

## Production of monoclonal antibodies against the Protozoa, *Perkinsus marinus*: estimation of parasite multiplication in vitro

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**Abstract** – Among the different stages in the protozoan *Perkinsus marinus* life cycle, the trophozoite stage is known to be the most infective stage in marine molluscs. To develop a direct method for in vitro studies of *P. marinus* proliferation under various environmental conditions, monoclonal antibodies (MAbs) specific for this pathogen were produced. Inbred strains of mice BALB/c were immunised with a trophozoite prepared from cloned isolate *Perkinsus* 1 cultured on JLODRP1 medium. The mouse polyclonal antiserum showing the highest antibody titre for pathogen trophozoites was chosen for lymphocyte hybridisation. The screening of positive hybridoma by indirect enzyme-linked immunosorbent assay (ELISA) revealed two probes (17B<sub>2</sub>D<sub>5</sub> and 19G<sub>3</sub>H<sub>6</sub>) detecting *P. marinus* trophozoites and their protein lysates but also trophozoites from *P. atlanticus*. These MAbs belonged to the immunoglobulin IgG1 subclass. Their binding specificity was investigated by ELISA and fluorescein (FITC) methods. Both immunoreacted with trophozoite stage as well as hypnospore and zoospore stages of *P. marinus*, but neither with hemolymph and tissues of oysters, *Crassostrea gigas* and *C. virginica*, nor with parasites, *Bonamia ostreae* and *Marteilia refringens*. A competitive ELISA method was developed, using 17B<sub>2</sub>D<sub>5</sub> MAb to evaluate parasite multiplication in culture media and to estimate the parasite burdens from infected oysters. This method is sensitive enough to detect 10<sup>3</sup> trophozoites in 50 µL assay sample. © 2001 Ifremer/CNRS/INRA/Cemagref/Éditions scientifiques et médicales Elsevier SAS

monoclonal antibodies / trophozoites / immunoassays / *Perkinsus*

**Résumé** – Production d'anticorps monoclonaux contre le protozoaire *Perkinsus marinus*: estimation de la multiplication parasitaire in vitro. *Perkinsus marinus* (stade trophozoïte) est un protozoaire qui provoque d'importantes mortalités chez l'huître américaine *Crassostrea virginica*. Dans le but de mettre au point un dosage immunologique sensible et spécifique permettant d'évaluer le nombre de stades trophozoïtes cultivés (in vitro) dans des milieux de cultures de composition différente, nous avons produit par hybridation lymphocytaire deux sondes anticorps monoclonaux 17B<sub>2</sub>D<sub>5</sub> et 19G<sub>3</sub>H<sub>6</sub>, appartenant à la sous-classe des immunoglobulines IgG1. Au moyen de tests immunoenzymatiques (ELISA) et de la fluorescéine (FITC) ces sondes ont une réactivité sur les stades trophozoïtes (l'immunogène) et les lysats protéiques correspondants ainsi que sur deux autres stades parasitaires de *Perkinsus marinus*: hypnospores et zoospores. Cette spécificité réactionnelle pourrait être élargie au niveau du genre *Perkinsus* puisqu'une réaction croisée est observée pour les stades trophozoïtes de *P. atlanticus* parasite de la palourde *Ruditapes decussatus*. Ces deux sondes anticorps ne réagissent ni contre l'hémolymphe et les tissus des huîtres *Crassostrea gigas* et *C. virginica* ni contre les lysats protéiques d'autres parasites de l'huître: *Bonamia ostreae* et *Marteilia refringens*. Sur la base de ces résultats, un dosage de type inhibition compétitive, C-ELISA, a été réalisé par utilisation de l'anticorps monoclonal 17B<sub>2</sub>D<sub>5</sub>. Cette méthode est assez sensible pour détecter 10<sup>3</sup> trophozoïtes dans un échantillon de 50 µL. Cet anticorps nous permet de dénombrer d'une manière simple la prolifération des stades trophozoïtes de *P. marinus* in vitro. © 2001 Ifremer/CNRS/INRA/Cemagref/Éditions scientifiques et médicales Elsevier SAS

anticorps monoclonaux / trophozoïtes / immunoanalyse / *Perkinsus*

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## 1. INTRODUCTION

The protozoan parasite *Perkinsus marinus* causes the most important disease of *Crassostrea virginica*, along the coast of the United States (Burreson and Ragone-Calvo, 1996; Ford, 1996; Krantz and Jordan, 1996). In spite of many investigations spanning some 40 years, the pathogenesis of the disease, the life cycle of the pathogen, and the dynamics and mechanisms of its dissemination are poorly understood. To date, three stages have been described in the pathogen's life cycle: trophozoite, hypnospore and zoospore (Perkins, 1996). Each of them can infect oysters by direct injection, but trophozoites have been shown to be the most efficient in initiating serious mortalities (Mackin, 1962; Perkins and Menzel, 1966; Volety and Chu, 1994).

