Journal of Invertebrate Pathology 114 (2013) 324–328

Contents lists available at ScienceDirect

Journal of Invertebrate Pathology

journal homepage: www.elsevier.com/locate/jip

Short Communication

Nocardiosis in Mediterranean bivalves: First detection of *Nocardia crassostreae* in a new host *Mytilus galloprovincialis* and in *Ostrea edulis* from the Gulf of Naples (Italy)

Francesca Carella [a,*], Noelia Carrasco [b], Karl B. Andree [b], Beatriz Lacuesta [b], Dolors Furones [b], Gionata De Vico [a]

[a] Department of Biological Sciences, University of Naples Federico II, 80134 Napoli, Italy
[b] IRTA-Sant Carles de la Ràpita and Catalonia's Aquaculture R&D and innovation Reference Network (XRAq), Ctra. Poble Nou Km 5, 43540 St. Carles de la Ràpita, Spain

ARTICLE INFO

Article history:
Received 23 July 2013
Accepted 1 October 2013
Available online 16 October 2013

Keywords:
Nocardia
Ostrea edulis
Mytilus galloprovincialis
Gulf of Naples
Nocardia crassotreae
Aquaculture

ABSTRACT

In this work *M. galloprovincialis* and *O. edulis* specimens were surveyed for a pathological study in the Gulf of Naples (Mediterranean sea, Campania Region, southern Italy). Clusters of *Nocardia* sp.-like cells were observed in histological slides. PCR amplification, sequencing and *in situ* hybridization were carried out in order to corroborate *Nocardia* species identification for both hosts. Blast results showed a 99% of maximum identity with *Nocardia crassostreae* sequences in Genbank. This is the first report of *N. crassostreae* in the new host *M. galloprovincialis* and, in a new area, the Mediterranean Sea.

1. Introduction

The genus *Nocardia* is composed of Gram-positive, partially acid-fast actinomycete bacteria present in both aquatic and terrestrial environments (Goodfellow and Lechevalier, 1986). *Nocardia* spp. are pathogenic for a large variety of animals (Beaman and Sugar, 1983), among which are included mammals, humans (Beaman and Beaman, 1994), fish (Austin and Austin, 1999) and crustaceans (Alderman et al., 1986). In molluscs, nocardiosis associated with Pacific oyster (*Crassostrea gigas*) mortalities, has been reported since the mid-twentieth century, linked with mass mortality episodes in Matsushima Bay, Japan (Takeuchi et al., 1955), and later reported in California, Washington, and British Columbia (Elston, 1993). Furthermore, studies indicate that this bacterium is also pathogenic and lethal to flat oysters (*Ostrea edulis*) (Eglesma et al., 2008; Bower et al., 2005; Lauckner, 1983). Recently, in Netherlands an extensive mortality of Pacific oysters *Crassostrea gigas* occurred. Stressful environmental conditions along with the presence of pathogenic *Vibrio* sp. and *N. crassostreae* suggested a likely aetiology for an oyster disease (Eglesma et al., 2008). From an epidemiological point of view, the knowledge of the susceptibility to *Nocardia* infection of other bivalve species, which could act as potential reservoirs of infection, is limited to hypotheses of *Mytilus edulis* in New Zealand (Diggles et al., 2002), without any further reference. Regarding the Mediterranean basin, the literature lacks baseline data about distribution of *Nocardia* spp. in molluscs, with very few records in marine mammals and fish (Degollada et al., 1996; Elkesh et al., 2012). This hinders any possible strategy of disease prevention in this critical geographic area which is generally associated to mussel farming (FAO, 2007), and where oyster aquaculture is an emergent, rapidly growing economic sector of the shellfish culture industry. In this work we report the first histological and histochemical description, accompanied by genetic analyses (16S rDNA sequencing and *in situ* hybridization [ISH]), of *N. crassostreae* infection in the Mediterranean mussel *Mytilus galloprovincialis* for two consecutive years, June 2011 and July 2012, and in *Ostrea edulis* in 2012, in Gulf of Naples (Mediterranean sea, Campania Region, southern Italy).

2. Materials and methods

2.1. Sampling and histology

In June 2011 and June 2012, 20 mussels (*Mytilus galloprovincialis*) per month for each year (40 animals) were sampled from raft-culture farms at Capo Miseno in the Gulf of Naples (Italy); moreover, in July 2012, 20 mussel specimens were collected from natural

* Corresponding author.
E-mail address: francesca.carella@unina.it (F. Carella).

0022-2011/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.jip.2013.10.001

banks of Naples harbour and five specimens of flat oysters (*Ostrea edulis*) were sampled in a natural bank of Procida island. From each animal, a transverse section, including digestive gland, mantle and gills, were fixed in Davidson's solution and preserved for at least 48 h at room temperature. Subsequently, tissues were embedded in paraffin blocks, sectioned at 5 μm, stained with haematoxylin and eosin (H&E), in addition to Gram and Ziehl-Neelsen staining. Moreover, from each animal, a tissue slice was also stored at −20 °C for eventual further molecular analysis.

