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A Fungal Infection Caused by *Lagenidium sp.* and its Control Measures in Hatchery Reared Shrimp Larvae *Penaeus monodon* in Bangladesh

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The shrimp farm industries in Bangladesh have long been facing problems with the supply of poor quality seeds being produced in shrimp hatcheries. In light of this, the present study was conducted between February to August 2012, concerning the fungal diseases of *P. monodon* larvae reared in a commercial shrimp hatchery in Cox's Bazar, Bangladesh. The causative fungus was identified as a member of the genus *Lagenidium* (Oomycetes, Lagenidiales). High mortalities up to 50% was observed soon after infection. The affected larvae were whitish and filled with numerous aseptate hyphae and larvae lost equilibrium and exhibited respiratory difficulties. The fungal growth was observed on PYGS agar medium at 25°C. Infected untreated populations of nauplii, zoea and mysis stages showed mortalities of 15.75±0.76%, 31.25±3.12%, and 49.5±3.9% respectively. A 0.75 ppm treatment with trifluralin significantly reduced the mortality of the infected larval population. The pathogenicity tests of the infected fungi against the larvae of *P. monodon* by immersion method showed that the isolates were pathogenic causing 50%, 80% and 82% mortality in nauplii, zoea and mysis stage respectively in 96 hours post exposure at 10^4 zoospores/mL. This is the first report of *Lagenidium* sp. infection in shrimp larvae in Bangladesh.

Key words: *Lagenidium sp.*, Fungal infection, *Penaeus monodon* larvae, Trifluralin, Bangladesh.

It has been reported that a total of 56 shrimp hatcheries have been established along the coastal areas of Bangladesh (Aftabuddin and Kader, 2006), particularly in the southeastern part of the country. Presently, 36 shrimp hatcheries are operational, producing seeds of black tiger shrimp *Penaeus monodon*. While farmed and captured crustaceans contribute a considerable proportion with annual production exceeding 10 M metric tonnes with first sale value of $40bn, the decline is due to the outbreak of different diseases caused by bacteria, viruses and fungi, resulting in 40% loss of tropical shrimp production (Stentiford *et al*. 2012). Fungal infection is often reported to be found in hatchery reared eggs and larvae of *P. monodon*. When the fungal infections appear in these hatcheries, they have been attributed to problematic infections or conversion problems because fungal infections cause high mortality of the larvae, (reaching upto 100%) and inhibit larval molting process. Karunasagar *et al*. (2004) reported that nearly 500 fungal species have been isolated from marine and estuarine environment, some of

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them are pathogenic to shrimps. Mostly larval stages of shrimps are commonly affected by *Lagenidium callinectes* and *Serolpidium* spp. (Ramaiah, 2006). The clinical signs such as lethargy and mortality due to fungal infections can be detected in protozoea and mysis stages. Usually, fungal spores and mycelia can be observed in affected tissue. Ramasamy *et al*.(1996) observed 90% of mortalities due to fungal infections in *P. monodon* larvae at nauplii, zoea and mysis stages.

The high incidence of mycosis disease outbreak in recent years has been attributed to the poor quality of hatchery reared larvae and postlarvae. The shrimp farmers raise a question about the quality of hatchery reared seeds. Consequently, the supply of poor quality hatchery reared seeds poses a severe threat to shrimp culture industry of Bangladesh. There are no scientific reports on fungal infection of black tiger shrimp larvae in Bangladesh. Therefore, this study was undertaken for the first time to isolate and identify the pathogenic fungi from *P. monodon* larvae and their remedial measures in Bangladesh.

MATERIALS AND METHODS

The present study was undertaken from February 2012 to August 2012. Samples were collected and the experiment was conducted at a commercial shrimp hatchery, named 'Prime Shrimp Hatchery', located in Cox's Bazar (southeastern coastal district of Bangladesh). Concurrently, water parameters were recorded from the hatchery. The identification test was carried out at 'Shrimp and Fish Disease Diagnosis Laboratory' in the institute of Marine Sciences and Fisheries, University of Chittagong.

