

A direct monoclonal antibody sandwich immunoassay for detection of *Bonamia ostreae* (Asctospora) in hemolymph samples of the flat oyster *Ostrea edulis* (Mollusca: Bivalvia)

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ABSTRACT: Currently, recognition of molluscan diseases relies heavily upon light microscopic examination of stained histological sections of molluscan tissues. Such histopathological methods are time-consuming and relatively inefficient in the study of ongoing epizootics. Alternatively, more efficient investigative tools are needed to accelerate disease recognition and quantification. Such methods are needed to prevent, control and eradicate molluscan diseases. This paper reports the development of a direct sandwich enzyme-linked immunosorbent assay (ELISA) for detecting the ascetosporan intrahemocytic parasite *Bonamia ostreae* of the flat oyster *Ostrea edulis*. Bonamiasis is a commercially important epizootic disease of oysters. A *Bonamia*-specific monoclonal antibody is employed in the assay. The assay is performed in antibody-coated microplates employing standardized reagent solutions and 50 µl oyster hemolymph samples. This procedure was developed into a commercial product, available as a kit. Based upon clinical studies, there was a 90% agreement in test results when ELISAs were compared to the reference standard, histopathologic light microscopic examination for the parasites.

INTRODUCTION

Bonamia ostreae is a protozoan parasite that infects hemocytes of the flat oyster *Ostrea edulis* (Pichot et al. 1980). Initially described in France, it has spread to numerous oyster farming areas in Europe since 1976 (Bannister & Key 1982, Van Banning 1982, Polanco 1984). Recently, a similar disease was observed in several populations of *O. edulis* on the west coast of North America (Elston et al. 1986, Farley et al. 1988). Bonamiasis is the disease caused by the pathogen and is responsible for substantial oyster mortalities (Grizel 1985). In order to continue flat oyster farming in infected areas of the world, it is essential to develop methods for the management and prevention of bonamiasis. Currently, epizootiological surveys of the disease are based upon histopathological diagnoses by molluscan pathologists. These surveys are very

demanding, requiring the processing of great numbers of specimens and utilizing many valuable professional man-hours in order to obtain a detailed estimation of disease prevalence. More efficient alternative diagnostic methods must be developed for greater progress.

Following the elaboration of a *Bonamia ostreae* purification protocol (Mialhe et al. 1988a), specific polyclonal and monoclonal antibodies were prepared (Rogier et al. 1991) and utilized in the development of an indirect immunofluorescent assay for detection of the parasites in tissue smears (Boulo et al. 1989). Despite the increased sensitivity of the newly developed method when compared to classical histologic methods, this assay requires several time-consuming steps for sample preparation and microscopic examination.

Enzymatic immunodiagnostic methods have been elaborated for several infectious diseases of animals

and plants (Yolken 1982, Kingsbury & Falkow 1985). These methods offer an attractive alternative, since the tests can be standardized and automated and the results of a large number of samples can be read simultaneously.

This paper describes the development and clinical evaluation of a direct sandwich-monoclonal antibody assay suitable for quantitative diagnoses of *Bonamia ostreae* in oyster hemolymph samples.

MATERIALS AND METHODS

Specimens. Japanese oysters *Crassostrea gigas* were obtained at La Tremblade, France. Flat oysters *Ostrea edulis* were collected in Quiberon Bay, where bonamiasis is chronically observed, and in Thau Pond, which is considered free of the disease. Hemolymph samples were taken from sacrificed oysters either by direct aspiration of hemolymph from the pericardial cavity using a pipet, or by venapuncture of the branchial vein using a syringe.

Reagents. Specific mouse immune sera were prepared against *Bonamia ostreae* as previously described (Mialhe et al. 1988a). Monoclonal antibody (MAB) 20B2, specific for the parasite-membrane epitopes, was produced in ascitic fluid and purified by affinity chromatography on immobilized protein-A (Rogier 1987). Alkaline-phosphatase conjugate was prepared using glutaraldehyde as a coupling agent (Avrameas 1969).

Light microscopy. Heart smears were fixed, colored (Hemacolor, Merck) and examined microscopically for *Bonamia ostreae*.

ELISA sample preparation. For preliminary experiments, large samples were prepared first by homogenizing healthy or highly infected oysters in sterile seawater (20 g in 500 ml) and second by pooling several hemolymph samples exhibiting similar infection levels. These samples were kept frozen until use. Individual hemolymph samples were also frozen and

used either immediately after being thawed or preferably processed by selected protocols: dilution with sterile distilled water (v/v) or treatment with Nonidet P40, according to the method used for *Plasmodium vivax* (Wirtz et al. 1985).

