Research article

Anti *Perkinsus olseni* Monoclonal Antibody Generation Using Hypnospores as Antigens

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Abstract

Antibodies are valuable for the detection, identification, and diagnosis of pathogens in many fields, including aquaculture. In this study, we aimed to produce monoclonal antibodies (mAbs) against the parasite *Perkinsus olseni,* isolated from the clam *Paphia undulata* in Thailand. The mAbs were characterized using dot blotting and immunohistochemistry techniques in order to identify their class and their ability to detect the hypnospores and zoospores of *P. olseni*. Immunoreactions between the obtained mAbs and *P. olseni* produced black or grey spots according to the concentration of parasites detected by the mAbs. The quantitative detection thresholds of hypnospores for the two most strongly reactive mAbs were 10^5 cells/mL (producing grey spots) to 10^7 cells/mL (producing black spots), and more than 10^7 cells/mL (grey spots) for zoospores. Both mAbs showed immunoreactivity at the surface of trophozoite cells of *P. olseni* in infected clam tissues as assessed by immunohistochemistry analysis, indicating that they could be used for the detection and identification of *P. olseni* infection in commercial *Pa. undulata* production.

Keywords

dot blot;

hypnospore;

monoclonal antibody;

Paphia undulata;

Perkinsus olseni

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1. Introduction

Protozoa belonging to the genus *Perkinsus* cause a disease known as Perkinsosis in molluscs, such as oysters, clams, abalone, and scallops. This disease is believed to be associated with mass mortality of wild and farmed abalone, with consequent economic losses to farmers of the molluscs [1]. The presence of pathogenic *Perkinsus* species has been reported from the coastal areas in several countries: *P. marinus* in the eastern and southeastern United States of America [2], *P. olseni* and *P. chesapeaki* in Spain [3] and *P. olseni* in Korea [4], Japan [5] and Thailand [6]. Within the genus *Perkinsus*, only *P. marinus*, *P. olseni* and *P. atlanticus* are listed in the Old Classification of Diseases Notifiable (List B) of the World Organization for Animal Health (OIE). Infections by these species have a significant adverse impact on the health of molluscs and can lead to markedly decreased commercial mollusc production according to manual of diagnostic tests for aquatic animals [7, 8].

The life cycle of all *Perkinsus* species consists of three stages: trophozoite, hypnospore and zoospore. Trophozoites occur in the tissues of the living host. When host tissues infected with *Perkinsus* sp. were incubated in Ray's fluid thioglycollate medium (RFTM), the trophozoites became enlarged, developed a thick wall, and became hypnospores. When hypnospores were isolated and transferred into seawater, they became zoospores [9].

Several different diagnostic methods have been used to detect *Perkinsus* infections in molluscs. A commonly used method is the RFTM. After being cultured for at least 4 days in RFTM, *Perkinsus* spp. trophozoites in the host tissue become enlarged, and when the tissue is macerated to allow penetration of aqueous Lugol's iodine solution, the enlarged trophozoites, previously known as hypnospores or prezoosporeangia, readily take up iodine and become easily visible under low-power magnification because of their bluish-black coloration and spherical shape [8].

Detection using the RFTM approach is more sensitive, simple, and inexpensive for *Perkinsus* diagnosis than histological methods that involve staining sections of the host tissues with haematoxylin and eosin (H&E). However, neither method can distinguish between different species of *Perkinsus*. Consequently, many PCR methods have been developed to detect and differentiate between *Perkinsus* species [3, 10, 11]. Although PCR is highly sensitive and specific for the pathogen, its use requires the availability of expensive equipment and highly skilled personnel. Thus, immunological methods have been widely applied in aquaculture for identifying the species of pathogens [12-15]. Immunological methods based on specific interactions between antibodies and antigens are highly sensitive and specific, and do not require in-depth training of the personnel involved in testing. Polyclonal antibodies (pAbs) and monoclonal antibodies (mAbs) specific for *Perkinsus* species were developed against hypnospores and trophozoites and can be used for detecting *Perkinsus* infection. Polyclonal antibodies (pAbs) were produced from *P. marinus* hypnospores from infected oysters (*Crassostrea virginica*). Both pAbs and mAbs specific for *P. marinus* were produced from the same oyster species [16, 17]. For mAb production, mice were immunized with trophozoites or a complex set of extracellular products to produce mAbs specific for *P. marinus* [18, 19]. There are several limitations to the use of pAbs. These limitations include cross reactivity, variable lot-to-lot quality, limited production levels per animal and unwanted background reactions. These issues can be solved using mAbs. Although the initial production of mAbs is expensive and the selection of desired hybridoma cells can be time consuming, once mAbs are obtained, their production is cheap and easy in the long term, and it is possible to produce an unlimited amount of mAbs with unchanged properties. In one study, mice were immunized with the zoospores of *P. olseni* but useful mAbs were not successfully obtained, principally due to the weak primary immune response (IgM) being elicited [20]. In the present study, we produced mAbs using hypnospores of *P. olseni* for immunization. Dot blotting was used for the selection and

