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Identification of *Enterocytozoon hepatopenaei* in Mud Crabs (*Scylla serrata*) Co-infected with White Spot Syndrome Virus in Taiwan

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ABSTRACT

Background: The high mortality rate and stunted growth of marine shrimp in the farm have raised concerns about the outbreak of co-infection involving *Enterocytozoon hepatopenaei* (EHP) and white spot syndrome virus (WSSV), which could lead to significant economic loss. In a mixed-culture farm consisting of *Scylla serrata* and *Peneaus vannamei* located in Yunlin Country, Taiwan, were reported mortality cases on culture animals.

Methods: Based on clinical signs, histology and molecular analyses, the disease was diagnosed in this study. In the histopathology examination, infected organs, such as the carapace and gills, were stained with hematoxylin and eosin (H and E), then observed under microscope. For the molecular analysis, *Sal*I DNA fragment (WSSV) and spore wall protein (SWP) gene (EHP) were targeted to detected the pathogen in animal host.

Result: The cumulative mortality data for this co-infection indicates that *S. serrata* (1.5% mortality) exhibits higher susceptibility compared to *P. vannamei* (>99% mortality). Phylogenetic tree analysis reveals that the spore wall protein (SWP) gene of EHP and the *Sal*I DNA fragment of WSSV strain extracted from *S. serrata* are genetically similar to those extracted from *Peneaus* spp., indicating the potential for cross-species infection. Histopathological examination of lesion tissue observed the presence of WSSV basophilic intranuclear inclusion bodies in the carapace tissue and gill fragments of *S. serrata*. The source of the disease outbreak is highly suspected to be the feed used, which consisted of raw shrimp and fish. This study provides the first documented case of natural EHP infection with co-infection of WSSV in *S. serrata*.

Key words: *Enterocytozoon hepatopenaei*, Marine mud crab, Natural infection, White spot syndrome virus.

INTRODUCTION

Mud crab (*Scylla* sp.), also known as mangrove crabs, are valuable in aquaculture and fishing across the Indo-Pacific (Ma *et al*., 2010; Petersen *et al*., 2016). Among *Scylla* species, Scylla serrata has the widest global distribution (Keenan *et al*., 1998). According to global statistics from Volza (2022), the top three importers of *S. serrata* are China, Thailand and Taiwan. Due to the high demand, the farming of *S. serrata* has become a mainstream practice in crab culture. However, a major obstacle to mud crab production is the spread of disease in large-scale, high-density farms (Parenrengi *et al*., 2022).

Recent studies found that white spot syndrome virus (WSSV) causing significant economic loss in *Scylla* species cultures (*S. serrata*, *S. paramamosain* and *S. tranquebarica*) (Chen *et al*., 2000; Gopalakrishnam *et al*., 2011; Diggles *et al*., 2020; Lai *et al*., 2020). Unfortunately, WSSV infection does not exhibit any clinical symptoms in the external morphology of mud crabs (Raja *et al*., 2015); instead, only white spots on the inner surface of the carapace (Gopalakrishnam *et al*., 2011; Raja *et al*., 2015; Diggles *et al*., 2020). This hidden disease causes significant mortality, posing challenges in crustacean aquaculture. Studies on WSSV in *S. serrata* have reported varying mortality rates: 40-43% with the immersion method (Chen *et al*., 2000; Gunasekaran *et al*., 2018), 90% with the injection method (Sahul Hameed *et al*., 2003) and and 100% with the ingestion method (Gunasekaran *et al*., 2018). Infected mud crabs exhibit histological changes, including round foci in the exocuticle and endocuticle,

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hypertrophied nuclei in tissues (gill, hepatopancreas and heart) and cytoplasmic granules in the hepatopancreas (Chen *et al.*, 2000; Gunasekaran *et al.*, 2018; Diggles *et al.*, 2020). In addition to being used for WSSV early detection (Gunasekaran *et al.*, 2018; Diggles *et al.*, 2020), molecular analysis is also crucial for the development of antiviral products aimed at combating WSSV in mud crab production (Wang *et al.*, 2017; Lin *et al.*, 2020; Hu *et al.*, 2021).

