acid, pH 8.0) and visualized by ethidium bromide staining. The DNA molecular weight marker was a 100 bp ladder (Vivantis).

2.2.3 Sensitivity and specificity of m-PCR

Sensitivity of the m-PCR was assessed with nucleic acid from pure cultures of *S. agalactiae*, *S. iniae* and *L. garvieae* reference strains. Each strain was standardized to contain 5 ng/µl of nucleic acid. The nucleic acid was a 2-fold serially dilution and 5 µl of each dilution was used as a template in the m-PCR assay. Specificity of m-PCR was confirmed by sequencing of the amplified m-PCR products with a single-nucleotide polymorphism assay with Mega BACE DNA Analysis system. The sequences were compared with the sequences in the GenBank database using the Basic Local Alignment Search Tool (BLAST, http://www.ncbi.nlm.nih.gov/BLAST/) (Altschul *et al*., 1997).

2.3 Detection of bacterial pathogens in natural infected fish

2.3.1 Fish epizootics and m-PCR assay

Mortalities of Nile tilapia (*O. niloticus*) and red tilapia (*O. niloticus* x *O. mossambicus*) from affected commercial farms in Hua Sai, Nakhon Si Thammarat Province and Bangkaew, Phatthalung Province were observed in April-May

Table 1. Oligonucleotide primers used for m-PCR assay.

Primer pair	Sequences (5'-3')	Target gene (bp)	PCR amplicon	Pathogen	Reference
F1 IMOD	GAGTTTGATCATGGCTCAG ACCAACATGTGTTAATTACTC	16S rRNA	220	*S. agalactiae*	Martinez *et al.* (2001)
LOX-1 LOX-2	AAGGGGAAATCGCAAGTGCC ATATCTGATTGGGCCGTCTAA	*lctO*	870	*S.iniae*	Mata *et al.* (2004a)
pLG-1 pLG-2	CATAACAATGAGAATCGC GCACCCTCGCGGGTTG	16S rDNA	1,100	*L. garvieae*	Zlotkin *et al.* (1998)

2010. Clinical signs of the infected fish were recorded and kidney and brain were aseptically obtained from the fish. These organs were blended with TE buffer (10 mM Tris and 1 mM EDTA) and processed for DNA extraction using the phenol-chloroform method. The total extract DNA was dissolved in sterile distilled water and 200 ng of DNA was used for the m-PCR experiment. Kidney and brain tissue samples from healthy fish from Sichon, Nakhon Si Thammarat Province and Paphayom, Phatthalung Province were examined to serve as a control.

2.3.2 Bacteriological culture assay

Tissue samples of kidney and brain of infected and healthy fish were cultured on TSA and incubated at 30ºC for 24-48 hrs. Bacterial isolates selected from almost pure colonies on TSA were inoculated onto TSA and incubated overnight to obtain a pure culture. Biochemical characteristic of the pure isolates were determined using conventional method as well as the API 20 STREP system (bioMérieux®, France) according to the manufacturer's instructions. The classification of bacterial genus and species was as described in Bergey's Manual of Systematic Bacteriology (Hardie, 1986) and the APILAB PLUS program (bioMérieux®, France).

3. Results

3.1 Optimization of the m-PCR

Based on the results, the optimum PCR reaction in a final volume made up to 50 µl contained 1.25 U of *Taq* polymerase (Invitrogen), 1 x PCR buffer, 2.0 mM of $MgCl_2$, 200 µM of each dNTP, 0.2 µM each of F1/IMOD and LOX-1/LOX-2 primers and 0.4 µM of each of pLG-1/pLG-2 and 50 ng of DNA template. Amplification of target DNA was performed with the following parameters: an initial denaturation step of 94ºC for 4 min; 35 serial cycles of a denaturation step at 94ºC for 1 min, annealing at 58ºC for 1 min, and extension at 72ºC for 2 min; and final extension step of 72ºC for 10 min. The amplification of target genes in the m-PCR assay permitted an appropriate identification of the three bacterial species. An amplification product of the expected size was observed for the *S. agalactiae* (220 bp), *S. iniae* (870 bp), and *L. garvieae* (1,100 bp) (Figure 1).

3.2 Sensitivity and specificity of m-PCR

The sensitivity of the technique indicated that the lowest detection limit of the m-PCR amplified DNA from each of the targeted species was 9.76, 39.06, and 19.53 pg for *S. agalactiae*, *S. iniae,* and *L. garvieae*, respectively (Figure 1). Sequencing results revealed the similarity of each DNA to *S. agalactiae* AB297817 (100%), *S. iniae* EU086698 (98%) and *L. garvieae* AF352163 (99%).

