Development of Two Quantitative Real-Time PCR Diagnostic Kits for HPV Isolates from Korea

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Viral pathogens, alongside other pathogens, have major effects on crustacean aquaculture. Hepatopancreatic parvovirus (HPV) is an emerging virus in the shrimp industry and has been detected in shrimp farms worldwide. The HPV genome has greater diversity than other shrimp viruses owing to its wide host range and geographical distribution. Therefore, developing diagnostic tools is essential to detect even small copy numbers from the target region of native HPV isolates. We have developed two easy to use quantitative real-time PCR kits, called Green Star and Dual Star, which contain all of the necessary components for real-time PCR, including HPV primers, using the primers obtained from the sequences of HPV isolates from Korea, and analyzed their specificity, efficiency, and reproducibility. These two kits could detect from 1 to $1 \times 10^9$ copies of cloned HPV DNA. The minimum detection limits obtained from HPV-infected shrimp were $7.74 \times 10^5$ and $9.06 \times 10^5$ copies in the Green Star and Dual Star assay kits, respectively. These kits can be used for rapid, sensitive, and efficient screening for HPV isolates from Korea before the introduction of postlarval stages into culture ponds, thereby decreasing the incidence of early development of the disease.

Keywords: Real-time PCR kit, Green Star, Dual Star, hepatopancreatic parvovirus

Since the first report of viral disease by Couch in 1974, shrimp viral disease of cultured shrimp has become an important issue in terms of economic impact in Asia, the Indo-Pacific region, and America [18]. Approximately 60% of disease losses in shrimp aquaculture are associated with viral pathogens, with the remaining 40% of disease caused by bacteria, fungi, and other pathogens [5]. The production of Fenneropenaeus chinensis, the native shrimp species in Korea, reached a maximum of 2,400 metric tons in 2004, but decreased rapidly thereafter because of white spot syndrome virus (WSSV) infection (http://fs.fips.go.kr). Although F. chinensis has been replaced with the Pacific white shrimp Litopenaeus vannamei which is less susceptible to WSSV, since 2002, introduction of this new species has resulted in the occurrence of new viral diseases [10]. In fact, our preliminary survey of 350 L. vannamei individuals collected in 2009, 2010, and 2011 showed high incidence of WSSV and hepatopancreatic parvovirus (HPV) (data not shown).

Two small shrimp parvoviruses, infectious hypodermal and hematopoietic necrosis virus (IHHNV) and hepatopancreatic parvovirus (HPV), which share the primary characteristics of the family Parvoviridae, have been reported [1]. HPV, one of the major shrimp-infecting viruses among the 20 shrimp viruses identified to date, infects epithelial cells of the digestive gland (hepatopancreas) and midgut [21]. Electron microscopic analysis of HPV-infected cells revealed intranuclear inclusion bodies containing viral particles 22–24 nm in diameter [2, 35]. This virus can cause significant mortality and depressed growth in infected larval, postlarval, and early juvenile stages of the shrimp life cycle. However, infection of adult shrimp does not cause 100% mortality, but shrimp that do not show clear signs of disease can act as carriers of the virus and therefore may mask the infection and pose a risk to commercial aquaculture [36].

The 6.3 kb linear single-stranded DNA (ssDNA) genome of HPV contains three open reading frames (ORFs); the left ORF and mid-ORF encode nonstructural proteins (NS2 and NS1, respectively), and the right ORF encodes
virus structural protein (VP) [33]. Owing to its wide host range, including *Peneaus monodon*, *Peneaus esculentus*, *Peneaus japonicus*, *Peneaus semisulcatus*, *Peneaus indicus*, *Peneaus penicillatus*, *Peneaus schmitti*, *Peneaus merguiensis*, *Peneaus stylirostris*, *Litopenaeus vannamei*, *Fenneropenaeus chinensis*, and *Macrobrachium rosenbergii*, and wide geographical distribution, this virus shows a high degree of genome structure variation in different isolates [1, 7, 20]. To date, four complete HPV genomes have been characterized [12, 14, 30, 33], which differed in total genome size (6,321, 6,310, 6,299, and 6,336 bp, respectively), genome sequence, and genome structure, such as the loop-like structure at the 5’ and 3’ termini. In addition, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of purified virus particles revealed a single band of about 54 kDa from a HPV strain isolated from *F. chinensis* in Korea (HPVchin) in contrast to the doublet bands (54 and 57 kDa) from a strain isolated from *P. monodon* (PmDNNV) [2, 33].

