Dense populations of the microsporidian *Enterocytozoon hepatopenaei* (EHP) in feces of *Penaeus vannamei* exhibiting white feces syndrome and pathways of their transmission to healthy shrimp

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Abstract

White feces syndrome (WFS) is an emerging problem for penaeid shrimp farming industries in SE Asia countries, Thailand, Malaysia, Vietnam, Indonesia, China, and in India. This occurrence of this syndrome is usually first evidenced by the appearance of white fecal strings floating on surface of the shrimp ponds. The gross signs of affected shrimp include the appearance of a whitish hindgut and loose carapace, and it is associated with reduced feeding and growth retardation. To investigate the nature of the white feces syndrome, samples of white feces and shrimp hepatopancreas tissues were collected from *Penaeus vannamei* in affected farms in Indonesia, and these were examined histologically. Within the white feces, we found densely packed spores of the microsporidian *Enterocytozoon hepatopenaei* (abbreviated as EHP) and relatively fewer numbers of rod-shaped bacteria. From WFS ponds, hepatopancreas samples from 30 individual shrimp were analyzed by histology and in situ hybridization. The results showed that all of the shrimp examined were infected with EHP accompanied by septic hepatopancreatic necrosis (SHPN). Midgut epithelial cells were also infected and this increased the number of tissue types being affected by EHP. By PCR, EHP was detected in all the samples analyzed from WFS-affected ponds, but not in those sampled from healthy shrimp ponds. To determine the modes of transmission for this parasite, we performed feeding and coinhibition bioassays, the results showed that EHP can be transmitted through *per os* feeding of EHP-infected hepatopancreas tissue to healthy shrimp and through coinhibition of infected and healthy shrimp. In addition, we found the use of Fumagillin-B, an antimicrobial agent, was ineffective in either reducing or eliminating EHP in infected shrimp.

Key words: shrimp disease, *Enterocytozoon hepatopenaei*; EHP; white feces syndrome, in situ hybridization; PCR.

Introduction

Marine shrimp farms in Asia have been increasingly reporting stocks that are exhibiting white feces syndrome (WFS) with concomitant economic losses (Ha et al., 2010; Thitamadee et al., 2016). Shrimp from affected ponds grow slowly and have increased food conversion ratios. There are no specific clinical signs displayed by the affected shrimp; and currently, WFS is usually first diagnosed through the appearance of whitish fecal strings floating on the pond surfaces. It has not been determined if this syndrome is caused by an infectious agent; but, based on its rapid spread, a contagion is suspected. Although the cause of WFS has not been established, *Vibrio* bacteria and aggregated, transformed, microvilli (ATM) resembling gregarines have been reported to be associated with this syndrome in Thailand (Limsuwan 2012; Sriurairatana et al., 2014). The microsporidian *Enterocytozoon hepatopenaei* (EHP) was also reported from *Penaeus monodon* and *P. vannamei* with WFS in Vietnam, Thailand and India (Ha et al., 2010, Tangprasittipap et al., 2013; Rajendran et al., 2016).

EHP is an intracellular spore-forming parasite that replicates within the cytoplasmic area of the tubule epithelial cells in the hepatopancreas (Tourtip et al., 2009; Tangprasittipap et al., 2013; Tang et al., 2015), and it is emerging as a critical threat to the shrimp farming industry in SE Asia. In Indonesia, EHP has been detected in farmed *P. vannamei* populations, including in East Java, West Java, North Sumatra, Lampung province at the southern tip of Sumatra, Bali, Lombok, and Sulawesi (discussions with producers and Indonesian researchers). It has been suggested that EHP is not the only agent causing WFS based on studies showing that EHP-infected shrimp, as determined by PCR and in situ hybridization analyses, did not produce white feces (Tangprasittipap et al., 2013). In this study, we collected samples (fecal strands and shrimp hepatopancreas) from the WFS-affected ponds in Indonesia to determine the presence of EHP by

PCR, histology and in situ hybridization. Furthermore, we performed experimental infections to demonstrate the routes that EHP can be transmitted.