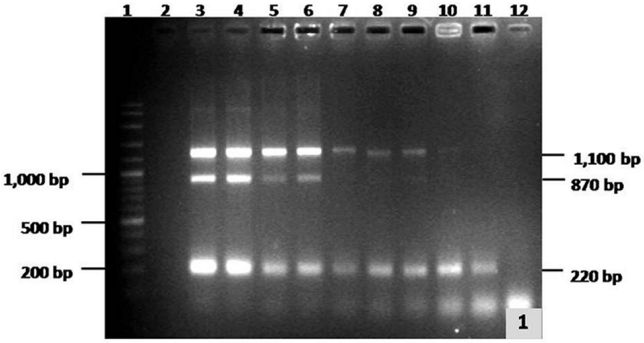

Figure 1. Agarose gel showing 1,100, 870 and 220 bp m-PCR amplification products generated with different DNA concentration for detection of *L. garvieae*, *S. iniae* and *S. agalactiae*. Lane 1, 100 bp DNA Ladder; Lane 2, Negative control (DDW); Lane 3-10, m-PCR products generated with DNA concentration of 25, 12.5, 6.25, 3.125, 1.563, 0.781, 0.391, 0.195, 0.098, and 0.049 ng, respectively.

3.3 Detection of bacterial pathogens in natural infected fish

During sample collection, a variety of symptoms typical of streptococcal infection were observed, including loss of appetite, lethargy, unilateral and bilateral exophthalmia, together with clouding of the cornea. Some fish showed accumulated fluid in the peritoneal cavity, pale liver and haemorrhaging of the internal organs.

The majority of tissues gave positive m-PCR results in agreement with bacteriological culture. Bacteriological culture of the tissues from diseased tilapia cultured in Hua Sai and Bangkaew revealed the fish pathogen as *S. agalactiae,* while m-PCR successfully detected *S. agalactiae* from kidney and brain tissues of infected fish from the same culture areas (Figure 2 and 3, Table 2 and 3). Further detection of bacterial isolates from infected fish cultured in Hua Sai and Bangkaew by m-PCR showed similar results with the tissue homogenates. *S. agalactiae*, *S. iniae* or *L. garvieae* were not detected from healthy tilapia cultured in Sichon and Paphayom by m-PCR and bacteriological culture method.

4. Discussions

Diagnostic techniques for detection of pathogenic bacteria responsible for streptococcosis of fish are usually based on the cultured technique, which requires several days to arrive a definitive diagnosis resulting in an increased potential for a disease outbreak. PCR can target unique genetic sequences of microorganisms and has previously been developed for the identification of fish pathogenic bacteria using a primer specific to target a gene segment of a given bacterium (Zlotkin *et al.*, 1998; Martinez *et al.*, 2001; Mata *et al.*, 2004a). In recent years, m-PCR assay has been

Table 2. Detection of *S. agalactiae*, *S. iniae* and *L. garvieae* in tilapia tissues by m-PCR technique[1].

Area	Kidney			Brain		
	S. agalactiae	*S. iniae*	*L. garvieae*	*S. agalactiae*	*S. iniae*	*L. garvieae*
Paphayom, Phatthalung	0	0	0	0	0	0
Bangkaew, Phatthalung	3	0	0	5	0	0
Hua Sai, Nakhon Si Thammarat	1	0	0	4	0	0
Sichon, Nakhon Si Thammarat	0	0	0	0	0	0

[1]Five fish from each area were used in this study.

Table 3. Detection of *S. agalactiae*, *S. iniae* and *L. garvieae* in tilapia tissues by microbiological method[1].

Area	Kidney			Brain		
	S. agalactiae	*S. iniae*	*L. garvieae*	*S. agalactiae*	*S. iniae*	*L. garvieae*
Paphayom, Phatthalung	0	0	0	0	0	0
Bangkaew, Phatthalung	2	0	0	3	0	0
Hua Sai, Nakhon Si Thammarat	3	0	0	3	0	0
Sichon, Nakhon Si Thammarat	0	0	0	0	0	0

[1]Five fish from each area were used in this study.

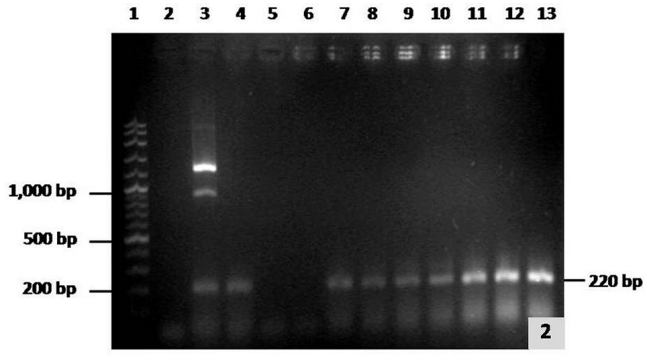

Figure 2. Agarose gel showing m-PCR products of *S. agalactiae* from kidney and brain tissues of tilapia cultured in Bangkaew. Lane 1, 100 bp DNA Ladder; Lane 2, Negative control (DDW); Lane 3, Positive control *L. garvieae* FK 040708, *S. iniae* and *S. agalactiae* DMST 17129; Lane 4-8, Kidney tissues; Lane 9-13, Brain tissues.

