Production and characterization of monoclonal antibody against *Perkinsus olseni* in undulated surf clams *Paphia undulata*

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Abstract

Perkinsosis is a mollusk disease caused by protozoan parasite belonging to the genus Perkinsus. Perkinsosis has been found in some commercially important mollusks including oysters, clams and abalones. Heavy infection with Perkinsus often results in tissue inflammation and mass mortalities. To date, three stages have been described in the pathogen's life cycle: trophozoite, hypnospore and zoospore. The infections in mollusks cannot be diagnosed without specialized testing. Various methods have been applied in the diagnosis of these infections including the fluid thioglycollate medium (FTM) technique, immunological and PCR assays. In this study, monoclonal antibodies (MAb) for immunological assay against Perkinsus olseni were produced and characterized. Three ICR mice were immunized with P. olseni in the zoospore stage. The polyclonal antiserum showed the antibody titer of 1:128,000 by dot blotting. After three fusions of the splenocytes and myeloma cells, two hybridoma clones producing MAbs (2/B4/A2 and 8/H11/F2) against zoopores were obtained. The isotype of the MAbs 2/B4/A2 was IgG1 while that of 8/H11/F2 was IgM subclass. Only MAb 8/H11/F2 showed immunoreactivity with the zoospores and hypnospores stage. Both MAbs can be used to identify zoospores P. olseni from clams by dot blotting with the sensitivity range of 10^8–10^9 cell/ml.

Keywords: Perkinsosis, *Perkinsus olseni*, *Paphia undulata,* monoclonal antibody, dot blotting

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1. Introduction

Perkinsosis is a bivalve disease caused by protozoan parasite belonging to the genus *Perkinsus*. The disease is also believed to be associated with mass mortalities of economically mollusks such as oysters, clams, abalones and scallops (Choi and Park, 2010). Infection with *Perkinsus* spp. is frequently lethal in susceptible mollusks, though an infected animal may live for several years before showing clinical signs of disease or abruptly dying. In Asian oceans, the prevalence of *Perkinsus olseni* were reported in the clams *Ruditapes philippinarum* (Korea, China and Japan). In addition, *P. olseni* could be identified in the undulated surf clams *Paphia undulata* in Thailand (Leethochavalit *et al.,* 2004).

Life cycle of *Perkinsus* species consists of three stages; two within the host, the trophozoite and hypnospore stages, and one outside the host, the zoospore stage (Figure 1). All of stages cause tissue damage in the mollusks and are infective. *Perkinsus* trophozoite multiplies in a vegetative form in host tissue. When trophozoites release immature trophozoite, they may continue to infect other tissues, or they may be released into the water in feces or when the host died. In an anaerobic condition such as in fluid thioglycollate medium or necrotic tissues, the trophozoite greatly enlarges and develops a new form of hypnospore or prezoosporangia. Hypnospores are characterized as enlarged cell size and thick cell wall that stain dark blue or brown with iodine. When hypnospores are transferred in aerated seawater, they release zoospores. The motile zoospore has two flagella, which are used to swim to new host. And when the host ingests zoospore, they become trophozoite (Denise, 2010). Experimental infection with *P. marinus* hypnospores and trophozoites could be found in oysters. One of stages which are believed to be infective stage is zoopores. *P. olseni* zoospores could be experimentally infected in the clams (Soudant et al., 2008).

Various methods are used to detect such as fluid thioglycollate medium (FTM) technique, immunological and PCR assays. As compared with other methods, immunological assay requires short analyzed time and gives high specificity. Polyclonal antibodies against *P. marinus* hypnospores in oysters have been reported (Choi *et al*., 1991). Nevertheless, these antibodies cross-reacted to several dinoflagellate species, particularly many of the parasitic dinoflagellates. Highly specific and sensitive monoclonal antibody (MAb) against *P. marinus* trophozoites was also produced. These MAb reacted against all parasite stages (Romestand *et al*., 2001). In addition, reported MAb production against hypnospores that did not react with other parasite stages was also reported (Dungan and Robertson, 1993).

Base on the specificity of antibody-antigen reaction, this research attempted to produce MAb against *P. olseni* using zoospores as immunogen and applied MAb to detect *P. olseni* in clam

tissues. Moreover, the obtained MAb will be useful for the parasite diagnosis in mollusk tissues by immunohistochemistry.

