

Molecular methods for the diagnosis of *Marteilia refringens*

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Abstract

A nested polymerase chain reaction (PCR) has been developed for the diagnosis of *Marteilia refringens* in mussels (*Mytilus galloprovincialis*) from Galicia (NW Spain). Specific primers were used in two consecutive amplification reactions, that were able to detect 15 fg of *Marteilia* DNA. The nested PCR yielded a 550 bp fragment that was used to generate a non-radioactive probe, specific for *Marteilia*, as determined by in situ hybridization. This probe was also used in a dot-blot developed for the diagnosis of the parasite. This method alone was much less sensitive than the nested PCR, however, its use after the nested PCR significantly increased the sensitivity of the PCR. When comparing these molecular diagnosis tools with traditional diagnosis methods (smears and histology), we determined that the nested PCR could detect *Marteilia* in 65% of the mussels screened, while only 20-25% of the mussels were identified as infected by traditional methods.

Introduction

Marteilia refringens (Grizel *et al.*, 1974), is the etiological agent of "Aber Disease". This paramyxean parasite is one of the most significant pathogens of European flat oyster, *Ostrea edulis* (Grizel *et al.*, 1974; Alderman, 1979; Grizel, 1985; Figueras and Montes, 1988; Robert *et al.*, 1991) and has also been detected in the mussels *Mytilus edulis* (Tigé and Rabuin, 1976) and *Mytilus galloprovincialis* (Villalba *et al.*, 1993).

The taxonomy of the genus *Marteilia* is unclear. The diagnosis of the two species of *Marteilia* found in Europe (*Marteilia refringens* and *Marteilia maurini*) was performed using ultrastructural characteristics and host specificity (Grizel *et al.* 1974, Comps *et al.*, 1982, Figueras and Montes, 1988). However, host specificity was disproved when *Marteilia refringens* was found in *Mytilus*

galloprovincialis (Figueras and Robledo, 1993; Villalba *et al.*, 1993). There is also some doubt concerning the taxonomic status of *Marteilia maurini*, and some authors have postulated that is really *Marteilia refringens*.

Marteilia refringens has caused serious mortalities in flat oysters cultured in Europe but no mortalities have been detected in parasitized mussels. Therefore it is important to establish whether there are one or several species of *Marteilia* in Europe, using a method that allows the discrimination of *Marteilia* species and/or strains.

In spite of numerous papers published on the subject, the transmission route of *Marteilia refringens*, and subsequently its life cycle, also remain unresolved. The existence of an indirect life cycle for *Marteilia* sp. was postulated by many authors (Balouet *et al.*, 1979; Van

Banning, 1979; Grizel, 1985; Lester, 1986; Perkins, 1988). Recent studies give experimental support to the existence of an intermediate host or free-living stages in the life cycle of *M. refringens* (Berthe *et al.*, 1998). However, the search for intermediate stages of the parasite remains a difficult task. It appears that prior to such a study, there is a need for both a *Marteilia refringens* infection model and a sensitive tool to screen for the presence of the parasite in potential intermediate or alternative hosts.

The currently used diagnostic methods are based on cytological or histological examinations. Balouet *et al.* (1979) searched for possible *M. refringens* stages in animals collected in areas in which the parasite was enzootic. However, histological examinations of numerous invertebrates and ultrastructural investigations of plankton samples failed to evidence any *M. refringens* stage. Because they are time consuming, due to sample preparation, these techniques appear less suitable for large-scale applications as those needed in life cycle investigations.

Molecular diagnosis of *M. refringens* could prove to be more useful in the elucidation of its life cycle. Monoclonal antibodies were prepared and are known to recognize *M. refringens* (Robledo *et al.*, 1994). Previous studies highlighted the limitation of this approach, mainly due to the specificity of parasite stages (Roubal *et al.*, 1989; Anderson *et al.*, 1994; Robledo *et al.*, 1994). Recent advances in the use of DNA probes and related assays could provide powerful detection tools. Anderson *et al.* (1995) set up a polymerase chain reaction (PCR) assay to detect *Marteilia sydneyi*

from infected oysters, *Saccostrea commercialis*. Unfortunately the published primers seem to be highly specific for *M. sydneyi* and cannot be used for the detection of *M. refringens*. Recently, a PCR method was developed for the diagnosis of *M. refringens* in *Ostrea edulis* and *Mytilus edulis* using specific primers (Le Roux *et al.* 1999).

