Journal of Invertebrate Pathology 200 (2023) 107968

Contents lists available at ScienceDirect

Journal of Invertebrate Pathology

journal homepage: www.elsevier.com/locate/jip

Detection of a novel microsporidium with intranuclear localization in farmed *Penaeus vannamei* from Latin America

Arun K. Dhar [a,*], Roberto Cruz-Flores [a,b], Hung N. Mai [a], Luis Fernando Aranguren Caro [a], Pablo Intriago [c], Xavier Romero [c]

[a] Aquaculture Pathology Laboratory, School of Animal & Comparative Biomedical Sciences, The University of Arizona, Tucson, AZ, USA
[b] Centro de Investigación Científica y de Educación Superior de Ensenada, (CICESE), Carretera Ensenada-Tijuana No. 3918, Zona Playitas, 22860 Ensenada, Baja California, Mexico
[c] South Florida Farming Corp, Southwest Ranches, FL, USA

ARTICLE INFO

Keywords:
Enterospora
Penaeus vannamei
Nuclear microsporidium

ABSTRACT

Microsporidia are emerging intracellular parasites of most known animal phyla in all ecological niches. In shrimp aquaculture, the microsporidium *Enterocytozoon hepatopenaei* (EHP) is a major cause of concern inflicting tremendous losses to shrimp producers in southeast Asia. During a histopathological examination of *Penaeus vannamei* samples originating in a country from Latin America presenting slow growth, we observed abnormal nuclei in the epithelial cells of the hepatopancreas. A PCR screening of the samples using DNA isolated from paraffin embedded tissues for the SSU rRNA gene of EHP provided a 149 bp amplicon. *In situ* hybridization using the SSU rRNA gene probe provided a positive signal in the nuclei instead of the cytoplasm. Sequence analysis of the SSU rRNA gene product revealed a 91.3 %, 89.2 % and 85.4 % sequence identity to *Enterocytozoon bieneusi, E. hepatopenaei* and *Enterospora canceri* respectively. Furthermore, phylogenetic analysis revealed the newly discovered microsporidium clustered with *E. bieneusi*. Considering the intranuclear location of the novel microsporidium and the differences in the sequence of the SSU rRNA, we tentatively consider this parasite a new member of the genus *Enterospora* sp. The pathogenicity and distribution of the shrimp *Enterospora* sp. are currently unknown. Our future efforts are focused on the characterization and development of diagnostic tools for this parasite to understand if it acts as an emergent pathogen that might require surveillance to prevent its spread.

1. Introduction

Since the formal description of *Enterocytozoon hepatopenaei* (EHP) by Tourtip et al., (2009), the studies on microsporidia that affect shrimp have increased significantly. The heavy losses attributed to hepatopancreatic microsporidiosis (HPM) caused by EHP and the association of this microsporidium to other gastrointestinal disorders like white feces syndrome (WFS) highlight the extensive negative impact of this pathogen and other related pathogens on shrimp culture in Latin America and elsewhere in the world (Aranguren Caro et al., 2020; Chaijarasphong et al., 2020; Sathish Kumar et al., 2022). To prevent the spread and mitigate losses due to EHP, histopathology and molecular diagnostic tools have been developed that are both sensitive and specific.

The detection of EHP by light microscopy can be easily performed by a trained shrimp pathologist. However, due to the preparation time this approach is not recommended for routine diagnostic purposes (Chaijarasphong et al., 2020). For many of the shrimp diseases enlisted by the World Organization for Animal Health (WOAH, Paris, France), PCR is considered as the gold standard for diagnosis (OIE, 2019, 2018). Recently, EHP has been listed as an emerging disease in the WOAH list of crustacean diseases and it is likely that PCR would be one of the recommended method of choice for a rapid identification of this parasite should the disease is listed in the near future. Currently, several PCR methodologies are available for the detection of EHP including one-step PCR (Tang et al., 2015; Tourtip et al., 2009), nested PCR (Jaroenlak et al., 2016; Tangprasittipap et al., 2013; Han et al., 2018), qPCR (Liu et al., 2018; Piamsomboon et al., 2019), and recombinase polymerase amplification combined with later flow dipstick (Pang et al., 2022). The PCR diagnostics based on the SSU-rRNA were widely used for EHP

* Corresponding author.
E-mail address: adhar@arizona.edu (A.K. Dhar).

https://doi.org/10.1016/j.jip.2023.107968
Received 24 December 2022; Received in revised form 30 June 2023; Accepted 5 July 2023
Available online 8 July 2023

detection until it was revealed that they show substantial cross-reactivity with closely related microsporidians. Subsequently, additional methodologies were developed targeting genes with higher sequence diversity (e.g. spore wall protein gene) to avoid false positives (Jaroenlak et al., 2016).

