Epizootiology and Detection of Nocardiosis in Oysters

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ABSTRACT

Nocardiosis is a bacterial disease of oysters caused by *Nocardia crassostreae*. This disease has many common names, some of which describe its pathology, including: fatal inflammatory bacteraemia, multiple abscesses, focal necrosis and Pacific oyster nocardiosis (PON). Infection with *N. crassostreae* induces a massive accumulation of haemocytes resulting in the formation of green coloured lesions or pustules up to 1 cm in diameter in the mantle, gill, adductor muscle, and heart of oysters. However, histological evidence suggests that some oysters are capable of ridding themselves of infection by the process of diapedesis (haemocyte migration through intact epithelium). Reported in association with high mortalities among Pacific oysters (*Crassostrea gigas*) in Matsushima Bay, Japan in the1950s, *N. crassostreae* has since been observed in various locations along the west coast of North America but its true geographic distribution is not known. Recent investigations in British Columbia clearly indicate that this bacterium is also pathogenic and lethal to flat oysters (*Ostrea edulis*). Infection and mortalities seem to be highest among beach cultured oysters, especially those on a muddy substrate. However, other environmental factors such as reduced water circulation in shallow embayments, warm temperatures and high nutrient levels also increase the prevalence of infection and severity of nocardiosis. Although eradication of nocardiosis is not feasible, off-bottom culture seems to mitigate the disease. The potential negative impact of this disease on oyster culture around the world warrants further investigations and precautions against the transplantation of infected oysters. A polymerase chain reaction (PCR) assay, developed as a tool for monitoring *N. crassostreae* in oyster populations and the environment, proved equally as sensitive as histological examination techniques for the detection of nocardiosis but should be useful for studying the epizootiology of *N. crassostreae*.

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INTRODUCTION

Nocardia is a genus of Gram-positive actinomycete bacteria that reproduce by mycelial fragmentation. Most species in the genus are soil saprophytes but some strains and species are pathogenic to animals (Goodfellow and Lechevalier, 1986). In the aquatic environment, *Nocardia* spp. isolates were reported as pathogenic in fish (see Fryer and Rohovec, 1993 for summary) and crayfish (Alderman *et al*., 1986). Actinomycete bacteria, possibly *Nocardia* spp., have also been reported from oysters on both sides of the Atlantic Ocean. In western Europe, they were observed in European flat oysters (*Ostrea edulis*) during the early 1920s (see Lauckner, 1983, pg. 500). On the east coast of the United States, actinomycete bacteria were reported from the eastern oyster (*Crassostrea virginica*) (Mackin, 1961, pg. 205-213; Meyers, 1981). The biology of these pathogens was not extensively studied. However, the morphology of the organism(s) and associated pathology seem different from that described for nocardiosis in Pacific oysters (*C. gigas*).

Since the late 1940s, nocardiosis has been observed in association with summer mortality in Pacific oysters at several warm locations along the coasts of Japan and subsequently North America. Over the years, this disease was given various names including multiple abscesses, focal necrosis, fatal inflammatory bacteraemia and Pacific oyster nocardiosis (PON). However, the actinomycete bacteria and associated haemocytic infiltration lesions characteristic of nocardiosis were usually not accused of causing the mortalities. Although nocardiosis was reported from oysters in Hiroshima Bay and Matsushima Bay during mass mortality episodes (Fujita *et al*., 1953; 1955; Takeuch *et al*., 1955; Numachi *et al*., 1965), researchers concluded that physiological disorders induced by environmental conditions and not infectious disease caused the mortalities (Tamate *et al*., 1965; Imai *et al*., 1965; 1968). Nocardiosis was also detected in association with high mortalities of Pacific oysters in several bays of Washington State and California during summer in the 1960s but again not identified as the cause of the losses (Lindsay, 1969; Sindermann and Rosenfield, 1967; Glude, 1975). In a review of the Pacific oyster summer mortality issue, Beattie *et al*. (1988) proposed two hypotheses for the cause: one ascribed to the physiological stress associated with spawning and the other attributed to nocardiosis. From results of field sampling and laboratory transmission studies, Friedman *et al*. (1991) suggested that nocardiosis was a causal factor in summer mortalities of Pacific oysters in certain areas. In the late 1990s, Friedman *et al*. (1998) described *Nocardia crassostreae* as the cause of nocardiosis in the Pacific oyster thereby facilitating the initiation of a full assessment of the significance of this oyster pathogen.

Many questions remain concerning the significance of nocardiosis and the potential impact of the disease on future oyster culture operations. The purpose of the present paper is to summarise the current knowledge on the pathology of nocardiosis in oysters and the biology of *N. crassostreae*. Standard diagnostic techniques and our development and validation of a new molecular assay will be described. We will also suggest how the new molecular assay could be used in assessing the significance of this pathogen to oyster culture.

