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In vitro propagation of the alveolate protozoa *Perkinsus olseni* isolated from Manila clam *Ruditapes philippinarum* in Korea

Hyun-Sil Kang[1,2], Hyun-Ki Hong[1], Young-Ghan Choi[1,3], Hye-Mi Lee[1] and Kwang-Sik Choi[1]

[1]*Department of Marine Life Science (BK21 PLUS) and Marine Science Institute, Jeju National University, 102 Jejudaehakno Jeju 63243, Republic of Korea*

[2]*Subtropical Fisheries Research Institute, National Institute of Fisheries Science (NIFS), 6 Yeondaemaeul-gil, Jeju 63068, Republic of Korea*

[3]*Tidal Flat Research Center, National Institute of Fisheries Science (NIFS), 177-1 Sandannambuk-ro, Gunsan 54001, Republic of Korea*

ABSTRACT

The alveolate protozoan parasite *Perkinsus olseni* has a unique life cycle, including a mobile zoospore in an aerobic water column, vegetative trophozoite in the host tissues, and dormant hypnospore in an anaerobic environment such as decomposing host tissues or subsurface of the sediment. In this study, *P. olseni* trophozoites were induced from the zoospores *in vitro* using a Dulbecco Modified Eagle's:Ham's F-12 (DME/Ham's F-12, 1:2) fortified with antibiotics and supplemented with 5% fetal bovine serum, 50 mM HEPES buffer, 3.5 mM sodium bicarbonate, and 200 mM L-glutamine. In the growth media, *P. olseni* zoospores developed into trophozoites and reproduced within two weeks at 25 °C room temperature. During two weeks of culture, the trophozoites increased their cell size from a few microns to 34.4 ± 14.1 μm in diameter. Numerous small-sized daughter cells of the trophozoites could be observed 4 and 6 days after incubation, suggesting that the doubling time of the trophozoites in the media can be 4 to 6 days. The hypnospore stage and subsequent zoosporulation could also be induced from the trophozoite stage developed in the growth media, confirming that the trophozoites are vital, although they were produced *in vitro*. The Dulbecco's modified Eagle's:Ham's F-12 (DME/Ham's F-12, 1:2) growth medium was considered a method of choice in the mass production of *P. olseni* trophozoites *in vitro*, as previously applied in *in-vitro* culture of *Perkinsus* spp.

Keywords: *Perkinsus olseni*; *Ruditapes philippinarum*; *in vitro* culture, hypnospore zoosporulation

INTRODUCTION

Since the first report of *Perkinsus olseni* infection in Manila clam *Ruditapes philippinarum* in Korean waters, several studies have reported lethal and sublethal impacts of the alveolate protozoan pathogen (Park and Choi, 2001; Park *et al*., 2006a; Lee *et al*., 2021). Recent studies also reported that *P. olseni* infection is not limited to Manila clams, *P. olseni* can infect the blood cockle *Anadara kagoshimensis* and the venerid clam *Prothothaca jedoensis* inhabiting the shallow subtidal soft bottom on the south coast (Park *et al*., 2006b; Cho *et al*., 2022). For the diagnosis of the infection, the fluid thioglycollate medium (FTM) assay developed by Ray (RFTM, Ray, 1953, 1966) has been adopted and used widely (Park and Choi, 2001; Leethochavalit *et al*., 2004; Waki *et al*., 2018). During the incubation in FTM, the trophozoite stage of *P. olseni* in the host tissue develops into a dormant hypnospore stage characterized by markedly increased cell size and a thick and robust cell wall within a few days. As the hypnospores are placed in aerated seawater, the hypnospores produce and discharge the

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Corresponding author: Kwang-Sik Choi
Tel: +82 (10) 3699-9000, e-mail: skchoi@jejunu.ac.kr
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biflagellated motile zoospores to the water column (Azevedo, 1989; Park and Choi, 2001). According to Villalba *et al*., (2004), the hypnospore stage is also infectious to an uninfected host, although the hypnospores have rarely been observed in a natural environment (Park *et al*., 2010).

