Asian Fisheries Science ***31S*** (2018): 257–269

ISSN 0116-6514
E-ISSN 2071-3720
https://doi.org/10.33997/j.afs.2018.31.S1.018

Asian Fisheries Science
The Journal of the Asian Fisheries Society

Recent Research on Acute Hepatopancreatic Necrosis Disease (AHPND) and *Enterocytozoon hepatopenaei* in Thailand

T.W. FLEGEL[1,*] and K. SRITUNYALUUCKSANA[2]

[1]Center of Excellence for Shrimp Molecular Biology and Biotechnology Faculty of Science, Mahidol University, Bangkok, Thailand and National Center for Genetic Engineering and Biotechnology (BIOTEC) Khlong Nung, Khlong Luang District Pathum Thani, Thailand
[2]National Center for Genetic Engineering and Biotechnology (BIOTEC) Khlong Nung, Khlong Luang District Pathum Thani, Thailand and Shrimp-virus Interaction Laboratory (ASVI) BIOTEC Yothi office, Bangkok, Thailand

Abstract

This review (up to 20 October 2016) covers two newly emerging, serious diseases in Asian shrimp aquaculture since 2009. The first (recognized in 2013) is acute hepatopancreatic necrosis disease (AHPND) caused by isolates of *Vibrio parahaemolyticus* (VP_{AHPND}) that carry a pVA plasmid containing genes for $PirA^{vp}$ and $PirB^{vp}$ toxins. The second is hepatopancreatic microsporidiosis (HPM) caused by *Enterocytozoon hepatopenaei* (EHP). AHPND causes high, early mortality, but prevalence may be overestimated if mistakenly equated with early mortality syndrome (EMS), a practice that is not problematic for farmers or the popular press, but is unacceptable for science. Progress on AHPND research in Thailand has focused on characterization of VP_{AHPND} isolates and development of molecular detection methods based their toxin proteins and respective genes. Additional work on AHPND outbreak ponds has revealed bacterial partners that have a potent synergistic effect on VP_{AHPND} virulence. Unlike AHPND, losses from HPM result from growth inhibition rather than mortality. However, this was not immediately recognized because growth inhibition from HPM is not easily detectable until the second or third month of cultivation, after the period of highest risk for AHPND. Work in Thailand has focused on the characterization and detection of EHP in broodstock and postlarvae (PL), and on development of laboratory infection models.

Keywords: acute hepatopancreatic necrosis, AHPND, EHP, EMS, *Enterocytozoon hepatopenaei*, HPM, Pir^{vp} toxins, shrimp disease, Thailand, *Vibrio parahaemolyticus*

[*]Corresponding author. E-mail address: tim.flegel@gmail.com

Introduction

Acute hepatopancreatic necrosis disease (AHPND) of shrimp is caused by unique isolates of *Vibrio parahaemolyticus* (VP_{AHPND}) (Tran et al. 2013) and is one of several diseases that can cause early mortality in shrimp rearing ponds. Thus, it is included under the heading "early mortality syndrome" (EMS) by shrimp farmers. This farmer practice can lead to confusion, if the term EMS is taken as equivalent to AHPND. For example, it can result in overestimation of the prevalence of AHPND outbreaks and underestimation or ignorance regarding early mortality caused by other agents or pathogens.

At least in scientific forums we must be aware of the distinction. The critical feature in diagnosing AHPND by histological analysis using the currently accepted case definition is the occurrence of massive sloughing of hepatopancreatic (HP) tubule epithelial cells in the absence of bacterial cells or other possible pathogens (Fig. 1). If this feature is not observed for at least one individual shrimp in a sample set, then the sampled population cannot be confirmed histologically as a case of AHPND.

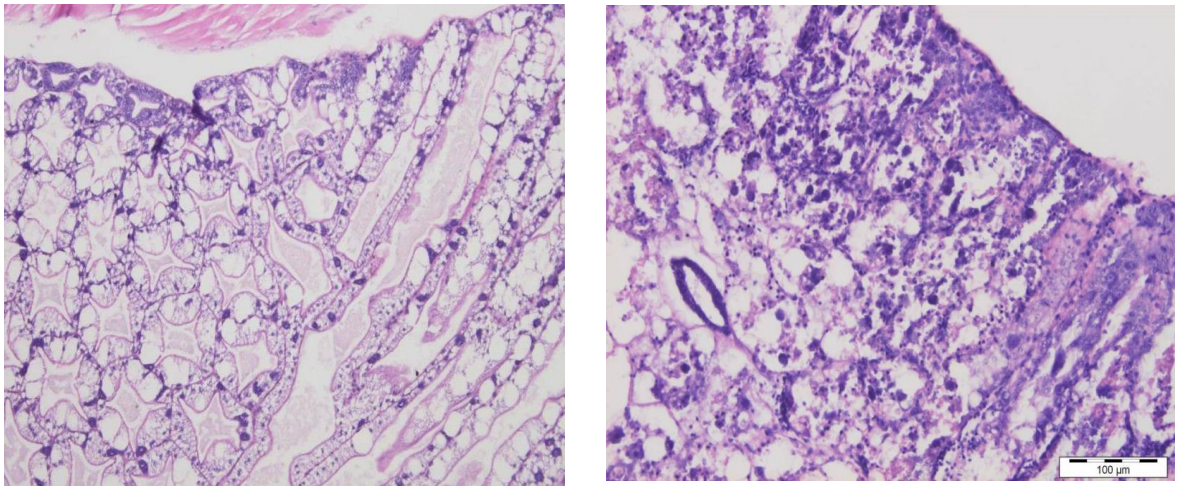

Fig. 1. Photomicrographs of normal histology of the shrimp hepatopancreas (left) compared to the pathognomonic lesion of AHPND (right) characterized by massive sloughing of tubule epithelial cells in the absence of bacteria or other pathogens.

