

養殖ブリのLactococcus garvieae, Streptococcus iniaeおよびS.dysgalactiae検出のための複合PCR

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著者名	畑井,喜司雄 Hussein, M.M.A.
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Multiplex PCR for Detection of *Lactococcus garvieae*, *Streptococcus iniae* and *S. dysgalactiae* in Cultured Yellowtail

Mortada M. A. HUSSEIN^{1, 2} and Kishio HATAI^{1, *}

Abstract: Streptococcosis is one of serious disease in cultured yellowtail, *Seriola quinqueradiata*, amberjack, *S. dumerili* and salmonids, and economically is problematic. *Streptococcus iniae*, is known as a pathogen of the marine and freshwater fishes. On the other hand, *Lactococcus garvieae*, and recently *S. dysgalactiae* are known as the pathogens of the yellowtail and amberjack. Mixed infection due to the pathogens, however, occurs very often in yellowtail. Therefore, the correct diagnosis is sometimes confused. In an attempt to elucidate the main pathogen, a multiplex PCR was newly designed in this study. The new multiplex PCR assays involves amplifying the three multiple gene products in a single reaction based on primers designed from the 16S rRNA, 16S-23S rDNA intergenic spacer region and lactate oxidase (*lctO*) genes of *L. garvieae*, *S. dysgalactiae* and *S. iniae*, respectively. The specificity of the multiplex PCR using the primer sets was confirmed by the fact that specific bands were only amplified equivalent to 1,100, 870 and 259-bp for *L. garvieae*, *S. iniae*, and *S. dysgalactiae*, respectively. In addition, the specific positive amplifications in used all templates were consistently observed only for each corresponding pathogen. Multiplex PCR did not produce any non-specific amplification products when tested against pure DNAs, bacterial suspensions or tissue homogenates from rainbow trout, *Oncorhynchus mykiss*, artificially infected with the three pathogens. Multiplex PCR was an effective tool for the rapid and specific detection of *L. garvieae*, *S. dysgalactiae* and *S. iniae* from fish tissues.

Key words: *Lactococcus garvieae*; Multiplex PCR; *Streptococcus dysgalactiae*; *Streptococcus iniae*

Streptococcus infection of fishes has become a major problem with the development of intensive aquaculture in Japan, particularly, yellowtail, *Seriola quinqueradiata*, amberjack, *S. dumerili* and salmonids. These fishes are of importance because of their high economic value. *Streptococcus iniae*, *Lactococcus garvieae*, and recently *S. dysgalactiae* subsp. *dysgalactiae* are known as pathogens of the fishes, and sometimes cause mixed infection (Kusuda et al. 1991; Kitao 1993; Nomoto et al. 2004). Yellowtail with streptococcosis exhibits very similar symptoms and clinical signs regardless of the etiological agents (Zlotkin et al. 1998; Eldar and Ghittino 1999; Kusuda and Salati 1999), there-

fore, a definitive diagnosis of the etiological agent was based on the microbiological characteristics of bacteria isolated from diseased fish (Mata et al. 2004a).

Recently, molecular methods based on DNA probes or PCR have overcome problems associated with culture-based techniques, enabling the detection of microorganisms directly from clinical samples without the isolation (González et al. 2004). However, a large number of individual PCR assays would be necessary if single primer sets are used on a large number of clinical samples and that can be a relatively costly and time-consuming process. The detection of several pathogens using a multiplex PCR

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¹Laboratory of Fish Diseases, Nippon Veterinary and Life Science University, 1-7-1 Kyonan-cho, Musashino, Tokyo 180-8602, Japan.

²Department of Fish Diseases and Managements, Faculty of Veterinary Medicine, Beni-Suef University, Beni-Suef 62511, Egypt.

*Corresponding author: Tel. & Fax: +81-422-31-6796. E-mail: hatai@nvlu.ac.jp

(m-PCR) would be rapid and cost-effective (Osorio et al. 2002).

In this present study we attempted to develop a m-PCR for the concurrent detection of *Streptococcus iniae*, *Lactococcus garvieae*, and *S. dysgalactiae*, the most prevalent gram positive cocci (GPC) pathogens incriminated in fish streptococcosis incidence in Japan, from the pure cultures as well as tissues of fish artificially infected with the pathogens.