The goal of this study was to develop a new DNA-based diagnosis assay that would help to elucidate in the future the *M. refringens* life-cycle and to clarify the taxonomy of the European *Marteilia* species by comparison of DNA sequences. A comparison between traditional detection methods and these DNA-based diagnosis methods was also performed.

Materials and Methods

Marteilia cells purification

Marteilia refringens cells were obtained from naturally infected European flat oysters (*Ostrea edulis*) and mussels, *Mytilus galloprovincialis*. Purification of the parasite was carried out following the protocol described by Robledo *et al.* (1995) with minor modifications. Digestive glands were homogenized in sterile sea water with 1% Tween 80 (SSWT) using an ultraturax (Janke & Kunkel, G.M.B.H.) The homogenates were filtered through 250 and 75 µm filters and the resulting filtrate centrifuged at 3000 rpm, for 30 minutes at 8°C. The pellet was then resuspended in 5 ml SSWT, placed on 5 / 35% sucrose gradients and centrifuged (3000 rpm, 30 min, 8°C). The pellet fraction containing *Marteilia refringens* sporangia was collected, resuspended in SSWT and placed on a 10/ 20/ 30/ 40% Percoll (NaCl 0.4M) gradients to be centrifuged (1800 rpm, 30 min, 8°C). The 10/

20% fraction was then collected, washed and pelleted before DNA extraction.

DNA extraction

Genomic DNA was extracted from purified *Marteilia* sp. sporangia purified from *Mytilus galloprovincialis*. DNA extracted from non-infected flat oyster (*Ostrea edulis*) and mussel (*Mytilus galloprovincialis*) were included as negative controls. For DNA extraction from tissues, the whole animal was ground. Cells or tissues were suspended in 500 µl of extraction buffer (100mM NaCl, 25 mM EDTA, 0.5% SDS, pH=8) with 10µl of proteinase K (10 mg / ml) to make a final concentration of 0.2 mg / ml of proteinase K. After an overnight incubation at 37°C, DNA was extracted using a standard protocol involving phenol/chloroform extraction and precipitation with 2.5 volumes of ethanol and 0.1 volumes of 3M ammonium acetate. After treatment with RNase A, quality and quantity of genomic DNA was evaluated by electrophoresis in a 0.8% agarose gel.

Nested PCR

A nested PCR was developed using primers previously designed by Lubat (1990) by random sequencing of *Marteilia refringens* DNA. The primers used for the first round of PCR were B1 (forward) (5'-CTAACGTCGCTTACCCATGC-3') and B2 (reverse) (5'-CCACCGAACATCTTTCCTGGTTCG-3'). For the second round of PCR B3 (forward) (5'-CCCATGCAATAGAAAACACCCTGCTC-3') and B4 (reverse) (5'-CGTCTGTGCGAGGCCACTCCATGAC-3') were used. Amplification was performed in 50 µl volume using 1 µl dNTP (final concen-

tration 0.2 mM), 2 units Taq polymerase (Pharmacia), 5 µl Taq buffer containing MgCl₂ and 3 µl bovine serum albumin (BSA, 1.5 mg / ml) to make a final concentration of 0.09 mg / ml. All primers were used at 10 pM / ml. The PCR was conducted with denaturation at 94°C for 3 min and amplification performed with 30 cycles of 94°C for 60s, 55°C for 60s, and 72°C for 60s, followed by extension at 72°C for 7 min. After amplification, products were separated in a 0.8% agarose gel and visualized by staining with ethidium bromide.