The development of an effective diagnostic method for HPV is crucial for preventing the introduction of asymptomatic infected juveniles into culture ponds and secondary infection by these carriers. Traditional diagnostic methods, such as histopathology and electron microscopy, are time-consuming and have limited sensitivity to analyze the virus load; also, genetically variable strains of HPV were initially used [16, 20]. To overcome these limits, highly sensitive rapid molecular- and immunology-based diagnostic methods, such as in situ hybridization, gene probes, polymerase chain reaction-enzyme linked immunosorbent assay (PCR-ELISA), Western blotting, loop-mediated isothermal amplification (LAMP), and PCR-based methods, have been developed [17, 22, 23, 25, 32, 34, 35].

In addition to traditional PCR methods, real-time PCR, which makes it possible to monitor the process of DNA amplification, has been applied in shrimp virus amplification [14]. In real-time PCR, the synthesis of DNA is determined based on the strength of fluorescence produced during the PCR process, which can be monitored on the screen [4]. Three real-time PCR methodologies are commonly used based on the generation of fluorescent signals. First, fluorescent agents, such as SYBR Green I and SYBR Green ER dye, the fluorescence of which increases markedly when incorporated into double-stranded DNA (dsDNA), are added to the reaction and their incorporation during DNA synthesis is monitored. In the second method utilizing light upon extension fluorogenic primer (LUX real-time PCR), the fluorogenic primer has a short sequence tail of four to six nucleotides on the 5’ end that is complementary to its 3’ end. The resulting hairpin secondary structure provides optimal quenching of the fluorophore. When the primer is incorporated into dsDNA during PCR, the fluorophore is dequequenced and the signal intensity increases by up to 10-fold. In the third method, a hydrolysis probe (20–30 bases) labeled with a fluorescent reporter dye at the 5’ end with a quencher at the other end is added to the reaction mixture. While the probe is intact, the proximity of the quencher dye greatly reduces the fluorescence emitted by the reporter. During DNA synthesis, the 5’ to 3’ exonuclease activity of Taq DNA polymerase will separate the reporter dye from the quencher dye, and the reporter dye signal will increase proportionally [4, 39].

Although molecular diagnostics methodologies are specific and sensitive, they may lose efficiency in detecting all HPV isolates owing to the wide range of genome variation and may allow the pathogen to escape detection. Phylogenetic analysis based on the amino acid sequence of the right ORF from seven HPV isolates revealed three genotypes in the HPV isolates [37]. Therefore, developing rapid and sensitive diagnostic tools for native isolates of HPV is important. In this study, we developed two reliable, time-saving, easy to use quantitative real-time PCR kits for effective screening of HPV isolates from Korea, using primers obtained by sequence comparison of HPV strains isolated in this country.

**MATERIALS AND METHODS**

**DNA Isolation**

HPV-associated DNA was isolated from the pereiopods of *F. chinensis* from an aquaculture farm located in Seocheon-Gun, Chungnam Province, in 2008, and *L. vannamei* collected from an aquaculture farm located in Sinan-Gun, Chenonnam Province, in 2010. Approximately 20 mg of pereiopod was removed from an individual shrimp, and homogenized in 500 µl of buffer containing 0.1 M NaCl, 20 mM Tris-HCl, EDTA, and 0.5% SDS. DNA was extracted directly from the homogenate using the same volume of phenol/chloroform/isoamylalcohol (25:24:1) mixture. DNA was precipitated with 0.1 volume of 3 M NaOAc (pH 5.3) and 2 volumes of absolute ethanol. The pellet was washed with 70% ethanol, and the air-dried pellet was redisolved in 100 µl of TE buffer. The DNA concentration was determined by measuring the ratio of the optical density at 260 nm to that at 280 nm (OD<sub>260</sub>/OD<sub>280</sub>) with an ASP-3700 Spectrophotometer (ACTGene, Inc., Piscataway, NJ, USA).

