Occurrence of Perkinsus Species (Protozoa, Apicomplexa) from Manila Clam Tapes philippinarum in Japan

Yukio Maeno¹*, Tomoyoshi Yoshinaga² and Kazuhiro Nakajima¹

¹National Research Institute of Aquaculture, Nansei, Mie 516-0193, Japan
²National Research Institute of Aquaculture, Tamaki, Mie 516-0423, Japan

(Received April 19, 1999)

ABSTRACT — In April 1998, unidentified parasites were found in Manila clam Tapes philippinarum in an inner bay of the western part of Japan. Ray’s fluid thioglycollate medium (RFTM) culture of tissues from infected Manila clams yielded prezoosporangia of the parasite. Many free motile zoospores were released from the prezoosporangia when they were transferred to seawater. Histologically two types of trophozoites were observed: cells in a cluster and free single cells. They were found in the digestive gland, gill, mantle and foot of the clam. In immunohistochemical assays using an antiserum against Perkinsus marinus, the antibody reacted to both types of trophozoites. These results indicated that these parasites in Manila clam were species belonging to the genus Perkinsus. However, they differed from previously described species in the morphology of trophozoites and prezoosporangia.

Key word: Perkinsus, Manila clam, Tapes philippinarum, Apicomplexa

The species of the genus Perkinsus are known to parasitize marine and estuarine molluscs and have been associated with massive mortalities in cultured and natural populations of molluscs (Andrews, 1988; Perkins, 1993). Perkinsus marinus infects the American oyster Crassostrea virginica along the east coast of America and Gulf of Mexico (Mackin et al., 1950; Andrews, 1988; Andrews and Ray, 1988). Perkinsus olseni has been associated with mortality in abalone Haliotis ruber from Australia (Lester and Davis, 1981). In Canada, Perkinsus karlsoni was described in scallops Argopecten irradians (McGladdery et al. 1991), and Perkinsus qugwadi was reported in cultured Japanese scallops Patinopecten yessoensis (Blackbourn et al., 1998). In Portugal and Spain, Perkinsus atlanticus was described in cultured clams Tapes decussatus (Azevedo, 1989; Auzoux-Bordenave et al., 1995; Ordas and Figueras, 1998). Perkinsus spp. have been reported in 67 species of molluscs, all bivalves except four species of gastropods (Perkins, 1993). However, little is known about the epizootiology of Perkinsus sp. or spp. from these molluscs.

In April 1998, samples of Manila clam Tapes philippinarum were collected in an inner bay in the western part of Japan, and examined histologically for parasites. Unidentified organisms were found in the clam tissues. In this paper, we describe the development of prezoosporangia in Ray’s fluid thioglycollate medium (RFTM) culture, zoosporulation in seawater, histological features of the organisms from Manila clam and immunoassays with anti-Perkinsus marinus polyclonal antibodies. Moreover, taxonomical relationships are discussed between the present species and five previously described Perkinsus species.

Materials and Methods

Clams

Manila clams Tapes philippinarum used in this study were collected in cultured clam beds in an inner bay of the western part of Japan in April 1998.

Ray’s fluid thioglycollate medium (RFTM) culture

Pieces of the gill and mantle of clams were incubated in RFTM containing 500 units/mL each of penicillin and streptomycin at 20°C in the dark for 8 days to induce the formation of the prezoosporangia. The incubated pieces were stained with 20% Lugol’s iodine solution for detection of prezoosporangia and observed under a microscope. A whole body diagnosis technique developed for P. marinus in oyster (Fisher and Oliver, 1996)
was employed with some modifications to quantify the parasites in each clam. Briefly, each shucked clam was weighed and homogenized in 20 mL of the RFTM in a 50 mL tube. The tube was incubated in the dark for 7 days at 20°C and centrifuged. The sediment was digested in 2 M NaOH at 60°C and washed with distilled water. The prezoosporangia in each tube were stained with 4% Lugol's iodine solution and counted by serial dilution in a 96 well plate.

Observation of zoosporulation

Gill and mantle of clams were incubated in RFTM and minced with scissors. Prezoosporangia were collected from the minced tissues and washed in sterilized seawater containing 500 units/mL each of penicillin and streptomycin by repeated transfer with micropipettes. Washed prezoosporangia were incubated at 25°C in antibiotics-containing seawater in 96 well plates for up to 14 days and observed with a microscope everyday.

