International Journal of Applied Research
Journal HP: www.intjar.com; ISSN: 2411-6610

Detection of microsporidia and gregarine from shrimps in Bangladesh

Abdur Rahman, Anisuzzaman, Md. Shahiduzzaman

Department of Parasitology, Bangladesh Agricultural University, Mymensingh-2202

ARTICLE INFO

Article history

Received: 03 November 2021
Accepted: 27 December 2021

Keywords

Microsporidia, Gregarine, Shrimp, Bangladesh

Corresponding Author

M Shahiduzzaman
Email: szaman@bau.edu.bd

ABSTRACT

Microsporidian and Gregarine are zoonotic parasites have emerged as a serious pathogen reported to be associated with retarded growth in cultured shrimp in many of the shrimp growing countries in Asia. This study investigated shrimp for the occurance of microsporidian and gregarine parasites in Bangladesh using light microscopy and polymerase chain reaction (PCR). In this study a total of 50 black tiger (Baghda) shrimp (*Paenus monodon*) and 40 local shrimp (Prawn small, Deshi Chingri) were collected from Sathkhira and Mymensingh districts respectively. Baghda shrimp reared in river in Sathkhira (Shymnagar Upazila) and small prawn from beal and river of Gouripur upazila of Mymentsingh district. Microsporidia was detected from squash preparation of hepatopancreas of shrimps using microscopy after Giemsa stain and polymerase chain reaction (PCR). DNA extracted from hepatopancreas and guts were subjected to PCR amplification using primers targeting microsporidian and gregarine SSU rRNA and SSU rDNA genes, respectively. The PCR yielded an expected product of ~1100 bp and 1000 bp, respectively. The microscopic examined revealed the oval shape microsporidian spores. The PCR product of expected band size confirms the presence of microsporidia in shrimp. None of the samples were positive for gregarines. This is the first report of identifying microsporidian parasite in shrimp from South-west region especially Satkhira and Mymensingh districts in Bangladesh.

Introduction

In aquatic animals, infection of microsporidia and gregarine in fish leads to reduction in growth rate and productivity (Rodriguez-Tovar et al., 2011). Recently, shrimp farms in Asia and other areas have been reporting heavy infection with a microsporidian parasite, *Enterocytozoon hepatopenaei* (EHP) in cultured shrimp impacting the production due to severe growth retardation (Newman, 2015). To date, approximately 187 genera and over 1300 species of microsporidia have been described as parasites infecting a wide range of vertebrate and invertebrate hosts, among which almost half infect aquatic species (Stentiford et al., 2013; Vavra et al., 2013). Several microsporidians have been reported as pathogens of penaeid shrimp as well as finfish (Vavra et al., 2013). Recently, a new species of microsporidian *Enterocytozoon hepatopenaei* (EHP) has been reported to cause hepatopancreatic microsporidiosis in penaeid shrimp in Asian countries like China, Indonesia, Malaysia, Vietnam and Thailand and India (Tourtip et al., 2009; Tangprasittipap et al., 2013; NACA 2015). EHP infections have been reported in both black tiger shrimp *Penaeus monodon* and Pacific white leg shrimp *Penaeus* (Litopenaeus) vannamei causing severe economic loss to shrimp aquaculture (Thitamadee et al., 2016). The parasite has been confirmed in P. vannamei in India (CIBA, 2014-15) during 2014, leading to detailed studies in farmed shrimp (Rajendran et al., 2016).

Currently, there are 200 known genera of microsporidia, with over 80 known to infect fish and aquatic invertebrates. Although the most common pathology associated with microsporidians is a whitish discoloration in muscles due to spores that can stunt growth and cause other types of problems, EHP is different. It only infects the tubules of the hepatopancreas in shrimp, which damages the ability of this critical organ to gain nutrition from feed. It is widely understood that EHP does not cause mortality but heavily limits growth. Formerly, classified as protozoa, genomic taxonomy has determined that microsporidians are closely related to fungi. About 100 genera of microsporidians are known to infect crustaceans and fish.