**Preparation of Standard Plasmids**

DNA fragments of approximately 1.7, 1.5, and 1.8 kb were amplified from extracted total DNA using primers F2/R2, F3/R3, and F4/R4, respectively, the sequences of which are shown in Table 1, and cloned into the pCR8/GW/TOPO vector (Invitrogen, Carlsbad, CA, USA). Recombinant plasmids were isolated using a Plasmid Mini Prep Kit (GeneAll, Seoul, Korea) and digested with EcoRI followed by electrophoresis on a 1% agarose gel to screen for the insert DNA. The sequences were analyzed at G&C Bio (Daejon, Korea). The concentration and purity of recombinant plasmid were determined by quantification of OD<sub>260</sub>/OD<sub>280</sub> using a Victor<sup>®</sup> Multi-label Plate Reader (PerkinElmer, Shelton, CT, USA) and converted to copy number.
Primers for Real-Time PCR

Nucleotide sequences of the six plasmids (3 from *F. chinensis* and 3 from *L. vannamei*) were determined and compared to design a set of real-time PCR primers from each DNA fragment. Three sets of primers were designed for real-time PCR using Primer3 software (available at http://www.genome.wi.mit.edu/cgi-bin/primer/primer3). The sequences of the primers are shown in Table 1, and their locations on the HPV strain from Korean *F. chinensis* (JN082231) are as follows: RT-1F/RT-1R, 2325–2346 (162 bp); RT-2F/RT-2R, 3476–3604 (129 bp); and RT-3F/RT-3R, 5232–5251 (201 bp). These primer sets were tested for their specificity and annealing temperature by conventional PCR. Reactions were carried out in PCR premix tubes (Prime Taq premix2X; Genetbio, Nonsan, Korea) containing 10 pmol of each primer and 40–100 ng of DNA template. PCR was conducted using a Gradient Thermal cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with the following cycling conditions: 3 min of initial denaturation at 95°C followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 52°C–55°C for 40 s, and extension at 72°C for 1 min. Annealing temperatures for each primer set are indicated in Table 1.

Test of Primers by SYBR Green Real-Time PCR

Three standard curves were generated independently using serial dilutions from 1 × 10^9 to 1 × 10^3 copies of the HPV-containing gene to determine the sensitivity and reproducibility of the assay. DNA extracted from HPV-infected tissue and no-template controls, or approximately 80 ng of HPV-associated DNA. The thermal cycle pattern consisted of an initial incubation at 95°C for 7 min, followed by a two-step cycle pattern consisting of 40 cycles at 95°C for 30 s and 52°C–55°C for 40 s (Table 1).

Sequence Analysis of the Selected Target

The selected target sequence of 129 bp produced by amplification with the RT-2F and RT-2R primers was aligned using Clustal W2 (EBI) with other HPV sequences available in GenBank [JN082231 (FrDNV, Korea), AY008257 (HPVvchin, Korea), EU588991 (Tanzania), EU247528 (Madagascar), DQ002873 (PnDNV, Thailand), DQ458781 (PmerngDNV, Australia), FJ410797 (PnDNV, India), and GU371276 (China)] to compare the variations at the primer and probe binding sites.

Development and Test of Green Star Quantitative Real-Time PCR Diagnostic Kit

The Green Star quantitative real-time PCR diagnostic kit, which uses the SYBR Green I dye, was designed and produced by Bioneer Corporation with the RT-2F/RT-2R primer set that showed best performance in the primary SYBR Green real-time PCR test. This kit is composed of 15 pmol of RT-2F and RT-2R primers, SYBR Green I dye, and the necessary components for real-time PCR. The test kit was analyzed using standard plasmids, along with HPV-infected and HPV-free samples. In each reaction of 20 µl, a dilution series of the HPV plasmid standard (1 × 10^0 to 1 × 10^3) was used to produce a standard curve, and 80 ng of total DNA extracted from HPV-infected or HPV-free shrimp was used as test samples. The real-time PCR thermal cycling was conducted using Exicycler (Bioneer Corporation), with a program consisting of pre-denaturation at 95°C for 7 min followed by 40 cycles of denaturation at 95°C for 30 s and primer annealing and DNA synthesis at 55°C for 40 s. Melting curve analysis was performed by increasing the temperature from 60°C to 95°C at a rate of 0.1°C/2 s. The specificity of quantitative real-time PCR was tested by melting peak analysis and 2% agarose gel electrophoresis of the amplified PCR products. The cycle threshold (Ct) values were determined by automated threshold analysis with Exicycler3 ver. 3.547. The minimum detection limit was analyzed using 1 to 1 × 10^3 copies of cloned HPV DNA.

