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A novel report of fungal pathogen *Aspergillus awamori* causing black gill infection on *Litopenaeus vannamei* (pacific white shrimp)

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ABSTRACT

Litopenaeus vannamei (pacific white shrimp) is the most cultured shrimp species which is also susceptible to microbial diseases like other shrimps. In the present study, the fungi, *Aspergillus awamori* KM434331 caused black gill disease to pacific white shrimp. It was first reported from *L. vannamei* in shrimp grow out pond located at Vellapallam, Nagapattinam District, Tamil Nadu, India. *A. awamori* KM434331 was isolated from affected gill of shrimp. Further, its morphological, cultural and phylogenetic characteristics were identified. The histopathological depiction is inflammatory response of *L. vannamei* against *A. awamori* KM434331 are haemocytic infiltration, encapsulation, melanization and collagen-like fibre deposition in the gill. In addition to that, *Aspergillus awamori* KM434331 cause dysfunction of gills that leads to chronic mortality in the grow-out pond of shrimps.

1. Introduction

Aquaculture is an important economic activity globally (Kathiresan et al., 2012) and contributes one-third of global production (Food and Agriculture Organization, 2009). *Litopenaeus vannamei* (white leg shrimp), is one of the economically important Penaeid shrimp currently being cultured in many countries, especially in Southeast Asia (Alcivar-Warren et al., 2007). It is mandatory to have a disease free yield for successful culture and economic viability for shrimp aquaculture globally. However, fungal infection is one of the foremost disease problems in shell and fin fish aquaculture (Khoa et al., 2005). Earlier report stated that the primary mycosis was caused by *Saprolegnia parasitica* in larvae of shrimp *Palaemonetes kadiakensis* (Hubschman and Schmitt, 1969). Black gill disease was first reported on American spiny lobster *Homarus americanus* (Lightner and Fontaine, 1975).

In general, black gill condition in shrimp caused by *Fusarium* species initially produces generalized "gill discoloration" which gradually develops into "blackened gill" condition and eventually leading to death of affected species (Khoa, 2005). *Fusarium moniliforme* has been reported on early stages of *Penaeus japonicus* where gills appeared to show a slight change in colour from opaque white to black spots. Further, *F. moniliforme* conidia was intramuscularly inoculated to prove whether isolated fungus was capable of causing black gill disease and leads to death of shrimp (Rhoobunjongde et al., 1991).

Mantis shrimp (*Oratosquilla oratoria*) was experimentally infected with fungi and was similar to those of naturally infected shrimp. Histopathologically, the hyphae and conidia were found in the gill filaments, the heart and the hyphae were encapsulated by hemocytes in the gill filaments and the base of the gills. The result confirmed that these two anamorphic fungi *Plectosporium oratosquillae* and *Acremonium* sp. were pathogenic to mantis shrimp (Duc et al., 2009). However, there are no reports of *Aspergillus awamori* KM434331 fungal causing black gill infection to *L. vannamei* shrimps so far. Hence the present study of *A. awamori* isolated from black gill infected *L. vannamei* shrimps is to be reported foremost. The current study reveals the infection of *A. awamori* KM434331 in the gill of *L. vannamei* cultured in brackish water environment of shrimp grow-out ponds.

2. Materials and method

2.1. Study area

Vellapallam (Lat. 10°32′49.42″N; Long. 79°50′29.45″E) is a coastal village located between Vellankanni and Vedharaniyam, Nagapattinam district, Tamil Nadu, India. In this area, a total of 123 shrimp grow out farms extensively cultured *L. vannamei* between 2013 and 2014.

2.2. Sample collection

The black gill disease affected shrimps (*L. vannamei*) of 12–16 g of body weight which were collected from the pond during May 2013 to April 2014 by operating cast net. For each sampling, 30 to 45 infected shrimps were collected and brought to the laboratory for further investigation.

