Ultrastructure of the microsporidian *Thelohania* (*Agmasoma*) *penaei* in the Pacific white shrimp (*Litopenaeus vannamei*)

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ABSTRACT

The structure of *Thelohania* (*Agmasoma*) *penaei*, a microphoridian parasite of farm-reared Pacific white shrimp (*Litopenaeus vannamei*) was examined with electron microscopy. Infected abdominal muscles and hepatopancreas were used for this study. Development was found in the cells of both tissues of infected shrimp. Earliest stages observed were limited to diplokaryotic cells. Sporogony was a series of three binary fissions, each producing unikaryotic cells within the sporophorous vesicle. Mature spores were ovoid, measuring 3.61 x 2.09 µm. The polar filament had eight coils at 0.19-1.06 µm in diameter.

Key words: *Thelohania, Agmasoma,* microsporidian, *Litopenaeus vannamei*

INTRODUCTION

Cotton shrimp or milk shrimp disease is caused by three genera of microsporidians: *Agmasoma* (*Thelohania*), *Amesoma* (*Nosema*) and *Plistophora* (*Plistophora*). These microsporidians have been reported from wild penaeid species and linked to serious impacts on commercial fisheries (Lightner, 1996). Although microsporidiosis caused by microsporidian parasites is not considered a major disease in cultured shrimp, opacity of abdominal musculature is a marketing problem (Owens and Glazebrook, 1988). In Thailand, *Agmasoma* (*Thelohania*) *penaei* is the most frequently reported microsporidian in wild banana shrimp (*Penaeus merguiensis*) as well as cultured black tiger shrimp (*P. monodon*) (Limsuwan, 1991; Flegel *et al*., 1992). The microsporidian *Thelohania* is a tiny, single-celled, spore-forming, intracellular parasite that causes milk shrimp or white-back shrimp disease in *P. monodon* and *P. merguiensis.*

Since the Department of Fisheries, Ministry of Agriculture and Cooperatives, Thailand, allowed the introduction of specific pathogen-free (SPF) Pacific white shrimp (*Litopenaeus vannamei*) into Thailand for commercial culture in 2002, microsporidian *Thelohania* infections have been reported from farm-reared *L. vannamei* using normal sea water along the coastal areas. Affected shrimp had whitish or milky body appearance especially dorsally from the cephalothorax to the middle of the body (Limsuwan *et al*., 2008a). The prevalence of infection reached 25% in ponds which did not use calcium

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hypochlorite ($Ca(OCl)_2$) for water treatment before stocking the postlarvae (Limsuwan *et al*., 2008b). Light microscopic examination of fresh specimens and scanning electron microscope revealed each sporophorus vesicle contained eight spores belonging to the genus *Thelohania* or *Agmasoma* (Limsuwan *et al*., 2008a). The aim of this study was to describe the ultrasructure of *Thelohania* (*Agmasoma*) *penaei* from farm-reared *L. vannamei.*

MATERIALS AND METHODS

Specimen collection

L. vannamei were caught by castnet from intensive ponds located in Nakornsrithammarat province, southern Thailand. Live shrimp with clinical signs of white-back disease, indicated by opaque, whitish musculature and hepatopancreas (Fig.1) were examined for spores using transmission electron microscopy.

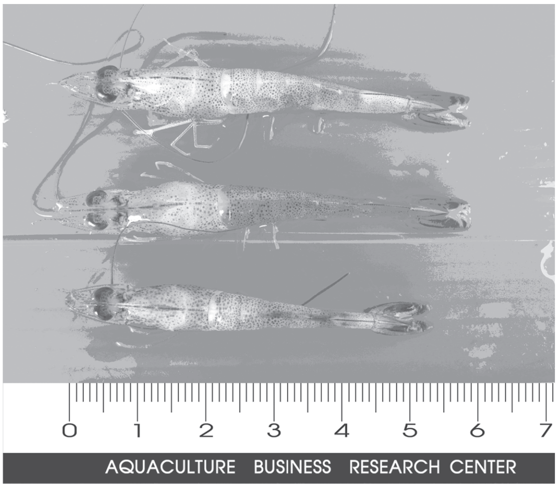

Figure 1. White shrimp infected with microsporidia had whitish or milky appearance on various parts of their bodies

