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Pythium insidiosum as a new opportunistic fungal pathogen for Pacific white shrimp, *Litopenaeus vannamei*

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The moribund shrimps were exhibiting yellow discoloration of the cephalothorax, blackening of gills and ulcers in the uropod and telson region. PCR for OIE listed viral pathogens ruled out known viral infections. No bacteria was present in haemolymph however, low level of *Vibrios* i.e. *Vibrio harveyi, V. parahaemolyticus* and *V. alginolyticus* was found both in the water collected from the broodstock tanks and affected tissue region. Histopathological examination of affected tissues revealed presence of highly invasive fungal hyphae both by routine and specific fungal stain. PCR amplification of the ITS region (approximately 900 bp) and sequencing confirmed presence of *Pythium insidiosum*. Phylogenetic analysis of this isolate placed it among the environmental isolates.

[Keywords: Broodstock, Hatchery, Histopathology, Fungus, *Pythium insidiosum*, Phylogentic tree.]

Introduction

Shrimp aquaculture in India was developed as a traditional practice dominated by a single species, the tiger shrimp (*Penaeus monodon*). The high export values however motivated the stakeholders to adopt more scientific and innovative approaches and convert this practice to a successful industry. With the expansion of culture area and stocking density, the production started increasing gradually and towards 2006-07 it reached the highest level. The rapid expansion and modification however brought severe pressure on the delicate balance of the ecosystem. As a result, starting from the early 90's, the industry started facing regular threat from emerging diseases. Amongst all, the impact of white spot syndrome virus (WSSV) was the most severe and its impact in terms of crop failures and economic losses in several parts of India[1] were similar to the impact in other parts of the world[2]. Suffering from this multiple crop failures and continuous losses, the stakeholders started looking for an alternate species. Based on several positive attributes[3] and the success story in other parts of the Asian countries, the Pacific white shrimp, *Litopeaneus vannamei* was looked as a preferred one for introduction in 2009 into Indian aquaculture system.

Through several attempts, it was made possible to develop pathogen free stocks. Culture of penaeid shrimp opened up new avenues through the development of specific pathogen free (SPF) stocks[4, 5]. Further, successful expansion of the *L. vannamei* industry has been possible mainly because of the development of domesticated broodstock and rapid expansion of selective breeding programs[6, 7]. Because of this, several multiplication centres were operated in a number of countries as a source for the supply of these SPF broodstocks. Upon the request from stakeholders, Govt. of India introduced *L. vannamei* to Indian system through a strict regulatory body called Coastal Aquaculture Authority of India (CAA) after proper screening at a well-established quarantine facility[8]. As a regular practice, the hatcheries receive these broodstocks after thorough screening in the quarantine facility and afterwards the shrimps are reared them under strict biosecurity conditions for induced maturation, spawning and subsequent larval production.

Optimal environmental conditions along with proper nutrition are very much essential for the maturation of broodstocks. A number of literatures pertaining to these technologies have been published and can be found through a review by Browdy[9]. One of the very important requirements is the quality sea water which should be almost near to the natural sea water and free of pollutants. Sometimes hatchery operators breach the biosecurity and use direct sea

water for hatchery operations, which increases the risk of development of infections either by direct pathogens or by opportunistic pathogens. In the present study, one such case report is presented where SPF broodstocks of *L. vannamei* got infection and suffered mortality. A systematic investigation of the case study revealed that an opportunistic fungal pathogen, *Pythium insidiosum* is responsible for the cause of mortality of broodstocks of *L. vannamei*.

Materials and Methods

Mature broodstocks of *L. vannamei* (n=10) comprising both male and female were included for sampling. The samples included both infected, moribund and apparently healthy looking animals. For DNA and RNA extraction, samples were collected in 90% ethyl alcohol and RNAlater, respectively and for histological analysis the tissue samples (gill, telsion, hepatopancreas, muscle) were fixed in Davidson's fixative.

DNA extraction, PCR reaction and primers used for the detection of both the DNA viruses as well as the entire method for the detection of RNA viruses were as per the description of Otta *et al.*[10,11]. PCR amplified products were electrophoresed on 1.2% agarose tris-acetate-EDTA (TAE) gel incorporated with 0.5 mg ml^{-1} ethidium bromide and Gel doc 2000 UV transilluminator (BioRad) was used for photography as well as recording of the results.

