

# Efficiency of different monoclonal antibodies in immunological assays developed for the detection of *Marteilia* sp. isolated from *Mytilus galloprovincialis*

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## Abstract

Six monoclonal antibodies directed against *Marteilia* sp. isolated from *Mytilus edulis* in France have been characterized and proven to be specific for *Marteilia* sp. isolated from *Mytilus galloprovincialis* in Galicia (NW Spain). Several immunological tests (indirect immunofluorescence, tissue immunoperoxidase, immunodot and enzyme-linked immunoassay, ELISA) have been applied obtaining various results with the different antibodies in all of them. These methods may be used as potential tools for efficiently diagnosing *Marteilia* in the future.

## Introduction

*Marteilia refringens*, the etiological agent of Aber disease, has caused serious mortalities in the flat oyster (*Ostrea edulis*) in Europe since 1968 (Alderman, 1979). Many species of the genus *Marteilia* have been described in molluscs of commercial importance in several regions of Europe. *Marteilia* sp. has been also reported from mussels (*Mytilus edulis* and *Mytilus galloprovincialis*) from Galicia (NW Spain) (Gutierrez, 1977; Figueras *et al.*, 1991) and *Marteilia maurini* was described in the mussel *M. galloprovincialis* by Comps *et al.* (1982). This parasite was differentiated from *Marteilia refringens* by host specificity, shape of the haplosporosomes and structure of the spore envelope (Comps *et al.*, 1982). However, host specificity can no longer be used as a characteristic of *Marteilia maurini* because this same parasite was also reported in *Mytilus edulis* (Auffret and Poder, 1985).

Parasites of the genus *Marteilia* are easily diagnosed by staining smears of digestive gland and histology. However it is difficult to examine a great number of samples since these techniques are time consuming and require experience. Furthermore, some interpretations could be erroneous. Therefore, a need arose to apply more sensitive and fast immunological techniques for the detection of protozoal infections in invertebrates. Recently, monoclonal antibodies have been developed against several molluscan pathogens such as *Bonamia ostreae* (Rogier *et al.*, 1991), *Vibrio P1* (Noel *et al.*, 1991), *Marteilia* sp (Robledo *et al.*, 1994) or *Perkinsus atlanticus* (Goggin *et al.*, 1991). These antibodies have been used in the development of indirect immunofluorescence assay (IIFA) (Miahle *et al.*, 1988) or enzyme linked immunoassay (ELISA) (Cochenec *et al.*, 1992) and were suitable for both diagnostic and taxonomic purposes.

In this paper, we report the use of six monoclonal antibodies (MAbs) raised against *Marteilia* sp isolated from *Mytilus edulis* in France (Robledo *et al.*, 1994) in diagnosis assays for the detection of *Marteilia* sp. in the mussel, *Mytilus galloprovincialis* from Galicia.

## Materials and Methods

### *Mussels and monoclonal antibodies*

*Marteilia* cells were purified from mussels (*Mytilus galloprovincialis*) collected from Galicia following the protocol previously described by Robledo *et al.* (1995). Mussels from a non-infected area (Cork, Ireland) were used as negative controls.

The monoclonal antibodies (Mabs) (1/1-1, 3/1-1, 4/1-1, 6/2-3, 9/1-1, 12/1-1) used in the assays described in this study are those generated in our earlier study (Robledo *et al.*, 1994). These had been obtained against *Marteilia* sp. isolated from the mussel *M. edulis* in France.

### *Indirect Immunofluorescence assay (IIFA)*

For the fixing of either purified *Marteilia* cells or digestive gland smears, poly-L-lysine-treated slides were used. Samples from infected and non-infected controls were air dried and fixed for five minutes by immersion in acetone. Slides were then overlaid with the undiluted Mabs for 30 minutes in a humidified chamber at 37°C. Following two washes with phosphate buffered saline (PBS) slides were incubated with FITC-conjugated anti-mouse IgG-fluorescein (Sigma) diluted 1/50 in PBS buffer containing 0.1% Evans blue (bioMérieux). Slides were washed twice in PBS, dried, mounted and observed under a microscope equipped with epifluorescence

(Nikon) looking for green specific immunofluorescence. Negative controls without the monoclonal antibodies were included in the assay. To calculate the MAb titres, the assay was repeated using serial dilutions of the different MAbs. Titres were calculated as the inverse of the last dilution that gave a positive signal per ml.

