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In vitro propagation of two Perkinsus species from the softshell clam Mya arenaria

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IN VITRO PROPAGATION OF TWO *PERKINSUS* SPECIES FROM THE SOFTSHELL CLAM *MYA ARENARIA*

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Summary :

Two continuous, axenic cultures of *Perkinsus* spp. (H49 and G117) were obtained from the softshell clam *Mya arenaria* collected from Swan Point in the Chester River, Chesapeake Bay (Maryland).

Isolate H49 was obtained from the hemolymph of a heavily infected clam. Except for their larger size, H49 trophozoites and schizonts exhibited the characteristic morphology of *Perkinsus marinus* and divided by schizogony. Isolate G117 was obtained from a combined gill/palp sample of a moderately infected clam. Unlike H49, vegetative forms (trophozoites and schizonts) of G117 were present along with prezoosporulation stages in the same culture. In culture, G117 cells multiplied by both schizogony and successive bi-partition.

Both H49 and G117 cells reacted positively with anti-*Perkinsus marinus* polyclonal serum and formed hypnospores upon incubation in Ray's fluid thioglycolate medium that stained black with Lugol's iodine. This is the first isolation of *Perkinsus* species from the softshell clam. Studies to determine the infectivity, pathogenicity, enzyme activities, and genotyping of both softshell clam *Perkinsus* spp. are ongoing.

KEY WORDS : *Perkinsus* spp., softshell clam (*Mya arenaria*), *in vitro*, zoosporulation.

Résumé : PROPAGATION *IN VITRO* DE DEUX ESPÈCES DE *PERKINSUS* DE LA PALOURDE À COQUILLE LISSE, *MYA ARENARIA*

Deux cultures axéniques permanentes de Perkinsus *spp. (H49 et G117) ont été obtenues de la palourde à coquille lisse* Mya arenaria, *récoltée à Swan Point dans le fleuve Chester, dans la baie de Chesapeake (Maryland).*

L'isolat H49 a été obtenu de l'hémolymphe d'une palourde hautement infestée. Mise à part leur grande taille, les trophzoïtes et les schizontes H49 présentent la morphologie caractéristique de Perkinsus marinus *et se divisent par schizogonie. L'isolat G117 a été obtenu à partir d'un échantillon combiné de lamelles et de palpes d'une palourde modérément infestée. Contrairement à H49, les formes végétatives (trophozoïtes et schizontes) de G117 co-existent avec des stades de pré-zoosporulation dans la même culture. En culture, les cellules G117 se multiplient par schizogonie ainsi que par des divisions en deux successives. H49 et G117 ont réagi positivement avec un serum polyclonal anti-*Perkinsus marinus *et ont formé des hypnospores lors de l'incubation dans le milieu fluide au thioglycolate de Ray, qui se sont colorées en noir sous Lugol. Il s'agit du premier isolement d'une espèce de* Perkinsus *à partir de palourde à coquille lisse. Les études se poursuivent pour déterminer l'infectivité, la pathogénicité, l'activité enzymatique et le génotypage des deux espèces de* Perkinsus *obtenues de la palourde à coquille lisse.*

MOTS CLÉS : Perkinsus *spp., palourde à coquille lisse* (Mya arenaria), in vitro, *zoosporulation.*

INTRODUCTION

Protozoa of the genus *Perkinsus* cause serious diseases in several bivalve mollusks such as: *Perkinsus marinus* in the eastern oyster *Crassostrea virginica* (Mackin *et al.*, 1951), *P. olseni* in the blacklip abalone *Haliotis ruber* (Lester & Davis, 1981), *P. atlanticus* in the Portuguese clam *Ruditapes decussatus* (Azevedo, 1989), and *Perkinsus*. sp in the bay scallop *Argopecten irradians* (McGladdery *et al.*, 1991).