Because *Perkinsus* species are pathogens of numerous mollusc species of economical interest, most studies have concentrated on the host response and little information is available on pathogen physiology. Recently, Gauthier and Vasta (1994) showed that iron is required for *P. marinus* growth. Ahmed et al. (1997) reported the presence of iron-containing superoxyde dismutase in *P. marinus*. Volety and Chu (1997) showed that acid-phosphatase played a crucial role in *P. marinus* physiology. To enlarge such studies relating to the effect of culture conditions on the growth and proliferation of parasites, techniques to estimate total parasite numbers within individual host or in culture samples are needed. Previously, most studies had employed microscope examination and counting but this approach did not supply precise and reliable data.

The thioglycollate medium assay based on transformation of trophozoites into hypnospores, stained with lugol and counted under a microscope was successfully used by Ford et al. (1999) in *in vivo* studies of *P. marinus* dynamics during infections of *C. virginica*. But this technique is indirect and, because hypnospores were rarely observed in infected tissues, it requires a time-consuming culture of trophozoites on Ray's fluid thioglycollate medium (RFTM).

In an effort to overcome the limitations of these methodologies, the production of antibodies directed against the parasite was attempted. Choi et al. (1991) and Dungan and Roberson (1993) produced antibodies against *P. marinus*. However, hypnospore epitopes are not present in all life-stage of the parasite since hypnospores are rarely seen in live oyster tissues (Mackin, 1962). To overcome this limitation, we attempted to produce MAb directed against trophozoites which are easily cultured *in vitro* and commonly observed in infected molluscs.

## 2. MATERIALS AND METHODS

### 2.1. *Perkinsus marinus* culture

The cloned isolate *Perkinsus* 1 (gift of M. Faisal), was cultured on JLODRP1 medium according to the

method of La Peyre et al. (1993). Pellets of trophozoites were obtained after centrifugation for 15 min at 500 g, washed three times with phosphate buffered saline (PBS) pH 7.2, and counted with a Malassez hemocytometer  $1 \times 10^7$  cell·mL<sup>-1</sup>. Trophozoites suspension in PBS were then frozen at -80 °C.

### 2.2. Mice antibody production

#### 2.2.1. Immunisation

Four inbred strains BALB/c (female) mice were each immunised three times (15 d apart) with 0.3 mL trophozoites of *P. marinus*. Two weeks after the last immunisation, serum titres (50 % binding titre) were estimated for all mice, by ELISA (technique described below). Pre-immune sera were employed at the same dilution as negative controls. The mouse presenting the highest antibody titre (more than 1/10 000) was chosen for the production of MAbs by lymphocyte hybridisation.

#### 2.2.2. Hybridoma production

Monoclonal antibodies were produced using the Kohler and Milstein (1975) method with modifications. Briefly, 4 d after the final booster intraperitoneal injection ( $0.5 \times 10^6$  *P. marinus* trophozoites, in 0.3 mL PBS), spleen cells were collected and fused with a non-secreting myeloma cell line, P3X63-Ag 8.653. The cells were distributed into 96-well tissue culture plates (Nunc) at a dilution of  $1.5 \times 10^5$  cells·well<sup>-1</sup> in a selective medium containing hypoxanthine, aminopterin and thymine (HAT) and held in a humidified incubator at 37 °C and 5 % CO<sub>2</sub>. After 10–15 d, the culture supernatants were screened by ELISA in plates coated with *P. marinus* trophozoites or trophozoite protein lysates. Positive hybridomas were cloned in 96-well tissue culture plates (Nunc) using the rapid limiting dilution method (Harlow and Lane, 1988) and screened by ELISA. Selected hybridomas were sub-cultured into tissue culture flasks and maintained in complete RPMI (Roswell Park Memorial Institute) medium.