### *Immunoperoxidase assay*

Histological sections of infected and non-infected mussels were dewaxed and rehydrated. Slides were incubated with 0.1% trypsin in 1% CaCl<sub>2</sub> (pH 7) for one hour, followed by a treatment with blocking solution consisting in Tris buffered saline (TBS, pH 7.5) supplemented with 0.2% BSA and 0.05% Tween 20. As controls, some slides were not treated with trypsin. After incubating with undiluted Mabs for one hour at 37°C, peroxidase-conjugated anti-mouse IgG diluted 1/50 in PBS was added. Slides were incubated with the enzymatic substrate 3-3'-diaminobenzidine (DAB) for 5-10 minutes and then stained with Mayer's hematoxylin.

### *Immunodot*

Purified *Marteilia* cells were spotted onto a TBS-hydrated nitrocellulose membrane using a vacuum manifold apparatus. After a treatment with blocking solution (TBS with 1% bovine serum albumin, BSA), the MAbs were added and incubated for one hour at room temperature. Unbound antibodies were removed by three consecutive washes in TBS. Peroxidase-conjugated anti-mouse IgG (Sigma) diluted 1:1250 was added and incubated for one hour. After eliminating the unbound antibody, the membrane was immersed in peroxidase substrate solution, 4-

	Monoclonal antibodies used						
	1/1-1	3/1-1	4/1-1	6/2-3	9/1-1	12/1-1	Mix
IIFA	-	+	+	+	+	+	+
Tissue Immunoperoxidase	-	+	-	+	+	-	+
Immunodot	-	-	+	+	+	-	+
ELISA	-	+	+	+	+	+	+

Table 1. Efficacy of the different MAbs in the different immunological assays developed (Mean OD  $\pm$  SD).

chloro-1-naphthol (Sigma), until the color was developed.

#### *ELISA (enzyme-linked immunoassay)*

Fifty microlitres of purified *Marteilia* cells were applied onto a polystyrene microtiter plate and allowed to stand overnight at 4°C. After five washes with TBS, plates were blocked for two hours with a TBS-T1 buffer (TBS, 0.02% Tween 20) plus 5% powdered milk. Following five washes with TBS-T2 (TBS, 0.05% Tween 20), Mabs were added and incubated for two hours at 37°C. After five washes with TBS-T2, samples were incubated for one hour with anti-IgG peroxidase conjugated antibody diluted 1/2000. Five washes with TBS-T1 buffer, followed by five washes with TBS were performed, just before adding the enzyme substrate, orthophenylendiamine (Sigma). After 20 minutes, the reaction was stopped with the addition of 3M sulphuric acid. The reaction intensity was measured by reading the optical density at 492 nm. To calculate the MAb titres, the assay was repeated using serial dilutions of the different MAbs. Titres were calculated as the inverse of the last dilution that gave a positive signal per ml.

In order to test the sonication effect, purified *Marteilia* samples were applied either sonicated or without sonication. Mussel cells non-infected by *Marteilia* and PBS buffer were included as negative controls in the assays. A sample without Mabs was included as a blank.

#### **Results**

MAbs produced against *Marteilia* sp. cells isolated from *Mytilus edulis* in France, were capable of reacting with *Marteilia* sp. cells isolated from *Mytilus galloprovincialis* collected from Galicia. These antibodies never reacted with non-infected mussel cells.

#### *Indirect immunofluorescence assay (IIFA)*

The IIFA method applied to the detection of purified *Marteilia* cells isolated from *Mytilus galloprovincialis* showed two different patterns of fluorescence. The Mabs 3/1-1, 4/1-1 and 6/2-3 reacted against the sporoblast membrane while the Mabs 9/1-1 and 12/1-1 produced an intense fluorescence emission in the spore cytoplasm of the parasite. The monoclonal antibody 1/1-1 did not react with the *Marteilia* cells (Table 1).

	Monoclonal antibodies used					
	3/1-1	4/1-1	6/2-3	9/1-1	12/1-1	Mix
Sonicated <i>Marteilia</i>	0.648±0.005	0.358±0.025	0.114±0.016	0.960±0.007	0.314±0.013	1.048±0.00
Non sonicated <i>Marteilia</i>	0.437±0.006	0.268±0.006	0.088±0.00	0.600±0.089	0.148±0.001	0.530±0.00

Table 2. Optical density values (mean ± standard deviation) measured by ELISA obtained before and after the sample sonication

When a mix of all Mabs was used (in 1: 1 proportions), an intense fluorescence distributed in the sporoblast as well as the spore of the parasite was observed. The same reactivity was observed when this technique was applied to digestive gland smears infected by *Marteilia*.

