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Four-Year Surveillance of *Marteilia refringens* in Shellfish Farms in the Gulf of La Spezia (Liguria, Italy)

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Abstract

The paramyxean protistan parasites *Marteilia refringens* and *M. maurini*, two *Marteilia* species detected in Europe, infect several bivalve mollusc species of economic interest. Marteiliosis, caused by infection with *M. refringens*, is included in the World Organization for Animal Health (OIE) list of diseases (OIE Aquatic Animal Health Code 2014) and is listed as a nonexotic pathogen under European Union legislation (2006/88/EC). As mandated by Italian Legislative Decree 148/2008, an annual sampling program is in place to monitor the health status of the Mediterranean mussel *Mytilus galloprovincialis* farmed in the Gulf of La Spezia (northwest Italy), test for the presence of the parasite in mussels, and evaluate abnormal mortality as an indicator of disease presence. During the four-year period 2010-2013, 4460 samples of *M. galloprovincialis* were collected from six farming areas and analyzed by cytological methods for the detection of *M. refringens*. Suspected positive diagnosis was confirmed by PCR assay performed according to the Manual of Diagnostic Tests for Aquatic Animals (OIE, 2012). Although infection rates generally decreased between 2010 and 2013, samples from all six areas tested positive for *M. refringens*, with an overall positivity rate of 4.05%.

Keywords: Tissue imprint, cytology, PCR, *Mytilus galloprovincialis*, *Marteilia* spp.

Introduction

The genus *Marteilia*, phylum Paramyxea, groups protistan parasites that affect several species of commercially important bivalve molluscs, including the Australian flat oyster (*Ostrea angasi*), the Chilean oyster (*O. chilensis*), the European flat oyster (*O. edulis*), the Argentinean oyster (*O. puelchana*), and mussels (*Mytilus edulis* and *M. galloprovincialis*). Two molecular types or species of *Marteilia* have been identified in Europe: *M. refringens* (type O) and *M. maurini* (type M), infecting oysters and mussels, respectively (OIE, 2012, Le Roux *et al*., 2001; López-Flores *et al*., 2008). Whether they constitute two different strains of *M. refringens* remains debated. Moreover, recent data suggest the existence of a new molecular type of *M. refringens* (type C) infecting the common edible cockle *Cerastoderma edule* (Carrasco *et al*., 2012b).

M. refringens has been the agent responsible for recurrent mass mortalities of flat oysters in Europe over the last four decades. Recognizing *M. refringens* as a significant pathogen of bivalve molluscs (OIE, 2014), the World Organization for Animal Health (OIE) has issued recommendations on shellfish movement between countries, assumptions for declaring zones free from infection, and operating procedures to diagnose marteiliosis. Sampling should be carried out within one month after the coastal marine water temperature has reached 17°C, the temperature necessary for infection with *M. refringens* to develop.

M. refringens is listed as a nonexotic pathogen under European Union legislation (2006/88/EC). As mandated by Italian law (Legislative Decree 148/2008), annual sampling programs are in place to detect the presence of the parasite and to limit its spread as much as possible.

Although infection with *M. refringens* generally causes less damage in *Mytilus* spp., the mortality rate may be as high as 40% in areas where the parasite is found. Detection of infection in molluscs is performed using screening techniques (tissue imprints and histopathology) followed by confirmatory techniques (PCR, *in situ* hybridization, transmission electron microscopy, and genome sequencing). On macroscopic examination, infected animals show emaciation, pale digestive gland, stunted growth,

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tissue necrosis and death. In some individuals, however, the parasite can be present without causing disease. The body appears contracted and emaciated, and digestive gland tissues are translucent. Because the macroscopic lesions are identical to those observed in haplosporidiosis, diagnosis is confirmed with histological or cytological examination.

The two most commonly farmed mussel species in Europe are the blue or common mussel (*Mytilus edulis*) in the north and the Mediterranean or Spanish mussel (*M. galloprovincialis*) in the south. In Italy, shellfish farming makes up the bulk of aquaculture production, based largely on mussels (*M. galloprovincialis*) and the Manila clam (*Tapes philippinarum*) followed by limited amounts of clams (*Tapes decussatus*) and oysters (*Crassostrea gigas* and *Ostrea edulis*). The main production area in northwest Italy is the Gulf of La Spezia, where mussels are farmed by small operators granted aquaculture site leases in coastal marine areas divided into 6 zones (Figure 1). Cultivation is by the pile or raft technique. The estimated annual production is 6,000 tons. Here we report the results of four-year surveillance of marteiliosis caused by *Marteilia refringens* in *M. galloprovincialis* from the Gulf of La Spezia.

