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Short Communication

# Application of PCR-based diagnostic tools that target *Enterocytozoon hepatopenaei* for the molecular detection of a *Vittaforma*-like microsporidium that infects *Penaeus vannamei* from Costa Rica

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ABSTRACT

Several PCR methodologies are available for the detection of *Enterocytozoon hepatopenaei* (EHP) that target the SSU rRNA gene. However, these methodologies are reported as unsuitable for the detection of EHP due to specificity issues. Here, we report the applicability of two commonly used SSU rRNA methodologies for the detection of additional microsporidia from the genus *Vittaforma* that is present in cultured *Penaeus vannamei* from Costa Rica. The molecular detection of DNA of the novel microsporidia can only be achieved using SSU rRNA targeting methodologies and does not cross-react with the highly specific spore wall protein gene PCR detection method.

## 1. Introduction

The continuous spread of *Enterocytozoon hepatopenaei* (EHP) around the globe is a major concern among shrimp farming nations. This microsporidian pathogen causes Hepatopancreatic Microsporidiosis (HM) which severely hinders the growth of shrimp causing size disparity in ponds that translates to high economic losses (Chaijarasphong et al., 2020; López-Carvallo et al., 2022). To mitigate the negative effects and to limit the spread of this pathogen several PCR and qPCR-based methodologies have been developed to detect EHP and prevent its inadvertent introduction to naïve regions (Tourtip et al., 2009; Tangprasittipap et al., 2013; Tang et al., 2015; Jaroenlak et al., 2016; Liu et al., 2018; Piamsomboon et al., 2019).

While some PCR methodologies have proven to be highly specific for EHP diagnostic purposes others have been shown to be less selective with the ability to detect other shrimp infecting microsporidia. Methods that target the spore wall protein gene and the beta-tubulin gene are considered to have great diagnostic characteristics for specific EHP detection (Jaroenlak et al., 2016; Han et al., 2018; Piamsomboon et al., 2019). In contrast, methodologies based on the SSU-rRNA gene have been shown to, in some occasions, cross-react with closely-related aquatic microsporidians such as *Enterospora canceri* and *Hepatospora eriocheir* making them unsuitable for EHP detection (Jaroenlak et al., 2016; Stentiford et al., 2011, 2007).

In October of 2022 *Penaeus vannam*ei shrimp from three farms in Costa Rica presented the typical signs associated with EHP infection including slow growth and size disparity (Fig. 1A). Shrimp from these farms were sampled and two commonly used SSU-rRNA based PCR methodologies were employed to evaluate the presence of EHP. Our results indicate that DNA from a previously unknown hepatopancreatic infecting *Vittaforma*-like microsporidia that is associated with the diseased shrimp can be detected utilizing two commonly used SSU-rRNA based PCR methods and DNA from this microsporidium would otherwise go undetected if tested by more specific methods.

## 2. Material and methods

### 2.1. Sampling

During routine molecular-based health surveys shrimp, *P. vannamei*,

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**Fig. 1.** Cultured *Penaeus vannamei* from Costa Rica presenting slow growth and size disparity. Affected animals displayed a pale to whitish hepatopancreas, reddish uropods with green tips. The animals presented in the image are from the same grow-out cycle and pond.

collected from three different farms from Costa Rica that are located near the Gulf of Nicoya, at two different time points. The first sampling (farm 1) was carried-out in October of 2022 and thirty shrimp were sampled. The second sampling (farm 2 and 3) was performed in November of 2022 and eight shrimp were sampled from each farm. From each shrimp the hepatopancreas was aseptically dissected and placed in a sterile 1.5 ml tube containing 300 µl of molecular grade ethanol. In both samplings shrimp presenting slow growth and shrimp with a normal appearance, with an average size of 5.3 g considering both groups, were collected.