Materials and Methods

Bacterial strains and the preparations

Eight strains used in this study were isolated from diseased fishes with clinical signs of streptococcosis (Table 1). All strains were inoculated in Todd Hewitt Broth and incubated at 25°C for 48 h (THB, Sigma, Missouri, USA), and then the bacterial pellets were collected by centrifugation at 3000 rpm at 4°C for 10 min. Subsequently, the obtained pellets were 10-fold serially diluted using sterile phosphate-buffered saline starting with 3×10^8 (McFarland No. 1) till 3×10 cells/ml. Bacterial suspensions were used for either m-PCR (with and/or without DNA extraction) or artificial infection.

Artificial infection and tissue homogenates

Eighteen apparently healthy rainbow trout used in this study were 70 ± 5 g in body weight and 20 ± 2 cm in body length, which were obtained at a private farm in Tokyo, Japan. After

acclimatization for 10 days, fish were divided into 8 groups and kept separately in 10 l aquaria at 15°C. Two groups were designated as A and B ($n = 3$ each), 2 groups as C and D ($n = 2$ each) and 4 control groups as E, F, G, and H ($n = 2$ each). Each fish of the group A was injected with single dose of 0.1 ml of either *L. garvieae* NJB E2, *S. iniae* NJB 02-011, or *S. dysgalactiae* NJB 0442 with bacterial suspension of 3×10^7 cells/ml. Group B was treated as group A but with bacterial suspensions at concentrations of 3×10^2 cells/ml. On the other hand, fish in the group C were injected with 0.1 ml mixed bacterial suspensions of the three selected isolates at a concentration of 3×10^7 cell/ml while fish in the group D were injected with 0.1 ml of the same bacterial mixtures but at a concentration of 3×10^2 cell/ml. Control groups were injected with 0.1 ml sterile bacterial free phosphate-buffered saline. Fish in all groups were monitored for 10 d post injection and tissue samples including spleen, kidney, brain, and muscles were collected from moribund fish, and homogenized for DNA extraction.

In parallel, a loopful of each tissue homogenates was streaked onto the TH agar supplemented with 3% sheep blood and incubated at 25°C for 48 h. Live fish were scarified at the end of the experimental period (10 d) and the same tissues were sampled. Fish used as control were processed in the same way with the experimentally infected ones.

Table 1. Isolates used for m-PCR assay

Isolate	Year	Fishes	Site	Location
<i>Lactococcus garvieae</i>				
KGLA 0415	2004	Amberjack	Brain	Kagoshima, Japan
NJB B1	2005	Yellowtail	Brain	Kagoshima, Japan
NJB K2	2005	Yellowtail	Kidney	Kagoshima, Japan
NJB E2	2005	Yellowtail	Eye	Kagoshima, Japan
<i>Streptococcus dysgalactiae</i> subsp. <i>dysgalactiae</i>				
Kdys 0442	2004	Amberjack	Kidney	Kagoshima, Japan
Kdys 0432	2004	Amberjack	Kidney	Kagoshima, Japan
Kdys 0440	2004	Amberjack	Caudal fin	Kagoshima, Japan
<i>Streptococcus iniae</i>				
NJB 02-011	2002	Rainbow trout	Kidney	Nagano, Japan

Table 2. Oligonucleotide primer sets used for m-PCR amplification and the expected amplicon sizes

Primer pairs	Sequences (5' to 3')	Target gene	PCR amplicon (bp)	Pathogen	Reference
pLG-1	CATAACAATGAGAATCGC	16S rRNA	1,100	<i>Lactococcus garvieae</i>	Zlotkin et al. (1998)
pLG-2	GCACCCTCGCGGGTTG				
LOX-1	AAGGGGAAATCGCAAGTGCC	<i>lctO</i>	870	<i>Streptococcus iniae</i>	Mata et al. (2004)
LOX-2	ATATCTGATTGGGCCGTCTAA				
STRD-DyI	TGGAACACGTTAGGGTCG	16S-23S rDNA	259	<i>S. dysgalactia</i> subsp. <i>dysgalactia</i>	Hassan et al. (2003)
dys-16S-23S-2	CTTAAGTAGAAAACTCTTGATTATTC				

Design of multiplex PCR (m-PCR)

The new multiplex PCR assay involves amplifying the three multiple gene products in a single reaction based on primers deduced from the regions carrying the 16S rRNA (Zlotkin et al. 1998), 16S-23S rDNA intergenic spacer region (Hassan et al. 2003; Nomoto et al. 2004) and lactate oxidase (*lctO*) genes (Mata et al. 2004a) of *L. garvieae*, *S. dysgalactiae* and *S. iniae*, respectively.