The basic procedure consists of a direct sandwich immunoenzymatic assay using polyclonal or monoclonal antibodies. Optimal concentrations of capture and detection antibodies were determined by checkerboard titrations.

All washing procedures were performed 5 times with addition and vacuum suction of 200 μ l NaCl solution (0.3 M), containing Tween 20 (0.1 %).

Flat-bottomed polystyrene microtiter plates (Nunc, high protein adsorption quality) were coated with 100 μ l of polyclonal or monoclonal antibodies diluted in sterile PBS (phosphate buffer solution). Plates were stored either at 4 °C for at least 14 h before use, or at 37 °C for 3 h. After 3 washes, 200 μ l of phosphate-buffered saline with 20 % gelatin hydrolysate (PBS-G) was added to the wells (3 h, 37 °C). After this saturation step, the sample was added (1 h at 37 °C or 1 night at 4 °C), the plates were again washed and 100 μ l of alkaline-phosphatase labeled monoclonal antibodies, diluted in PBS-G, were added. After incubation (1 h, 37 °C) and washes, 100 μ l of nitrophenyl-phosphate (NPP) solution (1 mg of 5 % diethanolamine buffer ml^{-1} , pH 9.8) was added and allowed to react (90 min, 37 °C).

The amount of color resulting from hydrolysis of the substrate by enzyme bound to the well was measured at a wavelength of 405 nm using a microplate colorimeter.

RESULTS

Selection of capture and probe antibody concentrations

In order to perform direct sandwich immunoassays, the 20B2 monoclonal antibody was selected because of

Table 1. Comparison of different concentrations of monoclonal antibody (MAB20B2) as capture and detection antibodies, according to the ratio P/H, with P and H corresponding to optical density values obtained for parasitized and healthy oysters respectively

MAB detection ($\mu\text{g well}^{-1}$)		MAB capture				
		0.062 $\mu\text{g well}^{-1}$	0.125 $\mu\text{g well}^{-1}$	0.250 $\mu\text{g well}^{-1}$	0.500 $\mu\text{g well}^{-1}$	1 $\mu\text{g well}^{-1}$
0.8	P	0.388	0.448	0.442	0.479	0.590
	H	0.175	0.174	0.174	0.243	0.315
	P/H	2.2	2.6	2.5	2.0	1.9
1.6	P	0.301	0.317	0.286	0.260	0.322
	H	0.086	0.069	0.046	0.090	0.155
	P/H	3.5	4.6	6.2	2.9	2.1

the high representation of its specific epitope on the parasite membrane (Mialhe et al. 1988b). The respective efficiencies of different concentrations of this antibody as a capture and probe were determined by checkerboard titrations using homogenates of infected and non-infected oyster bodies as antigens (Table 1). The most significant signals were obtained for 0.125 and 0.250 $\mu\text{g well}^{-1}$ for the capture antibody and 0.8 $\mu\text{g well}^{-1}$ for the probe antibody.

Experiments were then performed using hemolymph samples as antigens. Hemolymph is particularly suitable for detection and quantification of *Bonamia ostreae* because of the intrahemocytic location of the parasite and the feasibility and quantitative reproducibility of hemolymph sampling. The results shown in Fig. 1 confirm the efficiency of the capture and probe monoclonal antibody selection, allowing differential recognition between low-incidence infection and non-infected hemolymph samples. When the lowest capture antibody concentrations were inadequate, all of the other samples appeared equivalent. The specificity of signals was also compared according to the incubation conditions of the antigen suspensions (1 h at 37 °C or 1 night at 4 °C). The results corresponding to the latter incubation regimen showed a decrease in the non-specific part of the signal for non-infected and low-intensity-infected samples, and an increase in optic densities for higher-intensity-infected samples (Fig. 1).

Purified immune sera were tested as capture antibodies taking into account their large epitope specificity range and their possible better retention of parasite antigens. Using infected hemolymph pools serially diluted in healthy hemolymph pools as antigen suspensions (incubation for 1 night at 4 °C), several concentrations of polyclonal antibodies (PABs) were compared to MAB 20B2 (0.125 $\mu\text{g well}^{-1}$) as a capture antibody. The results (Fig. 2) evidenced the superiority of monoclonal antibody, whatever the quantity of polyclonal antibody employed.

