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Short communication

New set of PCR primers for SYBR green-based qPCR detection of IMNV in India

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ABSTRACT

Emerging and existing diseases are the major havoc to the shrimp aquaculture industry. Outbreaks of viral epidemics severely hinder the sustainable farming system with significant economic losses worldwide. The Pacific white shrimp, *Penaeus vannamei* farming was seriously affected by infectious myonecrosis caused by a double-stranded RNA virus, infectious myonecrosis virus (IMNV). Early and rapid diagnostics is the priority on considering the efficient management and prevention measures. So, the present study utilized the accurate, rapid and specific detection capabilities of real-time PCR on SYBR Green platform to diagnose and quantify the viral load from the infected tissues by designing an efficient PCR primer set. The developed PCR could detect the virus at 98% efficiency with 10 viral copy number as the limit of detection. The standard curve analysis and amplification arithmetics have shown that it can even detect even less than 10 copy numbers of virus in a sample. The standard curve of the assay has shown R^2 value of 0.98 and slope of −3.3834 without any significant variations in inter- and intra- assays. The validated PCR primer pairs and developed SYBR green-based real-time PCR is highly specific, equally sensitive and comparatively economic than the existing TaqMan probe-based PCR for detection of IMNV.

1. Introduction

Infectious myonecrosis is an Office Internationale des epizooties (OIE-world organization of Animal health) notifiable disease (OIE, 2017), caused by a double stranded RNA virus belonging to the family Totiviridae. Penaeid shrimp aquaculture was hampered by infectious myonecrosis in 2002 (Nunes et al., 2004; Lightner et al., 2004a, 2004b; Poulos et al., 2006; Prasad et al., 2017) and the disease outbreak was restricted to Brazil and Indonesia (Senapin et al., 2007; Senapin et al., 2011; Prasad et al., 2017), till the virus was reported from India in 2017 (Shyam et al., 2017; Sahul Hameed et al., 2017). Besides *Penaeus vannamei*, the natural host and the species acutely susceptible to IMNV (Tang et al., 2005), *P. monodon, Litopenaeus stylirostris* and *Farfantepenaeus subtilis* were also proven to be experimentally susceptible to the virus (Lightner et al., 2004a, 2004b; Tang et al., 2005; Coelho et al., 2009). The 40 nm diameter- non enveloped virus possesses a non-segmented genome of size 8226–8230 bp (Poulos et al., 2006; Dantas et al., 2016). The genome encompasses two overlapping ORFs- ORF1 and ORF2, where ORF1 codes for the structural proteins *viz* major capsid protein (MCP) and two small proteins- SP1&SP2 and a presumed RNA binding protein. ORF2 region codes for the virus specific RNA dependent RNA polymerase (RdRp) (Poulos et al., 2006; Melo et al., 2011; Dantas et al., 2016). Based on the RdRp gene homology, the virus is classified into *Giardiavirus* clade of arthropod *Totivirus* family (Nibert, 2007; Oliveira et al., 2014).

The acute phase of infectious myonecrosis (IMN) is characterized by extensive white necrotic areas in abdominal segments and tail fan of the shrimps. Chronic phase is characterized by coagulative and liquefactive necrosis as evident in histology. The skeletal muscles and lymphoid organ are considerably affected in the advanced chronic stage (Nunes et al., 2004; Lightner et al., 2004a, 2004b). Even though the lethal effect of IMN is comparatively less, associated mortality may escalate upto 70% in favourable environmental cues (Nunes et al., 2004; Tang et al., 2005; Poulos et al., 2006).

PCR is the gold standard for aquatic animal disease diagnosis among other molecular diagnostic methods used in the field. At present, nested RT-PCR is currently employed and is being recommended for the detection and surveillance for the IMNV from infected shrimp tissues (Prasad et al., 2017). Poulos and Lightner (2006) have developed a rapid and highly sensitive nested RT-PCR assay for the detection of the IMNV. First step of the PCR assay, targets a 328 bp fragment, is proven to detect almost 100 IMNV copies and the nested primer targeting

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139 bp fragment is known to detect as low as 10 IMNV particles from infected tissues.

Real time PCR (qPCR) is the most sensitive and reproducible method for detection and quantification of the shrimp viruses. qPCR commonly employs SYBR Green and Taq Man chemistries for routine diagnostic assays and for quantitation of viruses in infected samples. TaqMan chemistry is probe –based, more sensitive and can detect up to a single copy of virus present in the sample. The detection limit of real time PCR can reach one copy number/μL virus DNA in several shrimp viruses such as Hepatopancreatic parvovirus (HPV), Monodon baculovirus (MBV) (Yan et al., 2009a, 2009b, Yan et al., 2010). Andrade et al. (2007) developed a TaqMan based real-time RT–PCR assay and da Silva et al. (2011) has standardized a SYBR Green dye probe based qPCR reaction for the specific detection of IMNV. The sensitivity of TaqMan probe is reported to be 10 IMNV copy number/μL RNA whereas the SYBR Green based assay could detect 100 copies/ μL IMNV cDNA (da Silva et al., 2011). Another pair of real-time PCR primers and analogous TaqMan probe was developed by Liu et al. (2013) which could amplify a 101 bp fragment of IMNV cDNA. Other established molecular based or antibody-based diagnostic methods include LAMP and Immunochromatographic test (Arunrut et al., 2013; Puthawibool et al., 2009; Widowati et al., 2012; Wangman et al., 2016; Chaivisuthangkura et al., 2013). In the field of disease diagnosis, scope for improving the sensitivity and specificity of existing assays remain always a challenge, hence development of new sets of primers and assays with improved specificity and sensitivity are the need of the hour. SYBR green-based real time detection is equally sensitive and is more cost-effective than probe-based detection assays. Thus validation of a new set of PCR primers for SYBR green-based real time PCR detection will be an attribute to the field of aquatic viral disease diagnostics. Aim of the present study was to design and validate a set of PCR primers for SYBR green-base qPCR assay to augment the IMN disease detection in India.