Among the three life stages in *P. olseni*, the trophozoite stage is an intra-cellular stage eliciting sublethal impacts, including tissue inflammation and necrosis (see review of Villalba *et al*., 2004). Such virulence of *P. olseni* is often tested by challenging uninfected host organisms with the zoospores induced from hypnospores developed in FTM or trophozoites developed in growth media (Shimokawa *et al*., 2010; Waki *et al*., 2012; Waki and Yoshinaga, 2018, 2019). Accordingly, *in vitro* propagation of the zoospores or trophozoites is crucial in experiments testing *P. olseni* virulence. The host-free *Perkinsus* trophozoite culture technique was reported by Gauthier and Vasta (1993), Kleinschuster and Swink (1993), and La-Peyre *et al*. (1993) independently, who formulated or adapted and modified various growth media used in the cell-line culture to induce the trophozoites of *P. marinus*. Gauthier and Vasta (1995) also reported the continuous culture technique propagating *P. marinus* trophozoites *in vitro* using Dulbecco Modified Eagle's (DME) base medium fortified with Ham's F12 nutrient mixture. The DME:Ham's F12 cell culture medium used in *P. marinus* trophozoite culture was applied successfully in the host-free culture of *P. atlanticus* (=*P. olseni*) infecting the carpet shell clam *R. decussatus* in the European waters (Ordas and Figueras, 1998; Casas *et al*., 2002).

The host-free development of *P. olseni* trophozoite is crucial in understanding the life cycle and the virulence of different strains of *P. olseni* in Korean waters. In this study, we report the development of the trophozoite stage of *P. olseni in vitro* using the modified DME growth media and subsequent zoosporulation in the media.

MATERIALS AND METHODS

In October 2013, we collected Manila clams from the tidal flat in Gomso Bay on the west coast of Korea to obtain *P. olseni* stock for the *in vitro* culture. The previous study reported that Manila clams in Gomso Bay are heavily infected by *P. olseni* with an infection range of 3.7×10^5 to 2.2×10^6 cells in late summer to early fall (Yang *et al*., 2012). The gill tissues of Manila clams were removed and incubated in FTM to obtain hypnospores. Subsequently, the trophozoites in the gill tissues increased the cell size and developed into hypnospores in the anaerobic media. After decanting FTM and washing several times by centrifugation in filtered and sterilized seawater, the hypnospores developed in FTM were harvested for inducing zoospores to be used to propagate trophozoites *in vitro*.

For the *in vitro* culture, we prepared the Dulbecco's modified Eagle's:Ham's F-12 medium (DME/Ham's F-12, 1:2) supplemented with antibiotics, 5% fetal bovine serum, 50 mM HEPES buffer, 3.5 mM sodium bicarbonate, 200 mM L-glutamine according to Ordás and Figueras (1998) and Reece *et al*. (2008). The harvested hypnospores in 100 μL aliquot were first inoculated in 2 mL of DME:Ham's F-12 (1:2) medium in a 24-well microplate and incubated at 25ºC for 7 to 10 days to induce zoospores and subsequent trophozoites.

As microscopy revealed zoosporulation inside the hypnospores, 100 μL aliquot of the growth medium containing the sporulating hypnospores was transferred into 2 mL of the DME:Ham's F-12 (1:2) medium and cultured for two weeks to induce the trophozoite. After the culture in the medium, the *in vitro* developed trophozoites were isolated and diluted serially at 0.1 mL/well in the 96-well microplate until a single cell or poly cells (2-3 cells) were identified. The diameters of the trophozoites growing in the media were determined at 4, 6, 9, and 14 days after incubation using an image analyzing software. Microscopic features of the growing hypnospores in the media during the incubation were photographed using an inverted microscope equipped with Hoffman modulation contrast optics.

