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Managing *Enterocytozoon hepatopenaei* (EHP), microsporidial infections in vannamei shrimp farming: An Advisory

Enterocytozoon cytopenaei (EHP) belongs to a group of microbes called microsporidia classified as fungus, and was first reported in tiger shrimp in 2009 in Thailand. There are no specific signs and symptoms in shrimp due to EHP infection. However, EHP has been reported to be associated with growth retardation and white faeces syndrome (WFS) in shrimp (needs to be proved). The target organ of EHP is hepatopancreas and affects its digestive and absorptive functioning resulting in poor growth and immunity. EHP can be detected microscopically and by polymerase chain reaction technique. There is no drug for the control of EHP infection in shrimp. SPF broodstock, live feed such as polychaete worms, clams, oysters, *Artemia* and other inputs have to be screened and ensured free from EHP. The EHP spores in fecal pellets or dried cadavers can remain viable up to six months and retain infectivity for over a year under aqueous conditions. Application of lime and maintaining the soil pH to 12 has been suggested for the disinfection of ponds. Better management practices (BMPs) and proper biosecurity is the only way to keep this parasite away from the aquaculture ecosystem.

The Indian aquaculture production has considerably improved during the last five years, especially after the introduction of the exotic Pacific white shrimp. Availability of imported specific pathogen free (SPF) broodstock provided the much needed growth rate to India's brackishwater aquaculture sector. However, the intensification of vannamei farming has exacerbated the epizootics and disease issues are becoming a major constraint affecting productions and profitability. Among the listed viruses of the farmed crustaceans, the white spot syndrome virus (WSSV) and infectious hypodermal hemotopoetic necrosis virus (IHHNV) have been frequently reported from Indian subcontinent and, both these pathogens can be considered as endemic in India. Other viral pathogens such as infectious myonecrosis virus (IMNV), Taura syndrome virus (TSV), yellow head virus (YHV) which have been responsible for causing losses to aquaculture in the Americas and the Southeast Asian countries have so far not been scientifically confirmed from India. During the last couple of years, a number of disease syndromes such as running mortality syndrome (RMS), white faeces syndrome (WFS)/ white gut syndrome and growth retardation have been negatively impacting shrimp aquaculture in India. In the Southeast Asian countries, emergence of a new microsporidian parasite "*Enterocytozoon hepatopenaei* (EHP)" has been reported since 2009. Studies conducted at CIBA during the last one year also indicated widespread occurrence of EHP in Indian shrimp farming systems. Considering its reported association with growth retardation resulting in reduced farm productivity, this advisory has been brought out for the benefit of shrimp farming community to prevent production losses

associated with this emerging pathogen in Indian shrimp farming.

What are microsporidia and what is *Enterocytozoon hepatopenaei* (EHP)?

EHP is an yeast-like fungus belonging to a group called "microsporidia", which are obligate intracellular parasites. Microsporidia are ubiquitous pathogens and are important components of terrestrial and aquatic ecosystems worldwide. The microsporidia are found in all kinds of environments, from deep-sea methane seeps, terrestrial environments including hospitals. Microsporidian infections have been reported to occur in aquatic arthropods inhabiting a wide array of habitats such as freshwater lakes, rivers, estuaries, temporary water bodies, rocky shorelines, brackish, and marine environments including stenohaline deep ocean floor. They have been described as diverse, dynamic, with pathogenic capabilities in aquatic systems and are known to infect a wide range of organisms, from the most primitive protists to humans. They are very small in size, ranging between 1 and 40 microns in diameter. Over 190 genera have been described to date and almost half of them are known to infect aquatic organisms. Approximately 20 genera infect fish, 50 genera infect aquatic arthropods, and at least 21 genera infect aquatic non-arthropod invertebrates, protists, and hyperparasites of aquatic hosts (Corradi, 2015). So, it appears that the aquaculture ecosystem including shrimp broodstock, PL, etc are carriers of these microbes. It appears that cryptic infections of microsporidia were common and no one gave attention until recently, wherein, the individual level effects have got extrapolated to populations and ecosystems now with increasing load of the microbe.