Sample processing

The intrahemocytic location of *Bonamia ostreae* made it necessary to test different sampling processes for the release of the parasites from hemocytes, which might influence the efficiency of the assay. The initial hemolymph process consisted of freezing-thawing cycles either alone or associated with an incubation with 0.25 % Nonidet P40, a non-ionic detergent which has been successfully used with intraerythrocytic *Plasmodium* parasites (Wirtz et al. 1985). In practice, the latter method seemed to destroy the parasite-specific epitope of 20B2, since no signal was obtained for any antigen concentration.

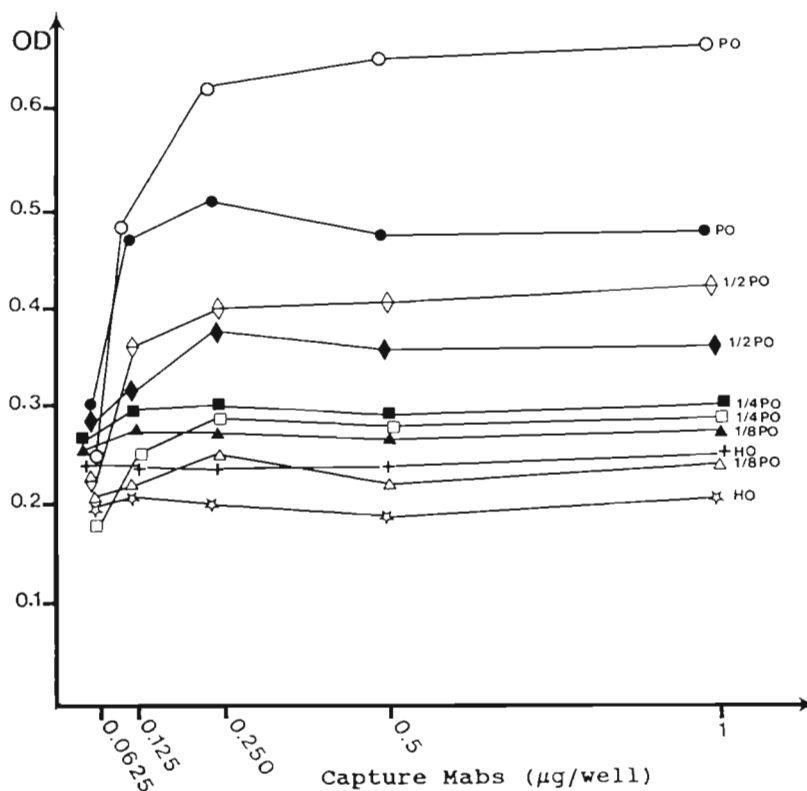


Fig. 1. Immunoassay for detecting *Bonamia ostreae*. Effect of capture antibody quantity and parasitic antigen concentration on optical density (OD) values for 2 antigen incubation regimens: 1 h at 37 °C (●, ■, ◆, ▲, +) or 1 night at 4 °C (○, □, ◇, △, ☆). PO: Parasitized oyster homogenate; HO: healthy oyster

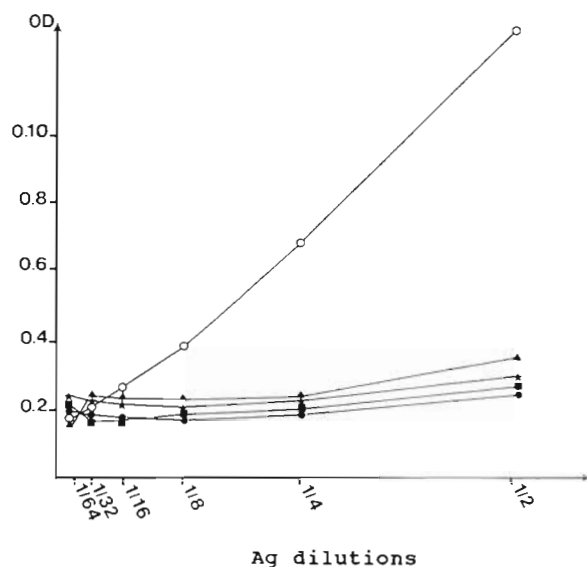


Fig. 2. Sandwich immunoassay for *Bonamia ostreae*. Optical density (OD) values obtained for different relative concentrations of parasite antigens (Ag; incubated for 1 night at 4 °C) to different capture antibodies: 20B2 MAB at 0.125 µg well⁻¹ (○); PAB dilutions: 1/10 (▲), 1/50 (★), 1/100 (■) and 1/500 (●)

