Detection of *Enterocytozoon hepatopenaei* using an invasive but non-lethal sampling method in shrimp (*Penaeus vannamei*)

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A B S T R A C T

The detection of enteric pathogens that cause diseases in shrimp involves the sacrifice of the host to obtain tissue samples for diagnosis. In this study, we describe an invasive but non-lethal sampling methodology using a syringe to collect biopsy samples from the hepatopancreas (HP) of *Penaeus vannamei* to detect the microsporidian pathogen, *Enterocytozoon hepatopenaei* (EHP), by qPCR and transmission electron microscopy. EHP was detected in all the infected shrimp by qPCR. The shrimp infected by the microsporidian showed 65% survival at 7 days post-sampling. Transmission electron microscopic examination of the biopsy samples revealed numerous spores of the pathogen. The presence of EHP was further confirmed by histology and *in situ* hybridization from HP tissue samples. The data shows that a hepatopancreas biopsy could be a viable means of detecting enteric pathogens in shrimp, and the method could be valuable in sampling broodstock and natural populations without the need to sacrifice the animals.

1. Introduction

The detection of pathogens that cause diseases in shrimp usually involves the sacrifice of the organism to obtain the tissue samples for histological and molecular diagnosis (Lightner and Redman, 1998; Cervellione et al., 2017). However, this practice is not ideal and it becomes expensive when assessing the health status of candidate Specific Pathogen Free (SPF) broodstock in captive breeding programs. In particular, obtaining samples to test for enteric pathogens of shrimp is challenging because this practice involves the removal of tissue from the hepatopancreas (HP), stomach or gastrointestinal tract (Bell and Lightner, 1988). Therefore, there is an urgent need to develop a tissue sampling method that overcomes the need to sacrificing genetically valuable broodstock.

Enterocytozoon hepatopenaei (EHP) is a microsporidian parasite that infects the hepatopancreas of *Penaeus monodon* and *Penaeus vannamei* and is associated with a disease known as slow growth syndrome (SGS) (Tourtip et al., 2009; Tang et al., 2015, 2017; Kummari et al., 2018). The economic losses attributed to SGS are significant and are now considered to critically affect the shrimp industry (Tang et al., 2015). The detection of EHP can be carried out by PCR, qPCR, histology, transmission electron microscopy (TEM) and *in situ* hybridization using tissue samples of the HP (Tourtip et al., 2009; Tang et al., 2015, 2017; Qiu et al., 2018). However, this practice becomes expensive when

assessing the SPF status since in most cases the animals will be sacrificed to remove the HP tissue and each broodstock shrimp can cost US $50.00 or more. The collection of the HP tissue is fundamental to test for pathogens by molecular techniques (PCR, RT-PCR and qPCR) because these are considered the gold standard for the detection of shrimp pathogens due to their sensitivity and are recommended protocols by The World Organization for Animal health (OIE) (Bondad-Reantaso et al., 2001; Lightner, 2005). More recently, this issue was addressed by Kerdmusik et al. (2018) that developed a method to detect *Penaeus monodon* densovirus form large mature black tiger shrimp. To date, no methods have been reported to obtain tissue samples from the HP of the smaller sized *P. vannamei* that are infected with EHP. In this study we describe an invasive non-lethal sampling methodology to obtain tissue from the HP of *P. vannamei* to detect the microsporidian pathogen, EHP, by qPCR and TEM.

2. Material and methods

2.1. Needle biopsy

Twenty EHP infected *P. vannamei* shrimp (1.5–2.0 g) and 10 SPF shrimp (~1.2 g) were used for this study. The sampling methodology consisted of using a 1 ml TB syringe with a 25G X 5/8 needle to collect the HP tissue. The carapace over the HP of the shrimp was surface
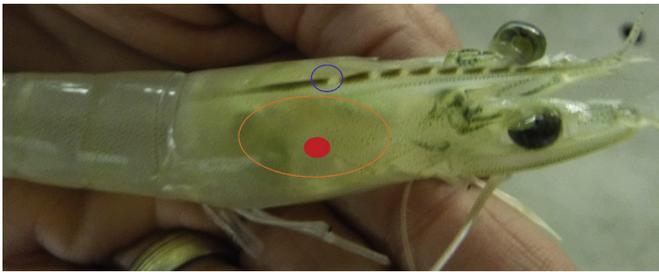
Fig. 1. Location of the area where the needle was inserted for the biopsy. The needle was inserted in the lateral part of the center of the hepatopancreas (HP). The orange circle indicates the HP area, the blue circle indicates the last rostrum spine and the red dot indicates the location where the needle was inserted. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

sterilized using 70% EtOH before the needle was inserted in the lateral part of the center of the HP (Fig. 1). The needle was inserted about 3 mm into the HP. A single draw was made from the HP and half of the content of the needle was expelled into a 1.5 ml sterile microcentrifuge tube that was placed immediately in liquid nitrogen. The remaining half of the sample was expelled into another 1.5 ml sterile microcentrifuge tube containing 1 ml of 3% glutaraldehyde in sodium cacodylate. After the biopsy collection the shrimp were placed in the appropriate separate holding tanks. The animals were observed for seven days post-sampling to determine if the shrimp would survive the biopsy, and then they were sacrificed to collect HP tissue for qPCR, histology and *in situ* hybridization to confirm the presence of EHP.

