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Enterocytozoon hepatopenaei: A microsporidian in the midst of serious threat to shrimp aquaculture

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Abstract
Enterocytozoon hepatopenaei (EHP) is a group of microorganisms belonging to microsporidia which is now classified as fungus. It was first reported in tiger shrimp in 2009 in Thailand. Shrimp farm industry in Southeast Asia have been reported gradually increasing cases of rigorous growth retardation in shrimp populations with high prevalence of microsporidian. Generally, there is absence of specific signs and symptoms in shrimp due to EHP infection but its association with growth retardation and white faeces syndrome (WFS) in shrimp has been reported. EHP is mainly restricted to tubule epithelial cells of hepatopancreas and it interferes with the digestion and absorption of food hampering the growth of shrimp. However, till now there is no drug recommended for the control of EHP infection in shrimp. This study reveals the occurrence and distribution of EHP in farms along with their pathogenecity, diagnosis and control measures in shrimp farms and hatcheries.

Keywords: *Enterocytozoon hepatopenaei*, microsporidia, prevalence, diagnosis, control

1. Introduction
Shrimp aquaculture has been considerably developed and is still expanding rapidly with long term potential, especially after the introduction of the exotic Pacific white shrimp in India. However, the intensification of shrimp farming has introduced various ecological and physiological stresses in culture system leading to the introduction of various infectious agents hampering the health and economy of the farms. Diseases in shrimp can be caused by various pathogenic agents like viruses, bacteria, parasites, fungi, nutritional deficiencies and changes in environmental parameters. Among these pathogens, bacterial and virus infection in shrimps has been mostly studied. In India, occurrence of white spot syndrome virus (WSSV) and infectious hypodermal hemotopoetic necrosis virus (IHHNV) are most frequent and are prevalent. Beside these other viral pathogens of shrimp incluse Yellow Head Virus (YHV), Covert Mortality Disease (CMD) caused by covert mortality nodavirus and Macrobrachium rosenbergii nodavirus causing White Tail Disease (WTD) have been responsible for causing losses to aquaculture in the Americas and the Southeast Asian countries (Thitamadee *et al*., 2016) [1]. Among bacterial infections, Vibrio sp. are known to act as threat for shrimp farming causing acute hepatopancreatic necrosis disease (AHPND) by Vibrio parahaemolyticus (Tran *et al*., 2016) [2]. Besides this, several parasitic microsporidians have also been reported as pathogenic on penaeid shrimp (Lightner, 1996) [3]. Agmasoma previously called Thelohania infecting muscle tissue and connective tissue of *Penaeus monodon* and *Penaeus merguiensis* has been reported from Thialand (Flegel *et al*., 1992; Pasharawipas *et al*., 1994; Pasharawipas and Flegel, 1994) [4, 5, 6]. Recently it was also reported infecting the same tissues in *Penaeus vannamei* in Thailand (Limsuwan *et al*., 2008; Laisutisan *et al*., 2009; Prasertsri *et al*., 2009) [7, 8, 9]. The other microsporidian reported from Thailand in penaeid shrimp was a newly described species (Tourtip, 2005) [10] which were found restricted to tubule epithelial cells of the hepatopancreas. The parasite was isolated from *P. monodon* in Thailand and was described and named as *Enterocytozoon hepatopenaei* (EHP) (Tourtip *et al*., 2009) [11]. It hampers the digestive and absorptive ability of shrimp leading to growth retardation *P. vannamei* and *P. monodon* (Newman, 2015) [12] and do not cause mortality in population. Studies conducted at CIBA during the last one year also indicated widespread occurrence of EHP in Indian shrimp farming systems. In India EHP was first reported by Rajendran *et al*., (2016) [13]. The economic loss in shrimp production due to EHP appears to be significant and causing great economical and production loss.