#### 2.2.3. Production and purification of monoclonal antibodies (MAbs)

Cloned selected hybridomas ( $4\text{--}5 \times 10^6$  cells) were injected intraperitoneally into BALB/c mice that had been previously treated for 7 d by daily injection of 500 µL pristane. The ascitic fluid was withdrawn 10–20 d after injection and clarified by centrifugation. Ascites fluid were tested by ELISA. MAbs purification was performed on Protein A-Sepharose Hi-trap column (Pharmacia) and the purity was determined on sodium-dodecyl sulphate/polyacrylamide gel electrophoresis (SDS/PAGE).

### 2.3. Protein lysates of trophozoites and oyster samples

Protein lysates from *P. marinus*, *Bonamia ostreae* and *Martelia refringens* were obtained by shaking trophozoites in PBS with glass beads (0.1 mm in diameter) in a Mini Bead Beater (Biospec. Product, Bartlesville, OK). Hemolymph samples and gills were aseptically collected from unparasited *C. gigas* and *C. virginica*. Gills were homogenised in sterile PBS. Protein concentration of all the samples was measured after centrifugation using the dye-binding method described by Bradford (1976).

### 2.4. Immunoassays

#### 2.4.1. ELISA for screening and dilution tests

Plates (Nunc ELISA) were coated overnight at 60 °C with 50  $\mu\text{L}\cdot\text{well}^{-1}$  trophozoite suspension of  $1 \times 10^6$  trophozoites $\cdot\text{mL}^{-1}$  in PBS or with trophozoite protein lysate at 10  $\mu\text{g}\cdot\text{mL}^{-1}$  overnight at 4 °C (three rinses with PBS were performed between all steps).

The wells were blocked with 250  $\mu\text{L}\cdot\text{well}^{-1}$  5 % skimmed milk in PBS (PBS/5 % SM) for 1 h at 37 °C. Culture supernatant (50  $\mu\text{L}$  screening) or purified MAbs 17B<sub>2</sub>D<sub>5</sub>-19G<sub>3</sub>H<sub>6</sub> (dilution tests) were serially diluted with PBS/0.5 % SM and incubated for 2 h at room temperature. Fifty microlitres PBS/0.5 % SM were used as non-specific control (NS). Finally, 50  $\mu\text{L}$  anti-mouse IgG horseradish peroxidase conjugate (Pierce Interchim) was added (at 1/4 000 in PBS/0.5 % SM) and plates were incubated for 1 h at 37 °C. The assay was developed by adding 50  $\mu\text{L}$  chromogen (orthophenylenediamine), in substrate buffer (0.1 M citric acid, 0.1 M sodium acetate, pH 5.4 containing 0.33 % H<sub>2</sub>O<sub>2</sub>). The reaction was stopped after 15 min by adding 25  $\mu\text{L}$  4 N H<sub>2</sub>SO<sub>4</sub> to the wells. Optical density was read at 492 nm using an ELISA plate reader (Dynatech MR 5000). The values were plotted against serial dilution of antibodies to obtain curves from which the 50 % points could be calculated to determine the titers and corresponding concentration for dilution tests.

#### 2.4.2. Enzyme-linked immunosorbent assay (ELISA)

The specificity of the two monoclonal antibodies was also assessed by indirect ELISA using protein lysates of various oyster pathogens (*P. marinus* trophozoites, *B. ostreae* and *M. refringens*) or unparasited oyster tissues (gills and hemolymph from *C. gigas* and *C. virginica*). Microtitration plates were coated overnight at 4 °C with 50  $\mu\text{L}\cdot\text{well}^{-1}$  protein lysates diluted in PBS alone at 10  $\mu\text{g}\cdot\text{mL}^{-1}$ . After blocking the wells and washing, 50  $\mu\text{L}$  mouse MAbs at concentration of 10  $\mu\text{g}\cdot\text{mL}^{-1}$  were added and incubated for 2 h at 37 °C. The assay was developed, stopped and measured as described above.

#### 2.4.3. Immunofluorescence

For immunofluorescence, free hypnospores were obtained by culturing trophozoites on Ray's fluid thioglycollate medium (RFTM) for 15 d in the dark (Ray, 1952). After several washes in sterile seawater, hypnospores were transferred to sterile seawater for 15 d in the dark to allow zoospores production.