PATHOLOGY OF NOCARDIOSIS IN OYSTERS

Visible signs of nocardiosis include yellow-to-green pustules (raised from the surface) or ulcers (surface depressions) up to 1 cm in diameter, referred to as multiple abscesses by earlier workers, in the mantle, gill, adductor muscle, and heart. Although characteristic of nocardiosis, these lesions are not pathognomonic. For example, they can be reminiscent of lesions induced by *Mikrocytos mackini*, the cause of Denman Island disease in oysters (Farley *et al*., 1988), but are often larger in size. The lesions are yellowish-green in colour due to the accumulation of haemocytes, containing greenish pigments, at the site of infection. Histological examination is required to reveal the dense clusters of basophilic, Gram-positive mycelia of branching bacteria within the core of haemocytic infiltration (Figs. 1, 2, and 3). In heavy infections, multiple foci of infection can occur throughout the oyster and the massive accumulation of the haemocytes can cause considerable disruption to the surrounding tissues (Fig. 2). Large lesions in the adductor muscle of the oyster may compromise the ability of the oyster to close thereby providing access to predators or dehydration when the oyster is out of water.

In most cases, the presence of *N. crassostreae* in the oyster tissues induces an infiltration of numerous haemocytes. Even a small colony of *N. crassostreae* can attract a large number of haemocytes. In heavy, advanced infections, the individual areas of haemocytic infiltration coalesce to produce larger zones of haemocyte accumulations that form the visible lesions. At the terminal stages of nocardiosis, the connective tissue and accumulated haemocytes begin to show degenerative changes (Elston, 1993). However, infected oysters are capable of riding themselves of *N. crassostreae* via diapedesis, a process by which haemocytes with phagocytosed or encapsulated bacteria migrate through epithelial layers to the outside (Morse and Zardus, 1997) or through the intestinal wall (Fig. 4) into the lumen of the gut and are excreted from the body.

The portal of entry of *N. crassostreae* is not known. Lauckner (1983) proposed that *N. crassostreae* may enter via the alimentary canal because of the accumulation of colonies in the connective tissue around the digestive gland. Elston (1993) suggested that the reproductive tract may be another route because *N. crassostreae* has been observed in the gonadal follicles. Friedman and Hedrick (1991) were able to experimentally produce nocardiosis and mortality in *C. gigas* by inoculation with a relatively high number of *N. crassostreae* (150 µl of 1.4 x 10^5 colony forming units per ml of saline). However, *N. crassostreae* was not transmitted between oysters by cohabitation of disease-free oysters with infected oysters or by incubating oysters with sediments from enzootic areas (Friedman *et al*., 1991). This apparent resistance to nocardiosis and the occurrence of the disease only in populations of oysters during periods of physiological stress suggest that *N. crassostreae* may be an opportunistic pathogen. Nevertheless, the presence of *N. crassostreae* must be considered as a threat to oyster health and survival when water temperatures become elevated.

SIGNIFICANCE OF NOCARDIOSIS

Elston *et al*. (1987) reported nocardiosis in about 30% of the oysters in Puget Sound, Washington in the falls of 1984 and 1985 and Friedman *et al*. (1991) observed a prevalence of 58% in Pacific oysters from Oakland Bay, California in September 1986. In British Columbia, the highest confirmed prevalence of *N. crassostreae* was about 30% (Table 1). These prevalences could be extremely significant if the disease is one of rapid progression to death.

Table 1. Detection of *Nocardia crassostreae* in *Crassostrea gigas* and *Ostrea edulis* from various locations in British Columbia. Examinations conducted because of reported mortalities or evidence of pustules during processing for market (rejection of oyster at shucking) are indicated by asterisk.

