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EVALUATION OF A DETECTION SYSTEM OF YELLOW HEAD DISEASE VIRUS (YHV) USING Q-PCR

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Abstract - Yellow head disease is caused by the yellow head virus (YHV). This important disease affects *Penaus monodon* farms in Thailand. In Mexico, reports of this disease in *L. vannamei* have been issued. This has not been officially declared by the World Organization for Animal Health (OIE). This study reports a method of reverse-transcription polymerase chain reaction for the detection of this virus in shrimp. A certified sample was analyzed with a commercial detection system for YHV. We obtained a differential sequence of genotypes that cause both YHV and gill-associated virus (GAV) by comparison with ClustalW. Primers were designed for amplification of the fragment by a TaqMan probe with which a positive standard to YHV was amplified. Tests were negative for other pathogens. A survey of shrimp farms in Mexico in 2009 showed negative results for YHV presence. These results demonstrate that the system developed in this study is a specific diagnostic method, sensitive and reproducible for detection of YHV.

Key words: Probe; q-PCR; prevalence.

INTRODUCTION

The yellow head virus (YHV) is an emerging disease in cultured shrimp and was first reported in Thailand in 1992, attacking black tiger shrimp, *Penaeus monodon* (Limsuwan, 1991). However, YHV has been present in shrimp farms of various Asian countries. Currently, in Mexico the presence of YHV has been reported on the northwest coast of Sonora, Sinaloa and Nayarit (De la Rosa Velez et al., 2006; Castro Longoria et al., 2008; Barajas Sanchez, 2009), however, this is an apparently avirulent genotype (Lightner, 2012). This virus has caused large economic losses in countries such as Thailand and Vietnam. In 1995 it reached 5 000 tm, which about $40 million in losses (Lightner, 1996). It has been described in the People's Republic of China, India, Indonesia, Malaysia, Philippines, Sri Lanka, Taiwan, Thailand, and Vietnam (Lightner, 1996; Lightner and Pantoja, 2001; Owens, 1997). Now white spot syndrome virus (WSSV) is considered the most important pathogen in terms of production losses due to the fact that it is lethal to all species and also for its fast growth and high mortality (Flegel., 2006a). YHV is the second most severe (Dhar et al., 2004; Sittidilokratna., 2008; Walker et al., 2005) as it causes rapid and high mortality for both *P. monodon* (Boonyaratpalin et al., 1993; Chantanachookin et al., 1993) and *L. vannamei* (Senapin et al., 2010; Sittidilokratna et al., 2009). For the latter, there are reports that show the presence of shrimp

with signs of disease on farms in the central part of Thailand where they produced economic losses up to $3 million in late 2007 and early 2008 (Senapin et al., 2010).

Yellow head disease is caused by YHV, which is an RNA virus of positive single-stranded, enveloped, bacilliform about 40-60 nm x 150-200 nm with prominent projections and a striated nucleocapsid. It presents 26 662 bp, and contains three structural proteins – gp116 (110-135 kDa), gp64 (63-67 kDa) and p20 (20-22 KDa). Gp116 and gp64 are glycoproteins of the envelope, while p20 is the viral nucleocapsid protein (Stentiford et al., 2009). It is classified within the genus *Okavirus*, *Roniviridae* family within the order *Nidovirales* (Liu et al., 2009). It is known to be related to *Torovirus*, *Arterivirus* and *Coronavirus* (Jitrapakdee, et al., 2003). In addition, it is closely related to GAV, a genotype that occurs commonly in healthy shrimp in Australia, Thailand and Vietnam and has been associated with the syndrome of the average harvest mortality (Sittidilokratna et al., 2009; Walker et al., 2001) which together with YHV and four more genotypes make up the yellow head complex. YHV and GAV, although closely related viruses, present in their genome considerable differences by which they have been cataloged as geographic topotypes.

According to Wijegoonawardane et al. (2008), there are at least six distinct genetic lines of YHV distributed in most parts of the natural geographic range of *P. monodon* from Africa through South and Southeast Asia to eastern Australia. The external signs associated with yellow head disease include yellowish coloration of the cephalothorax and gills, cessation of feeding and erratic swimming on the surface of ponds (Jitrapakdee, et al., 2003). The lymphoid organ of dying bodies shows evidence of necrosis and cells with nuclear hypertrophy and basophilic cytoplasmic inclusions (Lu et al., 1995; Longyant et al., 2005; Jitrapakdee et al., 2003).

Although clinical signs of both YHV and GAV genotypes, which are the main components of the yellow head complex, are very similar and can be differentiated by molecular techniques, in this study, a detection system alternative to the already existing was performed to achieve the detection of YHV without interference from other genotypes existing inside the complex of yellow head, and this will allow a timely and effective detection of the pathogen.