The protozoa are amongst the main causing agents of diseases in shrimp, being the ones from the Genus *Nematopsis* Schneider, 1892 (Apicomplexa: Eugregarinida). Septate gregarines are common intestinal parasites of both natural and cultured shrimp (Feigenbaum 1975). In particular the genera Nematopsis (Schneider 1892) has a worldwide distribution in cultured penaeoid shrimp (Lotz & Overstreet 1990). There is concern that heavy infection with these gregarines has the potential to cause economic losses through host

Cite this article: *Rahman, A., Anisuzzaman & Shahiduzzaman, M (2021). Detection of microsporidia and gregarine from shrimps in Bangladesh. International Journal of Applied Research, 7 (1): 46-50. DOI: 10.5281/zenodo.8146931*

mortality and reduced growth of the host in ponds (Ball 1959; Jimenez 1991).

Since microsporidia and gregarines are the threats to the global shrimp industry, their identification and molecular characterizations are important for better understanding their infection mechanisms and virulence in order to facilitate the development of preventative and therapeutic strategies. However, information on identification and molecular characterizations of microscopridia and gregarine associated with shrimp industry in Bangladesh yet to be explored. Therefore the present study was conducted to detect and characterize microsporidia and gregarines affecting shrimps in Satkhira and Mymensingh districts of Bangladesh.

Materials and Method

Study Area

The study was conducted on shrimps present in coastal area Satkhira and Mymensingh districts of Bangladesh. The samples were examined and processed for microscopic examination and molecular detection in the Laboratory of the Department of Parasitology, Bangladesh Agricultural University, Mymensingh.

Sample collection

In this study a total of 50 Baghda shrimp (*Paenus monodon*) and 40 local shrimp (Prawn small, Deshi Chingri) were collected from Sathkhira and Mymensingh districts of Bangladesh. Baghda shrimps were reared in river of Shymnagar Upazila under Satkhira District and small prawn were from beal and river area of Gouripur upazila of Mymentsingh district. The shrimps were transferred to the Department of Parasitology, Bangladesh Agricultural University, Mymensingh by maintaining a cool-chain using ice-box. The gut content and heaptopancreas were removed from the shrimps and examined for identification of microscopidia and gregarine. The samples were stored at -20°C prior to DNA extraction.

Microscopic examination of shrimp sample

The collected samples were examined at the laboratory of Department of Parasitology. A conventional thin smear was prepared and examined under microscope for morphological identification of microsporidian and gregarine parasites.

DNA extraction

DNA was extracted from collected samples gut and hepatopancreas of shrimp using Purelink^{TM} Genomic DNA Mini kit (Invitrogen) following manufactures instructions. The DNA was quantified using NanoDrop One Spectrophotometer (NanoDrop One Microvolume UV-Vis Spectrophotometer, Thermo Fisher Scentific Inc., USA) and stored at 20°C.

PCR detection of E. hepatopenaei (EHP)

A fragment of the ssrRNA gene from the microsporidian infecting the shrimp was amplified in duplicate from the four genomic DNA samples using previously described primers (Tourtip *et al.,* 2009): forward primer MF1 (5'-CCGGAGAGGGAGCCTGAGA-3') and reverse primer MR1 (5'-GACGGGCGGTGTGTACAAA-3').

Amplification reactions were carried out in a thermo cycler (MJ Research Inc., Waltham, MA, USA) and contained 10 mMTris–HCl, pH 8.3, 50 mMKCl, 1.5 mM MgCl2, 0.1 mM each of dNTP, 2.5 l M of each primer, 1 unit of Taq polymerase (Applied Biosystems, Foster City, CA, USA), and 20–50 ng genomic DNA in a total volume of 25µl. The mixture was subjected to following the thermo cycling conditions were as follows: an initial denaturation at 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 1 min, primer annealing at 52°C for 1 min, chain extension at 72°C for 2 min, with a final extension at 72°C for 5min. The amplified product of PCR assay was analyzed by electrophoresis on a 1.5% agarose gel and stained with Ethidium Bromide.

PCR detection of Gregarines

Small subunit (SSU) rDNA sequences were PCR-amplified using primers: F1 5′-GCGCTACCTGGTTGATCCTGCC-3′ and R1 5′-GATCCTTCTGCAGGTTCACCTAC-5′- (Leander et al., 2003). Thermal cycler was performed in a volume of 25 µl containing: 2.5 µl of genomic DNA, 0.5 mM of each primer, 0.2 mM of each dinucleotide, 2.5 mM of MgCl2, 5 µl of PCR buffer and 1 unit of Taq polymerase (Applied Biosystems, Foster City, CA, USA).

