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Application of a competitive real time PCR for detection of *Marteilia refringens* genotype "O" and "M" in two geographical locations: The Ebro Delta, Spain and the Rhine-Meuse Delta, the Netherlands

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ABSTRACT

Species belonging to the genus *Marteilia* are protozoan parasites of bivalves. The species *Marteilia refringens*, jeopardizing the health of European bivalves, is included on the list of OIE notifiable pathogens. Two genotypes of *Marteilia refringens* are distinguished: type "O" affecting mainly oysters, and type "M" affecting mainly mussels. Historically, detection of *Marteilia* species is primarily carried out by histology. In recent years molecular assays are more frequently used for the detection of mollusc pathogens, also in routine monitoring. In the present work, a competitive real-time PCR assay was developed for rapid and sensitive detection of *M. refringens* and discrimination between "M" and "O" genotypes of *M. refringens*. The real-time PCR assay was shown to be analytically sensitive and specific and has a high repeatability and efficiency. Subsequent application of the assay on collected bivalves from two geographical locations, the Ebro Delta in Mediterranean Spain and the Rhine-Meuse Delta in the Netherlands resulted in detection of *M. refringens* type M in *Mytilus galloprovincialis* and *M. refringens* type O in *Ostrea edulis* from Spain. In two *O. edulis* specimen both *M. refringens* type O and type M were detected. In the Netherlands *M. refringens* was not observed in any of the tested *Mytilus edulis* and *O. edulis*. The results obtained by real time PCR were in correspondence with the results obtained by histopathology and a substantial agreement with the results obtained by conventional PCR.

In conclusion, the developed real time PCR assay facilitates rapid detection and subtyping of *M. refringens* and could be applied for further studies on epidemiology of the parasite, geographical distribution and host specificity.

1. Introduction

Marteilia refringens is a protozoan parasite belonging to the Order Paramixyda and Phylum Cercozoan (Cavalier-Smith and Chao, 2003a, 2003b; Chatton, 1911; Grizel et al., 1974) with the potency to negatively affect the health of bivalves species such as the European flat oyster *Ostrea edulis*, and the mussel species *Mytilus galloprovincialis* and *Mytilus edulis*. Because of the detrimental effects associated with the presence of the parasite, *M. refringens* is listed as a notifiable pathogen by the World Animal Health Organization (OIE) and the European Union (directive 2006/88/EC). Two types of *M. refringens* have been described based on differences in the ITS-1 region: type "O" affecting mainly oysters, and type "M" affecting mainly mussels (Le Roux et al., 2001). However, the genotypes have been reported to have no strict host specificity (Lopez-Flores et al., 2004; Novoa et al., 2005). Furthermore, other bivalves with economical interest such as the striped venus clam *Chamelea gallina* and the razor clam *Solen marginatus*, have been reported to be infected with *M. refringens* (Lopez-Flores et al., 2008a, 2008b). Recently, *Marteilia cochillia*, a closely related species to *M. refringens* and in literature also referred to *Marteilia* sp. type C, has been described in cockles *Cerastoderma edule* from European waters (Carrasco et al., 2013).

Since the first observation of *M. refringens* in 1969 in flat oysters from French Brittany (Grizel et al., 1974; Herrbach, 1971) detection of the parasite has been primarily carried out through histological and cytological observation. In order to improve rapid and specific detection of *M. refringens*, molecular tools have been developed more recently, among which a number of conventional PCR assays targeting 18S rRNA, ITS-1 and IGS regions (Le Roux et al., 1999, 2001; Lopez-Flores et al., 2004). Subtyping of *M. refringens* is also based on detection

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Table 1
Primer and probe sequences for real-time PCR and conventional PCR detection of *Marteilia* sp. used in the present study.

	Primer/Probes	5′-Sequence-3′	Amplicon size	Reference
qPCR	Mare.F01	YCAGGCGAGTGCTCTCGTT	70 bp	*Present paper*
	Mare.R01	TGATCTGATATTATTCAGCTGTTCGA		
	MareO.p01	FAM CCTTTCCCCGACGGC MGB NFQ	–	
	MareM.p01	VIC GCTTGCCCTACGGCC MGB NFQ	–	
IGS PCR	MT-1	GCCAAAGACACGCCTCTAC	425 bp	Lopez-Flores et al. (2004)
	MT-2	AGCCTTGATCACACGCTTT		
	MT-1B	CGCCACTACGACCGTAGCCT	358 bp	
	MT-2B	CGATCGAGTAAGTGCATGCA		