Materials and Methods

Shrimp and fecal samples. Shrimp hepatopancreas and feces were sampled from 3 shrimp farms displayed WFS in Indonesia in 2016. From each of the farms #1 and #2 (both located in Lampung, Indonesia), samples (shrimp weighted 3-5 g at 50-60 days culture) were collected from one pond exhibited WFS and another pond without WFS. From farm #3 (located in Situbondo), only a WFS-pond was sampled. For histology analyses, the hepatopancreas samples were fixed in the Davidson's AFA fixative, the fecal samples were submerged in 95% ethanol. These samples were processed for histological and in situ hybridization at University of Arizona. For PCR analyses, samples of hepatopancreas, hindgut and feces were preserved in 95% ethanol.

DNA extraction and EHP PCR. From hepatopancreas and fecal samples, total DNA was extracted using a High-pure DNA template preparation kit (Roche Bioscience) and followed by EHP PCR. PuReTaq Ready-To-Go PCR beads were used. The primers for detecting the EHP are EHP-510F (5'-GCCTGAGAGATGGCTCCACGT) and EHP-510R (5'-GCGTACTATCCCCAGAGCCCGA). Amplifications were performed with the following cycling parameters: initial denaturation at 94°C for 3 min, followed by 35 cycles of 94°C for 15 s, 60°C for 15 s, and 72°C for 30 s, and a final extension at 72°C for 5 min (Tang et al., 2015).

Probe labeling and in situ hybridization. The pEHP-1 plasmid DNA was used to generate a probe for ISH. The gene probe was labeled with digoxigenin-11-dUTP (Roche) in a

PCR reaction as described by Tang et al. (2015). Following PCR, the digoxigenin-labeled DNA probe (1.1 kb) was precipitated with ethanol, re-suspended in water, and stored at -20°C.

The Davidson's AFA fixed shrimp were processed, embedded in paraffin, and sectioned (4 µm thick) in accordance with standard methods (Lightner 1996). After deparaffinization, hydration, proteinase K digestion, and post-fixation, sections were overlaid a hybridization solution containing the EHP probe (0.2 µg/ml). Slides were placed on a heated surface at 90°C for 10 min and hybridized overnight at 37-42°C. Final detection was performed with anti-digoxigenin antibody conjugated to alkaline phosphatase (Roche) that was visualized using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

EHP laboratory infection

Per os bioassay. 30 specific pathogen free (SPF, determined by histology and PCR analyses for major shrimp pathogens including EHP) *Penaeus vannamei* indicator shrimp (mean weight: 1 g) were fed with minced EHP-infected hepatopancreas at 10% of their body weight once for 2 days (day 1 and day 2) in a 90-L glass tank, then maintained on a pelleted ration, Rangen 35% (Rangen, ID). Samples of 3 shrimp were taken at each time point (days 0, 5, 10, 15, 20, and 24) and stored at -20°C. At day 24, 3 shrimp were fixed in Davidson's AFA fixative for histological analyses. The feeding tissue, from a population of EHP-infected shrimp (mean weight: 30 g), were from Thailand and quarantined in our laboratory as described in Tang et al. (2015).

Coinhibition bioassay. 30 SPF *P. vannamei* (mean weight: 1 g) tagged with visible elastomers (Northwest Marine Technology, WA) and 24 EHP-infected shrimp (30 g, from Thailand) were stocked in a 1000-L tank, then these shrimp were fed with Rangen 35% for 90

days. Samples of 3 shrimp were taken at each time point (days 35, 42, and 90), one fecal sample was sampled on day 35.

Fumagillin-B treatment. The Fumagillin-B (Glory Bee, Inc. OR; for veterinary use) was incorporated into feed to produce a diet with a ratio of 1g/kg. This dose was chosen based on published methods used to treat microsporidia and other infections in fishes and insects. A 0.6 g of Fumagillin-B was combined with 240 ml of water which was pre-heated to 30-35°C and then mixed thoroughly. After mixing, the Fumagillin-B solution was added to 600 g of Rangen 35% commercial shrimp premix (Rangen Inc., Idaho) and 18 g of carboxymethyl cellulose (3%, used as a binder) and allowed to thoroughly mix prior to cold extrusion through a meat grinder. The extruded feed were dried overnight at 40°C and then broken into appropriately sized pellets.