### 2.2. DNA extraction, PCR and Sanger sequencing

DNA was extracted from ~ 30 mg of hepatopancreatic tissue. The tissues from shrimp collected during the first sampling were pooled for a total of six pools (5 shrimp per pool). Hepatopancreases from the second sampling (farm 2 and 3) were processed individually. PCR for the detection of a 510 bp fragment of the SSU rRNA gene of EHP was performed for all samples as described by Tang et al., (2015). Additionally, the protocol of Liu et al., (2018) was adapted to conventional PCR. Two pools (1 and 2) from the first sampling and all positive samples from the seconds sampling were tested by the latter methodology. The conventional PCR amplification was carried out in a total volume of 50 µl containing 2 µl of template DNA (50–100 ng/µl), 10 µl of MyTaq Reaction Buffer (Bioline®), 0.6 µl of MyTaq DNA polymerase, 3.5 mM $MgCl_2$, and 350 nM of each primer. The PCR conditions consisted of an initial denaturation at 95 °C for 2 min, followed by 40 cycles at 95 °C for 10 s, 53 °C for 10 s and 72 °C for 10 s with a final elongation step at 72 °C for 2 min. To corroborate that only SSU-rRNA based methodologies react with novel microsporidium, we employed the nested-PCR method described by Jaroenlak et al., (2016) since it is widely considered as the most sensitive and specific diagnostic methodology to detect EHP. The cycling conditions for the first step consisted of an initial denaturation at 95 °C for 5 min, followed by 30 cycles at 95 °C for 30 s, 58 °C for 10 s and 68 °C for 45 s with a final elongation step at 68 °C for 5 min. For the second step the conditions consisted of an initial denaturation at 95 °C for 5 min, followed by 20 cycles at 95 °C for 30 s, 64 °C for 30 s and 68 °C for 20 s with a final elongation step at 68 °C for 5 min. As a positive controls two plasmids containing fragments of 1146 bp and 514 bp of the SSU-rRNA and spore wall protein genes of EHP were utilized respectively. The PCR products were run on a 2% agarose gel and were visualized on a GelDoc™ XR+ (Bio-Rad). All positive products were sent for Sanger Sequencing at Eton Biosciences, San Diego, California. Sequences were compared with BLASTn and alignments were performed in Geneious Prime software version 2023.0.4 using the Geneious Aligner.

### 2.3. Phylogenetic analysis

The SSU rRNA gene fragment of the novel microsporidia from Costa Rica was compared with other members of the order Microsporidia to deduce its taxonomic affiliation. We used the SSU rRNA sequences described in Tourtip et al., (2009) as references for our phylogenetic analysis. The sequences were aligned using the Geneious Prime Aligner tool and the phylogenetic analysis were conducted using MEGAX software (Kearse et al., 2012; Kumar et al., 2018). A phylogenetic tree was constructed using the Maximun-likely hood method (Kumar et al., 2018). The bootstrap consensus tree was inferred from 1000 replicates.

## 3. Results

### 3.1. Molecular detection and identification of a Vittaforma-like microsporidia

Five out of the six pools from the first sampling generated PCR products of slightly lower molecular weight when compared to the EHP control using the methodology of Tang et al., (2015). From the second sampling similar results were obtained for five shrimp (three shrimp from farm 2 and two shrimp from farm 3), amplicons were ~ 30 bp smaller than the control. Utilizing the modified methodology of Liu et al., (2018) pools 1 and 2 from the first sampling and all five positive shrimp from the second sampling generated amplicons of ~ 140 bp. PCR amplicons from both methodologies appeared smaller than the reported expected size (Fig. 2). Sequence analysis of the amplicons produced using the methodology of Tang et al., (2015) from both samplings revealed a fragment size of 482 bp (GenBank accession: OQ331021) with a 97.1% and 92.3% identity to an uncharacterized microsporidian (GenBank accession:MF374875) and *Vittaforma corneae* (GenBank accession: OP703353) respectively (Table 1). Amplicons generated using the methodology of Liu et al., (2018) were 146 bp and showed a 95.2% identity to *V. corneae* (MH333251.1). No amplification of the spore wall protein gene was observed in any of the tested samples using the methodology described by Jaroenlak et al., (2016) (Fig. 2).

The results of the phylogenetic inferences utilizing representative member of the microsporidia showed that the *Vittaforma*-like microsporidium from *P. vannamei* is closely related to *Vittaforma corneae*, a human pathogen, and *Cystosporogenes legeris*, an insect pathogen (Fig. 3). According to these results the novel shrimp microsporidian possibly represents a new member of the genus *Vittaforma*.

