A PCR Method to Detect *Nocardia seriolae* in Fish Samples

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ABSTRACT—Species-specific polymerase chain reaction (PCR) targeting the 16S rRNA gene of *Nocardia seriolae* was developed. The PCR targeted nucleotide #609 to 1038 (Escherichia coli numbering), which gave a 432 bp-length product. This method could detect *N. seriolae* type strain (JCM3360) and eight clinical isolates of this species from yellowtail *Seriola quinqueradiata* and Japanese flounder *Paralichthys olivaceus*, but not those of other bacterial species including 5 other *Nocardia* spp. and 4 yellowtail pathogens. The detection limit of the PCR was $10^2$ CFU. Eight diseased yellowtail were employed for detection of the bacterium by the PCR. Positive results were obtained from all fish.

Key words: *Nocardia seriolae*, PCR, 16S rDNA, *Seriola quinqueradiata*

Fish nocardiosis was first reported in cultured yellowtail *Seriola quinqueradiata* and amberjack *Seriola dumerili* in 1967 in Mie Prefecture, Japan (Kariya et al., 1968). The economic damage caused by the nocardiosis was not large at that time, however, in recent years, the damage to fish culture industry caused by nocardiosis has been increasing. This disease is one of the re-emerging infections. The bacterium that causes nocardiosis is *Nocardia seriolae* (Kudo et al., 1988), a Gram-positive, acid-fast aerobic actinomycete with branched hyphae. Fish nocardiosis is characterized by the formation of abscesses in the epidermis and tubercles in the gill, kidney, and spleen (Kariya et al., 1968; Kumamoto et al., 1985; Kusuda and Taki, 1973; Kusuda et al., 1974). Since the tubercle forms a barrier to drugs, chemotherapy is not effective for this disease. Therefore, rapid detection of the bacterium is needed to prevent the bacterium spreading to other cages. The standard methods of diagnosis include microbiological techniques using selective media and the fluorescent antibody technique (FAT) (Kawahara et al., 1986). However, *N. seriolae* takes 4 to 5 days for colony formation on an agar plate, and the FAT sometimes causes nonspecific reaction with other bacteria. Thus, the standard methods have problems in time and specificity.

Polymerase chain reaction (PCR) is frequently used to detect causative bacteria of diseases in cultured fish (Mirian et al., 1997; Talaat et al., 1997; Zlotkin et al., 1998; Bin Kingombe et al., 1999). Chun and Goodfellow (1995) and other researchers determined the 16S rRNA gene (16S rDNA) sequence of *Nocardia* spp., including *N. seriolae*. The information of the deposited 16S rDNA sequences is useful to design species-specific PCR primers. In this study, we develop a PCR method for detecting the 16S rRNA gene of *N. seriolae* and detect this gene from diseased fish.

**Materials and Methods**

**Bacterial strains and growth conditions**

The standard strain used in this study was *Nocardia seriolae* JCM3360T. Eight clinical strains isolated from affected fish with nocardiosis (yellowtail and Japanese flounder *Paralichthys olivaceus*) that originated from different fish farms in Japan were used. The strains from yellowtail were EY01-1, EY01-2, EY01-3, EY01-4, EY01-5, EY01-6 and EY01-7, and the strain from flounder was KF01-1. Five *Nocardia* species (*N. asteroides, N. brasiliensis, N. farcinica, N. nova* and *N. otitidiscaviarum*) were also used. Other bacteria examined were *Myco-bacterium* sp. ATCC49159, *Lactococcus garvieae* SSS91-014N, *Vibrio alginolyticus* NCMB1903, *V. anguillarum* V-1037 and *Escherichia coli* JM109. All *Nocardia* species and *Myco-bacterium* sp. were grown on 1% Ogawa media (Nissui, Japan) at 25°C for 4 days and 10 days, respectively. Other bacterial species were cultured as follows: *L. garvieae* was on brain heart infusion (BHI) (Difco, USA) agar at 37°C for 1 day, *Vibrio* species were on marine broth 2216 (Difco, USA) agar at 25°C for 1 day.

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day, and *E. coli* was on Luria Bertani (LB) (Difco, USA) agar at 37°C for 1 day. All cultures were stored at 4°C after harvest until DNA extraction.

