Nocardial Infections in Fish:
An Emerging Problem in Both Freshwater and Marine Aquaculture Systems in Asia

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ABSTRACT

Rising numbers of infections with *Nocardia seriolae* have caused increasing damage to the yellowtail and amberjack industry in Japan. However, nocardial infections in fish are not limited to Japan. Over the last few years, routine disease investigations in cultured fish in the Southeast Asian region revealed that nocardiosis is omnipresent in countries such as Malaysia, Taiwan, Indonesia and China. The disease was observed in marine species such as pompano, four-finger threadfin, big-eye trevally, snapper and grouper, but also in the freshwater fish tilapia. Various levels of mortality could be found in association with the disease. Typical clinical signs included nodules in gills, spleen, kidney and liver, with or without multiple skin ulcers and nodules. Isolation of the pathogen was difficult unless samples were taken from fresh lesions and cultured on nutrient-rich media. Although clear clinical signs were present in all cases and impression prints showed massive presence of typical *Nocardia*-like cells, only in 55% of cases could *Nocardia* be recovered by culture method. Impression prints represented a fast and reliable method to demonstrate the presence of *Nocardia* sp. Histopathologically, the observed nodules were typical granulomas. A selected set of biochemical tests allowed us to identify the causative agent as *N. seriolae* and allowed differentiation from other pathogens. Specific PCR primers were designed and used to confirm the identity up to species level. Experimental infection has demonstrated the role of *N. seriolae* as a primary pathogen in fish.

INTRODUCTION

Nocardiosis caused by *Nocardia* sp. in fish was first described by Rucker (1949) as *Streptomyces salmonicida* infecting sockeye salmon (*Oncorhynchus nerka*). Later, the bacterium was transferred to the genus *Nocardia* as *Nocardia salmonicida,* based on the presence of meso-diaminopimelic acid, arabinose and galactose in whole organism hydrolysates and subsequently confirmed by an almost complete sequence of 16S rDNA analysis (Pridham *et al.*, 1969; Isik *et al.*, 1999). Since then, four species of *Nocardia* [*N. asteroides*, *N. seriolae* (formerly called *N. kampachi*), *N. salmonicida* and *N. crassostreae*] have been isolated from diseased aquatic animals (Valdez *et al.*, 1963; Kusuda *et al.,* 1974; Kudo *et al.*, 1988; Chen, 1992; Friedman *et al.*, 1998; Brandsen *et al.*, 2000; Chen *et al.*, 2000; Wang *et al.*, 2005). *N. seriolae* has by far been the most frequently isolated *Nocardia* sp. from fish and in recent years its damage to the fish industry has been increasing. In particular, the yellowtail (*Seriolae quinqueridiata*) and amberjack (*S. dumerelli*) industries in Japan have suffered from *N. seriolae* infections. The economic losses are significantly high as outbreaks tend to occur several months after stocking when fish are between 300 to 1,000 g (Kariya *et al.*, 1968; Kubota *et al.*, 1968; Kusuda *et al.*, 1974; Kudo *et al.*, 1988). However, not only Japan appears to suffer from Nocardiosis as *N. seriolae* has been found to affect Japanese sea bass (*Lateolabrax japonicus*) and yellow croaker (*Larimichthys crocea*) in Taiwan and China, reportedly causing more than 15% mortality in each species (Chen *et al.*, 2000; Wang *et al.*, 2005).

Nocardiosis is a systemic bacterial disease caused by a Gram-positive, partially acid-fast, aerobic, filamentous bacterium. Typical disease signs include nodules in gills, spleen, kidney and liver with or without multiple skin ulcers/nodules. Histopathologically, the observed lesions are typical granulomas (Egusa, 1992).

From 2002 to 2006, we have investigated the causes of mortality associated with typical clinical disease signs indicative of nocardial infections in Indonesia, China, Singapore and Malaysia. This isolation, identification and prevalence of nocardial infections in South East Asia will be discussed to illustrate the role of *N. seriolae* as a primary pathogen of fish.

MATERIALS AND METHODS

Sampling

In this study, the samples were taken at fish farms in China (4 sites), Singapore (1 site), Indonesia (1 site) and Malaysia (3 sites). The sample sites in China included Guandong and Hainan provinces. In Indonesia, samples were taken at fish farms in Sumatra and in Malaysia, samples were taken at fish farms in the states of Selangor and Johor. Sampling covered the period from February 2002 to June 2006.

