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TECHNICAL ADVISORY ON STEPS FOR FIRST TIME CONFIRMATION OF AN EXOTIC DISEASE – A CASE STUDY WITH AHPND/EMS

Summary

A step by step protocol for the first time confirmation of an exotic disease taking AHPND / EMS as an example has been described. Until the identification of the etiological agent of AHPND, histopathology was regarded as a sole diagnostic method to declare the presence of AHPND/EMS.PCR protocols have been described to identify the causative agent. However, both these tools require to be supported with experimental infection to prove Koch's postulates when the disease is declared for the first time. A systematic mechanism for disease validation along with diagnostic protocol has been provided in this advisory. This document is expected to be used both by researchers and policy makers while taking a final decision on the status of any exotic disease.

Introduction

There are no specific guidelines for reporting exotic disease for the first time in the country. Due to this, both researchers and policy makers many times provide inconclusive and conflicting statements. Sometimes the status of an exotic disease in a country is based on a publication which has not been validated in another laboratory. One example is the stated presence of yellow head disease of shrimp in India in OIE publications which was published based on histopathology investigations (Mohan *et al*., 1998, Dis.Aquat. Org., 34: 9-12). However, as per OIE Manual of Diagnostic Tests for Aquatic Animals (2013), histopathology should be used for presumptive diagnosis of YHD, and a combination of diagnostic tests is necessary for confirmation of any disease. Subsequent studies have revealed presence of only genotype 4 of yellow head complex of viruses in the country (NBFGR Annual Report, 2009-2010; Wijegoonawardane *et al*., 2008, Virology, 380: 213-225). This YHV genotype 4 occurs commonly in healthy *Penaeus monodon* and is rarely or never associated with the disease (OIE 2013). Taking this into consideration, we want to highlight the issues regarding declaration of any exotic disease for the first time in the country. In the wake of conflicting reports on the presence of AHPND/EMS in India, we here propose technical guidelines for systematic diagnosis and validation in more than one laboratory before declaring the presence of this or any other new disease.

Early Mortality Syndrome (EMS) / Acute Hepatopancreatic Necrosis Disease (AHPND) has caused mass mortalities of shrimp in some countries in Asia. Central Institute of Brackish water Aquaculture (CIBA) along with other partners in India, are constantly engaged in surveillance for the detection of aquatic animal diseases. Emerging issues in Asian region including EMS/AHPND in shrimp has also been taken care by initiating targeted surveillance. So far we have not come across any case that conclusively establishes the presence of EMS/AHPND in India. The outcome of this surveillance on AHPND has been published (CIBA e-publication series 19; originally published in April 2013 and subsequently revised in January 2014 and recently in 2016) in CIBA's website (www.ciba.res.in).

The present technical advisory is primarily targeted towards researchers who are working on or likely to work on AHPND/EMS in India. This document can be also used by the policy makers to take a decision when conflicting reports on AHPND/EMS from different sources in India are received and evaluate the mechanism suggested to officially confirm the presence AHPND/EMS in the country. The advisory is intended to:

1. Highlight limitations of histopathology and PCR for diagnosis of AHPND/EMS.
2. Increase awareness on PCR methods available for detection of AHPND/EMS.
3. What diagnostic steps to carry out for confirmation of AHPND/EMS in the country for the first time *vs* subsequent routine diagnostics for general disease surveillance.

1. Limitations of histopathology and PCR for diagnosis of EMS/AHPND

Histopathological features of the late stage of* AHPND/ EMS *may be confused with other bacterial diseases

Initially it was proposed that histopathology is the only method to confirm detection of AHPND/EMS and is very specific to this disease. However, there is some possibility of confusion, if the pathognomonic histopathology of AHPND at the acute stage of the disease is not seen in the specimens examined. The confusion results because histopathology for the late stage of AHPND is very similar to that for other bacterial diseases infections (e.g., Anderson, 1988, Asian Fish. Sci., 2: 93-108; Robertson *et al*., 1998, Dis.Aquat. Org., 32: 151–155; Song *et al*., 1993, J. Invert.Pathol., 61: 24-31; Lavilla-pitogo *et al*., 1998, Aquaculture, 164:337-349; Esteve and Herrera, 2000, J. Invert. Pathol.,76: 1-5; Martin *et al.,* 2004, Dis. Aquat. Org., 60: 21–29; Alavandi *et al.,* 2008, Dis. Aquat. Org., 81: 163–171). Thus, confirmation of histopathology for the acute stage of infection is necessary to differentiate AHPND from other bacterial diseases. Opinion sought from Dr. D.V. Lightner and Prof. T. W. Flegel indicates that massive sloughing of epithelial cells into the lumen in the acute phase [early infection when bacteria are not found in the hepatopancreas (HP)] is the characteristic feature that distinguishes AHPND from other bacterial diseases. Moreover as suggested in the FAO-MARD EMS/AHPND conference and reiterated by Prof. Flegel, a minimum of 10 and possibly more shrimp from a single suspected AHPND outbreak pond may be necessary to find at least one specimen that clearly shows this characteristic histopathology needed to confirm a diagnosis for EMS/AHPND. Only the acute phase of AHPND clearly distinguishes from other bacterial diseases. ***Therefore, only massive sloughing of HP epithelial cells in the absence of bacteria can differentiate AHPND from other bacterial diseases / infections. However, for the first confirmed report of AHPND / EMS in the country, the steps and institutional mechanism given in this advisory should be followed.***