Since the Food and Agriculture of the United Nations (FAO) meeting on EMS/AHPND in Panama in June 2015, the countries reporting the occurrence of AHPND outbreaks (Thitamadee et al. 2016) have changed only by the addition of Australia, where two AHPND outbreaks were reported from giant tiger prawn (*Penaeus monodon* Fabricius 1798) cultivated in Queensland. In the Australian report to the World Organisation for Animal Health (OIE) in early 2016, the bacterium isolated was identified as *V. harveyi* carrying the Pir^{vp} toxin genes, but possibly on the chromosome rather than in a pVA plasmid as reported for VP_{AHPND} isolates (Lee et al. 2015). However, there are rumours that outbreaks have also occurred but not been reported from India and from countries in Central America in addition to Mexico (Enríquez-Espinoza et al. 2016; Nunan et al. 2014; Soto-Rodriguez et al. 2015).

There is also one published report of a *Vibrio harveyi* (VH) isolate that causes AHPND and carries a pVA plasmid containing the $PirA^{vp}$ and $PirB^{vp}$ binary toxin genes (Kondo et al. 2015), possibly as a result of horizontal gene transfer from a VP_{AHPND} isolate. This suggests that additional isolates of VH and perhaps isolates of other *Vibrio* species or even species of other bacterial genera may eventually be found to carry this plasmid and cause AHPND.

In addition, there are two reports from Thailand that describe presence of the pVA plasmid in many different serotypes of VP from environmental samples (Chonsin et al. 2015; Kongrueng et al. 2015). These results suggest that pVA transfer among bacteria is relatively frequent. The review of work on hepatopancreatic microsporidiosis (HPM) caused by *Enterocytozoon hepatopenaei* (EHP) presented at the FAO meeting on EMS/AHPND in Panama in June 2015 cited reports of its occurrence in Thailand (Chayaburakul et al. 2004; Tangprasittipap et al. 2013; Thitamadee et al. 2016; Tourtip et al. 2009), the People's Republic of China *(*Liu et al. 2016) and Viet Nam (Ha et al. 2010), but it has now been reported also from India (Biju et al. 2016; Santhoshkumar et al. 2016; Rajendran et al. 2016) and Indonesia (Tang et al. 2016).

These and earlier reports of unidentified but morphologically similar hepatopancreatic microsporidians in *P. monodon* from Malaysia (Anderson et al. 1989; Baticados and Enriques 1989) and in *Penaeus japonicus* Spence Bate 1888 from Australia (Hudson et al. 2001) suggest that this pathogen is enzootic in the region and has been transmitted to imported, exotic stocks of *Penaeus vannamei* Boone 1931 that were derived from specific pathogen free (SPF) stocks known to be free of EHP.

Acute Hepatopancreatic Necrosis Disease (AHPND)

Variation in VP_{AHPND} Virulence

Recent work has shown variation in the virulence of VP_{AHPND} isolates for reasons as yet unknown (Joshi et al. 2014; Lai et al. 2015). It has been suggested that such variation may be related to differences in pVA copy number (e.g. 7 to 121 copies per cell) (Han et al. 2015) or to other VP_{AHPND} virulence factors that may or may not be carried by the pVA plasmid (Sirikharin et al. 2015). However, Dr Bruno Gomez-Gil (personal communication, 2016), whose group has sequenced full genomes of many VP_{AHPND} isolates has stated that the read frequencies in their next generation sequencing data for all of the isolates indicated only one pVA plasmid copy in each isolate.

There is evidence that there are other factors (e.g. toxins) that may act to potentiate the Pir^{vp} binary toxins or may kill shrimp directly without causing the pathognomonic histopathology characteristic of AHPND (see Fig. 1). Support for this proposal can be found in a newly characterized VP isolate from Viet Nam (unpublished). It contains a mutated $Pir^{vp}A/Pir^{vp}B$ gene region on its pVA plasmid and does not produce either of the Pir^{vp} toxins, but still causes 50 % shrimp mortality without characteristic AHPND histopathology. Instead, the moribund shrimp show collapsed HP tubule epithelia (like those in Fig. 2).

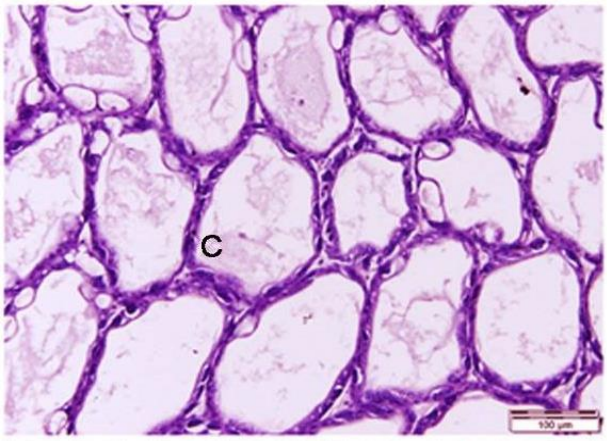

Fig. 2. Photomicrograph of a lesion resulting from shrimp challenged with a virulent VP_{AHPND} mutant that produces no Pir^{vp} toxins but still causes 50 % shrimp mortality. The lesions are characterized by collapsed HP tubule epithelia rather than the pathognomonic AHPND lesions shown in Fig. 1.

Similarly, VP isolate (2HP) obtained from an AHPND outbreak pond in Thailand also causes only 50 % shrimp mortality accompanied by lesions characterized by collapsed HP tubule epithelia (Joshi et al. 2014) similar to those seen in Fig. 2 for the Vietnamese VP_{AHPND} mutant. Thus, it is being further characterized to determine whether it is also a VP_{AHPND} mutant. At the same time, it is known that dilution of VP_{AHPND} isolate 5HP results in reduced shrimp mortality with the moribund shrimp also showing collapsed HP tubule epithelia (like those in Fig. 2) instead of pathognomonic AHPND lesions (Joshi et al. 2014).

Similar results are obtained when mixed, heterologously expressed $Pir^{vp}A$ and $Pir^{vp}B$ toxins are diluted in shrimp challenge tests (Sirikharin et al. 2015). More important, the concentration of the combined, heterologously expressed $Pir^{vp}A$ and $Pir^{vp}B$ toxins needed to cause AHPND (20 $\mu g.g^{-1}$ shrimp) was 20 times higher than the 1 $\mu g.g^{-1}$ of crude ammonium sulfate protein precipitate fraction obtained from the culture broth of the VP_{AHPND} isolate (5HP) used as the positive control (Sirikharin et al. 2015). Since the crude precipitate fraction contained many proteins, the proportion of the total 1 $\mu g.g^{-1}$ attritutable to $Pir^{vp}A$ and $Pir^{vp}B$ toxins would necessarily be much less than 1 $\mu g.g^{-1}$ each. All these examples support the proposal that other proteins produced by VP_{AHPND} potentiate the virulence of its $Pir^{vp}A/B$ toxins.