Samples for bacteriology included aseptically drawn hemolymph directly from the heart of broodstocks through syringes, swabbing the appendages exhibiting clinical signs (after a thorough wash with sterile PBS) and water from the rearing tanks. Samples were plated both on Zobell Marine Agar (ZMA) and Thiosulphate Citrate Bile salt Sucrose (TCBS) agar plates and incubated at 30 ºC. Isolated colonies grown on plates were selected for identification by 16s rRNA amplification and sequencing.

Fixed tissue samples were processed as described in Bell &Lightner[12]. The paraffin embedded tissues were cut into 5µm sections (Leica, Germany) and stained with hematoxylin and eosin[13]. Slides were observed and photographed with a camera attached microscope (Olympus, Japan). Selected slides were stained with Grocott's Methenamine Silver strain for specific demonstration of fungal pathogen[14].

For detection of fungal pathogen, the infected gills of shrimp with black discoloration were used for DNA extraction. Method for DNA extraction was similar to that for shrimp DNA viruses as described above. The internal transcribed spacer (ITS) region primers and PCR conditions as described in White *et al.*[15] were used for PCR amplification of fungi. A pure culture of *Penicillium citrinum* was used as a positive control

Amplified PCR products were purified as per the manufacturer's instructions in the kit (BioBasic) and this purified product was cloned into pTZ57R/T vector based on the protocol provided in the InsTAclone kit (Thermo scientific). Positive clones as verified by PCR were sequenced and NCBI Blast search was done to identify the species. The sequence information obtained was used for building the phylogenetic tree using MEGA 5 software as per the protocol described by Hall[16].

Results and Discussion

Mature broodstocks of L. vannamei were imported and initially monitored in the quarantine facility, once it reached India. After the preliminary screening, samples were then certified for transporting to a specified hatchery located in the east coast of India. The animals were further acclimatized to hatchery conditions and during this period, low mortality (1-2 animals/day) was reported. However, the rate of mortality was increased after the eye stalk ablation and its effect was more in females. Samples were collected during the period of increased mortality. The infected shrimps exhibited ulcers in the uropod region (Fig. 1a), yellowish discoloration of the carapace and blackening of the gills (Fig. 1b)

Ulcer and white discoloration of the telson are observed in infectious myonecrosis virus (IMNV)

Fig. 1a — Clinical signs of infected *L.vannamei* brooders (a) ulcer (black arrow) and necrosis (white arrow) found in the telson region of shrimp.

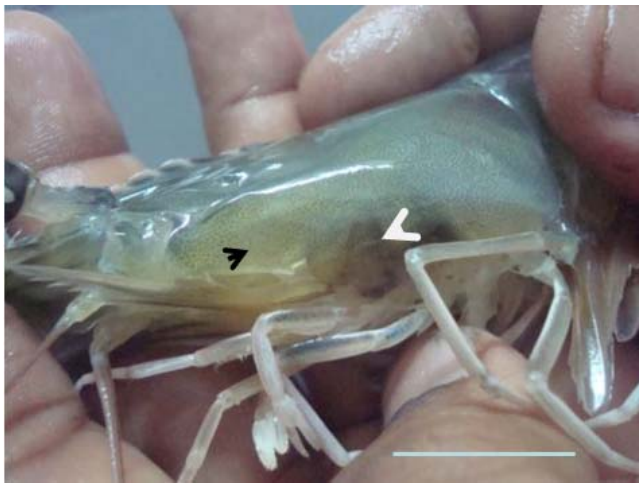

Fig. 1b — Yellow discoloration of cephalothorax (black arrow head) and black discoloration (white arrowhead) of infected gills Bar: 2.5cm.