Isolation and Identification

Larvae (nauplius, zoeal and mysis stages) of black tiger shrimp *P. Monodon* showing signs of fungal disease were examined by a simple microscope. The suspected larvae with fungal infection were washed in sterile physiological saline (0.85% NaCl) and directly inoculated onto PYGS agar plates (peptone 1.25 g, yeast extract 1.25 g, glucose 3 g, agar 12 g and sterilize sea water 1 L) containing ampicillin and streptomycin sulphate (supplied by a pharmaceutical company named 'Square', Bangladesh) 2 mg each, to prevent bacterial growth and incubated at 25°C for 4 days. After 3-4 days, with the appearance of fungal growth, mycelial masses were then transferred onto new PYGS agar to make a pure culture (Muraosa *et al*. 2006). For morphological observations, the fungal culture was transferred to PYGS broth by inoculating mycelial masses with solid medium for sporulation, followed by the incubation of the culture at 25°C for 4 days. A part of mycelia in PYGS broth was rinsed with sterilized seawater and incubated at 25°C for 36 hours. The single spore culture method of Ho and Ko (1997) was applied to obtain pure isolates. The fungus was identified following the works of Sparrow (1960), Bian *et al*. (1979) and Karling (1981).

Larval rearing operation and applying the fungicide

Five larval rearing tanks (each with 30 ton capacity) were filled with 22 tons of UV treated seawater. Three tanks were kept as experimental tank, treated with trifluralin and another two were kept as a control tank. 10 ppm EDTA (Ethylene diamine tetra acetate) along with 10 ppm ampicillin and streptomycin sulphate was given to all tanks (both experimental and control) two hours before the stocking as a prophylactic treatment. The EDTA is a chelating agent which can remove chemical pollutants like trace metals and other fine debris. Just before stocking, algae (*Skeletonema* sp.) were added at a density of 100,000 cells/ml. The nauplii (N5 /N6) were harvested from hatching tanks and were released into the larval rearing tanks (LRT) slowly in small quantities at different points of the tank at 100,000 nauplii/ton.

Twenty four hours after stocking, the nauplii converted into protozoea I (called zoea I) stage. First feeding was started when the zoea I appeared. The zoeal stages (I to III) were fed with *Skeletonema* sp. twice daily both in control and in experimental tanks. The mysis stages (I to III) were fed with algae at a density of 4×10^5 cells/ml in both control and experimental tanks. In addition to algae, mysis stages were fed with commercially available microencapsulated feed like CAR, CD-2 etc. three times a day. Newly hatched Artemia nauplii were also given as a live feed in mysis III stage. Continuous aeration was maintained at a level appropriate for each larval stage to provide sufficient oxygen and to keep feed and larvae in suspension. Siphoning was performed routinely

(daily from mysis stage onwards) to clean the unconsumed food and fecal materials on the tank bottom. The fungal infection analysis was conducted routinely under a microscope and using PYGS agar to assess the evolution of the fungal zoospores in each tank to help make a decision on any fungicides required. After observing the fungal zoospores, the experimental tanks were treated with trifluralin at 0.75 ppm twice a day, while control tanks left untreated and larval population was counted randomly. Each experiment was carried out once in each cycle, continuing up to 4 cycles. The water quality parameters of the experimental and control tanks were regularly monitored.

Experimental challenge test

Shrimp larvae (n=50 each stage), nauplii, zoea and mysis of *P. monodon* were held in a 100 ml glass beaker, filled with 75 ml of autoclaved seawater, maintaining temperature 25°C, pH 7.8, salinity 25 ppt. There was no water exchange during the challenge test. The number of zoospores in the seawater was adjusted to 1.0×10^5, 1.0×10^4 and 1.0×10^3 zoospores/ml. In the control group, each glass beaker with autoclaved seawater had only 50 nauplii, zoea and mysis. To minimize the bacterial contamination, 200µg/ml of ampicillin together with the same amount of streptomycin sulphate was added to the seawater. The larvae were examined under an inverted microscope at every 24 hours for 4 days after exposing to zoospores. The experiment was conducted at 25° C without feeding. Before and after the challenge test, water and shrimp larvae samples were processed for fungal examination. *Lagenidium* sp., recovered from the infected larvae, was reidentified to confirm identity with the test strains. The fungal infection experiment was performed based on the method described by Muraosa *et al*. 2006.