## 4. Discussion

In recent years HM caused by EHP has become one of the biggest challenges for shrimp farming worldwide. Several PCR-based methodologies that target different genes have been developed to detect EHP as an effort to limit its spread among shrimp farming nations. EHP diagnosis has slowly moved away from the SSU-rRNA targeting PCR-based methods due to their potential cross-reactivity (Stentiford et al., 2007; Jaroenlak et al., 2016). However, we have confirmed that these methodologies have value in shrimp diagnostics due to their ability to detect additional uncharacterized microsporidia that cause disease in commercially important shrimp species. Recently, Dhar et al., (in press)

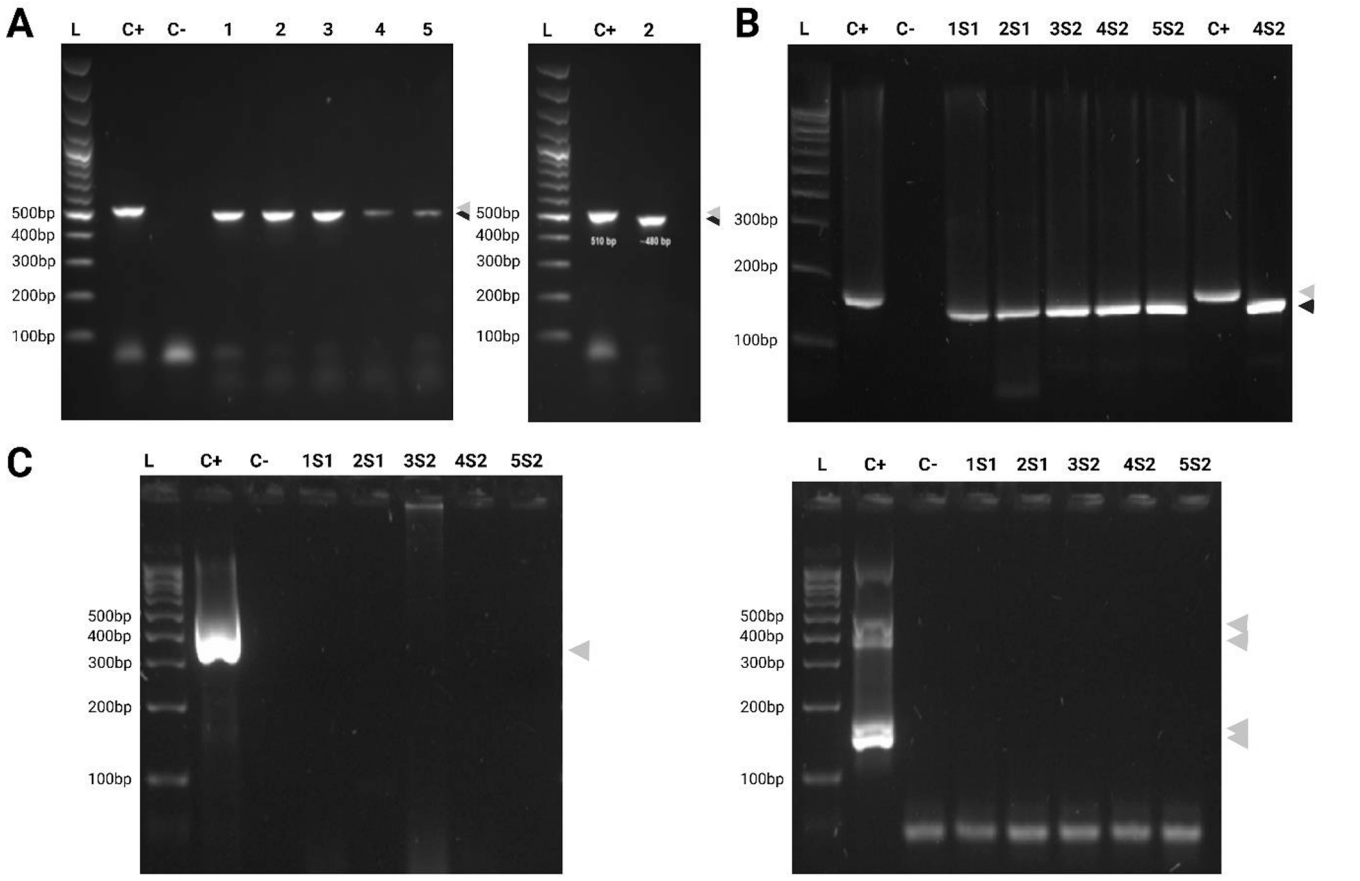


**Fig. 2.** Gel electrophoresis of PCR products generated utilizing the methodologies reported by Tang et al., (2015), Liu et al., (2018) and Jaroenlak et al., (2016). (A) EHP Positive control (C + ) shows the expected PCR product of 510 bp corresponding to EHP. Lanes 1–5 show PCR products of a slightly smaller molecular weight (~480 bp) corresponding to shrimp showing signs similar to Hepatopancreatic Microsporidiosis. At the right side of the image the EHP positive control and pool 2 were run in parallel to highlight the difference in size. (B) PCR products of ~ 145 bp from a selected number of samples from both samplings utilizing the modified methodology of Liu et al., (2018). Lanes labeled as 1S1 and 2S1 represents pools 1 and 2 from the first sampling. Lanes labeled as 3S2, 4S2 and 5S2 represent individual shrimp from the second sampling. At the right-hand side, the positive control was ran next to sample 4S2 to exemplify the difference in the size of the products. (C) On the left side of the image the first step of the nested-PCR methodology of Jaroenlak et al., (2016) is shown and no amplification was observed in any of the samples (1S1, 2S1, 3S2, 4S2 and 5S2). On the right side of (C) the second step of the nested-PCR methodology of Jaroenlak et al., (2016) is shown and no amplification was observed in any of the samples (1S1, 2S1, 3S2, 4S2 and 5S2). The small grey arrows on the side of the gels indicate products generated by the positive plasmid controls while the small black arrows indicate PCR products generated from the DNA of *Vittaforma*-like microsporidium.

**Table 1**
Similarity of the partial fragment of the SSU rRNA of the *Vittaforma*-like microsporidia to other know microsporidia from GenBank. Accession numbers KR303711.1, MF374875.1, MF374852.1 and MF374898.1 represent BLASTn-hits 1, 2, 3 and 4 respectively. While Accession numbers OP703353.1 and OP703350 represent BLASTn-hits 8 and 9 respectively.