After 4 cycles of initial denaturation at 94 °C for 4.5 min, 45 °C for 1 min and 72 °C for 1.75 min, 34 cycles of 94 °C for 30 sec (denaturation), 50 °C for 1 min (annealing), 72°C for 1.75 min (extension), followed by a final extension period at 72 °C for 10 min were performed.

Gel electrophoresis

The amplified product of PCR assay was analyzed by gel electrophoresis on 1.5% agarose gel at 100V for 30 minutes, stained with EtBr solution and the image was captured using gel documentation system. PCR products corresponding to the expected size were gel isolated under transilluminator.

Results

Occurrence of Gregarine

No samples were positive for gregarine parasite in microscopy and PCR examination.

Occurrence of Microsporidia

During this study, a total of 90 shrimp samples were examined microscopically (Figure 1), of which 9 positive samples (22.5%) (pooled to 3) from Mymensingh and 15 positive samples (30%) (pooled to 5) from Satkhira were found. The pooled samples subjected to DNA extraction were amplified with target gene sequence showed 1 PCR positive from Mymensingh and 2 PCR positive from Satkhira. The study shows more infection in Baghda shrimp (*Paenus monodon*) than local shrimp (Prawn small, Deshi Chingri). The study also shows that the occurrence of *Microsporidia* in Satkhira is higher than Mymensingh as shown in Table 1.

Table 1: Occurrence of *Microsporidia* in shrimp in Satkhira and Mymensingh district

Study area	No. of samples examined	Microscopically positive	PCR positive	Positive rate
Satkhira	50	15 (Pooled to 5)	2	30%
Mymensingh	40	9 (Pooled to 3)	1	22.5%
Total	90	24 (8 pooled)		

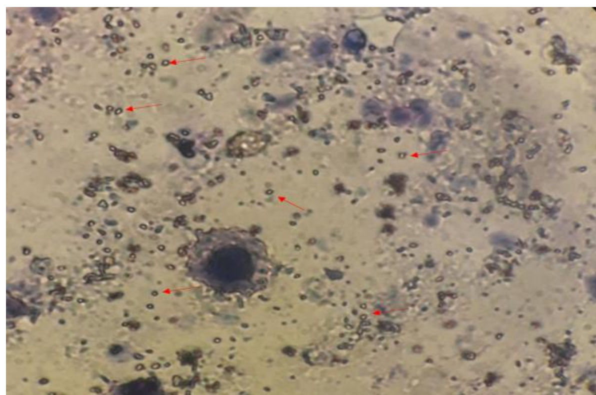

Figure 1: Microsporidia under microscope, 40X Microscopy, Giemsa staining

Detection of Microsporidia by PCR

In the first step, extracted DNA samples from microscopically positive samples 15 (Pooled to 5) from Satkhira and 9 (Pooled to 3) from Mymensingh were subjected to initial PCR. During this study, microscopically positive pooled samples were examined of which 1 PCR positive was found in Mymensingh out of microscopically positive 9 samples (pooled to 3) and 2 found out of microscopically positive 15 samples (pooled to 5) in Satkhira. PCR amplified product was ~1100 bp confirming the presence of Microsporidia organism in the samples (Figure 2). PCR amplification products were analyzed by running them on electrophoresis gel. Fifteen microlitres of PCR products and DNA ladder of 1kbp were run on a 1.5% agarose gel at 100 volts for 30 minutes.

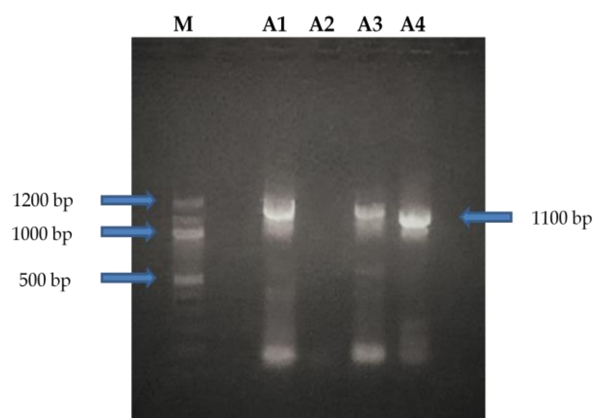

Figure 3: Detection of Microsporidia by PCR: Expected band size of Microsporidia is 1100bp. M (Ladder-1kbp), A1-A4 are samples.