Table 1 — Bacterial count and species composition from different sources of *L. vannamei* hatchery samples. (TNTC: Too numerous to count)

Samples	Bacterial count on ZMA	Bacterial count on TCBS	Major Vibrio species identified
Haemolymph	Nil	Nil	-
Swab from telson	TNTC	TNTC	*V. harveyi*, *V. parahaemolyticus*, *V. alginolyticus*
	1.3×104- 3.2×105	2.2×10^2- 8.9×102^2	*V. harveyi, V. parahaemolyticus, V. alginolyticus*

infected shrimps[17]. Since the brooders were passed through two quarantine processes, one at the place of origin and the other in India, it was highly unlikely that the shrimps were carrying IMNV. Through a regular monitoring programme, Otta *et al.*[11] did not find the presence of IMNV or other exotic viruses in India. It was not possible that the shrimps could have got infected through a breach in biosecurity. However, to further rule out the chance of IMNV infection, PCR was carried out for detection of IMNV and all the samples were found to be negative. Additionally, the brooders were also negative for other two exotic viral pathogens i.e. Taura Syndrome Virus (TSV) and Yellow Head Virus YHV). White Spot Syndrome Virus is another highly virulent prevalent shrimp virus which can cause mortality to all stages of shrimp. In spite of its SPF status, *L. vannamei* shrimps are highly sensitive to WSSV and suffer mortality[11]. It is also possible that very low level of virus that present in brooder may multiply to higher number due to spawning stress[18] and then bring mortality. We therefore tried to detect the WSSV in the brooders by nested PCR. Our result indicated absence of WSSV in both the steps and therefore, it was considered that the mortality was not due to WSSV infection. PCR result also indicated that the brooders were negative for IHHNV. Hence, any known virus related stress and mortality was ruled out.

As ulcers were observed in the body surface (telson region) of the broodstock, it was assumed that there might be bacterial infection. Bacteriological investigation indicated presence of vibrios in affected telson region and also in water samples collected from broodstock rearing tanks (Table 1). The major species were identified to be *V. harveyi*, *V. parahaemolyticus* and *V. alginolyticus*. Vibrios in general and *V. harveyi* in particular have been found to be a major pathogen both for shrimp larvae[19] and juveniles[20]. Due to the presence of pathogenic vibrios in affected parts of shrimp and in water and because of their chitinolytic nature, it was considered that the ulcer in the telson region might be due to vibrio infection. Many a times matured and fully grown shrimp and brooders though do not suffer mortality, they show different clinical signs. It was informed that antibiotics were already applied through feed to check bacterial infection. Hence low level of bacteria that was found through our investigation might be the reason for that. Since mortality continued irrespective of the antibiotic application, it was assumed that mortality of shrimps might not be due to bacterial infections.

As a general practice activated charcoal is used during transport of shrimp broodstocks to reduce ammonia and other toxic gases from the water[21]. Therefore, it was initially assumed that black discoloration might be due to carbon particle deposition on gill filaments. Microscopic observation of wet mount preparation of gill filaments however did not provide any indication of carbon particle or presence of parasites. Importantly, histopathological examination of the gill filaments through H&E staining indicated presence of highly invasive fungal hyphae in tiisue sections (Fig. 2A & B). Hence, to confirm the presence of fungal hyphae in the gills, Grocott's Methenamine Silver strain was used to demonstrate the fungal infection (Fig. 2C).

Further, PCR was used to molecularly confirm the fungal infection by targeting amplification of the ITS 1 region. PCR yielded a product of approximately 900