It was previously shown by Jaroenlak et al., (2016) that SSU-rRNA PCR based method for the detection of EHP non-specifically reacted with crab microsporidia, namely *Enterospora canceri* and *Hepatospora ericheir*. *Enterospora* spp. are interesting parasites, and until now they are the only known microsporidia that are parasitic within the nuclei in invertebrates (Stentiford et al., 2007). The *Enterospora* genus, to date, contain only two species *E. canceri* and *Enterospora* spp. which are a parasites of *C. pagurus* and *E. bernhardus,* respectively. The prevalence of these parasites is low and neither has been assessed as mortality drivers in farmed or wild crustacean populations (Stentiford et al., 2007). On the other hand, all other know microsporidia within the Enterocytzoonidae that infect the nuclei are fish parasites (Stentiford et al., 2007; Stentiford and Bateman, 2007). Recent exploration of parasites that infect crustaceans of commercial and ecological interest has shed light on the diversity of these fascinating intracellular parasite. Two new hepatopancreas infecting Enterocytozoonida, *Pseudohepatospora borealis* and *Parahepatospora carcini*, have been recently described from crabs of fishery and ecological interest (Bojko et al., 2023, 2017). These studies show our limited knowledge of members of the Enterocytozoonidae that might require control due to the negative effects inflicted on the host.

In this study, during a health survey of cultured *Penaeus vannamei* from Latin America using routine histology we identified unusual inclusion bodies and abnormal nuclei in the epithelial cells in the hepatopancreases. Additional assays including *in situ* hybridization (ISH) test revealed the presence of the microsporidium within the nucleus. To our knowledge, this is the first microsporidium with intranuclear localization in penaeid shrimp.

2. Materials and methods

2.1. Histopathology

Six adult shrimp (*Penaeus vannamei,* three males and three females) samples fixed in Davidson's alcohol-formalin-acetic acid (AFA) preservative (X1, X2, X3, X4, X5 and X6) were sent to Aquaculture Pathology Laboratory for screening by routine histopathology as a part of disease surveillance. The samples originated in a shrimp farm in Latin America where the animals experienced unusual slow growth in a grow-out pond. The samples were processed for H&E histology following a standard procedure (Bell and Lightner, 1988). Briefly, after fixation, Davidsoń s fixative was removed and replaced with an equal volume of 70 % ethanol. For histological processing, samples were washed in a series of alcohol/xylene solutions, embedded in paraffin, section at around 4 μm and stained with H&E following standard procedures (Bell and Lightner, 1988). Histological slides were examined using a bright field light microscope.

2.2. Nucleic acid extraction from Davidsoń s-fixed paraffin embedded tissues

DNA was extracted from three paraffin embedded *P. vannamei* tissue samples (X3, X4 and X5) using a commercial kit FFPE DNA Purification Kit (NORGEN BIOTEK CORP) in accordance with the manufacturer's recommendations with the modifications described by Cruz-Flores et al., (2020). Briefly, during the deparaffinization step, the xylene washes were doubled, and the pellet was air dried for 20 min. Finally, during the lysate preparation step, the incubation at 90 °C was increased from 1 h to 1 h 15 min. The DNA was quantified using a NanoDrop™ 2000/2000c Spectrophotomer before taking for the PCR analysis.

2.3. Real-time PCR, conventional PCR, cloning and sequence analysis

Genomic DNA isolated from three paraffin embedded *P. vannamei* tissue samples (X3, X4 and X5) were taken for the detection of EHP using a real-time PCR method. A TaqMan™ Fast Virus 1-Step Master Mix (Applied Biosystems™) was used for the detection of the parasite using the primers and probe (F157: 5-AGT AAA CTA TGC CGA CAA-3′ and R157: 5-AAT TAA GCA GCA CAA TCC-3′ and a TaqMan probe 5′-FAM-TCC TGG TAG TGT CCT TCC GT-TAMRA-3′) and following a published method (Liu et al., 2018). In order to clone the amplicon, the protocol of Liu et al., (2018) was adapted for a conventional PCR using the same primers. The conventional PCR amplification was carried out in a total volume of 25 μl containing 1 μl of template DNA (50–100 ng/μl), 12.5 μl of DreamTaq™ Hot Start Green PCR Master Mix (ThermoFisher) and 350 nM of each of forward and reverse primers. The PCR conditions consisted of an initial denaturation at 95 °C for 2 min, followed by 40 cycles at 95 °C for 10 s, 60 °C for 10 s and 72 °C for 10 s with a final elongation step at 72 °C for 2 min. The PCR products were run on a 2 % agarose gel and were visualized on a GelDoc™ XR+ (Bio-Rad). The amplified fragment was cloned into the pDrive Cloning Vector (QIAGEN®). The recombinant plasmid was sequenced using a Sanger sequencing method at the University of Arizona Genetics Core. Sequence analysis was performed with BLASTn and alignments were performed in Geneious Prime using the Geneious Aligner (Altschul et al., 1990; Kearse et al., 2012).