EHP disease condition

Enterocytozoon hepatopenaei (EHP) is an obligate, intracellular microsporidian parasite, first characterized and named in 2009 from the giant or black tiger shrimp *Penaeus monodon* from Thailand (Tourtip et al., 2009). There are no specific signs and symptoms of EHP infection in shrimp. It was discovered in slow growing shrimp and also from shrimp that exhibited mortality associated with white faeces syndrome (WFS, Fig 1)). However, Tangprasittipap et al (2013) later reported that EHP was not the causative agent of WFS. EHP is confined to the shrimp hepatopancreatic tubular cells. The other microsporidian described from *P. monodon* and *P. vannamei* in Asia is *Agmasoma penaei* (also called *Thelohania penaei*) that targets shrimp muscles and connective tissue but not the tubule epithelial cells of the hepatopancreas (Flegel et al., 1992; Prasertsri et al., 2009). Since the main organ affected in shrimp is hepatopancreas, it is most likely that the digestive functions are hampered and this would in turn affect the digestive and absorptive functioning of the hepatopancreas resulting in poor growth and immunity.

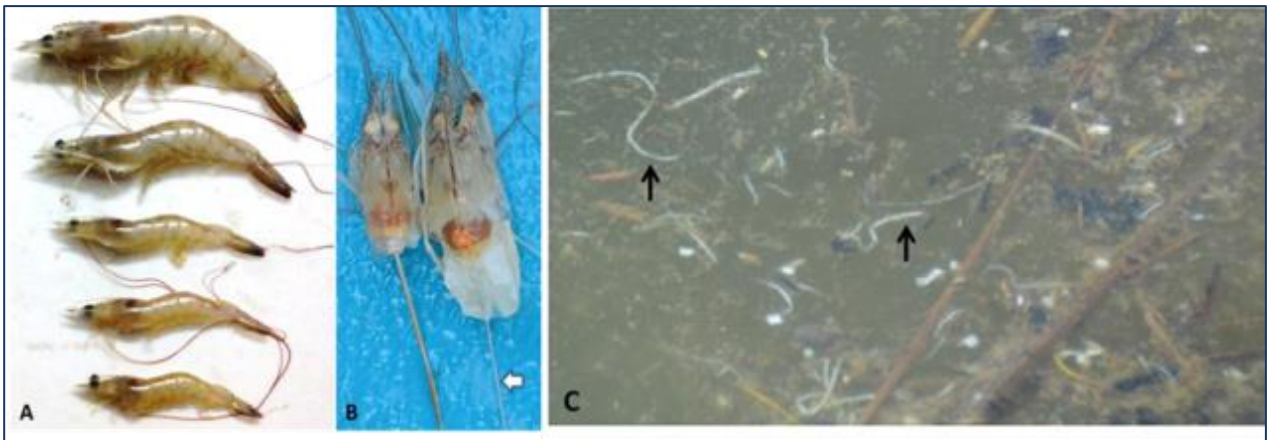

Fig. 1. Observations on EHP infected samples of *Penaeus vannamei*. A. Retarded growth observed after 90 days of culture; B. White/empty gut (arrow) and discolouration of hepatopancreas; C. Floating white faeces in the pond water.

Which species of shrimp are susceptible to EHP?

So far, *Penaeus monodon, P. japonicas, P. vannamei, and P. meruuiensis* are reported to be susceptible to EHP infection.

What is the status of Occurrence/ prevalence of EHP in India?

EHP is reported to occur widely in China, Indonesia, Malaysia, Vietnam and Thailand. However, no data exist on prevalence. Disease

surveillance carried out at CIBA has indicated that occurrence of EHP was in as much as 15.6% of over 100 farms investigated. So far we have seen that EHP was associated with about 16% shrimp farms affected with growth retardation and in as high as 50% of the farms affected with white feces syndrome (WFS). More work is required to have a clear understanding of its role in growth retardation / white faeces syndrome and its morbidity potential to inflict mortality.

How to detect / Diagnose EHP?

The EHP spores can be detected by light microscopy using 100 x magnification in hepatopancreas (HP) tissue sections prepared by histological techniques (Fig 2A and B) or HP squash preparations using specific vital stains. Spores of EHP could also be seen under the microscope in squash preparations of shrimp faecal matter stained with Giemsa stain (Fig 2 C). Due to their very small size, the microscopic detection of EHP spores is possible under oil immersion in heavily infected shrimp. Spores purified from infected hepatopancreas and faecal matter measure about 1.7x 1.0 µm and appear oval with smooth surface with dent-like curvature under a scanning electron microscope (Fig 2 D). Specific detection of EHP can be done using DNA based molecular diagnostic tools such as 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). A commercial diagnostic kit is also available from M/s GeneReach Biotechnology Corp., Taiwan. The AAHED lab of CIBA has been testing shrimp samples under the disease surveillance programme, using PCR protocols of Tourtip et al., 2009; Tangprasittipap et al., 2013 and Tang et al., 2015) (Fig 3) and further improved with specific primers (Rajendran et al., 2016).

