

# Multiple Infections in Pacific White Shrimp (*Penaeus vannamei*) with Black Gill Disease

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## ABSTRACT

*Penaeus vannamei* (Pacific white shrimp) is the most extensively farmed among the crustaceans globally. Disease is the major impeding factor in the commercial culture of *P. vannamei* undertaken in freshwater, brackish and marine aquaculture systems. In this study, *P.vannamei* samples were collected from a commercial shrimp farm at Tiruvallur district, Tamilnadu that has reported black gill disease. The clinical symptoms observed in the shrimp were melanized black gills, reduced feeding, surfacing and mortality in the early morning. Samples of gills were collected aseptically and diagnosed for bacterial Acute Hepatopancreatic Necrosis Disease (AHPND); viral diseases viz., Gill Associated Virus (GAV), White Spot Syndrome Virus (WSSV), Yellow Head Virus (YHV), Infectious Hypodermal and Hematopoietic Necrosis Virus (IHHNV) and fungal infections by conventional diagnostic methods and PCR following confirmation by nucleotide sequencing. The results of the study showed that *P.vannamei* infected with black gill disease had multiple infections with fungus, *Aspergillus niger* and virus WSSV.

**Key words:** *Penaeus vannamei*, Black gill diseases, WSSV, *Aspergillus* sp.

## INTRODUCTION

Sea foods are important and cheap source of protein. As shrimp is considered a delicacy among the consumers in many

countries, there is a growing demand for shrimp in the world market (Belton and Thilsted, 2014). India is the one of the leading producer of cultured *P.vannamei* (MPEDA, 2017). Although shrimp farming has grown rapidly, its development and expansion is seriously affected due to various diseases caused by diverse pathogens (Lightner *et al.*, 1997; Durand *et al.*, 2003). Among viral pathogens infecting shrimp, WSSV is considered a highly virulent pathogen causing mortality in shrimp within 3 to 7 days of infection resulting in severe economic losses to the farmers (Zhan *et al.*, 1998; Corbel *et al.*, 2001; Lightner, 2011). Other viral

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pathogens *viz.*, Gill-associated virus (GAV), a single-stranded RNA virus and Yellow head virus (YHV) have been reported to cause mass mortalities in penaeid shrimps cultured in Australia and Asia (Spann *et al.*, 1997; Cowley *et al.*, 2000). Diseases caused by *Vibrio* sp. are the most common among the bacterial diseases infecting shrimp (Chatterjee and Halder, 2012). Fungal pathogens have often been reported to be associated with the discoloration and diseases of gills (Rhoobunjongde *et al.*, 1991). Black gill associated with *Fusarium* sp. in Japanese kuruma prawn, *P. japonicus* (Ishikawa *et al.*, 1968) and *Aspergillus flavus* has been reported in *P.vannamei* (Dewangan *et al.*, 2015). The objective of this work is to identify the causative of black gill in *P.vannamei* sample from a commercial shrimp farm.

## MATERIALS AND METHODS

### Sample collection

*P. vannamei*, Pacific white shrimp (20±2g) exhibiting symptoms of black gill condition were collected from a commercial shrimp farm that had reported a disease outbreak in Tiruvallur district, Tamilnadu (13°19'25.2"N 80°13'44.7"E), India during November 2017. The details *viz.*, stocking density, days of culture (DOC), physico-chemical parameters of water (pH, salinity, DO, alkalinity, hardness, ammonia and H<sub>2</sub>S) were recorded at site. Samples of gills from live *P.vannamei* samples (n=10) were collected aseptically for microbiology or fixed in Davidson's fixative for histopathology and 70% ethyl alcohol for PCR.