Development and Test of Dual Star Quantitative Real-Time PCR Diagnostic Kit

The Dual Star quantitative real-time PCR kit, which uses a fluorescent probe, was produced in lyophilized form by Bioneer Corporation. The FAM-conjugated TaqMan probe (5′-CGGAGG GAGCTAAAGCHAAATACGAG-3′) was designed using Primer3 software (available at http://www.genome.wi.mit.edu/cgi-bin/primer/primer3) from the sequence of a Korean HPV strain (FrDNV).

### Table 1. Primer nucleotide sequences, location, and Tm.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Nucleotide sequences (5′–3′)</th>
<th>Locations</th>
<th>Tm</th>
</tr>
</thead>
<tbody>
<tr>
<td>RT-1F</td>
<td>CAG CAG GCA GAT TAT ATT GAG G</td>
<td>2325–2346</td>
<td>52°C</td>
</tr>
<tr>
<td>RT-1R</td>
<td>TCT GTG TGC TCT GTG CAA TCA</td>
<td>2487–2467</td>
<td>52°C</td>
</tr>
<tr>
<td>T-2F</td>
<td>ACG ATC AGA GCA ATC CGA AG</td>
<td>3476–3495</td>
<td>55°C</td>
</tr>
<tr>
<td>T-2R</td>
<td>TGG GTC TCT CCA GGT ATT CG</td>
<td>3604–3585</td>
<td>55°C</td>
</tr>
<tr>
<td>T-3F</td>
<td>CTC ATT GGA GTG ATG CGA AG</td>
<td>5232–5251</td>
<td>52°C</td>
</tr>
<tr>
<td>T-3R</td>
<td>GAA TAT CGG AGC ACC CTC TG</td>
<td>5432–5413</td>
<td>52°C</td>
</tr>
<tr>
<td>F2</td>
<td>AGC CAA TAA AGT ATG TAA GAA CAT ATA TG</td>
<td>1696–1725</td>
<td>52°C</td>
</tr>
<tr>
<td>R2</td>
<td>ACT GTG TCT GCA ACG ACC TG</td>
<td>3421–3440</td>
<td>52°C</td>
</tr>
<tr>
<td>F3</td>
<td>AGG ATG ATT CCA ATC AAG AAG AA</td>
<td>3051–3073</td>
<td>52°C</td>
</tr>
<tr>
<td>R3</td>
<td>ACA CAT TGT CTC CAA TGT GTT TG</td>
<td>4541–4563</td>
<td>52°C</td>
</tr>
<tr>
<td>F4</td>
<td>GAG GGC GAA GAT ACG ATG G</td>
<td>4347–4563</td>
<td>52°C</td>
</tr>
<tr>
<td>R4</td>
<td>GAG GGC GAA GAT ACG ATG G</td>
<td>6138–6155</td>
<td>52°C</td>
</tr>
</tbody>
</table>

*The numbers indicate the locations on the nucleotide sequence of JN082231.
Each lyophilized tube with a final reaction mixture of 20 µl contained all of the necessary components for real-time PCR, 15 pmol of RT-2F and RT-2R primers, and 15 pmol of the probe. A dilution series of the HPV plasmid standard (1 × 10^3 to 1 × 10^9) was used to produce a standard curve, and aliquots of 80 ng of total DNA extracted from HPV-infected or HPV-free shrimp were used as test samples. Real-time PCR thermal cycling was conducted using Exicycler (Bioneer Corporation) with a program consisting of pre-denaturation at 95°C for 7 min followed by 40 cycles of denaturation at 95°C for 30 s and primer annealing and DNA synthesis at 55°C for 40 s. The standard amplicons were analyzed by 2% agarose gel electrophoresis for specificity assay. The minimum detection limit of the Dual Star kit was analyzed using 1 to 1 × 10^8 copies of cloned HPV DNA.

**Determination of Detection Limits**

Serial dilutions of 1 to 10^9 copies of HPV plasmid DNA were prepared. The assays were carried out in 20 µl of Green Star and Dual Star reaction tubes containing the serially diluted HPV plasmid DNA and the rest of the reaction components. Real-time PCR thermal cycling was conducted using Exicycler (Bioneer Corporation) with a program consisting of pre-denaturation at 95°C for 7 min followed by 40 cycles of denaturation at 95°C for 30 s and primer annealing and DNA synthesis at 55°C for 40 s.