Most recently, McLaughlin & Faisal (1998) reported infection with *Perkinsus* spp. in the softshell clam *Mya arenaria* of the upper Chesapeake Bay. The disease was characterized by the presence of clusters of *Perkinsus* spp. cells embedded in structureless, eosinophilic substrates, with their peripheries walled by granulocytes. The gills appeared to be the major site of infection by *Perkinsus* spp. in the softshell clam; however, abscesses containing *Perkinsus* spp. trophozoites were also noticed in the digestive gland and gonads of heavily parasitized clams. The species of *Perkinsus* causing this infection, however, remains to be identified.

The recent success in propagating *Perkinsus spp. in vitro* has allowed the study of their life cycles (La Peyre *et al.*, 1993; Kleinschuster *et al.*, 1994; La Peyre & Faisal, 1995a), identification of virulence factors (La Peyre & Faisal, 1995b; La Peyre *et al.*, 1995), determi-

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nation of the role of secretory products for establishment of infection (Garreis *et al.*, 1996; La Peyre et *al.*, 1996), and has provided a basis for chemotherapeutant trials (Faisal *et al.*, in press). Therefore, the major thrust of this study was to isolate *Perkinsus* spp. from tissues of infected softshell clams and to develop immortal axenic cultures of these isolates.

MATERIALS AND METHODS

Collection of softshell clams

Softshell clams *Mya arenaria* ranging in shell length from 62 mm to 85 mm (average length = 70.1 ± 5.5 mm) were collected by hydraulic escalator dredge from the Chester River (Maryland, USA) at Swan Point (39° 08' 00.1" N and 76° 16' 24.1" W) in October of 1996. Water temperature at Swan Point was 15.6 °C and salinity was 2 ppt at the time of collection. The clams were transported on ice to the wet laboratory facility at the Cooperative Oxford Laboratory (COL), Oxford, Maryland, and held until processed in 76-L glass aquaria supplied with either running Tred Avon River water or artificial seawater at 6-15 °C and 11-12 ppt salinity. A subsample of Swan Point clams was transported on ice to the Virginia Institute of Marine Science (VIMS) and was either held in the refrigerator at 4 °C or placed in recirculating aquaria containing artificial seawater (15 ppt) until processed. Clams were allowed to depurate for three to four days in sterile-filtered (0.22 µm) artificial seawater. In order to minimize microbial contaminants, the clams were brushed thoroughly twice daily and then transferred to sterile seawater.

Initiation of cultures from the hemolymph

Hemolymph was withdrawn with a sterile 3 ml hypodermic syringe, equipped with a 20-gauge needle, from the anterior adductor muscles of the softshell clams. The needle was inserted 5-10 mm into the muscle and 0.5-2 ml of hemolymph was obtained. Hemolymph samples were centrifuged (250 g, 10 min) and the pellet resuspended in an antibiotic mixture that consisted of chloramphenicol (50 mg/L), gentamycin (500 mg/L), kanamycin (1 g/L), penicillin (1,000,000 U/L), polymyxin B (500 mg/L), streptomycin (1 g/L), and rifampicin (50 mg/L) dissolved in sterile JL-ODRP-1 growth medium (La Peyre *et al.*, 1993). All antibiotics were purchased from Sigma Chemical Co. (St. Louis, Missouri). Following a 60-min incubation in the antibiotic mixture at room temperature, the cells were washed three times with JL-ODRP-1, and then resuspended in 10 ml medium. The cells were seeded in 25-cm^2 culture flasks (Corning Glass Works, Corning, New York, 4 ml/flask). The flasks were placed in atmospheric chambers supplied with ambient, 5 % carbon dioxide, or a gas combination of 80 % nitrogen, 10 % oxygen, and 10 % carbon dioxide. Incubation was attempted at 17, 20, or 28 °C for up to 15 days, and the flasks were examined daily.

Initiation of cultures from hypnospores using gills and palps

Gills and/or palps of infected clams were excised, rinsed in antibiotic mixture, minced finely with a razor blade, and then added to 3 ml fluid thioglycolate medium (RFTM, Sigma Chemical Co.) containing penicillin (50 units/ml) and streptomycin (500 µg/ml) as described by Ray (1952). The flasks were incubated for nine days at 23 °C, tissues were then further macerated, placed in 5 ml JL-ODRP-1 growth medium, and incubated at 28 °C for six days. Cultures were centrifuged and resuspended in fresh JL-ODRP-1 culture medium at a concentration of 10^5 hypnospores/5 ml medium. A subsample of macerated tissues was stained with Lugol's iodine solution and examined for characteristic blue-black cells. Incubation then continued at 28 °C (with or without 5 % carbon dioxide tension) for up to 15 days and the flasks were examined daily.