Samples were only taken when abnormal behaviour and mortality were observed and no apprently normal fish were sampled. Lesions and clinical signs were recorded, as well as the weight and length of each fish sampled. Fish were sampled on-site, at the farm and affected areas such as abscesses, internal organs such as spleen, kidney, liver or

brain displaying granulomatous lesions were plated on selective (Ogawa agar) and non-selective media (tryptone soy agar, Oxoid) supplemented with 1.5% NaCl (w/v), brain heart infusion agar (Becton Dickinson) supplemented with 1.5% NaCl (w/v), blood agar (Biomedia laboratories) and Eugon agar (Becton Dickinson). Affected organs (including skin ulcers/lesions) were plated using disposable loops and the agar plates were incubated at 26°C until growth was observed. Thereafter, the colonies were purified by sub-culturing on Eugon agar and incubating at 26 °C for 3-5 days. From affected organs, organ imprints were made by sampling the organ and imprinting the cut-through section onto a clean microscope glass slide. Presumptive diagnosis was achieved based on the characterisation of clinical signs, Gram-staining of the impression prints and, where appropriate histology. Samples for histology were fixed in 10% (v/v) formalin, embedded in paraffin wax and sectioned at 5 µm. The sections were stained by haematoxylin and eosin (H&E). Some sections were stained with Gram stain, Ziehl-Neelsen (ZN) and modified ZN (Fite-Faraco (FF) (Faraco, 1938; Fite *et al*., 1947).

Phenotypic/biochemical characterisation

The purified bacterial cultures recovered were Gram-stained (MERCK commercial staining kit, according to manufacturer instructions) and selected cultures identified as representative strains were examined for acid fastness using a modified ZN stain.

For biochemical characterisation, bacterial colonies were collected after 5 days of incubation. The isolates were tested for oxidase by placing some growth (using a platinum loop) on filter paper impregnated with oxidase reagent (Biomerieux). The catalase test was performed by placing some growth on a glass slide and flooding it with 3% H_2O_2. Urease production was tested by stabbing urease agar slants (urea: 20 g/L, NaCl: 5 g/L, KH_2PO_4: 2 g/L, peptone: 1 g/L, glucose: 1 g/L, phenol red: 0.01 g/L, agar 15 g/L) with some collected growth, followed by streaking the slanted surface of the agar. Results were read after 5 days incubation at 26°C. Colonies were suspended into sterile 0.5% NaCl (w/v) and adjusted to turbidity equivalent of 5-6 McFarland standard Nitrate reduction was tested by inoculating three drops of the above suspension in nitrate broth (Difco) supplemented with 1.5% NaCl (w/v). Results were read after 3 and 5 days of incubation at 26°C. McConkey agar (Oxoid) was inoculated with the bacterial suspension and incubated for 5 days at 26°C. The same suspension was used to inoculate API ZYM (Biomerieux) which was read after 5 days incubation at 26°C according to the manufacturer's instructions.

Confirmation of identity by PCR

A specific PCR test was used to confirm identity of the strains. DNA extraction was performed using QIAamp DNA mini kit (QIAGEN) according to the manufacturer's recommendation. The partial sequence (~1.2 kb) of the *N. seriolae* 16S ribosomal RNA gene was determined and gene-specific primers (N5F1 and N5R1) were designed. Primer sequences were tested for specificity using Primer Designer 4 software.

The forward and reverse primers used were N5F1: 5'-TGA GCC TGA ACT GCA TGG TTC-3' and N5R1: 5'-ACG GTA TCG CAG CCC TCT GTA-3'. Two PCR mixes were used namely puReTaq Ready-To-Go PCR beads (Amersham Biosciences) or a cocktail

consisting of 0.2μm of forward and reverse primers each, DNA template 1.0μl, 2.5μl of 1X PCR buffer minus $MgCl_2$, 1.5mM of $MgCl_2$, 0.16mM dNTP, 1U Taq DNA Polymerase in a total reaction volume of 25μl. Amplification was carried out using Hybaid Thermal Cycler with the following cycling parameters: 95ºC (2 mins) x 1 cycle, followed by 30 cycles at 95 ºC for 30 sec; 58ºC for 1 min and 72ºC for 1 min and finally 72ºC for 5 mins. The expected specific amplified product was 1069 bp. PCR products were analysed on 1.2% (w/v) agarose gel (BST Techlab) in TAE buffer. The gels were run at 120V for 75 min, stained with ethidium bromide and photographed using the SynGene Bio Imaging System. The specificity of the primers was tested on isolates, type strains *N. seriolae* CCUG 46828T and *N. asteroides* CCUG 10073T, and unrelated species, as shown in Table 1.

Table 1. Strains used for specificity testing of PCR primers.