2.4. In situ hybridization (ISH)

All paraffin embedded tissue sections were processed as described previously in section 2.1. However, for ISH, sections were dried on to a positively charged microscopic slides and ISH was carried out following a protocol described by Cruz-flores et al., (2019) with an equivolume mixture of two primers, EHP 510F and EHP 510 R (Tang et al., 2015). These primers were tailed at 3′-end with digoxigenin-11-dUTP (Sigma-Aldrich™). After deparaffinization, hydration, proteinase K digestion, and pre-hybridization, the sections were overlaid with 500 μl of hybridization solution containing DIG-labeled primers (100 fmol). The slides were placed on a heated surface at 90 °C for 10 min and hybridized overnight at 50 °C. Final detection was performed with an anti-digoxigenin antibody conjugated to alkaline phosphatase (Roche), which was visualized using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate [27]. A paraffin block containing previously known EHP-affected shrimp was used as positive control. As a negative control, paraffin blocks of specific pathogen free (SPF) shrimp were used. Slides were examined by light microscopy for the presence or absence EHP hybridizing with the DNA probe, and those slides that showed blue to purple precipitates were considered as positive.

2.5. Phylogenetic analysis

The SSU sequences representative of microsporidia infecting cytoplasm and nucleus of fish, crustacean and mammalian hosts were acquired from NCBI database, and used to develop a maximum likelihood phylogenetic tree. Multiple alignment was done by MAFFT (algorithm = auto; Scoring matrix = 200 PAM/k = 2; Gap open penalty = 1.53; Offset value = 0.123) and the alignment was trimmed using clipkit (kpic mode). The resulting alignment was then analyzed using a best-fit model using Jmodel (number of substitution = 40). MrBayes was employed to build a phylogenetic tree (substitution model = GTR + I + G; Chain length = 1,100,000 cycles; subsampling freq = 200).

3. Results

3.1. Histopathology and in situ hybridization

Histopathological examinations of H&E stained sections revealed

basophilic cytoplasmic inclusion bodies in the epithelial cells within the hepatopancreas tubule which are suggestive of early plasmodium stage of EHP, in four out of six shrimp examined. These lesions were categorized at severity grade of G1-G3 (Fig. 1 A-B). One sample contained no lesion in the hepatopancreas and in one sample both cytoplasmic and intranuclear inclusion bodies were observed within the R cells of hepatopancreas tubule (Fig. 1 C-D, Sample X3). Also, in one out of six shrimp samples examined, septic hepatopancreatic necrosis (SHPN) within the hepatopancreas tubule cells was observed. This was probably due to infection by opportunistic *Vibrio* sp. or other opportunistic bacterial species. The R-cell activity within the hepatopancreas, an indication of nutrient absorption, digestion and storage, were below the normal range in two out of six shrimp examined. None of the shrimp examined displayed any histological lesion that are considered as hallmarks of infection by any of the ten WOAH (Paris, France)-listed crustacean pathogens.

3.2. Real-time PCR, conventional PCR and sequence analysis

EHP was not detected in samples 1X and 2X by a real-time PCR method and sample 3X provided a cycle threshold (Ct) value of 32.5. Using the conventional PCR method, with modifications of Liu et al., (2018) method as described in the method section, a faint DNA band was observed in sample 3X that appeared to be slightly smaller than the DNA band of the EHP positive control. Cloning and sequence analysis of the fragment revealed a size of 149 bp (the expected size for EHP is 157 bp). A Blastn search and sequence alignments revealed a 91.3 % identity to *Enterocytozoon bieneusi*, a 89.2 % identity to *Enterocytozoon hepatopenaei* and a 85.4 % identity to *Enterospora canceri* (Fig. 2) When phylogenetic analysis was performed using a maximum likelihood method, the newly discovered microsporidium clustered with *Enterocytozoon bieneusi* (Fig. 3).

3.3. In situ hybridization

The EHP positive control slide shows the characteristic signal observed in the cytoplasm of the infected cells from the hepatopancreas (Fig. 1). Slides from sample 3X showed a positive reaction to the digoxigenin labeled probes, but interestingly the signal was not observed in the cytoplasm of the cells which is a hallmark of EHP infection. Instead the signal observed from sample 3X was intranuclear (Fig. 1). This indicates this is a different species of microsporidium and not EHP. μm.