characterization of the mAbs. The obtained mAbs were applied to the sectioned tissues of infected *P. olseni* from the clam *Paphia undulata* using a standard immunohistochemical (IHC) method.

2. Materials and Methods

2.1 *Perkinsus olseni* culture

Hypnospores and zoospores of *P. olseni* were isolated from infected undulated surf clams *Pa. undulata*, as reported previously [6]. Hypnospores were obtained as pellets after centrifugation for 8 min at 450×g, followed by 125×g and finally 30×g for 3 min. Some of the pellets were suspended in 0.02 M phosphate-buffered saline pH 7.4 (PBS) and then frozen at -80°C. Some pellets were cultured in filtered seawater with 400 µg mL^{-1} streptomycin and 400 U mL^{-1} penicillin G until the zoospores were released. The free zoospores were then harvested, washed, centrifuged, and kept in PBS at -80°C.

2.2 Immunization

Three eight-week-old female ICR mice were immunized intraperitoneally with a 100 µL emulsion of a 1:1 (v/v) mixture of formalin-fixed hypnospores (2.0 x 10^7cell/mL) in sterile PBS: complete Freund's adjuvant (Sigma, USA). The mice were subsequently immunized at two-week intervals, as described above, except for the use of incomplete Freund's adjuvant (Sigma, USA) for three of the boosts. Antibody titres from the collected sera were determined by dot blotting, as described below. The mice were given a final boost without Freund's adjuvant three days before sacrifice and hybridoma generation. All procedures involving laboratory animals were approved by, and conducted, according to the guidelines of the Institutional Animal Care and Use Committees of the Institute of Biotechnology and Genetic Engineering, Chulalongkorn University.

2.3 Production of mAbs

2.3.1 Hybridoma generation

To generate hybridomas, a mouse was euthanised, and its spleen was removed and splenocytes were harvested. The mouse myeloma cells (P3X63/Ag8.653) were grown in Rosewell Park Memorial Institute medium (RPMI 1640) supplemented with 20% (v/v) foetal bovine serum (FBS; Biochrom AG, Germany) and collected. The myeloma cells were then fused with splenocytes at a ratio of 1:2 pellet cells to splenocytes, using 50% (w/v) polyethylene glycol (MW 3-3.7 kDa, Sigma USA) as the fusogen. The fused cells were cultured in hypoxanthine-aminopterine-thymidine (HAT) medium (RPMI 1640 supplemented with 20% (v/v) FBS, 10 µM hypoxanthine, 0.4 µM aminopterine and 16 µM thymidine (Sigma, USA), and placed into 96-well culture plates (Corning, USA) at 200 µL/well. The plates were incubated at 37°C and 5% (v/v) carbon dioxide. After culturing in HAT medium for 10-14 days to select for splenocyte-fused myeloma cells (hybridomas), the culture supernatant from each well was screened for antibodies against hypnospores by dot blotting, as described below. Positive hybridomas (wells) were selected and subcloned at least twice using a limiting dilution method until monoclones were obtained. Clonal hybridomas were then expanded for the production of mAbs, and some were cryopreserved in liquid nitrogen.

2.3.2 Dot blot screening of hybridomas

Dot blotting was used to determine the antibody titre of the sera from immunized mice, and to screen for antibody-producing hybridomas. Briefly, 1 µL of hypnospores (10^7cells/ mL in PBS) were spotted onto nitrocellulose membranes (Bio-Rad, USA). The membranes were dried at 37°C for 30 min and blocked using 5% (w/v) skim milk in PBS (HiMedia, India) for 30 min. After washing with PBS containing 0.05% (v/v) Tween 20 (PBST), the membranes were incubated with antiserum from immunized mice or culture supernatants from hybridoma-growing wells for 2 h. The membranes were then washed with PBST and incubated in goat anti-mouse IgG-horseradish peroxidase (GAM-HRP; Jackson ImmunoResearch Laboratories, USA) at a dilution of 1:3000 in PBS for 2 h. After washing with PBST, the membranes were treated with a substrate mixture containing 0.03% (w/v) diaminobenzidine, 0.06% (v/v) hydrogen peroxide and 0.05% (w/v) cobalt chloride (Sigma, USA) in PBS for 5 min before the color reaction was stopped by washing with deionised water. Membranes showing black or grey dots were deemed to represent positive results.