Location[a] and date	Oyster species	Culture method	Substrate type	Sample size	Prevalence
Pendrell Sound (1)					
13 Nov. 1986	*C. gigas*	Beach	Rock	50	25%
19 Aug. 1987	*C. gigas*	Beach	Rock	20	15%
29 Sept. 1988	*C. gigas*	Beach	Rock	20	25%
Malaspina Inlet (2)					
5 Oct. 1999	*O. edulis*	Hanging		30	13%
2 Nov. 1999	*C. gigas*	Beach	Not indicated	– [b]	25%*
29 Aug. 2000	*O. edulis*	Beach	Sand/mud	30	13%
29 Aug. 2000	*C. gigas*	Beach	Sand/mud	30	27%
29 Aug. 2000	*C. gigas*	Beach	Gravel/rock	30	3%
31 July 2001	*O. edulis*	Beach	Gravel/rock	24	8%
31 July 2001	*O. edulis*	Beach	Sand/mud	20	30%
31 July 2001	*C. gigas*	Beach	Sand/mud	23	17%
12 Sept 2002	*C. gigas*	Beach	Sand/mud	60	12%
Nelson Is (3)					
15 Aug. 1989	*C. gigas*	Beach	Not indicated	40	15%*
15 Aug. 1989	*C. gigas*	Hanging		20	5%
5 Oct. 1994	*C. gigas*	Beach	Not indicated	– [b]	30%*
5 Oct. 1994	*C. gigas*	Hanging		26	12%
Baynes Sound (4)					
28 April 1986	*C. gigas*	Beach	Sand/gravel	40	3%
5 Aug. 1986	*C. gigas*	Beach	Sand/gravel	45	18%
25 Jan. 1987	*C. gigas*	Beach	Sand/gravel	40	15%
30 Oct. 1987	*C. gigas*	Beach	Not indicated	– [b]	4% with lesions, 25% mortality*
Nanoose Bay (5)					
12 Nov. 1985	*C. gigas*	Beach	Sand/mud	– [b]	Up to 50%*
20 June 1986	*C. gigas*	Beach	Sand/mud	40	5%
14 May 1987	*C. gigas*	Beach	Sand/mud	– [b]	5%*
21 Oct. 1987	*C. gigas*	Beach	Sand/mud	30	25% mortality*
Ladysmith Harbour (6)					
1 Oct. 1986	*C. gigas*	Beach	Pea gravel	40	28%*
2 April 1987	*C. gigas*	Beach	Not indicated	40	5%
30 Sept 1987	*C. gigas*	Beach	Not indicated	– [b]	30%*
3 June 1988	*C. gigas*	Beach	Sand/mud	40	3%
28 June 1988	*C. gigas*	Beach	Not indicated	40	8%
17 Sept. 1998	*C. gigas*	Beach	Not indicated	30	27%*
25 Sept. 2000	*C. gigas*	Beach	Mud	30	20%
4 Sept. 2002	*C. gigas*	Beach	Mud	75	27%
Saltspring Is. (7)					
12 May 1989	*C. gigas*	Beach	Not indicated	40	5%

[a] Numbers in parentheses refer to locations shown in Fig. 5.

[b] Random sample not examined. The prevalence of oysters with yellowish-green pustules was estimated by oyster shuckers or growers. Presence of nocardiosis was confirmed by histological examination of a few oysters.

Although approximately equal numbers of male and female oysters were found to be infected during the early summer, Elston *et al*. (1987) and Friedman *et al*. (1991) observed that during the late summer many more males and sexually undifferentiated oysters were infected than females. Elston (1993) proposed that infection in females may result in early, abnormal spawning, resorption of reproductive follicles and return to an undifferentiated state, or possibly inhibition of reproductive development. Alternately, females may experience disproportionately higher mortalities in comparison to males and immature oysters as a consequence of the higher metabolic demands of egg production. The effect of nocardiosis on oyster reproduction requires further study before the significance to oyster production can be fully evaluated.

In addition to being reported from several locations in Japan (Takeuchi *et al*., 1955; Imai *et al*., 1965), California, Washington and British Columbia (Elston, 1993), we have detected nocardiosis in association with oyster mortalities at four additional locations in the Strait of Georgia, British Columbia (Saltspring Island, Nelson Island, Malaspina Inlet and Pendrell Sound, see Table 1). It is interesting to note that in British Columbia, nocardiosis has only been detected in oysters cultured in the Strait of Georgia (Fig. 5). Likely, *N. crassostreae* occurs more widely than published records indicate and the potential distribution of nocardiosis may encompass all areas where the Pacific oyster is cultured.

AETIOLOGY AND EPIZOOTIOLOGY

Like all *Nocardia* spp., *N. crassostreae* is aerobic, Gram-positive, acid-fast; produces mycelia which fragment into irregular rod-like elements, has a peptidoglycan layer containing *meso*-diaminopimelic acid, arabinose and galactose as major sugars, mycolic acids with 46-58 carbon atoms and G + C-rich DNA. However, biochemical, chemical, morphological and physiological properties distinguish *N. crassostreae* from other *Nocardia* as described by Friedman *et al*. (1998).

Little is known about the epizootiology of *N. crassostreae*. Friedman *et al*. (1999) found that oysters challenged with *Nocardia* synthesized heat shock proteins in a pattern similar to control animals. However, the degree of induced thermal tolerance was reduced in oysters with nocardiosis. The reduction in thermal tolerance in infected oysters may help to explain why nocardiosis and associated mortalities usually occur during the late summer and fall months when water temperatures are highest.

The cycle of *N. crassostreae* in the environment between oysters is not known. Because most species of *Nocardia* are soil saprophytes, Elston (1990) and Friedman and Hedrick (1991) suggested that *N. crassostreae* may also be a saprophitic microbe that is acquired from the environment as an opportunistic invader of live oysters. Conversely, *N. crassostreae* may normally be a relatively benign parasite of oysters that becomes pathogenic when its oyster host is stressed during periods of elevated temperatures. The development of new molecular detection techniques as described below should help to resolve this issue.