DNA extraction and m-PCR amplification

DNA was extracted using DNAzol[®] reagent (Invitrogen Life Technologies, California, USA) from bacterial suspensions and fish tissue homogenates. The target gene and species-specific oligonucleotide primer sets used in the m-PCR are indicated in Table 2.

The m-PCR amplifications were performed in 25 μ l reaction mixtures with Ready-to-go PCR beads (Amersham Pharmacia Biotech, Ltd., Piscataway, USA) by adding 1 pg of each primer, 1 μ l of template (extracted DNA, bacterial suspension or fish tissue homogenate) and 18 μ l distilled water (DW). Samples were subjected to a designated regime of amplification in PCR thermal cycler (Takara Biomedical, Tokyo, Japan) with the following parameters: an initial denaturation at 94°C for 1 min, 30 serial cycles of a denaturation at 92°C for 1 min, annealing at 52°C for 1 min, extension at 72°C for 90 s, and a final extension at 72°C for 5 min. Negative control was included in each batch of m-PCR reaction. PCR-generated products were

Table 3. Recovery of the three streptococcal pathogens and their detection by m-PCR in different tissues from artificially infected fish

Organ/ Group	Bacterial re-isolation				m-PCR detection			
	A	B	C	D	A	B	C	D
Kidney	+	+	+ ^a	+	+	+	+	+
Spleen	+	+	$\pm$ ^b	$\pm$	+	+	+	+
Liver	+	+	$\pm$	$\pm$	+	+	+	+
Brain	+	+	$\pm$	$\pm$	+	+	+	+
Eye	+	+	$\pm$	$\pm$	+	+	+	+
Muscles	+	+	$\pm$	$\pm$	+	+	+	+

^a: Recovery of the three streptococcal pathogens.

^b: Recovery of one or two out of the three streptococcal pathogens.

simultaneously analyzed by electrophoresis of 5 μ l of each amplification mixture in 2% agarose in Tris-borate acid EDTA buffer (100 V, 30 min) gels containing ethidium bromide.

Results

Recovery of the bacteria from fish artificially infected with the three pathogens

Tissues of artificially infected fish were attempted to recover the injected pathogens with TH agar containing 3% sheep blood (Table 3). The injected three pathogens were recovered in pure cultures from fish in groups A and B. On the other hand, re-isolation of the three injected pathogens from groups C and D was varied considerably. In contrast, one or two pathogen (s) were recovered from selected organ homogenates except that of kidneys and they could be differentiated in their pure form after several subcultures, however, the three

injected pathogens were recovered purely from kidney homogenates.

Development of multiplex PCR

The specificity of the m-PCR assay was evaluated by testing the three primer sets with the pure DNA templates either from pure bacterial suspensions and/or tissue homogenates of the artificially infected fish. Specific positive PCR amplifications in all used samples including bacterial suspensions, tissue homogenates from different organs, and extracted DNAs from either bacteria or tissues were consistently observed for each corresponding pathogen. In contrast, positive PCR amplifications of DNA templates produced a single specific fragment bands for each pathogen with a size of 1,100, 870, and 259 bp for *L. garvieae*, *S. iniae*, and *S. dysgalactiae*, respectively (Fig. 1). Similarly, single specific positive PCR amplifications bands were obtained when m-PCR assay was performed with DNA templates including tissues homogenates and/or extracted DNA. In addition, the specificity of the m-PCR was clear not only with infected tissue samples obtained from groups A and B but also with groups C and D. The detection limit of m-PCR assay in fish tissue was equivalent to 3×10^2 cells/ml (Fig. 2). The m-PCR assays neither produce non-specific amplification products of used samples nor false positive ones of the uninfected controls.