Obviously, it will be important to identify and characterize the other virulence factors that may act synergistically with $Pir^{vp}A$ and $Pir^{vp}B$ in isolates that cause AHPND. A number of putative toxin genes have been identified in the pVA plasmid (Han et al. 2015), and these would be prime candidates to study for possible synergistic activity when combined with the Pir^{vp} toxins. In addition, the description of pathology from AHPND bacteria should be expanded to include information indicating that low bacterial or Pir^{vp} toxin concentrations may lead to reduced mortality accompanied by collapsed HP tubule epithelia rather than massive sloughing of HP tubule epithelial cells seen with acute AHPND. In addition, we will need to decide whether or not VP isolates that carry mutant pVA and do not produce Pir^{vp} toxins but still kill shrimp should be included under the heading AHPND.

These examples add to the data reinforcing the need to stop using the term "early mortality syndrome" (EMS) interchangeably with AHPND. We already know that some proportion of early mortality in shrimp ponds is not the result of AHPND. For example, it may be caused by viruses such as white-spot syndrome virus (WSSV) and yellow head virus (YHV), by environmental factors (e.g. low dissolved oxygen, pesticides) or by bacterial pathogens other than those carrying an intact pVA plasmid. Thus, farmer reports of EMS cannot be equated with AHPND, and reports of AHPND must be considered suspect if they are not accompanied by confirmatory laboratory tests.

Bacterial Partners that Potentiate VP_{AHPND} Virulence

In addition to variation in virulence of AHPND bacteria themselves, it has been reported that some bacterial genera not previously associated with shrimp occur in a higher proportion in EMS ponds than in normal ponds (FAO 2013). Using this information as a starting point, a new species of bacterium from the Order Berkholderiales has been isolated from AHPND ponds in Thailand (unpublished). This bacterium (tentatively called *Delftia*-like) is lethal to shrimp with virulence equal to that of VP_{AHPND} isolate 5HP (i.e. 100 % mortality in three days at a concentration of 10^5 cfu.mL^{-1} in bath culture). However, the *Delftia*-like isolate does not cause AHPND histopathology but instead a unique histopathology of its own. This consists of vacuolated cells in the epithelium of the anterior midgut caecum (AMC**) (**Fig. 3) and sometimes also in other tissues. However, when the two isolates were mixed together in a bath challenge at 10^3 cfu each (i.e. 2 000 cfu total), 100 % mortality also occurred within three days. The 50 times lower cfu required to obtain 100 % mortality revealed a synergistic effect on virulence by mixed bath challenge. Furthermore, this mortality was not accompanied by pathognomonic AHPND histopathology and might not be diagnosed as a case of AHPND based on histological analysis of moribund shrimp in an outbreak of early mortality. It is also possible that the level of VP_{AHPND} in such a sample might be too low for detection by one-step polymerase chain reaction (PCR) analysis.

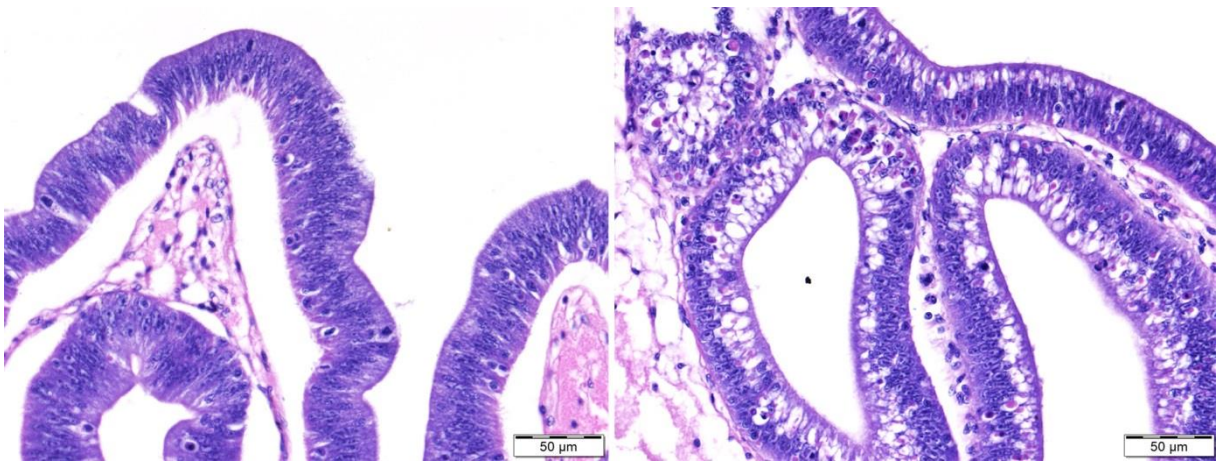

Fig. 3. Distinctive lesions found in moribund shrimp challenged by bath exposure to the *Delftia*-like bacterium. (A) Normal non-vacuolated epithelial cells of the anterior midgut caecum from negative control shrimp. (B) Abnormal vacuolated epithelial cells from a moribund shrimp specimen challenged with the *Delftia*-like bacterium.