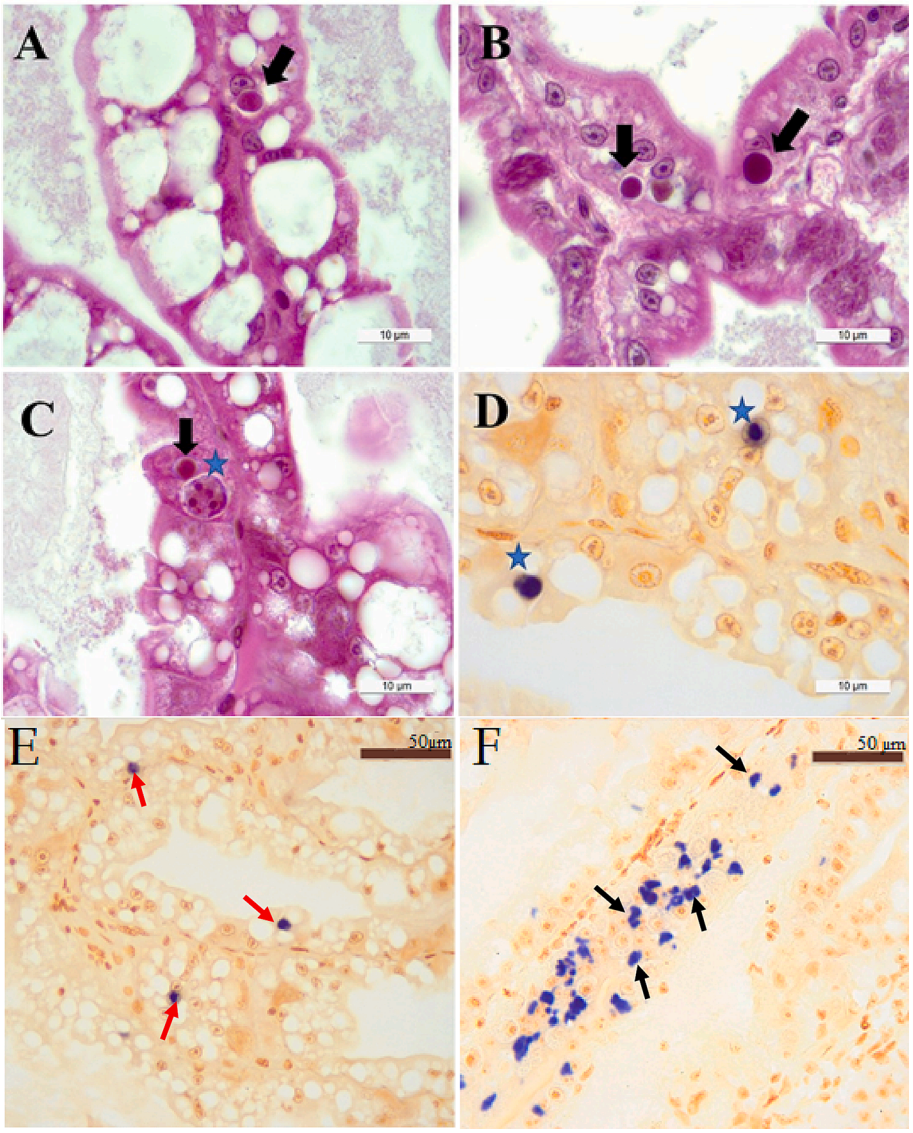

Fig. 1. (A and B) Photomicrographs of H&E (Mayer–Bennet hematoxylin and eosin-phloxine) stained sections of hepatopancreas tissue of *Penaeus vannamei* showing basophilic inclusion bodies in the cytoplasm of a hepatopancreatic epithelial cell, R cell, indicated by black arrows. (C) Basophilic inclusion bodies in the cytoplasm and in the nucleus of a hepatopancreatic epithelial cell (R cell) in the same sample (Sample X3) are shown by a black arrow and a blue asterisk, respectively. (D) *In situ* hybridization of a hepatopancreas tissue section of Sample X3. The intranuclear localization of the signal (indicated by blue asterisk) was observed in this sample within the nucleus of a R cell, whereas no signal was detected in the cell cytoplasm.. (E) Intranuclear localization (red arrows) of the signal in the epithelial R cells of the hepatopancreas from the sample 3X (*P. vannamei*). (F) Cytoplasmic signal of EHP (black arrows) in the EHP infected *P. vannamei* controls. White scale bars = 10 μm and brown scale bars = 50 μm.