**RESULTS**

**Selection of Best Primer Combination**

All three sets of primers (i.e., RT-1F/RT-1R, RT-2F/RT-2R, and RT-3F/RT-3R) amplified target DNA of the expected sizes (163, 129, and 201 bp, respectively) from cloned DNA by conventional PCR. None of the primers showed nonspecific amplification or primer dimer formation (data not shown). Therefore, these three primer sets were tested by real-time PCR for their sensitivity and efficiency with cloned DNA and DNA extracted from HPV-infected and HPV-free shrimp as templates. All three primer sets produced fluorescent signals from the cloned DNA and DNA extracted from HPV-infected shrimp, but no signals were detected using DNA from HPV-free shrimp. However, real-time PCR with the RT-3F/RT-3R primer set was not consistent and the slope value of the standard curve was -0.1866. The efficiency of the assay using primer set RT-2F/RT-2R was 99% followed by 87% for RT-1F/RT-1R and 57% for RT-3F/RT-3R primer sets. Therefore, we chose the RT-2F/RT-2R primers for diagnostic kit development.

**Fig. 1.** Variation of target nucleotide sequences with other isolates and primers, and the probe of the targeted region. FcDNV, L. vannamei, and HPVchin are Korean isolates. GenBank accession numbers: JN082231 (FcDNV), AY008257 (HPVchin), EU588991 (Tanzania), E1257528 (Madagascar), DQ200773 (PmDNV, Thailand), DQ5861 (PmerygDNV, Australia), FJ410797 (PmDNV, India), and GU371276 (China). The probe sequence is underlined. Forward and reverse primers are shaded.
Sequence Analysis
The target sequence of 129 bp produced by RT-2F/RT-2R primer amplification is shown in Fig. 1, with the primer sites and a site for the real-time PCR probe. The target sequence from three HPV strains isolated from Korea, FcDNV and HPVchin isolated from F. chinensis and one from L. vannamei, showed 100% nucleotide sequence identity. However, this region showed low sequence identity with HPV strains isolated from other geographical regions as follows: 94.6% with the Tanzanian strain, 93.8% with the Madagascan strain, 88.5% with the Chinese strain, 88.4% with PmagnDNV, 76.3% with the PmdNV Indian strain, and 72.4% with the PmdNV Thai strain.

Analysis of the Green Star Real-Time PCR Diagnostic Kit
The Green Star kit was analyzed using the standard HPV plasmids and randomly selected DNA samples from HPV-infected shrimp. Amplification curves and a standard curve were generated with standard plasmid from $10^9$ to $10^3$ copies per reaction (Fig. 2). The standard curves showed a slope of $-0.3257$ and the regression coefficient $R^2$ was 0.997. The specificity for the Green Star real-time PCR kit was confirmed by melting peak analysis, which showed only a single desired amplification peak per reaction without any additional peaks, due to nonspecific integration or primer dimer formation (Fig. 3). This was further confirmed by agarose gel electrophoresis of amplified PCR products (Fig. S1). Tests with randomly selected samples showed $7.74 \times 10^1$ to $1.49 \times 10^3$ copies of HPV from 80 ng of total DNA extracted from infected shrimp.

Analysis of the Dual Star Real-Time PCR Diagnostic Kit
The amplification curves and standard curves of the Dual Star real-time PCR kit with standard plasmid from $10^9$ to $10^3$ copies per reaction are shown in Fig. 4. The standard curves showed a slope of $-0.3408$ and a high $R^2$ of 0.9916. The amplification signals were detected only from the cloned DNA and infected samples. No amplification signal was detected from total DNA extracted from HPV-free shrimp. The specificity of the Dual Star real-time PCR kit was analyzed by 2% agarose gel electrophoresis of all amplified PCR products. As shown in Fig. S2, only a PCR product of 129 bp was detected from the cloned DNA and infected shrimp without any nonspecific amplification. Tests with randomly selected DNA samples showed HPV-specific amplification corresponding to between $9.06 \times 10^1$ and $2.16 \times 10^3$ copies of HPV from 80 ng of total DNA.