In *C. virginica* infected and uninfected by *P. marinus*, and in *Ruditapes decussatus* infected and uninfected by *P. atlanticus*, tissue sections were deparafinised and rehydrated through a graded ethanol series (Mitta et al., 2000). After equilibration in PBS, sections were blocked for 30 min, and incubated for 2 h with mouse 17B<sub>2</sub>D<sub>5</sub> at concentration of 10  $\mu\text{g}\cdot\text{mL}^{-1}$  in PBS/0.5 % SM. Sections were washed with PBS and incubated with the anti-mouse antibody FITC conjugated (10  $\mu\text{g}\cdot\text{mL}^{-1}$  in PBS/0.5 % SM) for 1 h at room temperature. The slides were washed with PBS/0.1 % Tween, mounted in buffered glycerol and observed under UV light microscope.

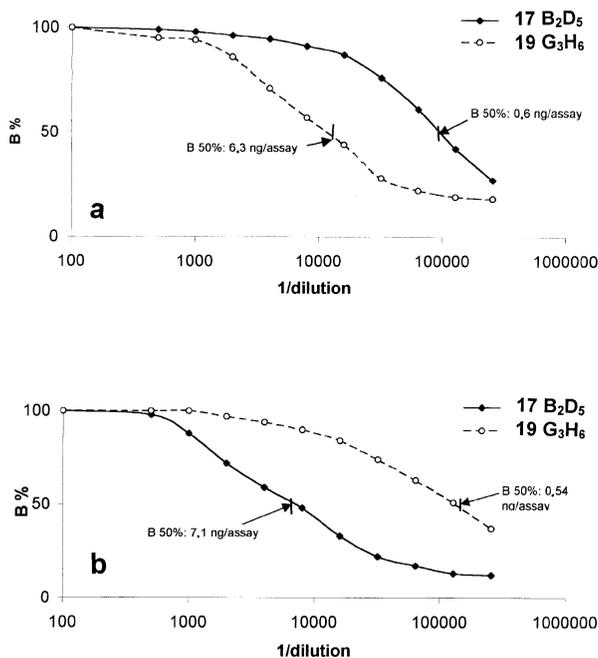
In order to visualise the cellular location of antibody binding, we used immunofluorescence on in vitro cultivated trophozoites, hypnospores and zoospores. Briefly,  $5 \times 10^5$  trophozoites or hypnospores of *P. marinus* or, for zoospores, 30  $\mu\text{L}$  culture medium JLODRP1 were deposited onto microscope slides, air dried for 2 h at 4 °C, and then fixed in an acetone bath for 10 min at room temperature. Slides were then prepared as described above, with the same controls, and observed under the same conditions.

#### 2.4.4. Inhibition ELISA (C-ELISA) and recovery experiment

For the detection of trophozoites in tissue homogenates (gills) and for enumeration tests (recovery experiments), ELISA plates were coated with 50  $\mu\text{L}\cdot\text{well}^{-1}$  trophozoite suspension containing  $1 \times 10^6$  cells $\cdot\text{mL}^{-1}$  in PBS. The plates were incubated and blocked as described above.

A standard curve was obtained by preincubation of progressive dilutions of trophozoites in PBS/0.5 % SM ( $5.3\text{--}0.04 \times 10^6$  cells $\cdot\text{mL}^{-1}$ ) with 17B<sub>2</sub>D<sub>5</sub> at concentration of 0.1  $\mu\text{g}\cdot\text{mL}^{-1}$ . After 1 h at room temperature, 50  $\mu\text{L}$  suspension from each tube were added and incubated at 37 °C for 2 h. The amount of bound mouse MAbs was detected by adding specific conjugate peroxidase and incubating for 1 h at 37 °C. The assay was developed and measured as described above. These experiments were performed five times. Correlation between numeric variables were assessed with the *R*<sup>2</sup>-test of Pearson.

For enumeration tests, known concentrations of *P. marinus* trophozoites (1 000 to 20 000) obtained in culture (corresponding to theoretic values) were mixed with *C. gigas* gill protein lysate solution at 10  $\mu\text{g}\cdot\text{mL}^{-1}$  and assays were conducted as described above. The results of three separated experiments were used to calculate the mean and the standard error of the mean (SEM). The corresponding trophozoites concentration for each test was estimated by comparison with the



**Figure 1.** Dilution and specificity tests by enzyme-linked immunosorbent assay (ELISA) of the two monoclonal antibodies, 17B<sub>2</sub>D<sub>5</sub> and 19G<sub>3</sub>H<sub>6</sub>. (a) *Perkinsus marinus* trophozoites, (b) *P. marinus* trophozoite protein lysates.

standard curve and the recovery percentage was calculated by comparison with theoretic value.