If *N. crassostreae* is a soil saprophyte, the prevalence and severity of nocardiosis could be reduced by culturing oysters in off-bottom systems as suggested by Elston (1993). However, the relationship between the substrate and infections is not always clear. In a review of the mass mortalities of oysters that occurred in Japan between 1945 and 1973, Koganezawa

(1975) noted that the mass mortalities appeared coincident with the development of hanging methods of culture and the increase in productivity. Koganezawa (1975) also indicated that the distance from shore and the depth of oyster beds seemed to bear no relation to mortality rates. During our investigations in British Columbia, Canada, *N. crassostreae* was detected in up to 13% of the oysters in hanging culture on three occasions at two different locations (Table 1). Nevertheless, at these locations, oysters being cultured on the beach had two to three times higher prevalence of *N. crassostreae* (15 to 30% infected). Although nocardiosis is most frequently reported from oysters cultured on a muddy substrate, oysters naturally set on the rocky shores of Pendrell Sound, British Columbia consistently had a high prevalence of *N. crassostreae* (Table 1). Possibly this occurrence could be attributed to the unique geographical features of Pendrell Sound that promote high oyster reproductive activity due to relatively warm nutrient rich waters. To complete our understanding of the dynamics of *N. crassostreae* in the environment, it will be necessary to sample the substrate, water and oysters throughout the year in areas where nocardiosis is enzootic.

Historical observations indicate that the Pacific oyster is the principal oyster to be affected by *N. crassostreae* and infection has been reported in six month old Pacific oyster seed (Sindermann and Rosenfield, 1967). However, Elston *et al*. (1987) detected nocardiosis in a few specimens of the European flat oyster (*Ostrea edulis*) cultivated near areas of infected Pacific oysters in Washington State. A similar scenario occurred in Malaspina Inlet, British Columbia where up to 30% of the flat oysters had nocardiosis from two locations where Pacific oysters were also infected (Table1). At one of these locations where both species of oysters were being cultured on a sand/mud beach, the prevalence of infection was about twice as high in the flat oysters.

Imai *et al*. (1968) was able to detect *N. crassostreae* in Pacific oysters being cultured in Matsushima Bay during most months of the year but prevalence of infection was highest during the late summer and fall months. In British Columbia, we have also detected N. crassostreae in oysters during the winter and spring with no evidence of the disease and mortalities. Problems associated with this bacterium usually occur only during the late summer and fall (Table 1). Thus, the presence of *N. crassostreae* is not sufficient to cause a disease that results in mortalities. Imai *et al*. (1968) reported that mass mortalities of oysters occurred during periods of intensive growth and maturation of the gonad under conditions of high water temperature and very high nutrition. Friedman and Hedrick (1991) also noted that elevated temperatures and nutrient levels occurred coincident with time of peak occurrence of the disease. Also, *N. crassostreae* can cause severe nocardiosis and mortalities when the oyster is experiencing periods of "stress."

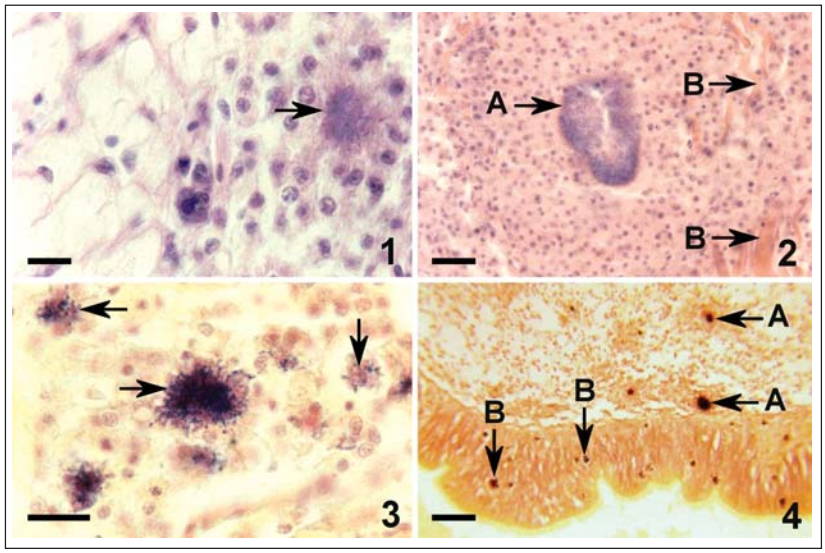

Figure 1. Histological section of *Crassostrea gigas* through the edge of a lesion in the mantle with normal vesicular connective tissue in the left part of the figure and an accumulation of haemocytes around a cluster of *Nocardia* sp. (arrow) on the right. This haematoxylin and eosin stained section does not reveal bacterial morphology. Scale bar = 10 µm.
Figure 2. Histological section through a large lesion in the adductor muscle of *Crassostrea gigas* with nocardiosis. Massive accumulation of haemocytes around a colony of *Nocardia* sp. (A) has displaced and disrupted the muscle fibers (B) of the adductor muscle. Haematoxylin and eosin stain. Scale bar = 20 µm.
Figure 3. Gram stained histological section through lesion containing several clusters of *Nocardia* sp. (arrows) in *Crassostrea gigas*. Because *Nocardia* sp. are Gram-positive, the bacterium is more evident in Gram stained sections than in sections stained with haematoxylin and eosin stain (see Fig. 1 above for comparison). Scale bar = 10 µm.
Figure 4. Histological section through wall of intestinal tract of *Crassostrea gigas* with nocardiosis. The clusters of Nocardia sp. are surrounded by haemocytes (A), many of which are in the process of migrating (B) through the epithelium of the gut wall. Gram stain. Scale bar = 50 µm.

