J. Exp. Zool. India Vol. 22, No. 1, pp. 563-568, 2019 | www.connectjournals.com/jez | *ISSN 0972-0030*

A MICROSPORIDIAN INFECTING BODY MUSCLE OF *PARAPENAEOPSIS STYLIFERA* : HISTOLOGICAL STUDIES

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(Accepted 20 November 2018)

ABSTRACT : In the present study, the microsporidian infecting wild shrimp were characterized by histological and histochemical studies. A six months survey to examine the microsporidian infection in feral shrimp revealed that only *Parapenaeopsis stylifera* was infected with a microsporean, *Perezia* sp. Other species examined (*Metapenaeus brevicornis*, *Metapenaeus affinis* and *Solenocera* sp.) did not reveal any infection. The prevalence of the microsporean, Perezia infecting *P. stylifera* was found to be very low (1.76%). The spores were found only in the skeletal musculature and the infected shrimp appeared milky white in colour. The spores measured 1.4-2.8 mm x 0.9-1.9 mm. The histological sections revealed extensive lesions that appeared more homogeneous and muscle fibers found to be completely replaced by spores. The spores were stained with different stains viz, Giemsa, Gram's, and hematoxylin & eosin (H&E) stain. The Gram's and H&E stained spores at higher magnification often showed a darkly reddish coloured circular body at the one end. Prussian blue stained histological sections showed bluish tinge near the necrotized area of muscle, due to growth of spores.

Key words : Microsporidia, histology, *Parapenaeopsis stylifera.*

INTRODUCTION

Microsporidians are obligate intracellular protozoan parasites, which infect a diverse group of organisms from protozoa to humans. Several genera of microsporidians parasitize shrimp and crabs, causing mortality or reducing market values (Couch, 1983; Sparks, 1985). Microsporidians seem to be ubiquitous in wild penaeid shrimp populations and when infections occur with high prevalence, they are believed to have serious impacts on commercial fisheries. Pond cultured shrimp with these parasites have been observed, with prevalence rates as high as 16% in some instances (Lightner, 1975) and have resulted in economic losses up to 20% (Lightner, 1988). Some of the most severe microsporean diseases of shrimp can be recognized grossly by a progressive white opacity of muscles and other tissues. They cause the abnormality known as "cotton shrimp" or "milky shrimp" in penaeids. Incidences of this disease have been reported in penaeid shrimp in different parts of the world (Overstreet, 1973; Owens and Glazebrook, 1988; Ramasamy *et al*, 2000; Canning *et al*, 2002; Toubiana *et al*, 2004) and in the natural population of pandalid shrimps namely *Pandalus borealis* from the northwest Atlantic and *P. jordani* from the north Pacific (Olson & Lannan, 1984; Parsons & Khan, 1986).

Majority of the total prawn landing in India is constituted of penaeid prawns. However, reports on microsporidian infection in shrimp are limited from this part of the world. Cotton shrimp diseases were reported in the natural populations of *Penaeus indicus*, *P. semisulcatus*, *Metapenaeus affinis*, *M. monoceros* and *M. brevicornis* (Soni, 1986; Ramasamy *et al*, 2000). Jose (2000) reported a microsporidian infection in *Parapenaeopsis stylifera* for the first time from India. In this background, the objective of the present study was to estimate the prevalence of microsporidian infection in various species of captured shrimp and to characterize the most prevalent microsporidian species infecting them. Using histological, histochemical studies, a microsporidian, resembling *Perezia nelsoni* was found to be the most prevalent microsporidian species infecting *Parapenaeopsis stylifera*.

MATERIALS AND METHODS

Collection of specimens

Various species of shrimp *P. stylifera*, *Metapenaeus affinis*, *M. brevicornis* and *Solenocera* sp. were collected from landing centers and fish markets in Mumbai, during 2002-2004, brought to the laboratory on ice and preserved at -80°C. Fresh specimens were also collected onboard

the vessel M.F.V. Narmada on a weekly basis. Preliminary screening of microsporidian infection was carried out by gross examination and wet mount preparations made from different tissues and observed under microscope for any microsporidian spore. For histopathological analysis, shrimp were dissected onboard and tissue samples were fixed in Davidson AFA fixative.