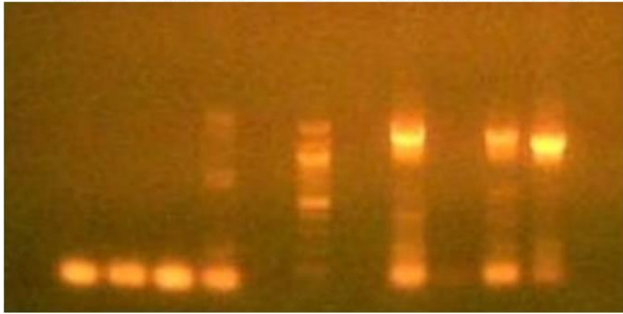

Figure 2: Gel image of PCR product. G1-G4 samples amplified for gregarine parasite (No band observed). Sample M1-M4 samples amplified for Microsporidia with expected band size of 1100bp. M (Ladder-1 kbp).

Discussion

Microsporidian parasites are a zoonotic pathogen, which are spore forming unicellular parasites that can affect aquaculture severely. The phylum Microsporidia consists of obligate intracellular parasites thought to be derived from the Fungi (Edling *et al.* 1996; James, 2006). They are known to parasitise most of the major phyla of invertebrates and five classes of vertebrates (Canning and Vavra, 2000).

Giemsa staining of tissue smears from squash preparation of hepatopancreas and white feacal white faecal strings (gut) with light microscopic examination are routinely used in the detection of microsporidia in clinical and field samples in Bangladesh. Light microscopic observation on white faecal matter revealed that the samples collected from different farms contained large number of microsporidian spores. Association of microsporidian with white faeces syndrome, had been reported previously by Ha *et al.,* (2010). Flegel (2012) has indicated that severe infection with a microsporidian morphologically similar to *E. hepatopenaei, which* associated with WFS of *P. vannamei*. On the contrary, later, Tangprasittipap *et al.,* (2013) reported that EHP is not the cause of WFS and, further, according to Sriurairatana *et al.,* (2014) the syndrome arises due to transformation, sloughing and aggregation of hepatopancreatic microvilli into vermiform bodies. Moreover, they have reported that the outbreaks of EHP are prevalent in China, Indonesia, Malaysia, Vietnam and Thailand, and the parasite was detected by PCR in samples of slow growing shrimp collected from India (Rajendran *et al.,* 2016).

A microsporidian parasite, initially reported from tiger shrimp (*Penaeus monodon*) and subsequently from whiteleg shrimp (*Penaeus vannamei*), has become one of the most serious emerging pathogens in all the shrimp growing countries in Asia. In the present investigation, EHP has been identified and characterised for the first time from farm-reared *P. vannamei* from Bangladesh. Microsporidians are unicellular eukaryotes that are obligate intracellular parasites infecting wide range of eukaryotic hosts, from protists to humans (Corradi, 2015). Several species of microsporidians have been reported from penaeid shrimp (Lightner, 1996). In the present study, typical microspodian spores could be identified in the smear preparation of hepatopancreatic tissues through light microscopy. However, only heavily infected HP showed the presence of spores in the smear preparation under the microscope. This indicates that light infection of EHP can go undetected if only microscopy is used for the detection. The morphology and morphometry of the spores showed close similarity with the spores of EHP reported from *P. monodon* (Tourtip *et al.,* 2009). However, spores observed in the present study were found to be larger than the previously reported EHP and the negligible variation in spore size could be due to the difference in the processing of the spores for microscopy.

In addition to conventional microscopic examination, we performed Polymerase chain reaction (PCR) which is a gold standard for diagnosis of many shrimp diseases as outlined in the protocols of the World Organization for Animal Health (OIE, 2019a, 2019b, 2019c). A number of PCR-based methods have been described for EHP detection, including one-step PCR (Tang *et al.,* 2015; Tourtip *et al.,* 2009), nested PCR (Jaroenlak *et al.,* 2016; Tangprasittipap *et al.,* 2013), and multiplex PCR that can detect EHP alongside other shrimp pathogens (Koiwai *et al.,* 2018). In general, one-step PCR is simpler to perform and requires only one set of primers, but the detection limit often ranges from 1000–10,000 copies per reaction.

PCR based methods have been used increasingly in the detection and characterization of Microsporidia. The detection specificity and sensitivity is high in PCR based methods. Organisms present at low infection levels could be masked by those with higher infection levels and could therefore be missed in traditional method. The SSU rRNA gene was the target since this gene

provides more accuracy to identify Microsporidia from shrimp (Noaman *et al.,* 2009).

