Black gill disease of Pacific white leg shrimp (Litopenaeus vannamei) by Aspergillus flavus

Naresh Kumar Dewangan1, Ayyaru Gopalakrishnan1*, Daniel Kannan2, Narayanasamy Shettu2, Ramakrishna Rajkumar Singh1
1Centre of Advanced Study in Marine Biology, Faculty of Marine Sciences, Annamalai University, Parangipettai 608502, Tamil Nadu, India
2PG & Research Department of Zoology, Pachaiyappa’s College, Chennai 600030, Tamil Nadu, India
3CP Aquaculture India Private Limited, Sambasivanagar, Ongole 523002, Andhra Pradesh, India

1. Introduction

Aquaculture is playing an important role as a prominent source of nutrition and also presents employment opportunity to millions. This sector is growing as a multi-million dollar industry and the farmed fish contributes 42% of total 158 million tons of fish produced by capture and culture fishery[1]. The share of fisheries sector in the national total export earning is about 6%, out of which approximately 85% is being contributed by shrimp alone[2]. Indian coastal zone has 1.40 million ha brackish water area, out of which 1.19 million ha area has been declared as suitable for shrimp aquaculture. India is the fastest growing nation in shrimp aquaculture with natural resources like brackish water, mud flats, swamps, marshes, lagoons, backwaters and estuaries[3].

The culture of shrimp received maximum importance due to high nutritional value, but the disease outbreak during the culture is a major concern[4]. Some environmental factors, infectious organisms (fungi and viruses, protozoans etc.), and pollutants (chemical and biological) also cause the mass mortalities in the pond[5]. These environmental factors negatively influence the sustainability of shrimp farming and also directly affect to the growth rate and survival in the grow-out culture system[6].

Black gill disease was first reported in Japanese Kuruma prawn, Penaeus japonicas (P. japonicas) by Ishikawa in 1968. Fusarium species was the causative agent for this black gill disease, and later Fusarium species was considered as the most detrimental pathogens for Kuruma prawn in Japan[7]. Chemical contaminants like oil, cadmium, copper, zinc, potassium permanganate, ozone, ammonia,
nitrate, ascorbic acid deficiency, heavy siltation, high organic load due to residual feed, debris, and fecal matter on pond bottom have also been reported to be responsible for black gill disease[8]. Fungal infections are secondary reason following the bacteria reason for economic losses in aquaculture[9]. Fungi which have ability to produce proteinase and phospholipase like enzyme facilitate the adhesion and invasion in host tissue, because they can easily degrade cell membranes which are composed mainly of proteins and lipids[10]. Gill is the most important organ which is responsible for the respiration in shrimps. Fungal infection in gill may be lethal for the shrimps as the fungi can block the respiration, increase the chronic mortality of shrimps and also pave the way for the susceptibility of shrimps to other diseases. Change in the colour of the gill is the first clinical symptom of fungal infection. In the early stages, the gills appear opaque white to yellow or brown in colour and finally it appears as black[11].

Although fungi occur widely in marine environment, the distribution of fungi in diseased shrimps has not been studied well as compared to the studies on fungi in freshwater and terrestrial ecosystems[12]. Therefore, the present study was undertaken with the objective to identify the major causative pathogens for the black gill disease in Litopenaeus vannamei (L. vannamei).

2. Materials and methods

Normal and diseased L. vannamei were collected from shrimp farms in Mahendrapalli, Tamil Nadu (11°21’36.9756” N, 79°48’37.6884” E) during March and June 2014. Shrimps weighed 15-25 g were collected on the basis of colour of gill shrimp. Total area of pond, days of culture, dissolve oxygen, pH, stocking density and temperature were recorded. Gills were isolated from normal and diseased L. vannamei by the help of sterile scalpel blade. Black gills and normal gills were fixed separately in Davidson’s fixative and transferred in 50% ethanol for preservation. Gill tissues were sectioned approximately 3 µm by the help of microtome and the processed tissues were stained with haematoxylin and eosin. Tissue was examined by using the light microscope under different magnifications.