Histology

Eleven clams (20–40mm in length along the major axis) were shucked and soft tissue were fixed whole in Davidson's solution. A transverse slice (approximately 5 mm thick) including digestive gland, gill, mantle and foot was removed from each clam. The tissue slice was embedded in paraffin wax, sectioned at 4 μm thickness, and used for histological and immunohistochemical examinations. For histological analyses, sections were processed according to standard techniques and stained with Mayer's hematoxylin and eosin (H-E).

Immunohistochemical assay

Immunohistochemical assays were conducted with anti-Perkinsus marinus polyclonal antibodies to determine if the organisms were antigenically similar to P. marinus. Antibodies were generously supplied by Mr. C. F. Dungan, Cooperative Oxford Laboratory, Oxford, MD, USA. The production and specificity of the antibody was described by Dungan and Roberson (1993). For immunohasay, 4 μm sections were affixed to separate glass slides, paraffin wax removed in xylene, and preparations hydrated in a graded ethanol series to water. The slides were incubated with anti-Perkinsus marinus IgG or normal rabbit serum as a control (10 μg/mL and 1:100 dilution, respectively) for 30 min at 37°C, and developed using a Histofine SAB-PO Kit (Nichirei, Japan). Immunostained sections were counterstained with Mayer's hematoxylin, dehydrated in ethanol, cleared in xylene and coverslipped as permanent preparations.

Results

RFTM culture

Perkinsus prezoosporangia, which were stained blue-black with Lugol's iodine solution, were observed in the mantle and gill of all of 14 clams examined by RFTM culture (Fig. 1). The prezoosporangia reached up to 60 μm in diameter after incubation for 8 days. The numbers of prezoosporangia ranged from 1.3 x 10^3 cells to 8.7 x 10^5 cells/g tissue.

Observation of zoosporulation

Prezoosporangia showed formation of zoospores, when incubated in seawater. Several stages of the zoosporulation are shown in Fig. 2. From the first day in seawater, the vacuole of prezoosporangia became granular, and a pore was found in the cell wall followed by formation of a short discharge tube (Fig. 2a). On the second day, the cytoplasm contracted, releasing intravacuolar fluid to the space between the cell wall and the cytoplasm, and moved into the center. On the fourth day after the beginning of the binary division, zoospores swam into the seawater through the discharge tube following breakage of a plug of wall material located in the pore at the base of the tube (Fig. 2d).

Histology

No external sign of disease was observed in the clams examined when shells were opened. Histological examination of stained sections revealed the presence of organisms in the digestive gland, gill, mantle and foot of Manila clams. The parasites were more frequently observed in the digestive gland and gill than in the mantle and foot. In digestive gland and gill, 100% (11/11) of the clams were infected with the parasites. Moderate infections were observed in mantle (55%) and foot (36%). The infected Manila clams contained two different types of trophozoites. One was comprised of a cluster (Fig. 3) of twenty cells (range 7.6–11.4 μm in
Perkinsus spp. from Manila clam in Japan

Fig. 2. In vitro zoosporulation of Perkinsus sp. following transfer from RFTM culture to seawater. Live preparation viewed by phase contrast microscopy. a, Formation of discharge tube (arrowhead); b, 2-cell stage; c, 16-cell stage; d, release of zoospores, arrow indicating a breakage of a plug located in the pore at the base of the discharge tube. Scale bar = 50 μm.

Fig. 3. Cluster of Perkinsus sp. in connective tissue of digestive gland, showing the cytoplasm occupied by vacuoles (arrows) and eccentric nucleus. Note infiltration of host hemocytes into affected tissues surrounding the parasite (arrowheads). H-E staining. Scale bar = 20 μm.

Fig. 4. Single cells (arrows) in gill tissue showing the eosinophilic cytoplasm. H-E staining. Scale bar = 20 μm. Inset, free single cells.

diameter; average 9.2 μm; S.D. 1.6 μm). These cells were spherical with a basophilic cytoplasm. They often contained a vacuole (range 3.8–7.6 μm in diameter; average 5.8 μm; S.D. 1.4 μm; n = 20) that almost completely filled the cell, and a nucleus with a distinct nucleolus lying close to the basophilic cell wall. Infiltration of hemocytes was found in affected tissues surrounding the cell clusters. The other type was composed of a single cell (Fig. 4), which was eosinophilic and spherical (range 5.7–11.4 μm in diameter; average 8.2 μm; S.D. 2.1 μm; n = 20). No hemocyte infiltration was observed around the cells of this type.