Similar to the *Delftia*-like isolate, an isolate of *Shewanella* obtained from an AHPND pond in Thailand (unpublished) acts in a synergistic manner to increase the virulence of 5HP even though it is not itself virulent for shrimp. The result of the mixed challenge also differs in that it does cause pathognomonic AHPND histopathology, but together with a histological feature consisting of vacuolated E-cells in the HP tubule epithelium, similar to the vacuolated cells seen in the AMC with the *Delftia*-like isolate above (see Fig. 3). Thus, unlike the case of the *Delftia*-like/5HP mixture, histological analysis of moribund specimens infected with a *Shewanella*/VP_{AHPND} would be diagnosed correctly as a case of AHPND in any EMS outbreak. However, if an accompanying PCR test gave a negative result for VP_{AHPND} because of its low-level concentration, it might seem to contradict the histological diagnosis. Both the *Shewanella* and *Delftia*-like isolates lacked pVA and gave negative PCR test results for the $PirA^{vp}$ and $PirB^{vp}$ toxin genes. Thus, we suspect that they have their own virulence factors or virulence potentiaters that increase the virulence of AHPND bacteria. These factors also need to be identified. Whole genome information of the *Shewanella* isolate has recently been obtained and should assist in unravelling this phenomenon.

In summary, the pathology of AHPND and virulence of VP_{AHPND} isolates appear to be somewhat complex and many questions remain to be answered. The situation may be further complicated by the possibility of pVA plasmid transfer to other *Vibrio* species or even other genera of bacteria.

Hepatopancreatic Microsporidiosis (HPM)

Hepatopancreatic microsporidiosis (HPM) caused by *Enterocytozoon hepatopenaei* (EHP) is the other disease of major concern in Asia at this time. EHP differs from the only other microsporidian (*Agmasoma penaei*) (Flegel et al. 1992; Pasharawipas et al. 1994) earlier reported to infect *P. monodon*, *P. merquiensis* and *P. vannamei* (Laisutisan et al. 2009) in the region. However, unlike EHP, *A. penaei* does not infect shrimp tissues of endodermal origin such as cells of the HP tubule epithelium and cells of the midgut epithelium that are targeted by EHP. On rare occasions, severe infections of *A. penaei* may include expansion into connective tissue between the tubules of the hepatopancreas, but the tubule epithelial cells (endodermal origin) never become infected. In addition, *A. penaei* is not transmitted horizontally among shrimp. Instead, they are proposed, based on PCR testing, to be infected by spores originating from an alternative fish host (Pasharawipas and Flegel 1994).

The proposal is supported by successful control of the pathogen by elimination of suspected fish species from the shrimp culture system. HPM from Thailand was first reported in 2004 as an unidentified hepatopancreatic microsporidian in *P. monodon* that morphologically resembled microsporidians reported from *P. monodon* in Malaysia (Anderson et al. 1989; Baticados and Enriques 1989) and *P. japonicus* from Australia (Hudson et al. 2001). In Thailand, no correlation was found between this infection and shrimp growth at that time. It was later characterized and named as a new microsporidian species (*Enterocytozoon hepatopenaei*) (Tourtip et al. 2009).

Although it was subsequently found in shrimp exhibiting white faeces syndrome (WFS) in Thailand, it did not appear to be the direct cause of WFS (Tangprasittipap et al. 2013). However, there is evidence that HPM is associated with retarded shrimp growth that may not become clearly visible until the second or third month of cultivation. It has also been found that there is no apparent impact on growth if the copy number by quantitative PCR (qPCR) is not above 10^3 per ng DNA, after which the degree of retardation is directly proportional to the increase in copy number (Liu et al. 2016) and may result in progressive, slow mortality at numbers above 10^8 per ng DNA (Robins McIntosh, personal communication, 2016). The best way to determine EHP severity of infection is to determine its copy number using quantitative PCR (Liu et al. 2016). Estimation by counting infected HP cells containing EHP spores by normal light microscopy is not recommended for two reasons. First, the spores are very small and it is not easy to detect and confirm their presence by normal light microscopy because an oil emersion lens must be used (Fig. 4A-C). Second and more important is the fact that estimation from counting infected HP cells containing EHP spores is unreliable in determining the severity of infection because heavily infected specimens sometimes show few or no cells containing spores.

This has been revealed by staining tissue sections with haematoxylin & eosin (H&E) or chromotrope stain and comparing the results to adjacent sections assayed by *in situ* hybridization (Fig. 5). Thus, counting infected cells by presence of spores alone can lead to a great underestimation of the severity of infections. An additional revelation from *in situ* hybridization was that the distribution of EHP-infected cells in the HP can be very uneven, such that a small HP tissue sample taken from different portions of the same hepatopancreas may give opposite results (i.e. positive and negative). With qPCR, this problem must be avoided by homogenization of the whole HP followed by removal of a measured proportional sample, so that a qPCR infection index can be calculated for the whole HP of each sample. This would be particularly important for studies on the effect of infection level on shrimp growth and for comparison of infections between PL and juveniles or between small and large juveniles.

The high prevalence of EHP was not realized until an epidemiological study of EMS outbreaks was carried out in Thailand covering the period 19/08/2013 to 23/04/2014 (Boonyawiwat et al. 2016). The study included analysis of ten shrimp samples (sufficient to detect pond prevalence of EHP at 26 % or more from each of 196 shrimp cultivation ponds randomly selected prior to shrimp stocking. The results revealed an unexpected overall pond prevalence of 119/196 (61 %) that prompted an immediate warning (Sritunyalucksana et al. 2015) and follow-up studies to determine the source of infections and their impact on shrimp cultivation. More information is available in a recent review (Thitamadee et al. 2016).

There is a danger that living shrimp stocks exported from Asia or translocated within Asia for aquaculture may carry EHP, so it should be added to the list of pathogens to be monitored by quarantine authorities. PCR detection methods based on the ssu rRNA gene of EHP are available (Tourtip et al. 2009; Tangprasittipap et al. 2013; Tang et al. 2015; Itsathitphaisarn et al. 2016; Liu et al. 2016) and are suitable for testing PL and hepatopancreatic tissue of cultivated shrimp specimens.