2. *Enterocytozoon hepatopenaei* (EHP) aetiology

Microsporidia group constitute a spore-forming unicellular eukaryotic parasites and were once considered in group of protozoans (Adl *et al*., 2012) [14], but are now known to be more closely related to yeast-like fungi (Newman, 2015) [12]. Approximately 20 genera are identified as infecting fish (Corradi, 2015) [15]. Microsporidia are ubiquitous, obligate intracellular parasites and are found in all kinds of environments, from deep-sea methane seeps to terrestrial environments. They lack motile structures, such as flagella, and produce highly resistant spores capable of surviving outside their host for up to several years. They are very small in size, ranging between 1 and 40 microns in diameter. Spore morphology is used for differentiating species. Spores produced by EHP are oval in shape and measured as 1.7-1.0 µm in diameter (Rajendran *et al*., 2016) [13]. Development of the parasite occurs within the cytoplasm of the host cell via spore formation (Sporogony) and nuclear proliferation (Lom & Dykoa 2002; Stentiford, *et al*., 2007) [16, 17].

3. Pathological changes

The pathological changes occurring in EHP infected shrimp populations are not specific and are mainly associated with retarded growth. Stunted shrimp are further followed by deterioration of juvenile shrimp health with reduced feed consumption (50-70%) with white faecal matter and discolouration in hepatopancreas of infected population (Kesavan *et al*., 2016) [18]. Since, EHP is confined to the hepatopancreatic tubular cells of shrimp, it destroys the digestive and absorptive capability of cells. Similar microsporidian isolated from *P. monodon* and *P. vannamei* in Asia was *Agmasoma penaei* that targets muscles and connective tissue of shrimp but not the cells of hepatopancreas (Flegel *et al*., 1992; Prasertsri *et al*., 2009) [4, 9]. Earlier, EHP was reported to be associated with white faeces disease (WFS) in cultured *P. monodon* (Ha *et al*., 2010) [19] but later the study reported that EHP is not the cause of WFS in *P. vannamei* (Tangprasittipap *et al*., 2013) [20]. This parasite generally takes about 14 days to commence infection in tubules of hepatopancreas (Salachan *et al*., 2017) [21]. EHP damages the ability of hepatopancreatic cells and hence deprives the shrimps from nutrition resulting in poor growth and production. Histologically, larger eosinophilic to basophilic inclusions were observed in distal end tubular epithelium of hepatopancreas indicating the presumptive developmental stages of EHP. Basal membranes of hepatopancreas were detached from tubular epithelium with sloughing of the tubular epithelial cell in case of heavy infections followed by cellular damage (Kmmari *et al*., 2018) [22].

4. Susceptibility to shrimp and prevalence of EHP in India

EHP is mainly the parasite of penaeid shrimp, and have been found in both black tiger prawns *P. monodon* and Pacific white shrimp *P. vannamei*. Most commonly susceptible shrimp populations to EHP are *Penaeus monodon*, *P. japonicas*, *P. vannamei*, and *P. meruuiensis* are reported to be susceptible to EHP infection. A very close association in prevalence of EHP and WFS was observed in shrimp farming. EHP is reported to occur widely in China, Indonesia, Malaysia, Vietnam and Thailand. In India, the prevalence of EHP without WFS (39.7%) and with WFS (96.4%) was identified in EHP infected ponds (Rajendran *et al*., 2016) [13]. Disease surveillance carried out at CIBA, 2016, reported EHP was associated with about 16% shrimp farms affected with growth retardation and in as high as 50% of the farms affected with white faeces syndrome (WFS). However, intensity of EHP prevalence depends on the culture conditions of the pond like in Greenhouse ponds the prevalence was 54.4% as compared to earthen ponds (79.5%) (Shen *et al*., 2017) [23].

5. Mode of Transmission

Main route of transmission of EHP in farms are observed orally however, infected live feeds like polychaetes can also be the major source of transmission. Live feeds can infect and spread EHP through faeces (Newman, 2015) [12] or it can also be directly transmitted to other shrimps horizontally via water (Salachan *et al*., 2017) [21]. EHP could be transmitted directly from shrimp to shrimp by the oral (horizontal) route (Tangprasittipap *et al*., 2013) [20] and does not require an intermediate host. It differs from the other microsporidian parasites such as *Agmasoma* and *Pleistophora*, previously reported from cotton shrimp, which require intermediate host for its transmission. Although horizontal transmission is the main route of transmission but some microsporidia like *Crangonyx* sp utilize both horizontal and vertical transmission (Dunn *et al*., 2001; Smith, 2009) [24, 25]. Hence, vertical transmission of EHP could also be a possibility. Generally infected epithelial cells of hepatopancreas slough off and release spores through faeces of shrimps which remain in an infective stage in water for some time. These released spores enter the environment and infect the entire shrimp population of farm.