RESULTS AND DISCUSSION

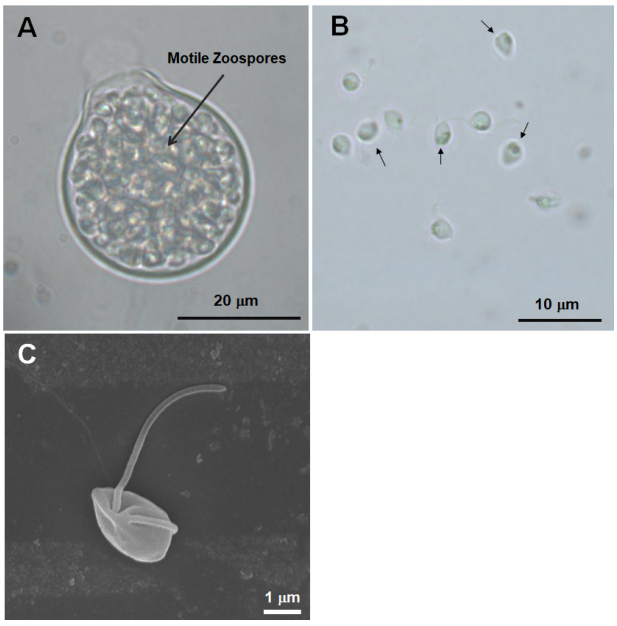

Fig. 1. **A**, Mature hypnospores developed in the growth media exhibiting numerous motile zoospores. **B**, The zoospores released from the hypnospores in the growth media. **C**, Scanning electron microscopic (SEM) view of the zoospores.

The isolated and purified hypnospores harvested from FTM successfully produced zoospores in Dulbecco Modified Eagle's:Ham's F-12 (DME/Ham's F-12, 1:2) supplemented with 5% fetal bovine serum, 50 mM HEPES buffer, 3.5 mM sodium bicarbonate, 200 mM L-glutamine (Fig. 1). In the growth media, we also added penicillin (500 unit/mL), streptomycin (500 μg/mL), and amphotericin B (1.25 μg/mL) as antibiotics, and the antibiotics seemed to prevent bacterial growth successfully, as no bacteria-contaminated growth media were observed.

Figure 2 shows different sizes of the trophozoites propagated in Dulbecco Modified Eagle's:Ham's F-12 (DME/Ham's F-12, 1:2) growth medium. In the growth media, the zoospores transformed into trophozoites shortly after the inoculation. For four to 6 days after the culture, asexually produced trophozoite schizonts could be seen in the media, and numerous small-sized daughter cells released from the schizonts were observed, suggesting that the doubling time of *P. olseni* trophozoites in the media at 25 °C could be 4 to 6 days (Fig. 3). The sphere-shaped trophozoites produced *in vitro* increased the diameter from a few