2.2. DNA extraction and qPCR

DNA was extracted from both sets of samples (biopsy samples and tissue samples) using the Genomic DNA Isolation Kit from Norgen Biotek Corp and a Maxwell® 16 Cell LEV DNA Purification Kit following the manufactures instructions. The qPCR analyses were performed using TaqMan™ Fast Virus 1-Step Master Mix (Applied Biosystems™) following the methods described by Qiu et al. (2017).

2.3. Transmission electron microscopy examination

The biopsy samples obtained for TEM were centrifuged for 20 min at 17,600*g* to form a visible pellet. The pellets obtained from the biopsy samples were processed and imaged at the Imaging Core Facility, Life Sciences, The University of Arizona, Tucson, AZ. The EHP spores were visualized and measured using an Advance Microscopy techniques high resolution camera system (4 Mpixel) at magnifications ranging from 16,500-43,000×.

2.4. Histology and in situ hybridization

The HP samples obtained at the end of the study were processed for histology following the standard method as described by Tang et al. (2017). For *in situ* hybridization, all sections were dried onto positively charged microscopic slides and *in situ* hybridization was carried out following the protocols described by Antonio et al. (1998) and Cruz-Flores et al. (2016) with an equivolume mixture of four primers, EHP 510 F, EHP 510 R (Tang et al., 2015), EHP F (GCAGTGATGGGTAACGGGAA) (this study) and EHP R (AATTTCCAACGGCCATGCAC) (this study). These primers were tailed at 3′-end with digoxigenin-11-dUTP (Integrated DNA Technologies®, San Diego, CA). A paraffin block containing previously known EHP affected shrimp was used as the positive control. As a negative control, paraffin blocks of SPF shrimp were used. Slides were examined by light microscopy for the presence or absence EHP hybridizing with the DNA probes, and those slides that showed blue to purple precipitates were considered positive.

3. Results

3.1. Shrimp survival

At the termination of the study (seven days post-sampling) the EHP infected shrimp and the SPF shrimp showed a 65% and 40% survival rate respectively. The EHP infected shrimp were larger (1.5–2.0 g) than the SPF shrimp (1.2 g). This could contribute to the survival percentage.
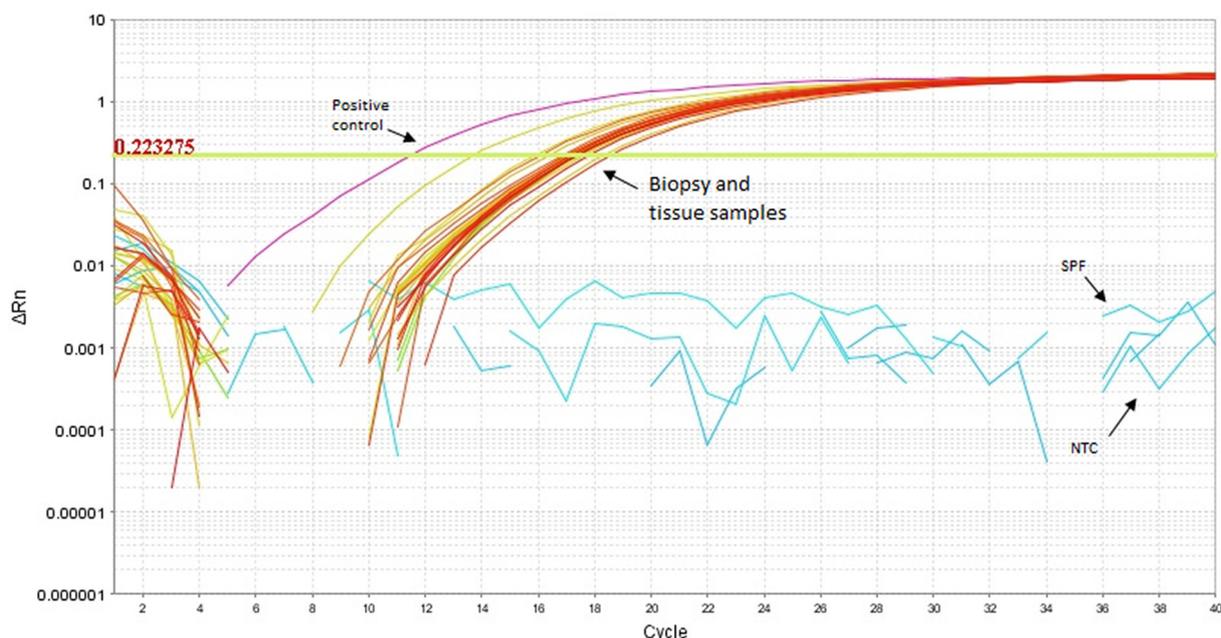

