Vol. 120: 173–177, 2016
doi: 10.3354/dao03023

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published July 7

NOTE

Isolation and genetic characterization of *Nocardia seriolae* from snubnose pompano *Trachinotus blochii* in Vietnam

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ABSTRACT: A total of 480 cage-cultured fish were collected from 4 coastal provinces in central Vietnam to investigate the causative agent of nocardiosis. Fish displayed unique characteristics such as paleness and lethargy and exhibited haemorrhages and ulcers on the skin. Prominent white nodules varying in size were observed in the spleen, kidney, and liver. Furthermore, histopathological sections showed typical granulomatous lesions in these organs. Using the Ziehl-Neelsen staining method, isolated bacteria exhibited acid-fast, bead-like filament morphology when cultured in brain-heart infusion medium or Ogawa medium. Phylogenetic analysis of 16S rDNA confirmed that the isolated bacterium was *Nocardia seriolae*. This study demonstrates for the first time an outbreak of *N. seriolae* in snubnose pompano in central Vietnam.

KEY WORDS: Pathogenicity · Nocardiosis · Snubnose pompano · Aquaculture

INTRODUCTION

Nocardia seriolae causes norcadiosis in various cultured marine and freshwater fish in several countries, e.g. sea bass *Lateolabrax japonicus* in Japan (Chen et al. 2000), yellow croaker *Larimichthy crocea* in Taiwan and China (Chen et al. 2000, Wang et al. 2005), and pompano *Trachinotus blochii* in Malaysia and China (Labrie et al. 2008). The clinical signs of infected fish include skin ulcers and tubercles on gills and in the spleen, liver and kidney (Kariya et al. 1968, Kubota et al. 1968, Wang et al. 2005, Labrie et al. 2008). In particular, histopathological examination reveals typical granulomas in the kidney, spleen and liver (Chen et al. 2000). Based on phenotypic and genotypic characterizations using α-glucosidase activity and biased sigmoidal field gel electrophoresis, the *N. seriolae* strains isolated from diseased fish have been divided into α-glucosidase-positive and -negative strains (Shimahara et al. 2006, 2008). The *N. seriolae* strains isolated from different countries have different genotypic characteristics (Shimahara et al. 2008, 2009).

In Vietnam, pompano *Trachinotus blochii* has been bred since 2009 (Lai et al. 2011). Recently, this species has been raised commercially throughout the country (Northern, Central and Southern Vietnam) and has yielded large profits for farmers. So far, there is only a single report describing nocardia-like bacteria related to disease in pompano raised in Nha Trang (Khanh Hoa province) (Giang et al. 2012). However, identification of pathogens by genetic analysis and

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detailed prevalence studies were not conducted. In this study we report on *Nocardia seriolae* in infected pompano and describe the pathology of the disease and the identification of the causative agent.

MATERIALS AND METHODS

Sampling

Samples were collected from cages experiencing fish losses in 4 coastal provinces (with salinity 32–35‰ and water temperature 26–28°C) in central Vietnam (Khanh Hoa, Phu Yen, Ninh Thuan, and Ba Ria–Vung Tau). A total of 480 fish with clinical signs (pale, lethargic with external necrosis and skin ulcers or spinal deformity) were sampled and transported alive to the laboratory for bacteriological examination during February 2014 to July 2015.

Pathology

The fish were sacrificed with an ethyl m-aminobenzoate (MS-222) overdose, and the kidney, spleen, liver and other internal organs with lesions were fixed in 10% buffered formalin and processed for paraffin sectioning. Sections were stained using haematoxylin and eosin (H&E) and the Ziehl-Neelsen (ZN) methods.

Bacteriology

Samples were taken from the kidney, heart, spleen, and liver and streaked on tryptic soy agar (TSA), blood agar (BA = TSA 5% sheep blood), brain heart infusion (BHI) agar and Ogawa medium (OM). Plates were then incubated at 26°C for 30 d.