RESULTS AND DISCUSSION

Morphology of the fungus

The isolated fungus was whitish, flat and filamentous on PYGS agar (Fig. 1), and the vegetative hyphae were septate and irregularly branched. Zoospore formation was observed after 48 hours of the mycelia were transferred into sterilized seawater. During the zoospore formation, discharge tubes were developed gradually from hyphae (Fig. 2). It severely destroyed the shrimp larvae (Fig. 3) and caused larval mortality up to 75%. The zoospores were typically 7-9 µm in diameter, monoplanetic and creatively biflagellate (Fig. 4). The vegetative hyphae were crooked, usually 18-25 µm in width, 90-130 µm in length with greenish yellow on refractive light and consisted of intense cytoplasm with numerous oil droplets (Fig. 5).

The class Peronosporomycetes (Oomycetes) has four genera i.e. *Lagenidium*, *Haliphthoros*, *Halocrusticida* and *Atkinsiella* which are known as pathogens in marine crustaceans (Roza and Hatai, 1999, Hatai *et al*. 2000). Recently, the class Perononsporomycetes

Table 1. Cumulative mortality observed from the pathogenicity test of *Lagenidium* sp. on *P. monodon* larvae.

P. monodon larval stages	Challenge dose (Spore/mL)	Percentage of mortality (n=50)			
		24 hrs	48 hrs	72 hrs	96 hrs
Nauplii	10^2	0	0	6	10
	10^3	0	0	10	20
	10^4	10	20	40	50
	Control	0	0	0	0
Zoea	10^2	0	0	10	20
	10^3	0	10	20	40
	10^4	20	40	70	80
	Control	0	0	0	0
Mysis	10^2	0	0	6	6
	10^3	10	30	50	60
	10^4	20	40	68	82
	Control	0	0	0	0

is transferred from the kingdom Fungi to the newly constructed kingdom Stramenopila (Chromista) based on molecular phylogenetic analysis (Muraosa *et al*. 2006). However, the infection caused by some members of Peronosporomycetes in marine crustaceans is still classified as fungal infection in the field of shellfish disease (Muraosa *et al*. 2006). The disease observed here in the larval

Table 2. Percentage of larval mortality (average percentage calculated by no. of control and experimental tanks)

	% of mortality at nuplius stage	% of mortality at zoeal stage	% mortality at mysis stage
Cycle 1			
Control tanks (without fungicide)	14±0.45	30±2.5	45±4.0
Experimental tanks (with fungicide)	5±0.25	10±1.2	12±1.5
Cycle 2			
Control tanks (without fungicide)	16±1.0	32±3.0	50±3.5
Experimental tanks (with fungicide)	7±0.5	8.5±1.0	15±2.0
Cycle 3			
Control tanks (without fungicide)	15±1.2	28±3.0	48±4.6
Experimental tanks (with fungicide)	6±0.4	12±1.0	15±1.2
Cycle 4			
Control tanks (without fungicide)	18±0.5	35±4.0	55±3.5
Experimental tanks (with fungicide)	8±0.4	13±1.0	18±1.0
Average mortality in control tanks	15.75±0.76	31.25±3.12	49.5±3.9
Average mortality in experimental tanks	6.5±0.39	10.88±1.05	15±1.42