MATERIALS AND METHODS

Isolated YHV

The YHV used in this study was obtained from a standard sample that was inoculated into the species *L. vannamei* in the facilities of the pathology laboratory of aquaculture at the University of Arizona. The pathogens WSSV, IHHNV and streptococcus were obtained from the aquatic health laboratory of the Technological Institute of Sonora.

RT-PCR in real time

Primer3 software was used to design the primers. TaqMan probe of the region located between ORF1b and ORF2 of the YHV genome (accession number in GenBank AF102829) YHVIF primers (5'-GGTTTTAGGTGACATTAGGGTCT-3') and YHVIR (5'-GATGATTGAAAGGGATTGTCG-3 ') produced a 268 bp fragment. YHV-TM probe (5'-6FAM CGAAGTGACTATGCGCCTTCCACC-BBQ-650) was synthesized and labeled with the dye 5-carboxyfluorescein (FAM) at the 5 'end and with 8-alkoxyjulolidine at the 3' end. The YHV fragment was amplified using Fast Real Time 7500 (Applied Biosystems). Before amplification, RNA was extracted with a commercial system, the reaction mixture containing 2.5 µl of RNA, 5 µl of master mix, 0.5 µl of a 10 µM TaqMan probe, 0.25 µl of each primer at a concentration of 5 µM and 1.5 µl of water, for a final reaction volume of 10 µl. The amplification profile of RT-PCR was a preincubation for 10 min at 95°C followed by 45 cycles of 10 s at 95°C, 30 s at 60°C and 1 s at 72°C.

Analysis of prevalence

A random sampling was performed using cast net capture during the growing season in 2009, in the

State of Sonora. These were used examined by real-time PCR and a commercial system for YHV detection.

RESULTS

Preparation of cDNA

Conversion to cDNA for amplification with the alternative system was performed with the two primers that have been reported, the oligo DT for amplification of the total mRNA, and Gy5 for amplifying YHV within the genome of the virus of the yellow head disease with which similar results were obtained as for subsequent amplification efficiency with different detection systems.

The quantification of the reference sample was carried out with the commercial system that was obtained in a concentration of $3.8x10^{u}$ copies/μL. The sample was analyzed with both systems, obtaining similar results between the two detection systems and finding no differences between them as to the identification of the presence of RNA and YHV in the samples analyzed at different dilution levels ranging from 10^6 to 10^1.

By the specificity test, several samples were analyzed in which only the sample known as positive to YHV presented amplification with the alternative system for detecting YHV, while the known specific samples to other pathogens as WSSV, NHP and streptococcus showed a nonspecific pattern of amplification.

Analysis of the samples was carried out obtaining similar results in each of the replicates performed with the alternative detection system specific for YHV by the quantitative PCR technique.

DISCUSSION

The YHV is located, according to the Committee on Taxonomy of Viruses, in the yellow head complex; this complex includes the GAV (gill-associated virus) and YHV, as well as six more genotypes. YHV and GAV, although closely related viruses, show considerable differences in their genomes, so it has been decided to catalog them as geographic topotypes. According to Wijegoonawardane et al. (2008), there are at least six different YHV genetic lines distributed in most parts of the natural geographical range of *P. monodon* from Africa through South and Southeast Asia to eastern Australia. It is due to this level of recombination that a detection system is needed to perform the search for this pathogen in a specific way. In addition, the suspicion of a Mexican isolate that could be avirulent (De la Rosa et al., 2006; Castro-Longoria et al., 2008; Cedano-Thomas et al., 2009; Sanchez-Barajas et al., 2009) and the possibility of occurrence of these simultaneous infections with WSSV/YHV is the reason why in some severe cases of WSSV where the YHV presence is suspected these samples must be analyzed later by some other method such as RT-PCR with specific tests to confirm or exclude the presence of YHV (Lightner 2011).

Although YHV is considered by the World Organization for Animal Health (OIE) as a disease of compulsory declaration, this does not have a specific classification as such, as it is located within the complex of the yellow head syndrome and is reported as GAV by the International Committee on Taxonomy of Viruses (May, 2002).