**DNA extraction from bacterial cells**

DNA was extracted from bacterial cells as follows. In *Nocardia* and *Mycobacterium* strains, one solid colony of the culture was suspended in 500 μL of ultra pure water containing 150 μL of InstaGene Matrix (Bio-Rad Laboratories, USA). To obtain a homogenous suspension, the mixture was vortexed for 2 min. After heating at 100°C for 30 min, the mixture was centrifuged at 9,000 × g for 8 min. The supernatant was diluted in ultra pure water at 1/50, and 5 μL of the dilution was used for PCR assay. In Gram-negative bacteria, one loop of the colony was suspended in an isolation solution (0.15 M NaCl, 0.1 M EDTA, 0.5 mg/mL RNase A and 0.5% SDS) and incubated at 65°C for 5 min. In Gram-positive bacteria, one loop of the colony was suspended in 100 μL of lysozyme solution (50 μg/mL) and incubated at room temperature for 15 min, followed by the addition of the isolation solution. The DNA was purified by extraction with phenol saturated with TE [10 mM Tris-HCl (pH 8.0) and 1 mM EDTA], TE-saturated phenol:chloroform:iso-amyl alcohol (25:24:1) (PCI) solution and then chloroform. The DNA was precipitated by the addition of ethanol, and the pellet was washed with 70% ethanol, dried and dissolved in 50 μL of TE.

**Primer design and polymerase chain reaction (PCR)**

The 16S rRNA gene (16S rDNA) was targeted for PCR amplification. The forward primer (NS1) was constructed for the specific region of the *N. seriolae* 16S rDNA sequence. The genus *Nocardia* specific primer (NG1) reported by Laurent et al. (2000) was used for the reverse primer. The primer sequences and characters are NS1: 5'-ACTCACAGCTCAACTGTGG-3' (Tm 56.1°C, *E. coli* # 609-627) and NG1: 5'-ACCGACCACAGGGGG-3' (Tm 54.0°C, *E. coli* # 1023-1038). The primer NS1 was based on the 16S rDNA sequences of 17 *Nocardia* spp. from the DDBJ/EMBL/GenBank database (Table 1). GENETIX-WIN (Version 5. 1. 1, Software Development Co., Ltd., Japan) software was used for genetic analysis. NS1 was determined based on the matching of Tm to NG1 and the specific sequence for *N. seriolae*. The primer set NS1-NG1 amplified the 432 bp-long section between nucleotide #609 and 1038 (*E. coli* numbering). PCR reaction was carried out in a 50 L reaction mixture containing PCR buffer [1 mM Tris-HCl (pH 8.3), 5 mM KCl and 0.15 mM MgCl2], 0.2 mM dNTP mixture, 0.25 μM of each primer and 2.5 U Ex-Taq polymerase (TaKaRa, Japan). After denaturation at 94°C for 3 min, the amplification reaction was run through 30 cycles of denaturation at 94°C for 1 min, annealing at 57°C for 20 s and extension at 72°C for 1 min. Final extension was performed at 72°C for 10 min. PCR products were analyzed by 1.5% agarose gel electrophoresis with TAE (40 mM Tris-acetate, 1 mM EDTA) running buffer and visualized by ethidium bromide staining. To confirm that the PCR products were derived from the target region of *N. seriolae* 16S rDNA, sequencing was performed for the product using an ABI PRISM BigDye Terminator Cycle Sequencing FS Ready Reaction Kit (Applied Biosoysystems, Japan) and ABI PRISM 310 DNA sequencer (Applied Biosoysystems, Japan) at the Gene Research Center, Ehime University.

**Detection limit of *N. seriolae* by PCR**

The detection limit of the PCR was examined. One loop of *N. seriolae* JCM3360 culture was suspended in 30 mL of BHI liquid medium in a 50 mL polypropylene tube. To avoid aggregation of the cells, the culture was shaken at 25°C for 4 days on a rotary shaker after 50 pieces of 3 mm-diameter glass beads were added, then the cells were precipitated by centrifugation at 3,500 × g for 20 min. The resulting pellet was suspended in phosphate buffered saline (PBS) and washed twice. Finally, the pellet was re-suspended in 20 mL of PBS. Serial 10-fold dilution was prepared, and each dilution was spread on BHI agar and incubated at 25°C for 4 days to determine the viable cell number in each diluted solution. Another 1 mL portion of the diluted solution was used for DNA extraction. The maximum dilution of the PCR positive was evaluated as the detection limit.