For bioassay to test the efficacy of Fumagillin-B against EHP infection, 25 EHP-infected shrimp (30 g, from Thailand) were stocked in each of 4 1000-L tank. Two (replicate) tanks was fed Fumagillin-B mixed shrimp feed, 2 tanks was fed pelleted feed, Rangen 35%. All animals in these tanks were fed their respective diets at a 1% bodyweight once a day for 30 days. At day 30, 2 shrimp from each tank were removed and pooled into one sample for EHP PCR analyses.

For these bioassays, shrimp were held at 25 ppt salinity and 28-29°C for the duration of experiments.

Results

Clinical signs of shrimp associated with white feces syndrome

The clinical signs of farmed shrimp with the white feces syndrome (WFS) are not visually apparent, apart from the production of white feces. The symptoms include reduced feeding, growth retardation and an increase in the food conversion ratio (FCR, mass of feed to

mass of shrimp produced). Based on the data obtained from WFS-ponds in Indonesia (personal com. with local farmers), white feces first appear 40-60 days after stocking postlarvae into the ponds. After the appearance of white feces, shrimp health will deteriorate if not treated. In general, the shrimp in the WFS-ponds show FCR of over 2.5-3.0 as compared to the range of 1.4-1.7 that is typical for healthy shrimp.

White feces: histology and *Enterocytozoon hepatopenaei* (EHP) in situ hybridization

Histological examination of white feces samples collected from 3 different WFS-ponds showed the presence of densely packed mature, basophilic, spores (Fig 1A, total magnification: 40X; Fig 1B, total magnification: 600X). We suspected these were *Enterocytozoon hepatopenaei* (EHP) spores, since a high percentage of EHP was detected in *Penaeus vannamei* cultured in Indonesia in recent years. Thus, we performed in situ hybridization (ISH) with an EHP-specific probe, the probe reacted intensely to the spores (Fig 1C, total magnification: 40X, insert: 600X). There were sloughing hepatopancreas tubules filled with EHP inclusions as well as large quantities of EHP spores embedded within the mucous-like substances within the white feces (Fig 1D). In addition, many basophilic, rod-shaped bacteria were present (Fig 1E). The EHP spores were not found in the fecal samples collected from the non-WFS ponds determined by histology and ISH (Fig 1G, 1H). By histology and ISH, we did not find the EHP in the normal fecal samples from EHP-infected shrimp those maintained in our laboratory; these fecal samples were positive by EHP PCR similar to that described in Tang et al. (2015).

During the histological preparation of fecal samples, we noticed that white feces were easier to cut than normal feces. This is because the white feces are not true feces which are composed of egested, non-assimilated, shrimp feed. Instead they were comprised of EHP spores, gut mucus, and necrotic tubule epithelial cells.

Shrimp hepatopancreas and midgut: histology and EHP ISH

Hepatopancreas samples from WFS-ponds were examined for EHP by histology, the results showed the presence of basophilic, cytoplasmic inclusions in all 30 samples (20 shrimp from WFS-pond #1, 10 from WFS-pond#3) examined. Hepatopancreatic tubules epithelial cells were enlarged, presumably filled with large numbers of EHP spores, and detached from the basal membranes (Fig 2A, a representative sample showing severe infection). The infected cells ruptured and discharged the mature spores to the lumen (Fig 2B, in situ hybridization Fig S1). We performed EHP ISH with 20 shrimp hepatopancreas from a WFS-pond #1, the results showed that all 20 of them were positive. The probe reacted intensely to the basophilic inclusions within the cytoplasm (Fig 2C), the blue-purple precipitates from ISH corresponded to the inclusions observed by H&E histological examination. In addition, all 20 shrimp were affected with septic hepatopancreatic necrosis (SHPN), where large numbers of rod-shaped bacteria were found in the inflamed tubule lumen. Focal inflammatory lesions were present, the cores of these lesions consisted of EHP-infected cells (positive by ISH, Fig S2) and they were surrounded with hemocytes (Fig 2D). Samples from WFS-pond #3 had a less severe EHP infection, and 4 out of 10 shrimp were affected by SHPN. EHP was also detected in the midgut epithelial cells from a sample from WFS-pond # 3 by histology and ISH (Fig 2E, 2F). This is unusual since EHP is known to infect only hepatopancreas.