Transmission electron microscopy (TEM)

Samples of abdominal muscle and hepatopancreas of infected shrimp were fixed in 3 % glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 4 h at 4°C, and post-fixed in 1% osmium tetroxide in the same buffer for 2 h. Tissues were then washed three times with distilled water, dehydrated in a graded series of ethanol from 50 to 100%, and then twice in 100% propylene oxide. Tissues were subsequently embedded in Epon 812 resin by successive 1 h infiltration of 1:1 and 2:1 resin: propylene oxide, and 100% resin. The tissues were polymerized by incubating at 48°C for 24 h and at 60°C for 48 h in fresh 100% epoxy resin. Semi-thin sections (1 μm thick) were stained with 1% toluidine blue and then observed with a light microscope. Ultrathin sections were stained in 2 % uranyl acetate and 0.3% lead citrate solutions for examination using a JEOL, JEM 1210 transmission electron microscope at 100 kV.

RESULTS AND DISCUSSION

Examination of semi-thin muscle and hepatopancreas sections stained with toluidine blue revealed heavy microsporidian infection with different sizes of spores (Figs 2,3). However, each sporophorous vesicle (SV) containing eight spores could not be clearly determined as formerly reported from fresh smear spores. The microsporidian parasite occurred in clusters throughout the infected tissues. Spores had an ellipsoidal or oval shape. There were no apparent host encapsulations or xenoma formation within the muscle fibers and hepatopancreatic tissues.

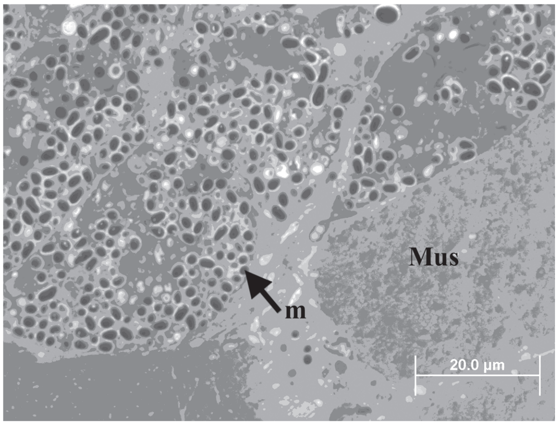

Figure 2. Semi-thin section of abdominal muscle of *L. vannamei* was largely replaced by microsporidian spores. m=microsporidian infection in muscle, Mus= normal muscle (scale bar=20µm)

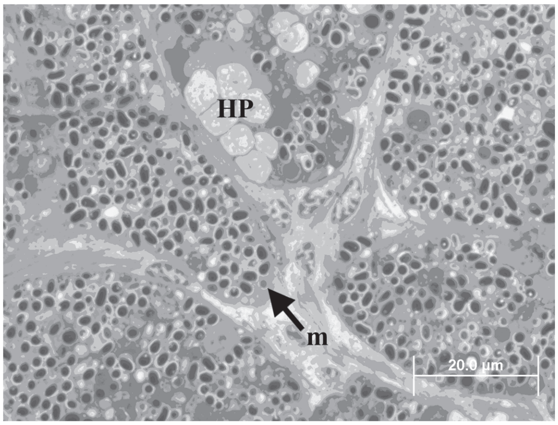

Figure 3. Semi-thin section of hepatopancreas of *L. vannamei* was largely replaced by microsporidian spores. m=microsporidian infection in hepatopancreas, HP=normal hepatopancreatic tubule (scale bar=20µm)

Transmission electron microscopy

Examination of ultrathin abdominal muscle and hepatopancreas sections revealed heavy microsporidian infection with different life cycle stages and mature sporoblasts and spores surrounded by a persistent SV membrane (Fig. 4)