The purified bacterial isolates were examined by Gram and ZN staining and subjected to phenotypic characterization (Kudo et al. 1988, Chen et al. 2000). In addition, the enzymatic profiles of isolated strains were obtained using API ZYM galleries (Bio-Merieux, France). Bacterial isolates taken from BHI agar were suspended in 2 ml of sterile saline (0.85% NaCl) and sonicated for 10 to 20 s (Sonics & Materials Inc.) to disperse aggregated cells. The turbidity of each suspension was adjusted to a No. 6 McFarland standard, and 65 µl of the bacterial suspension was added to each cupule of the strip as described in the manufacturer's protocol. Plates were then incubated at 26°C, and after 5 h of incubation, ZYM A and B reagents were added and the substrate reactions allowed to react for 5 min.

16S rDNA analysis

DNA was extracted from isolated strains using the QIAamp DNA mini kit (Qiagen) according to the manufacturer's recommendations. The species-specific 16S rDNA genes of *Nocardia seriolae* were amplified using the primers N5F1 (5′-TGA GCC TGA ACT GCA TGG TTC-3′) and N5R1 (5′-ACG GTA TCG CAG CCC TCT GTA-3′) (Labrie et al. 2008).

The PCR mixture contained template DNA (2 µl), 0.5 µl of each primer (0.2 µmol), and HotStarTaq Master Mix (12.5 µl), and the volume was made up to 25 µl with distilled water. Amplification was carried out using the following cycling parameters: 95°C for 2 min; followed by 30 cycles of 95°C for 30 s, 58°C for 1 min and 72°C for 1 min; and finally 72°C for 5 min. The PCR products were run on 1.5% agarose gels and visualized by ethidium bromide staining.

DNA products amplified using primers N5F1/N5R1 were sequenced (Macrogen Co.). The DNA sequences obtained were aligned with representative sequences from GenBank databases. The MEGA6 (Tamura et al. 2013) computer program was used for all analyses. An unrooted phylogenetic tree was inferred using the neighbour-joining (N-J) tree algorithm. Evolutionary distance matrices for the N-J methods were generated after Jukes & Cantor (1969). The resultant tree topologies were evaluated by bootstrap analyses (Felsenstein 1985) of the N-J method based on 1000 resamplings.

Experimental infection

Healthy snubnose pompano *Trachinotus blochii* (65–70 g) were obtained from a cage culture at Nha Trang Bay, Khanh Hoa province. For acclimatization to laboratory conditions, fish were maintained in continuously aerated 300 l aquaria which had a water volume of 250 l, water temperature of 28°C, and salinity of 30‰ for 7 d. The fish were fed commercial diets at 2% body weight twice daily, and waste was removed daily. The fish were left starved for 1 d before inoculation to avoid stress.

A representative *N. seriolae* isolate from snubnose pompano was grown in a flask, agitated at 175 rpm with glass beads (Shimahara et al. 2010) in BHI

broth (0.5% NaCl) at 25 to 30°C for 60 to 72 h and then harvested using normal saline. A bacterial suspension was prepared in saline solution (0.85% NaCl) to a final concentration of 1.3×10^6 CFU ml^{-1}. (The bacterial cell numbers were quantitated by optical density at 580 nm and viable plate counts). Then 0.1 ml (1.3×10^5 CFU) of the bacteria suspension was injected intraperitoneally into a group of 20 fish. The fish in the control group were injected with sterile saline (0.85% NaCl). The fish were continuously monitored for morbidity and mortality and sampled for histopathological and bacteriological analyses. The experiment was terminated 15 d after inoculation. Organ smears and reisolations were also performed for survivors.

RESULTS

Clinical signs and pathology

The naturally infected cultured pompano were characterized as pale, lethargic, with ulcers on the skin and at the base of the dorsal fin, and with swollen abdomens (Fig. 1). An examination of the internal organs revealed white foci ranging from 1 to 2 mm on the spleen, liver and kidneys. Fish having a spinal deformity (52 out of 480 sampled fish) often had a large nodule along the spine. The posterior kidneys of affected fish were swollen and about 2 to 3 times greater in size than observed in normal fish. Approximately 15 to 30% of the fish showed gross lesions.

Gram and Ziehl-Neelsen staining of internal organs revealed the presence of Gram-positive, acid-fast, coccoid or rod-shaped, filamentous bacteria branched (filaments, fragmented from 2 to 10 µm), in affected fish only. Healthy fish had no signs of infection.

