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Short Communication

Detection and quantification of hepatopancreatic parvovirus in penaeid shrimp by real-time PCR assay

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ABSTRACT

As one of the major pathogens, hepatopancreatic parvovirus (HPV) can cause severe diseases in penaeid shrimp. We developed a TaqMan-based real-time PCR assay for the HPV detection in China. A pair of primers (HPVF and HPVR) and a TaqMan probe were designed according to the HPV genomic sequence of Chinese isolate (GenBank: GU371276). Our data showed that the primers and TaqMan probe were specific for HPV, and they exhibited no cross-reaction with infectious hypodermal and hematopoietic necrosis virus (IHHNV), white spot syndrome virus (WSSV) and specific pathogen free (SPF) shrimp DNA. The assay had a detection limit of four plasmid HPV DNA copies per reaction. Furthermore, HPV was detected in 16 of 21 *Fenneropenaeus Chinensis*, 3 of 52 *Litopenaeus vannamei* and 2 of 2 *Marsupenaeus japonicus* penaeid shrimp samples. In addition, HPV was also detected in crabs. Therefore, this assay could be successfully used as a sensitive and rapid molecular-based diagnostic method to screen HPV-free animals and survey the prevalence of HPV in cultured populations of penaeid shrimp in China.

1. Introduction

As one of the major pathogens, hepatopancreatic parvovirus (HPV) can cause severe diseases in penaeid shrimp. Since the first infection case found in wild *Penaeus merguiensis* in 1984 from Singapore (Chong and Loh, 1984), many other infections have been reported in many countries, such as Australia, China, Korea, Philippines, Indonesia, Malaysia, Kenya and Israel, as well as North and South America (Bonami, 2008). HPV infection in cultured shrimp has been linked to chronic mortalities during the early larval or post-larval stages (Lightner et al., 1993; Spann et al., 1997), and it may be the major cause of stunted growth during the juvenile stages (Flegel et al., 1992, 1999; Limuswan, 1999).

HPV is a non-enveloped icosahedral virus of 22–23 nm in diameter with a linear ssDNA. It belongs to the Parvoviridae family and Densovirinae subfamily, which infects invertebrates (Bonami et al., 1995; Roekring et al., 2002). To date, the complete HPV genomes of five strains from *Penaeus monodon* (*Pm*DNV), *P. monodon* (*Pm*DNV), *P. merguiensis* (*Pmerg*DNV), *Fenneropenaeus Chinensis* (*Fc*DNV) and *F. chinensis* (*Fc*DNV) have been isolated from India, Thailand, Australia, Korea and China, respectively, and all of these genomes have been sequenced (Sukhumsirichart et al., 2006; Safeena et al., 2010; La Fauce et al., 2007a,b; Jeeva et al., 2012; Zhang et al., 2011). Genome sequence comparison showed that the highest sequence similarity to HPV is observed in the strains isolated from the same species, *F. chinensis* in Korea (GenBank: AY008257) and China (GenBank: GU371276) (Jeeva et al., 2012).

Highly sensitive and rapid molecular diagnostic methods have been established for the HPV detection, such as polymerase chain reaction (PCR) (Sukhumsirichart et al., 1999; Pantoja and Lightner, 2000; Phromjai et al., 2001), nested PCR (Manjanaik et al., 2005), PCR-enzyme linked immunosorbent assay (PCR-ELISA) (Sukhumsirichart et al., 2002) and loop-mediated isothermal amplification (LAMP) (Nimitphak et al., 2008). Most of these methods for HPV detection have been restricted to particular HPV geographic strains. TaqMan real-time PCR is a quantitative measure, which can approximately determine the number of viral copies in tissue samples. It has been used for the detection of the *P. merguiensis* strain of HPV from Australia and *F. chinensis* strain of HPV from Korea (La Fauce et al., 2007a,b; Subbiah et al., 2012; Yan et al., 2010). In the present study, we developed a quantitative real-time PCR assay based on HPV strains isolated from *F. chinensis* in China. Our data showed that this assay could be successfully used as a sensitive and rapid molecular-based diagnostic method to screen HPV-free animals and survey the existence of HPV in cultured populations of penaeid shrimp in China.

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2. Materials and methods

2.1. Sample preparation

Litopenaeus vannamei, *F. chinensis*, *M. japonicas*, *Exopalaemon carinicauda* and crab samples were collected from seven Chinese provinces in 2012. Infectious hypodermal and hematopoietic necrosis virus (IHHNV)-infected *L. vannamei*, white spot syndrome virus (WSSV)-infected *L. vannamei* and HPV-infected *F. chinensis* samples were stored at −80 °C prior to further analysis.

One penaeid shrimp and other crustaceans were considered as a separate sample for the real-time PCR analysis. In order to avoid cross-contamination, samples were separately processed using disposable equipment.

2.2. DNA extraction

Total DNA was extracted from 50 ± 5 mg hepatopancreas and gills of adult/juvenile shrimps and crabs (whole body of post-larval) using the TIANamp Marine Animals DNA kit (TIANGEN, China) according to manufacturer's instructions. The integrity and quality of purified DNA was determined based on the ratio of the optical density (OD) at wavelengths of 260 nm and 280 nm (OD_{260}/OD_{280}) using NanoDrop™ 2000c Spectrophotometer (Thermo Fisher Scientific, USA).