DIAGNOSTIC TECHNIQUES

Histological examination is the standard technique for the detection of nocardiosis. In histology, *N. crassostreae* appears as dense clumps of deeply staining Gram-positive, PAS-positive, branching, beaded, basophilic bacterial clusters surrounded by an intense accumulation of haemocytes and occurs in most organs (Figs 1 to 4). The bacterial clusters usually have dense cores but the branching nature of *N. crassostreae* is usually apparent at the periphery of the cluster. Clusters of *N. crassostreae* are more easily detected in tissue sections stained with Gram stain (see Fig 1 in comparison to Fig. 3). A strong presumptive diagnosis can be made if Gram-positive, acid-fast, branched and beaded colonies of filamentous bacteria are observed in tissue imprints (impression smears) of material from pustules. It is important to note that pustules may occur in oysters in the absence of *N. crassostreae* (false positives) and that not all infected oysters develop visible pustules (false negatives). Confirmatory diagnosis is based on the observation of characteristic histological signs of the diseases as described above, or isolation of *N. crassostreae* in culture as described below.

Many early attempts to culture *N. crassostreae* were unsuccessful (Glude 1975). However, Friedman and Hedrick (1991) were able to culture N. crassostreae on a variety of common microbiological media incubated at about 28 °C. On Brain Heart Infusion (BHI) agar plates, strains isolated from diseased Pacific oysters produced a well developed mycelium that fragmented into irregular rod-shaped forms. The colonies were dry, waxy and wrinkled but did not carry aerial hyphae. The chemotaxonomic characteristics of *N. crassostreae* are described by Friedman *et al*. (1998). However, culture procedures are not suitable as a standard or routine diagnostic method because isolates from infected oysters required prolonged periods of incubation (3 weeks to 3 months) before colonies of *N. crassostreae* became visible (Friedman and Hedrick, 1991).

In histological sections, it is typical to observe accumulations of haemocytes in which the core of *N. crassostreae* is not visible. Because haemocyte accumulations occur in oysters for numerous reasons, this observation without the presence of *N. crassostreae* can not be used for diagnosis. In an attempt to address this concern and to detect light (early) infections, a polymerase chain reaction (PCR) assay was developed and validation of the assay was conducted.

Table 2. List of species included in the alignment analysis used to design *Nocardia crassostreae* specific primers NC-990F and NC-1409R described in Table 3.

Nocardia spp.	Genbank accession number	Other members of the Corynebacterineae	Genbank accession number
N. crassostreae	U92799 and U92800	*Corynebacterium otitidis*	X73976
N. asteroides	X80686	*Cryobacterium aff. psychrophilum*	AJ297438
N. brasiliensis	X80608	*Dietzia maris*	Y08311
N. carnea	X80607	*Gordonia sp.*	X85245
N. pseudobrasiliensis	X84854	*Mycobacterium bohemicum*	AJ277284
N. farcinica	Z36936	*Rhodococcus erythropolis*	AJ250929
N. nova	Z36930	*Tsukamurella columbiensis*	AF272835
N. restricta	X80613	*Brevibacterium lactofermentum*	Y12792
Nocardia sp.	AB035565	*Skermania piniformis*	Z35435
N. brevicatena	X80600	*Williamsia murale*	Y17384
N. otitidiscaviarum	X80599		
N. petroleophila	X80596	Unrelated bacterium	
N. seriolae	X80592		J01859
		Escherichia coli	
N. transvalensis	X80598		
N. vaccinni	X80597		
N. paucivorans	AF179865		
N. croynebacteroides	X80615		
N. calcarea	X80618		
N. salmonicida	Z46750		
N. uniformis	Z46752		
N. flavorozea	Z46754		
N. pseudosporangifera	Z37136		
N. amarae	X80601		
N. pinensis	X82242		

Table 3. Sequences of primers used in *Nocardia* spp. general and *N. crassostreae* specific PCR.