| Microsporidia | Accession No. | Query Cover (%) | E-Value | Percent Identity (%) |
|---|---|---|---|---|
| Microsporidium sp. LVEI.SMW V3S3-FR | KR303711.1 | 99% | 0.0 | 97.05% |
| Microsporidium sp. isolate Q1-SML | MF374875.1 | 86 | 0.0 | 96.88 |
| Microsporidium sp. Q1-CCL-U | MF374852.1 | 86 | 0.0 | 96.88 |
| Microsporidium sp. Q2-GPR-D | MF374898.1 | 86 | 0.0 | 96.64 |
| Vittaforma corneae isolate 1115/21 | OP703353.1 | 99 | 0.0 | 92.34 |
| Vittaforma corneae isolate 1606/21 | OP703350 | 99 | 0.0 | 92.34 |

utilized the same primers as in this study (Tang et al., 2015; Liu et al., 2018) to detect the first microsporidium (*Enterospora* sp.) that infects the nucleus of shrimp hepatopancreatic cells. In this study, we detected DNA of a novel microsporidium from the genus *Vittaforma* that infects the hepatopancreas and is associated with growth retardation and size disparity in *P. vannamei* from Costa Rica. The nucleotide identity of both PCR products and the phylogenetic analysis concur that this parasite is a new member of the genus *Vittaforma*. This is the first report of a *Vittaforma*-like microsporidium infecting commercially important *P. vannamei* that present similar signs of HM.

The study of Dhar et al., (in press) and this study underscore the value of SSU-rRNA targeting PCR methodologies for the molecular detection of microsporidian parasites that could cause problems in shrimp culture. The utilization of the more specific spore wall protein gene did not result in amplification. Therefore, utilizing this highly specific methodology we would have failed to detect the *Vittaforma*-like microsporidium that is possibly associated with an EHP-like disease that warrants further examination. It is important to mention that in some cases related to the movement of broodstock and shrimp post-larvae for cultivation purposes it is imperative that highly specific PCR-based methodologies are employed to guarantee the absence of EHP. However, we propose that for routine monitoring during a production cycle it could be greatly beneficial to employ a more broadscale methodology that simultaneously allows the detection EHP and other opportunistic microsporidians. Differentiation between EHP and other microsporidian using these SSU-rRNA targeting methodologies is simple by gel