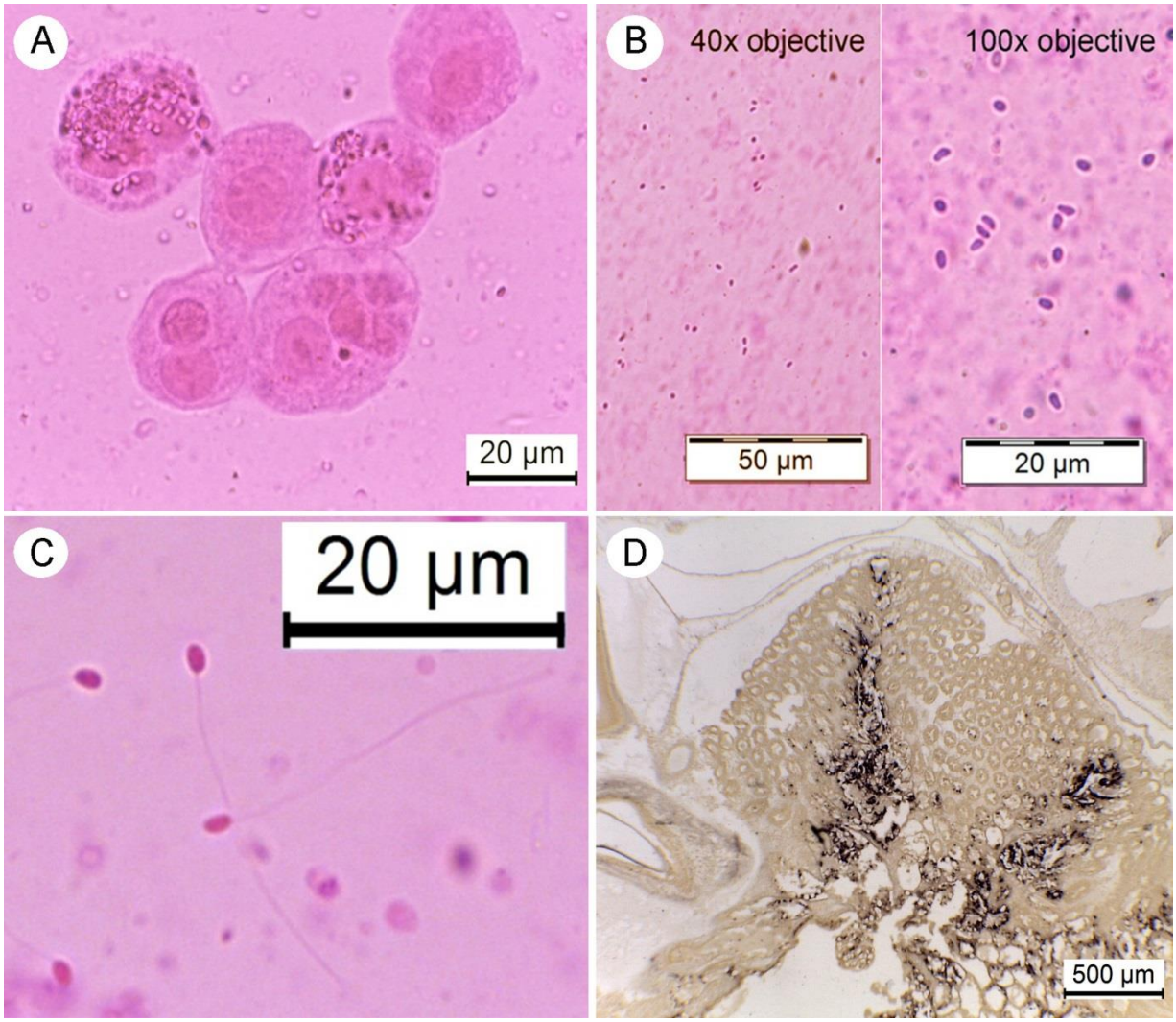

Fig. 4. Photomicrographs of EHP spores in HP squash mounts from infected PL and of *in situ* hybridization detection in HP tissue sections from an infected juvenile shrimp specimen. (A) Squash mount of HP tissue stained with 2 % phloxine B in distilled water and showing EHP spores in tubule epithelial cells. (B) Squash mount stained with phloxine B showing released spores using the 40x and 100x objectives. (C) Squash mount showing EHP spores with extruded polar tubes (also called polar filaments). (D) Positive *in situ* hybridization reactions (dark staining) with a probe targeting the EHP SSU rRNA gene and showing that the distribution of infected cells in the HP is not uniform such that the whole HP should be homogenized before subsampling to do qPCR.

However, we now know that closely related microsporidians give cross reactions with these detection methods and that they are therefore not suitable for use in testing shrimp feeds or feed ingredients when doing surveys for potential carrier species or for other environmental samples, since they may give false positive results. Instead, a recently developed nested-PCR method based on the spore wall protein gene of EHP is recommended. The method is described in detail at the website of the Network of Aquaculture Centres in Asia-Pacific, Bangkok, Thailand (Itsathitphaisarn et al. 2016) and a manuscript describing the development has been published (Jaroenlak et al. 2016).