Discussion

Identification based on biochemical and antigenic characteristics can be barely differentiating among *L. garvieae*, *S. iniae* and *S. dysgalactiae*, however, they are limited by the length of time required to complete the assays (Zlotkin et al. 1998). In addition, even isolation of those pathogens, particularly in a case of mixed infection, is considered as time and effort consuming and sometimes lead to misdiagnosis. The results represented herein showed that in a case of mixed infection with the three pathogens, they could be re-isolated in pure after several subcultures and not from all selected tissues (Table 3). That is probably due to special requirements by such fastidious microorganisms (Shoemaker et al. 2001; Hassan et al. 2003). Therefore, a rapid diagnosis is necessary to initiate prompt therapeutic and prophylactic measures in order to limit the economic losses caused by such pathogens. The molecular techniques have been the most effective methods for diagnosis because they are more specific and sensitive detection than other conventional or commercial assays (González et al. 2004). Alternatively, m-PCR assays using bacterium-specific gene can be used as targets for PCR amplification to permit more specific detection as well as subspecies and strain differentiation in a single reaction. Williams et al. (1999) previously developed m-PCR for the detection of

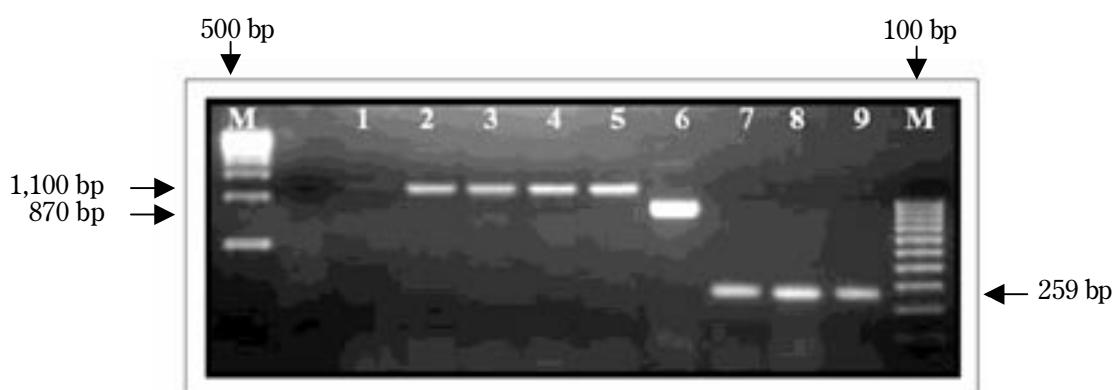


Fig. 1. Amplification products obtained using the m-PCR developed for the concurrent detection of *L. garvieae* (1,100 bp), *S. iniae* (870 bp) and *S. dysgalactiae* subsp. *dysgalactiae* (259 bp). Lanes M, 100 (right) and 500 (left) bp DNA ladder (Bio-Rad, Marnes-la-Coquette, France); lane 1, negative control; lanes 2-5, *L. garvieae* KGLA 0415, NJB B1, NJB K2, NJB E2; lane 6, *S. iniae* NJB 02-011; lanes 7-9, *S. dysgalactiae* subsp. *dysgalactiae* Kdys 0432, Kdys 0440, Kdys 0442.