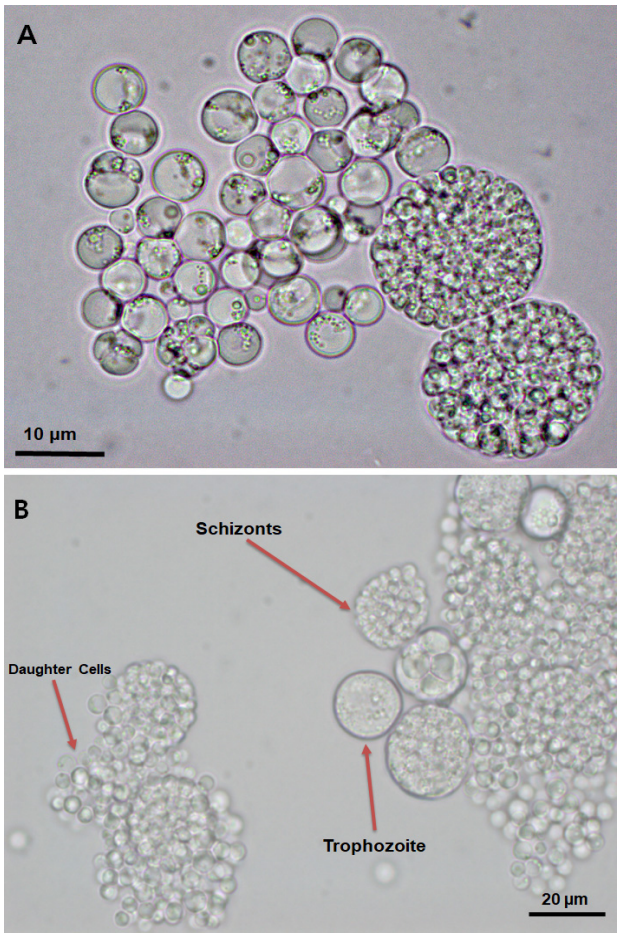

Fig. 2. The trophozoites of *P. olseni* produced *in vitro* using DME:Ham's F-12 (1:2) medium fortified with antibiotics. **A**, the trophozoites developed in the growth media. **B**, the schizont and the daughter cells released from the schizonts.

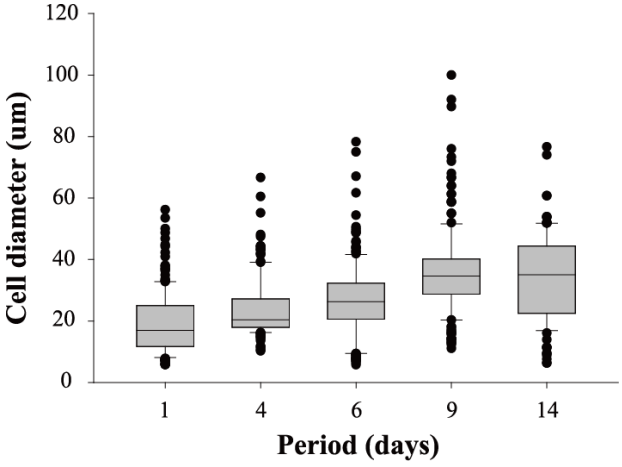

Fig. 3. The mean cell diameter of in vitro propagated trophozoites during two weeks of cultivation in the Dulbecco Modified Eagle's:Ham's F-12 (DME/Ham's F-12, 1:2) growth medium.

