Journal of Coastal Life Medicine

journal homepage: www.jclmm.com

Original article doi: 10.12980/jclm.3.2015j5-70

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

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ARTICLE INFO

Article history:
Received 1 Jun 2015
Accepted 28 Aug 2015
Available online 28 Sep 2015

Keywords:
Aquaculture
Litopenaeus vannamei
Black gill disease
Aspergillus flavus
Scanning electron microscopy

ABSTRACT

Objective: To study the epidemiology of black gill disease in white leg shrimp which is a major problem being faced by the commercial shrimp farmers who are culturing *Litopenaeus vannamei* (*L. vannamei*) in India.

Methods: The normal and infected shrimps were collected from shrimp pond and the gill was preserved in appropriate preservative for histopathological examination and scanning electron microscope analysis. Pathogenic fungus was isolated from black gill of *L. vannamei* in potato dextrose agar medium. Morphological study and fungal strain identification were done by using light microscopy and scanning electron microscope. Fungal DNA was amplified by ITS4 and ITS5 primers and gene sequencing was done by Macrogen Inc., Korea. Phylogenetic tree was prepared by using MEGA 6 software.

Results: Fungal spores and hyphae were observed both in internal and external gill surface of infected shrimps. Fungal spores were round in shape and mature sporangium was observed. The histopathology study showed clearly that infected gill was damaged by the fungi. Scanning electron microscopic study showed adherence of fungi in infected gill. Internal transcribed spacer gene sequencing revealed that it was caused by *Aspergillus flavus*.

Conclusions: The outcome of the present study would help to know the cause of black gill disease and to understand the effect of pathogenic fungi in shrimp culture. This study will initiate researchers for work in field of treatment or prevention of black gill disease in commercial *L. vannamei* culture.

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,

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Foundation Project: Supported by Science and Engineering Research Board of Department of Science and Technology, Government of India, New Delhi (Grant No. SR/SO/AS-012/2013).

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 transfered 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/m^2 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.

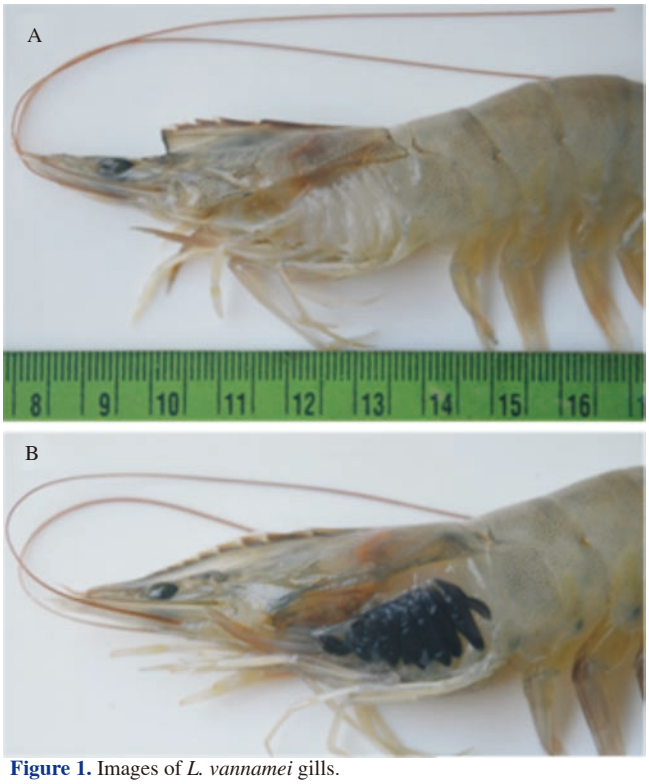

Figure 1. Images of *L. vannamei* gills.
A: Normal gill of *L. vannamei*; B: Black gill of diseased *L. vannamei*.

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.