Maintenance of cultures and cloning

Subcultures were performed according to the methods described by La Peyre & Faisal (1996). Viability was evaluated by staining with 0.005 % (w/v) neutral red. Following three subcultures, single cell cultures were initiated using a combination of the standard limiting dilutions and micromanipulation procedure (Freshney, 1994). Cultures dominated by *Perkinsus*-like protozoal cells were serially diluted using fresh growth medium. From appropriately diluted cultures (1 cell/200X microscopic field), a single cell was picked using a sterile micropipette. Each cloned cell was then placed in one well of a 96-multiwell tissue culture plate (Corning) to which 50 µL fresh and 150 µL conditioned tissue culture medium were added. Following three weeks of incubation at 28 °C (5 % CO_2), the cloning procedure was repeated on selected cultures. Subculturing of cloned isolates was repeated every four-six weeks.

Characterization of cloned isolates

Two continuously-propagated cloned isolates were selected for characterization: G117 (originated from the gills) and H49 (originated from hemolymph samples). Softshell clams from which the protozoal cells were isolated were processed for histological examination as described by McLaughlin & Faisal (1998).

The morphology and life cycle stages present in culture were identified using an Olympus (CK-2) inverted

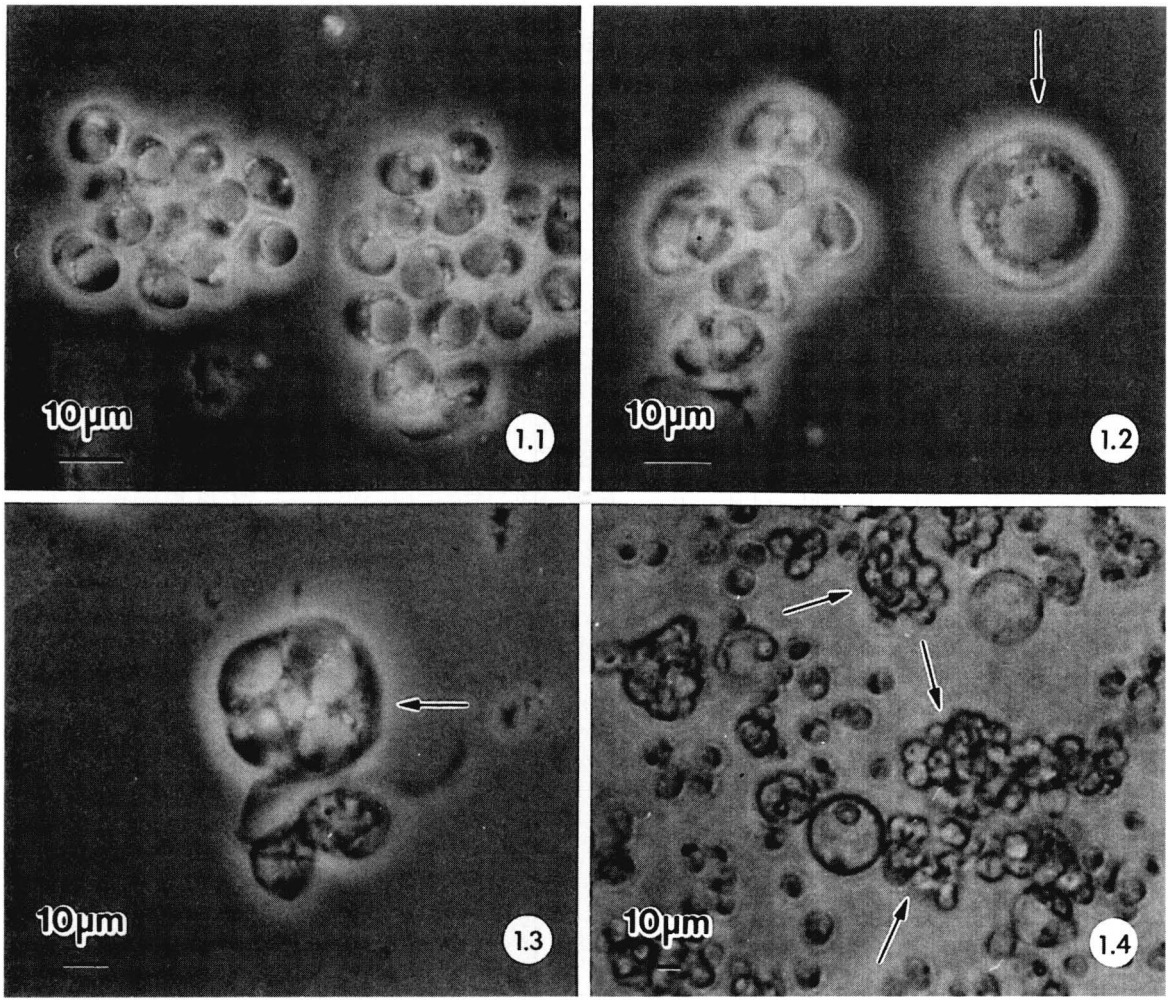