| Name of strain	Isolation	Country	Year
N. seriolae-YT-Japan	Yellowtail	Japan	2003
N. seriolae-SB-Taiwan	Japanese Sea bass	Taiwan	2003
N. asteroids-SB-Taiwan	Japanese Sea bass	Taiwan	2003
N. seriolae-TF-Malaysia-5	Threadfin	Malaysia	2002
N. seriolae-PF-Malaysia-2	Pomfret	Malaysia	2002
N. seriolae (CCUG 46828T)	Yellowtail	Japan	1988
N. asteroides (CCUG 10073T)	See J. Gen microbial 20 (1), 129-35, 1959		
Vibrio (Listonella) anguillarum	Yellowtail	Japan	2000
Lactococcus garvieae	Rainbow trout	Belgium	2003
Photobacterium damsela subspecies *piscicida*	Yellowtail	Japan	2001
Tenacibaculum maritimum	Asian sea bass	Singapore	2003
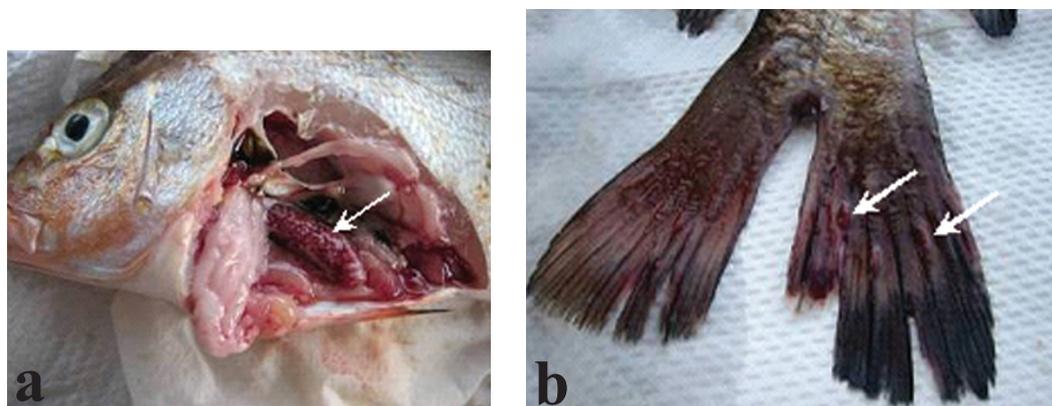
Figure 1. Red snapper (weight: 150 g) showing typical clinical signs of nocardial infections. a: ascites, splenomegaly, macroscopic nodules in spleen (arrow). b: typical nodules (arrow) in tail region.

Experimental infection

Experimental infection was performed on yellowtail (*S. quinqueradiata*) with average weight of 93 g. A *N. seriolae* strain from Japan was cultured in Eugon broth (100ml) for approx. 60 hr and bacterial suspensions of 1.2×10^6 CFU/ml, 1.5×10^7 CFU/ml and 8.5×10^7 CFU/ml were prepared in sterile 1.5% NaCl (w/v). Ten fish per group were injected intraperitoneally with 0.1 ml of the challenge suspension after sedation using AQUI-S (AQUI-S New Zealand Ltd.). Fish were held in a 50-L tank in full strength seawater (approx. 30 ppt) at 28 °C and observed for a period of 19 days.

RESULTS

Clinical signs

Common gross clinical signs included lethargy, multiple skin ulcerations and appearance of red spots with or without an ulcerative mouth. Other common clinical signs observed grossly, were brownish or haemorrhagic gills, abscess inside the operculum, greyish or haemorrhagic liver with white nodules often associated with a brittle texture, fibromatosis in the abdominal cavity, spleen necrosis associated with the presence of macroscopic white nodules, ascites, hemorrhagic brain and swollen kidney often associated with the presence of white nodules.

On-farm mortality associated with nocardial infections was difficult to estimate. Overall, nocardial infections seemed to induce low level chronic mortality in most of the sampled farms. Nonetheless, mortality was considered as economically important as the disease usually occurred several months after stocking, when fish were big. However, in some species, such as Pompano, cumulative mortalities of up to 30% were seen in farms in China causing very significant financial loss.

Bacterial recovery results

In Malaysia, mainly pompano (*Trachinotus blochii*) was affected. However, nocardial infections were also found in mangrove snapper (*Lutjanus argentimaculatus*), four-finger threadfin (*Eleutheronema tetradactylum),* tiger grouper *(Epinephelus fuscoguttatus)* and big-eye trevally (*Caranx sexfasciatus*). Samples from China and Singapore showed that these same fish species were affected there also (see Table 2). In Indonesia, nocardial infections were found in tilapia. *Nocardia* was typically isolated from larger fish (> 100 g, up to 600 g). The causative agent was often isolated from the skin, brain and spleen but could also be isolated from the liver and gills. For all the fish mentioned in Table 2, clinical signs were indicative of nocardial infection; however, in 20 out of 45 cases, no bacteria were isolated through culture method. Those 20 cases were presumptive infections based on clinical signs and impression prints (see Figure 2) where typical *Nocardia*-like cells were seen indicating that a *Nocardia* infection was present.

When clear clinical signs were observed in association with the presence of the bacteria on organ imprints, there was only a 55% chance that the organism was isolated from the diseased fish. In all cases where *Nocardia* was isolated, the bacteria could be seen in organ imprints but likewise the cases where no positive isolation was obtained. Table 2 shows

Table 2. Confirmed and presumptive diagnosis of nocardial infections found in China, Malaysia, Indonesia and Singapore.