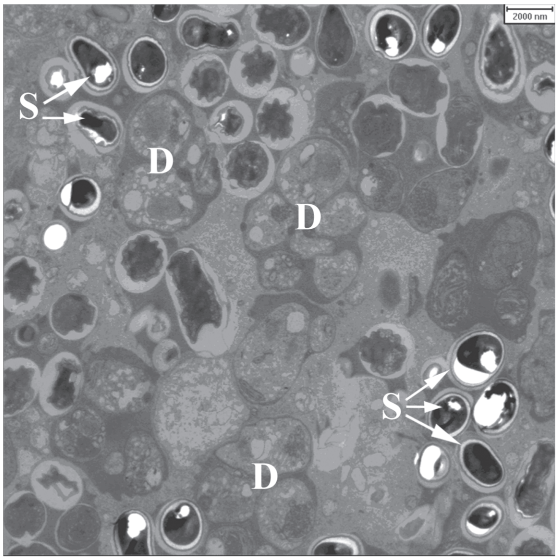

Figure 4. Transmission electron micrograph showing different stages of *Thelohania*. Mature spores (S) and developmental stages (D) (scale bar = 2000 nm)

The early stages of merogony were localized among muscle fibrils and hepatopancretic tissues. Uninucleated cells were not seen in this study. Diplokaryotic cells were the least mature stage of development observed. These cells were surrounded by irregular thin plasmalemma contour. The cytoplasm showed mainly free ribosomes while the nuclei were small and had moderate chromatin and a nucleolus (Fig. 5). Early sporonts were distinct from meronts as they possessed a thickened plasmalemma and a cytoplasm with a low ribosome content (Fig.6). Later, the differentiation of a parallel membrane, which became the sporophorous membrane was observed. In the final phase of the sporont, cytokinesis gave rise to eight uninucleate sporoblasts (eight-cell stage) within the SV. In ultrathin sections, the number of sporoblasts observed varied from three to six from a maximum of eight sporoblasts.

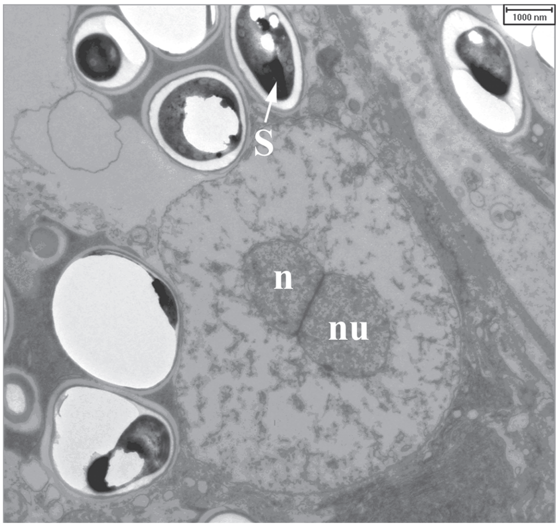

Figure 5. Transmission electron micrograph (TEM) of early stage of merogony showing two nuclei (n) in diplokaryotic arrangement, nucleolous (nu), mature spore (S) (scale bar=1000 nm)

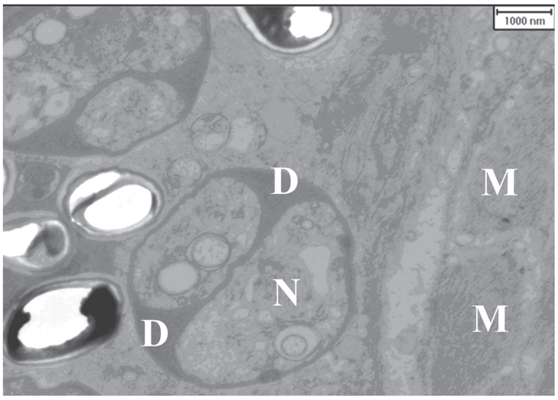

Figure 6. Developmental stages under transmission electron microscopy undergoing binary fission showing unikaryotic nucleus (N). Some muscle fibers are visible nearby (M). Dark, finely granular material occurs between stages (D). (scale bar=1000 nm)