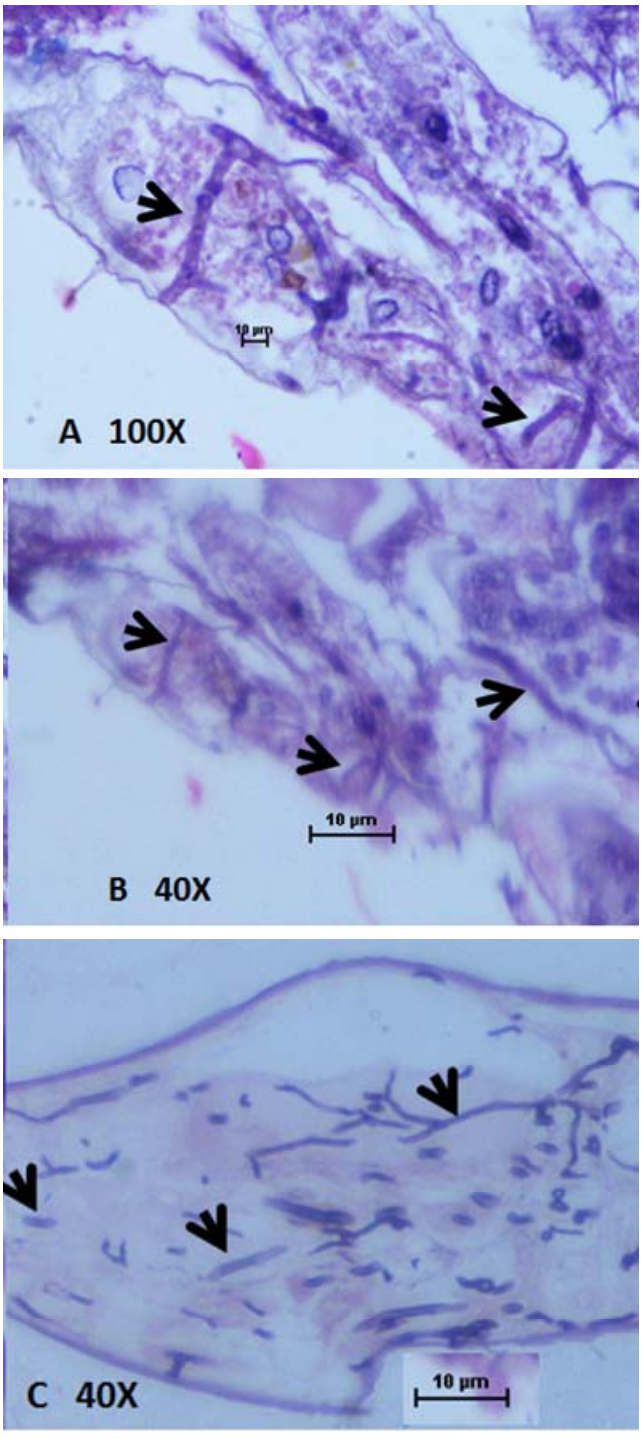

Fig. 2 — Histopathological section of gill filaments showing fungal spores and highly invasive fungal hyphae. A & B: H & E staining under different magnifications, C: Fungal specific stain.

bp (Lane 1 and 2, Fig 3) and upon sequencing it was confirmed to be *Pythium insidiosum*.

P. insidiosum, responsible for pythiosis in human, has worldwide distribution and usually infects horse, cattle, dogs, cats and fishes[22]. Several numbers of this

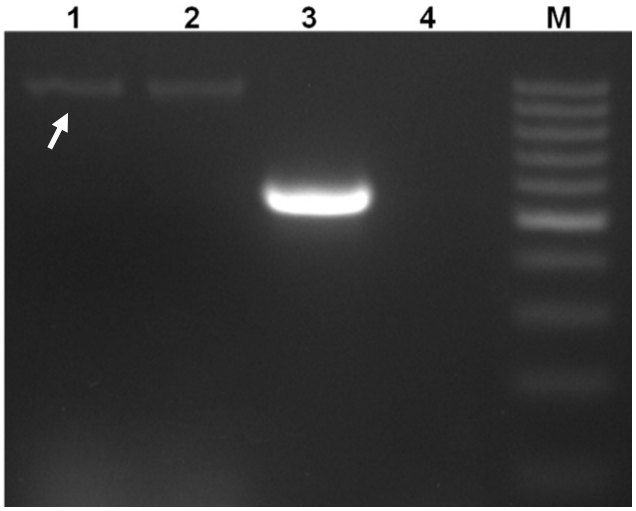

Fig. 3 — PCR amplification of ITS 1 region of fungus. Lane 1 & 2: Sample from infected gills (arrow indicating positive), Lane 3: Positive control, Lane 4: 100 bp molecular weight marker.