2. Materials and methods

2.1. Samples

Naturally infected tissue samples of *Penaeus (Lito)vannamei* preserved in *RNA Later* were kindly provided by CP Prima Laboratories, Indonesia. Natural samples of shrimps were collected from shrimp farms of West Bengal, India (Sahul Hameed et al., 2017).

2.2. RNA extraction and cDNA synthesis

Total RNA from infected *P. vannamei* muscle was extracted using the TRI*zol*® reagent (Life Technologies, USA) as per the manufacturer's protocol. Briefly, shrimp tissue (20 mg) was homogenized in 750 μL of Trizol reagent and allowed to phase separate by adding 200 μL of chloroform with 5 min room temperature incubation. The samples were centrifuged at 12000 rpm for 15 min, 4 °C. The aqueous phase was precipitated using 500 μL of isopropyl alcohol in fresh tube with 5 min incubation followed by centrifugation at 12000 rpm for 10 min, 4 °C. The obtained RNA pellet was washed twice with ice-cold 75% ethanol and centrifuged at 7500 rpm for 5 min, 4 °C. The pellet was air dried and dissolved in 30 μL DEPC treated water and stored at −20 °C.

The quality and the concentration of the extracted RNA were checked by Nanodrop 2000 Spectrophotometer (ThermoFisher Scientific, USA). About 2.5 μg of total RNA was subjected to cDNA synthesis using RevertAid First strand cDNA synthesis kit (ThermoFisher Scientific, USA) according to the manufacture's protocol. Synthesized cDNA were stored at −20 °C for further use.

2.3. Primers

Two sets of specific primers were designed from IMNV genomic sequence (GenBank accession No. KJ636784.1 and No. KJ636782.2) (Naim et al., 2014) targeting the major capsid protein (MCP) of ORF 1 of IMNV using GeneRunner Software V5.0.99 beta. The sequences of the primers designed for the present study are listed in table no. 1.

2.4. Construction of specific positive control plasmids and standards for quantification

IMNV ORF1 target amplicons of size 144 bp were amplified by conventional PCR. The amplicons were purified using GeneJet PCR purification kit (ThermoFisher Scientific, USA) and were ligated into pTZ57R/T vector (ThermoFisher Scientific, USA) and competent *E.coli* DH5α cells were transformed with the ligation mix as per the manufacturer's protocol. The culture was plated on to Luria Bertani (LB) agar plates supplemented with X-gal, IPTG and ampicillin (100 μg/mL). Blue white screening was employed for selection of the recombinant clones. The positive colonies were carefully selected and grown overnight in LB broth supplemented with ampicillin for extraction of plasmid DNA. The plasmid DNA were purified using Miniprep plasmid purification kit (ThermoFisher Scientific, USA) and the plasmid was sent for sequencing at Bioserve Biotechnologies India Pvt. Ltd., (Hyderabad, India) for further confirmation of the clones.

The copy numbers of quantified recombinant plasmids were calculated manually using the standard formula. The plasmids were serially diluted ranging from 10^{10} copies/μL to 10^{1} copies/ μL and these were used as positive controls for the absolute quantification in all the real-time PCR assays.

2.5. SYBR Green-based real-time PCR assay and standard curve analysis

Real time PCR amplifications were carried out in LC 96 Roche, (Germany). The standard curve for the reaction was generated automatically by the lightcycler. Initially, the primer concentrations were optimized with three different concentrations (5 pM, 10 pM and 15 pM). Real-time PCR reactions were carried out in a final volume of 10 μL containing 5 μL of SYBR Green I master mix (Takara, Japan), 0.2 μ L of (10 pM) each forward and reverse primers, 1 μL each of standard plasmid dilutions as template. The reaction was performed in triplicates along with non-template controls to rule out the cross contamination.

The thermal profile for the reaction consisted of initial denaturation at 95 °C for 30 s, followed by 45 cycles of denaturation at 95 °C for 10 s, annealing at 53 °C (first set of primers) and 60 °C (second set of primers) for 10 s and extension at 72 °C for 10 s.

2.6. Assay validation

The assays were optimized for its linearity, sensitivity, specificity and reproducibility. Standard curves were generated by plotting mean Ct value of replicates against copy numbers using serially diluted plasmid DNA from 10^{10} to 10^{1}copies/μL and compared the standard curves of assays of both set of primers. The limits of detection were ascertained by the point at which the assay lost its linearity. The intra-assay variation was determined using three different dilution series prepared from recombinant IMNV plasmids in a single plate in a single run. Similarly, inter-assay variations were determined using a dilution series of a single recombinant plasmid in three different runs. Statistical variance is taken as the factor to depict the repeatability and reproducibility of the assays. Cross reactivity of the developed assay was tested against available positive samples of shrimp viruses such as WSSV, MBV, HPV and IHHNV. A melt-curve analysis was performed at the end of the SYBR Green- assay to ascertain a single specific amplification.

2.7. Absolute quantification of experimentally infected samples

Quantification can be done by comparing the sample Ct values with