Fig. 1. – *In vitro* stages of the isolate H49 (7 days old, subculture 31). 1.1. Trophozoites. 1.2. Enlarged trophont (arrow). 1.3. Schizont containing daughter cells. 1.4. Clusters of daughter cells around ruptured mother cells.

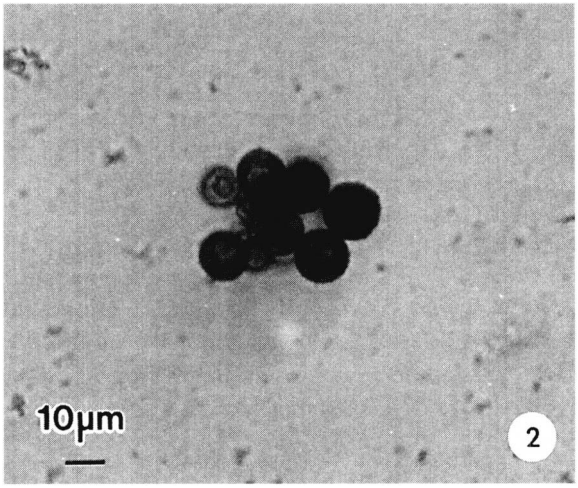


Fig. 2. — Hypnospores of H49 cells stained with Lugol's. Cultured cells in their fifth subculture were incubated in Ray's fluid thioglycolate medium for nine days.

light microscope equipped with phase contrast optics. The diameter of 200 cells from quadruplicate cultures of each isolate was measured using an ocular micrometer. The ability of cultured cells to form zoosporangia, which stained blue black with Lugol's solution after incubation in FTM, were tested as described above. The isolates were tested for reactivity with a polyclonal rabbit antiserum prepared against *Perkinsus marinus* Strain P1 (a kind gift from Dr. Stephen L. Kaattari, Virginia Institute of Marine Science, Gloucester Point, Virginia) using a standard ELISA assay.

RESULTS

Eight cultures of *Perkinsus* spp. were developed: one from the gills, one from the palps, one from a combined sample of gills and palps, and five from the hemolymph. Two of these cultures were selected for further subculturing and cloning; namely G117 originating from a combined sample of gills and palps from clam number 117, and H49, originating from the hemolymph of clam number 49. RFTM assays, and the examination of hematoxylin and eosin stained tissue sections, confirmed a moderate *Perkinsus* spp. infection in softshell clam # 117 and an advanced infection in clam # 49.