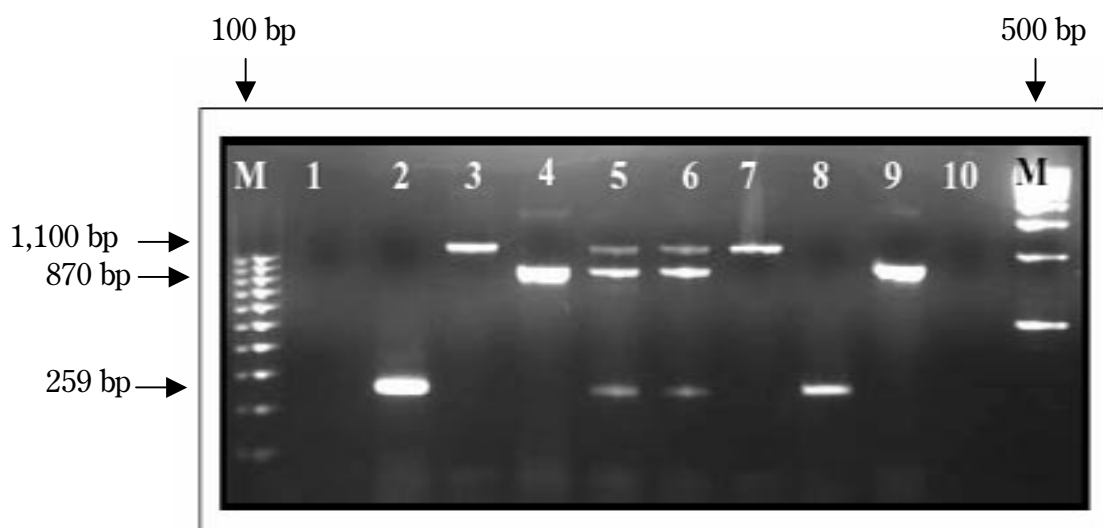


Fig. 2. Sensitivity of detection of *L. garvieae* (1,100 bp), *S. iniae* (870 bp) and *S. dysgalactiae* subsp. *dysgalactiae* (259 bp) by m-PCR of experimentally infected fish tissues homogenates. Lanes M, 100 (left) and 500 (right) bp DNA ladder (Bio-Rad, Marnes-la-Coquette, France); lanes 1 and 10, negative control; lanes 2 and 8, (3×10^7) and (3×10^2) of *S. dysgalactiae* subsp. *dysgalactiae*; lanes 3 and 7, (3×10^7) and (3×10^2) of *L. garvieae*; lanes 4 and 9, (3×10^7) and (3×10^2) of *S. iniae*; lanes 5 and 6, (3×10^7) and (3×10^2) of bacterial mixture of the three pathogens used.

fish viruses. In addition, a m-PCR for detection of *Aeromonas salmonicida*, *Yersinia ruckeri*, and *Flavobacterium psychrophilum* was established by del Cerro et al. (2002).

In this work, a m-PCR was developed for the concurrent detection of *L. garvieae*, *S. iniae*, and *S. dysgalactiae* in their pure cultures and the tissues from fish artificially infected with the pathogens. The specificity of our m-PCR using the above-mentioned primer sets was confirmed by the fact that only specific bands were amplified equivalent to 1,100, 870 and 259 bp, which are characteristics for *L. garvieae*, *S. iniae* and *S. dysgalactiae*, respectively. These results are in agreement with those obtained by Zlotkin et al. (1998) for *L. garvieae*, Mata et al. (2004a) for *S. iniae* and Hassan et al. (2003) for *S. dysgalactiae* subsp. *dysgalactiae* when they used single PCR. In addition, same results were reported by Mata et al. (2004b) for *L. garvieae* and *S. iniae* but not for *S. dysgalactiae* because the later was not included in their m-PCR. Moreover, our m-PCR could detect the three pathogens in the tissues of fish artificially infected with them at low concentrations of 3×10^2 indicating its specificity as well as sensitivity. Interestingly, this concentration in the artificially infected tissue samples is lower

than that reported by Mata et al. (2004b), who demonstrated that their m-PCR could detected *L. garvieae* and *S. iniae* in tissue at a concentrations of 2.5×10^3 and 5×10^3 , respectively.

From these data, it can be concluded that the proposed m-PCR assay can be useful not only as a diagnostic tool but also for epidemiological surveys and could be efficient to establish preventive measures on time. The m-PCR that we have developed is sensitive, fast, and simple technique that can be accomplished in 5 h for rapid and specific detection of *L. garvieae*, *S. iniae* and *S. dysgalactiae*, the main GPC pathogens responsible for fish streptococcosis in Japan.

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養殖ブリの *Lactococcus garvieae*, *Streptococcus iniae* および *S. dysgalactiae* 検出のための複合 PCR

モルタダ M. A. フセイン・畑井喜司雄

レンサ球菌症は、日本の養殖ブリ、カンパチおよびサケ科魚類の重要な病気の一つであり、経済的に問題となる。*Streptococcus iniae* は海産魚と淡水魚の病原体として知られている。いっぽう、*Lactococcus garvieae* と *Streptococcus dysgalactiae* はブリおよびカンパチの病原体として知られている。しかしながら、これらの病原体の混合感染症がブリで発生しているため、正確な診断は時に困難である。主たる病原体が何であるのかを特定するために、この研究の中で複合 PCR による診断の有効性について検討を行った。PCR は、*L. garvieae* では 16S rRNA から、*S. dysgalactiae* では 16S-23S rDNA の ITS 領域から、また *S. iniae* では lactate oxidase から設計されたプライマーを使用して行った。これらを使用した複合 PCR により、培養した *L. garvieae*, *S. dysgalactiae* および *S. iniae* から各々明瞭な 1,100, 259 および 870 bp の増幅産物を得られ、非特異的な増幅産物は得られなかった。また、ニジマスにこれら 3 種の細菌を接種し、各組織から複合 PCR により各細菌の検出を試みた結果、迅速で正確に各細菌の増幅産物を得ることができた。このことから本複合 PCR はブリのレンサ球菌症の診断に有効であると判断された。