The gene has a suitable length of about 1100bp which is large enough, with sufficient inter specific polymorphisms to provide distinguishing and statistically valid measurements. Besides, SSU rRNA gene is universally distributed in all parasites, thus relationships can be measured among them. The whole sequence of SSU rRNA is highly conserved and functionally constant through evolutionary history.

Conclusion

During this study, the PCR detection of microsporidia was found positive from most of the microscopically positive samples. In the PCR assessment, microscopically positive pooled samples were examined microsporidia were confirmed.

The study shows that the occurrence of microsporidia is more in Baghda shrimp (*Paenus monodon*) than local shrimp (Prawn small, Deshi Chingri). However, we could not compare our results due to paucity of relevant literatures.

Collectively, our study revealed the occurrence of microsporidia in shrimp and people are at risk to the infection.

Acknowledgement

The research work was supported by the special research grant (BS/86, 2019-20) from Ministry of Science and Technology, Government of Bangladesh.

References

Feigenbaum, D. L. (1975). Parasites of the commercial shrimp Penaeus vannamei Bcon and Penaeus brasiliensis Latreille. Bulletin of Marine Sciences. 25: 491–514.

Leander, B. S., Clopton, R. E. & Keeling, P. J. (2003). Phylogeny of gregarines (Apicomplexa) as inferred from small-subunit rDNA and beta-tubulin. Int J Syst Evol Microbiol 53, 345–354. Kruijssen, I. Froukje, H. Kelling, K. MeeChee, Jespersen and S. Ponte. 2012: Value chains of selected aquatic products from four Asian countries.

Lotz J. M., Overstreet, R. M. and Grimes, D. J. (2006). Aquaculture and Animal Pathogens in the Marine Environment with Emphasis on Marine Shrimp Viruses. Pp. 431- 451.

Rajendran, K.V., Shivam, Saloni, Ezhil Praveena, P., Joseph Sahaya Rajan, J., Sathish Kumar, T., Avunje, Satheesha, Jagadeesan, V., Prasad Babu, S.V.A.N.V., Pande, Ashish, Navaneeth Krishnan, A., Alavandi, S.V., Vijayan, K.K., Emergence of Enterocytozoon hepatopenaei (EHP) in farmed *Penaeus* (Litopenaeus) *vannamei* in India, Aquaculture (2016), doi: 10.1016/j.aquaculture.2015.12.03

Schneider (1892). Nematopsis spp. (Apicomplexa: Gregarinida) in bivalve molluscs off Ria de Vigo (Galicia, NW Spain).

Stentiford, G. D., Bateman, K. S., Small, H. J., Moss, J., Shields, J. D., Reece, K. S., & Tuck, I. 2010: *Myospora metanephrops* (ng, n. sp.) from marine lobsters and a proposal for erection of a new order and family (Crustaceacida; Myosporidae) in the Class Marinosporidia (Phylum Microsporidia). International Journal for Parasitology, **40** 1433-1446.

Tangprasittipap A, Srisala J, Chouwdee S, Somboon M, Chuchird N, Limsuwan C, Srisuvan T, Flegel TW, Sritunyalucksana K. 2013: The microsporidian *Enterocytozoon hepatopenaei* is not the cause of white feces syndrome in white leg shrimp *Penaeus (Litopenaeus) vannamei*. BMC Veterinary Research, **9** 139-139.

Thitamadee S, Prachumwat A, Srisala J, Jaroenlak P, Salachan PV, Sritunyalucksana K, Flegel TW, Itsathitpaisarn O. 2016: Review of current disease threats for cultivated penaeid shrimp in Asia. *Aquaculture* **452** 69-87.

Tourtip S, Wongtripop S, Stentiford GD, Bateman KS, Sriurairatana S, Chavadej J, Sritunyalucksana K, Withyachumnarnkul B 2009: *Enterocytozoon hepatopenaei* sp. nov. (Microsporida: Enterocytozoonidae), a parasite of the black tiger shrimp *Penaeus monodon* (Decapoda: Penaeidae): Fine structure and phylogenetic relationships. *Journal of invertebrate pathology* **102** 21–29.

Vávra, J., and Maddox, J. V. (1976). Methods in microsporidiology. In Biology of the Microsporidia (pp. 281-319). Springer, US.