Histology

Fresh infected specimens were fixed in Davidson's AFA (Humason, 1972) fixative by lifting the carapace slightly and giving an incision longitudinally for easy and rapid penetration of fixative. Histological preparations were made according to the method of Bell and Lightner (1988). The deparaffinised tissue sections were stained using Harry's haematoxylin, counter stained in eosin (Sheehan and Hrapchak, 1980) and mounted in DPX mountant. All the light microscopy studies including histopathological observations were made using a light microscope (Olympus, CX-31). Photomicrographs were taken using a microcamera (PM-C35DX) fitted to the microscope. Tissue sections prepared from the paraffin blocks were subjected to histochemical analysis also using Prussian blue staining method for ferric iron detection (Lillie, 1965; Carson, 1990).

Histochemical analysis

The tissue sections prepared from the paraffin blocks were subjected for histochemical analysis by using Prussian blue staining method for ferric iron detection (Lillie, 1965). The tissue sections were deparaffinised and hydrated to water. Then the slides were handled with nonmetallic forceps in subsequent steps. The slides were placed in freshly prepared mixture of equal parts of 2% potassium ferrocyanide plus 2% hydrochloric acid and heated for 20 minutes at 60°C. The sections were washed thoroughly in several changes of distilled water and counterstained in nuclear-fast red for 5 minutes. The slides were rinsed in running tap water for at least 1 minute followed by dehydration in 95% alcohol and two changes of absolute alcohol. The sections were cleared in three changes of xylene and subsequently mounted with synthetic resin (Carson, 1990).

Purification of microsporidian spores

The spores were isolated following the method of Pasharawipas and Flegel (1994). Briefly, the infected shrimp tissue (body muscle) was suspended in phosphate buffered saline (PBS) and homogenised using a glass tissue homogenizer. This suspension was layered on top of a discontinuous 20% plus 40% sucrose gradient solution and centrifuged at 3,000 rpm for 10 min (Himac preparative ultracentrifuge model CP80mx, rotor P40ST). The interface between the 20% and 40% phases, that constitutes the spore fraction, was collected and washed with PBS thrice. The spore pellet was suspended and re-purified by a second separation on a 20% plus 40% discontinuous sucrose gradient followed by 4 to 5 washings with PBS. The spores were stained with Gram's stain, Giemsa's, haematoxylin & eosin stains using standard protocols and observed under high power objective of a light microscope.

RESULTS

Prevalence of microsporidian infection

The prevalence of microsporidian infection was found to be quite low. During the study period, 851 *P. stylifera* individuals were examined and infection could be detected in 15 specimens giving the prevalence of 1.76%. However, 835 *M. brevicornis*, 40 *M. affinis* and 44 individuals of *Solenocera* sp. were also examined during the study and no microsporidian infection was detected in these species (Table 1- previous publication).

Gross and microscopic examination of sample

P. stylifera with microsporidian infection exhibited opaque whitish discoloration of the body muscle compared to the translucent appearance in the normal, uninfected shrimp. A squash preparation of the infected muscle examined under a microscope showed a milky appearance and juicy nature with negligible muscle fibers and large number of spores.

Fresh spores released from infected muscles appeared ellipsoid, measured 1.4-2.8 mm × 0.9-1.9 mm and dispersed on release from muscle with no evidence of clumping. The morphology of the spores could not be ascertained under the light microscope, as the spores were too small.

Histological observation

Histological sections of infected muscle of *P. stylifera* stained with haematoxylin and eosin showed extensive lesions that appeared more homogeneous and muscle fibers were completely replaced by the parasite. In addition to this, some parts of muscle were completely degenerated and necrotized when compared to normal uninfected muscle which showed fine skeletal muscle fibers without any damage (Fig. 1A & 1B). The tissue sections were also stained with Prussian blue stain for identifying the ferric iron in necrotized tissue. Ferric iron accumulation in the necrotized area of muscle caused by the microsporidian was evident as bluish stained area, whereas uninfected region of the muscle took reddish stain (Fig. 2A & 2B).