EHP PCR

PCR was performed with DNA extracted from fecal and hepatopancreas samples collected from farm #1 and #2, from each pond (WFS- and non-WFS-pond), 5-10 shrimp hepatopancreas were pooled into one sample. The results showed that EHP was detected in white feces and shrimp hepatopancreas, not detected in the samples from healthy, non-WFS, ponds

(Fig 3A, farm#1). From WFS-pond#3 (farm #3), EHP was detected in all 18 individual shrimp hepatopancreas, one hindgut sample, and the white feces sample (Fig 3B).

***Per os* feeding bioassay**

To determine if EHP can be transmitted to healthy *P. vannamei* by oral ingestions, the specific pathogen free (SPF) shrimp were fed with minced EHP-infected tissue for 2 days and then maintained on a pelletized ration for an additional 22 days. Shrimp were sampled at various time points, followed by EHP PCR analyses. The results showed that EHP was not detected in samples collected on day 5 and day10 post feeding. EHP was detected in shrimp collected on day 15 and afterwards (Fig 4A). The EHP challenge did not cause any mortality in these shrimp. Histological analysis showed 3 shrimp collected at day 24 were infected by EHP, as basophilic inclusion bodies and mature spores were found in their hepatopancreas tissues (data not shown).

Coinhibition bioassay

In a 90-day bioassay, we coinhibited a group of SPF shrimp with a group of EHP-infected shrimp to determine if EHP would be transmitted to the healthy shrimp. Shrimp and fecal samples from the SPF group were collected on days 35, 42, and 90 after the two groups were coinhibited. These samples were analyzed by PCR, and the results showed that EHP was detected in both hepatopancreas tissues and fecal samples of the SPF group sampled at day 35 and from that day on (Fig 4B).

Fumagilin-B treatment

To determine if the fumagillin, a chemical used to control microsporidia, *Nosema* sp., infection in honey bees, can be used to treat EHP, we applied Fumagillin-B in the EHP-infected *P. vannamei* for one month, then sampled for PCR analyses. The results showed that the level of EHP was not reduced in the Fumagillin-B treated shrimp comparing with the control groups (Fig

4C). On the contrary, the infection levels appeared increased in the treated groups based on the stronger band intensities seen.

Discussion

White feces syndrome has caused significant economic losses to shrimp farmers, because affected populations exhibit elevated food conversion ratios (FCR), growth retardation, and highly variable sizes of individual shrimp at harvest. We performed a direct analysis of the samples of white feces and found that they are not true feces. The white feces are composed, almost completely, of massive quantities of EHP spores, gut mucus, remnants of sloughed tissues from hepatopancreas tubules infected with EHP and rod-shaped bacteria (likely *Vibrio* spp.). It is likely the white feces syndrome is resulted from severe EHP infection, that EHP was rapidly multiplying in hepatopancreatic tubule epithelium cells, then the degenerated epithelial cells and the large numbers of EHP spores were released into the hindgut.

All of the shrimp sampled from the ponds with white feces syndrome tested positive for EHP, suggesting that EHP is highly infectious. Our *per os* and coinhibition bioassays showed that EHP transmission did not require the involvement of a vector as seen in some microsporidia such as *Thelohania duorara* (Roth and Iversen, 1971) or *Perezia* sp. (unpublished data) that cause cotton shrimp disease. In ponds, EHP can readily be transmitted among individuals through coinhibition and as well as through healthy shrimp cannibalizing those that were moribund or dead. Our findings are consistent with the report by Tangprasittipap, et al. (2013) that EHP can be transmitted horizontally among shrimp in rearing ponds.