Fig. 2. Amplification plots of the biopsy and tissue samples by qPCR. Both the biopsy and the tissue sample presented similar Ct's (17.07 ± 0.92). Ct: cycle threshold, SPF: DNA from Specific Pathogen Free shrimp; NTC: No Template Control.
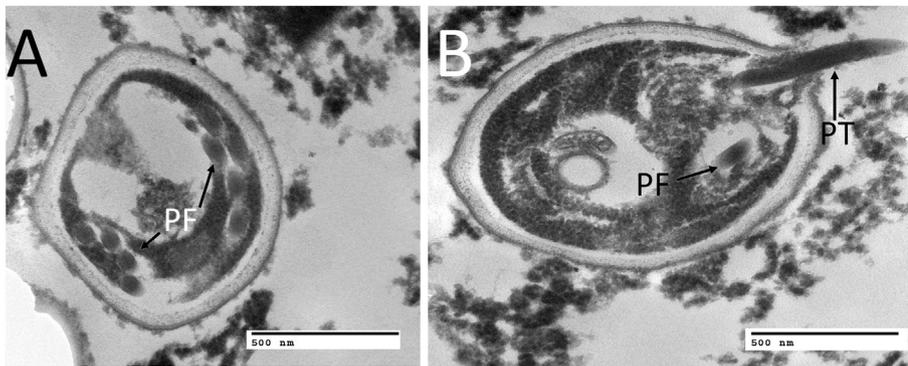
Fig. 3. Electron microscopy of *Enterocytozoon hepatopenaei* from the pelleted biopsy samples. (A) Mature spore showing a section of the coiled part of the polar filament (PF), five coils are observed on one side and four coils are observed on the other side. (B) Cross section of a mature spore were a coiled portion of the PF and the polar tube (PT) are observed. Magnification of both images is 43,000×.

3.2. qPCR results

All the biopsy samples were positive by qPCR. Furthermore, all the tissue samples from the surviving EHP infected shrimp were also positive by qPCR (13 surviving shrimp). The EHP biopsy and the tissue samples presented an average cycle threshold (Ct) of 17.07 ± 0.92 (Fig. 2). All the surviving SPF shrimp were negative for EHP by qPCR (four surviving shrimp).

3.3. Transmission electron microscopy, histology and in situ hybridization

The spores formed by EHP were detected in the biopsy samples by TEM (Fig. 3). The spores ($N = 30$) showed a width of 1.0 ± 0.8 μm by a length of 0.7 ± 0.4 μm. By histology all the tissue samples of the HP from the surviving shrimp (13 shrimp) showed the presence EHP inclusion bodies. While some organisms showed the presence of both, EHP inclusion bodies and spores in the tubes of the HP by histology (Fig. 4). The infection intensity of EHP was low/moderate in 85% of the surviving shrimp and only 15% of the organisms showed a high infection intensity. *In situ* hybridization of the HP of the surviving shrimp showed positive hybridization to the EHP Digoxigenin labeled probes (Fig. 4).

4. Discussion

The method described here allows the detection of EHP by qPCR and TEM from HP biopsy samples obtained by an invasive syringe biopsy. It is important to remark that not all the tested shrimp survived the biopsy (65% survival of the EHP infected shrimp and 40% survival of the SPF shrimp). However, it is also important to mention that the shrimp used for this study were all of a small size 1–2 g. The EHP infected shrimp were slightly larger (1.5–2.0 g) than the SPF shrimp (1.2 g). A preliminary study with larger SPF shrimp of a size of ~5.0 g yielded a 100% survival (unpublished results). Furthermore, Kerdmusik et al. (2018) carried out a needle biopsy to detect hepatopancreatic parvovirus (HPV) by fresh smears, histology, and PCR from 100 g *P. monodon* and obtained 100% survival. These observations and the study by Kerdmusik et al. (2018) suggest that larger shrimp have a higher possibility of survival, but further studies are needed to confirm these observations in *P. vannamei*. Nonetheless, these results are encouraging since this indicates that this method could be suitable to test broodstocks for the presence of enteric pathogens. The identity of the pathogen in this study was confirmed in the surviving shrimp by qPCR, histology and *in situ* hybridization.

