NOTE

*Marteilia refringens* infection in cultured and natural beds of mussels (*Mytilus galloprovincialis*) along the Campanian coast (Tirrenian sea, South of Italy)

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Abstract

Protozoan parasites belonging to genus *Marteilia* cause the mollusc disease named Marteiliosis. In Italy the parasite has only been rarely observed. The first Italian report was in Venice lagoon in 1980; consequently, has been detected in mussels from different Italian areas, most of the times only designating the genus of the parasite (*Marteilia* sp.), with no confirmation of the species involved. In this study, histological observations and molecular analysis revealed the presence of *Marteilia refringens* ‘M’ type in mussels (*Mytilus galloprovincialis*) with a prevalence of 15% at one natural bed and of 14% and 25% respectively at two of 14 sampled farms. The parasites occurred in epithelial cells of the digestive ducts and tubules with early stages and more advanced phases, usually accompanied by haemocyte infiltration. This is the first report of *Marteilia refringens* along the Campanian coast (Italy).

Italian aquaculture is based on different farming systems, reflecting the high geographical diversity offering a wide choice of locations for extensive and intensive culture. Mussel (*Mytilus galloprovincialis*) production has an important tradition in Italy, reaching 100.000 tonnes annually (t/y), firstly with Apulia (19.700 t/y), followed by Sardinia (9.753 t/y) and Campania (4.100 t/y), respectively. Today, Campanian mussel rearing occupies a significant territory of up to 2.800.000 m² of the area, representing 75% of the total regional aquaculture product (FAO, 2007).

Marteiliosis due to cercozoan *Marteilia refringens* is a notifiable mollusc disease (OIE, 2009) whose management is a central concern for shellfish rearing, recognised as a significant constraint to aquaculture production and trade, affecting both economic development and socio-economic revenue (Berthe et al., 1999). Marteiliosis prevalence, geographic distribution and associated mortality have been reported in different areas of the
Mediterranean basin (Pellumb et al., 2006; Zrncic et al., 2001; Alderman, 1979). However, to date, knowledge about this shellfish disease in Italy is very scarce, and available data on Marteilia sp. distribution in M. galloprovincialis and M. edulis, in most of the cases are limited to the genus only (Ercolini et al., 2004; Tiscar et al., 1993; Ceschia et al., 1990), except for the unique report of M. refringens infection in M. galloprovincialis in Italy recorded from the areas of Trieste, Venice, and La Spezia, respectively (North of Italy) (Balseiro et al., 2007).

In contrast, in other parts of the world parasitic infections of commercially exploited bivalves are well studied and knowledge of their impact is increasing with the developing aquaculture economy (Berthe et al., 2004; Bower et al., 1994; Lauckner, 1983). However, despite information that the parasite has potentially important local socioeconomic impact, records from Campania region have not been reported so far.

In this note, we describe the cytological, histological and molecular findings of a marteliosis due to M. refringens in M. galloprovincialis from mussel farms and from natural bank in the Campania Region (Tirrenian sea, south of Italy).

As a part of a monitoring project to assess animal health status, 14 mussel raft-culture farms and two natural banks of M. galloprovincialis of commercial size (5-6 cm) were seasonally sampled on the Campania littoral from Summer 2008 until Autumn 2009 (Figure 1). In order to limit any histological artifacts, that may result from the sampling and transport process, mussels were kept cool in an insulated sealed container for transit back to the laboratory (Dimitriadis and Koukouzika, 2003).

A total of 30 animals for each site were collected for diagnostic methods as cytological and histological procedures, with a total of 2880 processed animals; infected animals were used for DNA extraction and parasite identification.

After the individuals were removed from the shell, a little part of the digestive gland was used for tissue imprints, air dried and stained with MGG May Grünwald Giemsa quick stain (Bio Optica, Italy). Half of the animals was stored at -20°C and from the remaining animal, a single cut, including mantle, gonad, digestive gland, gills and foot, was taken from the middle of the body, fixed in 10% Formalin, embedded in paraffin wax, sliced (4 μm), stained with haematoxylin and eosin H&E and observed by light microscopy.

In order to attempt parasite species identification, digestive gland of the infected animals was used for genomic DNA extraction phenol/chloroform/isoamylcol performed according to Andres et al. (1994). DNA concentration and purity were estimated by measuring the 260/280 optical density (OD) ratio and DNA quality was verified by electrophoresis on 1% agarose gel stained with ethidium bromide.

The primers LEG1/PRO2 for Marteilia sydneyi (Kleeman and Adlard, 2000) and the primer set recommended by the Office International des Epizooties (OIE) ITS1-4/5 for M. refringens
Figure 1. Italy-Campania region: location of the 14 rearing raft cultures and 2 natural banks along Campanian coast, Italy.

and *M. maurini* were used in a PCR diagnostic method for parasite identification. The PCR was carried out at reaction and thermal cycling parameters recommended by OIE (OIE, 2009). Mussels samples that showed no infection in tissue sections were included as negative controls.

The PCR products were run on 1% agarose gel. One amplification fragment was gel eluted, cloned into pSCA plasmid using the StrataClone™ PCR Cloning Kit (Stratagene) and sequenced using the T3 and T7 universal primers and the BigDye Terminator Cycle Sequencing Kit v. 1.1 (Applied Biosystems). Sequence reactions were run on 310 Automated Sequencer (Applied Biosystems).

Polymorphism in the ITS region amplified by PCR was investigated by RFLP analysis. Eluted amplified products were digested with restriction endonuclease *HhaI* for two hours at 37°C and separated on a 2% agarose gel (Novoa et al., 2005).