**Detection of *N. seriolae* gene from clinical samples**

Eight samples of yellowtail with nocardiosis or bacterial hemolytic jaundice were employed for detection of *N. seriolae* by the PCR. Samples were collected in October, 2001 in Ehime Prefecture and November, 2002 Strains with asterisc are tested in Fig. 1.
DNA was extracted from tissues (brain, gill, heart, liver, kidney, spleen and muscle), intestinal contents and skin mucus using the method of Qasem et al. (2001) with slight modification. A 100 mg of the sample was homogenized in 500 μL of PBS, and a 100 μL portion was taken for further digestion with the addition of 1 μL of proteinase K (10 mg/mL) at 65°C for 1 h. Then, 900 μL of PBS containing 0.05% Tween80 was added to the solution, and the mixture was centrifuged at 14,000 × g for 10 min. The supernatant was discarded and the pellet containing nocardial cell debris with chromosomal DNA was suspended in 100 μL of TES [50 mM Tris-HCl (pH 8.0), 5 mM EDTA, and 50 mM NaCl] with 5 μL of 20% SDS and 2 μL of proteinase K. After incubation at 65°C for 1 h, extracted DNA was purified twice times of separation with an equal volume of PCI. The DNA was precipitated by adding two volumes of 100% ethanol at -20°C over night and then washed with 70% ethanol. After drying, the DNA was suspended in 40 μL of ultra pure water, and 3 μL was used for the PCR reaction. N. seriolae was isolated from various organs of all fish samples with nocardiosis.

**Results and Discussion**

A new PCR primer set was designed in this study. To examine the specificity of the PCR, N. seriolae JCM3360 and other bacterial strains were employed. As shown in Fig. 1, a 432 bp product was detected in N. seriolae but not in the other bacterial strains. The PCR product was sequenced, confirming that the present PCR with the primer set, NS1-NG1, amplified the target region of 16S rDNA of N. seriolae (data not shown). Eight clinical isolates of N. seriolae gave the same 432-bp bands (Fig. 2). These results indicate the PCR was specific for N. seriolae.

To determine the sensitivity of the PCR, N. seriolae cells were serially diluted, and the DNA from each dilution was used as a template. The amount of the PCR product decreased with decreasing cell number (Fig. 3). The detection limit of the present PCR was 10² cells. Kusuda and Nakagawa (1978) reported that N. seriolae detected using FAT near fish cages was 10⁵–
Clinical samples of fish with nocardiosis or bacterial hemolytic jaundice were employed for the PCR testing (Table 2). In the samples from Ehime and Oita Prefectures, *N. seriolae* was observed in various organs. All samples from Ehime Prefecture were positive and showed serious symptoms of nocardiosis, such as abscesses in the epidermis and tubercles in the gill, kidney, spleen and other organs. The bacterium infected almost all organs in the samples from Ehime Prefecture. In the samples from Oita Prefecture, the brain, liver and intestinal contents were negative, but the heart, gill, kidney and spleen were positive. It has been reported that the target organs of *N. seriolae* are the gill, kidney and spleen (Kariya et al., 1968; Kusuda and Taki, 1973; Kusuda et al., 1974; Kumamoto et al., 1985). Our results showed these organs were highly positive, confirming that they are targeted by *N. seriolae*. Nocardiosis is considered to be a chronic disease (Ikeda et al., 1976). Our PCR might be useful to detect small number of bacterium in various organs of the fish being chronic state. In sample A (Table 2) from Oita Prefecture diagnosed with bacterial hemolytic jaundice, the *N. seriolae* gene was detected in the gill and skin mucus. Since this sample was positive in PCR targeting bacteria causing hemolytic jaundice (Fukuda, personal communication), double infection by the bacteria of bacterial hemolytic jaundice and *N. seriolae* was suspected in this sample.

Nocardiosis is a disease occurring in two-year-old fish, which is market size. Such fish have high economic value. Therefore, to get information of infection from fish surface without killing the fish is very useful. It has been reported that the epidermis is one of the probable infectious routes of *N. seriolae* (Kusuda and Nakagawa, 1978). Our results suggest that it might be possible to diagnose this disease using the skin mucus collected from living fish. The detection of *N. seriolae* gene from skin mucus in this study is noticeable evidence from such viewpoint.

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References


antibody technique compared to standard media culture for detection of pathogenic bacteria for yellowtail and amberjack. *Fish Pathol.*, **21**, 39–45.