Comparison of the Two Kits
The detection limits of the two diagnostic kits were determined by using a serial dilution of the plasmid

Fig. 2. Green Star real-time PCR test kit analysis. Standard plasmids were serially diluted from $1 \times 10^9$ to $1 \times 10^3$ and randomly selected HPV-infected and uninfected DNA samples were used as test samples to generate amplification curves and standard curves. (A) Standard plasmids and sample amplification curve with threshold. (B) Standard curves of copy number versus threshold cycle ($C_T$) value. 10$^9$ to 10$^3$, standard plasmids; untagged samples, HPV-positive; negative, HPV-free shrimp DNA; $R^2$, regression coefficient; $Y$, slope; efficiency, PCR amplification efficiency.

Fig. 3. Specificity analysis of the Green Star test kit. The specificity of the Green Star real-time test kit was tested by melting curve analysis of the PCR products.
standard from 1 to $10^6$ copies per reaction. A standard curve was obtained from the reaction curves, which are shown in Fig. 5. The standard curves showed a slope of $-0.3238$ for the Green Star kit and $-0.314$ for the Dual Star kit. The $R^2$ was 0.997 with an assay efficiency of 111% for the Green Star and the signal was detected for all diluted plasmid DNA samples. The Dual Star assay also showed high efficiency of 106% with an $R^2$ of 0.9441, and the entire diluted plasmid DNA amplification signal was detected. These results indicated that both kits can detect as low as one copy of template DNA per reaction. The performances of the two detection kits were compared with the CT values and copy numbers determined by the kits using randomly selected samples and the results showed no significant deviation in one kit (Table 2).

Table 2. Comparison of two real-time PCR assay kits using identical randomly selected templates.

<table>
<thead>
<tr>
<th>Samples</th>
<th>$C_T$ values</th>
<th>Copy numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Green Star</td>
<td>Dual Star</td>
</tr>
<tr>
<td>1</td>
<td>25.19</td>
<td>27.28</td>
</tr>
<tr>
<td>2</td>
<td>25.31</td>
<td>28.91</td>
</tr>
<tr>
<td>3</td>
<td>23.25</td>
<td>27.13</td>
</tr>
<tr>
<td>4</td>
<td>28.48</td>
<td>31.32</td>
</tr>
<tr>
<td>5</td>
<td>29.13</td>
<td>31.28</td>
</tr>
<tr>
<td>6</td>
<td>27.31</td>
<td>30.15</td>
</tr>
</tbody>
</table>
DISCUSSION

Despite its worldwide distribution, including the Asia-Pacific region, HPV has not been regarded as a major shrimp pathogen, probably because of heavy infection and severe economic losses caused by WSSV. However, recent real-time PCR surveys of shrimp tissue and seawater sampled from culture ponds of *F. chinensis* and *L. vannamei* showed high incidence rates of HPV in Korea [9–11]. Although HPV does not cause mass mortality of adult shrimp, it can cause growth retardation and even high mortality rates via infection of early larval stages [6, 19, 31]; therefore, more careful monitoring of this virus is necessary. Furthermore, *L. vannamei*, which has been cultured in Korea since 2002 in place of *F. chinensis*, which is known to be susceptible to WSSV, was shown to be susceptible to HPV and other viruses, including infectious hypodermal and hematopoietic necrosis virus (IHHNV) and Taura syndrome virus (TSV) in China and TSV in Taiwan [41, 42].

Three genotypes of HPV have been reported to date, and in situ hybridization and nucleotide sequence analysis showed that the HPV strain from Korea belongs to genotype I [27, 37]. However, recent PCR analysis of shrimp showing HPV symptoms failed to produce any amplification product using a type I-specific 1120F/1120R primer set [11, 25]. HPV isolates from different hosts or geographical regions are known to show genetic differences [37]. Therefore, two easy to use and highly sensitive quantitative real-time PCR kits were developed to detect HPV isolates from Korean *L. vannamei*, which is currently the major cultured shrimp species in Korea.