To our knowledge, this is the first report of a successful invasive non-lethal biopsy of the HP of *P. vannamei* that allows the detection of EHP in shrimp. EHP is a pathogen that is becoming ever more important
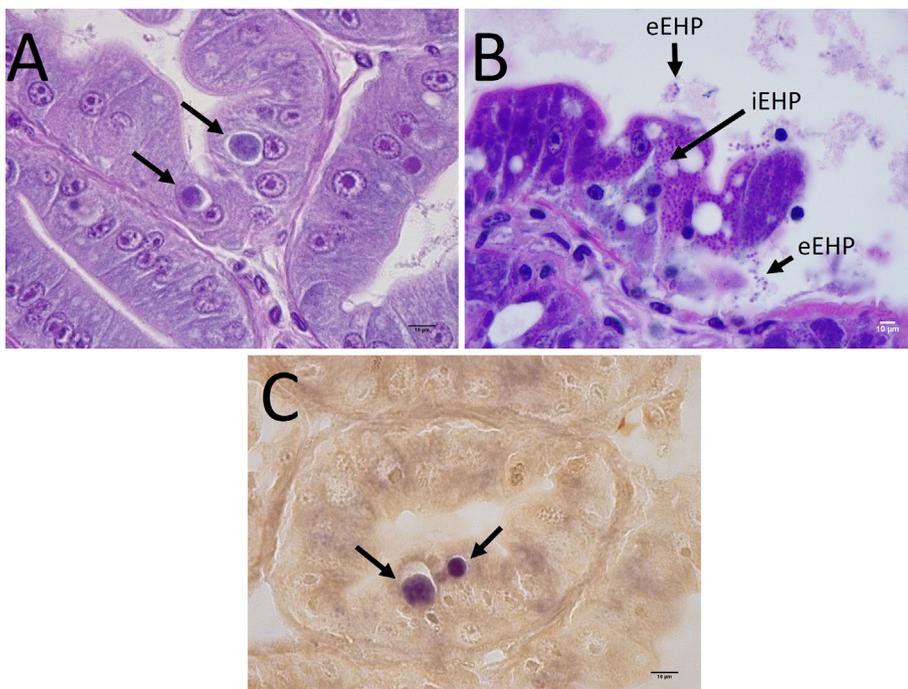

Fig. 4. H&E (Mayer-Bennet hematoxylin and eosin) histology and *in situ* hybridization of *Enterocytozoon hepatopenaei* (EHP) from the hepatopancreas (HP) tissue samples of the surviving shrimp. In (A) two inclusion bodies of EHP are shown (arrows). (B) A large number of EHP spores can be observed intracellularly (iEHP) and extracellularly (eEHP) in the lumen of the HP tubes. (C) Positive hybridization is observed in the EHP inclusion bodies in the HP tubules confirming the identity and presence of EHP in studied shrimp. Magnification of all images is 40×.

in shrimp farming globally (Qiu et al., 2017). Due to the chronic nature of EHP infection, EHP has been overlooked in the past resulting in the inadvertent spread of the pathogen (Tang et al., 2017). It is especially important to screen broodstock for EHP. Although the present study was performed with animals that are much smaller than broodstock (1.2–2.0 g compared to the 30–50 g size of the broodstock), the results clearly demonstrated the potential of using the HP biopsy as a means of detecting enteric pathogens in shrimp.

In other invertebrates such as bees (*Apis mellifera*) and in other aquatic organisms such as fish and toads (*Bufo cognatus*) invasive non-lethal sampling has been carried out successfully for distinct PCR based applications (Cummings and Thorgaard, 1994; Châline et al., 2004; Gonzalez et al., 2004). Previously, reports of non-invasive sampling methods involving DNA extraction of fecal samples for the detection of acute hepatopancreatic necrosis disease, Necrotizing hepatopancreatitis, and hepatopancreatic parvovirus have been reported (Pantoja and Lightner, 2000; Briñez et al., 2003; Han et al., 2017). These methods are limited by the presence of inhibitors in the fecal samples and are susceptible to false positives or false negatives (Monteiro et al., 1997; Pantoja and Lightner, 2000). In conclusion, we report a non-lethal invasive sampling methodology that can be employed to obtained HP biopsy samples to test for the presence of enteric pathogens of shrimp. This methodology can serve as a fundamental tool for the testing of precious and expensive broodstocks to assess their SPF status.

Acknowledgements

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