species have been isolated either from agricultural area or aquatic environment [23, 24] and have been extensively analysed. One of the species belonging to *Pythium myophilum,* earlier called as *Lagenidium myophilum*, has been reported to cause black abdominal infection to shimps[25, 26]. However, *P. insidiosum* has never been reported from shrimps or other invertebrates. In one of the experimental studies, Zanette *et al*.[27] proved that *Drosophila melanogaster* flies deficient with Toll like receptors are susceptible to *P. insidiosum* infection and mortality of more than 70% may occur. This indicates that invertebrates like shrimp with reduced immunity may be susceptible to this pathogen. It is predicted that the shrimps were under transport related stress and strict biosecurity measures were not adopted in the hatchery. As a result of this, the fungus got a chance to invade the shrimp. Moreover, as antibiotics were applied to control the bacterial infections, that might have given ideal space to the fungus for easy multiplication. Phylogentic tree (Fig. 4) indicated our isolate to be closer to environmental isolates further indicating breach in biosecurity in the hatchery.

Conclusion

The mortality of brooders in hatchery was therefore confirmed due to *P. insidiosum* infection. This is first report to indicate this fungal species to be an opportunistic pathogen for shrimp. Mortality might have resulted due to respiratory failure as the gills were filled with highly invasive fungal hyphae. The black and pale discoloration of the gills was due to the