2.2. DNA isolation, PCR amplification and sequencing

In order to complete molecular characterization of *Nocardia* sp. present in *M. galloprovincialis* and *O. edulis*, DNA was isolated as reported by Bower et al. (2005) for the enhanced recovery of bacterial DNA of the *Nocardia* genus. PCR amplification was carried out in the samples which had positive results by histology and in specimens which showed similar inflammatory lesions or macroscopic lesions resembling a *Nocardia* infection, but no visible pathogen. Specific primers were designed based on the 16S rDNA gene sequence of *Nocardia* sp. present in GenBank: Forward: NVPF-GCGCAAGTGACGGTACCTGTAG; Reverse: NVP3R-CGTTAGCTACGG-CACGGA. For each PCR reaction, a mixture containing 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of each deoxynucleotide (Fermentas), 0.4 μM of each primer and 1.25 units of Taq DNA Polymerase (Invitrogen) in distilled water was prepared. The amplification reaction was performed with an initial hot start of 95 °C for 4 min followed by 35 cycles of 94 °C for 45 s, annealing at 60 °C for 45 s and elongation at 72 °C for 1 min. A final elongation step at 72 °C for 10 min was performed. The amplicon obtained was visualised on a 2% agarose gel under UV light, and the purified product was directly sequenced bi-directionally on a 310 Automated Sequencer (Applied Biosystems). The identity of the sequences obtained was compared with sequences in the GenBank data base using BLAST.

2.3. Phylogenetic analysis

To assess the genetic affinity of the different 16S sequences obtained, rDNA sequences of *N. crassostreae* and other species of this genus, including outgroups, were downloaded from GenBank to compile into an alignment, and used in an evolutionary reconstruction. Phylogenetic analyses were conducted in MEGA4 (Tamura et al., 2007). The evolutionary history of 33 taxa was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). Bootstrap confidence values were calculated using 1000 replicates (Felsenstein, 1985). Bootstrap values are shown at the nodes (Fig. 2). The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980). All positions containing gaps and missing data were eliminated from the final total dataset.

2.4. In situ hybridisation (ISH)

In order to confirm that the observed pathogen detected by histology corresponded to the *Nocardia* sp., a digoxigenin-labelled probe specific for *Nocardia crassostreae* species was elaborated using the same primers as those from the PCR reaction with the Nocardia-positive mussel DNA. The Dig–ISH was conducted on a subset of 4 of the mussels examined by H&E staining of histological samples. Of these, 3 of them showed visible *Nocardia*-like structures, 1 with comparable inflammatory lesions. The temperature profile was the same as previously described for PCR with the primer pair in this work. The procedure was a modification of the method described by Carrasco et al. (2011). In particular, sections from the paraffin blocks of 7 μm thickness were placed on amino-alkyl silane coated slides (Sigma) and then treated with proteinase K (200 μg/ml) in TE buffer (50 mM Tris, 10 mM EDTA) at 37 °C for 20 min. Slides were then dehydrated by immersion in an ethanol series and air dried. Then they were placed in 150 μl hybridisation buffer (4× SSC, 50% formamide, 1× Denhardt's solution, 250 μg/ml yeast tRNA, dextran sulphate 10%) containing 15 ng of the labelled probe. Sections were placed on a heating block at 95 °C for 5 min, cooled on ice for 1 min and incubated overnight at 42 °C in a humid chamber. Sections were washed twice for 5 min in 2× SSC at room temperature, and once for 10 min in 0.4× SSC at 42 °C. The detection step was performed according to manufacturer's instructions (Dig Nucleic Acid Detection Kit, Boehringer Mannheim). Counter staining was carried out with Bismarck Brown Y.

An uninfected specimen of *M. galloprovincialis* was used as a negative control.

3. Results

In regards to mussel histology, 6/60 animals (10% prevalence) sampled in 2011 and 2012 showed *Nocardia*-like colonies. In these cases, the pathogen appeared as dense clumps of branching, eosinophilic bacteria surrounded by haemocytes located in tissues of the foot, digestive gland and mantle, and resulted as Gram-positive to the specific stain (Fig. 1A and B). Inflammatory lesions were characterized by both haemocytic capsules and nodules, and observed most frequently in the connective tissue surrounding the digestive tract, with scattered multifocal lesions resulting from formation of haemocytic nodules. Nodules were composed of both live and degenerating haemocytes, sometimes associated to necrotic foci. When large amounts of the pathogen were present, bacterial cells were arranged in polymorphic inclusions and became surrounded by typical haemocyte capsules. In some cases, the pathogen was not microscopically visible, but massive infiltration of haemocytes in the gonads, penetrating adipogranular tissue (ADG), was recorded. Regarding *O. edulis*, macroscopically, one specimen showed yellowish pustules on the surface of the mantle tissue. Histological observation revealed strong haemocytic infiltration accompanied by nodule formation and no visible pathogen (Fig. 1C).

As regards to the molecular analysis, the six mussel specimens found to be positive by histology and two more specimens showing comparable inflammatory lesions, along with the *O. edulis* specimen with macroscopic pustules, displayed a 360 bp amplicon when a *Nocardia* sp. 16S rDNA PCR reaction was carried out. The partial 16S rDNA sequences obtained, and analyzed using BLAST showed 99% of maximum identity with the previously published *N. crassotreae* sequences. Both of the new partial sequences reported here have been deposited in the GenBank database under the following accession numbers: **KF017424** (Oyster) and **KF017425** (Mussel). From the phylogenetic analysis of the sequences obtained, a single optimal tree was found which had a sum branch length of 0.30919290. The dendogram showing the evolutionary reconstruction of the included taxa, clearly places the new isolates described here within the *N. crassostreae* clade (Fig. 2).

In *M. galloprovincialis* tissue sections mounted onto slides, the ISH using the *Nocardia* sp. genus-specific probe, showed reaction with the bacterial colonies and yielded an unequivocal high intensity positive result in most of the cases (Fig. 1D).

4. Discussion

The histological and histochemical features described accompanied by the obtained sequences and ISH signal, confirm that *N. crassostreae* is infecting mussel, *M. galloprovincialis* and oyster, *O. edulis* in specimens from the Gulf of Naples. Elston et al.