How EHP is transmitted?

EHP could be transmitted directly from shrimp to shrimp by the oral (horizontal) route (Tangprasittipap et al., 2013). EHP differs from the other microsporidian parasites such as *Agmasoma* (=*Thelohania*), *Ameson* (=*Nosema*), *Pleistophora* (= *Plistophora*), previously reported from cotton shrimp, where the parasite required an intermediate fish host for its transmission, while EHP does not require an intermediate host.

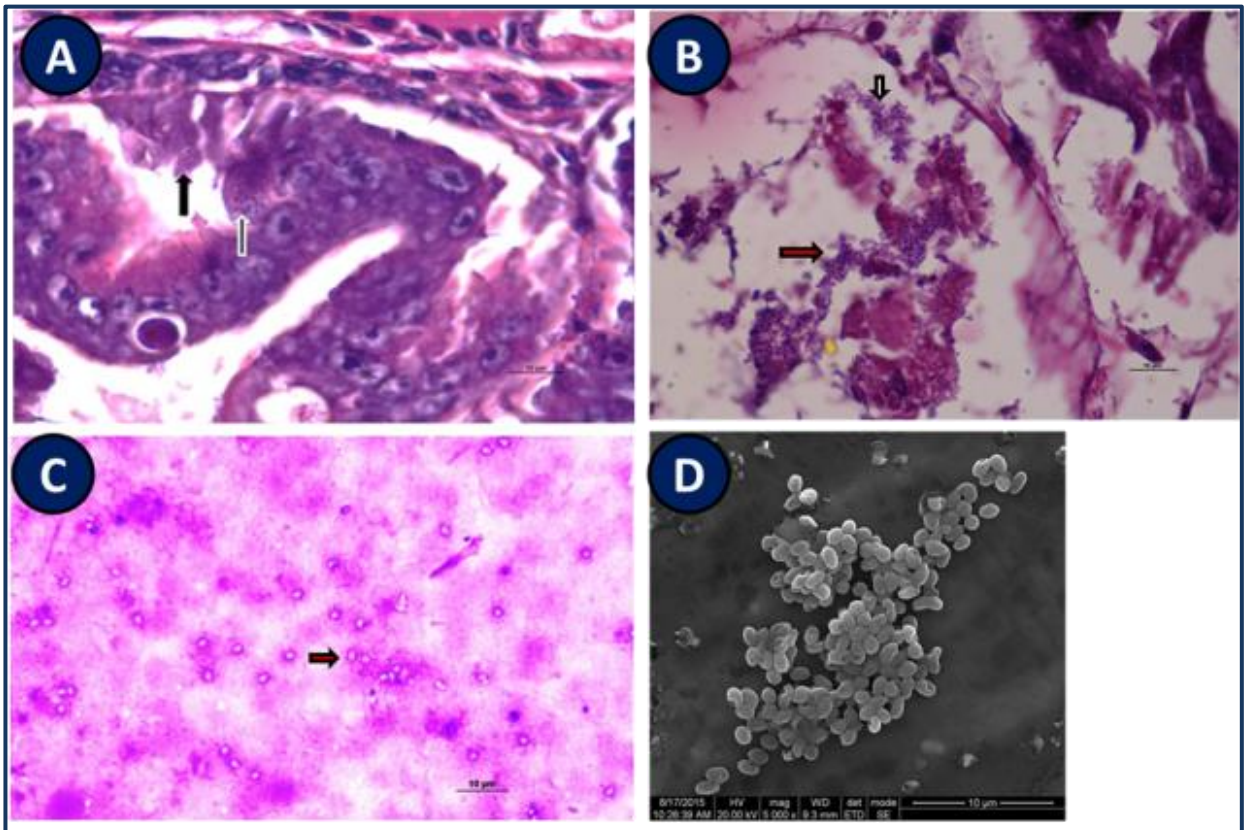

Fig 2. Photomicrograph of hepatopancreas stained with hematoxylin and eosine showing EHP spores: in the HP tubule epithelial cells (A) sloughed HP cells (B); faecal matter stained with Geimsa stain (C) and scanning electron micrograph showing oval spores of EHP.