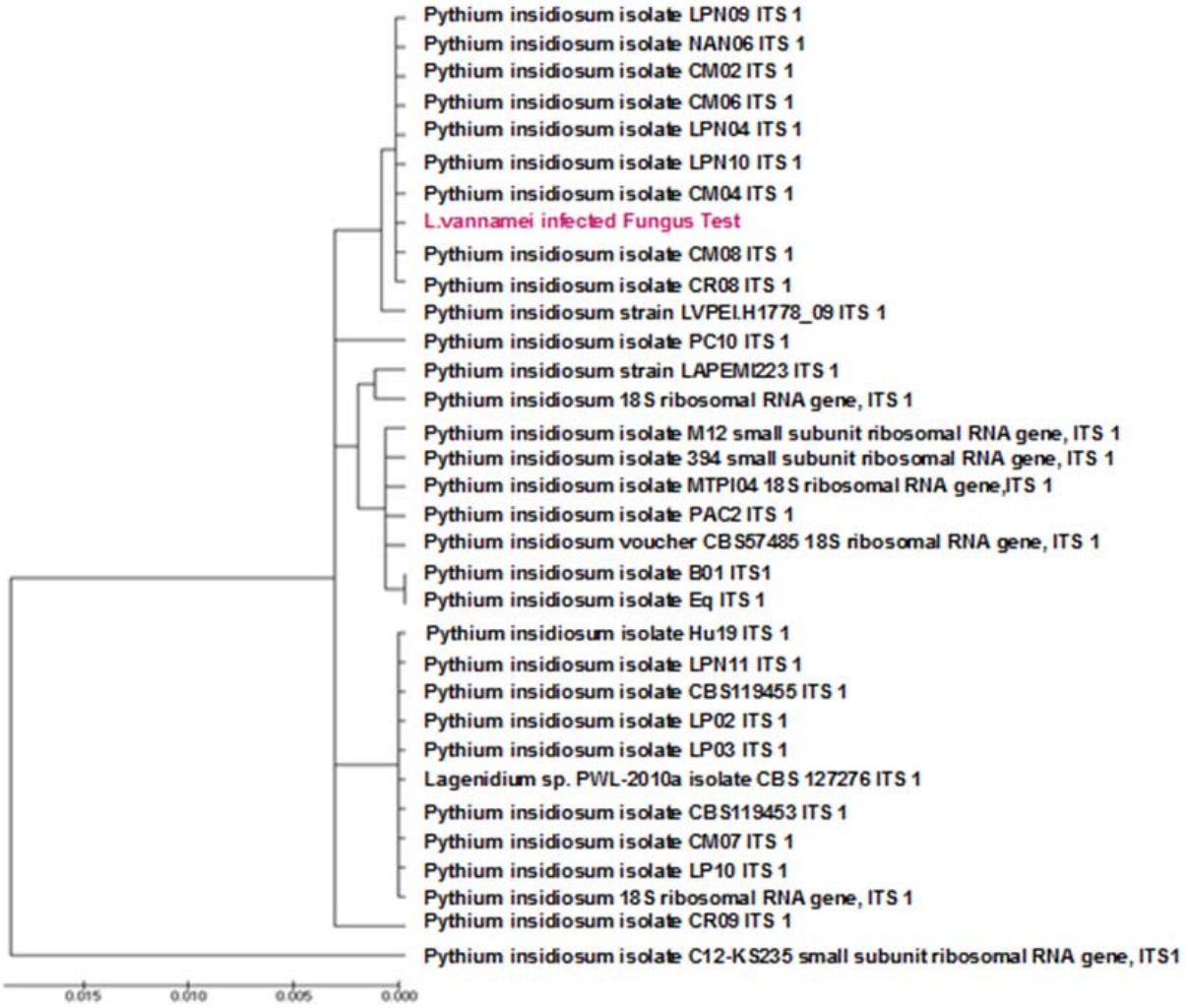

Fig. 4 — Phylogenetic tree of the fungal isolate from gill of *L.vannamei* compared to available information in the gene bank. Letter in red indicates the sample isolated in the present investigation.

presence of fungus as well as due to necrosis of the gill filaments caused by the fungus. As these brood stocks were imported as SPF stocks after proper quarantine and considered to be free from any known pathogens, it could have been infected through local contamination. Maintenance of strict biosecurity measures is therefore highly essential to prevent such infections from opportunistic pathogens. Application of BMP in hatcheries with focus on biosecurity measures, stress free environment and zero tolerance to contaminants and antibiotics would prevent diseases and ensure production of healthy shrimps.

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Reference

1 Kalaimani, N., Ravisankar, T., Chakravarthy, N., Raja, S., Santiago, T. C., Ponniah, A.G., Economic losses due to disease incidences in shrimp farms of India. Fish. Techno., 50(2013): 80-86.

2 Lightner, D. V., The penaeid shrimp viruses TSV, IHHNV, WSSV and YHV: current status in the Americas, available diagnostic methods, and management strategies, J ApplAquacult., 9(1999): 27-52.

3 Davis, D.A., Samocha, T.M., Boyd, C.E., Acclimating Pacific white shrimp, Litopenaeus vannamei, to inland, low-salinity waters. Southern Regional Aquaculture Centre (SRAC) publication no. 2601(2004): pp.230.

4 Wyban, J.A., Selective breeding of specific pathogen free (SPF) shrimp for high health and increased growth. In: Fulks, W., Main, K.L. (Eds.), Diseases of Cultured Penaeid Shrimp in Asia and the United States. Oceanic Institute, Honolulu, HI, USA. 1992, pp. 257–268.

5 Pruder, G.D., Brown, C.L., Sweeney, J.N., & Carr, W.H., High health shrimp systems: seed supply—theory and practice. In: Browdy, C.L. and Hopkins, J.S. (Eds.), Swimming Through Troubled Water, Proceedings of the Special Session on Shrimp Farming. World Aquaculture Society, Baton Rouge, LA, USA, (1995) pp. 40–52.

6 Briggs, M., Funge-Smith, S., Subasinghe, R.P., Phillips, M., Introductions and movement of two penaeid shrimp species in Asia and the Pacific. FAO Fisheries Technical, FAO, Rome, Italy. 476(2005): pp. 78.

7 Clifford, H.C., Preston, N.P., Genetic improvement of farmed shrimp. In: Boyd, C.E., Jory, D.E., Chamberlain, G.W., (Eds.), Operating Procedures for Shrimp Farming. Global Aquaculture Alliance, St. Louis, Missouri, USA, (2006) pp. 8–18.

8 Remany, M.C., Cyriac, D., Nagaraj, S., Rao, B., Panda, A.K., Kumar, J., Samraj, Y.C.T., Specific pathogen-free assurance of imported pacific white shrimp Litopenaeus vannamei (Boone, 1931) in the aquatic quarantine facility, Chennai. Current Science 99(2010): 1656-1658.

9 Browdy, C. L., Recent developments in penaeidbroodstock and seed production technologies: improving the outlook for superior captive stocks. Aquaculture 164(1998): 3-21.