Probe construction

A probe was constructed using the PCR product obtained by amplification of *Marteilia* DNA obtained from infected mussels (*Mytilus galloprovincialis*) from Galicia using the nested PCR described above. The PCR product was visualized on an agarose gel, removed from the gel with a scalpel and transferred to an eppendorf with distilled water. After homogenization, the sample was kept overnight at 4°C, and then centrifugated (14000 x g for 10 min). The supernatant was removed and precipitated with 2.5 volumes of absolute ethanol and 0.1 volumes of 3M ammonium acetate. The purified PCR product was then labelled with a random priming fluorescein labelling kit from Amersham.

In situ hybridization

In situ hybridization was performed with purified *Marteilia* cells obtained from parasitized *Mytilus galloprovincialis* to test the specificity of the probe. One hundred µl of purified *Marteilia* cells (2 x 10⁸ cells / ml) were incubated for 1h at 60°C in a slide, and then fixed with 4% paraformaldehyde for 20 min. The slides were then washed twice with phos-

phate buffered saline (PBS) and dehydrated with increasing concentrations of ethanol. Once dehydrated, the slides were incubated in prehybridization solution (SSCx2, 50% formamide, 5% dextran sulfate and 0.2% skim milk) for 30 min at 42°C. For the hybridization, 10 µl of the probe (1 µg / ml) were then added to the slides. A denaturation step was performed for 10 min at 95°C and the slides were then incubated overnight at 42°C. All the hybridization steps were performed in a PCR in situ apparatus (Perkin Elmer). The removal of the non-hybridized probe was made with washes in decreasing solutions of SSC at 42°C (30 min washes with SSCx1 and SSCx0.1). The slides were stained with Evans blue (10% in PBS) for 10 min at room temperature, mounted and visualized under a microscope equipped with epifluorescence.

Dot-Blot

For the dot-blot assay, extracted DNA or PCR products were denatured, first with 10 min at 94°C, followed by chemical denaturation with NaOH 1M for 2 min at room temperature. The samples were then treated with neutralizing solution (1.5M NaCl, sodium citrate 0.15M, Tris-HCl 0.25M, HCl 0.25M, pH 8) and kept in ice until used. The samples were applied in a SSC x 6 previously wetted nylon membrane (Amersham) using a Multiwell Biodot system and fixed for 2 h at 80°C. The Dot blot was then developed using the ECL^R kit (Amersham) following the manufacturer's instructions.

Comparison of traditional and molecular biology methods

Twenty mussels (*Mytilus galloprovincialis*) obtained from Galicia (NW Spain) were used for

the comparison of the different diagnosis methods. The digestive glands of these animals were used for the production of smears, fixed and included for histology and used for total DNA extraction following the protocol described above. The smears on slides were fixed with absolute ethanol and stained with Hemacolor (Merck). For the histology, the digestive gland was fixed in Davidson for 24 h. The samples were then included in paraffine and once the blocks were solidified, thin sections (5 µm) were obtained using a microtome (Reichert-Jung). The sections were stained with hematoxyline-eosin, mounted and observed under the microscope (Nikon).

Results

Nested PCR

Marteilia DNA purified from infected oysters (*Ostrea edulis*) or mussels (*Mytilus galloprovincialis*) was amplified with the nested PCR developed. The first round of PCR (primers B1 and B2) yielded a 650 bp fragment, while the second PCR yielded a 550 bp fragment. When DNA from non-infected host (*O. edulis* or *M. galloprovincialis*) was used, both PCR rounds were always negative. The sensitivity of the method was assayed using dilutions of the extracted Marteilia DNA. The first PCR was capable of detecting *Marteilia* up to the 1: 10 dilution that corresponded to 150 ng DNA. When the nested PCR was performed, the product was observed up to the 1: 10000 dilution that corresponded to 150 fg of DNA (Fig. 1). The sequence amplified with the nested PCR was submitted to the Gene Bank (Accession number 407819).