Primer	5'- Sequence - 3'	Location1
Nocgen-1F	GCG AAC GGG TGA GTA ACA CG	91
Nocgen-2R	ACC CCG ATC CGA ACT GAG AC	1282
NC-990F	GGA AAG CCG TAG AGA TGC	973
NC-1409R	CCT TAC GGG TTA GGC CAG	1426

[1] 5' nt. positions are based on alignment with *N. crassostreae* 16S rRNA gene (Genbank accession no. U92800)

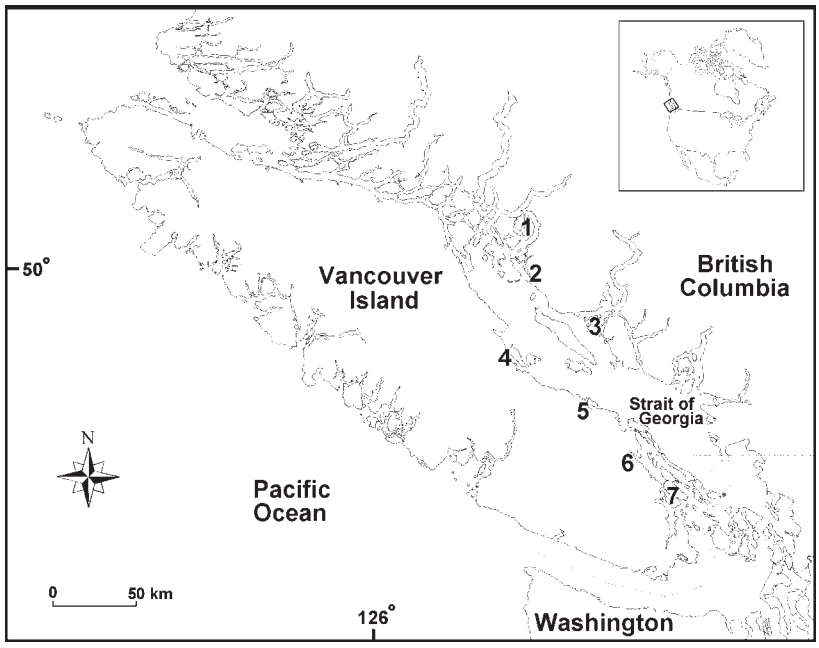

Figure 5. Localities (1-7) in British Columbia, Canada, from which *Nocardia crassostreae* were detected in oysters between November 1985 and September 2002 (see Table 1 for details).

Gee and Elston (1997) developed two sets of PCR primers and one oligonucleotide probe. One primer set was designed to amplify a portion of the 16S rDNA template from more than one genus of mycolic acid-producing bacteria, including *Nocardia*. Nested in the first set was a primer set specific for the genus *Nocardia*. The oligonucleotide molecular probe is a pathogen-specific sequence within the *Nocardia* specific amplicon. This observation was consistent with the sequence information that was used to describe the 16S ribosomal RNA gene of *N. crassostreae* and demonstrate that *N. crassostreae* belonged in the *Nocardia otitidiscaviarum* rRNA sub-group (Friedman *et al*., 1998). However, the general *Nocardia* primers (Nocgen-1F and Nocgen-2R, see Table 3) developed for this purpose were not specific for *N. crassostreae* (Fig. 6).

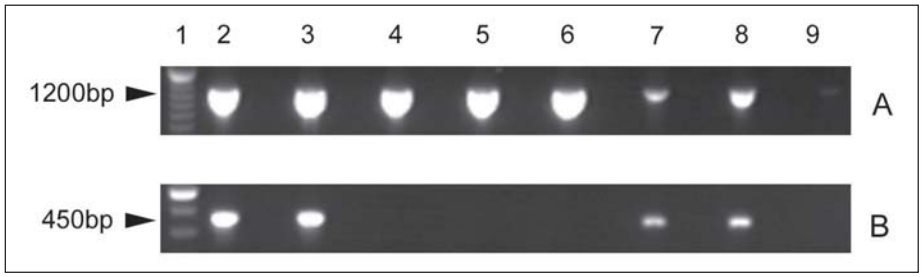

Figure 6. Specificity of *Nocardia crassostreae* PCR assay (Panel B with primers NC-990F and NC-1409R) in comparison to an assay using the general *Nocardia* primers (Panel A with primers Nocgen-1F and Nocgen-2R). See Table 3 for primer descriptions. For both panels, contents of the lanes are as follows: 1 = 100 bp DNA ladder; 2 and 3 = cultured *N. crassostreae*; 4 = cultured *N asteroides*; 5 and 6 = cultured *N. serioles*; 7 and 8 = *N. crassostreae* infected *C. gigas*; and 9 = *Mikrocytos mackini* infected *C. gigas* (negative control). Cultured *Nocardia* spp. DNA samples were provided by one of us (Dr. Arthur Gee).