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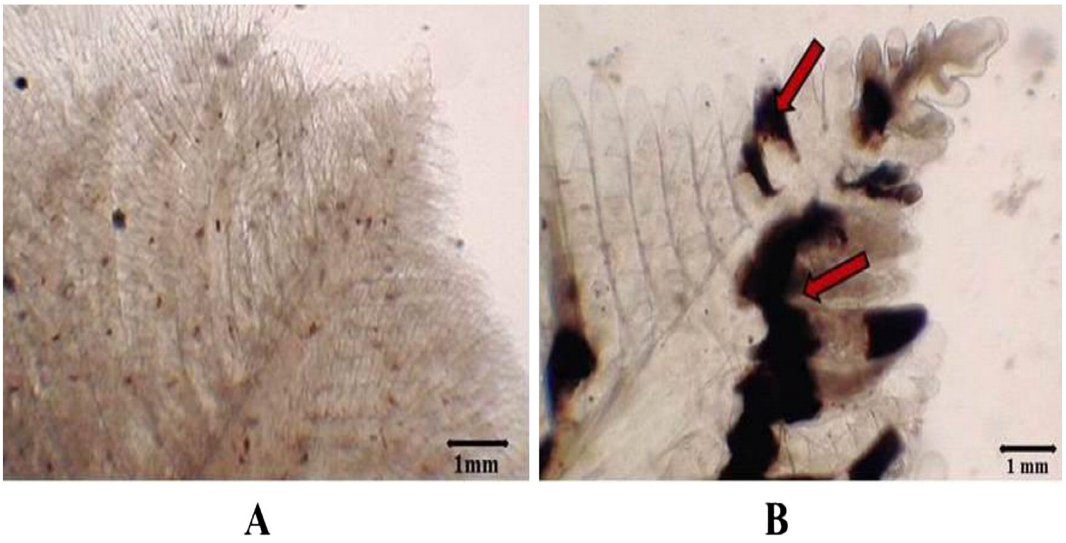

Fig. 1. (A) A wet mount preparation of the *L. vannamei* normal gill. (B) A wet mount preparation of the *L. vannamei* fungal infected gill surface observed under microscopy.

2.3. Histopathological study

Fungi infected moribund shrimps were injected with Davidson's fixative in the heapatopancreases and muscle of shrimp in order to avoid autolysis. After injection, the whole shrimp was immersed with the same fixative for 48 h before processing. The black gill infected portions of gills were dissected and immersed in Davidson's fixative for 48 h and transferred to 70% ethanol for further processing. Further, the sections were stained with usual Ehrlich's haematoxylin and eosin stains, the slides were observed under a 40× lens in light microscope (Olympus) for routine histopathological investigation to determine the internal lesions in the shrimps due to fungal invasion.

2.4. Microbiological investigations

The fungi infected black gills were removed from the infected shrimp and observed under a 40× lens in light microscope (Olympus). The black gill infected shrimps black gill lamellae were washed three times with sterile physiological saline (0.85% NaCl) and plated on Sabourad dextrose agar plates supplemented with Amphotericin-B and streptomycin sulphate (25 μg/ml) to inhibit unwanted bacterial contamination. Further, the plates were incubated for 4 days at 25 °C and the pure cultures were maintained at 25 °C on (give expansion) SDA slants for subsequent experiments. The fungal strain was identified through Lacto phenol cotton blue mount (LPCB) and the morphology was observed under light microscopy.

2.5. Scanning Electron Microscopy (SEM)

L. vannamei black gill caused *Aspergillus* sp. scraped to SDA plate using sterile blade, dehydrated and sputter-coated with gold and were examined using JEOL JSM-7401F scanning electron microscope at an accelerating voltage of 15 kV GB low.

2.6. Molecular identification and phylogenetic analysis

The amplification of DNA fragments was done by modified method of Iwamoto et al. (2002). Fungal spores were centrifuged at 13,000 ×g for 5 min and 1 μl of supernatant was used as template for amplification along with (give expansion) ITS5 primer pair (O'Donnell, 1992; White et al., 1990). The PCR conditions were as follows: initial denaturation at 95 °C for 15 min; followed by 45 cycles of denaturation at 94 °C for 20 s, annealing at 55 °C for 1 min, extension at 72 °C for 50 s; and final extension at 72 °C for 10 min. The DNA sequences from both strands were read on an ABI PRISM 377 DNA sequencer. The homology of the sequences was analyzed using BLAST algorithm (http://www.ncbi.nlm.nih.gov) and was aligned with reference taxa along with their GenBank accession numbers using ClustalW implemented in MEGA5 software (Tamura et al., 2011).

3. Result

3.1. Microscopical and histopathological observations

The external clinical symptoms of *L. vannamei* with black gill infection were observed as black colour appearance on the surface of the gill. The normal gill of *L. vannamei*, and conidia of attached fungal were encapsulated at the base of the gill observed in the infected shrimp under light microscope (Fig. 1). The results of histological examination evidenced that the normal gill lamella. Cross-section of haemocoel in gill tissue, and encapsulated hyphae were observed in the gills and at the base of the gills haemocytic infiltration is surrounded by large amounts of substances caused by coagulation necrosis (Fig. 2).