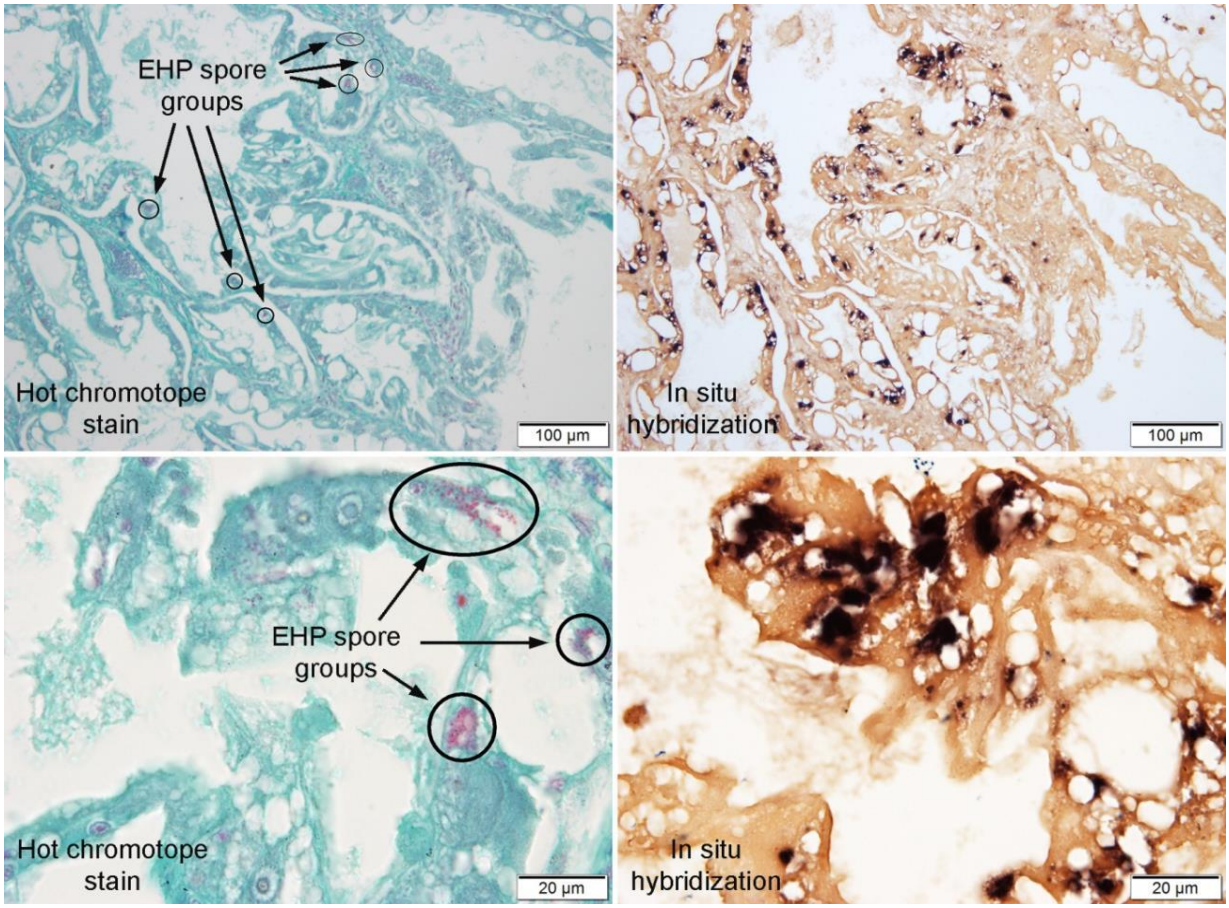

Fig. 5. Photomicrographs HP tissue of a juvenile shrimp specimen stained to reveal EHP-infected cells by using the chromotrope method (Weber et al. 1992; Moura et al. 1997) (green counterstain) for detecting the presence of spores (red staining) and contrasted with an adjacent HP tissue section stained to reveal EHP-infected cells by positive (dark staining) *in situ* hybridization reactions (Bismark brown counterstain). It is clear that using spore detection alone would give a gross underestimation of the number of infected cells in this specimen. The lower pair of photomicrographs are simply enlargements of matching portions of the upper pair.

It has been tested with DNA from microsporidian species closely related to EHP and does not cross-react with them as the ssu rRNA method does. The spore-wall protein gene sequence was obtained from DNA extracted from purified EHP spores obtained from infected shrimp by density gradient separation. The purified DNA was used to prepare a draft genome that has been submitted for publication (Boakye et al. submitted). It is hoped that sequence information will provide insights suitable for use in developing potential targets for HPM prevention and therapy.

The availability of the new spore wall protein PCR (SWP-PCR) method makes it possible to screen not only cultured shrimp but also suspected carriers and environmental samples for EHP with good assurance that false positive results will not be obtained from closely related microsporidians. At the same time, any PCR-positive, putative carriers should be tested by *in situ* hybridization to determine whether they are infected or mechanical carriers. This has not yet been done with the currently suspected carriers such as polychaetes but should now be possible using PCR-positive polychaetes from natural sources or using the co-habitation method of infected shrimp separated from uninfected polychaetes.

So far, there is no recommended or approved chemotherapy for HPM, and the main focus for control is on supply of PCR-negative PL cultivated in ponds where appropriate biosecurity measures have been taken to exclude EHP. However, EHP is horizontally transmissible by feeding of infected HP tissue or by cohabitation of infected shrimp separated by a mesh cage from naïve shrimp in the laboratory. The latter method is particularly suitable for testing of proposed chemicals and reagents designed to prevent transmission. Other methods for control of EHP in shrimp hatcheries and on farms have been summarized elsewhere (Sritunyalucksana et al. 2015; Thitamadee et al. 2016).

In summary, a highly specific and reliable nested PCR detection method is now available for use in screening broodstock, PL and juvenile shrimp for EHP infections and for testing environmental samples for EHP reservoirs. Thus, it should be possible to produce EHP-free PL to supply shrimp farmers by the proper use of SPF shrimp stocks with a history for freedom from EHP. SPF stocks of *P. vannamei* or *P. monodon* imported into Asian countries for production of PL in local hatcheries are at great risk of contamination by EHP unless held under strict hatchery quarantine. Work is urgently needed to find potential reservoirs for EHP and to test methods of prevention or therapy using the cohabitation model.

References

Anderson, I.G., M. Shariff and G. Nash. 1989. A hepatopancreatic microsporidian parasite in pond-reared tiger shrimp, *Penaeus monodon*, from Malaysia. Journal of Invertebrate Pathology 53:278–280.

Baticados, M. and G. Enriques. 1989. A hepatopancreatic microsporidian in pond-reared tiger shrimp, *Penaeus monodon*, from Malaysia. Journal of Invertebrate Pathology 53:278–280.

Biju, N., G. Sathiyaraj, M. Raj, V. Shanmugam, B. Baskaran, U. Govindan, G. Kumaresan, K.K. Kasthuriraju and T.S.R.Y. Chellamma. 2016. High prevalence of *Enterocytozoon hepatopenaei* in shrimps *Penaeus monodon* and *Litopenaeus vannamei* sampled from slow growth ponds in India. Diseases of Aquatic Organisms 120:225–230.

Boonyawiwat, V., T. Patanasatienkul, J. Kasornchandra, C. Poolkhet, S. Yaemkasem, L. Hammell and J. Davidson. 2016. Impact of farm management on expression of early mortality syndrome/acute hepatopancreatic necrosis disease (EMS/AHPND) on penaeid shrimp farms in Thailand. Journal of Fish Diseases DOI: 10.1111/jfd.12545.