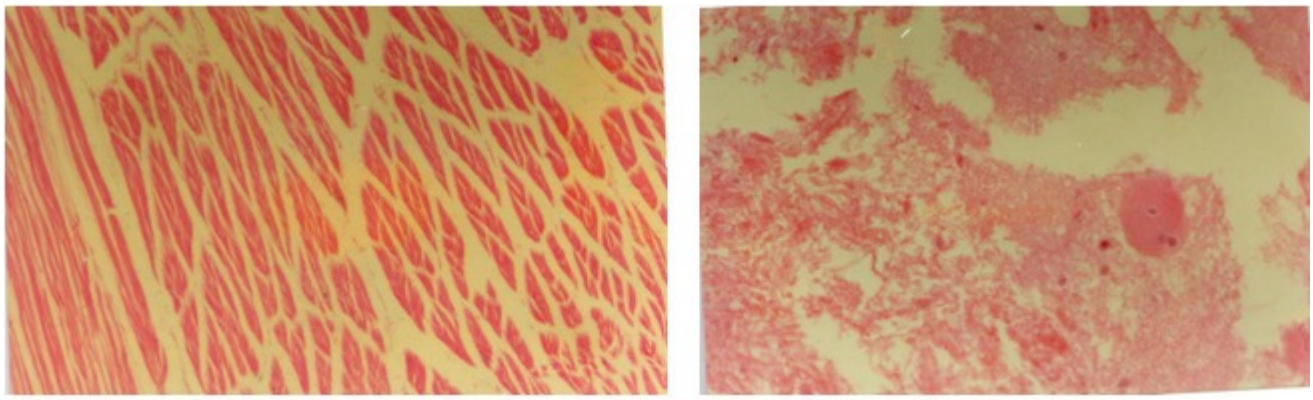

Fig. 1 : Normal (A) and infected shrimp histological section (B) showing the destruction of muscle fibers replaced by spore [Haematoxylin and eosin stain (400x)].

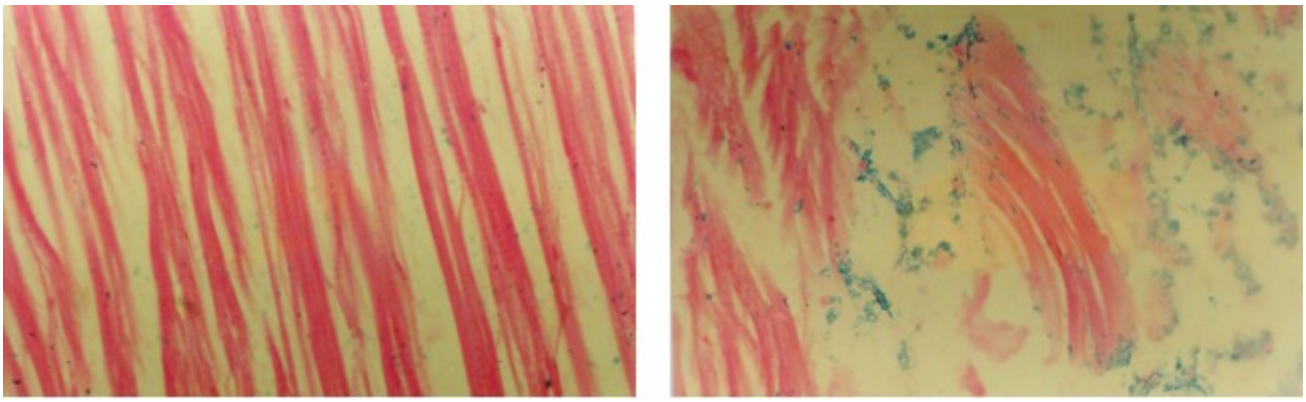

Fig. 2 : Prussian blue stained histological section of normal (A) and infected shrimp (B) showing degeneration of muscle and deposition of blue stain of Prussian blue (400x).