### Isolation and identification of pathogens

Aliquots of the fresh gill tissue were observed under the microscope as wet mounts (40x magnification). For isolation of fungi, the gill tissue were cut into small pieces, rinsed several times in sterile saline (0.85% NaCl) and inoculated in potato dextrose agar (PDA) supplemented with ampicillin and streptomycin and incubated at 25°C for one to three days. An agar block with the fungal mycelia was placed on PDA (with ampicillin and streptomycin) and incubated at 25°C in dark (Khoa *et al.*, 2004). The observations such as colour and colony characteristics were recorded. The fungal isolates grown on the media were stained with lactophenol cotton blue and the morphology was observed under the microscope (40x and 100x) and identified (Barnett and Hunter, 1999). Inocula from the gills with black discoloration were also plated on to TCBS agar and incubated for 24 h at 37°C to facilitate the growth of *Vibrio* spp., the most common bacterial pathogen infecting shrimp.

### Molecular identification of viral and fungal pathogens

Total genomic DNA was extracted from the gill samples and fungal isolates using QIAamp genomic DNA kits (Qiagen, Germany) following manufacturer's protocol for the identification of pathogens by PCR amplification and nucleotide sequencing. Total RNA was extracted (Trizol reagent, Sigma) and reverse transcribed to cDNA (High capacity cDNA synthesis kit, Applied Bio systems Inc., USA) following the manufacturer's instructions. PCR amplification was carried out in a thermal

cycler (Biorad T100 Thermal cycler, USA) in a total volume of 25µl reaction mixture containing 2X PCR mastermix (Ampliqon, Denmark), 1 µl (10 pmol) of forward and reverse primer each and 1µl of sample DNA. Published protocols were followed for the PCR diagnosis of WSSV (Takahashi *et al.*, 1996), GAV (Cowley *et al.*, 2000), IHNV (Tang *et al.*, 2007), and YHV (Cowley *et al.*, 2004). For fungal identification, ITS1 and ITS4 fungal rRNA gene-specific primers were used for PCR amplification (White *et al.*, 1990; and Gardes and Bruns, 1993). Nested PCR reactions were carried out similar to the first step PCR except for the nested primers and 1 µl of the first step product as the template. An aliquot of PCR product was separated on a 1.5 % agarose gel and visualised under UV illumination using a gel documentation system (Biorad, USA). The PCR amplified products of fungal ITS was purified and sequenced using both forward and reverse primers (Eurofins, Bangalore, India). The sequence was compared with the available sequences in the GenBank using BLASTn (Basic Local Alignment Search Tool) and the sequence information was submitted to the GenBank ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)).

## RESULTS AND DISCUSSION

The observed values of various physico-chemical parameters of the water samples collected from the farm is presented in Table 1. The clinical signs observed in the gills of *P. vannamei* shrimp samples include, black discoloration (Fig. 1), reduced feed consumption and mortality in the early morning. More than 50% of the shrimp population was observed to have black gills condition. Various factors have been reported to be associated with blackening and diseases of gill in shrimp. Presence of various parameters above optimum levels *viz.*, total ammonia (<1.0ppm), H<sub>2</sub>S (<0.02ppm), nitrite concentration (2-3 ppm) combined with high organic matter and deposition of debris (Lavilla-Pitogo *et al.*, 2000), nitrate, acids, crude oils, potassium permanganate, cadmium, copper and ozone results in discoloration of gills (Lightner, 1996). High ammonia and H<sub>2</sub>S levels in ponds are usually associated with poor pond bottom and favor the growth of fungi and other pathogenic microbes (Soegianto *et al.*, 1999).

**Table – 1. Physico-chemical parameters in the water sample from *P.vannamei* pond.**

Parameter	Value
Temperature(°C)	30°C
Dissolved oxygen (ppm)	3.5
Salinity (ppt)	30
pH	8.1
Alkalinity (ppm)	150
Total Hardness (ppm)	4500
Ammonia (ppm)	1.2
H <sub>2</sub> S (ppm)	0.05