Positivity limit

The range of negative ELISA reactions was determined by testing 2 series of 100 hemolymph samples from healthy oysters collected from the non-infected population in Thau Pond and previously confirmed as negative by microscopic examination. The A 405 nm values were determined for these negative samples. Mean values and standard deviations of the optical density (OD) were estimated (\bar{x} = 0.240 OD, SD = 0.045). Thus, a clinical sample would be considered positive if the optical density value of the sample was greater than the mean value and more than 3 standard deviations (e.g. A 405 nm = 0.375 OD).

Clinical studies

Initial studies on sample processing methods led to the conclusion that the freezing-thawing procedure for parasite release was most efficient. This method was subsequently retained because of its simplicity and reliability during numerous hemolymph sample analyses performed during the clinical studies.

Hemolymph samples (50 µl each) from 200 oysters collected in Quiberon Bay were analyzed by means of ELISA and compared to results obtained by direct microscopic examination of stained cardiac hemolymph smears. Five minutes were allocated for microscopic examination of each stained smear. Results were recorded as either positive or negative. In the case of

positive smears, the level of infection was estimated and recorded as follows (see Fig. 3): (B) only 1 parasite observed; (+) 2 to 10 parasites; (++) 10 to 100 parasites; (+++) > 100 parasites.

ELISA test results indicated that 30 oysters were positive and 167 were negative (Fig. 3). Among the ELISA negative samples, 15 corresponded to specimens for which only 1 *Bonamia ostreae* (▲ in Fig. 3) was detected by the histological method. In contrast, the 8 ELISA-positive specimens (★ and ○ in Fig. 3) corresponding to parallel initial negative histological diagnoses were reexamined; a second prolonged examination of the same heart hemolymph smear, sometimes after 15 min of observation, managed in 4 cases (★ in Fig. 3) to detect single *B. ostreae* cells. The ELISA was 90 % in agreement with the light microscope reference standard.

Sensitivity, defined as the ratio of the percentage of positive results obtained by the tested method to that obtained by the reference method, was 76.7 %. Specificity, defined as the ratio of the percentage of negative results obtained by the tested method to that of the reference method, was 106 %. Four of the ELISA-positive results were due to extended negative microscopic examinations and may be considered as false ELISA-positive.

DISCUSSION

We developed an ELISA method for detection of *Bonamia ostreae* in hemolymph samples. The assay utilized a monoclonal antibody, specific for a parasite-membrane-localized epitope, as the capture and probe antibodies.

A sandwich immunoassay (Yolken 1982) was utilized because of the broad application of this technique for the diagnosis of infectious diseases (Araujo 1982, Van Knappen & Panggabean 1982, Taylor et al. 1986). The specific retention of antigens by capture antibodies and their consecutive evidence by probe antibodies are the reasons why the sandwich immunoassay was successful. The method not only detected the antigen but also quantified antigens of such complex biological samples as hemolymph.

The successful application of this ELISA method was dependent upon the availability of monoclonal antibody, as demonstrated by the poor results obtained when polyclonal antibody was employed. The efficient use of monoclonal antibody as capture and probe resulted from preliminary comparative experiments with different types of monoclonal antibody preparations that yielded weaker signals (data not shown). The choice of monoclonal antibody was reinforced by the presence of the large and ubiquitous specific epitope of the parasite membrane (Mialhe et al. 1988b).