Country of isolation		Common fish name	Latin name	Isolation organ	Fish weight/ size	Isolation date (d/m/y)
Malaysia	1	Pompano	*Trachinotus blochii*	B S	20 cm	2/12/2002
	2	Pompano	*Trachinotus blochii*	B SK	19 cm	"
	3	Threadfin	*Eleutheronema tetradactylum*	SK	15 g	12/12/2002
	4	Pompano	*Trachinotus blochii*	B	120 g	"
	5	Threadfin	*Eleutheronema tetradactylum*	B	208 g	"
	6	Pompano	*Trachinotus blochii*	L[1]	133 g	"
	7	Pompano	*Trachinotus blochii*	B[1]	15 g	"
	8	Pompano	*Trachinotus blochii*	S[1]	32 g	"
	9	Wild fish	-	B SK[1]	25 g	"
	10	Pompano	*Trachinotus blochii*	S	> 200 g	23/01/2003
	11	Mangrove snapper	*Lutjanus argentimaculatus*	S[1]K	150 g	28/07/2003
	12	Grouper	*Epinephelus fuscoguttatus*	-[1]	8 g	"
	13	Pompano	*Trachinotus blochii*	B	150 g	"
	14	Trevally	*Caranx sexfasciatus*	SK	204 g	22/10/2004
	15	Pompano	*Trachinotus blochii*	S L[1]	150 g	28/07/2003
	16	Pompano	*Trachinotus blochii*	SK	100 g	13/01/2005
	17	Red snapper	*Lutjanus erythopterus*	L	50 g	07/10/2005
	18	Red snapper	*Lutjanus erythopterus*	K	25 g	"
	19	Red snapper	*Lutjanus erythopterus*	S	180 g	"
	20	Red snapper	*Lutjanus erythopterus*	B L K S[1]	100 g	"
China	21	Pompano	*Trachinotus blochii*	SK[1]	60 g	16/08/2003
	22	Snapper	*Lutjanus sp.*	B	400 g	17/08/2003
	23	Green grouper	*Epinephelus coicoides*	SK[1]	11 g	"
	24	Green grouper	*Epinephelus coicoides*	SK[1]	30 g	"
	25	Green grouper	*Epinephelus coicoides*	SK[1]	40 g	"
	26	Pompano	*Trachinotus blochii*	SK	220 g	6/11/2003
	27	Pompano	*Trachinotus blochii*	G	175 g	"
	28	Pompano	*Trachinotus blochii*	SK	200 g	"
	29	Pompano	*Trachinotus blochii*	SK	220 g	7/11/2003
	30	Pompano	*Trachinotus blochii*	S	70 g	27/12/2005
	31	Red snapper	*Lutjanus eryhtropterus*	B L	75 g	1/9/2005
	32	Red snapper	*Lutjanus eryhtropterus*	L[1]	50 g	"
	33	Pompano	*Trachinotus blochii*	K[1]	500 g	14/11/2005
	34	Pompano	*Trachinotus blochii*	SK	350 g	15/11/2005
	35	Pompano	*Trachinotus blochii*	L K[1]	350 g	"
	36	Pompano	*Trachinotus blochii*	K	320 g	"
	37	Pompano	*Trachinotus blochii*	L[1]	350 g	"
	38	Pompano	*Trachinotus blochii*	S[1]	-	10/4/2006
	39	Pompano	*Trachinotus blochii*	S L[1]	-	"
Singapore	40	Red snapper	*Lutjanus eryhtropterus*	S	46.3 g	20/01/2005
	41	Pompano	*Trachinotus blochii*	SK	12 cm	4/10/2005
Indonesia	42	Tilapia	*Oreochromis sp.*	SK	600 g	9/6/2005
	43	Tilapia	"	S	450 g	"
	44	Tilapia	"	S L[1]	700 g	"
	45	"	"	SK	200 g	15/6/2006

[1] Indicates presumptive diagnosis based on the dominant presence of typical Gram-positive filamentous branching bacteria seen in impression prints in association with typical clinical signs of nocardiosis. B: brain, S: spleen, L: liver, K: kidney, SK: skin ': same as above
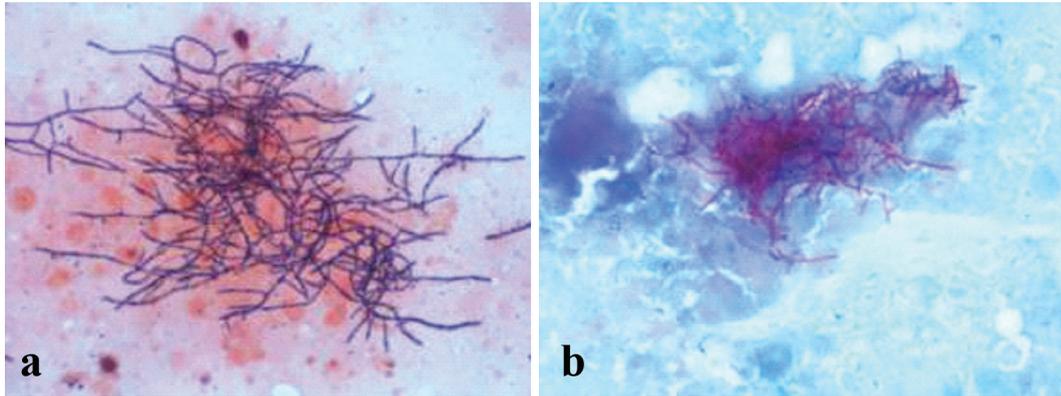
Figure 2. **a:** Gram-stain of impression print from infected fish brain showing typical branching Gram-positive hyphae indicative of nocardial infections (oil immersion X1000). **b:** Ziehl-Neelsen stain of impression print from infected fish brain showing typical acid-fast branching hyphae (oil immersion X1000).