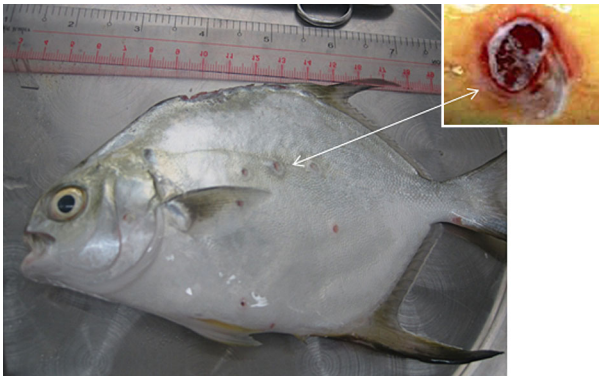

Fig. 1. *Trachinotus blochii.* Ulcers on the skin of infected fish

Bacteriology

Cultures from the heart, spleen, kidneys and liver were incubated for 30 d at 26°C. After 7 to 10 d, weakly pigmented, white adherent colonies 1 to 2 mm in diameter appeared as a heavy, almost pure growth on OM and TSA. Of the 480 sampled fish, 252 bacterial isolates resembling *Nocardia seriolae* (each from one fish) were successfully isolated from fish with clear clinical signs of the disease. The bacteria were Gram-positive, non-motile, non-sporulating rods which occasionally formed branching filaments, and were weakly acid-fast. The bacteria appeared as oval forms or long, slender, multiseptate rods. The strains produced a well-developed mycelium which fragmented into irregular rod-shaped forms. Colonies were dry, waxy and wrinkled with substrate hyphae, but did not carry aerial hyphae.

The physiological and biochemical characteristics of isolates from snubnose pompano in the current study are as follows: Catalase, nitrate reduction and aesculin hydrolysis were positive. Tests for oxidase, urease production, gelatin liquefaction, starch hydrolysis, and decomposition of casein, adenine, elastin, hypoxanthine, xanthine and tyrosine were negative. The organism could utilize citrate, but not mannitol, arabinose and sorbitol as sole carbon sources. It grew with 4% NaCl, but not at 45°C and did not survive at 50°C for 8 h. These characteristics were consistent with the other members of the genus *Nocardia*, and were identical with *N. seriolae* (Kudo et al. 1988, Chen et al. 2000).

The API ZYM profiles of the isolates from diseased snubnose pompano were similar to those reported for *N. seriolae* (Shimahara et al. 2008, Wang et al. 2009, 2014) except in the butyrate esterase and valine arylamidase activities which were positive.

Phylogenetic analysis

A representative isolate from an infected fish yielded an expected PCR product of 1069 bp using the N5F1 and N5R1 primers. The sequence had 100% identity with *N. seriolae* (GenBank accession number JF834066.1), 99% similarity to *N. concava* (GenBank accession number AB126881.1), 98% similarity to *N. nova* (GenBank accession number KP025810.1), and 98% similarity to *N. otitidiscaviarum* (GenBank accession number KP025741.1) (Fig. 2).

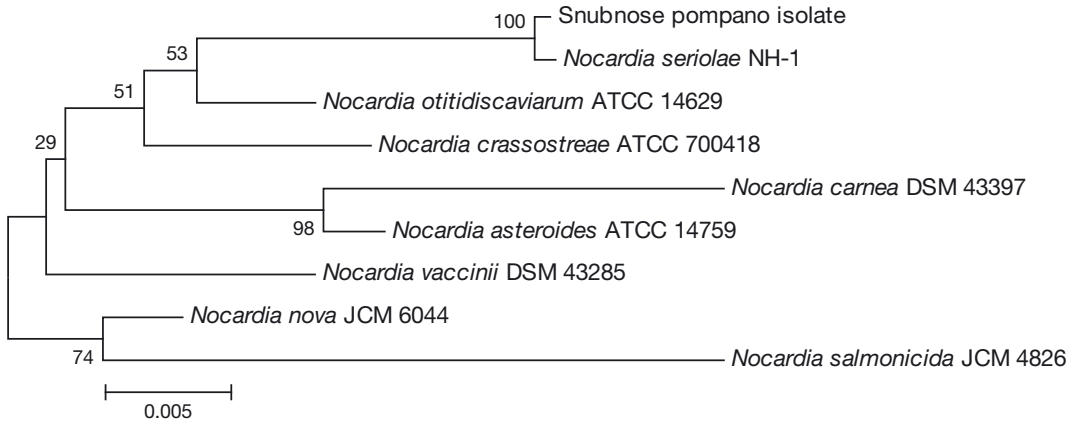

Fig. 2. 16S rDNA sequence-based phylogenetic tree of isolates from Vietnam and from GenBank. Bars indicate genetic distances. Numbers at each node indicate percent bootstrap values. Scale represents 0.005 nucleotide substitutions per position