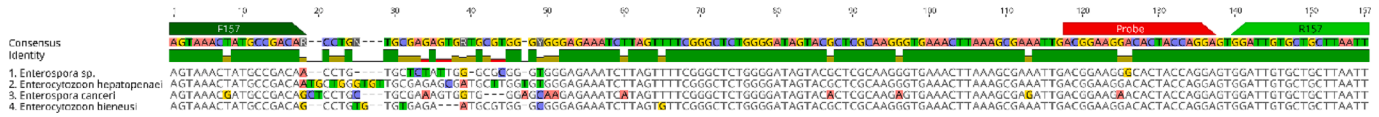

Fig. 2. Sequence alignment of the novel shrimp microsporidium (*Enterospora* sp.), EHP, *Enterospora canceri* and *Enterocytozoon bieneusi*. The alignment shows the primer binding site, the probe binding site and the consensus sequence on top.

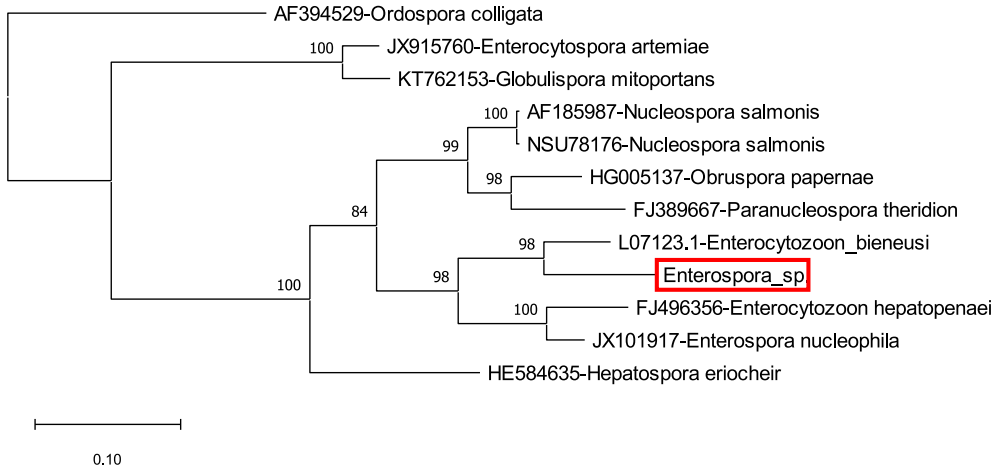

Fig. 3. A phylogenetic tree deduced using a fraction of the small subunit ribosomal RNA gene from *Enterospora* sp. and other microsporidia infecting invertebrates, fish and mammalian hosts. The numbers at nodes showed the posterior probability value. The new described microsporidia (Enterospora sp.) used in this study is enclosed in the red rectangular box.

4. Discussion

It is clear from the results of this study and previous studies (Chaijarasphong et al., 2020; Jaroenlak et al., 2016) that molecular diagnostic methods for EHP based on the SSU rRNA gene can yield false positives with closely related microsporidians. While PCR-based screening of EHP targeting the SSU rRNA gene is not adequate for a specific detection of EHP, they can be employed for preliminary screening, detection and potentially identification of other microsporidian species in shrimp, as was demonstrated through this disease surveillance case study. Using a combination of histopathology, *in situ* hybridization, real-time and conventional PCR and sequence analysis, a novel microsporidium was identified in *P. vannamei.* The newly identified microsporidium can be distinguished from EHP by the observation of an intranuclear inclusion bodies, positive signal obtained in ISH using SSU-rRNA gene probe, a slightly smaller amplicon (i.e. an amplicon of 149 bp size compared to a 157 bp) and sequence differences (deletions and substitutions) in the target amplicon.

Interestingly the microsporidium described here has only been observed infecting the R-cells of the hepatopancreatic tubules (Fig. 1C, 1D and 4A). It is worth noting that Stentiford et al. (2007) described a microsporidium localized in the nucleus and infecting the R, F, and E cells but not the B cells in European edible crab, *C. pagurus*. Considering we had access to limited number of samples to screen, it remains to be seen if this novel microsporidium infects other cell types.

To date, only two intranuclear microsporidian parasites have been characterized in Decapods, namely *E. canceri* and *Enterospora* sp. from *C. pagurus* and *E. bernhardus,* respectively (Stentiford et al., 2007; Stentiford and Bateman, 2007). The novel intranuclear microsporidium detected in this study represents the first *Enteropora* spp. to infect the family Penaeidae. We propose to tentatively assign this shrimp microsporidium in the family Enterocytzoonidae, genus *Enterospora* considering its site of infection, the nucleus of the epithelial cells of the hepatopancreas, the sequence divergence of a fragment of the SSU rRNA gene and phylogentic analysis. These characteristics align more with the type species of the genus, *E. canceri*, rather than to EHP. Further taxonomic comparisons based on ultrastructure and whole genome taxonomy are required to define the position of this new species of microsporidium within the family Enterocytozoonidae.