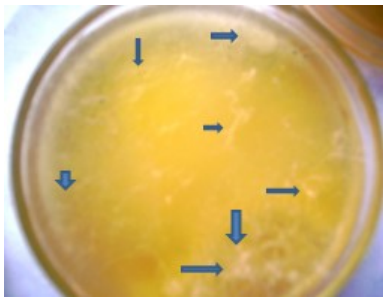

Fig. 1. Whitish, flat and filamentous fungus on PYGS agar plate

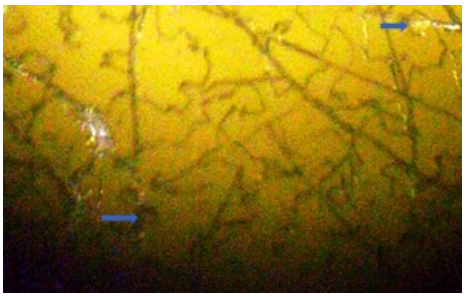

Fig. 2. Discharge tubes the top of the hyphae

Fig. 3. A protozoea of *P. monodon* larva infected with fungus

Fig. 4. Swimming zoospores with biflagella in sterilized seawater

shrimp *P. monodon* was remarkably similar to the mycosis recognized to *Lagenidium* described by Couch (1942) in ova of the blue crab, Lightner and Fontaine (1973) in the panaeid white shrimp, *Penaeus setiferus*, Bian *et al*., (1979) and Nakamura *et al*., (1995) in larvae of mangrove crab, *Scylla serrata*, Ramasamy *et al*., (1996) and Muraosa *et al*., (2006) in eggs and larvae of tiger shrimp *Penaeus monodon*. The isolate was creatively biflagellate zoospores, endobiotic and holocarpic thalli with segmented hyphae and irregularly branched.

Experimental challenge test

The results of cumulative mortalities of *P. monodon* larvae experimentally infected by immersion method with isolated fungi *Lagenidium* sp. were shown in Table 1. The results of pathogenicity experiments showed considerable differences in the virulence of various stages of *P. monodon* larvae. Different concentration of fungal zoospores caused varying rates of *P. monodon* larval mortality during the 96 hours exposure period. In all cases, the mortality rate was low at low concentrations and it increased with the increase of concentration and exposure time (Table 1). The pathogenicity study revealed that the isolated *Lagenidium* sp. was capable of causing 50%, 80% and 82% mortality in nauplii, zoea and mysis stage respectively in 96 hours post exposure at 10^4 zoospores/mL. The mortality was significantly different ($P<0.05$) at zoospore concentration 10^4 as compared to 10^2-10^3. In control group, no infection was observed in nauplii, zoea and mysis. The experimental exposure to the fungus severely damaged the *P. monodon* shrimp larvae and high mortality was observed up to 82% at mysis stage. Chien and Lin (1985) observed 85% mortality in *P. indicus* larvae by *Lagenidium* sp. in Taiwan, which closely related to present investigation.

Fig. 5. Irregularly branched vegetative hyphae of *Lagenidium sp*.

During the larval rearing operation, in control tanks (i.e. *Lagenidium* sp. infected), untreated populations of nauplii, zoea and mysis exhibited mortalities of 15.75%, 31.25% and 49.5%, respectively (Table 2). The present study observed that after treatment with trifluralin (0.75 ppm twice a day) significantly reduced the mortality of infected larval population i.e. 6.5% nauplii, 10.88% zoea and 15% mysis. Lightner and Fontaine (1973) also observed that a *Lagenidium* sp. was infective to larval white shrimp, *Penaeus setiferus*, and a brown shrimp, *Penaeus aztecus* while reared under laboratory conditions. The researchers observed that the natural mortality was 12.4% after the fungal mycelium had invaded and replaced nearly all the internal tissues, while 20% of the larval shrimp died after experimental exposure to the fungus. Ramasamy *et al*. (1996) reported that *Lagenidium callinectes* infected, untreated populations of nauplii, zoea and mysis, exhibited mortalities of 5% 25% and 48%, respectively in India. A 0.5 ppm treatment with trifluralin reduced the mortality of infected larval populations (i.e. 1.1% nauplii, 3.28% zoea and 5.21% mysis mortality) which fairly related to present investigation.

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