In addition to WSSV, *Enterocytozoon hepatopenaei* (EHP), the causative agent of hepatopancreatic microsporidiosis, has emerged as a parasitic disease affecting shrimp culture in several Asian countries, including China (Hou *et al.*, 2021), India (Behera *et al.*, 2019), Indonesia (Caro *et al.*, 2020), Taiwan (Lee *et al.*, 2022), Korea (Kim *et al.*, 2021) and Vietnam (Khiem *et al.*, 2022). The first characterized *E. hepatopenaei* was found in *Penaeus monodon* from Thailand in 2009 (Tourtip *et al.*, 2009) and later found in other *Penaeus* species (*P. japonicus* and *P. vannamei*) (Hudson *et al.*, 2001; Tangprasittipap *et al.*, 2013). Infected shrimp exhibited stunted growth and increased mortality (Rajendran *et al.*, 2016; Tang *et al.*, 2017). According to Chaijarasphong *et al.* (2021), the life cycle of EHP could be direct and simple or involve complex transmission. In the direct cycle, EHP duplicates and matures in the host and mature spores are released through host cell rupture, infecting neighboring cells or the environment via feces (Tourtip *et al.*, 2009). In the complex cycle, various hosts are involved and EHP is transmitted through the food web to the shrimp host, often due to biosecurity lapses like providing live feed (Flegel, 2012; Sritunyalucksana *et al.*, 2014). Infected shrimp ponds exhibit white feces with basophilic spores under histological examination (Tang *et al.*, 2017). Histopathological findings in shrimp show enlarged epithelial cells filled with EHP spores and basophilic inclusions within the cytoplasm, primarily in the hepatopancreas (Aranguren *et al.*, 2017; Rajendran *et al.*, 2016; Tang *et al.*, 2017). Although no natural infection of *E. hepatopenaei* in *Scylla* species has been reported, an experimental study in mud crabs showed similar histological changes in the shrimp host but no mortality (Mani *et al.*, 2022). However, However, EHP disease may weaken the host's immune system, making it susceptible to secondary bacterial or viral infections (Rajendran *et al.*, 2016). Co-infections of EHP and WSSV have been reported in Indian shrimp farming, resulting in high mortality (100%) (Thamizhvanan *et al.*, 2018; Krishnan *et al.*, 2021; Suryakodi *et al.*, 2022) and significant economic losses for farmers (Patil *et al.*, 2021).

Given the high demand for mud crabs in the global market, early prediction and detection of disease outbreaks are vital to mitigate aquaculture risks. Therefore, a study was conducted using diseased *S. serrata* mud crab obtained from a Taiwanese aquaculture farm. The study employed histopathological and molecular tests to pinpoint the causative agent, marking the first molecular detection of a naturally occurring EHP infection in *S. serrata*.

MATERIALS AND METHODS

A sample was taken from diseased animals at a mixed-culture farm in Yunlin County, Taiwan, with a stocking density of 30,000 pc/pond for both *S. serrata* and *Penaeus vannamei*. The disease outbreak occurred when the *S. serrata* were 5 months old, resulting in minor mortality (150 crabs/day) with a cumulative mortality of 1.5%. However, there was a significant die-off of over 99% of *P. vannamei* within 3 days. A total of 5 diseased *S. serrata* and 3 *P. vannamei* were sampled and their body length and weight are listed in Table 1. Both internal and external disease symptoms were documented.

The gills, hepatopancreas and other organs with lesions were fixed in 10% buffered formalin. After the paraffin sectioning, the organs were stained with hematoxylin and eosin (H and E). The stained samples were then observed under the electronic light microscope and significant disease morphology was captured and presented as figures.

The gills, hepatopancreas and other organs with lesions were preserved in 10% buffered formalin. Subsequently, these organs were subjected to paraffin sectioning and stained with hematoxylin and eosin (H and E). The stained samples were examined using an electronic light microscope and noteworthy disease-related morphology was documented and illustrated as figures.

DNA extraction from *S. serrata* involved muscle and hepatopancreas tissues and the manufacturer's protocol, along with Cheng *et al.* (2022), was followed. The extracted DNA was then employed in separate polymerase chain reactions using specific primers for the detection of white spot virus (WSSV) and *Enterocytozoon hepatopenaei* (EHP) separately: *Sal*I DNA fragment (WSSV) and spore wall protein (SWP) gene (EHP).

In the case of WSSV, the nested-PCR employed the primers 146-1 F/146-1 R and 146-2 F/146-2 R (Lo *et al.*, 1996). For EHP, the PCR utilized the primers SWP-1 F / SWP-1 R and SWP-2 F / SWP-2 R (Jaroenlak *et al.*, 2016).