ISOLATE H49 (Figs. 1 and 2)

The original culture was incubated at 28 °C with 5 % CO_2 atmosphere. This isolate was cloned in the fourth subculture, and has been subsequently subcultured 48 times. Most of the cultured cells were oval or spherical and measured 9.4 ± 2.1 µm in diameter, and often formed clusters of more than 30 cells. Trophozoites exhibited several cytoplasmic, minute refractive bodies, and a prominent vacuole (Fig. 1.1). Some trophozoites enlarged up to 40 µm in diameter with the vacuoles occupying 30-50 % of the protoplasm (Fig. 1.2). Protoplast of large-sized trophonts often cleaved progressively yielding daughter cells whose numbers and sizes varied greatly. Schizonts measured 37.5 ± 12.7 µm and contained several daughter cells (Fig. 1.3). Daughter cells formed clusters of large numbers around ruptured mother cell walls (Fig. 1.4). No prezoosporulation stages were observed in cultures. The doubling time of H49 cells in culture was 34.7 ± 4.5 hours. Cultured H49 cells enlarged upon incubation in RFTM for nine days and stained black with Lugol's iodine (Fig. 2). Polyclonal antibodies against *P. marinus* reacted positively with H49 cells.

ISOLATE G117 (Figs 3, 4, 5, 6)

The original culture was incubated at 28 °C in ambient atmosphere. This isolate was cloned in the ninth week

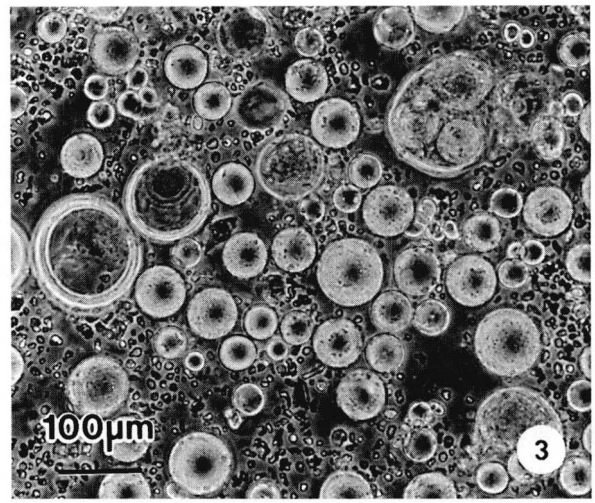


Fig. 3. – Culture of isolate G117. Notice the heterogeneity in cell size, cell wall thickness, and protoplasmic contents.

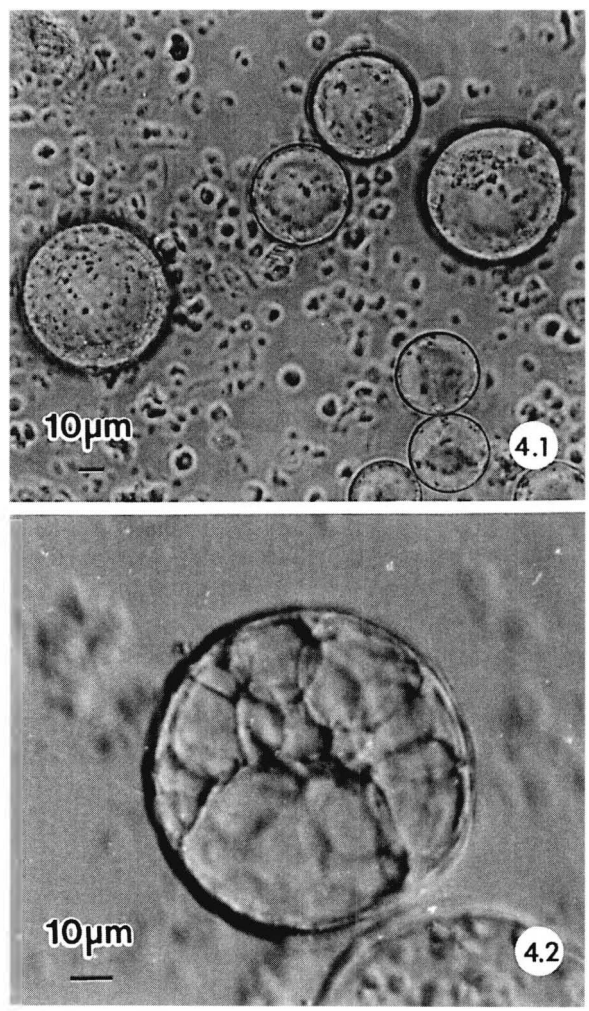


Fig. 4. – *In vitro* vegetative stages of the isolate G117 (14 days old, subculture 3). 4.1. Trophozoites. 4.2. Schizonts containing several daughter cells.