EHP, at the time an un-named, new species, was first reported in farmed shrimp in 2004 (Chayaburakul, et al., 2004). This initial report involved infections of populations of black tiger

shrimp *Penaeus monodon* cultured in Thailand. Later, this parasite was named as EHP (Tourtip et al., 2009). We found this parasite in the blue shrimp *P. stylirostris* cultured in Brunei during 2006 (Tang et al., 2015). At that time, the effects of EHP were largely ignored because EHP did not constitute a major threat to shrimp production, although it was thought to cause a reduction in growth of infected populations. Recently, severe EHP infections in shrimp farms have become common, and these infections are resulting in substantial economic losses to producers. Our findings of massive numbers of EHP spores in the "white feces" of WFS-affected ponds, not in the normal feces from the non-WFS ponds, suggest that EHP is associated with WFS. It appears that EHP has become more virulent over the last 12 years (2004 to present). As the spores can persist in the environment and, in high stocking density shrimp ponds, hosts are readily available, virulent strains would likely increase as there would be no selection pressure against increased virulence (Dunn and Smith, 2001). In addition, *P. vannamei* may be a new host for the EHP, and the shrimp stocks are supplied from clean broodstock in hatcheries, rather than from the reproduction of survivors from EHP infection, which results in a lack of opportunity for hosts to evolve resistance to this parasite.

EHP is a microsporidian parasite. Microsporidia are intracellular parasites, related to fungi, those infect a wide variety of vertebrate and invertebrate hosts (Wittner and Weiss 1999). A large proportion of microsporidian species infect aquatic hosts, such as crustaceans and fishes (Stentiford et al., 2013). The cause of the recent emergence of EHP as a pathogen of concern in the global shrimp farming industry is not known, although microsporidiosis, in general, is often linked with environmental stress or population immune suppression associated with inbreeding, that these factors may have played a role (Doyle 2016; Stentiford et al., 2015). However, it is unlikely that either of these factors, especially given that shrimp have only

a rudimentary immune system, would simultaneously affect a large number of production facilities over a wide geographic range, and which receive post-larvae from a variety of sources. Another possibility is that genetic changes allowed new strains of the parasite not only to infect *Penaeus vannamei*, but to also do well in shrimp ponds, where there is both a high density of hosts and the probability of serial infections of individuals is high.

WFS was reported to be associated with aggregated, transformed, microvilli (ATM) resembling gregarines in the *P. vannamei* cultured in Thailand (Sriurairatana et al., 2014). ATM is not a gregarine and was never claimed to be by Sriurairatana et al. (2014). In spite of this, their study has led to discussions in several publications concerning associations of gregarines with WFS (e.g. Mastan, 2015; Chaweepack, et al., 2015; Srinivas, et al., 2016). The report (Sriurairatana et al.), although providing no claim of the involvement of gregarines, has seemingly motivated shrimp diseases researchers to look in vain for gregarines in WFS-affected shrimp. Gregarines, and in particular the *Nematopsis* spp., *Cephalolobus* spp. and *Paraophioidina* spp., have been found in many cultured populations of penaeid shrimp around the world for some time (Lightner 1996), infestations of gregarines in farmed shrimp populations have never been shown to have any connection with WFS. Therefore, there is absolutely no evidence of any association of gregarines with WFS.