The PCR products of WSSV and EHP were sent to Genomics Bioscience Technology Co Ltd. (Taipei, Taiwan) for genetic sequencing. Forward and reverse sequences of *Sal*I DNA fragment (WSSV) and spore wall protein (SWP) gene (EHP) were assembled by using Bioedit Alignment Sequence Editor Ver. 7.2. All sequence was aligned using Bioedit Alignment Sequence Editor Ver. 7.2. The Maximum likelihood polygenetic treeswere constructed by using MEGA 11 software by bootstrap analyses using 1000 resampling's. Retrieved sequences had listed in Supplementary Table 1.

RESULTS AND DISCUSSION

The body weight range of *S. serrata* was approximately 6.7-7.5 cm and 88-108 g, while *P. vannamei* showed an average body length of 9.0±0.5 cm and an average body weight of 9.7±1.5 g (Table 1).

Co-infection with WSSV and EHP have been reported in India, although they are uncommon, having a significant detrimental impact on shrimp farms, often leading to 100%

Supplementary Table 1: WSSV and *Enterocytozoon hepatopenaei* (EHP) strains used in this study.

Species	Assessment code	Host	Country
WSSV	C1-M*	*Scylla serrata*	Taiwan
WSSV	C2-M*	*Scylla serrata*	Taiwan
WSSV	C3-M*	*Scylla serrata*	Taiwan
WSSV	C4-HP*	*Scylla serrata*	Taiwan
WSSV	C5-M*	*Scylla serrata*	Taiwan
WSSV	MN840357	*Panaeus vannamei*	USA
WSSV	MH663976	*Procambarus clarkii*	China
WSSV	KU216744	*Litopenaeus vannamei*	Mexico
WSSV	MG432482	*Penaeus vannamei*	Mexico
WSSV	KX686117	*Procambarus clarkii*	China
WSSV	KY827813	*Marsupenaeus japonicus*	China
WSSV	AF332093	*Penaeus japonicus*	China
WSSV	AF369029	*Penaeus monodon*	Thailand
WSSV	AF440570	*Penaeus monodon*	Taiwan
WSSV	MG702567	*Penaeus vannamei*	India
Metopaulias depressus WSSV-like virus	KR820241	*Metopaulias depressus*	Jamaica
Enterocytozoon hepatopenaei	C1-HP*	*Scylla serrata*	Taiwan
Enterocytozoon hepatopenaei	C1-M*	*Scylla serrata*	Taiwan
Enterocytozoon hepatopenaei	C2-HP*	*Scylla serrata*	Taiwan
Enterocytozoon hepatopenaei	C2-M*	*Scylla serrata*	Taiwan
Enterocytozoon hepatopenaei	C3-HP*	*Scylla serrata*	Taiwan
Enterocytozoon hepatopenaei	C3-M*	*Scylla serrata*	Taiwan
Enterocytozoon hepatopenaei	C4-HP*	*Scylla serrata*	Taiwan
Enterocytozoon hepatopenaei	C4-M*	*Scylla serrata*	Taiwan
Enterocytozoon hepatopenaei	C5-HP*	*Scylla serrata*	Taiwan
Enterocytozoon hepatopenaei	MW000458	*Penaeus vannamei*	Malaysia
Enterocytozoon hepatopenaei	KX258197	*Penaeus vannamei*	Thailand
Enterocytozoon hepatopenaei	MG015710	*Litopenaeus vannamei*	Thailand
Enterocytozoon hepatopenaei	KY483639	*Litopenaeus vannamei*	India
Enterocytozoon hepatopenaei	MW269619	*Macrobrachium rosenbergii*	China
Enterocytozoon hepatopenaei	MZ541056	*Penaeus vannamei*	South Korea
Enterocytozoon hepatopenaei	MW000459	*Penaeus vannamei*	Malaysia
Enterocytozoon hepatopenaei	MW000460	*Penaeus vannamei*	Malaysia
Enterocytozoon hepatopenaei	KY593133	*Penaeus vannamei*	Indonesia
Enterocytozoon hepatopenaei	KY674357	*Penaeus vannamei*	India
Spraguea lophii	ATCN01000405	*Lophius piscatorius*	United Kingdom

*represent strains from current study.