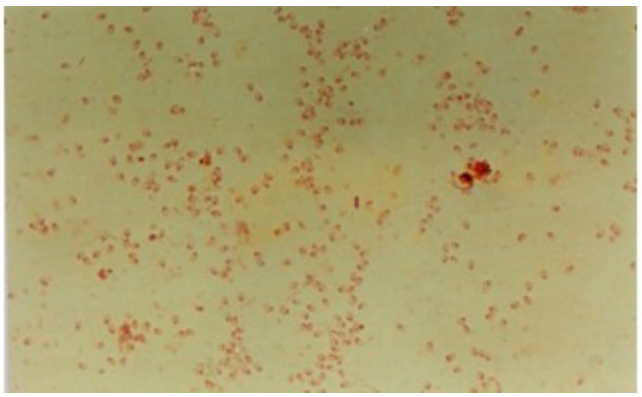

Fig. 3 : Spores stained with Gram's stain (1,000x).

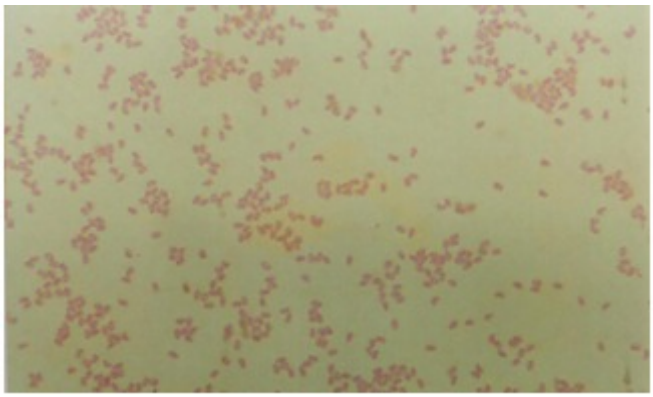

Fig. 4: Spores stained with Haematoxylin and Eosin stain (1,000x).

Isolation of spores

Usually, purified spores could be obtained after only two separations using sucrose gradients. In some cases, however, further separation was needed if microscopic examination of the preparation showed some contaminating material. The purity of the preparation was determined by comparison with unpurified samples using light microscopy (100 X objective) of Gram stained preparations. By this method, there was no indication of any contamination by the host tissue or by other organisms such as bacteria. The estimated recovery of spores was

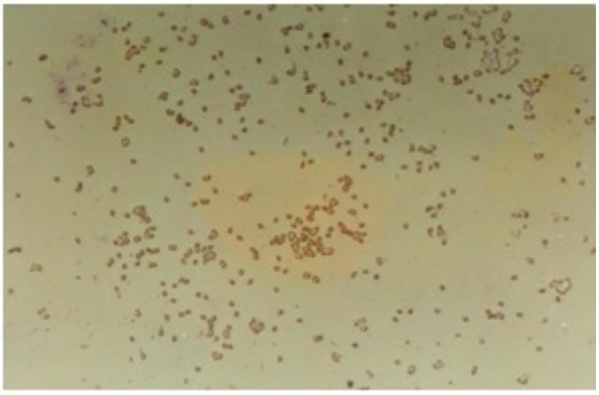

Fig. 5 : Spores stained with Giemsa stain (400x).

Table 1 : Prevalence of microsporidian infection in percentage.

Host species	Mean Length (cm)	Mean Weight (g)	Number examined	Number infected	Prevalence
Parapenaeopsis stylifera	8.85±0.03	5.41±0.05	851	15	1.76%
Metapenaeus brevicornis	9.59±0.06	7.95±0.08	835	nil	nil
Metapenaeus affinis	10.23±0.32	12.21±6.40	40	nil	nil
Solenocera sp	7.78±0.16	5.90±0.12	44	nil	nil

Source: Prakasha B K, Aparna Chaudhari, Rajendran K V and Naveen Kumar B T (2019). Prevalence of microsporidian infection in the wild caught shrimp population and its transmission mechanism. *Journal of Experimental, Zoology India*, Accepted for publication.

approximately 50-60%.