2. PCR methods available for the detection of *Vibrio parahaemolyticus* strain causing AHPND/EMS

Prof. T.W. Flegel, Center of Excellence for Shrimp Molecular Biology and Biotechnology, Faculty of Science, Mahidol University along with Prof. C. F. Lo, College of Bioscience and Biotechnology, National Cheng Kung University, Taiwan published a PCR protocol along with two primer sets for interim detection of AHPND bacterial isolates in December 2013, based on the sequence information generated by them from extra chromosomal DNA of the *Vibrio parahaemolyticus* isolates shown to cause AHPND/ EMS in bioassays. After performing several validation trials with a number of samples, they standardized the protocol and dedicated it to the scientific community for free use. This method and other information on AHPND/EMS was available in the Network of Aquaculture Centres in Asia-Pacific website (www.enaca.org). PCR test is carried out using DNA extracted from tissue samples (hepatopancreas/stomach) or DNA from bacterial isolates. The purpose of the test was to support results from histological analysis and to reduce the number of bacterial isolates that need to be subjected to bioassays. The initial targets (AP1 and AP2) were producing false positive to some extent and therefore one more specific primer (AP3) was designed by the same group and was made available since June 2014. Very recently a more sensitive and specific PCR method (AP4) has also been developed by the same group which will be more useful where enrichment protocol is not possible. Until now, no positive results were obtained from any of the shrimp samples tested by CIBA, College of Fisheries, Mangalore, C. Abdul Hakeem College, Melvisharam and Fisheries College and Research Institute, Thoothukudi.

Another PCR method has been developed by the University of Arizona, USA by Dr. D.V. Lightner's group which is available commercially and currently marketed by M/S Gene Reach Biotechnology Corporation, Taiwan.

However, sometimes the PCR method can provide false positive if all the protocols are not followed properly or through some contamination.

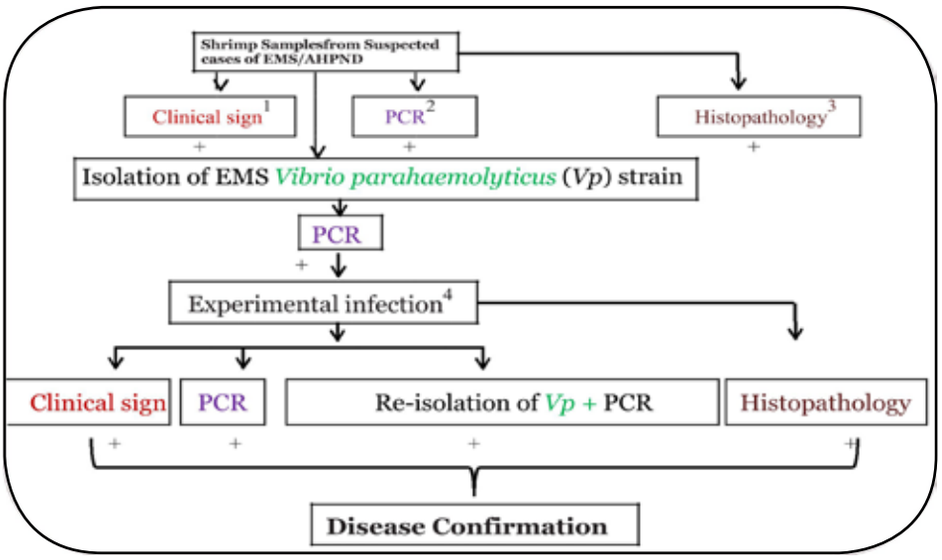
Vibrio parahaemolyticus is ubiquitous and there is frequent exchange of genetic material

V. parahaemolyticus strains are widely distributed in the estuarine and coastal environment and the ecology of the specific *V. parahaemolyticus* strain that cause EMS /AHPND is not understood. EMS / AHPND causing isolates are characterized by the presence of one or more extra chromosomal elements and the current diagnostic PCR methods are based on sequences in these elements. It is believed that false positive PCR test results for AHPND isolates sometimes arise because the extra chromosomal elements do not always contain the key virulence factor(s) that cause AHPND. It is well established that extra chromosomal elements are common in bacteria and can be frequently exchanged between them in the natural environment. Therefore, caution must be exercised in conclusions based simply on their presence or absence (Raghunath *et al.,* 2010, FEMS Microbiol.Lett., 307: 151-57; Ruwandeepika *et al.,* 2010, J. Appl. Microbiol., 109: 888-99). It has been shown in the AHPND research that many natural isolates of *V. parahaemolyticus* do not cause any disease in immersion challenges. Thus, it is likely that such isolates will also be found in India. Based on the work done so far, the new interim PCR methods have a high probability (i.e., have low false positive results) of detecting *V.parahaemolyticus* strains with the extra chromosomal element associated with AHPND and from those that do not. This will reduce the number of bacterial isolates that need to be subjected to immersion bioassays.