*Immunoperoxidase staining of histological sections*

When this technique was applied, Mabs 1/1-1, 4/1-1 and 12/1-1 did not react, while 3/1-1, 6/2-3 and 9/1-1, as well as the mix of all MABs reacted and showed the same pattern as with IIFA. Control slides not incubated with MABs were negative.

*Marteilia detection by immunodot assay*

The monoclonal antibodies 4/1-1, 6/2-3, 9/1-1 and the mix of all MABs, clearly reacted against the *Marteilia* cells. The rest of the antibodies did not react (Table 1). Negative controls never bound MABs.

*Monoclonal antibodies reactivity in ELISA*

All Mabs, except 1/1-1 gave a positive reaction in ELISA. Mabs 3/1-1, 9/1-1 and the mix of all MABs gave the highest values of optical density (OD). Neither the mussel cells nor the PBS gave positive values of OD.

When sonicated purified *Marteilia* cells were used in the assay, higher OD values were observed for all Mabs (Table 2). The higher difference obtained before and after the sonication was observed with the 9/1-1 antibody, directed against the spore cytoplasm.

Since the best results were obtained with either the IIFA or the ELISA, the MABs titres obtained using these two techniques were compared. The titres obtained in IIFA were always higher than the titres obtained with the ELISA (Table 3).

**Discussion**

Six monoclonal antibodies, generated against the sporoblasts and spores of *Marteilia* sp. iso-

	Monoclonal antibodies used						
	1/1-1	3/1-1	4/1-1	6/2-3	9/1-1	12/1-1	Mix
IIFA	0	666	1666	1666	666	3333	1666
ELISA	0	200	20	400	400	20	20

Table 3. MABs titres obtained in the IIFA and ELISA tests. Titres were calculated as the inverse of the last dilution that gave a positive signal per ml.

lated from *Mytilus edulis* by Robledo *et al.* (1994), have been applied for the detection of purified *Marteilia* sp. from *Mytilus galloprovincialis* collected from Galicia. These monoclonal antibodies reacted specifically against *Marteilia* cells isolated from *Marteilia* from Galicia, indicating that both *Marteilia* isolated from *Mytilus edulis* in France and from *Mytilus galloprovincialis* in Spain share common epitopes. The reactivity of these same monoclonal antibodies was assayed by Anderson (1994) against *Marteilia sydneyi* with negative results. The techniques developed with these antibodies could be used to distinguish isolates coming from several host and geographical areas worldwide. As a result, it could permit the clarification of the taxonomy and phylogenetic relationship of the *Marteilia* genus.

The immunoperoxidase technique allowed the detection of the parasite in histological sections. Positive results were never obtained without a prior enzymatic treatment. Probably, some antibodies are not capable of going through the sporangial wall to react with the parasite specific epitopes. As a result of the trypsin digestion, the cell permeability was increased allowing the monoclonal antibodies accessibility. This technique permitted not only detection of *Marteilia* but allowed some of the effects of the parasite on the host to be visualised. However, it is a time consuming technique for routine diagnosis.

The immunodot assay allowed the detection of *Marteilia* cells specifically. This technique permits the analysis of many samples simultaneously, which represents one of its main advantages and the results may be easily in-

terpreted. However, the ELISA technique, due to its sensitivity, automation, speed and quantitative qualities (Yolken, 1982), gave better results with these MAbs. No reaction was obtained when negative controls were assayed. On the other hand, best results were obtained after sample sonication, probably due to rupture of the sporangial membrane, that increases the reaction of the MAbs with the epitopes.

The monoclonal antibodies analysed reacted in different ways depending on the technique used (Table 1). Except for the antibody 1/1-1, all the antibodies could be used in the IIFA and the ELISA. These results have to be considered when the immunological test is to be used as a diagnostic tool.

Mussel culture is the most important aquaculture activity in Galicia, reaching values of production up to 200,000 metric tones per year. Although no mass mortalities have been observed, a decreasing production of cultured mussels during the last years has been reported. *Marteilia* infections may have contributed to this, since they have been associated with mortality, growth decrease, gonadal development, etc. (Figueras *et al.*, 1993; Villaba *et al.*, 1993) Furthermore, many aspects of the *Marteilia* life cycle and taxonomy remain unresolved.

A good diagnostic test is the best method to control the parasite spread. The new immunological techniques developed in this work were practical for the detection of the parasite *Marteilia* and could be used as a powerful diagnostic tool.

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