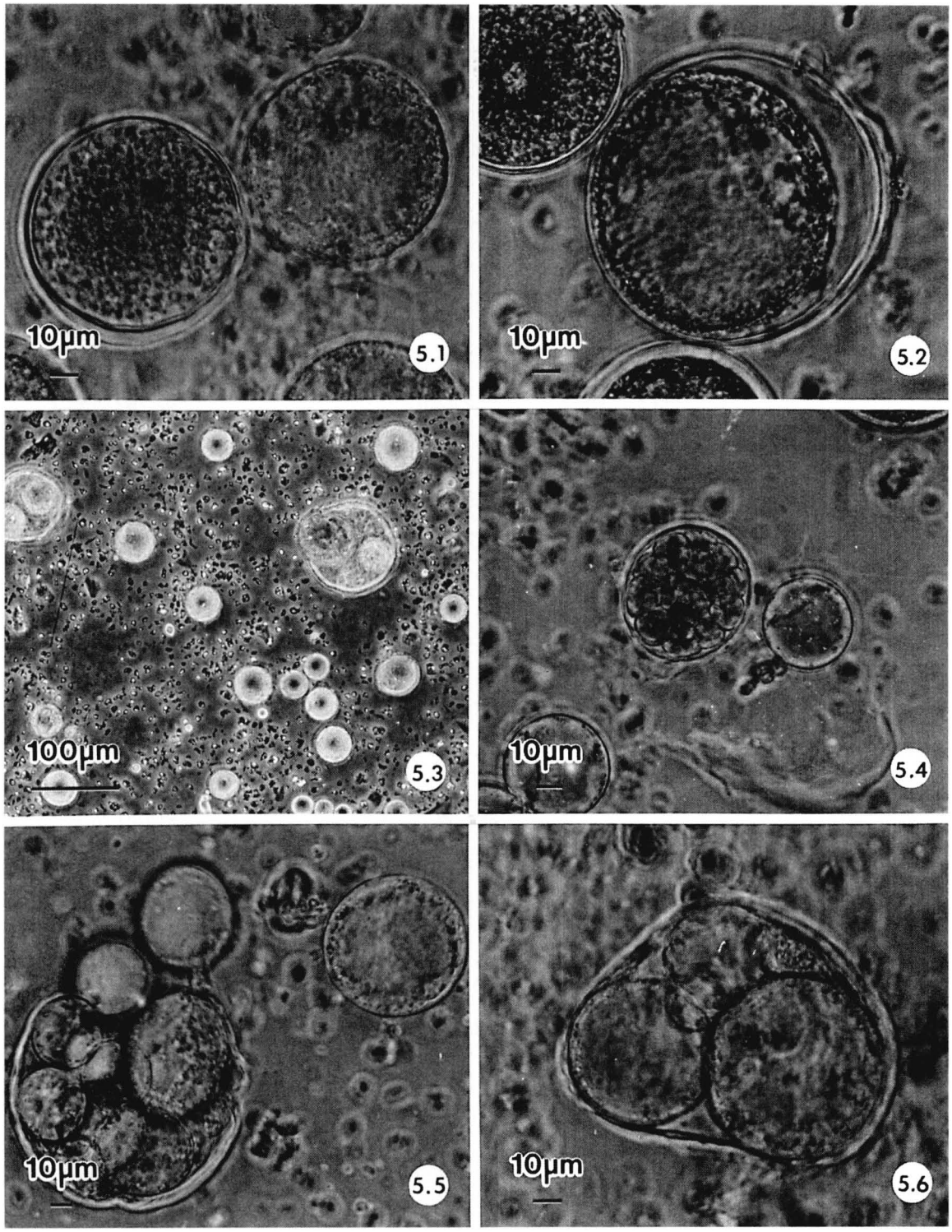
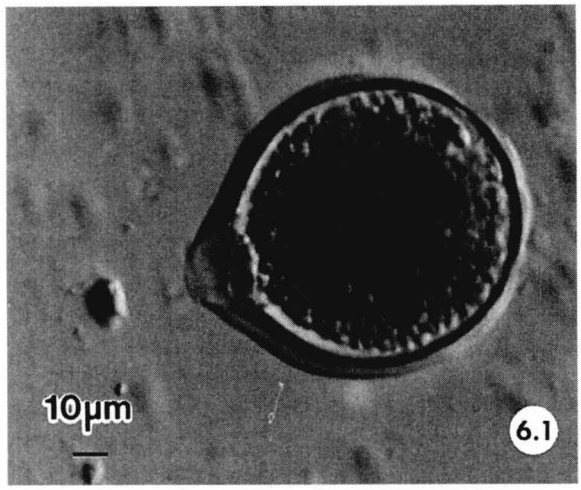
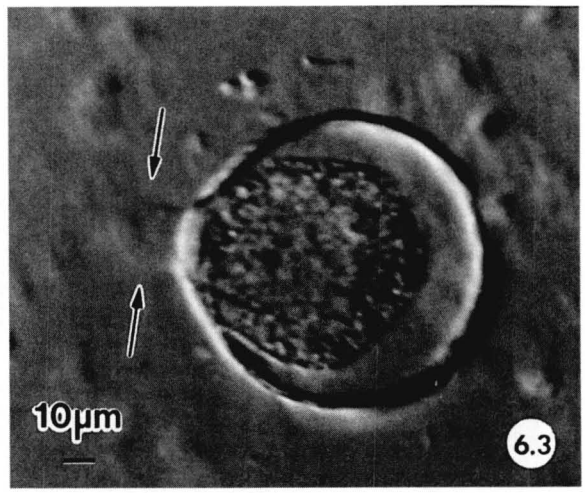