the isolation results over the sampling period showing confirmed and presumptive cases of nocardial infections. When *Nocardia* was isolated, it was always found in association with clinical signs of disease. From the original isolation plates, typical *Nocardia*-like growth was observed after 5-7 days of incubation. Optimum recovery was obtained with Eugon agar. After purification, colonies appeared matt to velvety and dry in appearance with a granular surface, irregularly shaped edges and were light brown in colour.

Histology was performed on several fish showing typical clinical signs of infection. Organs such as brain, liver, stomach, kidney, gills, spleen and skin were sampled. In the liver, mild to moderate diffuse fatty degeneration of the hepatocytes was seen in association with granulomata. Observation of Gram stained tissue sections showed these granulomata to have proliferating Gram-variable rod-shaped bacteria in the centre of the granulomata. The exocrine acinar cells of the pancreatic islets were devoid of zymogen granules. In the brain, large granulomata located at the base of the cerebellum were present; these consisted of necrotic cells, inflammatory cells and, in Gram stain tissue sections there was much proliferation of Gram-variable or Gram-negative mostly thin filamentous, segmented bacteria with a strong infiltration of inflammatory cells and macrophages. Similar granulomata were observed in other foci in the brain tissue. In the head and posterior kidney, parenchyma was often replaced by large, old granulomata or coagulated necrotic areas adjacent to the capsula unilateral. The granulomata were composed of necrotic cellular debris in the centre surrounded by a thick wall of epitheloid flattened cell layers in which proliferating, Gram-variable filamentous bacteria were observed. Remaining parenchyma showed mild necrosis and congestion in association with a large presence of either single or grouped melano-macrophages. The kidney seemed to be more or less affected depending on the case. Usually, the posterior kidney interstitial tissue and tubuli showed only minimal degenerative lesions. When gill lesions were seen, lamellar epithelium showed severe necrosis with the presence of proliferating coccoid rod- or filamentous-shaped bacteria. The capillaries of the primary lamellae showed thrombi composed of necrotic cells. If lesions in the operculum were seen, severe degeneration of the pseudobranchial epithelium

was noted and severe inflammation (consisting of necrosis, haemorrhage and leucocyte infiltration) were found in the surrounding tissue caused by a large number of proliferating Gram-positive filamentous bacteria. In addition, large granulomata composed of Gram-positive rod-shaped bacteria were found. In the stomach, multi-focal mild necrosis of the epithelium was more pronounced in the crypts. In the spleen, most of the pulpa was usually replaced by large old?? granulomata composed of necrotic cellular debris in the centre surrounded by epitheloid flattened cell layers. Younger granulomata were also found and showed the presence of proliferating thin filamentous Gram-negative bacteria. A mild to moderate diffuse presence of single or grouped melano-macrophages was noted and Gram stain revealed the presence of Gram-negative rod-shaped thin and filamentous bacteria in some of the granulomata. In the skin, the dermis showed focally severe inflammatory reactions around major granulomatous, i.e., necrotic foci that spread into the hypodermis. In Gram stain, the necrotic foci were composed of Gram-variable thin filamentous bacteria (see Figure 3).

Phenotypic/biochemical characterisation

All strains showed typical Gram-positive to Gram-variable (often with a bead-like appearance) branching cells after purification on Eugon agar. Some strains were used for acid-fast staining and gave partially acid-fast results (Figure 2).

The results of phenotypical and biochemical profiles of the isolated bacterial strains as well as *N. seriolae* CCUG 46828[T] and *N. asteroides* CCUG 10073[T] type strains are given in Table 3.

Overall, results indicated that the field isolates were closer to the *N. seriolae* type strain than *N. asteroides* (as determined by growth on McConkey agar, urease production, nitrate reduction, α-chymo trypsin negative, and α-galactosidase and β-galactosidase positive). Results amongst the strains were consistent; however, variable results were obtained when strains were tested for valanine arylamidase (19/25 strains positive), cystine arylamidase (20/25 strains positive), trypsin (18/25 strains negative) and α-galactosidase (22/25 strains positive). When the strains were analysed through cluster analysis (unweighted pair group average, percent disagreement, Statistica data analysis software system, version 6 - 2004, Stat Soft Inc.), the analysed strains formed a tight cluster (linkage distance < 0.2, Figure 4) and showed a clear difference with *N. asteroides* type strain (linkage distance > 0.3).