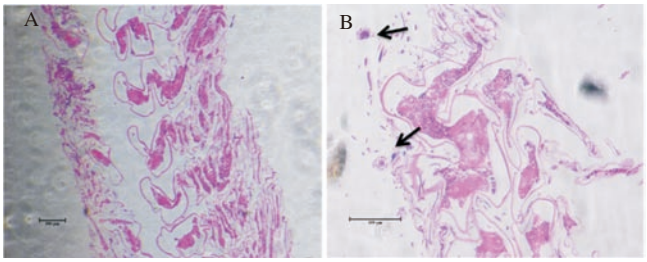

Figure 2. Images of tissue sections of gill lamella.
A: Normal gill lamella; B: Black gill lamella.

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) .

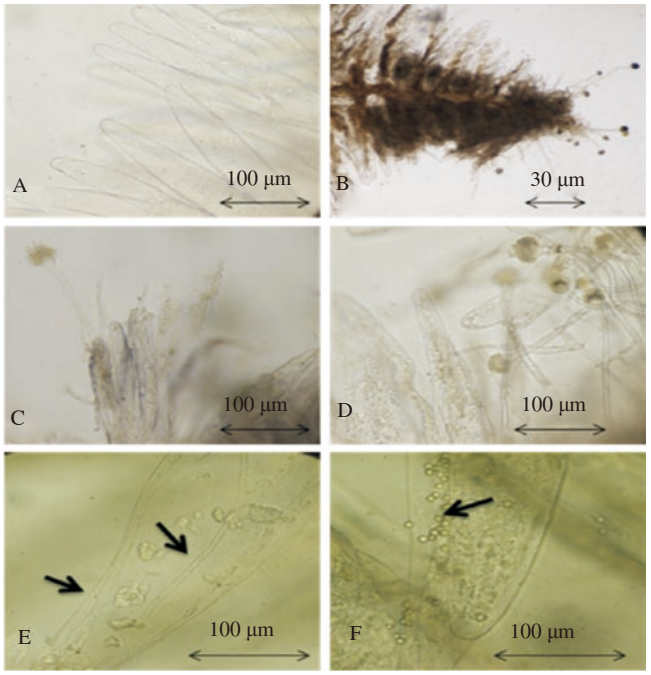

Figure 3. Light microscopic observation of gills.
A: Image of normal gill lamella; B: Fuzziness of black gill lamella infected with fungi; C: Outward growth of fungi from black gill lamella; D: Presence of fungi around the gill lamella; E: Presence of fungal hyphae inside the affected gill lamella; F: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

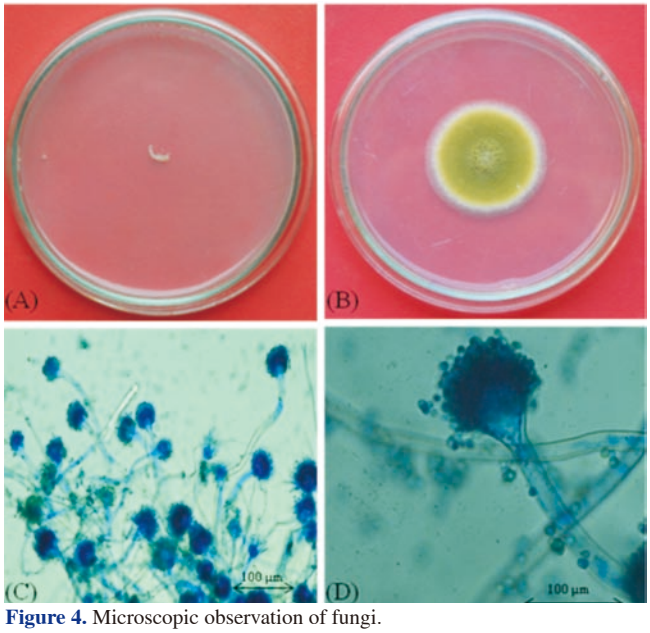

Figure 4. Microscopic observation of fungi.
A: Image of control PDA medium plate inoculated with normal gills; B: Colony of *A. flavus* NKD1 isolated from black gills of *L. vannamei* in PDA medium; C: Light microscopic view of *A. flavus* NKD1 stained with lacto phenol cotton blue; D: Close-up view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