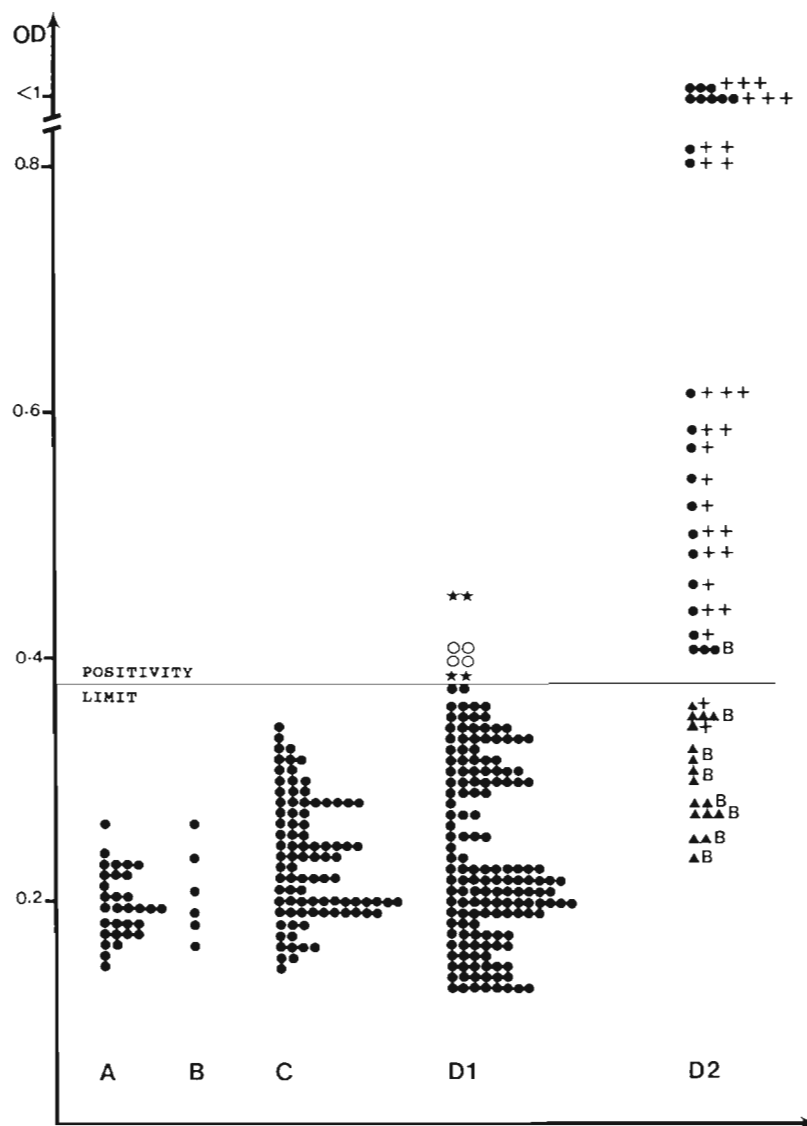


Fig. 3. ELISA diagnosis of *Bonamia ostreae* in different samples: (A) *Crassostrea gigas*, non-parasitized species. (B) Seawater. (C) *Ostrea edulis* collected from Thau Pond (*Bonamia*-free area). (D) *O. edulis* collected in Quiberon Bay; D1 and D2 represent respectively negative and positive diagnoses by the histological method. Capture antibody: $0.250 \mu\text{g well}^{-1}$; detection antibody: $0.8 \mu\text{g well}^{-1}$. Sample incubation overnight at 4°C . See 'Results - Clinical studies' for explanation of results and symbols

The sandwich ELISA offers a less laborious and time-consuming diagnostic alternative to microscopic examinations of tissue sections or smears. Also, the number of samples that can be examined during ongoing epizootics is extremely limited. This is especially true where low disease prevalence requires greater sampling for detection of infected animals (Mazurié 1988).

The comparative results demonstrated similar limits of sensitivity for both the ELISA and the reference microscopic methods, although they led to divergent diagnostics for very low-intensity infections, showing in such cases a possibly higher sensitivity of histological examination. Early infections are probably difficult to detect by either method. This fact should be kept in mind, especially in the case of oyster transfers from one oyster farm to another. It will be necessary to study the natural and experimental pathogenesis of bonamiasis

in order to estimate the time required for the inapparent stage of the disease. This information is needed to define the minimal quarantine period for prevention of introduction of the disease during animal transfers. ELISA is particularly suitable for this purpose, since it is preferable to detect and quantify *Bonamia ostreae* in the hemolymph without killing the oyster. The newly developed ELISA, associated with recent progress in purification of the parasite and experimental infections, will be especially valuable in future experimental studies of the disease (including pathogenesis), in immunity studies, and in the selection of resistant oyster stocks.

The development of a commercial ELISA assay (Sanofi, Libourne) will now allow professional and commercial scientists to utilize this method for the benefit of the oyster industry. This enzymatic

immunodiagnostic kit is the first employed in molluscan aquaculture. With the development of hybridoma technology and the production of specific monoclonal antibodies against other molluscan pathogens, important immunodiagnostic tools can be developed for the study and control of important molluscan diseases.

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