Because there are no drugs or vaccines for viral diseases in shrimp, a sensitive diagnosis is necessary to implement appropriate control measures of technologies using PCR or RT-PCR that have been used to analyze maternity and post-larvae stages for the presence of virus before the sowing. However, the amount of virus can be lower than the detection limit of the method resulting in a false negative test (Andrade et al., 2007). According to Jang et al. (2009), a sensitive and safe method was developed for detection with TaqMan in real-time PCR which provides a wide dynamic range of detection that can detect up to 10^9 copies of WSSV and to less than two copies per reaction, so its high sensitivity combined with its wide dynamic range is ideal for the detection of a wide range of infections of WSSV in hatcheries and postlarvae stage. Panichareon et al. (2011) men-

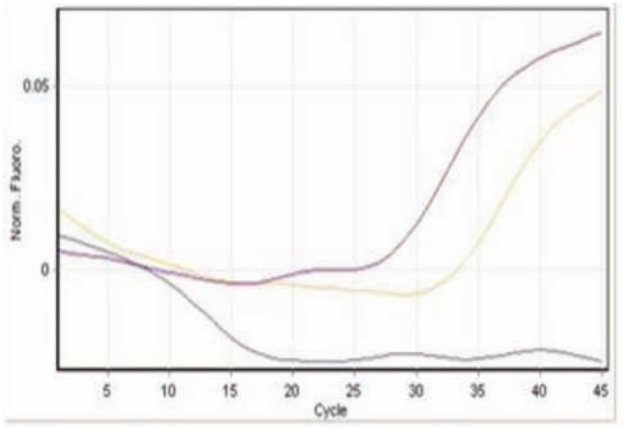

Fig. 1. DT oligo amplification and with primer GY5 for conversion to cDNA with an alternative detection system for YHV.

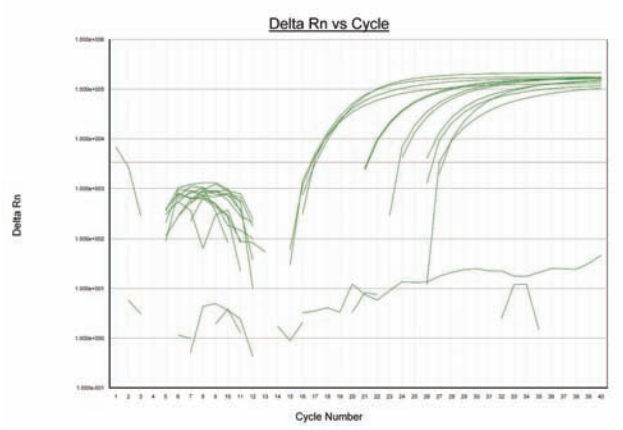

Fig. 2. Quantification of the sample with the trading system with dilutions from 10^6 to 10^2.

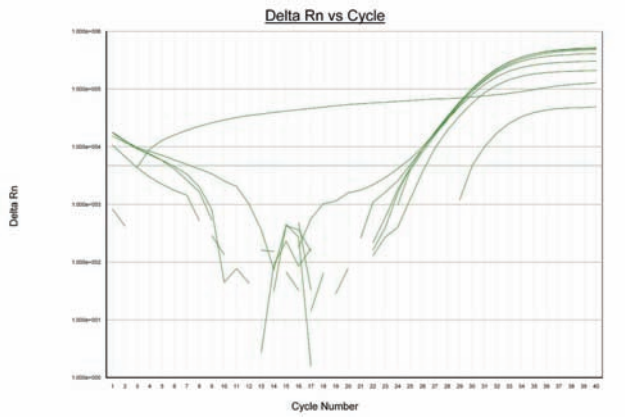

Fig. 3. Test of the sample amplified with the TM probe alternative system.

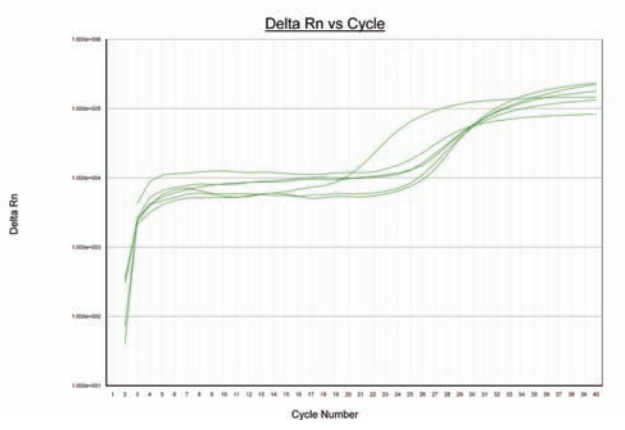

Fig. 4. Test of the sample amplified with the commercial system in dilutions of 10^6-10^1.

tions that in a multiplex detection for real-time PCR for YHV, this was less sensitive than conventional RT-PCR (Wongteerasupaya et al., 1997) and real-time PCR (Wijegoonawardane et al., 2010); however, this multiplex for real-time PCR provides a desirable sensitivity that potentially can be used as a diagnostic tool to detect virus in ponds or carriers. Thus, in the present study detection was implemented with RT-PCR in real-time using a TaqMan probe as this showed sensitivity and specificity higher than the nested detection system and equivalent to the commercial detection system for real-time.