### 3. RESULTS

#### 3.1. Monoclonal antibody production

To select antibodies with high specificity for *P. marinus* trophozoites, mouse sera were screened by indirect ELISA. The mouse chosen for lymphocyte hybridisation had a titre of  $4.8 \times 10^4$  for 50 % binding. After three screenings by means of ELISA dilution test, two hybridomas (17B<sub>2</sub>D<sub>5</sub> and 19G<sub>3</sub>H<sub>6</sub>), which reacted against *P. marinus* trophozoites or the corresponding protein lysates, were injected into pristane primed mice. After purification, two monoclonal antibodies appeared to be IgG1 isotype of 150 kDa molecular weight.

As shown in *figure 1a*, 17B<sub>2</sub>D<sub>5</sub> MAb (1 mg·mL<sup>-1</sup>) showed the highest reactivity for *P. marinus* tropho-

zoites with a 50 % binding corresponding to a 90 000-fold dilution, which represented approximately 0.6 ng per assay. In contrast, reactivity of the MAb 19G<sub>3</sub>H<sub>6</sub> (1.5 mg·mL<sup>-1</sup>) was lower since 50 % binding corresponded to a 12 000-fold dilution, representing 6.3 ng per assay.

With *P. marinus* trophozoites protein lysates (*figure 1b*), reactivities obtained were reversed; 19G<sub>3</sub>H<sub>6</sub> MAb gave the highest reactivity with a 50 % binding for 140 000-fold dilution (0.54 ng per assay) and 17B<sub>2</sub>D<sub>5</sub> showed the lowest reactivity with a 50 % binding at 7 000 (7.1 ng per assay).

#### 3.2. Specificity tests

When tested with ELISA, both antibodies showed no significant immune reactivity with proteins from gill lysates or hemolymph from uninfected oysters *C. virginica* and *C. gigas* (*table 1*).

*Figure 2A* shows the fluorescence obtained when both MABs were bound in situ to trophozoites, hypnospores and zoospores from trophozoites cultures. *Figure 2B* shows a large mature trophozoite within oyster mantle tissue. The fluorescence staining is observed on the parasite surface but not in the eccentric vacuole which is characteristic of mature trophozoite cells (*figure 2D, E*). Furthermore no reaction occurred with unparasited oyster tissues. Cross-reactivity of MAb 17B<sub>2</sub>D<sub>5</sub> in cross-sections of *R. decussatus* tissues infected with *P. atlanticus* revealed that such antibody is not specific to *P. marinus* (*figure 2F*).

#### 3.3. Inhibition ELISA (C-ELISA) and recovery experiments

The standard curve obtained after five C-ELISA tests gave the following results (*figure 3*). The linear regression performed with 17B<sub>2</sub>D<sub>5</sub> monoclonal antibody is:

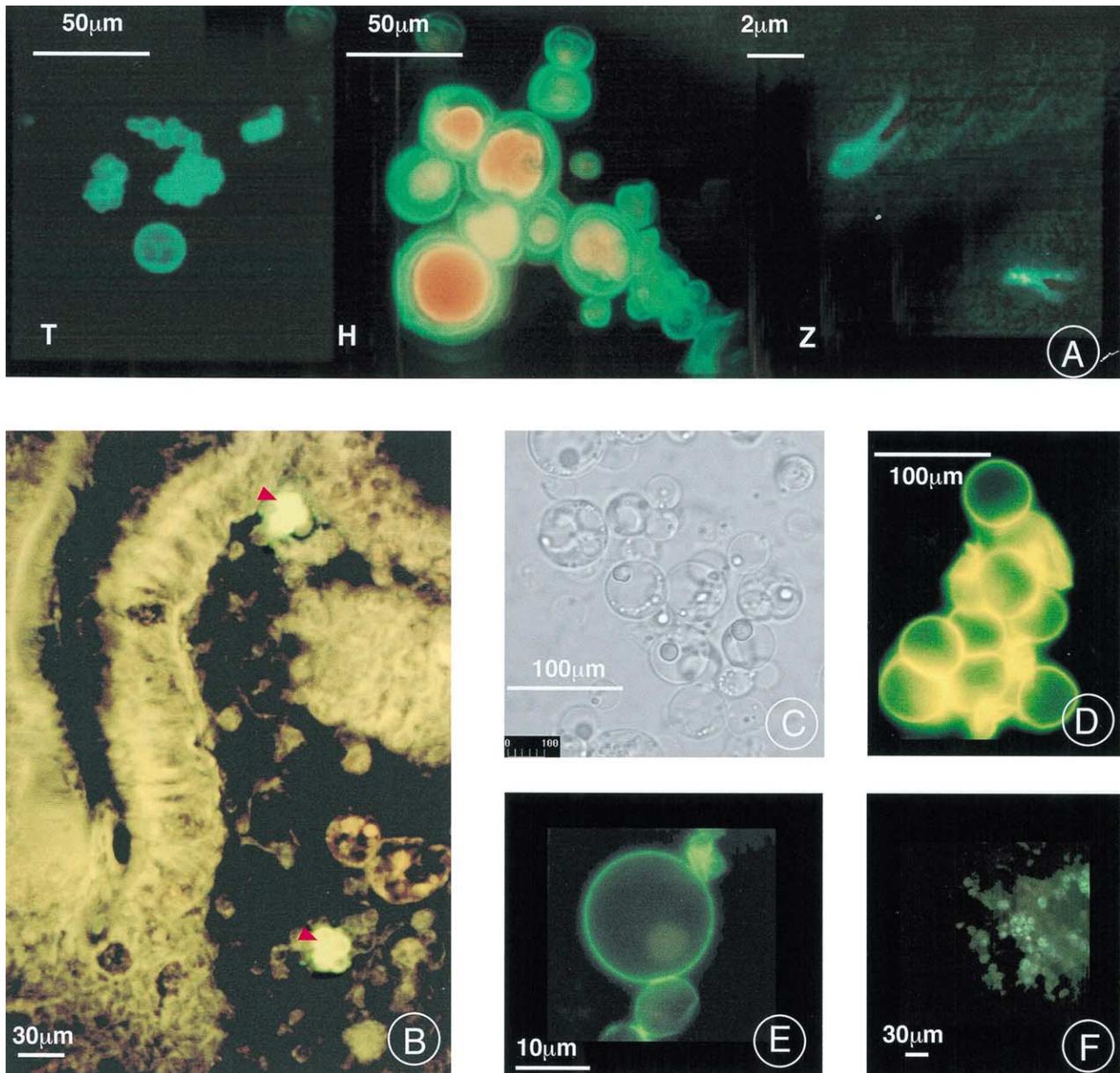
$$Y = -13.91 \ln(x) + 55.08$$

$$R^2 = 0.99, n = 89.$$

*Table 2* shows results of the three enumeration experiments performed with MAb 17B<sub>2</sub>D<sub>5</sub>. The experimental values obtained with added trophozoites (theoretical values) in oyster protein lysates were compared to the standard curve constituted by trophozoites diluted in buffer only. Recovery percentage were calculated, showing high value ranging between

**Table 1.** Optical density obtained with two monoclonal antibodies against hemolymph (HI), gill's protein lysates and lysates of oysters parasites of *Crassostrea gigas* and *C. virginica*.

Monoclonal antibody (2.5 µg·mL <sup>-1</sup> )	<i>C. gigas</i>		<i>C. virginica</i>		Lysates of oyster parasites		
	HI	Gills	HI	Gills	<i>Bonamia ostreae</i>	<i>Marteilia refringens</i>	<i>Perkinsus marinus</i>
17B <sub>2</sub> D <sub>5</sub>	0.14	0.18	0.14	0.16	0.18	0.11	0.54
19G <sub>3</sub> H <sub>6</sub>	0.17	0.16	0.16	0.16	0.17	0.12	0.76



**Figure 2.** Immunofluorescence with monoclonal antibodies (MAbs) 17B<sub>2</sub>D<sub>5</sub> and 19G<sub>3</sub>H<sub>6</sub>. **A:** *Perkinsus marinus* trophozoite (T), hypnospore (H), and zoospore (Z) labelled with MAb 17B<sub>2</sub>D<sub>5</sub>; **B:** histological cross-section of infected *Crassostrea virginica* showing *P. marinus* trophozoites labelled by MAb 17B<sub>2</sub>D<sub>5</sub> (arrows); **C:** *P. marinus* trophozoites cultivated in vitro unlabelled; **D** and **E:** *P. marinus* trophozoites cultivated in vitro labelled by MAb 19G<sub>3</sub>H<sub>6</sub>; **F:** *P. atlanticus* trophozoites in cross-sections of *Ruditapes decussatus* tissues.

