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Original Article

Duplex PCR for Simultaneous and Unambiguous Detection of *Streptococcus iniae* and *Streptococcus agalactiae* associated with Streptococcosis of Cultured Tilapia in Thailand

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Abstract

Warm-water streptococcosis outbreaks in Thai cultured tilapia are caused by *Streptococcus iniae* and *S. agalactiae*. However, distinguishing between these two species of streptococcus are very difficult due to their similar microbiological appearance and clinical signs of infected fish. In this study, we proposed a new duplex-PCR based method for simultaneous detection of these pathogens. The duplex-PCR amplified partial *lct*O and 16s rRNA gene of *S. iniae* and *S. agalactiae* at 870 bp and 220 bp, respectively. This technique gave 100% specificity while sensitivity of reaction was 100 fg of each bacterial genomic DNA. Detection limit of duplex-PCR applicable to clinical specimens is also evaluated as 10^6 bacterial cells per gram of fish tissue. This study suggested that this duplex-PCR based method might be a good candidate for easy, sensitive, specific and rapid detection of *S. iniae* and *S. agalactiae* associated with warm-water streptococcosis of cultured tilapia in Thailand.

Keywords: duplex PCR, *Streptococcus iniae*, *Streptococcus agalactiae*, Tilapia

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บทคัดย่อ

ปฏิกิริยาลูกโซ่โพลิเมอเรสแบบดูเพล็กซ์สำหรับตรวจหาเชื้อสเตรปโตคอคคัส อินิเอ้ และเชื้อสเตรปโตคอคคัส อกาแลคติเอ้ ที่ก่อโรคสเตรปโตคอคโคซีสในปลานิลเพาะเลี้ยงในประเทศไทย

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โรคสเตรปโตคอคโคซิสซึ่งพบในปลานิลเพาะเลี้ยงในประเทศไทย เกิดจากการติดเชื้อ สเตรปโตคอคคัส อินิเอ้และสเตรปโตคอคคัส อกาแลคติเอ้ การวินิจฉัยแยกแยะระหว่างเชื้อทั้งสองชนิดนี้ทำได้ยากในทางปฏิบัติเนื่องจากอาการป่วยของปลาที่ติดเชื้อรวมทั้งผลการทดสอบทางชีวเคมีนั้นมีความคล้ายคลึงกันมาก ด้วยเหตุนี้คณะผู้วิจัยจึงได้พัฒนาวิธีการตรวจวินิจฉัยด้วยเทคนิคปฏิกิริยาลูกโซ่โพลิเมอเรสแบบดูเพล็กซ์ขึ้น ซึ่งเทคนิคดังกล่าวอาศัยการเพิ่มปริมาณของยีนแลคเตทออกซิเดส (*lctO*) และไรโบโซม (16s rRNA) ซึ่งจำเพาะกับเชื้อ สเตรปโตคอคคัส อินิเอ้และสเตรปโตคอคคัส อกาแลคติเอ้ ตามลำดับ จากผลการทดลองแสดงให้เห็นว่าความจำเพาะในการตรวจสูงถึง 100 % และสามารถตรวจวินิจฉัยแม้มีดีเอ็นเอปริมาณเพียง 100 fg สำหรับปริมาณเชื้อต่ำที่ปนเปื้อนในตัวอย่างที่น้อยสุดเท่าที่จะสามารถตรวจพบได้ในตัวอย่างชิ้นเนื้อ คือ 10^6 เซลล์ของเชื้อต่อตัวอย่างหนึ่งกรัม จากผลการทดลองทั้งหมดแสดงให้เห็นว่าเทคนิคปฏิกิริยาลูกโซ่พอลิเมอเรสแบบดูเพล็กซ์นั้นเป็นวิธีการวินิจฉัยที่ทำได้ง่าย รวดเร็ว มีความไวและความจำเพาะสูง เหมาะสำหรับนำมาประยุกต์ใช้ในการตรวจหาสาเหตุของโรคสเตรปโตคอคโคซิสที่พบในปลานิลเพาะเลี้ยงในประเทศไทย