After separation of gills, wet mount slides were prepared separately for both normal and black gills which were then observed by using the light microscope under different magnifications. Gills were isolated from shrimps and washed thrice in 0.85% NaCl solution. After washing, the gills were inoculated in potato dextrose agar (PDA) medium supplemented with ampicillin and streptomycin to inhibit the bacterial growth and then incubated at 25 °C. After gill isolation, lacto phenol cotton blue was used for microscopic observation of fungi. Normal gills were also inoculated in the same medium as control. Experiment was performed in triplicate. Infected shrimp gills were first preserved in 3% glutaraldehyde, then dehydrated, coated with gold and observed under scanning electron microscope JEOL JSM 5610LV at 15 kV. DNA was amplified by using ITS4 and ITS5 primers[13]. DNA was sequenced by Macrogen Inc., Korea. The sequence similarity was analyzed by using basic local alignment search tool and the phylogenetic tree was constructed by using MEGA 6 software.

3. Results

3.1. Shrimp sampling

The shrimp farm was 0.66 ha where the dissolved oxygen level was 4.25 mg/L, pH was 7.4, stocking density was 30/m² and the temperature was 27–33 °C. The gills of 130-day-old normal shrimps were found white (Figure 1A) while the gills of diseased shrimps were black in colour (Figure 1B). We observed that the infected gills in the initial stage were slightly black and it gradually converted to dark black. Fungal spreading may be the reason for change in the colour of gill.

![Figure 1. Images of L. vannamei gills. A: Normal gill of L. vannamei; B: Black gill of diseased L. vannamei.](image)

3.2. Histopathology

In the case of normal gills, fungi were absent in gill lamella and physiology of gills was not changed (Figure 2A). However, in the case of black gills, the tissue was damaged and fungal hyphae, conidia, and whole sporangium were present inside the gill lamellae (Figure 2B). Normal gill filament was properly arranged at the central axis while black gill filament and central axis were in...
damaged condition.

**Figure 2.** Images of tissue sections of gill lamella. A: Normal gill lamella; B: Black gill lamella.

### 3.3. Microscopic observation of shrimp gills

Microscopic study revealed very clear and transparent gills in normal shrimps (Figure 3A). However, the affected gills were found to be more fuzzy and black in appearance and hyphae strongly attached to central axis of gills (Figure 3B). Outward growth of the fungi from the gill lamella was very prominent (Figure 3C). Mature sporangium was present around the gill lamella (Figure 3D). Fungal hyphae and conidia were also observed inside and in the outer surface of the affected gill lamellae (Figures 3E and 3F).

**Figure 3.** Light microscopic observation of gills. A: Image of normal gill lamella; B: Fuzziness of black gill lamella infected with fungi; C: Outward growth of fungi from black gill lamella; D: Presence of fungi around the gill lamella; E: Presence of fungal hyphae inside the affected gill lamella; F: Presence of conidia in the outer surface of black gill lamella.

### 3.4. Isolation and colony morphology of fungi

After 48-h incubation, no fungal growth was observed in the case of normal gills (Figure 4A), but in the affected gills, fungi became clearly visible in the petriplate, and after 72 h, it was transformed into matured colony. The fungus was lime green and yellowish in appearance. However, the reverse side was cream colour. The texture was cottony, granular with spherical shape, flat elevation with entire margin (Figure 4B).

### 3.5. Microscopic observation of fungi

Hyphae were septate and hyaline, while conidia was smooth, very finely roughened and loosely attached to the vesicles. Conidiophores were found long, coarsely rough and colorless, while vesicles were globose to subglobose. Phialides were present around the vesicle (Figures 4C and 4D).

**Figure 4.** Microscopic observation of fungi. A: Image of control PDA medium plate inoculated with normal gills; B: Colony of *A. flavus* NKD1 isolated from black gills of *L. vannamei* in PDA medium; C: Light microscopic view of *A. flavus* NKD1 stained with lacto phenol cotton blue; D: Close-up view of *A. flavus* NKD1 in light microscope (100×).