Table 1: The body length (cm) and body weight (g) of *Scylla serrata* and *Penaeus vannamei.*

Diseased animal	Sample code	Body length (cm)	Body weight (g)
Scylla serrata	C1	7.0	100
Scylla serrata	C2	7.0	90
Scylla serrata	C3	6.8	89
Scylla serrata	C4	7.5	108
Scylla serrata	C5	6.7	88
	Average	**7.0±0.3**	**95±8.7**
Penaeus vannamei	P1	9.5	11
Penaeus vannamei	P2	9.0	10
Penaeus vannamei	P3	8.5	8
	Average	**9.0±0.5**	**9.7±1.5**

mortality (Patil *et al.*, 2021; Suryakodi *et al.*, 2022). These diseases are challenging to detect during the early stages of infection in cultured animals due to the absence of significant clinical signs (Raja *et al.*, 2015; Rajendran *et al.*, 2016; Tang *et al.*, 2017). In the present study, the external appearance of *S. serrata* showed no signs of infection, such as discoloration or deformities. The internal organs appeared normal without any lesions. However, multiple white spots were observed on the internal carapace (Fig 1). These white spots were also present on the cephalothorax of *P. vannamei* from the same farm. Similar signs of infection have been reported in *S. serrata* infected with WSSV in previous studies (Gopalakrishnam *et al.* 2011; Raja *et al.*, 2015; Diggles *et al.*, 2020).

However, our histopathological analysis only revealed WSSV infection in the mud crab and the prevalence of EHP infection was undetectable. Only one research study has reported histopathological evidence of EHP infection in marine crabs, which illustrated the presence of EHP's spores and plasmodia in the lesion tissue (Mani *et al.*, 2022). Previous studies have investigated the occurrence of EHP infection due to biosecurity lapses, including the use of live feed or unprocessed raw shrimp or fish in shrimp farms (Flegel, 2012; Sritunyalucksana *et al.*, 2014). Mani *et al.* (2022) created artificial EHP infection through oral ingestion and EHP inoculum injection, discovering EHP infection in *S. serrata* from contaminated food sources. This finding aligns with the sources of EHP in our study, as farmers provided *S. serrata* with raw, unprocessed wild prawns and fish.

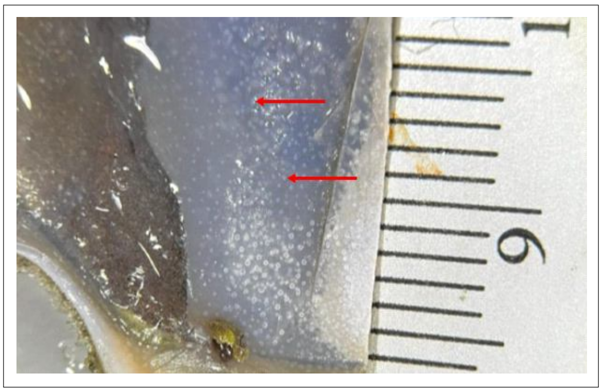

Fig 1: White spots observed on the inner carapace of *S. serrata*. The red arrow points to the white spot.

Therefore, early detection of WSSV heavily relies on molecular examination (Gunasekaran *et al.*, 2018; Diggles *et al.*, 2020). Using PCR tests and specific primers, the infection of *S. serrata* with WSSV and EHP was demonstrated in the current work. The *Sal*I DNA fragment gene of WSSV obtained in this study exhibited 100% similarity with all WSSV reference strains and 75.6% similarity with the outgroup strain (Metopaulias depressus WSSV-like virus). Given that the reference strains were isolated from the Penaeus family, the WSSV strains in our study were genetically related to those from the Penaeus family (Fig 2B). Moreover, all EHP strains showed high similarity (99.3-100%) with the SWP gene. The phylogenetic tree constructed using MEGA 11 software revealed that the EHP strain from our study was closely related to EHP strains from China (MW269619), Malaysia (MW000458) and Thailand (KX258197 and MG015710), despite being extracted from a different host (Fig 2A). The current WSSV and EHP strains appeared to be closely related to strains discovered in *Penaeus* spp. hosts on the phylogenetic trees (Fig 2), suggesting a common ancestor or the potential to cause disease.