Materials and Methods

During the four-year period from 2010 to 2013, samples were collected from all 6 mussel farming zones in the Gulf of La Spezia during the summer months when the coastal seawater temperature rises above the critical value of 17°C. The number of individuals collected per year was 860 in 2010, 900 in 2011, 900 in 2012, and 1800 in 2013. Approximately 150 samples from each of the 6 areas were examined for the presence of *Marteilia* spp. within 24 hours of collection. Detection of infection with *Martelia* spp. was performed by macroscopic examination of gross lesions followed by cytological analysis. Tissue imprints were made from a piece of digestive gland. After drying on blotting paper, sections of the target organ were put on glass slides. The slides were air-dried, fixed in absolute ethanol, and stained (Hemacolor® Kit, Merck) according to the manufacturer's instructions, then coverslipped and examined at 1000x in oil immersion using a Nikon Optiphot-2 microscope. Samples testing positive for *Marteilia* spp. underwent confirmatory PCR assay, as described in the Manual of Diagnostic Tests for Aquatic Animals for the diagnosis of marteiliosis in molluscs (OIE, 2012). The prevalence of *M. refringens* by farm and year of sampling was calculated with a 95% confidence interval.

Results

None of the samples showed gross lesions specific for marteiliosis. Microscopic examination of

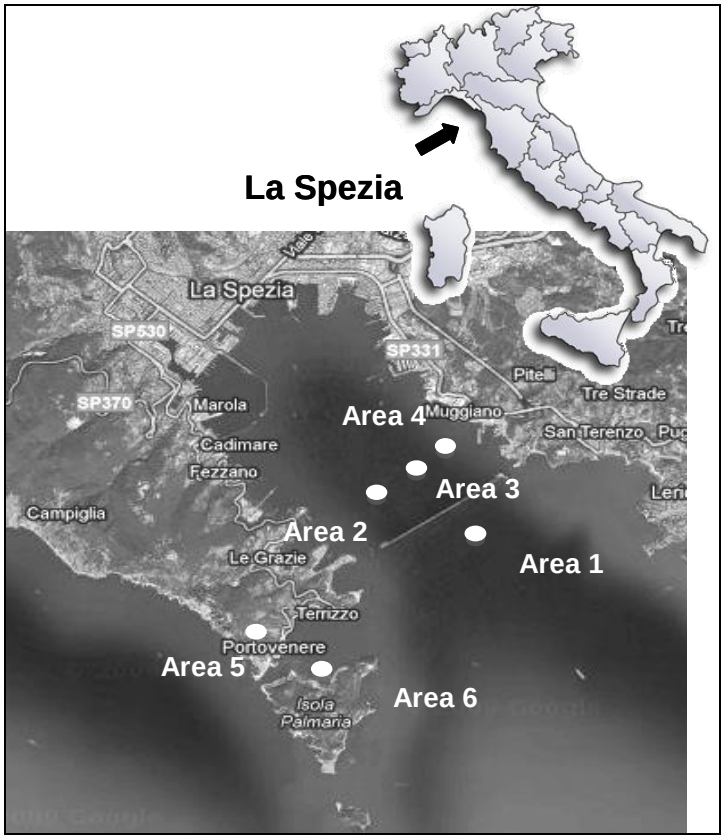

Figure 1. Mussel farming areas in the Gulf of La Spezia (Liguria, northwest Italy).

the digestive gland imprints revealed various life-cycle stages, including daughter cells, immature and mature sporonts. Different developmental stages of the parasite were observed in the stained imprints from samples collected over the four years of surveillance (total prevalence of 4.05%). The percentage by year of the presence of protozoan cells in the samples from all six farms was 9.07% in 2010, 6.9% in 2011, 2.2% in 2012, and 1.17% in 2013. Within farms the higher prevalence was recorded in farm 3 while the lower in farm 5 (Table 1). Although the samples from all farms tested positive for *M. refringens* infection, the percentage of positive samples decreased between 2010 and 2013. The same trend was observed within each farm (Table 1). No mortality of *M. galloprovincialis* was observed. PCR confirmed the presence of the genome of *M. refringens*.

Discussion

Infection with *M. refringens* in molluscs has been reported in northern Europe and along the Spanish, Tunisian, Greek, French, Italian and Croatian coasts of the Mediterranean basin (Elgharsalli *et al*., 2012; Carrasco *et al*., 2012a; Rayyan *et al*., 2006; Audemard *et al*., 2004; Zrnčić *et al*., 2001; Villalba *et al*., 1993). In the present study, the prevalence of infection with *M. refringens* in mussels was similar to that reported in a previous study on cultured *M. galloprovincialis* from Spain (Fuentes *et al*., 1995) but lower than that reported for the Thermaikos Gulf in northern Greece (Virvilis *et al*., 2003). A study using *in situ* hybridization and histological methods reported a prevalence of 5% in Croatia, fairly similar to the rate we estimated for the Gulf of La Spezia, although no cases of mortality occurred (Zrnčić *et al*., 2001) (Figure 2).