คำสำคัญ: ปฏิกิริยาลูกโซ่โพลิเมอเรสแบบดูเพล็กซ์ สเตรปโตคอคคัส อินิเอ้ สเตรปโตคอคคัส อกาแลคติเอ้ ปลานิล

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Introduction

Warm-water streptococcosis is now recognized as highly important infectious disease in several aquatic animal species which associated with significant morbidity and mortality in farmed fish. More than 50% of mortality can be found in diseased outbreak fish farm (Yanong and Francis-Floyd, 2002). Nowadays, several species of aquaculture have been found susceptible to streptococcosis, including salmon, mullet, golden shiner, pinfish, eel, sea trout, and tilapia, which outbreaks were reported in various country such as Kuwait, Israel, Brazil, Japan and Thailand (Perera et al., 1994; Bachrach et al., 2001; Duremdez et al., 2004; Agnew and Barnes, 2007; Suanyuk et al., 2008; Mian et al., 2009). Causative agent associated with fish streptococcosis worldwide are *Lactococcus garvieae, Streptococcus agalactiae, S. dysgalactiae, S. phocae, and S. iniae*. For Nile tilapia (*Oreochromis niloticus*) cultured in Thailand, *S. iniae* and *S. agalactiae* were the major causes of streptococcosis (Maisak et al., 2008; Suanyuk et al., 2008; Suanyuk et al., 2010). It is difficult to differential diagnosis of streptococcosis in tilapia caused by *S. iniae* and *S. agalactiae* because the clinical signs of tilapia infected with *S. iniae* are similar to *S. agalactiae*. Therefore, a definitive diagnosis of the causative agent should be based on the microbiological investigations of diseased tilapia. The pathogens can be identified by culture-based methods and biochemical tests, but they are the time-consuming and laborious. Although the phenotypic and biochemical characteristics of *S. iniae* and *S. agalactiae* are well characterized, the laboratory identification of the bacterium from diseased fish can still be difficult and mistakenly identified, especially when using the commercial identification systems, because currently available commercial identification system does not include *S. iniae* in its database (Mata et al., 2004). In order to solve any diagnostic problems of warm-water streptococcosis in Thailand, the genotypic-based identifications such as single individual PCR assays have been developed and used for identification of the fish pathogens associated with streptococcosis. However, the previous single individual PCR assays can used to detect only either *S. iniae* or *S. agalactiae* per one PCR reaction, which can be time-consuming and relatively costly. In order to develop easier and more rapid technique, a multiplex PCR was proposed as an effective method for simultaneous detection of several streptococci species (Mata et al., 2004). Unfortunately, application of this technique in Thailand and nearby region might

not be appropriate since *S. agalactiae* is not included for the detection. Therefore, a newly duplex-PCR for detection of two common pathogens of streptococcosis was developed in this study which offers a preferable application in Thailand and the country that *S. iniae* and *S. agalactiae* are involved with streptococcosis in tilapia.

Materials and Methods

***Bacterial strains and culture conditions*:** A total of 20 *S. iniae* strains and 15 *S. agalactiae* strains and 30 other related bacterial strains were used in this study as shown in Table 1. *S. iniae* ATCC 29178 and S. *agalactiae* ATCC 13813 were used as positive controls. Bacterial strains were cultured at 37^0C in Tryptic soy broth (TSB) and/or in Tryptic soy agar (TSA) (Difco, USA) with 5% sheep blood.

Isolation of bacterial DNA: Genomic DNA of bacteria was prepared by standard phenol-chloroform method (Ausubel et al., 2003). DNA were dissolved in 100 µl of distilled water and stored at -20^0C until use.

Duplex-PCR oligonucleotide primers: Duplex-PCR identification on the streptococcal DNA used *S. iniae* lactate oxidase genes (*lct*O) specific primers (Mata et al., 2004) and *S. agalactiae* 16S rRNA gene specific primers (Martinez et al., 2001). The primer sequences and expected sizes of amplicons for each PCR assay are described in Table 2.