### 3.6. Scanning electron microscope (SEM) analysis

SEM analysis revealed the absence of fungi in normal gills (Figure 5A) and also showed the presence of mature sporangium with long conidiophores in black gills (Figure 5B). After detachment of spores, clear phialides were visible. Microspores and hyaline mycelium were present in the black gills of *L. vannamei*. Fungal mat was observed all over the gills (Figure 5C). Conidia were spared on the gill surface (Figure 5D).

### 3.7. Phylogenetic analysis

Based on ITS gene sequencing, the pathogenic fungus was found to be *A. flavus* strain NKD1 (Genbank accession No. KM282572). In the analysis of basic local alignment search tool, fungi which were isolated from black gills showed 100% similarity to *A. flavus* (KJ175414) (Figure 6). In phylogenetic tree, it clustered in the
same clade of Aspergillus genus with the bootstrap support of 97. Neighbour joining tree showed the position and similarity of A. flavus NKD1 to other Aspergillus strains.

Figure 5. Scanning electron microscopic view of gills. A: Image of normal gill lamella; B: Mature sporangium of A. flavus NKD1 fungi; C: Black gill lamella covered by fungal mycelium; D: Small conidia present in black gill lamella.

Figure 6. The phylogenetic tree of the pathogenic fungus resulting in black gill disease.

4. Discussion

L. vannamei is one of the important cultivable shrimps all over the world; therefore, the healthy shrimp production is need of the hour. In the present study, we have isolated A. flavus from black gills of L. vannamei collected from a shrimp pond. Previously, A. flavus was also reported as the predominant species in L. vannamei out of 146 fungal isolates, but it was not isolated from black gills[10]. Apart of A. flavus, Fusarium oxysporium was also reported to be responsible for black gill disease in P. japonicas[14]. Black gill disease in cage-cultured ornate rock lobster Panulirus ornatus in Vietnam by Fusarium species has been observed[15]. Our present study was about L. vannamei in brakish salt water. Recently, a phytopathogenic fungus Gilbertella persicaria has been reported in shrimp Penaeus monodon cultured in brackish water along the east coast of India[16]. However, in the case of fresh water prawn Macrobranchium rosenbergii, the Fusarium infection has also been studied[17]. Histopathological observation in the present study clearly showed that gill tissue was damaged by A. flavus. The pathogenicity of anamorphic fungi in mantis shrimp Oratosquilla oratoria (O. oratoria) has also been reported[18]. In the present study, we have isolated A. flavus fungi from adult shrimps, while the fungal disease in larvae of Penaeus setiferus[19] and egg as well as larvae of Penaeus monodon by Legendium thermophilus has been reported previously[20]. Fungi can be secondary infection also in other diseased shrimps like loose shell syndrome[21]. Halioticida infection in wild mantis shrimp O. oratoria has also been reported in Japan[22]. Molecular phylogeny and morphology of Fusarium solani isolated from black gills were described[23], but because of similar morphological attributes, A. flavus was classified as separate species with respect to Aspergillus oryzae based on genetic diversity assessment of subtelomeric regions[24]. In the phylogenetic observation of present study, A. flavus showed close similarity to A. flavus, thereby clustering in the same clade of Aspergillus genus.

Black gill disease has been reported in shrimps like P. japonicas, rock lobster Panulirus ornatus, and mantis shrimp O. oratoria induced by Fusarium species and anamorphic fungi. However, no even a single report is available on this most economic and nutritional shrimp L. vannamei. In this context, we have isolated A. flavus fungi from black gills of L. vannamei which is a serious problem in shrimps growing in the ponds in the east coast of India. Microscopic and histopathological study clearly showed the presence of fungi inside the gill lamella. SEM analysis clearly showed the presence of fungi in the black gill shrimps. The fungal morphology along with ITS gene sequencing confirms the pathogenic fungus as A. flavus NKD1. Hence, we hypothesize that A. flavus could be the major pathogenic fungus causing the black gill disease in L. vannamei in India. Probiotics are used for quite a long time in aquaculture[25]. Hence, application of probiotics and chemistry of antifungal agents, though banned by Coastal Aquaculture Authority of India, is suggested to control this fungal disease in shrimp farms[26].

Conflict of interest statement

We declare that we have no conflict of interest.
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