3. Steps for declaring first confirmed report of EMS/ AHPND in the country and institutional mechanism for confirmation

Considering the above observations, a systematic and holistic approach is necessary for the detection and confirmation of a new disease. With this objective, the Central Institute of Brackishwater Aquaculture in consultation with collaborators suggests a method as outlined in the figure below.

Steps for first time confirmation of AHPND/EMS



1: NACA disease advisory on EMS, 2012.

2: PCR detection of EMS/AHPND, Sirikharin *et al.,* 2014, Dangtip *et al.,* 2015

3: Lightner *et al.*, 2012, Glob Aquacult Advocate Jan/Feb 2012: 40,

4: Tran *et al.*, 2013, Dis. Aquat Org., 105: 45-55

Fig 1: Flow chart for the 1st time confirmation of EMS/AHPND. The PCR detection method has been added to the original approach (FAO FIRA/R1053, June 2013)

It is necessary that all the steps outlined in Fig.1 need to be strictly adhered to while reporting AHPND for the first time. OIE always recommends a combination of methods for confirmation of any listed disease. Since this is a bacterial disease, it is compulsory to reproduce the disease by the isolated suspected agent and one should also be able to re-isolate the same causative agent again from the experimentally infected animals. This approach as recommended and suggested by FAO/MARD workshop on EMS/AHPND, was followed by Dr Lightner's group who attributed it to a specific strain of *V. parahaemolyticus* that was responsible for causing AHPND/EMS in Vietnam. AHPND/EMS affected shrimp can be colonized by a large number of different bacteria which might include both AHPND/EMS specific and nonspecific *V. parahaemolyticus*. Hence it is advisable to screen at least 10 green colonies to identify the specific strain of AHPND/EMS *V. parahaemolyticus* if suspected in the sample.

As an additional safety mechanism, it is also advisable to revalidate the findings of one institute/organization by at least one more institute/organisation, especially by a blind test, to further confirm the result. The positive samples and the bacterial isolates that are obtained by an institute should be maintained and shared with other institutes either for verification or use as a positive control.

In view of the uncertainties identified in the diagnosis regarding presence of AHPND/EMS, it will be appropriate to have all the requirements fulfilled before confirming the presence of this or any other new disease in the country. For subsequent routine surveillance, PCR can be used for the identification of the disease.

The declaration of the presence of AHPND/EMS or any new disease should be made only by the Competent Authority i.e. Department of Animal Husbandry, Dairying and Fisheries, Ministry of Agriculture and Farmer's Welfare, Government of India. Any publication regarding the first report of the disease in India should have prior approval of the Competent Authority.

CIBA and its collaborators are thankful to Dr T.W. Flegel for providing non-infectious positive controls and for clarifying technical aspects. For further clarification and assistance, contact:

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Research on brackishwater aquatic animal health and environment was initiated at the Central Institute of Brackishwater Aquaculture since 1990. Since then it has grown in terms of expertise, manpower and infrastructure. The Aquatic Animal Health and Environment Division or the AAHED in short, has scientists with all relevant specialities and expertise in Microbiology, Virology, Pathology, Parasitology, Biotechnology, Molecular Diagnostics, Soil and water Chemistry, Environment and Aquaculture. The AAHED has well established laboratory facilities for carrying out cutting edge research in molecular biology in addition to aquatic animal health and environment management including diagnostics, prophylactics and health management in brackishwater aquaculture. The advanced facilities have been developed with funding support from ICAR, National Agricultural Research Project (NARP), World Bank, National Agricultural Technology Project (NATP), Department of Biotechnology and National Fisheries Development Board with dedicated efforts of scientists. A well designed wet lab is also in place for carrying out live aquatic animal experiments and evaluating Koch's and River's postulates.

The AAHED, CIBA has the mandate to carry out research on (a) economically impacting diseases of brackishwater culture species and develop technologies for rapid diagnosis, prophylaxis and control; (b) brackishwater environment and develop mitigatory measures as required; and (c) provide technical and policy support to the Government on matters pertaining to aquatic animal health and environment management to improve productivity.

The AAHED of CIBA was the first to commercialise a white spot syndrome diagnostic kit to a premier Biotechnology company in the year 2002. The AAHED also produced kit for diagnosis of white tail disease in scampi in the year 2004. AAHED has the expertise and capacity to carry out all the proposed levels of Diagnostics of OIE listed Brackishwater pathogens and has been serving as a National Referral Laboratory.

The environment section of AAHED has the expertise to look into all aspects of abiotic parameters. Novel methods have been developed for the bioremediation and environmental monitoring of the brackishwater rearing systems, including hatcheries and farms. The unit also has expertise in climate related matters and has developed climate smart solutions for brackishwater farming systems. The section has also capacity for the environmental impact assessment and carrying capacity assessment of source waters for optimisation of brackishwater aquaculture development.

AAHED, CIBA has published over 60 research publications in peer reviewed national and international journals, produced 15 Ph. Ds, who are currently employed in key positions in various Institutions in India and abroad.

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