Fig. 5. – Formation of sporangia of G117 *in vitro*. 5.1. Enlarged trophozoite: note the granularity of the cytoplasm and the thickened cell wall. 5.2. Prezoosporangia: note contraction of the cytoplasm. 5.3 & 5.4. Prezoosporangia containing multiple cells. 5.5. Ruptured prezoosporangia releasing daughter cells. 5.6. Prezoosporangia with distorted walls due to enlarged daughter cells.





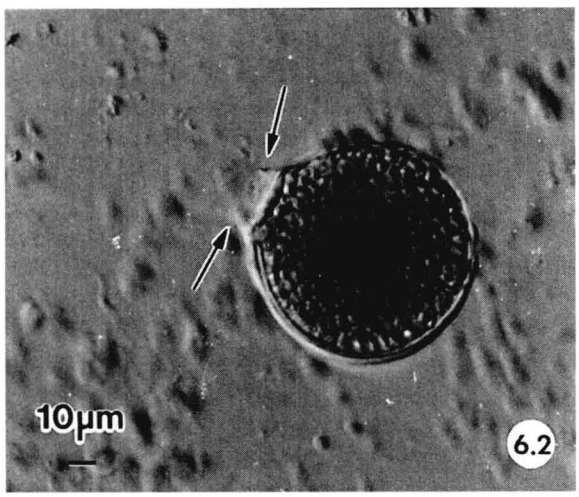


Fig. 6. – Zoosporulation of G117 *in vitro*. 6.1. Prezoosporangia filled with prezoospores. 6.2 & 6.3. Mature zoosporangia with discharge tubes.

of its primary culture and has been cultured 17 times to date. During incubation of the combined gill/palp sample of clam #117 in RFTM, parasitic cells appeared as spherical prezoosporangia that measured 33-87 µm in diameter, and stained black in Lugol's solution. Upon transfer of hypnospores to JL- ODRP-1 tissue culture growth medium, G117 cells developed vegetative stages, increased in number with a doubling time of 144 hours, and appeared extremely heterogenous in size, contour, thickness of cell walls, and cellular contents (Fig. 3). Unlike H49, vegetative forms (trophozoites and schizonts) of G117 were present along with prezoosporulation stages. Trophozoites were spherical in contour, possessed thin cell walls, varied in size from 8-50 µm (average 26.3 ± 17.2 µm) in diameter, and had vacuoles that occupied most of the protoplast (Fig. 4.1). Schizonts containing several daughter cells were observed and measured 69.3 ± 25.4 µm in diameter (Fig. 4.2).