The intensification of food production has the potential to drive increased disease prevalence in food animals (Stentiford et al., 2016). This is particularly true for microsporidia which are frequent in highly managed aquatic and terrestrial host that could be vulnerable to epizootics (Stentiford et al., 2016). Across the Enterocytozoonida there are examples of members affecting fisheries and aquaculture (Bojko et al., 2023). Some examples include EHP that has been devastating for shrimp production, *E. canceri* that affects edible crab *C. pagurus* and *Nucleospora salmonis* that impacts salmonid culture (Brown and Kent, 2002). Lately, the exploration of presence of microsporidians in crustaceans of commercial and ecological interest have expanded our knowledge on the diversity and host range of these intracellular parasites. *P. borealis* has been described infecting the *Cancer borealis* which is a species of commercial interest (Bojko et al., 2023). Two new microsporidians, *Cambaraspora faxoni* and *Parahepatospora carcini*, have been described infecting invasive crustacean species (Bojko et al., 2017; Stratton et al., 2023). In addition, a parasite, *Alternosema astaquatica*, of *Faxonius virilis* that causes a systemic infection has been discovered (Stratton et al., 2023). Currently, it is unknown if these parasites represent a threat to the host species. Therefore, surveillance, alongside of mortality assessment are urgently needed to understand the role of these microsporidian from a commercial and an ecological context.

The identification of a new species of microsporidium in the most important species of cultured shrimp, *P. vannamei* worldwide should be taken with care. Although the famer submitting the samples, used in this study, reported anecdotal evidence of slow growth, it is important to note that several pathogens from different groups, including viruses, bacteria and microsporidians have been associated to slow growth (Valappil et al., 2021). Considering that the hepatopancreas is not only an organ related to digestion of food (Ceccaldi 1997) but also a critical organ for immune functions in marine invertebrates (Rőster, 2014; Vogt 2019), any parasite infecting this multi-functional organ in shrimp deserves attention.

At this time, due to the limited number of samples screened we do not know if the shrimp *Enterospor*a sp. acts as an emergent pathogen or an incidental parasite that represents little to no threats to the host. However, the devastating impact that EHP has had on shrimp cultured in Asia (Chaijarasphong et al., 2020) and the subsequent discovery of a microsporidian with similar characteristics and tissue location in Latin America (Tang et al., 2017) underscores the importance to further characterize this newly identified microsporidium and develop species-specific diagnostic tools for the novel shrimp *Enterospora* sp. to limit its spread.

Author contributions

Roberto Cruz-Flores and Arun K. Dhar conceived the study and wrote the manuscript. Roberto Cruz-Flores performed histopathology, *in situ* hybridization and analyzed the sequence data. Hung N. Mai performed the PCR detection, cloning and sequencing, sequence analysis, and edited the manuscript. L. Fernando Aranguren Caro aided in histo-pathological examination, confirmed the initial observation of Xavier Romero (see below) and edited the manuscript. Xavier Romero initially observed this microsporidium during a routine health examination of shrimp in a grow-out pond in a Latin American country before sending them to the Aquaculture Pathology Laboratory in the University of Arizona. Xavier Romero and Pablo Intriago reviewed and edited the manuscript. Authors would like to express sincere thanks to Jasmine Millabas for preparing the H&E stained slides and Dr. Rod Russel R. Alenton for providing assistance in performing *in situ* hybridization. Partial funding for this work was provided by Aquaculture Pathology Laboratory- University of Arizona.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403–410. https://doi.org/10.1016/S0022-2836(05)80360-2.

Aranguren Caro, L., Mai, H., Pichardo, O., Cruz-Flores, R., Hanggono, B., Dhar, A., 2020. Evidences supporting Enterocytozoon hepatopenaei association with white feces syndrome in farmed Penaeus vannamei in Venezuela and Indonesia. Dis. Aquat. Organ. 141, 71–78. https://doi.org/10.3354/dao03522.

Bell, T.A., Lightner, D.V., 1988. A Handbook of Normal Penaeid Shrimp Histology, First, Edit. ed. World Aquaculture Society, Baton Rouge, Louisiana.

Bojko, J., Clark, F., Bass, D., Dunn, A.M., Stewart-Clark, S., Stebbing, P.D., Stentiford, G. D., 2017. Parahepatospora carcini n. gen., n. sp., a parasite of invasive Carcinus maenas with intermediate features of sporogony between the Enterocytozoon clade and other microsporidia. J. Invertebr. Pathol. 143, 124–134. https://doi.org/10.1016/j.jip.2016.12.006.