Duplex-PCR amplification condition: The duplex -PCR reaction mixture was performed in a PCR thermal cycler with 200 µl microcentrifuge tubes. Purified genomic DNA from all bacterial species was used as DNA template for duplex -PCR**.** The duplex-PCR was carried out in a 25 µl reaction mixture containing 2 µl of bacterial genomic DNA, 200 nM LOX-1 and LOX-2 primer, 400 nM F1 and IMOD primer, 250 µM of each deoxynucleoside triphosphate (dNTP), 1x*Taq* buffer with $MgCl_2$ and 0.75 Unit of *Taq* DNA polymerase. The appropriate annealing temperature, which did not interfere with the annealing of any of the primers, was 52^0C. The PCR thermocycling was one cycle of initial denaturation at 95^0C for 5 min, followed by 30 cycles of 95^0C for 1 min, 52^0C for 1 min, and 72^0C for 1 min, and subsequently extended by one cycle of 72^0C for 7 min.

Visualization of PCR products: After amplification, 3 µl of each reaction mixture was separated by electrophoresis on a 1.5% agarose gel containing 0.4 µg of ethidium bromide per ml. The gel was run in 1x Tris-Borate-EDTA (TBE) buffer at 100 V with standard DNA size markers for 25 min and visualized with a densitometer.

Sensitivity of duplex-PCR: The DNA concentration *S. iniae* ATCC 29178 and *S. agalactiae* ATCC 13813 were quantified by measuring the optical density at wave length 260 nm (*A*260 value) using spectrophotometer. The DNA template was added to each PCR reaction mixture by varying the DNA template concentration from 1µg to 1 fg/µl with 10-fold serial dilution in distilled water. Only the reaction which can be amplified all of 2 target DNA products was counted in positive.

Duplex-PCR for direct detection of DNA from clinical specimens: The duplex -PCR was applied to the direct detection of *S. iniae* and *S. agalactiae* from tilapia clinical samples. The direct detection was performed by using mixed DNA extracted from spleen, liver and kidney of tilapia with *S. iniae* and *S. agalactiae*. The tissues were isolated aseptically from freshly dead Nile tilapia and homogenized in sterile phosphate buffer saline (PBS) pH 7.4 with tissue mortar. The spleen, liver and kidney were pooled and mixed with bacteria approximately 10^7, 10^6, 10^5, 10^4, 10^3, 10^2 or 10^1 cells of each bacterial specie per gram of tissues. Then, 5 µl of lysozyme (10 mg/ml) was added to 50 µl of tissue mixture and incubated at 37^0C for 30 min prior to genomic DNA extraction by using High pure PCR template preparation kit (Roche, Germany). The DNA in each tube was used as a template for duplex-PCR. The detection limit was calculated by comparison with the bacterial cells present in 1 gram of tissue.

Table 1 Bacterial strains used in duplex-PCR.

Bacterial species	Collection code	Source of isolation
S. iniae	ATCC 29178	Amazon freshwater dolphin, *Inia geoffrensis*, Buffalo, NY.
S. iniae	J1-J2	Diseased fish, Japan
S. iniae	CUCR1-CUCR17	Diseased tilapia, Thailand
S. agalactiae	ATCC 13813	-
S. agalactiae	CUCR1-CUCR14	Diseased tilapia, Thailand
Streptococcus dysgalactiae	CU Vet Micro 1-CU Vet Micro 5	Mastitis cow, Thailand
Streptococcus uberis	CU Vet Micro 1-CU Vet Micro 4	Mastitis cow, Thailand
Streptococcus parauberis	CU Vet Micro 1	Mastitis cow, Thailand
Streptococcus suis	CU Vet Micro 1-CU Vet Micro 5	Pig tonsil, Thailand
Streptococcus canis	CUCR1	Canine skin, Thailand
Enterococcus faecium	CU Vet Micro 1	Chicken, Thailand
Staphylococcus aureus	CUCR1- CUCR5	Canine skin, Thailand
Lactococcus garviae	J1	Diseased fish. Japan
Flavobacterium sp.	CU Vet Micro 1	Diseased tilapia, Thailand
Aeromonas hydrophila	CUCR1-CUCR3	Diseased fish, Thailand
Aeromonas caviae	CUCR1-CUCR2	Diseased goldfish, Thailand
Edwardsiella tardar	CUCR1	Diseased goldfish, Thailand

Table 2 Primer names, oligonucleotide sequences and size of amplicons.