99 and 115 %. The sensitivity of the assay was conserved for 1 000 trophozoites·well<sup>-1</sup>.

#### 4. DISCUSSION

To develop an immunological diagnostic test for *P. marinus* infection in oysters based on trophozoite detection and enumeration, we produced MAbs directed against trophozoites cultivated in vitro. These

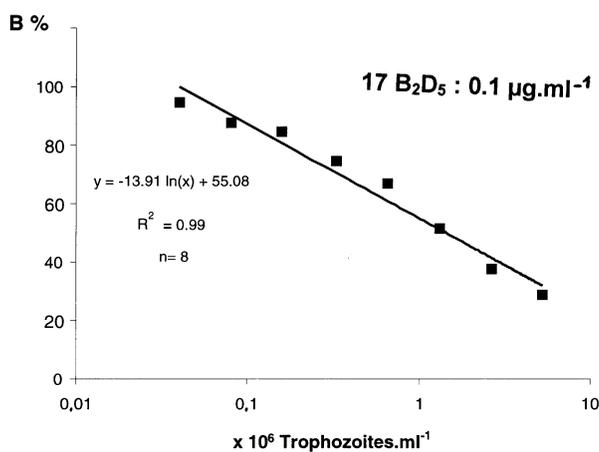
antibodies were successfully used in competition ELISA and immunofluorescence, hence providing sensitive and fast detection tools.

The monoclonal antibody 17B<sub>2</sub>D<sub>5</sub> provided the best results in competition ELISA method, allowing the detection and enumeration of 1 000 trophozoites/assay in oyster tissues samples. Compared to the classical FTM methods used for diagnosis and enumeration of *P. marinus* stages in oysters, our immunological assay

**Table II.** Overcharge tests and recovery percentage obtained by inhibition enzyme-linked immunosorbent assay (C-ELISA) using 17B<sub>2</sub>D<sub>5</sub> monoclonal antibody (trophozoites/assay).

Theoretical values Trophozoites added in well	Experimental values					
	Inhibition ELISA I	Inhibition ELISA II	Inhibition ELISA III	Arithmetical mean	SEM	Recovery %
20 000	16 000	22 200	23 000	20 667	3 372	103
10 000	8 600	11 700	11 300	10 553	1 686	105
5 000	4 900	6 150	6 200	5 750	737	115
2 000	1 940	2 400	1 650	1 997	378	99
1 000	1 050	900	1 200	1 050	150	105

SEM: standard error of the mean.

**Figure 3.** Standard curve by the competitive enzyme-linked immunosorbent assay (C-ELISA) for the detection of *Perkinsus marinus* trophozoites using monoclonal antibody 17B<sub>2</sub>D<sub>5</sub>.

is faster, more reliable, and allows the true enumeration of all parasite stages found in oyster tissues. In our assay, we directly enumerated living or dead parasite stages. The detection of parasites is based on a specific recognition by antibodies in contrast with lugol staining used in FTM methods, which is not specific for *P. marinus* and for which staining intensity depends on the dilution used (Fisher and Oliver, 1996).

The antibodies bind to all three known life stages of *P. marinus* cultivated in vitro and can also label parasite stages found in cross-sections of infected oysters without reacting with oyster tissues. This is a major difference with previous work that attempted to develop MAbs against this parasite using hypnospores (Dungan and Roberson, 1993). In these previous studies, MAbs failed to cross-react with parasite stages found in cross-sections of oysters tissues.

When used in similar experimental conditions, both antibodies successfully bound to trophozoites of *P. atlanticus* in cross-sections of *R. decussatus* tissues, indicating similarities between both *Perkinsus* species, as previously suggested by several authors (Dungan

and Roberson, 1993; Perkins, 1996; Ordàs and Figueras, 1998).

The competitive ELISA method developed using our antibody allows the evaluation of the parasite proliferation in culture media. Furthermore, our antibodies recognise two different *Perkinsus* species but do not cross-react with other pathogens such as *B. ostreae* and *M. refringens*. Although our antibodies do not possess the specificity and the sensitivity of molecular PCR-based diagnosis (Robledo et al., 1998), their capacity to recognise several *Perkinsus* species might provide useful tools to detect *Perkinsus* diseases.

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