Infected animals can release spore s that enter the environment by the decomposition of the dead animal, cannibalism, scavenging etc. Infected hepatopanctreatic cells and intestinal epithelial cells generally slough off and degrade within the digestive system, allowing spores to be released with the faeces which will remain infective for some time depending on environmental conditions. Polychaetes, being benthic feeders can be PCR positive for EHP and hence extreme caution should be exercised while using polychaetes as live feed in the hatcheries. Vertical transmission of microsporidia has been also reported in some crustacea such as *Crangonyx* sp. (ground water amphipod), by infection of gonads (Stentiford, 2014). Hence, possibility of vertical transmission of EHP could also be a possibility.

been reported that the spores from insects, placed on dry surfaces at room temperature, could remain infective for two weeks and even for an year. Further, the spores in fecal pellets or dried cadavers can remain viable up to six months under the same conditions but would retain infectivity for over a year under aqueous conditions. Application of lime (CaO) at the rate of 6 tons per hectare, followed by thorough ploughing and maintaining moist conditions for about a week to raise the soil pH to 12 has been also suggested for the disinfection of ponds. Nevertheless, controlling microsporidian infection could be a great challenge in aquaculture since it has been demonstrated that same species can infect different hosts such as insects and copepods and fish and their arthropod parasites (Weiss and Becnel, 2014).

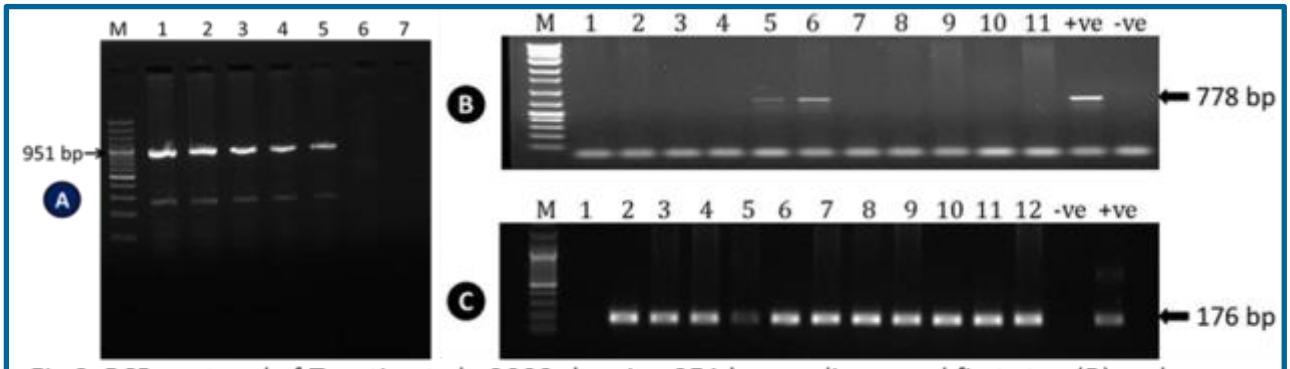

Fig 3. PCR protocol of Tourtip et al., 2009 showing 951 bp amplicon; and first step (B) and nested PCR (C) protocol of Tangprasittipap et al., 2013 showing 778 and 176 bp products.

Prevention / control of EHP

There is no drug for the control of EHP infection in shrimp. Hence, better management practices (BMPs) and proper biosecurity is the only way forward to keep this parasite away from the rearing system. It is possible that even the SPF shrimp broodstock can harbor EHP spores (observations by Flegel's group). It is advisable that ponds are stocked with EHP free seed. Shrimp seed can be ensured to be free by PCR testing. The most important measure is to prepare ponds appropriately as per the BMP protocols to ensure that the pond is free from EHP spores before the seed is stocked. The EHP spores have thick walls and cannot be easily inactivated even with high levels of chlorine. After every harvest, it is extremely important that the ponds are disinfected and thoroughly dried to make sure that the EHP spores in the ponds along with their carriers are destroyed before stocking ponds. Viability of microsporidian spores is also dependent upon environmental factors. It has

Do microsporidia have any public health importance?

While some microsporidia are known to cause infections in humans, so far there are no reports of EHP infections in humans.

What hatcheries could do?