Boakye, D.W., A. Prachumwat, P. Jaroenlak, T.A. Williams, K.S. Bateman, O. Itsathitphaisarn, K. Sritunyalucksana, A. Prachumwat, K.H. Paszkiewicz, K.A. Moore, G.D. Stentiford and B.A.P. Williams. Comparative genomics and disappearing glycolysis in the opportunistic and enigmatic Enterocytozoonidae. Environmental Microbiology 19:2077–2089.

Chayaburakul, K., G. Nash, P. Pratanpipat, S. Sriurairatana and B. Withyachumnarnkul. 2004. Multiple pathogens found in growth-retarded black tiger shrimp *Penaeus monodon* cultivated in Thailand. Diseases of Aquatic Organisms 60:89–96.

Chonsin, K., S. Matsuda, C. Theethakaew, T. Kodama, J. Junjhon, Y. Suzuki, O. Suthienkul and T. Iida. 2015. Genetic diversity of *Vibrio parahaemolyticus* strains isolated from farmed Pacific white shrimp and ambient pond water affected by acute hepatopancreatic necrosis disease outbreak in Thailand. FEMS Microbiology Letters DOI: 10.1093/femsle/fnv222.

Enríquez-Espinoza, T., F. Mendoza-Cano, T. Encinas-García and A. Sánchez-Paz. 2016. Molecular epidemiology of selected infectious diseases caused by bacteria in juveniles and post-larvae of the white shrimp *Penaeus vannamei* from the north-western coast of Mexico. Journal of Fish Diseases DOI: 10.1111/jfd.12473.

FAO. 2013. Report of the FAO/MARD Technical workshop on early mortality syndrome (EMS) or acute hepatopancreatic necrosis syndrome (AHPND) of cultured shrimp (under TCP/VIE/3304), Hanoi, Viet Nam, 25–27 June 2013. pp. 1–54. FAO Fisheries and Aquaculture Report No. 1053. FAO, Rome.

Flegel, T.W., S. Boonyaratpalin, D.F. Fegan, M. Guerin and S. Sriurairatana. 1992. High mortality of black tiger prawns from cotton shrimp disease in Thailand. In Diseases in Asian aquaculture I. (eds M. Shariff, R.P. Subasinghe and J.R. Arthur), pp. 181–197. Fish Health Section, Asian Fisheries Society, Manila.

Ha, N.T., D.T. Ha, N.T. Thuy and V.T.K. Lien. 2010 Occurrence of Microsporidia *Enterocytozoon hepatopenaei* in white feces disease of cultured black tiger shrimp (*Penaeus monodon*) in Vietnam. Aquatic Animal Health http://hadong86.wordpress.com/.

Han, J.E., F.F.J. Tang, D.V. Lightner and L. Tran. 2015. *Photorhabdus* insect related (Pir) toxin-like genes in a plasmid of *Vibrio parahaemolyticus*, the causative agent of acute hepatopancreatic necrosis disease (AHPND) of shrimp. Diseases of Aquatic Organisms 113:33–40.

Hudson, D.A., N.B. Hudson and S.B. Pyecroft. 2001. Mortalities of *Penaeus japonicus* prawns associated with microsporidean infection. Australian Veterinary Journal 79:504–505.

Itsathitphaisarn, O., P. Jaroenlak, P. Sanguanrut, P.V. Salachan, D. Wiredu-Boakye, B.A.P. Williams, G.D. Stantiford, T.W. Flegel, and K. Sritunyalucksana. 2016. A new and improved PCR detection method for *Enterocytozoon hepatopenaei* (EHP) based on a gene encoding a spore wall protein. Network of Aquaculture Centres in Asia-Pacific, Bangkok, Thailand. http://www.enaca.org/modules/library/publication.php?publication_id=1177 g.

Jaroenlak, P., P. Sanguanrut, B.A. Williams, G.D. Stentiford, T.W. Flegel, K. Sritunyalucksana, and O. Itsathitphaisarn. 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: e0166320.

Joshi, J., J. Srisala, V.H. Truong, I.T. Chen, B. Nuangsaeng, O. Suthienkul, C.F. Lo, T.W. Flegel, K. Sritunyalucksana and S. Thitamadee. 2014. Variation in *Vibrio parahaemolyticus* isolates from a single Thai shrimp farm experiencing an outbreak of acute hepatopancreatic necrosis disease (AHPND). Aquaculture 428–429:297–302.

Kondo, H., P.T. Van, L.T. Dang and I. Hirono. 2015. Draft genome sequence of non-*Vibrio parahaemolyticus* acute hepatopancreatic necrosis disease strain KC13.17.5, isolated from diseased shrimp in Vietnam. Genome Announcements 3:e00978-00915.

Kongrueng, J., M. Yingkajorn, S. Bunpa, N. Sermwittayawong, K. Singkhamanan and V. Vuddhakul. 2015. Characterization of *Vibrio parahaemolyticus* causing acute hepatopancreatic necrosis disease in southern Thailand. Journal of Fish Diseases 38:957–966.

Lai, H.-C., T.H. Ng, M. Ando, C.-T Lee, I.-T. Chen, J.-C. Chuang, R. Mavichak, S.-H. Chang, M.-D. Yeh and Y.-A. Chiang. 2015. Pathogenesis of acute hepatopancreatic necrosis disease (AHPND) in shrimp. Fish and Shellfish Immunology 47:1006–1014.

Laisutisan, K., S. Prasertsri, N. Chuchird and C. Limsuwan. 2009. Ultrastructure of the microsporidian *Thelohania (Agmasoma) penaei* in the Pacific white shrimp (*Litopenaeus vannamei*). Kasetsart University Fisheries Research Bulletin 33:41–48.

Lee, C.-T., I.T. Chen, Y.-T. Yang, T.-P. Ko, Y.-T. Huang, J.-Y. Huang, M.-F. Huang, S.-J. Lin, C.-Y. Chen, S.-S. Lin, D.V. Lightner, H.-C. Wang, A.H.J. Wang, H.-C. Wang, L.-I. Hor and C.-F. Lo. 2015. The opportunistic marine pathogen *Vibrio parahaemolyticus* becomes virulent by acquiring a plasmid that expresses a deadly toxin. Proceedings of the National Academy of Sciences of the United States of America 112:10798-10803.