These sporonts were closely pressed together and always associated with dark, amorphorous material in the episporontial space on the region between and partly surrounding the cells (Fig. 7). Each of the cells was bounded by a membrane which appeared to be trilaminate at first glance but seemed to be further divided when examined closely. Inside the sporoblast conspicuous changes were noted; the cytoplasm had become more dense, and an anchoring disc, the polar filament and posterior vacuole had appeared (Fig. 8). Eight mature spores within each SV were not observed in this study. The number observed varied upon the plane of sectioning. Spores had an ellipsoidal or ovoid shape and were 3.61 (2.33 to 4.42) µm x 2.09 (1.17 to 3.45) µm. The thick spore wall comprised a thin electron dense exospores and electrolucent endospore. The exospore consisted of alternating light and dark layers, with a combination thickness of 0.08 (0.03-0.14) µm. The endospore was of nearly uniform thickness of 0.18 (0.08 -0.27) µm. (Figs 9, 10)

Figure 7. Sporoblast or eight-cell stage (here only six cells are visible) with dark, finely granular material (D) between stages and partly surrounding cells. N=nucleus, PV= posterior vacuole (scale bar=900 nm)

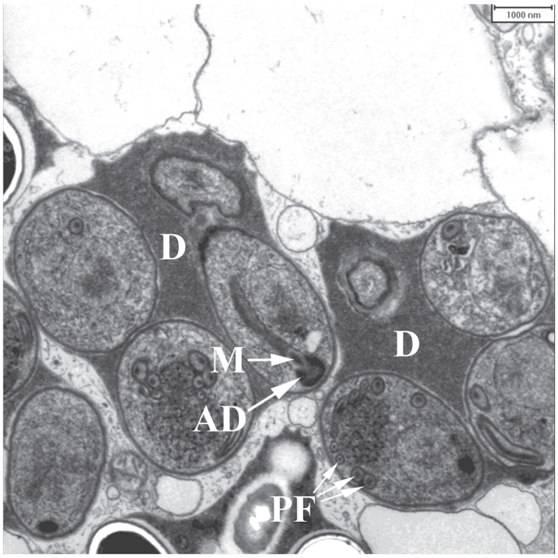

Figure 8. Sporoblasts or eight-cell stage with dark, finely granular material (D) between stages and partly surrounding cells. AD = anchoring disc, M= manubrium, PF = polar filament. (scale bar=1000 nm)

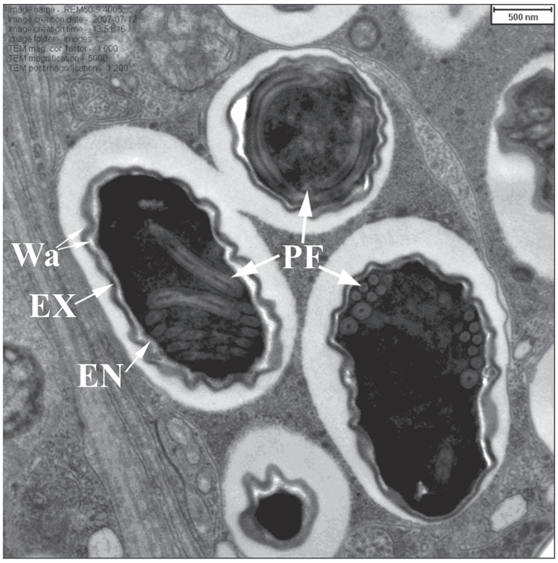

Figure 9. Several mature spores sections in different planes showing the wall (Wa), electron dense exospore (EX), electrolucent endospore (EN) and polar filament (PF) (scale bar=500 nm).