Testing of faecal samples of imported SPF broodstock in India during quarantine so far has indicated that they are free from EHP. However, once these broodstock are received and kept in the importers facility, the hatcheries must ensure BMP and strict biosecurity. It is strictly advisable that the live feed such as polychaete worms, clams, oysters, *Artemia* and other inputs need to be screened for pathogens before their use in the hatcheries. It has been suggested that freezing polychaete worms could help in reducing infectivity of microsporidia. Another preventive measure could be pasteurization of live feed before feeding, which can be performed by heating live feed for 10 min to 72°C. This will also

destroy most bacterial and viral pathogens. Washing eggs and nauplii with iodine, formalin etc., will help in reducing number of microsporidial spores if any.

While use of pond reared broodstock is illegal, considering the widespread occurrence in our aquaculture ecosystems along the coastline, such broodstock will most likely carry EHP, and hence, use of pond reared broodstock should never be practiced.

Disinfection of hatchery facility prior to starting new production cycle will help in minimizing EHP risk. Cleaning hatchery facility including all the surfaces, tanks, pipelines and implements, with 2.5 % sodium hydroxide solution with a minimum of three hours of contact time and then drying for about a week followed by rinsing with acidified chlorine (200 ppm) is advocated to prevent EHP.

How farmers could help?

Farmers must always buy seed from only registered shrimp hatcheries and insist on seed quality including testing for EHP by PCR, in addition to WSSV and IHHNV even though this may be an additional financial burden. To maintain farm productivity, farmers should adhere to better management practices, providing adequate time (at least 3-4 weeks) for the ponds to dry after every harvest. The ponds having history of disease should be disinfected using appropriate protocols.

Concluding remarks

The emergence and spread of infectious disease is usually the result of a series of linked events involving the interactions between the host, the environment and the pathogens. It is essential to focus efforts on producing high quality seed, follow improved better management practices, routine farm biosecurity measures and responsible trade practices to prevent epizootics in aquaculture. Further, health management is a shared responsibility, and each and every stakeholder's contribution is essential to the health management process to minimize losses and sustain productivity.

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Research on brackishwater aquatic animal health and environment was initiated at the Central Institute of Brackishwater Aquaculture since 1990. Since then it has grown in terms of expertise, manpower and laboratories. The Aquatic Animal Health and Environment Division or the AAHED in short, has scientists with all relevant specialities and expertise in Microbiology, Virology, Pathology, Parasitology, Biotechnology, Molecular Diagnostics, Soil and water Chemistry, Environment and Aquaculture. The AAHED has well established laboratory facilities for carrying out cutting edge research in molecular biology for diagnosis, prophylaxis and health management in brackishwater aquaculture. The advanced facilities have been developed with funding support from ICAR, National Agricultural Research Project (NARP), World Bank, National Agricultural Technology Project (NATP), Department of Biotechnology and National Fisheries Development Board with dedicated efforts of scientists. A well designed wet lab is also in place for carrying out live aquatic animal experiments and evaluating Koch's and River's postulates.

The AAHED, CIBA has the mandate to carry out research on (a) economically impacting diseases of brackishwater culture species and develop technologies for rapid diagnosis, prophylaxis and control; (b) brackishwater aquaculture environment and develop mitigatory measures as required; and (c) provide technical and policy support to the Government on matters pertaining to aquatic animal health and environment management to improve aquaculture productivity.

The AAHED of CIBA was the first to commercialise a nucleic acid-based diagnostic kit for the most devastating white spot syndrome of farmed shrimp to a premier Biotechnology company in the year 2002. The AAHED also developed a diagnostic kit for the white tail disease in scampi in the year 2004. AAHED has the expertise and capacity to carry out all the proposed levels of Diagnostics of OIE listed Brackishwater pathogens.

The environment section of AAHED has the expertise to look into all aspects of abiotic parameters especially with regard to aquatic ecosystem health management. Novel methods have been developed for the bioremediation and environmental monitoring of the brackishwater rearing systems, including hatcheries and farms. The unit also has expertise in climate related studies and has developed climate smart solutions for brackishwater farming systems. The section has also capacity for the environmental impact assessment and carrying capacity assessment of source waters for optimisation of brackishwater aquaculture development.

AAHED, CIBA has published over 60 research publications in peer reviewed national and international journals, produced 15 Ph. Ds, who are currently employed in key positions in various Institutions in India and abroad.

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