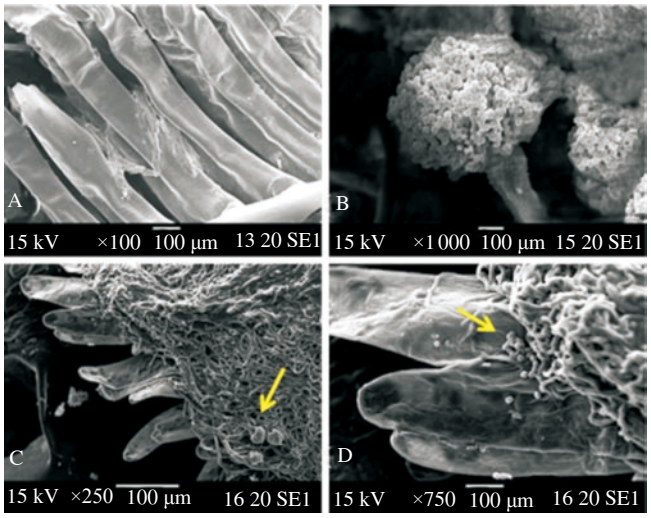

Figure 5. Scanning electron microscopic view of gills.
A: Image of normal gill lamella; B: Mature sporangium of *A. flavus* NKD1 fungi; C: Black gill lamella covered by fungal mycelium; D: Small conidia present in black gill lamella.

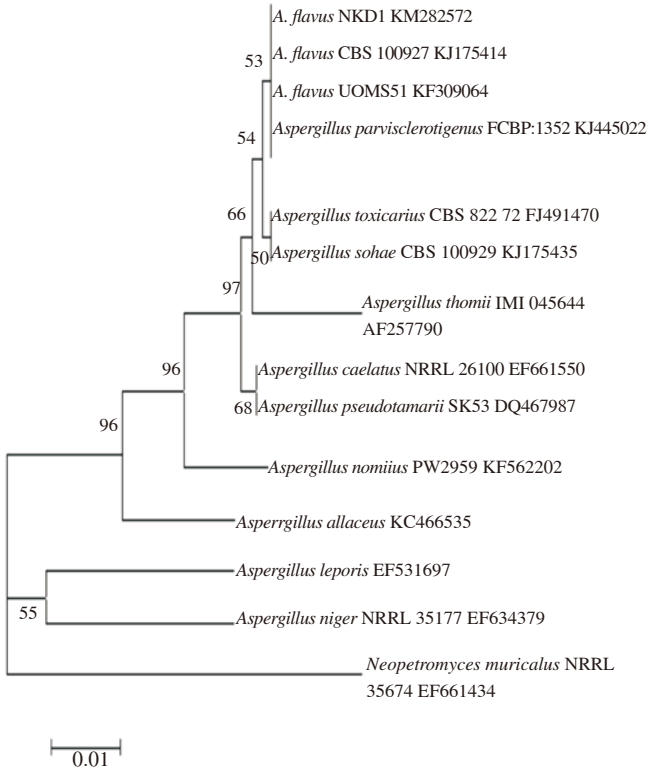

Figure 6. The phylogenetic tree of the pathogenic fungus resulting in black gill disease.

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 *Legenidium 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.

Acknowledgments

The authors would like to thank the Dean and Director, Center of Advanced Study in Marine Biology, Faculty of Marine Sciences, Annamalai University for providing facilities during the study period. This study was financially supported by Science and Engineering Research Board of Department of Science and Technology, Government of India, New Delhi (Grant No. SR/SO/AS-012/2013). We are also thankful to shrimp farmers, farm technicians for extending their help during the sample collection from their grow-out farms.

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