by conventional PCR (ITS-1 or IGS target) followed by PCR-RFLP or sequencing (Le Roux et al., 2001; Lopez-Flores et al., 2004). The conventional PCRs ITS-1 and IGS show also amplification of *M. cochillia*. Moreover, PCR-RFLP of ITS-1 using *HhaI* gives identical results between *M. cochillia* and *M. refringens* type M (Carrasco et al., 2012). A IGS PCR (Lopez-Flores et al., 2004) followed by the RFLP with *Bgl II* (Carrasco et al., 2012) allows the discrimination of *M. cochillia* from *M. refringens*. Recently, a specific PCR assay for *M. cochillia* detection has been developed by Villalba et al. (2014). Currently, real-time PCR assays gain interest over conventional PCR assays due to their improved sensitivity, reproducibility and reduced risk for carry-over contamination. Similar to other fields of research, the use of real-time PCR assays for detection is increasing in the field of shellfish pathology. For example, real-time PCR assays have been developed for detection of *Bonamia* species (Corbeil et al., 2006; Marty et al., 2006; Robert et al., 2009) and *Perkinsus marinus* (Audemard et al., 2004; De Faveri et al., 2009). Development of this technique for different pathogens support studies on epidemiology, host specificity and geographical distribution of the respective pathogen, and ultimately could help to improve health management of the disease. The present manuscript describes the development, evaluation and field application of a competitive Taqman real-time PCR assay based on the ITS-1 region of *M. refringens* for rapid detection and discrimination of *M. refringens* types “O” and “M”.

2. Material and methods

2.1. Field collection oysters and mussels

Bivalve specimens were collected from two geographical regions: the Mediterranean coast of Spain (a known area with *M. refringens*), and the Rhine-Meuse Delta in the southwest of the Netherlands (an area with no observation of *M. refringens* over the last decades). After collection, the soft tissue of the bivalves was prepared for histology as described below and tissue of the same individuals was stored in 96% ethanol or frozen at −20 °C for molecular analyses.

In Spain a total of 154 collected mussels and oysters were used for this study: 72 mussel specimens *M. galloprovincialis* collected between years 2005 and 2010 in Alfacs bay and Les Cases d́Alcanar in the Ebro Delta (Catalonia, Spain) and 82 samples specimens of European flat oysters *O. edulis* collected between years 2003 and 2011 in Alfacs bay in the Ebro Delta and Santa Pola Bay, (Alicante, Spain) were analyzed. Furthermore, five cockles positive for *Marteilia cochillia* type “C”, collected also in Alfacs Bay (Carrasco et al., 2012), were used in the present study.

In the Netherlands European flat oysters *O. edulis* and mussels *M. edulis* were collected as part of the annual monitoring program for shellfish diseases. For this study 50 flat oysters from Lake Grevelingen and 50 mussels from the Yerseke Bank area, Oosterschelde were collected in September-October over the years 2011, 2012 and 2013.

2.2. Histopathology

A transverse section (approximately 5 mm wide) through the body of the oyster was fixed in Davidsons solution for 24–48 h. The fixative was subsequently replaced by 70% ethanol and the tissues were processed using routine histological techniques. Deparaffinised 2–3 µm thick tissue sections stained with haematoxylin and eosin (H & E) were examined under a bright field microscope to check for the presence of *M. refringens*. Infection intensity level was evaluated qualitatively (+ + + heavy; + + moderate; + light) in positive histological slides. Furthermore in a subsample of qualitative infection intensity in 14 individuals was compared and correlated with levels (level 0–5) of infection intensity described by Villalba et al. (1993).

2.3. DNA extractions

DNA was extracted from ethanol fixed of frozen tissue using the QIAamp DNA Mini-kit (Qiagen) according to manufacturer’s instructions. DNA was eluted in 100 µl Elution Buffer supplied by Qiagen and stored at −20 °C until further processing.

2.4. Conventional PCR

A conventional nested-PCR was performed according to Lopez-Flores et al. (2004) amplify the IGS fragment using the two primer pairs; MT-1/MT-2 and MT-1B/MT-2B (see Table 1 for primer sequences). This essay detects *M. refringens* type “O” and type “M”, as well as *M. cochillia*.

2.5. Primer design

Real-time PCR primers Mare.F01 (5′-YCAGGCGAGTGCTCTCGTT-3′), Mare.R01 (5′-TGATCTGATATTATTCAGCTGTTCGA-3′) were designed with the Primer Express version 3.0 software program (Applied Biosystems) based on the Intergenic spacer region-1 (ITS-1) of 235 sequences of *M. refringens* available in GenBank (accession date 30 March 2010) and the sequences extracted from the manuscript of Le Roux et al. (2001). The sequences were aligned using ClustalX 2.1 (Larkin et al., 2007). Specified MGB probes (AppliedBiosystems) MareO.p01 (6-FAM 5′-CCTTTCCCCGACGGC-3′ MGB NFQ) and MareM.p01 (VIC 5′-GCTTGCCCTACGGCC-3′ MGB NFQ) were designed with Primer express for differential detection of *Marteilia* type “O” and *Marteilia* type “M” respectively (Fig. 1).

2.6. Construction of plasmids

In order to artificially test efficiency and competition between the probes, oligo nucleotide sequences containing the target sequences of the real time PCR for type O (5′-TCAGGCGAGTGCTCTCGTTGCCC TTTCCCCGACGGCCGTGCTGCGTCGAACAGCTGAATAATATCAGATCA-3′) and type M (5′-CCAGGCGAGTGCTCTCGTTGCGCTTGC CCTACGGCCGTGCTGCGTCGAACAGCTGAATAATATCAGATCA-3′) were ordered from Eurogentec. The sequences were amplified by conventional PCR using the primers Mare.F01 and Mare.R01 under the following cycling conditions 94 °C for 10 min, followed by 40 cycles of 94 °C for 60 s, 55 °C for 60 s, and 72 °C for 60 s, with a final elongation