Experimental infection

Snubnose pompano fish injected with a 1.3×10^5 CFU dose of *N. seriolae* experienced 100% mortality within 10 d. White nodules and histopathological changes from experimental infection were similar to what was observed with natural infection. Also, pure cultures of bacteria were re-isolated from the liver and spleen of moribund snubnose pompano fish. By comparison, there were no lesions observed in the control group.

DISCUSSION

This is the first study describing the pathology and genetic identification of the causative agent of nocardiosis in snubnose pompano *Trachinotus blochii* in Vietnam. The clinical signs observed among diseased fish were typical of nocardial infection. Similar clinical signs were seen in pond-cultured Japanese sea bass *Lateolabrax japonnicus* in Taiwan (Chen et al. 2000), yellow croaker *Larimichthys crocea* in China (Wang et al. 2005), and yellowtail *S. quinqueradiata* and amberjack S. *dumerilii* in Japan (Kariya et al. 1968, Kusuda et al. 1974, Kumamoto et al. 1985, Kudo et al. 1988, Shuzo 1992).

In this study, we identified white colonies on TSA and OM which appeared after 7 to 10 d of incubation. Colonies exhibited a dry to chalky and folded appearance. The bacterial growth characteristics, morphology and biochemical properties were almost identical to *N. seriolae* isolated from yellowtail (Kudo et al. 1988), sea bass (Chen et al. 2000), large yellow croaker (Wang et al. 2005), three striped tigerfish (Wang et al. 2009), and spotted butterfish (Wang et al. 2014).

Using phenotypic characterization by API ZYM, we found that all the isolated strains from diseased fish in Vietnam had the same characteristics as strain *N. seriolae* (Wang et al. 2009) and were negative for α-glucosidase. Our results are in agreement with another study which showed that the *N. seriolae* strains isolated in Japan during 2000 to 2005 were α-glucosidase-negative (Shimahara et al. 2008).

Several authors have developed specific PCR methods for identification of *Nocardia* to the genus level by 16S rDNA analysis (Laurent et al. 1999, Kono et al. 2002, Miyoshi & Suzuki 2003, Labrie et al. 2008). In this study we also amplified the 16S rDNA unit of *Nocardia* using PCR (Labrie et al. 2008). The amplified and sequenced product of 1069 bp had 100% identity to *N. seriolae* DNA (GenBank accession no. JF834066.1). In addition, 16S rDNA phylogenetic analyses confirmed that the organism belongs to the genus *Nocardia*. The unrooted evolutionary tree shows that the isolated strains form a monophyletic clade with *N. seriolae* (GenBank accession no. JF834066.1). This relationship was highlighted by the high nucleotide similarity value (100%) and the high bootstrap value (1000). Based on growth characteristics, morphology, physiological, biological and phylogenetic properties, the organism isolated from diseased snubnose pompano was identified as *N. seriolae*.

In our experimental infection studies on snubnose pompano with the bacterium isolated from a natural outbreak we demonstrated the same pathological changes as in naturally infected fish. In addition, the bacterium was re-isolated in pure culture from infected animals only. These observations confirm the specific bacterium as the aetiological agent of disease in cultured snubnose pompano. Most experimental fish exhibited various skin ulcers and granulomas in

their internal organs. The gross and histological features seen in this study are typical of the signs observed in other cases of nocardiosis in fish (Chen et al. 2000, Wang et al. 2005, 2009, 2014, Labrie et al. 2008).

The characterization of the phenotypic and genotypic properties of isolated *N. seriolae* from cultured snubnose pompano *Trachinotus blochii* in Vietnam will have a benefit for future vaccine development against nocardiosis in fish.

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Editorial responsibility: Alicia Toranzo, Santiago de Compostela, Spain

Submitted: February 3, 2016; Accepted: May 23, 2016
Proofs received from author(s): June 14, 2016