2.3.3 Characterization of mAb

The class and subclass of each selected mAb was determined using an isotyping kit, according to the manufacturer's instructions (Sigma, USA). The sensitivity and specificity of each mAb was investigated. One microlitre of each serial dilution of hypnospores and zoospores (approximately 10^7-10^3 cells/mL) was spotted onto nitrocellulose membranes, dried, and then processed for dot blotting.

2.4 Detection of *Perkinsus* by mAbs in infected clam tissues using IHC

Tissues from *P. olseni*-infected clams were collected, fixed in 4% (w/v) formalin (Carlo, Erba), embedded in paraffin and sectioned at 5 µm thickness, and mounted on slides. Deparaffinized and rehydrated sections were obtained followed immersion for 5 min in Ottix (Diapath, Italy), a butanol and 95%-70% (v/v) ethanol in PBS series and were then rinsed with PBS. Each section was blocked with 1% (w/v) bovine serum albumin (BSA; Sigma, USA) in PBS for 30 min before use. Each mAb obtained from a hybridoma culture supernatant at a dilution of 1:10 (v/v) was incubated with the sections at 4°C overnight, washed in PBS and then incubated in GAM-HRP diluted to 1:3000 for 4 h at 37°C, or overnight at 4°C. The sections were then washed in PBS and developed in a substrate solution containing 0.03% (w/v) 3, 3-diaminobenzididine-4HCl and 0.006% (v/v) hydrogen peroxide in PBS, before being counterstained with eosin Y (POCH S.A., Poland), dehydrated in a graded ethanol series, cleared in xylene, and mounted in permount. The immunoreaction was visualised under light microscopy as a brown coloration.

2.5 Data analysis

For the dot blotting, the black or grey spots were evaluated by visualization and each data represented from triplicate experiments. The clam samples were collected from Phetchaburi province and the infection of *P. olseni* was confirmed by Lugol iodine staining before the samples were used in immunohistochemical method.

3. Results and Discussion

Formalin-fixed hypnospores of *P. olseni* from *Pa. undulata* were used as the immunogen for immunization. After the fourth immunization of the three mice, antibody responses had titres ranging from 1: 512,000 to 1:1,102,000 (Figure 1). Three fusions were performed, and 2,976 hybridoma colonies were grown. Following screening and subcloning, eight hybridomas with an identified monoclonal origin were used to produce mAbs with immunoreactivity to the hypnospores of *P. olseni*, as detected by dot blotting. The mAbs generated in this study were isotyped. Three mAbs (nos.7, 12 and 20) were found to be IgG_{2b} and five were IgG_1.

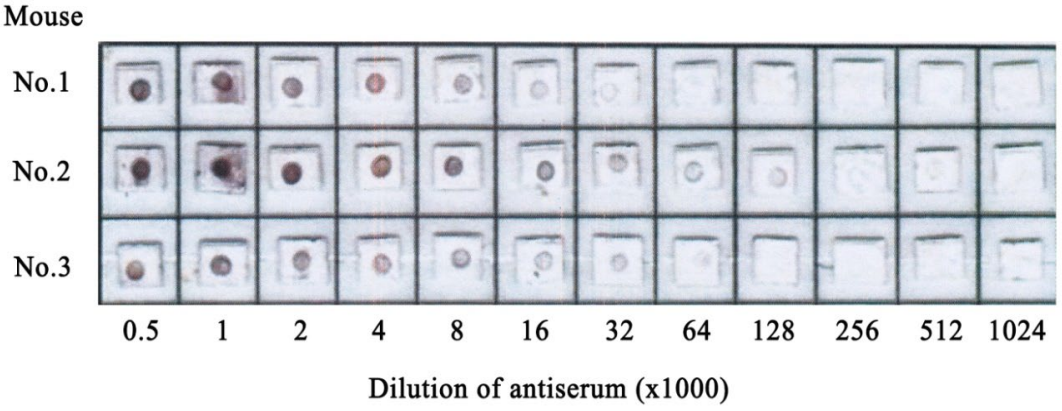

Figure 1. Representative dot-blots showing antibody titre against *P. olseni* hypnospores from the antisera of immunized mice

The sensitivity of each selected mAb was determined using a serial dilution of *P. olseni* hypnospores or zoospores, with a concentration ranging from approximately 10^3 to 10^7 cells/mL. The hypnospores or zoospores were then applied to a nitrocellulose membrane as the antigen target and incubated with each mAbs by dot blotting technique. The levels of sensitivity of each mAb were similar, with immunoreactivity against hypnospores detected at approximately 10^6 cells/mL, and with weak cross reactivity against zoospores identified at approximately 10^7 cells/mL (Figure 2).