There is a need to develop control measures to address the WFS problems in farmed shrimp populations. Fumagillin inhibits reproduction of the microsporidia spores, so it has been widely used in apiculture to control microsporidium, *Nosema* sp. (Katznelson and Jamieson, 1952; Bailey 1953). However, based on the shrimp bioassay and PCR analyses, it was found to be non-effective for suppressing EHP infection in shrimp. Some shrimp farmers are employing methods that they have come up with on their own. For example, we have found through

discussions with local producers, those in Indonesia, farmers have two main strategies: 1) attempting to reduce *Vibrio* spp. populations in the ponds through the frequent addition of probiotics such as *Bacillus* spp. or *Lactobacillus* spp. to the water; and 2) attempting to reduce pathogens in the shrimp digestive system through the use of feed additives. The feed additives being tried include: garlic, in the forms of freshly crushed or processed powders (10-30 g/Kg feed); Allicin (a major active component of garlic); vitamin C (2 g/Kg feed); and antiprotozoals, such as metronidazole. We recommend removal the white feces from the affected shrimp ponds, the white feces contain large quantities of EHP and as they can break down and sink to the pond bottom. The associated EHP can be ingested by shrimp, results in re-infection, and ultimately will increase the severity of the infection.

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Fig 1. H&E (Mayer-Bennet haematoxylin and eosin-phloxine) histology and *Enterocytozoon hepatopenaei* (EHP) in situ hybridization of *Penaeus vannamei* fecal samples. (A) H&E staining of white fecal samples collected from a white feces syndrome affected pond, (B) under a higher magnification; (C) in situ hybridization of the consecutive section with a digoxigen-labeled EHP probe, the presence of dark blue precipitates indicates the presence of EHP; (D) H&E staining of degenerated hepatopancreatic tubules and mature spores, (E) H&E staining of rod-shaped bacteria; (F) H&E staining of fecal samples from healthy shrimp, (G) EHP in situ hybridization of the fecal samples from healthy shrimp (a consecutive section). Scale bars = 25 μ m.

Fig 2. H&E histology and EHP in situ hybridization of hepatopancreas samples collected from *P. vannamei* exhibiting white feces syndrome. (A) H&E staining of hepatopancreas tissue, (B) under a higher magnification. Arrows indicate the inclusions, stars indicate the mature spores; (C) in situ hybridization of the consecutive section with a digoxigen-labeled EHP probe; (D) H&E staining of hepatopancreas displaying septic hepatopancreatic necrosis (SHPN), basophilic bacterial plaque colonized the lumen of the necrotic, inflamed, tubules; (E) H&E staining of midgut epithelial cells showing EHP inclusions, (F) in situ hybridization with EHP probe to the consecutive section. Scale bars = 25 μ m.

Fig 3. PCR detection of EHP in hepatopancreas and fecal samples. (A) PCR analyses for hepatopancreas (lane 1) and fecal samples (lane 2) from a healthy, non-WFS, pond. Hepatopancreas (lane 3) and fecal samples (lane 4) collected from a WFS-pond. (B) PCR analyses of 18 individual hepatopancreas, one hindgut (HG, from shrimp #3), and one fecal sample (F) collected from a WFS-pond. M: 1 kb plus ladder molecular weight marker. HP:

396 hepatopancreas. F: fecal sample. SPF: DNA from specific pathogen free (SPF) *P. vannamei*,
397 NTC: none-template control, +C: positive control.

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399 Fig 4. PCR detection of EHP in the hepatopancreas samples from laboratory infections. (A) *Per*
400 *os* feeding, lanes 1-3 represent days 15, 20 and 24 after feeding with EHP-hepatopancreas. (B)
401 Coinhibition bioassay, lanes 1-3 represent days 35, 42 and 90 after coinhibited specific
402 pathogen free shrimp with EHP-infected shrimp, lane 4: fecal sample (F) collected at day 35. (C)
403 Fumagillin-B treatment, lanes 1 & 3: EHP-infected shrimp were fed Fumagillin-B-mixed shrimp
404 feed (FTx: Fumagillin-treated), lanes 2&4: EHP-infected shrimp were fed Rangen 35% shrimp
405 feed (NT: not treated). M: 1 kb plus ladder molecular weight marker; SPF: specific pathogen free
406 shrimp DNA control; NTC: non-template control; +C: positive control.

Fig 1

Fig 2

Fig 3

Fig 4