6. Diagnosis of EHP

EHP can be diagnosed mainly by two methods i.e. molecular method using PCR or LAMP and microscopy method using different types of stain like Hematoxylin-eosin, giemsa, Scanning Electron Microscopy and Transmission Electron Microscopy (Karhikeyan *et al*., 2016) [26]. EHP spores can be detected by squash preparations of shrimp faecal matter stained with Giemsa stain and observing it into 100X magnification of light microscope. Spores morphology as observed appear to be oval with smooth surface with dent-like curvature under a scanning electron microscope and measure about 1.7x 1.0 µm. Further, EHP can be demonstrated with DNA based techniques like PCR and LAMP and are used widely for rapid diagnosis in shrimp farms and hatcheries. Various molecular methods like PCR, real time PCR, in situ hybridization (ISH) and loop mediated isothermal amplification (LAMP) methods (Tourtip *et al*., 2009; Tangprasittipap *et al*., 2013; Suebsing *et al*., 2013; Liu *et al*., 2014; Tang *et al*., 2015; Rajendran *et al*., 2016) [11, 13, 20, 27, 28, 29] has been employed for testing the presence of EHP in shrimps and farms. A commercial diagnostic kit is also available from M/s GeneReach Biotechnology Corp., Taiwan for detection of EHP. However, these described methods can be used to check shrimps but are not very specific and suitable for environmental samples because of possible cross reactions. Some microsporidians have rRNA sequences very similar to EHP, thus, a unique gene of EHP was needed to screen suspected carriers. Unique spore wall protein gene specific for EHP was identified and was used to develop a specific, nested PCR method for EHP (SWP-PCR) (Jaroenlak *et al*., 2016) [30]. SWP-PCR is more specific and more sensitive method and is now recommended as the best method for detecting EHP.

7. Prevention and treatment of EHP

To keep the parasites away, its recommended to use better management practices (BMPs) and proper bio security in the shrimp farms. After harvesting, disinfection, ploughing and drying of pond is important to make sure the destruction of EHP spores and their carriers before stocking. Even SPF shrimp broodstock can harbor EHP spores (Flegel, 1992) [4] hence only PCR tested EHP free seeds should be stocked. Use of live feed like polychaetes in shrimp farm should be avoided. If used, then live feed should be frozen and pasteurized (heating at 70 ^{0}C for 10 minutes) to kill all bacteria, virus and parasites (Sritunyalucksana *et al*., 2015) [31]. Viability of spores on dry surfaces at room temperature was found to be six months and it can retain infectivity for over a year under aqueous conditions due to its thick spore wall. Hence, liming @ six tons per hectare followed by thorough ploughing has been also suggested for the disinfection of ponds. Quarantining and following standard bio security principles are the key strategies to avoid entry of undesired pathogens.

Although, still no known drug is available till to date for control of EHP but farmers are using their own methods to treat the infected farms like by applying garlic and bitter guard paste (30-40 g/ kg of feed) in India. Indonesian farmers are applying probiotics, Garlic paste (10-20g/kg feed) and vitamin C (2g/kg feed) to avoid EHP and WFS in shrimp culture (Tang *et al*., 2016) [32].

8. Conclusion

Occurrence and prevalence of EHP in shrimp farm is of great concern for shrimp farmers due to its contribution in greater loss in economy and productivity. Increase in incidence of disease outbreak is mainly regulated by the interaction of ecological condition with shrimp health status and epizootics of EHP pathogen. Therefore, it is crucial to concentrate on better management practices for shrimp farming using high quality EHP free seeds, and strictly following farm bio security measures. It is required to prevent the spread of EHP pathogens to avoid the disease in subsequent culture operations and to improve the production and sustainability of shrimp culture. It is important for shrimp farmers to realise that following custom rules and regulations in shrimp farming is the need of the hour for responsible aquaculture practices.

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