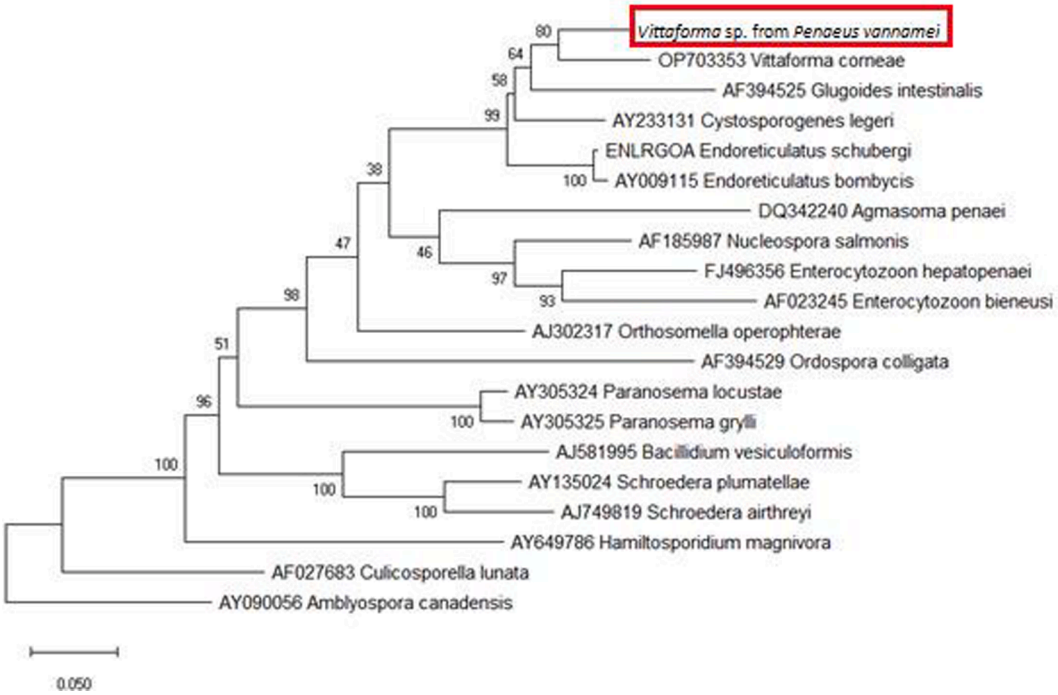


**Fig. 3.** The evolutionary history of the *Vittaforma*-like microsporidium that infects *Penaeus vannamei* was inferred using Maximun-likely hood method. The optimal tree with the sum of branch length = 2.46960742 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. This analysis involved 20 nucleotide sequences of SSU rRNA of members of the Microsporidia. There were a total of 2326 positions in the final dataset.

electrophoresis considering a differential PCR product size as observed in this study and in the study of Dhar et al., (in press). In addition, the taxonomic affiliation of other penaid-infecting microsporidians is easily determined by direct sequencing of the products and sequence analysis. This suggested course of action could help elucidate the etiology of slow growth in shrimp culture when EHP is not present.

Microsporidia are diversely distributed, opportunistic, and density-dependent parasites that are frequent in highly managed aquatic and terrestrial hosts (Stentiford et al., 2016). In recent, years there is strong evidence that microsporidiosis is increasing in prevalence in animals and humans in most biomes (Stentiford et al., 2016). The intrinsic characteristics of shrimp culture (suboptimal culture conditions, inbred populations, high density etc.) makes this activity particularly prone to the constant emergence and spread of infectious diseases (Cruz-Flores et al., 2022) particularly microsporidia. It is therefore greatly beneficial to have molecular diagnostic assays available, as the ones utilized here, that can potentially detect a broad number of microsporidia the infect cultured shrimp species as initial screening tools. These assays have potential to detect and identify disease causing microsporidia that would otherwise go unnoticed when using the more specific EHP diagnostic methods. In addition, the broad specificity of SSU-PCR offers significant advantages for targeting unknown microsporidian, particularly in developing countries with limited laboratory infrastructure. Detecting novel pathogens can be challenging in such settings, where resource constraints and a lack of advanced facilities or financial budget pose obstacles. However, these SSU-PCR targeting methodologies provide an inclusive and cost-effective approach that can help overcome these limitations. By enabling local pathogen detection, it eliminates the need for sample transportation, which is often complicated and, under some circumstances, may be delayed by sanitary filters. Therefore, the employed methodologies holds great potential as a valuable tools for detecting unknown microsporidians, especially in developing countries facing resource constraints. The application of these SSU rRNA targeting methods in this context can contribute to the improvement of disease surveillance and control measures, ultimately supporting the health and productivity of important food supply industries such as shrimp aquaculture.

**Authors contributions.**

Roberto Cruz-Flores conceptualized the study, wrote the manuscript, performed PCR diagnostics and sequence analysis. Nelson Peña-Navarro sampled the organism, performed PCR diagnostics and edited the manuscript. Brandon Chacón Perez performed PCR diagnostics and edited the manuscript. Antonio López-Carvallo wrote/edited the manuscript, designed the figures and performed PCR diagnostics.

## 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.

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