10 Sindhupriya, M., Saravanan, P., Otta, S.K., Amarnath, B.C., Arulraj, R., Bhuvaneswari T., Praveena, E.P., Jithendran, K.P., Ponniah, A.G., White spot syndrome virus (WSSV) genome stability maintained over six passages through three different penaeid shrimp species. Dis. Aquat. Organ. 111(2014): 23-9.

11 Otta, S.K., Arulraj, R., Praveena, P.E., Manivel, R., Panigrahi, A., Bhuvaneswari, T., Ravichandran, P., Jithendran, K.P., Ponniah, A.G., Association of dual viral infection with mortality of Pacific white shrimp (Litopenaeus vannamei) in culture ponds in India. Virus diseases 25(2014): 63–68.

12 Bell, T.A., Lightner, D.V., A handbook of normal penaeid shrimp histology. World Aquaculture Society, Special Publication, Baton Rouge, Louisiana 1988, pp.114.

13 Lightner, D.V., A handbook of pathology and diagnostic procedures for diseases of penaeid shrimp. World Aquaculture Society. Baton Rouge, LA 1996 pp. 256.

14 Bancroft, J.D., Gamble, M., Theory and Practice of Histological Techniques. (6thEdition., Churchill Livingstone. Elsevier) 2011 pp 320-321.

15 White, T.J., Bruns, T., Lee, S., Taylor, J., Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J., (Eds)PCR Protocols: a guide to methods and applications. Academic Press, New York, USA, (1990) pp. 315–322.

16 Hall, B.G., Building phylogenetic trees from molecular data with MEGA. Mol. Biol. Evol. 30 (2013): 1229–35.

17 Poulos, B.T., Tang, K.F.J., Pantoja, R.C., Bonami, J.R., Lightner, D.V., Purification and characterization of infectious myonecrosis virus of penaeid shrimp. J. Gen.Virol. 87(2006): 987-96.

18 Peng, S.E., Lo, C.F., Lin, S.C., Chen, L.L., Chang, Y.S., Liu, K.F., Su, M.S., Kou, G.F., Performance of WSSV-infected and WSSV-negative Penaeus monodon postlarvae in culture ponds. Dis. Aquat. Org., 46 (2001):165–172.

19 Karunasagar, I., Pai, R., Malathi, G.R., Karunasagar, I., Mass mortality of Penaeus monodon larvae due to antibiotic resistant Vibrio harveyi infection. Aquaculture 128(1994): 203–209.

20 Lavilla-Pitogo, C.R., Leano, E.M., Paner, M.G., Mortalities of pond-cultured juvenile shrimp Penaeusmonodon associated with dominance of luminescent vibrios in the rearing environment. Aquaculture 164 (1998):337–349.

21 FAO, FAO Fisheries Technical Paper. No. 446, Improving Penaeus monodon hatchery practices Manual based on experience in India. FAO, Rome (2007) pp. 101.

22 Thianprasit, M., Chaiprasert, A., Imwidthaya, P., Human pythiosis. Cur. Top Med. Mycol., 7 (1996): 43-54.

23 Supabandhu, J., Fisher, M.C., Mendoza, L.A. & Vanittanakom, N., Isolation and identification of the human pathogen Pythium insidiosum from environmental samples collected in Thai agricultural areas. Med. Mycol., 46 (2008): 41-52.

24 Presser, J.W., Goss E.M., Environmental sampling reveals that Pythium insidiosum is ubiquitous and genetically diverse in North Central Florida. Med. Mycol., 53 (2015): 674-683.

25 Hatai, K., Lawhavinit, O.R., Lagenidium myophilum sp. nov., a new parasite on adult northern shrimp (Pandalus borealis Kroyer). Trans. Mycol. Soc. Jpn. 29(1988): 175–184.

26 Muraosa, Y., Morimoto, K., Sano, A., Nishimura, K., Hatai, K., A new peronosporomycete, Halioticida noduliformans gen. et sp. nov., isolated from white nodules in the abalone Haliotis spp. from Japan. Mycoscience 50 (2009):106–115.

27 Zanette, R.A., Santurio, J.M., Loreto, E.S., Alves, S.H., Kontoyiannis, D.P., Toll-deficient Drosophila is susceptible to Pythium insidiosum infection. Microbiol. Immunol. 57 (2013):732-735.