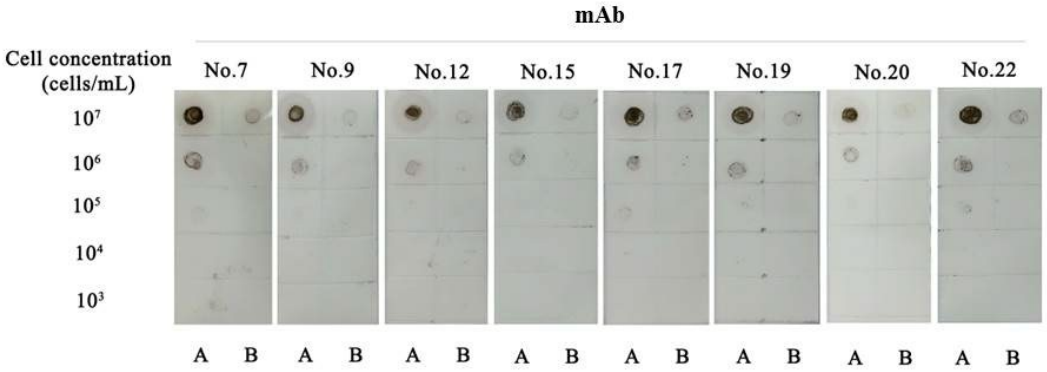

Figure 2. Representative dot-blots showing the sensitivity of the mAbs against different concentrations of A: hypnospores; and B: zoospores of *P. olseni*

To assess the possibility of the binding of mAbs to the trophozoite stage in *P. olseni*-infected clams using IHC, *P. olseni*-infected clams were sectioned and incubated with the mAbs obtained in this study. Monoclonal antibodies (mAbs) no. 7 and no. 20 showed the strongest immunoreactivity, with brown staining around the surface of trophozoite cells, while the other mAbs showed only weak staining. Representative images of the IHC staining are shown in Figure 3. We, therefore, used mAbs no. 7 and no. 20.

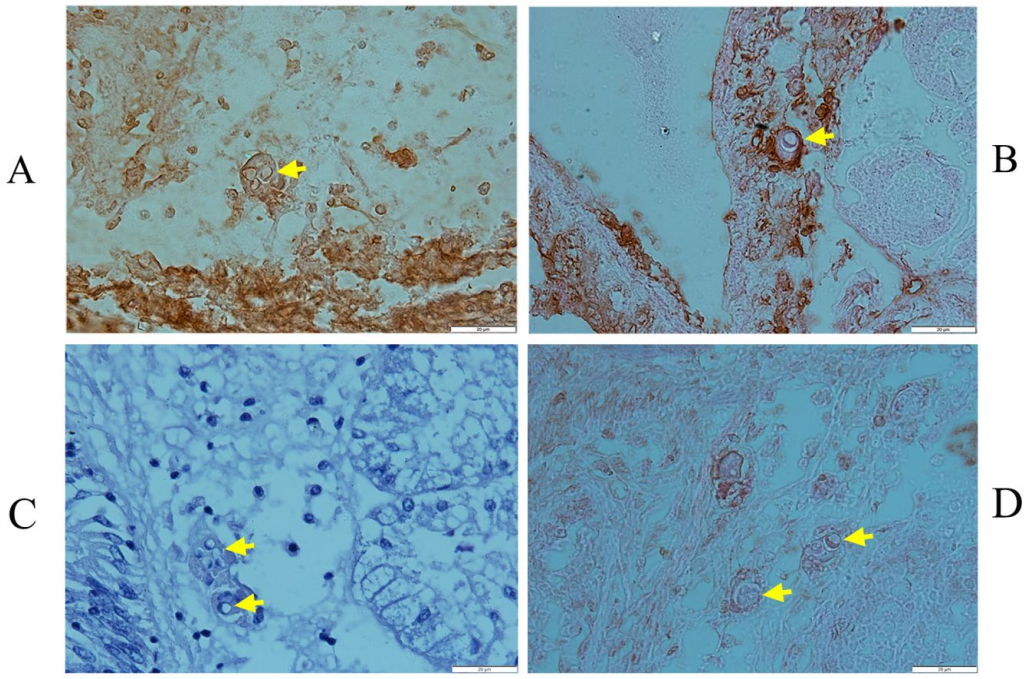

Figure 3. Representative IHC images showing a section of the digestive gland of *P. olseni*-infected clams (*Pa. undulata*) incubated with (A) mAb no. 7 or (B) mAb no. 20. Immunoreactivity is apparent as a brown stain surrounding the surface of trophozoite cells (yellow arrows) (C) H&E staining (D) No staining with mAb. Scale bar, 20 μm.

