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Short Communication

Development of a multiplex PCR assay to detect *Edwardsiella tarda*, *Streptococcus parauberis*, and *Streptococcus iniae* in olive flounder (*Paralichthys olivaceus*)

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A multiplex PCR protocol was established to simultaneously detect major bacterial pathogens in olive flounder (*Paralichthys olivaceus*) including *Edwardsiella* (*E.*) *tarda*, *Streptococcus* (*S.*) *parauberis*, and *S. iniae*. The PCR assay was able to detect 0.01 ng of *E. tarda*, 0.1 ng of *S. parauberis*, and 1 ng of *S. iniae* genomic DNA. Furthermore, this technique was found to have high specificity when tested with related bacterial species. This method represents a cheaper, faster, and reliable alternative for identifying major bacterial pathogens in olive flounder, the most important farmed fish in Korea.

Keywords: *Edwardsiella tarda*, multiplex PCR, olive flounder, *Streptococcus iniae*, *Streptococcus parauberis*

Edwardsiella (*E.*) *tarda*, *Streptococcus* (*S.*) *parauberis*, and *S. iniae* are major bacterial pathogens in olive flounder (*Paralichthys olivaceus*), the most valuable fish in Korean aquaculture that accounts for 56.5% of the total fisheries production in 2010 [7,8,11]. *E. tarda* is a Gram-negative bacterium and the etiological agent of edwardsiellosis in many fish species. Additionally, this microorganism causes enterohemorrhagic septicemia in amphibians, reptiles, marine mammals, and humans [8]. *S. iniae* and *S. parauberis* are Gram-positive bacteria that are responsible for streptococcosis in olive flounder, and can be distinguished from each other based on their respective hemolytic activities [7]. Olive flounder infected with these bacteria shows similar pathological symptoms such as darkening of skin, exophthalmia, rectal herniation, abdominal distension, ascites accumulation, and congestion of the liver, spleen, and kidney. Therefore, biochemical, molecular, or serological methods have been recommended for diagnosing these infections [4].

Variable phenotypes of *S. iniae* and *S. parauberis* have been identified using biochemical tests including an API 20 Strep kit (BioMérieux, France) and other diagnostic methods such as an Enzyme linked immunsorbent assay (ELISA), Western blotting, PCR assay, and random amplified polymorphic DNA (RAPD) analysis. However, these methods are technically difficult, labor-intensive, and time-consuming [4,7,11,13]. Thus, there has been an urgent need to establish a rapid yet accurate and sensitive tool for detecting these pathogenic bacteria [6]. Here, we developed a multiplex PCR protocol to simultaneously identify three major bacterial pathogens of olive flounder: *E. tarda*, *S. parauberis* and *S. iniae*. This may represent a rapid and cost-effective diagnostic technique capable of detecting different target genes within one reaction.

Bacteria were isolated from the head kidney of diseased olive flounder in Jeju (Korea) and identified using an API 20 Strep system for *S. parauberis* and *S. iniae* along with an API 20 E system for *E. tarda*. Ultimately, 36, 20, and 19 strains of *E. tarda*, *S. parauberis*, and *S. iniae*, respectively, were cultured on Tryptone Soya Agar (TSA; Oxoid, UK) or in Tryptone Soya Broth (TSB; Oxoid) supplemented with 2% (w/v) NaCl.

Bacterial genomic DNA was extracted using an Accuprep genomic DNA extraction kit (Bioneer, Korea) according to the manufacturer's protocol. Specific primer pairs for detecting the target genes of *E. tarda*, *S. parauberis*, and *S. iniae* are described in Table 1; the expected product sizes were 415, 718, and 300 bp, respectively [5,6,12]. Multiplex PCR was performed in 20 μL of Accupower PCR premix (Bioneer) containing DNA template (0.01 to

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Table 1. Primer pairs specific for target genes of *S. iniae*, *E. tarda*, and *S. parauberis* used for the multiplex PCR assay

Bacteria	Target gene	Primer pair	Product size (bp)	Sequence (5′ to 3′)	Reference
S. iniae	16S rRNA	Sin1bSi Sin2	300	F:CTAGAGTACACATGTAGCTAAG R:GGATTTTCCACTCCCATTAC	Zlotkin *et al.* [12]
E. tarda	gyrB1	gyrBF1 gyrBR1	415	F:GCATGGAGACCTTCAGCAAT R:GCGGAGATTTTGCTCTTCTT	Mata *et al.* [6]
S. parauberis	23S rRNA	Spa2152 Spa2870	718	F:TTTCGTCTGAGGCAATGTTG R:GCTTCATATATCGCTATACT	Lan J *et al.* [5]