Bojko, J., Behringer, D.C., Bateman, K.S., Stentiford, G.D., Clark, K.F., 2023. Pseudohepatospora borealis n. gen. n. sp. (Microsporidia: Enterocytozoonida): A microsporidian pathogen of the Jonah crab (Cancer borealis). J. Invertebr. Pathol. 197, 1–8. https://doi.org/10.1016/j.jip.2023.107886.

Ceccaldi H.J. (1997) Anatomy and physiology of the digestive system. In: Crustacean Nutrition Advances in World Aquaculture Vol. 6. L.R. D'Abramo, Douglas E. Conklin & D.M. Akiyama (eds). The World Aquaculture Society, Baton Rouge, LA, USA pp 261-291.

Brown, A.M.V., Kent, M.L., 2002. Molecular Diagnostics for Loma Salmonae and Nucleospora Salmonis (Microsporidia). In: Cunningham, C.O. (Ed.), Molecular Diagnosis of Salmonid Diseases. Springer, Dordrecht, Aberdeen, Scotland, pp. 267–283. https://doi.org/10.1007/978-94-017-2315-2_10.

Chaijarasphong, T., Munkongwongsiri, N., Stentiford, G.D., Aldama-Cano, D.J., Thansa, K., Flegel, T.W., Sritunyalucksana, K., Itsathitphaisarn, O., 2020. The shrimp microsporidian Enterocytozoon hepatopenaei (EHP): Biology, pathology, diagnostics and control. J. Invertebr. Pathol. 107458 https://doi.org/10.1016/j.jip.2020.107458.

Cruz-flores, R., Mai, H.N., Noble, B.L., Scho, P.J., Dhar, A.K., 2019. Detection of Enterocytozoon hepatopenaei using an invasive but non-lethal sampling method in shrimp (Penaeus vannamei). J. Microbiol. Methods 162, 38–41. https://doi.org/10.1016/j.mimet.2019.05.008.

Cruz-Flores, R., Mai, H.N., Kanrar, S., Aranguren Caro, L.F., Dhar, A.K., 2020. Genome reconstruction of white spot syndrome virus (WSSV) from archival Davidson's-fixed paraffin embedded shrimp (Penaeus vannamei) tissue. Sci. Rep. 10, 13425. https://doi.org/10.1038/s41598-020-70435-x.

Han, J.E., Tang, K.F.J., Kim, J.H., 2018. The use of beta-tubulin gene for phylogenetic analysis of the microsporidian parasite Enterocytozoon hepatopenaei (EHP) and in the development of a nested PCR as its diagnostic tool. Aquaculture 495, 899–902. https://doi.org/10.1016/j.aquaculture.2018.06.059.

Jaroenlak, P., Sanguanrut, P., Williams, B.A.P., Stentiford, G.D., Flegel, T.W., Sritunyalucksana, K., Itsathitphaisarn, O., 2016. A nested PCR assay to avoid false positive detection of the microsporidian enterocytozoon hepatopenaei (EHP) in environmental samples in shrimp farms. PLoS One 11. https://doi.org/10.1371/journal.pone.0166320.

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C., Thierer, T., Ashton, B., Meintjes, P., Drummond, A., 2012. Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28, 1647–1649. https://doi.org/10.1093/bioinformatics/bts199.

Liu, Y.-M., Qiu, L., Sheng, A.-Z., Wan, X.-Y., Cheng, D.-Y., Huang, J., 2018. Quantitative detection method of Enterocytozoon hepatopenaei using TaqMan probe real-time PCR. J. Invertebr. Pathol. 151, 191–196. https://doi.org/10.1016/j.jip.2017.12.006.

Oie, 2018. INFECTION WITH TAURA SYNDROME VIRUS. In: Manual of Diagnostic Tests for Aquatic Animals. OFFICE INTERNATIONAL DES EPIZOOTIES, Paris, France, pp. 1–18.

OIE, 2019. Infection with white spot syndrome virus, in: EPIZOOTIES, O.I. DES (Ed.), Manual of Diagnostic Tests for Aquatic Animals. Paris, France, pp. 1–16.

Pang, J., Zhao, C., Su, H., Liu, Z., Lu, Q., He, X., Weng, S., He, J., 2022. Rapid visual detection of Enterocytozoon hepatopenaei by recombinase polymerase amplification combined with lateral flow dipstick. Front. Mar. Sci. 9:985314. doi: 10.3389/fmars.2022.985314.