3.2. Microbiological investigations

The colonies on SDA plates were found to be grey at first, quickly becoming bright to light green. Conidiophores coarsely roughened up to 1 mm long, and loosely radiate or split or columnar, biseriate but having some heads with phialides borne directly on the vesicles; phialides 7–10 × 2–2.5 μm; conidia usually globose to sub-globose, occasionally elliptical, conspicuously roughened, and 3–6 μm diameter but mostly less than 4–5 μm diameter (Fig. 3).

3.3. Scanning Electron Microscopy

Scanning electron microscopy (SEM) observations reveal many hyphae, *A. awamori* KM434331 colonies with conidiophores and conidial heads, conidial heads typically radiate, splitting into several poorly defined columns, occasionally columnar heads, conidia are globose to sub-globose, conidia covering the whole surface of the conidiophore, conidia and spores *A. awamori* KM434331 (Fig. 4).

3.4. Phylogenetic analysis

Further identification of fungi *A. awamori* was deep-rooted through 18S rDNA sequencing and phylogenetic analysis. The homology analysis

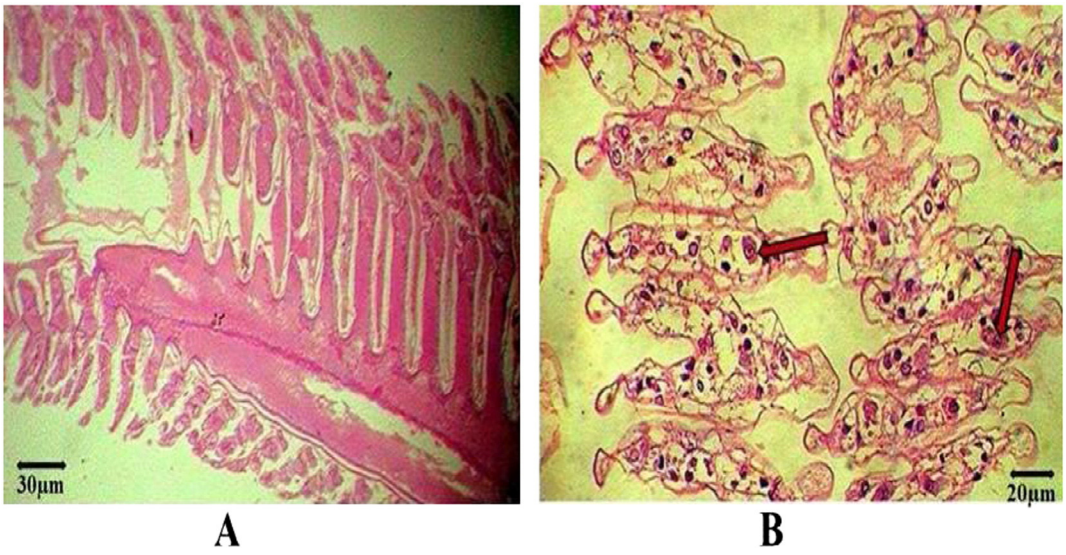

Fig. 2. (A) Normal gill lamella (B) Cross-section of haemocoel in gill tissue, haemocytes are surrounded by large amounts of substances caused by coagulation necrosis.

by BLAST concluded that the isolate casvk5 (KM434331) closely resembled *A. awamori* EU821242 (100%). In addition, phylogenetic tree analysis showed that the monophyly of isolate with respect to reference taxa and extremely short tree branches indicate minimal differences among the analyzed sequences (Fig. 5). As well, the phylogenetic tree indicated the close relationship between *A. brasiliensis* and *A. usamii*.

4. Discussion

The modernization and intensification of the aquaculture industry leads to outbreak of fungal infection resulting in huge loss of aquaculture industries throughout the world (Karthikeyan and Gopalakrishnan, 2014). In the present study, the pathogenic fungi *A. awamori* was first time reported in shrimp of black gill infected *L. vannamei* from grow out ponds. The current investigation reveals that the fungi *A. awamori* KM434331 was isolated and identified based on the morphology, phylogenetic, scanning electron microscopy and histopathology analysis. *A. awamori* was the most prevalent species, comprising 22% of all isolates from cultivable fungal diversity of shrimp (Silva et al., 2011). *Aspergillus* sp. is known to be the most powerful fungal species competent to produce aflatoxins, which are mycotoxins with carcinogenic potential (Lacaz et al., 2002). Aspergillomycosis is primarily a disease of tilapia *Oreochromis* sp. (Olufemi, 1983). These fungal species are presumably infectious through contamination of fish feed (Saleem et al., 2012).