Confirmation of identity by PCR

The N5F1 and N5R1 primers specifically amplified only *N. seriolae* and clearly distinguished it from the closely-related species *N. asteriodes*. The amplification was specific to *N. seriolae* and did not cross react with the other unrelated Gram-negative and Gram-positive bacteria tested (*Listonella anguillarum*, *Lactococcus garvieae* and *Tenacibaculum maritimum*; Figure 5). The primers also proved to be effective to confirm *N. seriolae* infection in clinically-infected fish. All amplifications yielded the expected specific amplification product of 1,069 bp indicated by arrow heads in Figure 5.

Table 3. Results of phenotypic/biochemical characterisation of *Nocardia* strains.

	A	B	1	2	3	4	5	10	13	14	16	17	18	19	22	26	28	27	29	30	31	34	36	40	41	42	43
Gram	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Nitrate reduction	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
McConkey growth	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Urease production	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Growth at 26°C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
API ZYM																											
Control	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Alk. Phos.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Esterase (C4)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Esterase Lip. (C8)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lipase (C4)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Leuc. Aryl.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Val. Aryl.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Cyst. Aryl.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Tryp.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
α-chymo trypsin	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Acid Phos.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
NAPHT	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
α–Gal.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
β-Gal.	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
β-Glucoron.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
α-GLucosidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
β-GLucosidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
NAC.β-Glucos.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
α-mannosidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
α-fucosidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

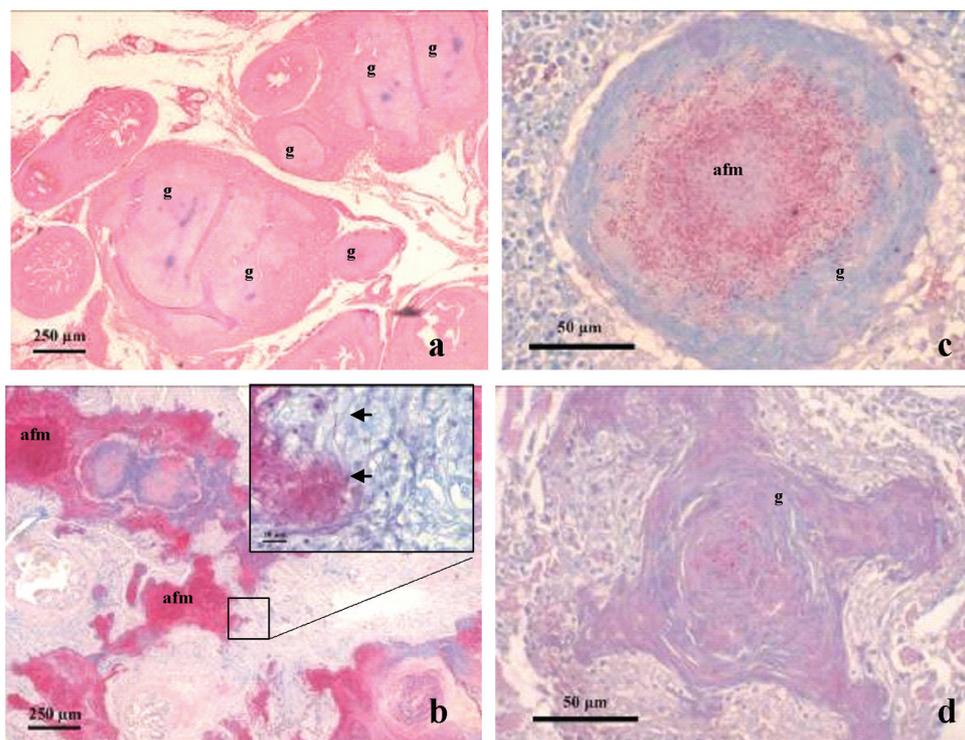A : N. asteriodes-type CCUG 10073T B : N. seriolae-type CCUG 46828T