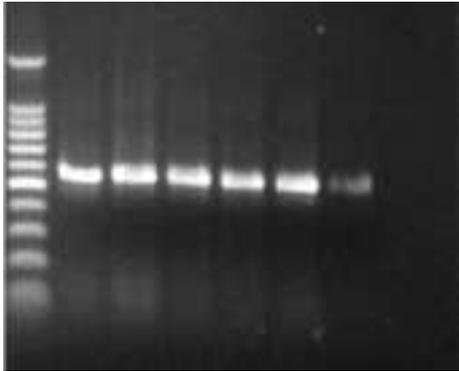


Figure 1. Sensitivity of the *Marteilia* nested PCR. Lane 1 corresponds to the molecular weight marker and lanes 2 to 8 to 1.5 µg, 150 ng, 15 ng, 3 ng, 1.5 ng, 150 fg and 15 fg of *Marteilia* DNA respectively.

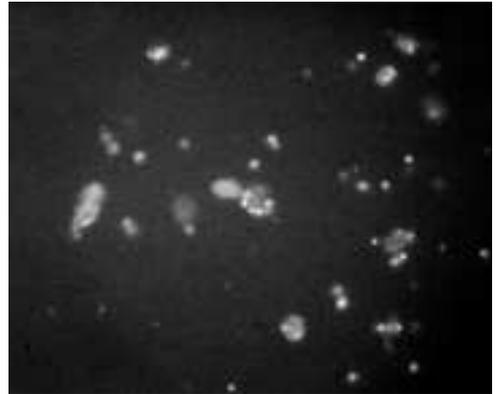


Figure 2. Specific fluorescence of purified *Marteilia* cells obtained by in situ hybridization with the specific probe.

In situ hybridization

The specificity of the nested PCR was confirmed with in situ hybridization using the PCR-generated fragment as a probe. When the probe was used for in situ hybridization techniques with purified *Marteilia* cells an intense reaction was observed (Fig. 2). The specific fluorescence was observed in the nucleus of *Marteilia* cells and never in mussel cells. No fluorescence was observed on the *Marteilia* cells not treated with the probe.

Dot-Blot

The PCR fragment obtained after amplification with the primers B3 and B4 was also used as a probe for Dot-Blot assays. Only DNA from *Marteilia*, and never DNA from the host, gave a positive result. The sensitivity of the

reaction was assayed with the same DNA as the PCR sensitivity assay. In this case, the detection limit was between 75 and 7.5 µg / ml DNA.

However, when dilutions of the PCR products obtained with the first round PCR and the nested PCR were applied on the membrane, the detection limit decreased to 3 ng / ml and 15 fg / ml, respectively (Table 1).

Comparison of traditional and molecular biology diagnosis methods

The results obtained when the traditional techniques (histology and smears) were compared to molecular biology methods (PCR and Dot-Blot performed with extracted DNA) are shown in Table 2. When a total of 20 mussels were screened for *Marteilia*, 25% and 20% of

Amount of DNA	1.5µg	150ng	15ng	3ng	1.5ng	150fg	15fg
Martelia DNA	-	-	-	-	-	-	-
First round PCR	+	+	+	+	-	-	-
Nested PCR	+	+	+	+	+	+	+

Table 1. Results obtained with Dot-blot technique applied to different dilutions of *Marteilia* DNA, or dilutions of the PCR products after one or two round of amplification. The first round of PCR was conducted with primers B1 and B2, while for the nested PCR, a second round of amplification with primers B3 and B4 was performed.

Sample	Smears	Histology	Dot Blot	PCR
1	+	+	+	+
2	+	-	-	+
3	-	-	-	+
4	-	-	-	+
5	+	+	+	+
6	-	-	-	+
7	-	-	-	-
8	-	-	-	-
9	+	+	-	+
10	?	+	+	+
11	?	-	-	-
12	-	-	-	-
13	-	-	-	+
14	-	-	-	-
15	-	-	-	-
16	-	-	-	+
17	+	-	-	+
18	-	-	-	+
19	-	-	-	-
20	-	-	-	+
Total (%)	5/20 (25%)	4/20 (20%)	3/20 (15%)	13/20 (65%)
Uncertain (%)	2/20 (10%)	0/20 (0%)	0/20 (0%)	0/20 (0%)

Table 2. Comparison of the different molecular biology methods with traditional diagnosis methods for the diagnosis of *Marteilia* in twenty mussel tissue samples.

infected individuals were detected with the smears and histology, respectively. However, when these same samples were screened for *Marteilia* by PCR, 65% of the mussels were identified as infected. The use of Dot-Blot in diagnosis gave worst results than traditional methods.