Microscopic observation of wet mounts of affected gills showed dark discolouration with numerous fungal hyphae attached to the gills. Fungal growth was observed in the PDA plates after 3 days. Microscopic observation of the lactophenol blue stained fungal colonies that were yellowish or yellowish green in colour on plates revealed hyaline, septate hyphae, with long conidiophores with globose tip and Phialides around the vesicle which are characteristic of *Aspergillus* sp. (Fig. 2). In this study, we have isolated *A.niger* from *P.vannamei* with black gills from ponds with higher ammonia (1.2ppm) and H<sub>2</sub>S (0.05ppm) levels. Infection with fungus viz., *Fusarium* sp., (Hatai and Egusa, 1978; Colorni *et al.*, 1989). *A.niger* (Fernand *et al.*, 2017) and *A. flavus* (Dewangan *et al.*, 2015) have been reported to cause melanized gill filaments resulting in black gill condition or disease. Gills are crucial for respiration, excretion, acid–base balance, and osmotic and ionic regulation (Ramaiah, 2006). As gills are immediately exposed to the external environment and are the first organs to be exposed to pollutants, gill diseases in shrimp may result in their death due to destruction of gills or suffocation caused by mechanical blockage of gas exchange across the surface of the gill lamellae (Lightner, 1996).



Fig.1 *P. vannamei* with black gill condition.

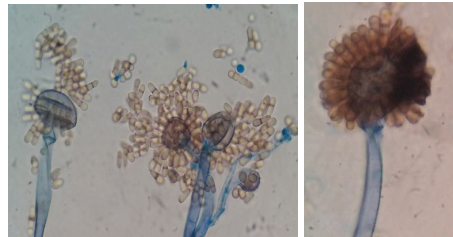


Fig.2 Microscopic observation of *Aspergillus* showing fungal hyphae and conidia (100×).

Histopathological sections of gills showed congestion, fusion of gill lamellae and multifocal mild degeneration and necrosis of secondary gill epithelium with multifocal mild mononuclear cell infiltration in the gill rakers (Fig. 3). Sequence comparison of the fungal isolate from black gill confirmed it as *Aspergillus niger* (Genbank Accession No. MH521172) as it showed higher similarity with other *A.niger* sequences in the BLAST analysis. PCR diagnosis for viral diseases resulted in the amplification of WSSV of expected product sizes (bp) (Fig. 4). No bacterial growth was observed in TCBS thus ruling out the role of *Vibrio* sp. infection. Hence, *P.vannamei* with black gill condition has been confirmed to have multiple infections with the fungus, *Aspergillus niger* and WSSV. Black gill disease in *P.vannamei* caused by *A.flavus* has been reported from India (Dewangan *et al.*, 2015). In addition, shrimps with black gills, when harvested, are not preferred for head-on packing and hence fetch very less market price. Treatment in shrimp with black gills may be treated effectively by bath treatment with 2 to 3 ppm concentration of furazolidone for 2 to 4 days (Shigueno, 1975). Fungal infections pose a significant threat to shrimp culture as

infection in gills would affect the respiration and subsequently their health status, making them susceptible to other diseases. Multiple infections in penaeid shrimp with bacteria and fungi (Kusumaningrum and Zainuri, 2015) and infection due to multiple virus have been reported earlier (Manivannan *et al.*, 2002; Anshary and Baxa, 2017). In our study, we observed that *P.vannamei* shrimp with black gill disease had multiple infections with *A.niger*, and virus WSSV. Reducing the organic load following good management practices (GMPs) will provide a pollution-free healthy environment to ensure the health status in the culture of *P.vannamei* and improves its sustainability in farming practices.

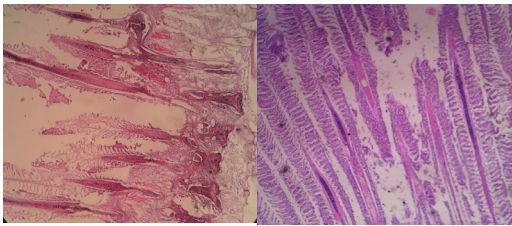


Fig. 3 Tissue sections of gill lamella from black gills showing changes.

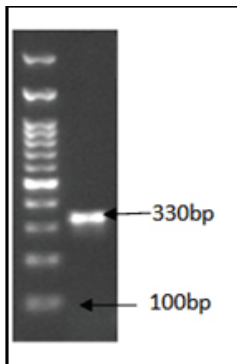


Fig. 4 PCR amplified product WSSV (330bp) in *P.vannamei* with black gill condition.

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