Fusarium solani causing black gill disease in cage-cultured *Panulirus ornatus* in Vietnam and attempts have been made to recover 97 fungal strains from 97 ornate rock lobsters cultured in cages with black gill condition (Nha et al., 2009). However, *F. moniliforme* was isolated from gill lesions of kuruma prawn *P. japonicus*, with black gill disease (Rhoobunjongde et al., 1991) and two kinds of anamorphic fungus, *P. oratosquillae* and *Acremonium* sp. were reported independently or together in the infected gills of mantis shrimp (Duc and Hatai, 2009). Although, *Aspergillus* sp. was found to be pathogen of numerous fishes and shrimps, no reports have been made from elsewhere about shrimp by the black gill disease and thus it appears to be the first report on *A. awamori* KM434331 isolates from shrimp black gill disease.

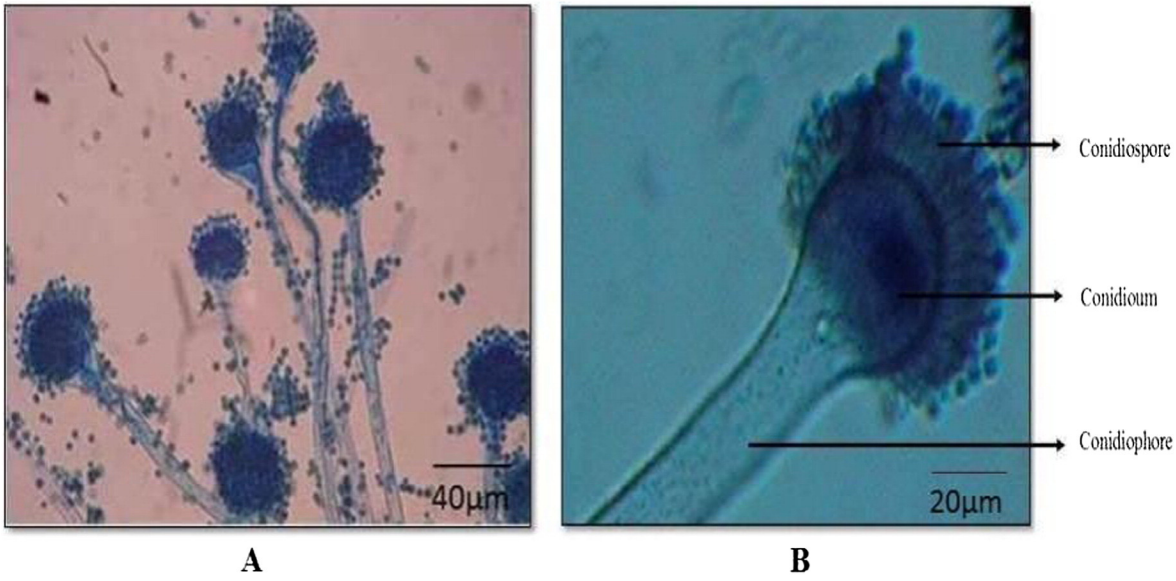

Fig. 3. (A) Photomicrograph reveals mature conidiophores of *A. awamori* KM434331 observed by LPCB mount. (B) LPCB mount showing conidiophores, conidiospore and conidioum of *A. awamori* KM434331.