2.3. Primers and probe

A set of specific primers/probe was designed according to the HPV sequence from *F. chinensis* (non-structural protein 2 (NS2), FcDNV, GenBank: GU371276) using AlleleID 6.0 software (PREMIER Biosoft International, Palo Alto, CA, USA). A 149-bp fragment was amplified using primers as follows: HPVF, 5′-TAA GGT GAA ATG GTT GGG AGT TGC-3′; and HPVR, 5′-CCG GCT CAT CCT CCT TCT TCT C-3′. The TaqMan probe was 5′-FAM-TCT TCT GTT GTT CCT TCT TCT CCT TGC TCT TCA TGT-TAMRA-3′. The primers and TaqMan probe were synthesized by TaKaRa Bio.

2.4. Construction and cloning of plasmid standard

A DNA fragment containing the 149-bp HPV NS2 gene (757–905 bp of GU371276) was cloned into the pMD18-T vector (TaKaRa Bio.) and designated as pHPV. The plasmid was purified using the E.Z.N.A.® Plasmid Mini Kit I (OMEGA) and then sequenced using above-mentioned PCR primers. Subsequently, the concentration of pHPV was determined according to the OD at a wavelength of 260 nm. The size of pHPV (2841 bp) was confirmed by agarose gel electrophoresis, and 1 ng plasmid DNA contained approximately 3.2×10^8 copies.

2.5. Real-time PCR amplification and analysis

Concentrations of primers and TaqMan probe were optimized prior to the amplification. PCR reaction was performed in a 25-μL reaction system containing 1 μL DNA template, 1×Premix Ex Taq (Probe qPCR, TaKaRa Bio.), 0.4 μM of each primer (HPVF/R) and 0.2 μM of TaqMan probe. Briefly, after a denaturation step at 95 °C for 30 s, amplifications were carried out with 46 cycles at a melting temperature of 95 °C for 5 s, and an annealing-extending temperature of 60 °C for 30 s. PCR amplification and data analysis were conducted on Rotor-Gene 3000 (Corbett Robotics, Australia). Amplified PCR products were subjected to electrophoresis on a 2% EtBr-agarose gel and then photographed.

2.6. Specificity of real-time PCR assay

DNA from specific pathogen free (SPF) shrimp (*L. vannamei*), IHHNV-infected, WSSV-infected and HPV-infected penaeid shrimp was used as template in order to determine the specificity of the real-time PCR reaction. All these samples were confirmed by PCR.

2.7. Generation of standard curve

A 10-fold serial dilution of purified plasmid pHPV was prepared in order to construct the standard curve. The concentration of plasmid DNA ranged from 2 to 2×10^9 copies per reaction. DNA sample of each dilution and a no-template control (NTC) were tested in triplicate under optimal conditions. Finally, the standard curve was generated by Rotor-Gene Software (Version 6.0.27).

2.8. Reproducibility of real-time PCR assay

The reproducibility of the real-time PCR assay was assessed by intra- and inter-assays using the 10-fold serial dilution of HPV-positive DNA samples. The intra-assay included three replicates of each dilution. The inter-assays were independently performed on different days. DNA samples of each dilution were aliquoted into three EP tubes and stored at −80 °C. The reproducibility was evaluated according to the CV values (coefficient of variation, which equals to the ratio of the standard deviation (SD) to the mean of Ct value).

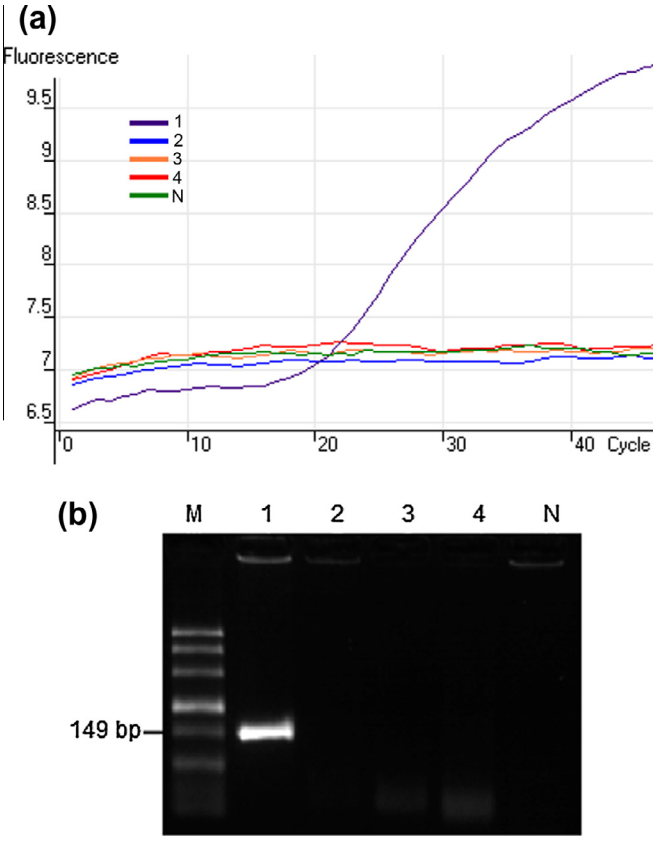

Fig. 1. Specificity of the real-time PCR. (a) The amplification plots. (b) Gel electrophoresis of real-time PCR products M: DL500 DNA Marker (TaKaRa Bio.); 1: HPV DNA; 2: WSSV DNA; 3: IHHNV DNA; 4: SPF shrimp DNA; N: NTC (distilled water).