Staining of spores

The spores were stained with different stains *viz*, Giemsa, Gram's stain and Haematoxylin & eosin stain. The Gram's (Fig. 3) and H&E stained spores (Fig. 4) at higher magnification (100x objective) often showed a darkly and densely reddish circular body at the one end. Our study with Geimsa stain (Fig. 5) to the spores didn't stain nuclei specifically instead the spores appeared ellipsoid with green colour only.

DISCUSSION

The present species of microsporidian closely resembles *Perezia nelsoni* reported from marine shrimp by Sprague (1950), in morphology, morphometry and ultrastructural details. *P. nelsoni* was first described as *Nosema nelsoni* from *F. aztecus* collected from the Lousiana coast, USA (Sprague, 1950). It has also been reported from *Litopenaeus setiferus* and *Farfantepenaeus duorarum* from southern USA and from South Africa (Sprague and Couch, 1971; Overstreet, 1973) and later in *Parapenaeus longirostris* from the Mediterranean (Loubes *et al*, 1977), *Penaeus semisulcatus*, *Fenneropenaeus merguiensis* and *Penaeus esculentus* from Australia (Owens and Glazebrook, 1988) and *Farfantepenaeus notialis* from Senegal (Clotilde-Ba and Togubaye, 1995, 1996). The spore dimensions of this species (measured fresh or fixed), provided by several authors are in accordance with that (2.5 × 1.5 µm) given in the original description (Sprague, 1950). In the present study, the spore dimensions observed were 1.4 - 2.8 µm × 0.9 - 1.9 m and fresh spores released from infected muscles appeared ellipsoid and is different from *Pleisthophora* sp which were slightly pyriform with 2.3-3.0µm × 1.7-2.5µm in dimension, pyriform shaped *Thelohania duorara* with 4.7-6.8µm × 3.0-4.2µm and most distinctly pyriform shaped *Thelohania penaei* microspores with 2.5-4.7µm × 2.0-3.5µm and megaspores with 5.5-8.2µm × 3.5-4.2µm.(Overstreet, 1973). The observation of *P. nelsoni*-like infection in *P stylifera* from Indian waters shows that it is likely that the microsporidian has a worldwide distribution, in a wide range of penaeid hosts but confirmation of this will rest with molecular studies.

The prevalence of the microsporidian in wild shrimp population appeared to be low. Olson and Lannan (1984) reported extremely low prevalence of microsporidian infection (0.19%) in wild caught *Pandalus jordani* during a 6 years study period. Nevertheless, Canning *et al* (2002) reported that the microsporidian infection has not exceeded 0.5% for several years when 50 specimens each of *Litopenaeus setiferus* and *Farfantepenaeus aztacus* collected from the coastal waters were examined on a monthly basis. The present study also revealed that the prevalence of microsporidian infection in wild shrimp *P. stylifera* is very low at about 1.8%. However, Lightner, (1975) had reported 16% prevalence of a microsporidian infection in pond reared *F. aztacus* from Texas and 15% in *Litopenaeus setiferus* reared in a net-enclosed bay in Florida. It has been reported that a high prevalence of microsporidian infection in wild shrimp cannot be ruled out. This could be attributed to the fact that the shrimps with very low infection and in the early stage of development and do not show any discolouration and if the infection is restricted to a small portion of the muscle that may go undetected. However, Vidal-Martinez *et al* (2002) reported that the prevalence of *Agmasoma penaei* (*Thelohania penaei*) was 2% in addition to other parasites in cultured shrimp *Litopenaeus vannamei* from a shrimp farm at Sisal. Ramasamy *et al* (2000) also recorded the 2% prevalence with *Thelohania* sp. in *Penaeus indicus* from India. Rajendran (1997) also reported a very low prevalence of microsporidian infection in cultured shrimp during 4 years study period. Ramasamy *et al* (2000) attributed the low prevalence of microsporidian infection in wild caught shrimp to the death of infected prawns, or to the vulnerability of infected prawns to predation, hence greatly reducing the numbers of infected individuals available for sampling. However, the effect of microsporidian on natural host population has yet to be determined.