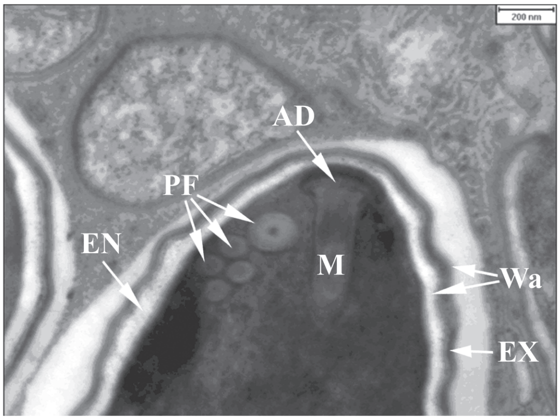

Figure 10. A longitudinal section of a mature spore showing the wall (Wa), electron dense exospore (EX), electrolucent endospore (EN), anchoring disc (AD), manubroid (M) and polar filament (PF) (scale bar =200 nm)

The polar filament was attached to the anterior end of the spore by an anchoring disc and was divided into two regions: the manubroid or straight portion, and the posterior region forming a variable number of coils around the sporoplasm (Fig. 10). The polar filament was slightly enlarged at the manubroid base but uniform in diameter throughout the rest of its length. The polar filaments had eight coils, arranged in a single row at the posterior end of the spore. In cross sections, alternating light and dark concentric layers were distinguishable. The coils were 0.55 (0.19-1.06) µm in diameter (Fig. 9). The nucleus was indistinct. The posterior vacuole was not evident in most spores observed. The polarroplast was indistinguishable.

The ultrastructure of microsporidians *Thelohania* from shrimps have been described by Vernick *et al*. (1977) and Johnston *et al*. (1978) while the sporogony and stages in the host tissues were identical to that described for *Agmasoma penaei* from *P. sertiferus* by

Hazard and Oldacre (1975) and Clotilde-Ba and Togubaye (1994). Descriptions of the ultrastructure of *Thelohania* presented here indicate that this microsporidian shares characteristics common to the family Thelohaniidae: formation of octospores by budding of a sporogonial plasmodium within a persistent pansporoblast membrane (Tuzet *et al*., 1971; Hazard and Oldacre, 1975; Sprague, 1977). Larsson (1999) used the term 'sporophorous vesicles' to describe a more or less persistent sac-like structure containing a specific number of spores. However, the term 'vacuole' instead of 'vesicle' has been used by some researchers (Azevedo and Canning, 1987; Sprague *et al*., 1992; Azevedo *et al*., 2000). *Thelohania* (*Agmasoma*) *penaei* spores differ from other Thelohania-like spores in decapod Crustacea in the number of turns made by the polar filament. Cytoplasmic vacuoles, similar to those found in *Thelohania*, are also commom in Microspora, and often contain electron-dense granules that have been implicated in polar filament formation (Weidner, 1970; Szolloski, 1971; Jensen and Wellingd, 1972; Walker and Hinsch, 1972). Mercier (1909), who described the life cycle of the type species *Thelohania giardi*, believed that small uninucleate cells were isogometes which underwent first cytoplasmic and then nuclear fusion, producing cells consistent with a single nucleus. The sporont observation by Johnston *et al*. (1978) who reported of apparently uninucleate cells followed by diplokaryotic cells, was consistent with Mercier's view that there was cytoplasmic fussion of two uninucleate cells. Unlike Mercier (1909) though, Johnston *et al*. (1978) found no evidence for karyogamy. In our study, we did not observe this life cycle stage.

The kind of sporogony occurring in *Thelohania* was previously reported by Henneguy and Thelohan (1892). Later, more detailed descriptions by Mercier (1909) clearly indicated that sporogony is a series of three cell divisions, each complete before the next begins. The important character of the genus *Thelohania* is the possession of a sporogonial plasmodium. However, Johnston *et al*. (1978) suggested that the definition of the genus *Thelohania* should be amended to include sporogony by a series of three binary divisions and absence of a sporogonial plasmodium.

More ultrastructure data related to life cycle stages are needed to elucidate this and other problems relating to sexuality in *Thelohania*.

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