Piamsomboon, P., Choi, S.K., Hanggono, B., Nuraini, Y.L., Wati, F., Tang, K.F.J., Park, S., Kwak, D., Rhee, M.H., Han, J.E., Kim, J.H., 2019. Quantification of enterocytozoon hepatopenaei (Ehp) in penaeid shrimps from southeast asia and latin america using taqman probe-based quantitative PCR. Pathogens 8, 4–9. https://doi.org/10.3390/pathogens8040233.

Rőster, T., 2014. The invertebrate midintestinal gland ("hepatopancreas") is an evolutionary forerunner in the integration of immunity and metabolism. Cell Tissue Res. https://doi.org10.1007/s00441-014-1985-7.

Sathish Kumar, T., Makesh, M., Alavandi, S.V., Vijayan, K.K., 2022. Clinical manifestations of White feces syndrome (WFS), and its association with Enterocytozoon hepatopenaei in Penaeus vannamei grow-out farms: A pathobiological investigation. Aquaculture 547, 737463.

Stentiford, G.D., Bateman, K.S., 2007. Enterospora sp., an intranuclear microsporidian infection of hermit crab Eupagurus bernhardus. Dis. Aquat. Organ. 75, 73–78. https://doi.org/10.3354/dao075073.

Stentiford, G.D., Bateman, K.S., Longshaw, M., Feist, S.W., 2007. Enterospora canceri n. gen., n. sp., intranuclear within the hepatopancreatocytes of the European edible crab Cancer pagurus. Dis. Aquat. Organ. 75, 61–72. https://doi.org/10.3354/dao075061.

Stentiford, G.D., Becnel, J.J., Weiss, L.M., Keeling, P.J., Didier, E.S., Williams, B.A.P., Bjornson, S., Kent, M.L., Freeman, M.A., Brown, M.J.F., Troemel, E.R., Roesel, K., Sokolova, Y., Snowden, K.F., Solter, L., 2016. Microsporidia – Emergent Pathogens in the Global Food Chain. Trends Parasitol. 32, 336–348. https://doi.org/10.1016/j.pt.2015.12.004.

Stratton, C.E., Kabalan, B.A., Bolds, S.A., Reisinger, L.S., Behringer, D.C., Bojko, J., 2023. Cambaraspora faxoni n. sp. (Microsporidia : Glugeida) from native and invasive crayfish in the USA and a novel host of Cambaraspora floridanus. J. Invertebr. Pathol. 199, 107949 https://doi.org/10.1016/j.jip.2023.107949.

Tang, K.F.J., Pantoja, C.R., Redman, R.M., Han, J.E., Tran, L.H., Lightner, D.V., 2015. Development of in situ hybridization and PCR assays for the detection of Enterocytozoon hepatopenaei (EHP), a microsporidian parasite infecting penaeid shrimp. J. Invertebr. Pathol. 130, 37–41. https://doi.org/10.1016/j.jip.2015.06.009.

Tang, K.F.J., Fernando, L., Piamsomboon, P., Eun, J., Maskaykina, I.Y., Schmidt, M.M., 2017. Detection of the microsporidian Enterocytozoon hepatopenaei (EHP) and Taura syndrome virus in Penaeus vannamei cultured in Venezuela. Aquaculture 480, 17–21. https://doi.org/10.1016/j.aquaculture.2017.07.043.

Tangprasittipap, A., Srisala, J., Chouwdee, S., Somboon, M., Chuchird, N., Limsuwan, C., Srisuvan, T., Flegel, T.W., Sritunyalucksana, K., 2013. The microsporidian Enterocytozoon hepatopenaei is not the cause of white feces syndrome in whiteleg shrimp Penaeus (Litopenaeus) vannamei. BMC Vet. Res. 9 https://doi.org/10.1186/1746-6148-9-139.

Tourtip, S., Wongtripop, S., Stentiford, G.D., Bateman, K.S., Sriurairatana, S., Chavadej, J., Sritunyalucksana, K., Withyachumnarnkul, B., 2009. Enterocytozoon hepatopenaei sp. nov. (Microsporida: Enterocytozoonidae), a parasite of the black tiger shrimp Penaeus monodon (Decapoda: Penaeidae): Fine structure and phylogenetic relationships. J. Invertebr. Pathol. 102, 21–29. https://doi.org/10.1016/j.jip.2009.06.004.

Valappil, R.K., Stentiford, G.D., Bass, D., 2021. The rise of the syndrome – sub-optimal growth disorders in farmed shrimp. Reviews in Aquaculture, 1-19 https://doi: 10.1111/raq.12550.

Vogt, G., 2019. Functional cytology of the hepatopancreas of decapod crustaceans. J. Morphol. 280, 1405–1444. https://doi.org/10.1002/jmor.21040.