In June and July 2009, typical forms of the cercozoan *Martelia* sp. parasite were cytologically visible in the animals of two stations (Naples Centre and Salerno) and one natural bank (Bagnoli) (Figure 1-2).

Prevalence ranged from 15% in the natural beds of Bagnoli to 14% (Salerno) and 25% (Naples Centre) of aquaculture areas. Different life cycle stages of the parasite were observed, by cytology and histology, in the visceral mass during the months of June, July and September (Table 1). In particular, primary cells (nurse
Figure 2. *Martelia refringens* in digestive gland imprint: cluster of mature sporonts containing spores (arrowhead) and refringent bodies appearing blue-grey (arrow) and polinucleated cells (big arrowhead) 150 X.

Figure 3. *Martelia refringens* life cycle stages and defensive response: a. Two nurse cell at the apical border of gut epithelium with low inflammation (arrow); H&E 80X. b-c-d. Mature stages of the parasite containing two secondary cells up to eight secondary cells (arrowheads), and sporangiosorus-like structure in which spore-like structures are developing (arrow); the infection was accompanied by moderate to heavy haemocyte infiltration of digestive gland; H&E 50X, 60X, 40X.
cells), consisting of spherical to elongated cells containing one or two nucleus, were detected in the apical border of gut epithelium with a low haemocyte reaction (Figure 3a); conversely, a variable number of mature stages of the parasite like one or more secondary cells (up to 8 sporonts) and sporangiosorus-like structure with developing spores, were also visible; in that case, the infection was accompanied by moderate to heavy interstitial haemocyte infiltration throughout the digestive gland (Figure 3 b-c-d).

In the infected animals, no PCR product was observed for primer pair LEG1/PRO2 and the negative controls, while a fragment (410 bp in length) was obtained with primer pair ITS1 4/5. BLAST analysis revealed the nucleotide sequence of these fragments (GenBank Accession nos Salerno-AB513427; Bagnoli-AB534169, Naples Centre-AB534170) corresponding to a region of the ribosomal internal transcribed sequence 1 (ITS1) of *M. refringens*, with an identity of 99% between all the sequences, and ranging between 96-100% with data bank, confirming the protozoan parasite was infecting the mussel *M. galloprovincialis* in the studied area.

Restriction analysis after HhaI digestion showed one profile referred as *Martelia* strains ‘M’ for all the infected areas (Le Roux et al., 2001) (Figure 4).

This is the first report of *M. refringens* along the Campanian coast. Previous observations have not reported prevalence levels detected in this study: other described cases of *Martelia* sp. in 2008 show a low value of infection (2-3%) in Veneto, Liguria and

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Table 1. Prevalence (%) of *Martelia refringens* in infected areas and related life cycle stage.

<table>
<thead>
<tr>
<th>Location</th>
<th>June Prevalence (%)</th>
<th>July Prevalence (%)</th>
<th>September Prevalence (%)</th>
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<tbody>
<tr>
<td>Naples</td>
<td>n.r.</td>
<td>n.r.</td>
<td>25 early and advanced phases</td>
</tr>
<tr>
<td>Bagnoli</td>
<td>14 early and advanced phases</td>
<td>13 early and advanced phases</td>
<td>n.r.</td>
</tr>
<tr>
<td>Salerno</td>
<td>13 early and advanced phases</td>
<td>15 early phases</td>
<td>n.r.</td>
</tr>
<tr>
<td>n.r.</td>
<td>Not Recorded</td>
<td>Not Recorded</td>
<td>Not Recorded</td>
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Lazio regions, while higher data (10-15%) in Sardinia (Arcangeli, 2009; pers. comm.), underlining a variability on its occurrence and distribution. In our study, samples collected at the two cultured stations the previous year at the same season (June 2008), didn’t reveal the presence of the parasite, suggest its recent introduction probably due to seed transplantation. In fact, most of the mussel cultivation systems in the world are based on the transplantation of seed from different natural beds or culture sites to rearing areas, where mussels are grown to commercial size (Fuentes et al., 2002). In this context, pathogen transfers through movements of aquatic organisms appear to be an important underlying cause of such epizootics, with the consequent need to establish effective Italian precautionary surveillance based on collection of epidemiological data for controlling disease spread from infected to non infected areas (Berthe et al., 2004). This parasite is considered lethal and its pathogenicity for flat oysters is well documented (Berthe et al., 1998); however, mussel populations of different areas has shown distinct susceptibility to Marteilia related to their genetic origin (Fuentes et al., 2002).

Marteiliosis in other countries has been shown to cause significant production losses at national and international level in many of the susceptible species (OIE, 2006); the direct economic impact of the disease is linked to its morbidity, mortality and effect on product quality, responding to some of the criteria for being listed by OIE, requiring to declare its presence in host species.

The parasite has digestive tropism, physically interfering with feed, absorption and, in heavy infections, with reproduction for the resultant
adipogranular cells development inhibition (Villalba et al., 1993). Defensive processes are mostly represented by focal and massive haemocyte concentration, accompanied by tissue necrosis of the target organ. All these symptoms combined, could eventually lead to death of the host (Berthe et al., 2004).

The above results show the need to increase the collection of epidemiological data about the prevalence of this parasite in the studied region and in the whole Italian coast, applying a sanitary monitoring programme particularly to natural banks as well as mussel farms of *M. galloprovincialis*, in order to prevent its spread to other populations.

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References