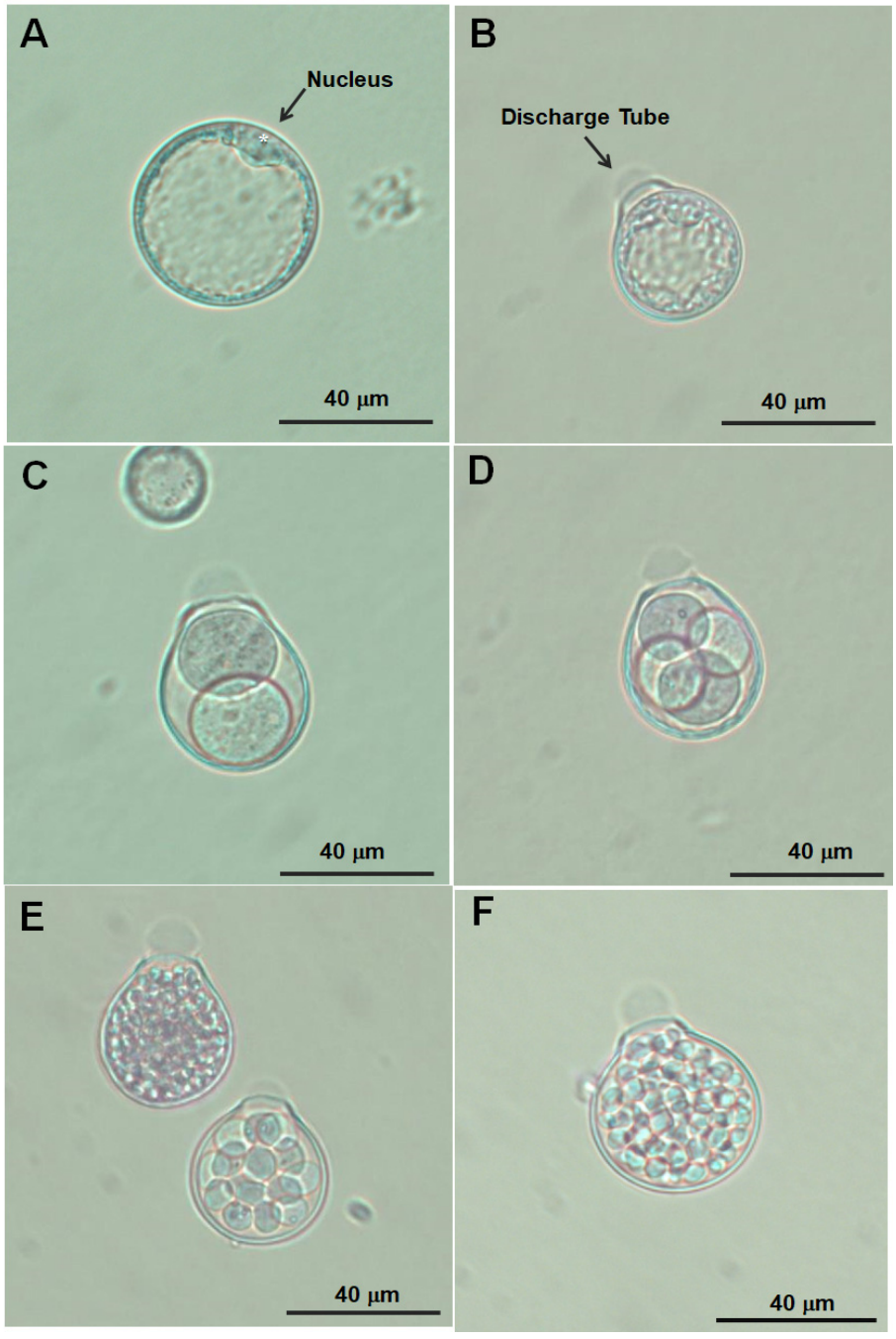

Fig. 4. *In vitro* cultured *P. olseni* cells in different life stages. **A**, The zoosporangium stage induced in the DME/Ham's F-12, 1:2 growth medium. **B**, The hypnospore stage of *P. olseni* exhibiting the discharge tube. **C, D**, The two and four-cell stages of hypnospore, **E, F**, Eight and 32-cell stages of the hypnospores.

microns shortly after the transformation to 34 μm after two weeks in the media.

To test the viability of the *in vitro*-produced trophozoites, we also induced the hypnospore stage and subsequent zoosporulation in the growth media at a laboratory condition. Figure 4 shows the hypnospores developing the zoospores, exhibiting the discharge tube. The zoosporangium propagated the zoospores in

the growth media, showing various multiplying stages. At the end of the culture, the numerous zoospores released from the hypnospores could be observed in the media, confirming that the trophozoites produced in the growth media were vital.

Several studies have reported that *P. olseni* infection intensity in Manila clams reached its annual peak in late summer when the water and sediment temperature stays over 25 °C in tidal flats on the west coast of Korea (Park *et al*., 2006a; Nam *et al*., 2018; Lee *et al*., 2021). Such high infection intensity recorded in late summer could be associated with the fast doubling time of *P. olseni*, which is closely linked to the hydrographic condition in the tidal flats of high temperature and salinity (Nam *et al*., 2018; Lee *et al*., 2021; Yang *et al*., 2022). In this study, the doubling time of *P. olseni* at 25 °C is estimated as four to six days, suggesting that *P. olseni* may reproduce at a faster rate in late summer when the temperature is elevated at a faster rate.

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