Target gene	Primer name	Oligonucleotide primer sequence	Size of Amplicon
Lactate oxidase (*lct*O)	LOX-1	5'-AAG GGG AAA TCG CAA GTG CC-3'	870 bp
	LOX-2	5'-ATA TCT GAT TGG GCC GTC TAA -3'	
16S rRNA/IMOD	F1	5'-GAG TTT GAT CAT GGC TCA G-3'	220 bp
	IMOD	5'-ACC AAC ATG TGT TAA TTA CTC-3'	

Results

Sensitivity and specificity of duplex-PCR: Target amplicons of PCR amplification from positive control of bacterial DNA were presented as expected (870 and 220 bp for *S. iniae* and *S. agalactiae*, respectively). Even in PCR reaction containing both *S. iniae* and *S. agalactiae* DNA, the positive amplicons band were still clearly seen, no difference in DNA band intensities from individual detection of each pathogens species as shown in Figure 1. For specificity of this assay, this duplex-PCR could specifically detect *lct*O gene and 16S rRNA gene in all isolates of *S. iniae* (n=20) and *S. agalactiae* (n=15), respectively. The assay did not produce any targeted amplicons when tested against other related bacterial species (n=30).The results revealed that the specificity of this duplex-PCR is 100%. For the sensitivity of the assay, this duplex-PCR was successful in the amplification *lct*O gene and 16S rRNA gene in the reaction contained as little as 100 fg of each bacterial genomic DNA template per reaction.

Detection of bacterial pathogens in clinical specimens: Artificially inoculated tilapia tissues with bacterial pathogens were applied in this duplex-PCR to determine sensitivity and specificity of the assay in clinical specimens. Amplification of bacterial targeted DNA in tissue homogenate was successful in the reaction containing at least 10^6 CFU/g of fish tissue for both streptococcus species as shown in Figure 2.

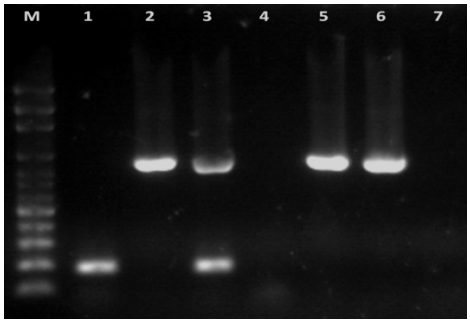

Figure 1 Duplex-PCR simultaneous amplification of S. iniae lactate oxidase genes (*lct*O) and S. *agalactiae* 16S rRNA gene. The duplex-PCR products showed the DNA bands of predicted sizes of 870 and 220 bp with primer *lct*O and 16S rRNAF1/ IMOD, respectively. Lanes M: DNA markers, lane 1: *S. agalactiae* ATCC 13813 DNA, lane 2: *S. iniae* ATCC 29178 DNA, lane 3: *S. agalactiae* ATCC 13813 DNA and *S. iniae* ATCC 29178 DNA, lane 4: *S. dysgalactiae* DNA, lane 5: *S. iniae* strain CUCR1 DNA lane 6: *S. iniae* strain J2 DNA, lane 7: no DNA template (negative control).

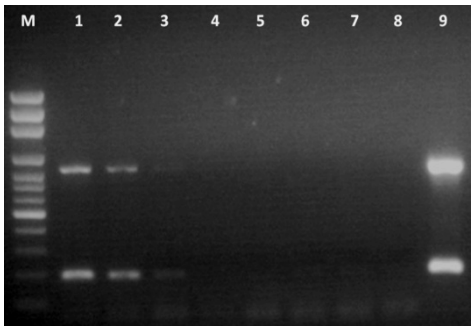

Figure 2 Detection limit of duplex-PCR applicable to clinical specimens. The duplex-PCR products showed the DNA bands of predicted sizes of 870 and 220 bp with primer *lct*O and 16s rRNA F1/IMOD, respectively. Lane M, 100 bp DNA markers; lane 1 through 7: duplex-PCR products of DNA extracted from tilapia's spleen, liver and kidney artificially infected with *S. iniae* and *S. agalactiae* at the concentration of 10^7 to 10^1 CFU of each bacterial specie/g of fish tissue, respectively, lane 8: no DNA template (negative control); lane 9, duplex-PCR product of mixed DNA of *S. iniae* and *S. agalactiae* (positive control).