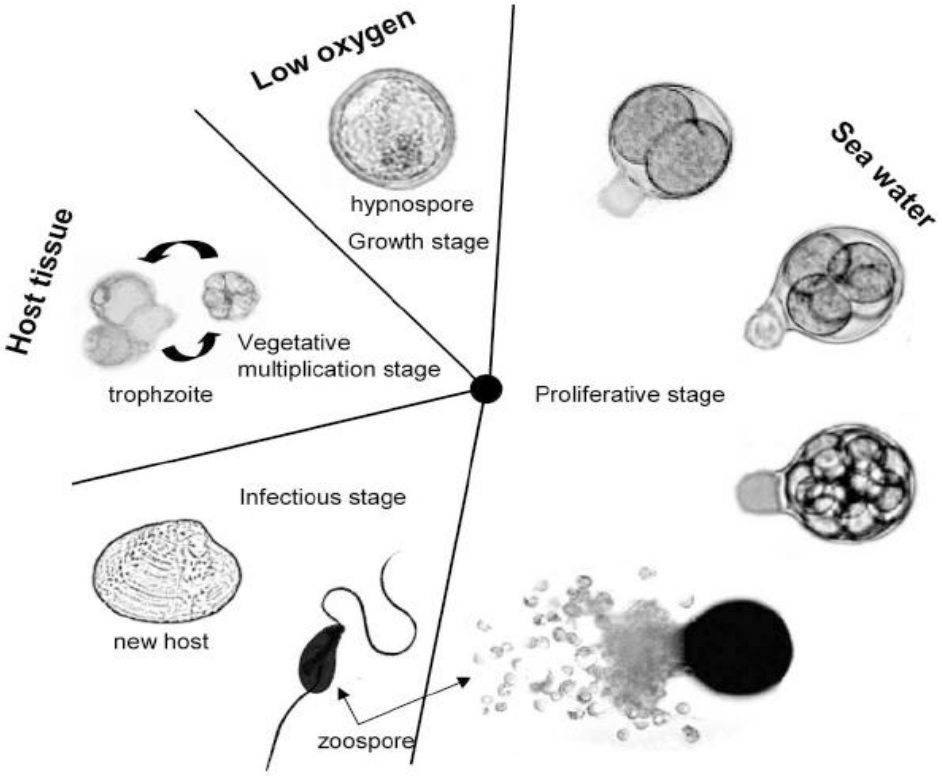

Figure 1 The life cycle of *P. olseni* (Choi and Park, 2010)

2. Materails and Methods

2.1 *Perkinsus olseni* culture

Perkinsus olseni hypnospores and zoospores were obtained from Institute of Marine Science, Burapha University.

2.2 Immunization of Mice

Three ICR female mice, 6 week old (National Laboratory Animal Centre, Mahidol University, Thailand) were immunized by intraperitoneally injections with 50 µl of zoopores (10^8 cell ml^{-1}) mixed with 50 µl of Freund's complete adjuvant (Sigma) at 1:1 (v/v). After three boosted doses of zoopores (50 µl) in Freund's incomplete adjuvant at 2 week intervals. One week after the fourth injection, mouse antisera were collected and then determined antiserum titer by dot blotting. Mice were final boosted with zoospores in PBS 3 days before hybridoma production.

2.3 Production of hybridoma

The immunized mice were sacrificed and their spleens were removed. The splenocytes were fused with myeloma cells using method described by Khamjing (2011). P3X myeloma cells were fused with spleen cells of the selected mouse using 50% (w/v) PEG as a fusogen. Fusion products were plated in 11 microculture plates (96 wells/plate). Hybridoma culture supernatants from each clone were subjected to screening by dot blotting against *P. olseni* zoospores. After identification of positive clones, cells were cloned by the limiting dilution method and stored in liquid nitrogen.

2.4 Screening of hybridoma by dot blotting

Culture supernatants from hybridoma cells were tested by dot blotting. The nitrocellulose membrane was spotted with *P. olseni* zoospores approximately 10^9 cell/ml at 1 µl. The membrane was dried at 37 ºC for 30 min and blocked in 5% skim milk at room temperature for 30 min. After washing with PBS containing 0.05% (v/v) tween-20 (PBST), the membrane was incubated with hybridoma culture supernatant from each culture for 2 h. The membrane was washed extensively with PBST and then membrane was incubated in horseradish peroxidase-conjugates goat anti mouse IgG heavy and light chain specific antibody (GAM-HRP) in the dilution of 1:3000 in PBS for 2 h. After washing with PBST, the membrane was treated with substrate mixture containing 0.06% hydrogen peroxide, 0.03% diaminobenzidine (DAB), 0.05% cobalt chloride in PBS for 5 min and the color reaction was stopped by washing with deionized water. All steps were incubated at room temperature. The positive result shows dark or reddish grey spot on nitrocellulose membrane. Positive control was sera of immunized mouse and negative control was sera of nonimmunized mouse.