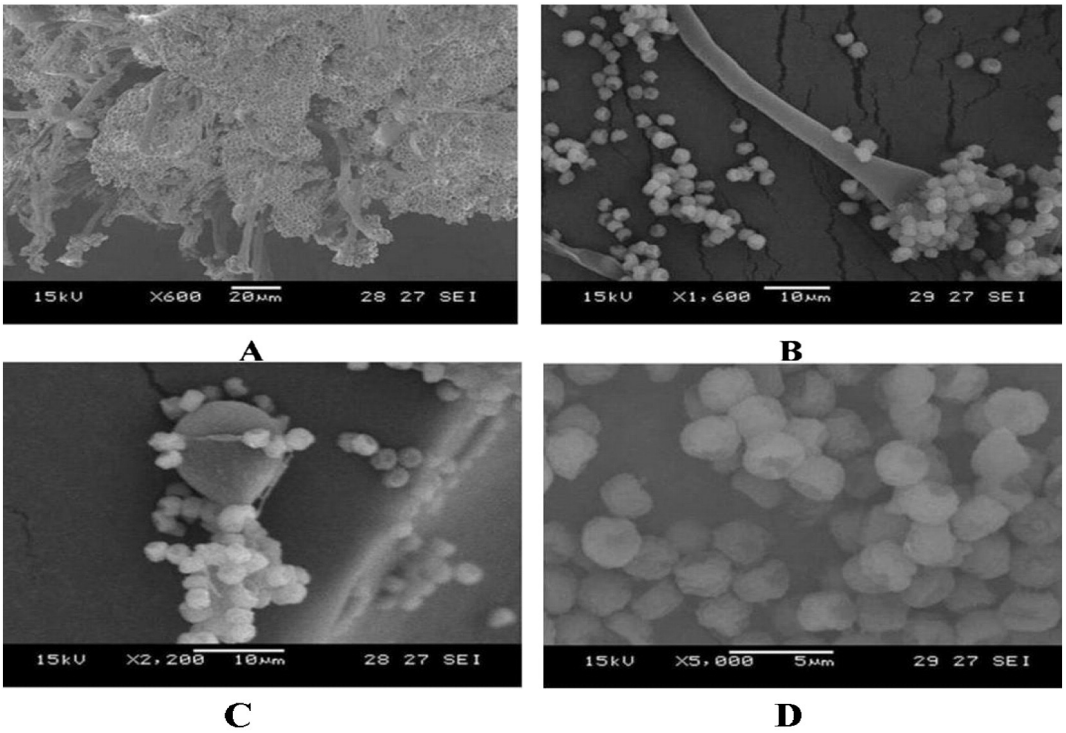

Fig. 4. (A) Photomicrograph reveals many hyphae, mature conidia and conidiophores of *A. awamori* KM434331. (B) A SEM image of conidia covering the whole surface of the conidiophore. (C) Photomicrograph view on conidia with spores of *A. awamori* KM434331. (D) A SEM close up view on spores of *A. awamori* KM434331.

In the present study, histopathological investigation depicted that the fungus *A. awamori* KM434331 produced a carcinogenic toxin which leads to black spot lesions on the gill and poses damage to the respiratory function of shrimp by haemocytic encapsulation and melanization finally leads to mortality. However, these pathological symptoms were almost similar to that of other reports for fungal diseases, the positive Lillie's Fe^{2+} ion uptake reaction causes black spot lesions (Bian and Egusa, 1981).

The SEM analysis evidenced the *A. awamori* KM434331 colonies with conidiophores, conidial heads, globose to sub-globose, conidia covering the whole surface of the conidiophore which were similar to that of pathogenic fungi *A. awamori* BTMFW032 (Beena et al., 2010 and the identity of phylogenetic results showed 100% sequence similarity with the previously available sequences of *A. awamori* (Basheer et al., 2011).

5. Conclusion

The results of the present study seem to be the first report of pathogenic fungi *A. awamori* on cultured shrimp *L. vannamei*. In the shrimp culture system, stunted growth, vulnerable to other diseases and poor marketability were caused by *A. awamori* due to unfavourable conditions such as polluted water, high density and overfeeding. Good farm practices and better planning are highly recommended for successful

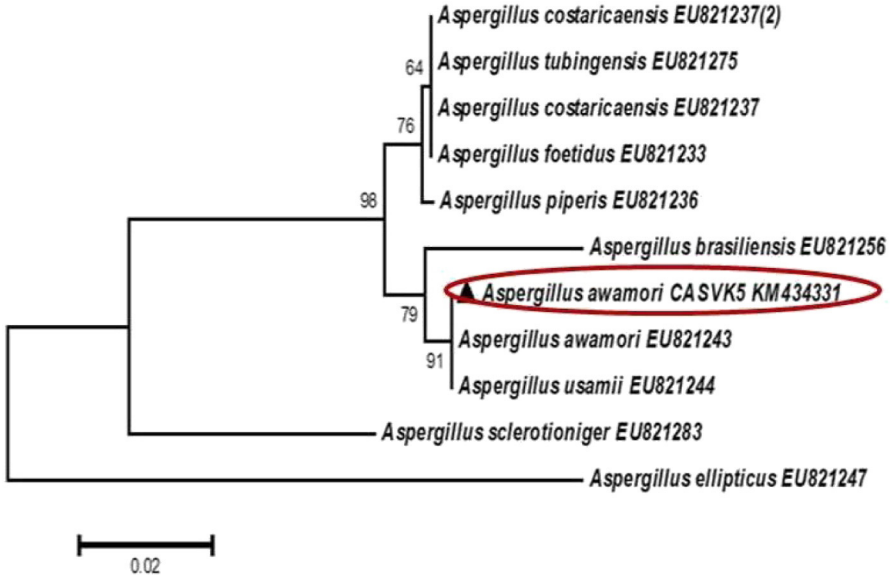

Fig. 5. Phylogram showing the relationship between *Aspergillus awamori* CASVK5 KM434331 and other related species of *Aspergillus*.

L. vannamei cultured in aquaculturists. Nevertheless, the present investigation provides amble information about the infection of pathogenic fungi *A. awamori* in cultured shrimp.

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