Discussion

The availability of specific primers or DNA probes is of great importance in the development of a sensitive and specific diagnosis

method for *Marteilia refringens* that would permit a better understanding of its life cycle and the antigenic differences among isolates. The design of PCR methods has permitted the diagnosis of a great number of protozoan pathogens. Ko et al. (1995) designed primers that detected *Haplosporidium costale* specifically, and not *Halposporidium nelsoni*. *Marteilia sydneyi*, parasite of the oyster, *Saccostrea commercialis*, was diagnosed using specific primers obtained from the ITS region of the rRNA (Anderson et al., 1995). A specific PCR

was also developed for the detection of a rickettsia-like organism (Kellner-Cousin *et al.*, 1993). In the case of *Marteilia refringens*, DNA probes have been developed for diagnosis in *Mytilus edulis* and *Ostrea edulis* (Le Roux *et al.*, 1999).

The nested PCR developed with the B1-B2 and B3-B4 primers proved to be highly specific for *Marteilia* DNA, isolated from *Mytilus galloprovincialis* from Galicia (NW Spain). The first round of PCR yielded a 650 bp fragment and was capable of detecting 150 ng of DNA, while a fragment never appeared with the host DNA (neither oyster nor mussel). The sensitivity for this PCR is lower than that observed in other PCR developed for the diagnosis of protozoan parasites (Anderson *et al.*, 1995; Kellner-Cousin *et al.*, 1993; Stokes *et al.*, 1995a, b), however, the second round of PCR opens the possibility of a highly increased sensitivity (15 fg DNA). The nested PCR not only increases the sensibility, but also the specificity of the reaction since primers B3 and B4 are contained in the first amplified product.

The specificity of the probe constructed with the 550 bp fragment amplified in the nested PCR also was confirmed by *in situ* hybridization. The non-radioactive probe specifically hybridized with *Marteilia* cells and never with cells from the host. However, *in situ* hybridization was never positive when performed on smears, despite the different treatments assayed to increase the permeability of the cells. This may mean that the process of *Marteilia* purification implies an increased permeability of the cellular membrane.

When the probe was used as a diagnosis tool, in the dot-blot assay, the sensitivity of the technique was less than that observed by PCR (75 µg *Marteilia* DNA). Other authors have developed dot-blot assays that may be used on their own as diagnosis tools (McCutchan *et al.*, 1988; Stokes and Burreson, 1995). However, in our case, when the dot-blot is used after nested PCR, the sensitivity of the combination is significantly higher. This is of great interest, since the dot-blot technique is a fast method, in which a great number of samples can be tested at the same time, and by testing the different PCR products, one would greatly increase the sensitivity and confirm the specificity of the reaction at the same time.

When traditional and the molecular diagnosis tools developed in this work were compared, we found that the nested PCR was the more efficient diagnosis tool, detecting *Marteilia* in 65% of the samples studied, whereas with traditional methods (smears and histology) only 20-25% of the samples were identified as infected. Therefore this PCR is much more sensitive than histology diagnosis, however it should be taken into account that histology gives much more information about the location of the parasite in the host, as well as the damage induced in the different tissues by the infection. However amplification by PCR gives us a powerful tool to indicate the presence or absence of the parasite. Its main disadvantage is that this technique can not quantify the grade of infection, however some authors have developed quantifiable PCR methods (Marsh *et al.*, 1995).

The movement of species has caused the dispersion of a great number of diseases in bi-

valve molluscs. The diagnosis of these diseases, prior to importation of seed stocks, would be a prevention strategy that would help control the spread of these infectious diseases. The nested PCR developed in this work could be used for that purpose since it is a sensitive and fast diagnosis tool. This PCR is also a good method to screen different species in the search for an intermediate host.

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