Liu, Z., Q.-L. Zhang, X.-Y. Wan and J. Huang. 2016. Development of real-time PCR assay for detection of microsporidian *Enterocytozoon hepatopenaei* and detection in shrimp samples under different growth rates. Progress in Fishery Sciences 37:119–126.

Moura, H., D.A. Schwartz, F. Bornay-Llinares, F.C. Sodre, S. Wallace and G.S. Visvesvara. 1997. A new and improved "Quick-Hot Gram-Chromotrope" technique that differentially stains microsporidian spores in clinical samples, including paraffin-embedded tissue sections. Archives of Pathology and Laboratory Medicine 121:888–893.

Nunan, L., D. Lightner, C. Pantoja and S. Gomez-Jimenez. 2014. Detection of acute hepatopancreatic necrosis disease (AHPND) in Mexico. Diseases of Aquatic Organisms 111:81–86.

Pasharawipas, T. and T.W. Flegel. 1994. A specific DNA probe to identify the intermediate host of a common microsporidian parasite of *Penaeus merguiensis* and *P. monodon*. Asian Fisheries Science 7:157–167.

Pasharawipas, T., T.W. Flegel, S. Chaiyaroj, S. Mongkolsuk and S. Sirisinha. 1994. Comparison of amplified RNA gene sequences from microsporidian parasites (*Agmasoma* or *Thelohania*) in *Penaeus merguiensis* and *P. monodon*. Asian Fisheries Science 7:169–178.

Rajendran, K., S. Shivam, P.E. Praveena, J.J.S. Rajan, T.S. Kumar, S. Avunje, V. Jagadeesan, S.P. Babu, A. Pande and A.N. Krishnan. 2016. Emergence of *Enterocytozoon hepatopenaei* (EHP) in farmed *Penaeus (Litopenaeus) vannamei* in India. Aquaculture 454:272–280.

Santhoshkumar, S., S. Sivakumar, S. Vimal, S. Abdul Majeed, G. Taju, P. Haribabu, A. Uma and A. Sahul Hameed. 2016. Biochemical changes and tissue distribution of *Enterocytozoon hepatopenaei* (EHP) in naturally and experimentally EHP-infected whiteleg shrimp, *Litopenaeus vannamei* (Boone, 1931), in India. Journal of Fish Diseases DOI: 10.1111/jfd.12530.

Sirikharin, R., S. Taengchaiyaphum, P. Sanguanrut, D.C. Thanh, R. Mavichak, P. Proespraiwong, B. Nuangsaeng, S. Thitamadee, T.W. Flegel and K. Sritunyalucksana. 2015. Characterization and PCR detection of binary, Pir-like toxins from *Vibrio parahaemolyticus* isolates that cause acute hepatopancreatic necrosis disease (AHPND) in shrimp. PLoS ONE 10:e0126987.

Soto-Rodriguez, S.A., B. Gomez-Gil, R. Lozano-Olvera, M. Betancourt-Lozano and M.S. Morales-Covarrubias. 2015. Field and experimental evidence of *Vibrio parahaemolyticus* as the causative agent of acute hepatopancreatic necrosis disease of cultured shrimp (*Litopenaeus vannamei*) in northwestern Mexico. Applied and Environmental Microbiology 81:1689–1699.

Sritunyalucksana, K., P. Sanguanrut, P.V. Salachan, S. Thitamadee and T.W. Flegel. 2015. Urgent appeal to control spread of the shrimp microsporidian parasite *Enterocytozoon hepatopenaei* (EHP). Network of Aquaculture Centres in Asia-Pacific (NACA). http://www.enaca.org/modules/news/article.php?article_id=2039.

Tang, K.F., J.E. Han, L.F. Aranguren, B. White-Noble, M.M. Schmidt, P. Piamsomboon, E. Risdiana and B. Hanggono. 2016. 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. Journal of Invertebrate Pathology 140:1–7.

Tang, K.F.J., C.R. Pantoja, R.M. Redman, J.E. Han, L.H. Tran and D.V. Lightner. 2015. Development of *in situ* hybridization and PCR assays for the detection of *Enterocytozoon hepatopenaei* (EHP), a microsporidian parasite infecting penaeid shrimp. Journal of Invertebrate Pathology 130:37–41.

Tangprasittipap, A., J. Srisala, S. Chouwdee, M. Somboon, N. Chuchird, C. Limsuwan, T. Srisuvan, T.W. Flegel and K. Sritunyalucksana. 2013. The microsporidian *Enterocytozoon hepatopenaei* is not the cause of white feces syndrome in whiteleg shrimp *Penaeus (Litopenaeus) vannamei*. BMC Veterinary Research 9:139.

Thitamadee, S., A. Prachumwat, J. Srisala, K. Sritunyalucksana, T.W. Flegel and O. Itsathitphaisarn. 2016. Review of current disease threats for cultivated penaeid shrimp in Asia. Aquaculture 452:69–87.

Tourtip, S., S. Wongtripop, G.D. Stentiford, K.S. Bateman, S. Sriurairatana, J. Chavadej, K. Sritunyalucksana and B. Withyachumnarnkul. 2009. *Enterocytozoon hepatopenaei* sp. nov. (Microsporida: Enterocytozoonidae), a parasite of the black tiger shrimp *Penaeus monodon* (Decapoda: Penaeidae): fine structure and phylogenetic relationships. Journal of Invertebrate Pathology 102:21–29.

Tran, L., L. Nunan, R.M. Redman,L.L. Mohney, C.R. Pantoja, K. Fitzsimmons and D.V. Lightner. 2013. Determination of the infectious nature of the agent of acute hepatopancreatic necrosis syndrome affecting penaeid shrimp. Diseases of Aquatic Organisms 105:45–55.

Weber, R., R.T. Bryan, R.L. Owen, C.M. Wilcox, L. Gorelkin and G.S. Visvesvara. 1992. Improved light-microscopical detection of microsporidia spores in stool and duodenal aspirates. The Enteric Opportunistic Infections Working Group. New England Journal of Medicine 326:161–166.