Furthermore, the close resemblance of the EHP-C3-M strain to MV269619 from the freshwater host *Macrobrachium resenbergii* (Fig 2A) suggests the potential for this strain to infect both freshwater and marine crustacean hosts. Notably, EHP infections can occur in a wide range of salinities, spanning from 2 ppt to 30 ppt (Aranguren *et al.*, 2021). Thus, to better control EHP infections in aquaculture farms, it would be beneficial to

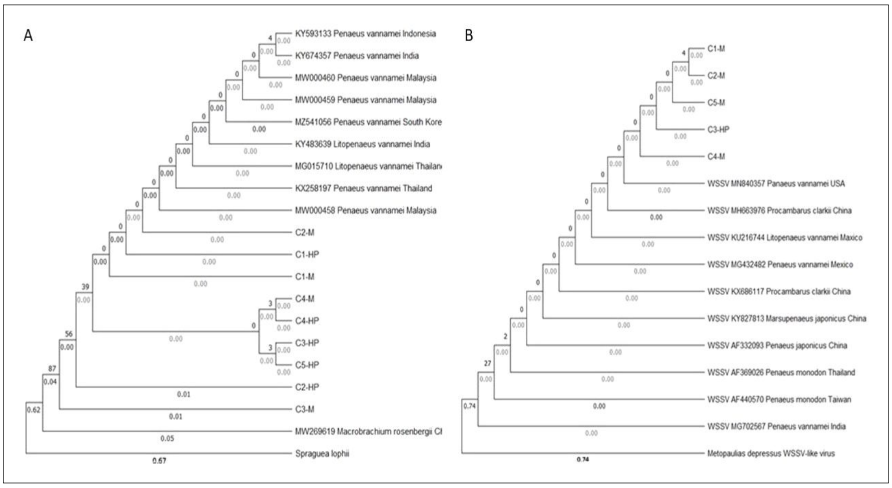

Fig 2: The maximum likelihood phylogenetic tree of the nucleotide sequence of SWP gene in *Enterocytozoon hepatopenaei* (A) and *Sal*I DNA fragment gene in WSSV (B) generated using the Kimura 2-parameter model of the MEGA 11 software. Metopaulias depressus WSSV-like virus was used as the outgroup.

conduct research on the susceptibility and pathogenicity of EHP strains under various environmental conditions.

Experimental studies of EHP infection in marine crabs did not result in mortality; instead, the replication of EHP spores was inhibited (Mani *et al.*, 2022). Limitation of case reports on natural EHP infection in marine crabs limits reference data for mortality comparisons. In shrimp culture, no significant mortality has been reported, but there is evidence of stunted growth (Rajendran *et al.*, 2016; Tang *et al.*, 2017). This suggests that the mortality of *S. serrata* and *P. vannamei* in the current study was possibly caused by WSSV infection. Furthermore, Rajendran *et al.* (2016) stated the suppression of the host's immune system by EHP infection, makes it more vulnerable to other infections, which can lead to higher mortality in the host population. This phenomenon is demonstrated by the co-infection of EHP and WSSV in *P. vannamei* (resulting in >99% mortality in 3 days) in the current study, but not in *S. serrata* (resulting in 1.5% mortality in 3 days). This indicates that the susceptibility and pathogenicity of EHP and WSSV strains in the current study are higher in *P. vannamei* and lower in *S. serrata*, which correlates with the inhibition of EHP spore replication in the marine crab, as reported (Mani *et al.*, 2022). Even though this was the first instance of EHP and WSSV co-infection in *S. serrata* farm with a mortality rate, the stunted growth of *S. serrata* lengthens the culture period and results in higher treatment and management costs (Patil *et al.*, 2021). Therefore, farmers should implement biosecurity procedures, such as pond disinfection with lime after cultivation and freezing the raw feed for 48 hours before feeding. Local departments should collaborate with scientific experts to monitor the spread of farm diseases and assist farmers in enhancing disease management.

CONCLUSION

This study represents the first documented natural infection of EHP in *S. serata* co-infected with WSSV, as determined by molecular analysis. Basophilic intranuclear inclusion bodies of WSSV were observed in the carapace and gill fragment, but no signs of EHP infection were found in the histopathology study. *S. serata* was observed to be more susceptible to infection with WSSV and EHP than *P. vannmei*. However, the pathogenic impact was manifested solely as stunted growth. Prolonging the culture period would result in higher management costs and an increased risk of contamination within the cultured species, potentially affecting nearby farms. Therefore, it is imperative to implement a robust biosecurity plan to enhance aquaculture practices and disease management.

Conflict of interest: None.

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