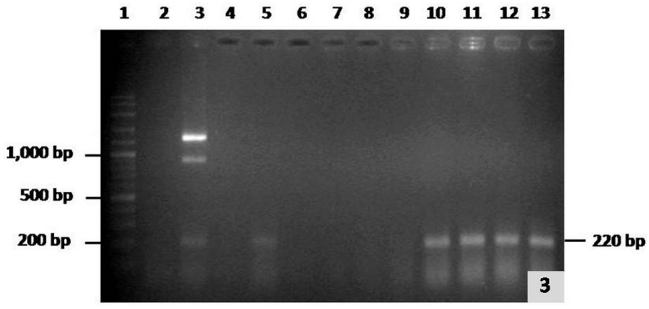

Figure 3. Agarose gel showing m-PCR products of *S. agalactiae* from tilapia cultured in Hua Sai. Lane 1, 100 bp DNA Ladder; Lane 2, Negative control (DDW); Lane 3, Positive control *L. garvieae* FK 040708, *S. iniae* and *S. agalactiae* DMST 17129; Lane 4-8, Kidney tissues; Lane 9-13, Brain tissues.

developed for simultaneous detection of streptococcosis caused by *S. iniae*, *S. difficilis, S. parauberis,* and *L. garvieae* (Mata *et al.*, 2004b) and *S. iniae*, *S. parauberis* and *L. garvieae* (Baeck *et al.*, 2006). The present study proposed the m-PCR assay for detection of streptococcosis caused by *S. agalactiae*, *S. iniae,* and *L. garvieae,* which can cause great loss and can be a threat to the Thai fish farmers.

Three sets of primers specific to the 16S rRNA, *lctO* and 16S rDNA region of *S. agalactiae*, *S. iniae,* and *L. garvieae* (Zlotkin *et al.*, 1998; Martinez *et al.*, 2001; Mata *et al.*, 2004a) were used for m-PCR analysis. All primers have been used for detection of these pathogenic bacteria isolated from infected fish cultured in various countries, including Thailand (Al-Harbi, 2011; Chen *et al.*, 2002; Duremdez *et al.*, 2004; Suanyuk, 2009; Suanyuk *et al.*, 2010). The m-PCR was sensitive and specific for detecting representative pure isolates of *S. agalactiae*, *S. iniae,* and *L. garvieae* with the detection limits of 10-40 pg. Mata *et al.* (2004b) also reported the sensitivity of the m-PCR using purified DNA in a similar range of 12.5-50 pg for detection of *S. iniae*, *S. difficilis*,

S. parauberis, and *L. garvieae,* while the lower detection limit of 20 pg was reported by Panangala *et al.* (2007) for *Flavobacterium columnare*, *Edwardsiella ictaluri,* and *Aeromonas hydrophila*.

Panangala *et al.* (2007) reported the m-PCR sensitivity threshold for detection of *F. columnare*, *E. ictaluri,* and *A. hydrophila* in fish tissue ranged between $3.4x10^2$ and $2.5x10^5$ cells/g of tissue. In the detection of warm-water streptococcosis, sensitivity threshold for detection of *S. iniae*, *S. difficilis*, *S. parauberis* and *L. garvieae* ranged between $2.5x10^3$ and $1.2x\ 10^4$ cells/g of tissue (Mata *et al.,* 2004b). Although the sensitivity of m-PCR for detecting specific bacteria from fish tissue was not fulfilled in this study, our m-PCR analysis allowed detection of *S. agalactiae* from the infected tilapia cultured in southern Thailand using crude DNA concentration of 200 ng.

Several species of *Streptococcus* spp. have been reported as fish pathogens. In the present study, a small number of Gram-positive cocci bacteria were isolated from some healthy fish and identified as *S. dysgalactiae* and *S. equinus*. These bacteria have been reported as pathogen in Nile tilapia (Netto *et al*., 2011) and human (Bump, 1977). Although no impact of these bacteria on tilapia cultivation observed from the present study, further study on pathogenicity of these bacteria in fish as well as m-PCR technique for diagnosis of these bacteria should be investigated.

In summary, the present study demonstrates that the m-PCR assay is a sensitive and specific diagnostic tool for simultaneous detection of streptococcosis of fish caused by *S. agalactiae*, *S. iniae,* and *L. garvieae*. Further studies on the application of the m-PCR on the seasonal variation and distribution of *S. agalactiae*, *S. iniae,* and *L. garvieae* in cultured fish are necessary to elucidate the damage caused by these pathogenic bacteria.

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