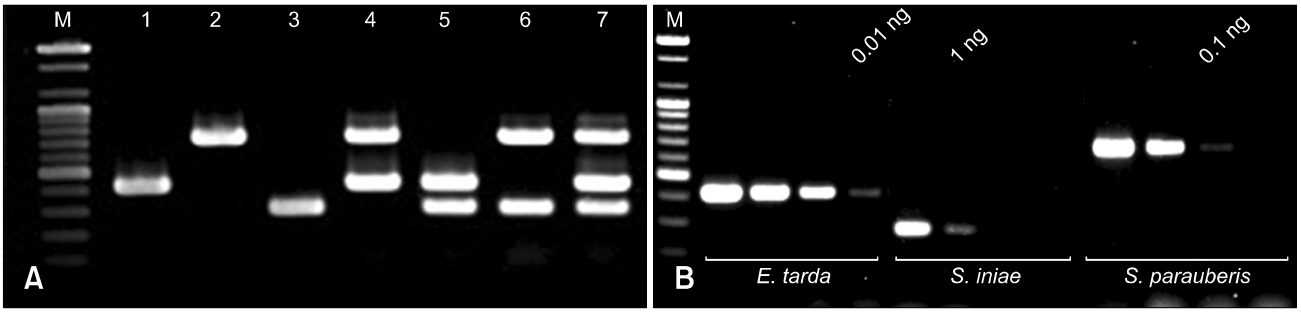

Fig. 1. Sensitivity of the multiplex PCR for detecting *Edwardsiella* (*E.*) *tarda* (415 bp), *Streptococcus* (*S.*) *parauberis* (718 bp), and *S. iniae* (300 bp). (A) Lane M, 100 bp DNA ladder; Lane 1, *E. tarda*; Lane 2, *S. parauberis*; Lane 3, *S. iniae*; Lane 4, *E. tarda* and *S. parauberis*; Lane 5, *E. tarda* and *S. iniae*; Lane 6, *S. parauberis* and *S. iniae*; and Lane 7, *E. tarda*, *S. parauberis* and *S. iniae.* (B) The multiplex PCR detected 0.01, 1, and 0.1 ng of *E. tarda*, *S. iniae*, and *S. parauberis* genomic DNA, respectively.

10 ng of genomic DNA for the sensitivity assay, and 10 ng of genomic DNA for simultaneous detection and the specificity assay) and 0.5 μL (10 μM) of each primer. Amplification was performed in a C1000 Thermal Cycler (Bio-Rad, USA) with one cycle at 94°C for 5 min followed by 25 cycles of 94°C for 30 sec, 50°C for 30 sec, and 72°C for 30 sec, and concluding with one cycle of 72°C for 7 min. The PCR products were separated by electrophoresis in a 2% agarose gel and visualized with ethidium bromide in an E-graph (ATTA, Japan).

Multiplex PCR using three primer pairs enabled the amplification of fragments 415 bp in size for *E. tarda*, 718 bp for *S. parauberis*, and 300 bp for *S. iniae* when testing genomic DNA from the 36, 20, and 19 strains of *E. tarda*, *S. parauberis*, and *S. iniae*, respectively (data not shown). The fragments specific for each bacterium (Fig. 1A) were amplified when the multiplex PCR was performed using a mixture of two or all three bacterial DNA templates (*i.e., S. parauberis* and *S. iniae*; *E. tarda* and *S. parauberis*; *E. tarda* and *S. iniae*; or *E. tarda*, *S. parauberis*, and *S. iniae*).

For the sensitivity assay, genomic DNA from *E. tarda*, *S. parauberis*, and *S. iniae* was serially diluted ten-fold (0.01 to 10 ng). The results showed that the minimum detectable levels of DNA from the three bacteria were 0.01 ng for *E. tarda*, 0.1 ng for *S. parauberis*, and 1 ng for *S. iniae* (Fig. 1B). These findings were the same for triplicate assays (data not shown).