In RNA viruses, a variety of different genomes and replication mechanisms are involved. Among the four types of RNA viruses, only the class that contains single stranded viruses of positive polarity exploits their entire genome. Members of this class are also the most numerous in terms of number of families and number of groups recognized by the International Committee on Taxonomy of Viruses (Fauquet et al., 2005); the yellow head syndrome virus is located within the group of Virus of RNA described above. The roniviruses have only 4 ORFs. In all the nidoviruses the two main and larger ones

Fig. 5. Specificity of the alternative system for detecting YHV.

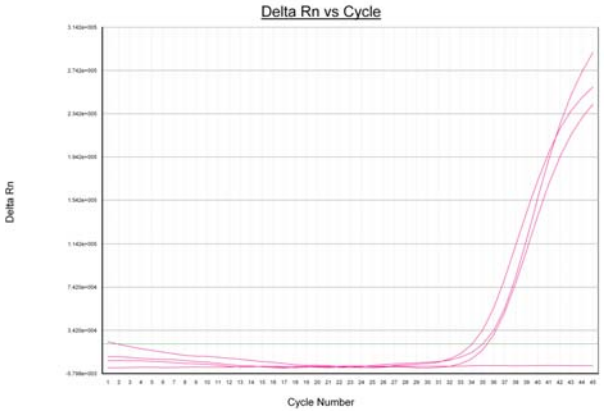

Fig. 6. Repeatability of results with the alternative detection system for YHV.

are at the 5' end which are ORF1a and ORF1b, occupying between two and three quarters of the total genome; they overlap in a small area containing a ribosomal reading frame that provides the signal for a translation of ORF1b by a fraction of ribosomes that initiate synthesis of proteins of the AUG initiator of ORF1a. These two ORFs encode subunits of the replicase machinery. The ORFs located downstream of ORF1b encode nucleocapsid and envelope proteins; the number of these differ between different branches of nidovirus. In families and specific groups, ORFs of this region may encode accessory and nonstructural proteins of virion. These ORFs located in the 3' end are expressed as a nested set of subgenomic RNAs, a property that is reflected in the name of the order of these viruses (Cavanagh, 1997). At present, only the organization and composition of the multidomain of the replicase gene can be used to discriminate nidovirus from other RNA viruses. The ORF1b encodes two domains that have not been identified in other RNA virus families and are therefore classified as molecular markers for this virus. These are the (putative) multinuclear domain for interaction with Zinc (ZBD) (Cowley et al., 2000; Gorbalenya et al., 1989; Seybert et al., 2005; Snijder et al., 1990a; Van Dinten et al., 2000) and the specific domain of endoribonuclease-uridylate domain (NendoU) (Bhardwaj et al., 2004; Ivanov et al., 2004b; Posthuma et al., 2006; Snijder et al., 1990a, 2003). These two domains are part of a conserved arrangement of domains whose sequential disposition may be abbreviated as follows: NH2-TM1, TM2, TM3-CL3^{pro}-RFS-RdRp-ZBD-HELI-NendoU-COOH (Gorbalenya, 2001). Most of the primers used for detection of the yellow head syndrome virus are designed within the area described above. The design was performed in the YHV genome fragment.

Selection, collection, preparation and management of the samples are critical variables in the design and development of an assay. Other variables, such as transport, sample follow-up, and the information management system of laboratory are also major sources of variation when the assay is performed for routine testing. The integrity of experimental results during assay development is as important as the quality of the samples used in the experiment or routine diagnostic test. Reference samples used in assay development should be in the same matrix used in the test (e.g. serum, tissue, whole blood) being representative of the species to be tested by the resulting test (OIE, 2012). In this study, it was used as reference sample that was provided by the University of Arizona, which is in the list of reference laboratories of OIE for disease detection in aquatic organisms.

Repeatability is the level of agreement between the results of replicas of a sample, both within and between runs of a same test method. Repeatability is estimated by evaluating a minimum of three sam-

ples, representing the activity of the analyte between the linear operating ranges of an assay. As a minimum, the duplicates of these samples are treated as individual samples (OIE, 2012). The development of this study conforms to the requirements of the OIE. As regards the repeatability of the test, it is clear that performing more repetitions was not possible due to the fact that in the area where this study was conducted the yellow head disease is so far an undetected disease.

Specificity is the degree to which the assay distinguishes between the target analyte and other components present in the problem sample (OIE, 2012). In this study, specificity of the assay was assured by using conserved sequences of the genome of the pathogen as well as the utilization of specific genetic probes for alignment with the chosen fragment of the genome. As reported by the OIE in the health code manual for aquatic animals, the technique of real-time PCR is listed as recommended for its availability, utility, specificity and sensitivity in the diagnosis in any type of samples (OIE, 2012).

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