Figure 3. Examples of histological findings in internal organs of a pompano infected with *N. seriolae*. Picture a: H&E stain of granulomata (g) in peritoneal cavity - note severe granulomata with necrotic centre (X40). Picture b: Modified Ziehl Neelsen stain (Fite-Faraco; FF) of severe granulomatous lesions in peritoneum - note massive presence of acid-fast material (afm) at centre and surrounding granulomata (X400). Insert: details of acid-fast typical *Nocardia*-branched cells at edges of granulomata (arrow). Picture c: granulomata in kidney with FF stain - note clear acid-fast material at centre (X400). Picture d: details of a typical granuloma found in the heart (FF, X400).
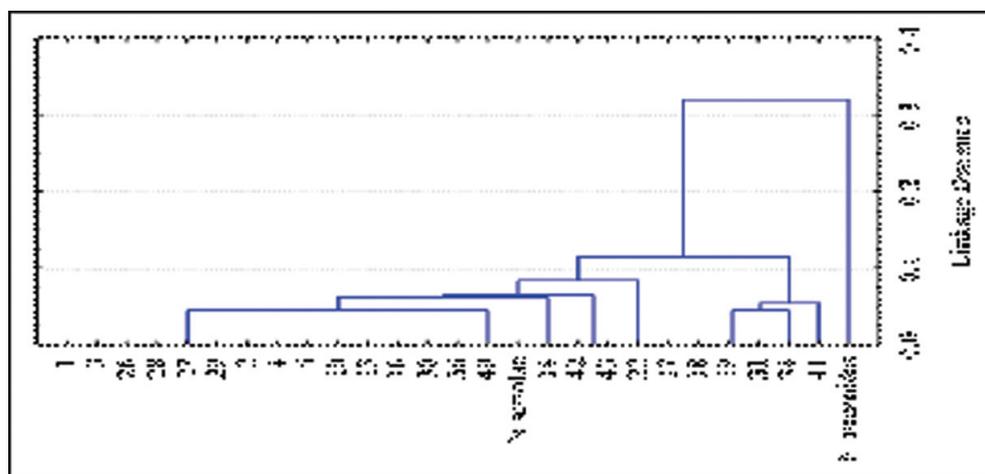
Figure 4. Linkage distance of field isolates and type strains *N. seriolae* and *N. asteroides* (Unweighted pair group average, percent disagreement).
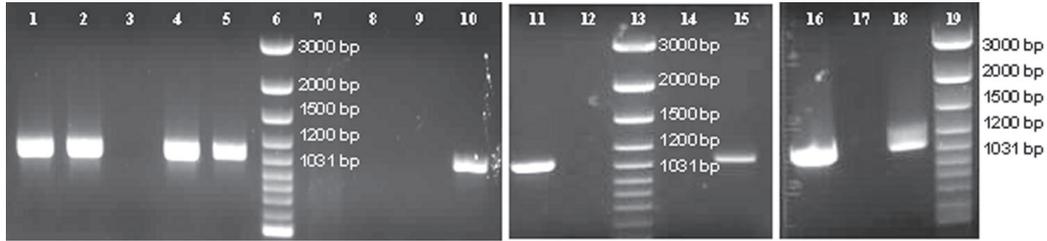
Figure 5. 1.2% agarose gel electrophoresis of PCR products of purified strains from field isolates identified as *N. seriolae* through biochemical/phenotypical identification, other closely-related bacteria and unrelated bacteria. Lanes: 1, *N. seriolae*-YT-Japan; 2, *N. seriolae*-SB-Taiwan; 3, *N. asteroides*-SB-Taiwan; 4, *N. seriolae*-TF-Malaysia-**5;** 5, *N. seriolae*-PF-Malaysia-**2**; 6, DNA ladder; 7, *L. anguillarum*; 8, *L. garvieae*; 9, *T. maritimum*; 10, *N. seriolae*-YT-Japan; 11, *N. seriolae* CCUG 46828T type strain; 12, *N. asteroides* CCUG 10073T type strain; 13, DNA ladder; 14, *N. asteroides*-SB-Taiwan; 15, *N. seriolae*-YT-Japan; 16, *N. seriolae*-infected tissue from clinically-infected fish; 17, *N. asteroides*-SB-Taiwan; 18, *N. seriolae*-YT-Japan; 19, DNA ladder.

Experimental bacterial infection

Experimental infection was performed on yellowtail (*S. quinqueradiata*) originating from Japan. Fourteen days after challenge, all fish showed severe granulomata in the internal organs and some fish developed typical skin and/or mouth ulcers and nodules.

The injection of *N. seriolae* at a concentration of 1.2 x 10^6 CFU/ml, 1.5 x 10^7 CFU/ml and 8.5 x 10^7 CFU/ml resulted in a clear dose response (Figure 6). The injection of 8.5 x 10^7 CFU/ml resulted in the highest mortality, reaching 90% at Day 19. With this dose, mortality started on Day 4 and continued until Day 15. With a six times lower dose (1.5 x 10^7 CFU/ml), mortality started one day later and peak mortality was seen at Day 8. The end mortality was 80%. With a dose of only 1.2 x 10^6 CFU/ml, mortality started only at
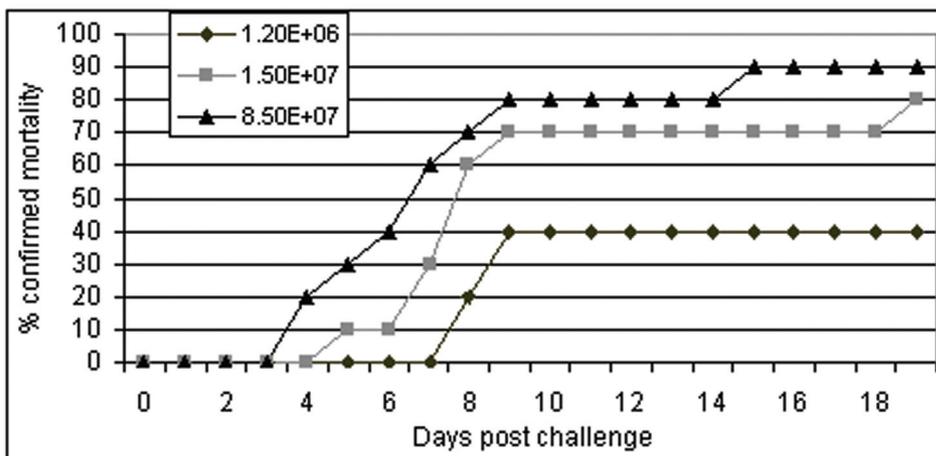

Figure 6. Cumulative percent mortality in 93-g yellowtail (*S. quinqueradiata*) after intraperitoneal injection with different concentrations of *N. seriolae*.