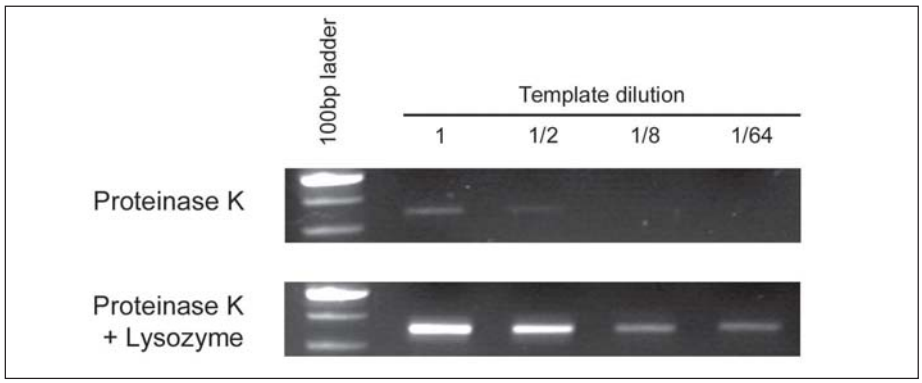

Figure 7. Effect of lysozyme treatment on extraction of *Nocardia crassostreae* DNA. The DNA was extracted from an oyster tissue pustule that contained numerous *N. crassostreae* in the presence or absence of lysozyme treatment. The resulting extractions were serially diluted and used in the *N. crassostreae* specific PCR.

To design *N. crassostreae* specific PCR primers, a SSU rDNA alignment using Clustal W (European Bioinformatics web site: http://www.ebi.ac.uk/clustalw/) was created from sequences provided in Genbank for the species listed in Table 2. Regions variable among the Corynebacterineae were selected for *N. crassostreae* primer development, and primer candidate sequences (i.e., NC-990F and NC-1409R as given in Table 3) were evaluated against the Genbank database using a Blast search (to identify matches to oyster or potential environmental contaminants). Once the likelihood of spurious amplification could be discounted, primers were purchased (as were all other PCR reagents, from Invitrogen Canada, Inc., Burlington, Ontario) and PCR optimisation was conducted. The final, optimised, *N. crassostreae*-specific PCR assay included primers (NC-990F and NC-1409R) at 50 nM, 1x PCR buffer, 1.25 mM $MgCl_2$, 200 µM nucleotides, 0.05units/ul Platinum *Taq* DNA polymerase, and template DNA at 1/10 the reaction volume. Cycling began with an initial denaturation at 94(C for 10 min, followed by 40 cycles of 94°C (1 min)/58°C (1 min)/72°C (1 min), and ended with a final extension at 72°C for 10 min. Amplification products were electrophoresed on an agarose gel, stained with ethidium bromide, and visualized under

UV light. The PCR assay was tested against two other *Nocardia spp*., one closely related and the other distally related to *N. crassostreae* (Friedman *et al*., 1998), and found only to be specific for *N. crassostreae* (Fig. 6).

During development of the PCR assay, problems with the DNA extraction procedures were revealed. Only 56% of the oyster tissue samples (n = 32) known to be heavily infected with *N. crassostreae* (category 3 and 4 infections as categorised by Elston *et al*. (1987)) were positive by PCR when the DNA was extracted using a standard procedure (DNeasy Tissue Kit, Qiagen Inc., Mississauga, ON). Because *N. crassostreae* is known to have a trilaminar cell wall (Friedman et al. 1998), the standard extraction procedure was modified as follows. Oyster tissue samples (2-3 mm^3) preserved in ethanol were rehydrated in double distilled water and digested with 200ul of 2 mg/ml proteinase K (Qiagen) in lysozyme lysis buffer (20 mM Tris-Cl, 2 mM EDTA, 1.2% Triton X-100, pH 8.0), overnight at 55°C. The pellet of digested tissue was washed with 500 ul of lysozyme lysis buffer to remove any residual proteinase K. Pellets were then treated with 200 ul of 20 mg/ml lysozyme (Sigma-Aldrich, St. Louis, MO) in lysozyme lysis buffer and incubated at 37°C for 30 min. The DNeasy Tissue Kit protocol for animal tissues was then continued from step 3. In order to test the effect of lysozyme treatment on DNA extraction and subsequent PCR assay, a tissue sample (5 mm^3) from a heavily infected oyster was digested in 2 volumes (400ul) of proteinase K in lysozyme lysis buffer, overnight at 55°C. The next day, one aliquot was treated with lysozyme, as described above, while the other was left at 55°C. After lysozyme treatment, both aliquots were processed, starting at step 3 of DNeasy protocol for animal tissues. The lysozyme step resulted in at least a 64-fold increase in the sensitivity of the *N. crassostreae*-specific PCR assay (Fig.7). Thus, the lysozyme step was incorporated into the PCR assay for *N. crassostreae* in oysters.

In an attempt to validate the PCR assay, 130 oysters from two locations (Malaspina Inlet and Ladysmith Harbour) known to have oysters with nocardiosis were assayed via standard histopathology and PCR. Precautions used to prevent cross contamination between samples included: scrubbing and rinsing oysters with freshwater prior to shucking, cleaning and sterilising shucking knives and all dissecting tools between each oyster by wiping each instrument with tissue followed by successive dip baths in diluted bleach solution (3% sodium hypochlorite), water, and 100% methanol followed by flame sterilisation, and using four layers of paper towel (absorption barrier) on the cutting board.