Closer examination of the culture, however, showed that some of the trophozoites enlarged, their cytoplasm became more granular (Fig. 5.1), and formation of prezoosporangia was initiated. Prezoosporangia possessed thickened cell walls and contained several developmental stages of zoosporulation. As displayed in figure 5.2, the cytoplasm contracted centrally leaving a transparent zone between the cell wall and the protoplast. A series of successive bi-partitions then took place, with the subsequent development of prezoosporangia containing 2, 4, 8, 16, 32, 64 or more cells (Fig. 5.3, 5.4). Cell walls of 50 % of the formed prezoosporangia often ruptured during the cleavage process releasing several daughter cells (Fig. 5.5). The released daughter cells were heterogenous in size, measured from 4-50 µm, and had thin cell walls. Some daughter cells enlarged within the prezoosporangia, thus distorting the spherical appearance of the wall (Fig. 5.6).

Bi-partition often continued in prezoosporangia with the formation of hundreds of much smaller daughter cells (prezoospores, Fig. 6.1). Mature zoosporangia possessed one or more discharge tubes through which they eventually released zoospore stages (Fig. 6.2, 6.3). The cell walls of empty zoosporangia collapsed and persisted in the cultures.

G117 cells reacted positively with anti-*Perkinsus marinus* polyclonal serum.

DISCUSSION

The characteristic morphology, reactivity with anti-*P. marinus* serum, enlargement in RFTM, and division patterns clearly indicate that cultures G117 and H49 possess the characteristic features of *Perkinsus* spp. This is the first isolation of *Perkinsus* species from the softshell clam and is an important step towards understanding the pathogenic mechanisms of this group of Protozoa in bivalve mollusks. There were several morphological differences between the gill/palp isolates and those isolated from the hemolymph, despite the fact that some of these isolates originated from the same clam. Hemolymph isolates exhibited only the vegetative forms and schizogony exhibited by *P. marinus* in culture (La Peyre *et al.*, 1993). Trophozoites and schizonts of H49 were, however, larger than those of *P. marinus*.

Life cycle stages exhibited by isolate G117 share morphological similarities with those of a *Perkinsus* spp. isolated from the Baltic clam *Macoma balthica* collected from the Chesapeake Bay (Kleinschuster *et al.*, 1994). The same authors noted similarities between the *M. balthica Perkinsus* sp. and *P. atlanticus* isolated from *Ruditapes decussatus* (Azevedo, 1989). Despite the relatively high protein concentration in our culture medium, active zoosporulation was observed in G117 cultures. This process was, however, often interrupted and daughter cells were released from ruptured prezoosporangia. Moreover, all of G117 prezoosporulation stages observed in this study were identical to those described for *P. atlanticus* by Auzoux-Bordenave *et al.* (1995). In addition, several aspects of *P. atlanticus* pathology and response by host hemocytes (Chagot *et al.*, 1987; Montes *et al.*, 1995) are similar to what was observed in infected softshell clams (McLaughlin & Faisal, 1998). The transition between schizogony and successive bi-partition (zoosporulation) exhibited by the G117 isolate is noteworthy and further attests to the direct life cycle of *Perkinsus* spp.

Based only on data observed in this study, the species of softshell clam isolates cannot be fully identified. It is obvious, however, that H49 and G117 are distinct from each other. This observation was further confirmed by molecular typing (Kotob *et al.*, submitted), in which the sequences of the small subunit ribosomal RNA gene of the two isolates showed discrepancies. This is the first evidence that a coinfection by two *Perkinsus* spp. can exist in one molluscan species. Studies to determine the infectivity, pathogenicity, enzyme activities, and genotyping of both softshell clam *Perkinsus* spp. are ongoing.

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