In this work, we were able to establish IgG class mAbs to *P. olseni* with a high antibody titre, using mice immunized with hypnospores. This finding differs from the findings of previous research, in which mice were immunized with zoospores resulting in the antibody titre shown in the lower range and identified as IgM class mAbs [20]. This difference may be due to the effects of the size of the immunogen; it is possible that the larger hypnospores cells induce a stronger immune response than that induced by smaller cells. Our results were similar to those presented in a previous report, which indicated that subcellular components of *P. marinus* hypnospore cells were good immunogens [17]. Most of the generated in this research showed reactivity against both hypnospores and zoospores in dot blot assays, and it is therefore possible that they may bind to a common surface epitope. However, only mAbs no. 7 and no. 20 appeared to be suitable for recognising trophozoite cells in *P. olseni*-infected clam tissue using IHC.

The standard examination method for detection of *Perkinsus* spp. in marine bivalves is the RFTM culture assay, due to its low cost and simplicity of use [21, 22]. However, this assay is not

species specific [23] and also stains the hypnospores of phytoplankton [24]. Recently, novel mAbs against *Perkinsus* spp. were developed, including mAbs against *P. marinus* [18, 19] and *P. olseni* [20].

Monoclonal and polyclonal antibodies to *Perkinsus* spp. were developed in the course of several previous investigations. Both mAbs and pAbs were produced against the oyster pathogen *P. marinus* using hypnospores as the immunogen. Polyclonal antibodies (pAbs) bound to hypnospores produced *in vitro* were produced as well as those against mature trophozoites and proliferating immature trophozoite rosettes in fixed tissue sections. The mAbs thus produced recognised epitopes that appeared unique to the hypnospore [17]. Two mAbs specific for *P. marinus* were generated as immunogens against trophozoites but were immunoreactive with not only *P. marinus* trophozoites and their protein lysates, but also with trophozoites from *P. atlanticus* and against the hypnospore and zoospore stages of *P. marinus* [19]. Monoclonal antibodies (mAbs) were produced using extracellular products from an *in vitro* culture of the oyster parasite *P. marinus* as the immunogen. The mAbs specifically bound to trophozoite and tomont walls. These mAbs were used to investigate morphological and antigenic changes in the walls during the RFTM-induced formation of hypnospores [18].

Since a *Perkinsus* species is known to occur in commercial and natural cultures of undulated surf clams, *Pa. undulata*, in the Gulf of Thailand, we attempted to find a method with which to detect this pathogen specifically. Immunological methods are widely used for pathogen detection in aquaculture, and this approach was considered to be promising, due to its high sensitivity and specificity of antibodies to species-specific antigens. Monoclonal antibodies (mAbs) are homogenous in specificity and can be produced in unlimited quantities. The mAbs obtained in this study, generated from mice immunized with *P. olseni* hypnospores, can also be applied to the detection of *P. olseni* trophozoites in infected clam tissues using IHC. Therefore, the mAbs generated against *P. olseni* in this study can be used as a simple tool for identifying species of *Perkinsus* using IHC, in contrast to the traditional H&E staining method. However, a limitation of these mAbs is that they have only been tested on *P. olseni* in the clams that were reported [6] and could be cultured in our laboratory. We did not have other *Perkinsus* species that could be used for testing cross reactivity. In order to prove the reliability and specificity of the method, the results showed no cross reactivity with phytoplankton sample in preliminary study.

4. Conclusions

In the study reported here, eight monoclonal antibodies for *P. olseni* from mice immunized with hypnospores of *P. olseni* were successfully produced and characterized. The mAbs no. 7 and no. 20 were immunoreactive with trophozoite cells of *P. olseni* in infected clam tissues by IHC. These findings indicate that the developed mAbs to *P. olse*ni can be useful for *P. olseni* detection and for monitoring *P. olseni* infections in commercial cultures of *Pa. undulata* clams. Additionally, identification of *Perkinsus* species is important for risk assessment and disease management in commercial clam aquaculture in Thailand and elsewhere.

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