2.5 Characterization of monoclonal antibodies (MAbs)

2.5.1 Isotyping of MAbs

The Class and subclass of immunoglobulins secreted by hybridoma cell lines were determined by the sandwich enzyme-linked Immunosorbent assay (Sandwich ELISA) using Sigma's Mouse Monoclonal Antibody Isotyping Kit (Sigma-Aldrich, USA).

2.5.2 Cross reactivity of MAbs

The cross-reactivity of MAbs was tested with *P. olseni* zoospores and hypnospores by dot blotting.

2.5.3 Sensitivity of MAbs by dot blotting

Serial dilution of zoospores (begin with 10^9 cell ml^{-1}) in PBS was performed and 1µl of each dilution was spotted onto a nitrocellulose membrane, then fixed for 30 min and processing for dot blotting.

3. Results and Discussion

3.1 Production of MAbs

After the fourth immunization, the polyclonal antibody titers from three mice were determined by dot blotting. The results were showed at the titer of 1:128000 (Figure 2). The fusion No. 1, 2 and 3 produced 950, 634 and 739 viable hybridoma clones (approximately 60-90% well yielded hybridomas), respectively. Two hybridoma, 2/B4/A2 and 8/H11/F2, which produced MAbs binding *P. olseni* zoospores clones were selected and recloned (Table 1).

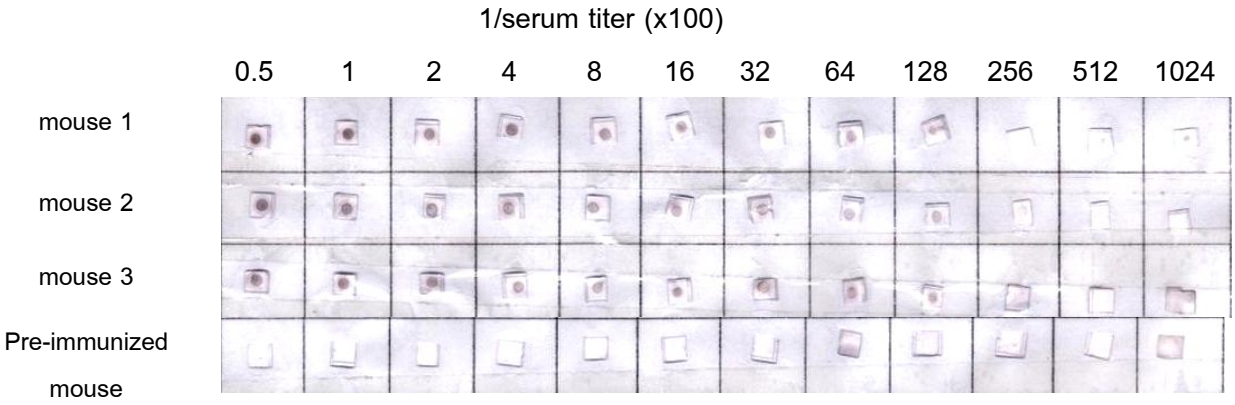

Figure 2 The antibody titers from immunized mouse No. 1-3 by dot blotting. Pre-immunized mouse serum was used as negative control.

Table 1 Numbers of hybridoma wells during fusion process

Hybridoma	Number of wells or clones		
	Fusion 1	Fusion 2	Fusion 3
Cultured hybridoma	1056	1056	1056
Obtained hybridoma	950	634	739
Hybridoma producing antibody against *P. olseni* zoospores	11	51	14
Stable hybridoma producing antibody against *P. olseni* zoospores	1 (2/B4/A2)	0	1 (8/H11/F2)

3.2 Characterization of MAbs

3.2.1 Isotyping of MAbs

The isotype of MAbs was determined using Sigma's Mouse Monoclonal Antibody Isotyping Kit. The adsorbance values of all MAbs for each fusion were measured at 450 nm. The highest adsorbance value represented the isotype of the MAb. From Table 2, MAb 2/B4/A2 was identified as IgG1 while MAb 8/H11/F2 was found as IgM.