Jose (2000) performed the histochemical studies using bathophenonthroline method and observed the presence of iron in the necrotized regions as red coloured, whereas microsporidean appeared blue and non-necrotized tissue

almost colourless. On the contrary, our histochemical study by prussian blue stain method (Lillie, 1965) showed the bluish tinge in the necrotized area where ferric iron deposited and microsporidian spores appeared red in colour.

The gram staining is useful for revealing mature microsporidian spores that are usually gram positive. This characteristic is particularly useful in distinguishing mammalian microsporidia from other tissue parasites (e.g. *Toxoplasma gondi*) that are gram negative (Vavra and Maddox, 1976). Vivares and Sprague (1979) reported that the gram's stained spores under light microscopy gave very little structural details and some times it was possible to discern one or two chromophilic granules, probably part of the polaroplast and /or nucleus. In our study also the purified spores stained with gram's stain showed red coloured ellipsoid spores with reddish circular mass at one end of the spore (both are red in colour). The haematoxylin and eosin stained spores showed the same results.

Giemsa staining after acid hydrolysis is a useful method for revealing nuclei in microsporidian spores if nuclei are obscured by the dense spore content after normal staining. Giemsa staining after hydrolysis is always specific for DNA but usually gives more satisfactory results than Feulgen's reaction (Vavra and Maddox, 1976). In contrary, our study with Geimsa stain to the spores didn't stain nuclei specifically instead the spores appeared ellipsoid with green colour only.

In the present study with *Perezia nelsoni* infecting skeletal muscle of *Parapenaeopsis stylifera* showed the extensive degeneration and replacement of muscle fibres by spores, where the lesion is more homogeneous in nature. Canning *et al* (2002) also reported that the histological section of skeletal muscle from *Litopenaeus setiferus* infected with *Perezia nelsoni* showed extensive lesions which appeared more homogeneous and opined that the homogeneous nature of infection may be attributed to the small size of spores. The histological lesions were different from lesions of *Tuzetia weidneri* in *Litopeneaus setiferus* as described by Canning *et al* (2002), because the infection with *Tuzetia weidneri* was characterized by the elongated lesions in the direction of the myofibrils tapering at the ends. Similar type of infection from *Nosema nelsoni* occurring singly were found typically surrounding the bundles of abdominal muscle in brown, *Penaeus aztecus,* Ives, and white shrimp, *P. setiferus* (L.) (Overstreet, 1973). Our study also showed that it was different from infection from *Thelohania penaei* Sprague (1950) as described by Overstreet (1973) that *Thelohania penaei* Sprague (1950) is usually located along the dorsal midline of the white shrimp and it infects smooth muscles of the blood vessels, foregut, hindgut, and the germinal tissue of the gonads. Rajendran (1997) found the infection of *Agmasoma* sp in *P. indicus* collected from Tamil nadu and Andhra Pradesh of India and reported that the histological sections of *Penaeus indicus* infected with microsporidian showed heavy degeneration of striated muscle and replacement of functional tissue by accumulation of numerous spores. Jose (2000) in their study found spores of microsporidia occupied the entire muscle fibres and replaced the nuclei and sarcoplasm in the histological sections of *Parapenaeopsis stylifera*. Our study also showed that no apperant host-inflammatory response to the infection of microsporidia. The same observation was also noted by Rajendran (1997) and Jose (2000) during the study. The observation of *P. nelsoni*-like infection in *P stylifera* from Indian waters shows that it is likely that the microsporidian has a worldwide distribution, in a wide range of penaeid hosts but confirmation of this will rest with molecular studies.

ACKNOWLEDGEMENTS

The authors are thankful to the Director, Central Institute of Fisheries Education (CIFE), Mumbai, for the facilities provided for the study.

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