Day 8 and an end mortality of 40% was obtained (see Figure 6). However, upon dissection, an additional 50% of the fish showed the presence of severe granulomas in the internal organs, resulting in 90% of severely affected fish even in the lowest dose group.

DISCUSSION

From 2002 to 2006, cases associated with the typical clinical disease signs indicative of nocardial infections have been observed in countries such as Indonesia, China, Singapore and Malaysia. The causative agent of those infections was indeed found to be *N. seriolae*.

The clinical signs observed amongst the affected fish were typical of nocardial infection, nocardial infection having already been described in fish species in various countries in the Asian region. Chen *et al*. (2000) described multiple yellowish white nodules in the gills, heart, spleen and kidney in pond cultured Japanese sea bass (*Lateolabrax japonicus*) in Taiwan. Similar clinical signs were seen in yellow croaker (*Larimichthys crocea*) in China (Wang *et al.*, 2005) and have often been described in yellowtail (*S. quinqueradiata*) and amberjack (*S. dumrelii*) in Japan (Karyia *et al.*, 1968; Kusuda *et al.,* 1974; Kumamoto *et al*., 1985; Kudo *et al.*, 1988; Shuzo, 1992). From our observations in the field, a clear indication of the presence of the disease was seen based on impression prints, where typical branching, beaded, filamentous Gram-positive bacteria were present in high numbers in association with typical clinical signs. However, isolation results suggest that, even when an infection is present in the population, there is only a 50% chance that the bacteria will be isolated. Although positive differentiation with similar pathogens (like *Mycobacterium* sp.) can only be made by isolation and identification of the causative agent, impression prints of affected organs were a useful tool for the establishment of a presumptive diagnosis. Morphologically, *Nocardia* appear filamentous, branched and beaded and are 5-50 µm long, while mycobacteria are usually shorter (1-3 µm) (Wolke *et al.*, 1987). In addition, *Nocardia* will only give a positive acid-fast staining when FF acid-fast stain is used (Woo *et al.*, 1999), whereas mycobacteria would readily give a positive acid-fast reaction with the (unmodified) ZN stain. Histology can also be used for the establishment of a presumptive diagnosis and is best interpreted when counter stained with Gram or FF stain.

Identification tests indicated that all the isolates were close to *N. seriolae* and phenotypically and biochemically distinct from *N. asteriodes*. Reaction profiles of our field isolates were similar to those of *N. seriolae* described by Goodfellow (1971), Kusuda *et al.* (1974) and Holt *et al.* (1994). Confirmation of identification was done by PCR. Specific primers N5F1 and N5R1 were used to specifically differentiate *N. seriolae* from *N. asteroides* and from other Gram-positive and Gram-negative bacteria, kidney cells and virus. As the isolation of *N. seriolae* is unreliable by culture method, PCR can be a good alternative for confirmation of the presence of the disease. Laurent *et al.* (1999) described a rapid method to identify clinically-relevant *Nocardia* sp. to genus level by 16S rRNA gene PCR. Kono *et al.* (2002) have described the use of a specific PCR method to identify *N. seriolae*. Recently, a loop-mediated isothermal identification technique has been described for the identification of *N. seriolae* based on 16S-23S rRNA internal transcribed spacer region and was found to be more sensitive than PCR (Itano *et al.*, 2006).

The economical importance of the disease was difficult to estimate as only low level chronic mortality was frequently observed. However, it was clear from our isolation data that mainly bigger fish (> 100 g) were affected and, therefore, the economical impact on the farm can be very significant, even at the lower levels of mortality. In addition, it seemed that species such as silver pomfret (*P. argenteus*) and pompano (*T. blochii*) were more susceptible to nocardial infections and could suffer up to 30% mortality during a single outbreak. Furthermore, it was shown that experimental infection was able to induce a significant level of mortality within a relatively short observation period of 19 days, indicating the role of *N. seriolae* as a primary pathogen. There are few, if any, effective treatments against the disease and antibiotic treatment is often ineffective in controlling the disease. Indeed, the abundant use of some drugs has been implicated in the increasing occurrence of antibiotic resistance amongst *N. seriolae* isolates (Itano *et al.*, 2002).

Our findings suggest that it is very likely that nocardial infections due to *N. seriolae* are largely underestimated in South East Asia due to the difficulty of isolation. The significance and prevention of *Nocardia seriolae* in economically-sustainable aquaculture in this region must be given serious attention.

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