Table 2 The adsorbance values at 450 nm of MAbs for isotype determination

MAb	A450					
	IgG1	IgG2a	IgG2b	IgG3	IgM	IgA
2/B4/A2	**3.142**	0.333	0.202	0.208	0.434	0.180
8/H11/F2	0.329	0.531	0.223	0.272	**3.659**	0.306

3.2.2 Cross-reactivity of MAbs

The MAb-producing clone 2/B4/A2 shows immunoreactivity with only zoospores whereas 8/H11/F2 shows immunoreactivity with both zoospores and hypnospores (Figure 3).

The binding of MAb-producing clone 8/H11/F2 *P. olseni* at the two difference stages was similar to the binding in the previous reports that attempt to develop PAbs against *P. marinus* hypnospores (Dungan and Roberson, 1993) and MAbs against *P. marinus* trophozoites (Romestand et al., 2001). Since each life stages of *Perkinsus* spp. has linked-differentiation, the binding specificity of the MAb indicates the presence of common epitopes shared by zoopores and hypnospores (Dungan and Roberson, 1993). Another reason is that the IgM isotype of 8/H11/F2 has a lot of antigen-binding fragments (Fab) in its pentamer, thus increasing the binding probability of antibody to several antigen epitopes. On the contrary, the MAb-producing clone 2/B4/A2 can bind to only zoospores without reacting against hypnospores.

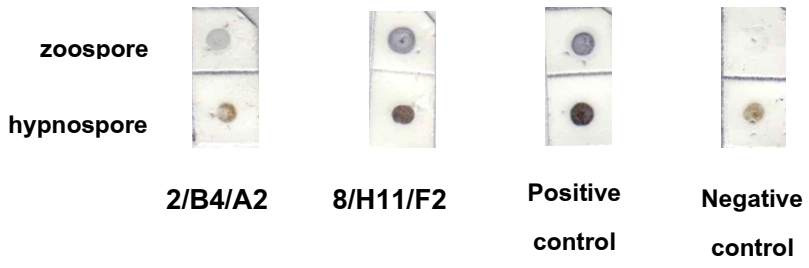

Figure 3 Cross reactivity of MAbs assayed by dot blotting. One microliter of the parasites (approximately 10^9 cells/ml) was spotted onto nitrocellulose membranes which were later treated with each MAbs. Positive control was polyclonal antibody and negative control was culture media.

3.2.3 Sensitivity of MAbs by dot blotting

Sensitivity of MAbs 2/B4/A2 and 8/H11/F2 were determined by dot blotting method. The positive results were based on the lowest antigen concentration causing the black or gray dots. The sensitivity of clones 2/B4/A2 and 8/H11/F2 was determined at approximately 10^9 and 10^8 cells/ml, respectively, as shown in Figure 4.

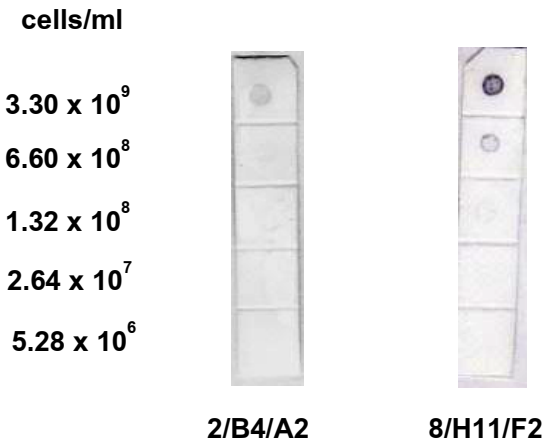

Figure 4 Sensitivity of the MAbs assayed by dot blotting method. One microliter of five-fold serial dilution of *P. olseni* zoospores was spoted onto nitrocellulose membrane which was later treated with MAbs at a dilution of 1:2.

4. Conclusion

This research was successful in the production of hybridoma clones producing MAb against *P. olseni* zoospores in undulated surf clams *Paphia undulata*. Two of MAb-producing hybridoma clones, 2/B4/A2 and 8/H11/F2**,** were selected and characterized. Both MAbs reacted to zoospores while only 8/H11/F2 also reacted to hypnospoes. All of monoclonal antibodies will be further characterized by immunohistochemistry technique to ensure the detection of *P. olseni* in mollusk tissues.

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