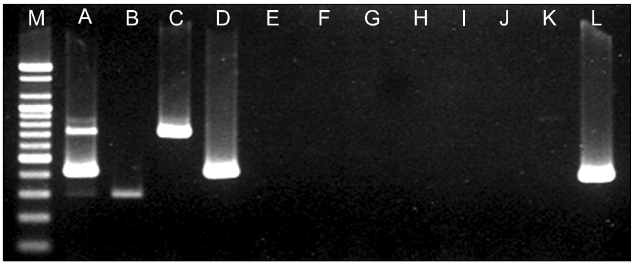

Fig. 2. Specificity of the multiplex PCR. Lane M, 100 bp DNA ladder; Lane A, *E. tarda*, *S. parauberis*, and *S. iniae*; Lane B, *S. iniae*; Lane C, *S. parauberis*; Lane D, *E. tarda*; Lane F, *Lactococcus garivieae*; Lane G, *Aeromonas hydrophila*; Lane H, *Aeromonas salmonicida*; Lane I, *Vibrio alginolyticus*; Lane J, *Vibrio anguillarum*; Lane K, *E. hoshinae*; and Lane L, *E. ictaluri*.

Specificity of the multiplex PCR was evaluated using genomic DNA from ten phylogenetically related bacterial species and other fish-specific pathogenic bacteria such as *Lactococcus garivieae* (ATCC 49156), *Aeromonas* (*A.*) *hydrophila* (ATCC 49140), *A. salmonicida* (ATCC 14174), *Vibrio* (*V.*) *alginolyticus* (ATCC 17749), *V. anguillarum* (ATCC 19264), *E. hoshinae* (ATCC 35051), and *E. ictaluri* (ATCC 33202). As expected, the target genes of *E. tarda*, *S. parauberis*, and *S. iniae* were amplified while no products

were generated for any of the other related bacteria except for *E. ictaluri* (Fig. 2).

Multiplex PCR can detect specific target bacteria simultaneously. Thus, several assays have been evaluated for their ability to identify prevalent pathogens in aquatic species. For example, Chang *et al*. [2] described a multiplex PCR assay for the detection of *A. hydrophila*, *E. tarda*, *Photobacterium damselae*, and *S. iniae* in fish species from subtropical Asia. Additionally, a multiplex PCR protocol was developed for the concurrent detection of pathogens that cause streptococcal diseases in warm-water fish including *S. iniae*, *S. difficilis*, *S. parauberis*, and *L. garivieae* [6]. In the present study, a multiplex PCR assay was designed to simultaneously detect major bacterial pathogens in olive flounder. This technique can detect bacterial genomic DNA at minimum concentrations. Our results demonstrated that the PCR assay was able to detect 0.01 ng of *E. tarda* DNA, 0.1 ng of *S. parauberis* DNA, and 1 ng of *S. iniae* DNA.

Based on previous studies, several primer sets were considered before identifying three target primer pairs for our investigation [3,4,5,6,9,10,12]. PCR amplifications produced product for all of the *S. iniae* and *S. parauberis* isolates using the primer pairs specific for *S. iniae* described by Zlotkin *et al*. [12] and primer sets for *S. parauberis* described by Mata *et al*. [6]. In the case of *E. tarda*, primer sets for the gyrase gene were selected from five primer pairs for this bacterium that did not interfere with the primer pairs for *S. iniae* and *S. parauberis* [5]. The primer sets also amplified fragments using genomic DNA from *E. ictaluri* since sequences of *E. tarda* share 96 to 100% identity according to Basic Local Alignment Search Tool (BLAST) searches for *E. ictaluri* [1]. Indeed, the amplicons produced for *E. ictaluri* genomic DNA showed a 95% similarity with the sequences of *E. tarda* LTB-4 (GenBank accession No. EU259313.1; data not shown). However, *E. ictaluri* infections have been reported mainly in fresh water fish and not in olive flounder [4]. On the other hand, the primer pairs for *E. tarda* published by Chen and Lai [3] did not generate any bands while primer sets described by Sakai *et al*. [9] produced two bands using genomic DNA from 36 *E. tarda* isolates. The primer pairs from Sakai *et al*. [9] were ruled out since they produced numerous bands when the multiplex PCR was performed using three kinds of genomic DNA and primer pairs. In addition, the amplicons (268 bp) generated using the primer pairs from Sakai *et al*. [10] were difficult to differentiate from the band specific for *S. iniae* (300 bp). Therefore, primer pairs specific for the *E. tarda* gyrase gene were selected for our study. The three primer pairs successfully amplified the target gene of each bacterium within one reaction.

In summary, the current study was performed to address the need for a less time-consuming but specific and cost-effective diagnostic method for identifying bacterial pathogens in olive flounder. For this, a multiplex PCR was developed to simultaneously detect *E. tarda*, *S. parauberis*, and *S. iniae*. This method can be useful for diagnosing prevalent pathogens and preventing disease outbreaks in olive flounder, the most valuable cultured fish species in Korea.

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