Discussion

In Thailand, *S. agalactiae* is a majority cause of warm-water streptococcosis in cultured tilapia. However, streptococcosis by *S. iniae* was sporadically reported in some region (Suanyuk et al., 2010). Differentiation between these two streptococcal species is mainly based on conventional microbiological method, i.e. bacterial isolation and biochemical investigation, which is laborious and time-consuming. It might be difficult to confirm streptococcus species by this conventional method considering from previous scientific studies which suggested that variation of some biochemical test results could occur among bacterial strains (Agnew and Barnes, 2007; Evans et al., 2008). In this work, a novel duplex-PCR for simultaneous and unambiguous detection of *S. iniae* and *S. agalactiae* was developed.

Each primer pair targeted on *lct*O and 16s rRNA gene of both *S. iniae* and *S. agalactiae* was referred to studies of Mata and Martinez, respectively (Martinez et al., 2001; Mata et al., 2004). In those original studies, a single PCR was tested for specificity with various species of streptococcus and other related bacterial genus. A 100% specificity in this duplex-PCR assay had no difference regardless of individual single PCR. While the intensity of amplicons from duplex-PCR contained separated or mixed streptococcal genomic DNA also show similarity (Fig 1). This result suggested that each

mixed streptococcal genomic DNA also show similarity (Fig 1). This result suggested that each primer pairs and amplification products have no deleterious effect when apply in duplex-PCR assay.

Detection of bacterial pathogens by duplex-PCR is still effective even in clinical specimens. The detection limits are as little as 10^6 CFU/g of fish tissue, this sensitivity of the assay is highly enough to detect bacterial target in diseased fish since distribution of *S. agalactiae* in experimental infected fish tissue was higher than 10^6 CFU/g (unpublished data) and 10^7 CFU/g for *S. iniae* (Lahav et al., 2004). For the application to disease diagnostic purpose, we suggested that brain, kidney, or liver tissue are appropriate due to the fact that streptococcus is overwhelmed in those tissues (Lahav et al., 2004).

Various species of bacteria are associated with warm-water streptococcosis in fish and previously developed duplex-PCR for detection of major causative agent of streptococcosis includes 4 species of related pathogens, i.e. *S. iniae*, *S. difficilis*, *S. parauberis*, and *L. garvieae* (Mata et al., 2004). Specific primer targeting *S. difficilis* also success to amplified *S. agalactiae* genomic DNA. This was expected since *S. difficilis* was reclassified as a synonym of *S. agalactiae* (Kawamura et al., 2005). In our novel duplex-PCR assay, we tried a different pair of primer for *S. agalactiae* detection which referred to the previous study (Martinez et al., 2001). The original article suggested that F1/IMOD primer was specified to several serotype of *S. agalactiae* including type I, II, III, IV, V and non-typable from both human and bovine isolate. Our duplex-PCR assay should be appropriate for simultaneous and unambiguous detection of streptococcosis in cultured tilapia in Thailand since *S. iniae* and *S. agalactiae* (serotype Ia and III) were occupied among cultured tilapia in Thailand (Suanyuk et al., 2008; Suanyuk et al., 2010). Additionally, reports of *S. parauberis* and *L. garvieae* associated with streptococcosis aquaculture have not been available in Thailand. Thus, limitation on target species for duplex-PCR detection, only *S. iniae* and *S. agalactiae,* should be enough and appropriate for detection of causative agents of streptococcosis in Thai cultured tilapia in order to reduce the PCR cost and simplify the method of the detection.

In conclusion, duplex-PCR developed in this study can be a rapid, sensitive, and specific method for simultaneous and unambiguous detection of *S. iniae* and *S. agalactiae* from pure cultures and fish clinical samples. Therefore, it can be a good PCR method candidate which applied for detection of *S. iniae* and *S. agalactiae* associated with streptococcosis in cultured tilapia in Thailand.

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