Immunohistochemical assay

Assayed sections of Manila clam contained both types of trophozoites, i.e., cells in clusters and single cells. The trophozoite cells in clusters strongly reacted with anti-Perkinsus marinus antibodies (Fig. 5). Although the cytoplasm, nucleus and external surface of the cell wall were positively stained, the vacuole in the cytoplasm was not stained. A single trophozoite cell within the gill is shown in Fig. 6. Antibody strongly bound to the whole cytoplasm of the trophozoite. No color development of the trophozoite was seen for sections assayed by normal rabbit serum.
In recent years, the presence of Pekinsus sp. and Perkinsus-like organisms have been reported in the clams Ruditapes species (Figueras et al., 1992; Sagrista et al., 1995; Figueras et al., 1996). In this study, the organisms greatly enlarged in RFTM and stained blue-black with Lugol’s iodine solution. When the enlarged organisms were transferred to seawater, they developed zoospores. The formation of prezoosporangia in RFTM and the development of zoospores in seawater were similar to those of P. marinus (Perkins, 1996), P. olseni (Lester and Davis, 1981), and P. atlanticus (Azevedo, 1989; Auzoux-Bordenave et al., 1995; Ordas and Figueras, 1998). The results suggest that the organisms found in Manila clam in this study belong to the genus Perkinsus.

The histological observations in Manila clam indicated two types of trophozoites, i.e., cells in clusters and free single cells. The cells in clusters were spherical and some of them had an eccentric nucleus and large vacuole. They have morphological similarities to those of P. olseni (Lester and Davis, 1981), P. atlanticus (Azevedo et al., 1990), and the Perkinsus-like organisms from carpet-shell clams Ruditapes decussatus in Spain described by Figueras et al. (1992).

In immunohistochemical assays the anti-Perkinsus marinus antibodies bound to both types of trophozoites. The antibodies used for the immunohistochemical assays recognized P. atlanticus, P. olseni, and P. qugwadi as well as several Perkinsus species, but did not bind to P. karlssoni (Dungan and Roberson, 1993). The present immunohistochemical assays indicated that the two types of trophozoites in Manila clam were antigenically similar to those of Perkinsus species. However, no explanation could be given for the relationship between the two types of trophozoites in tissues of Manila clam in the present study. Furthermore it is unclear whether the present prezoosporangia found in RFTM and seawater were two types or not. There is a need for further studies to know whether they belong to one species or two different species.

The present species differs from P. marinus in several respects. The trophozoites of P. marinus have a smaller size ratio of vacuole to cytoplasm than the one in the present trophozoites (Perkins, 1988). Although prezoosporangia of Perkinsus species in Manila clam developed a short discharge tube in seawater, those of P. marinus had a well developed discharge tube. In the case of P. karlssoni, the size range of 4.3–7.6 μm given for trophozoites of P. karlssoni is similar to that of the present species (Table 1). However, the present species had a large vacuole in the cytoplasm. Polyclonal antibodies to P. marinus recognized the present species, but did not bind to P. karlssoni (Dungan and Roberson, 1993), indicating that the present species can be distinguished from P. karlssoni. There were some similarities between P. qugwadi and the present species in morphology of the trophozoites, however, P. qugwadi did not develop during culture in RFTM (Blackbourn et al., 1998). Therefore the present species are different form P. qugwadi. The present species had some resemblances to those described as P. olseni (Lester and Davis, 1981) and P. atlanticus (Azevedo, 1989; Auzoux-Bordenave et al., 1995). The trophozoites were spherical with a large vacuole, and greatly enlarged in anaerobic medium, and when returned to seawater developed zoospores as in the present species. However, the difference between the present species and P. olseni and P. atlanticus was the average diameter of the trophozoites (Table 1). The average of trophozoite of the present species is much smaller than that of both Perkinsus species. Recently, an infection of Perkinsus was found in Tapes philippinarum in southwestern Japan (Hamaguchi et al., 1998). The histological features, RFTM culture, and the nucleotide sequences in the internal transcribed spacer regions of the prezoosporangia suggested that the Perkinsus
protozoan was *P. atlanticus* or a closely related species. The trophozoites of the *Perkinsus* protozoan are larger than those of the present species (Table 1). In addition, the maximum size of the prezoosporangia in RFTM culture is much larger (6.3–225.3 μm) than that of the present species. Thus, from the comparison of morphological characteristics of trophozoites and prezoosporangia, it is probable that the present species differ from all the previously described *Perkinsus* species.

The pathogenicity of the present species in Manila clam has yet to be clearly established. Further studies should be conducted to clarify its possible role as a potential pathogen for clam culture.

**Acknowledgement**

The authors gratefully acknowledge Mr. C. F. Dungan, Cooperative Oxford Laboratory, for providing polyclonal antibodies to *Perkinsus marinus*.

**References**