For histological examination, two tissue cross sections (about 2 mm in thickness) were cut through each oyster using a scalpel. The first tissue cross section was cut through the anterior portion of the body approximately in the mid region of the labial palps (resulting in mantle, gonad, digestive gland, stomach, intestine and labial palps tissue representation). The second tissue cross section was cut through the posterior portion of the body approximately in the mid region of the adductor muscle (resulting in adductor muscle, kidney, gonad, intestine, mantle and gill tissue representation). For some of the larger oysters it was necessary to downsize the second section to only include the adductor muscle and gills in order to achieve consistent single cassette processing per oyster. Tissue sections were fixed in Davidson's solution and standard 5 µm-thick paraffin embedded sections were stained with haemotoxylin and eosin stain. Each entire section was examined via compound microscope at 100X to 400X magnification (about 10 to 15 minutes for a negative specimen).

For the PCR assay, the entire heart was excised. In addition, a small piece of gonad (3-4 mm^3) was removed from just beneath the dorsal surface of the mantle and immediately adjacent to where the first histology cross-section was cut. If pustules characteristic of nocardiosis were observed, a maximum of two were also sampled. Each tissue sample was preserved separately in 95% ethanol. Preserved tissues were subsampled to about 2 to 3 mm^3 in size for DNA extraction.

Table 4. Comparison in detection of *Nocardia crassostreae* in 130 *Crassostrea gigas* using standard histopathological examination (two sections through mid-body of oyster as described in the text) and the PCR assay for *N. crassostreae* [combined results of separate assays on tissues (2-3 mm^3) from the heart, gonad and up to two pustules (if present) from each oyster].

Histolopathology Rating[1]	Histology results	PCR positive	PCR negative
Category 4	1	1	0
Category 3	18	18	0
Category 2	6	4	2
Category 1	3	1	2
Category 0	102	2	100

[1] Categorised according to Elston *et al*. (1987) with Category 0 indicating that there was no histological evidence of infection and Category 4 for oysters in the terminal phase of nocardiosis.

Histological examination and the PCR assay proved to be about equally sensitive in detecting *N. crassostreae* (Table 4). Both techniques appeared equally reliable in detecting heavy infections [categories 3 and 4 of Elston *et al*. (1987)]. However, detection sensitivity was variable in lighter infections. Because the apparent sample size of oysters with light infections in this validation trial were low (about 8% of the oysters sampled), the comparative sensitivity between the two techniques can not be reliably evaluated. But, preliminary results suggest that histological examination may be comparable in sensitivity to PCR, owing to the ease with which individual *N. crassostreae* colonies can be observed histopathologically at even low magnifications (Table 4). The real advantage of PCR relative to histopathology, in theory, may be in detecting small numbers of *N. crassostreae* cells initially colonizing oyster tissue before the onset of the host response. The two PCR-positive, histopathology-negative oysters (Table 4) could suggest the observation of such early, prepatent infections. Alternatively, these and the four PCR-negative, histopathology-positive oysters could reflect sampling error (i.e., the chance occurrence of the bacterium in the small pieces of tissue examined).

CONCLUSION

The specific geographic distribution of nocardiosis is not known. However, it appears to be widespread and given the historical movements of Pacific oysters around the Pacific Rim, no areas can be assumed to be disease free. Although the mortality rate due to nocardiosis has not been accurately measured, the severity of infection in some oysters and the high prevalence in some oyster populations suggest that it is a significant mortality factor (Elston, 1993).

Oysters with nocardiosis should not be transplanted and oysters from enzootic areas should not be transplanted to areas believed to be free of the disease agent. Eradication is not feasible, because the bacteria probably occur naturally and may be widely distributed in substrate of areas where infected oysters occur. However off-bottom culture systems may reduce the prevalence and severity of the disease. Moving oysters out of warm shallow embayments during the summer months may also reduce the impact of the disease.

Detection of infection should continue to be based on histological examination. Histological sections stained with Gram stain usually make *N. crassostreae* more visible (e.g., Fig. 1 in comparison to Fig. 3). However, the massive haemocyte infiltration response induced by *N. crassostreae*, a key to identifying the bacterium, is not usually as evident in Gram stained tissue sections (depending on the type of counter stain used). The new PCR assay described above does not appear to increase the sensitivity of detecting *N. crassostreae*. However, this assay should prove useful in confirming the identity of Nocardia spp. detected in other organisms from enzootic areas and for confirming the distribution of *N. crassostreae*. This PCR assay could also prove valuable in identifying the life cycle of *N. crassostreae* between oysters and the environment thereby providing a better understanding of the epizootiology of this pathogen.

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