In Italy, data on the presence/absence of *M. refringens* in farmed mussels are scant. As compared to other Italian regions (1.72% in Friuli-Venezia Giulia, 0.03% in Veneto, 0% in Emilia Romagna; data base 2002), the total percentage estimated from the present surveillance in the Gulf of La Spezia was higher (data base 2010-2013). The presence of *Marteilia* spp. (0% to around 1%) in mussel farms located in the northern Adriatic is far lower than that reported for southern Italy (10-20%; data base 2009).

According to surveillance carried out in the Gulf of La Spezia between 2002 and 2003, the prevalence was 4.61%, or slightly higher than the present rate of 4.05%, but markedly lower than the rates reported for 2010 and 2011 (9.07% and 6.9%, respectively). This increase could be explained by the recent rise in coastal marine water temperature, which may have created weather conditions conducive for survival of

Table 1. Prevalence of *Marteilia refringens* infection by farm and year of sampling

Farm	Year of sampling	Total samples	Positive samples	Prevalence%	Estimated prevalence (95% CI)
	2010	130	16	12.3	17.9-6.7
	2011	150	8	5.3	8.9-1.7
1	2012	150	7	4.7	8.1-1.3
	2013	300	4	1.3	2.6-0.02
	Total	730	35	4.8	6.4-3.2
	2010	130	11	8.5	13.3-3.7
	2011	150	9	6.0	9.8-2.2
2	2012	150	3	2.0	4.2-(-0.2)
	2013	300	7	2.3	4.0-0.6
	Total	730	30	4.1	5.5-2.7
	2010	150	26	17.3	23.4-11.2
	2011	150	15	10.0	14.8-5.2
3	2012	150	3	2.0	4.2-(-0.2)
	2013	300	4	1.3	2.6-0.0
	Total	750	48	6.4	8.2-4.6
	2010	150	13	8.7	13.2-4.2
	2011	150	18	12	17.2-6.8
4	2012	150	3	2.0	4.2-(-0.2)
	2013	300	3	1.0	2.1-(-0.1)
	Total	750	37	4.9	6.4-3.4
	2010	150	4	2.7	5.3-0.1
	2011	150	3	2.0	4.2-(-0.2)
5	2012	150	2	1.3	3.1-(-0.5)
	2013	300	1	0.3	0.9-(-0.3)
	Total	750	10	1.3	2.1-0.5
	2010	150	8	5.3	8.9-1.7
	2011	150	9	6.0	9.8-2.2
6	2012	150	2	1.3	3.1-(-0.5)
	2013	300	2	0.6	1.5-(-0.3)
	Total	750	21	2.8	4.0-1.6

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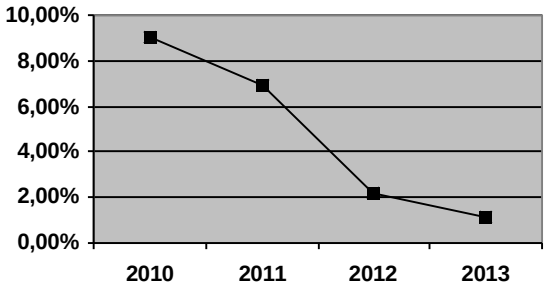

Figure 2. Percentage of the presence of protozoal cells.

the parasite, and by the introduction in local farms of seeds from other areas endemic for marteiliosis. Similarly, the decrease noted for 2012 and 2013 may probably reflect less conducive weather conditions in those years and an unusual drop in local coastal marine water temperatures. Despite the relatively high presence of *Marteilia* spp. recorded for the four years of surveillance, no mass mortality occurred.

The significance of *M. refringens* needs to be further investigated, particularly in relation to rising water temperature consequent to global warming and environmental stressors, such as elevated water temperature over extended periods of time or poor water quality, and severe parasitic infestation which could threaten mollusc viability, leading to mass mortality as occurred in August 2003. This study provides much needed long-term data on surveillance of infection with *M. refringens* in *M. galloprovincialis*, which inevitably depict more realistic dynamics that might pass unobserved in a one-year study. Because marteiliosis due to infection with *M. refringens* can cause serious recurrent mortalities in culture areas, monitoring for the disease by annual sampling programs can help to prevent economic damage to the local aquaculture industry.

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