Specific gene probes and primers are important factors for effective screening of pathogens in the development of molecular diagnostic tools. Owing to the wide host range and large degree of nucleotide sequence variation of HPV, specific primer sets or probes cannot be applied to other isolates. For example, the primer designed from HPVchin showed reduced sensitivity to another HPV isolate, *PmDNV* [27], and failed to detect the HPV isolate from India [38]. Therefore, specific primers for native HPV isolates have been applied in previous studies [15, 22, 26]. Nucleotide sequence analysis showed high degrees of sequence variation in the target region used in this study, which is located between ORF2 and ORF3 [12]. The target region showed 100% nucleotide sequence identity to the HPV isolate from Korean *F. chinensis*. It also showed 94.6% and 93.8% sequence identity to Tanzanian and Madagascan isolates, both of which belong to genotype I. In contrast, the target region showed high degrees of sequence variation with other distinct isolates: 72.4% with *PmDNV*, 88.4% with *PmergDNV*, and 88.5% with a Chinese strain (Fig. 1). Therefore, these real-time PCR assays are suitable for effective screening of HPV strains from Korea with nucleotide sequences distinct from genotypes II and III [37].

Although many sensitive diagnostic methods have been developed, quantitative real-time PCR is generally preferred for diagnostic purposes, as it can be applied to quantify even a single copy of the pathogen. Real-time PCR has various advantages, including rapid processing times, real-time monitoring of amplification, high sequence specificity, reliable results, quantitative results, no need for endpoint gel analysis, and low detection limit of the target sequence [8]. The detection limit and sensitivity of all of the molecular diagnostic methods are not uniform. The detection limit of *PmDNV* by LAMP-lateral flow dipsticks (LFD) was 1 ng [23]. PCR-ELISA could detect even 0.01 fg of target sequence, which is more sensitive than hematoxylin and eosin (H&E) staining, Southern hybridization, and conventional PCR [34]. The limit of detection was 1.0 pg to 40 fg by multiplex real-time PCR assay [13, 24], and the minimum limits of detection by TaqMan real-time PCR and Duplex real-time PCR were 1–10 copies of virus particles [15, 36, 40]. The detection limits of our real-time PCR kits were as low as one copy of viral DNA, which was similar to a previous report regarding TaqMan assays for HPVchin and *PmDNV* in Thailand [36, 40]. This detection limit is 10 times greater than that of the TaqMan assay for *PmergDNV* [15].

Two real-time PCR kits were developed in this study. The Green Star kit contains SYBR Green I dye, which emits fluorescence only when it binds to dsDNA. Therefore, the increase in SYBR Green I signal is correlated with the amount of amplified products during target DNA amplification. The Green Star kit requires no probe and can be used to diagnose the target sequence even in the presence of variations in the middle of the target. The Dual Star kit was developed based on a TaqMan probe quantitative PCR assay. The specific probe was labeled with both fluorescent reporter and a fluorescence quencher located in close proximity to each other. The PCR mixture contains Taq DNA polymerase, which hydrolyzes the probe during PCR, thus allowing the reporter dye to emit fluorescence due to the 5’ to 3’ exonuclease activity of polymerase, and the emitted fluorescence can be recorded automatically in the real-time PCR machine.

To compare the sensitivity of the two kits, serial dilutions of HPV-positive plasmid controls and HPV-infected tissues were tested. Both assays showed equal sensitivities and had similar linear ranges (Fig. 2 and 4). The efficiency of a real-time PCR assay can be determined from the slope of the linear regression line. The ideal slope is −3.32, which is correlated with an amplification efficiency of 100%, and slopes in the range of −3.60 to −3.10 are generally considered acceptable for real-time PCR [29]. These slope values correspond to amplification efficiencies between 90% and 110%. The standard curves
of the Green Star kit showed a slope of ~0.3257, and the Dual Star kit showed a slope of ~0.3408, both of which were within the acceptable range. The R² of the Dual Star kit was 0.9916 and that of the Green Star kit was 0.997; both kits have regression coefficients within the acceptable range reported previously (R² > 0.980) [29]. Therefore, the efficiency and sensitivity of these two kits were within the acceptable ranges and showed no significant differences.

The performance of the two kits was compared using randomly selected infected samples. As shown in Table 2, one kit showed high copy number in one sample and vice versa. The differences may have been due to minute differences during assay preparation rather than intrinsic differences between the kits. This comparison suggested that both diagnostic kits are efficient, and they show essentially no differences in performance.

All of the fluorescent methods have their own advantages and disadvantages. SYBR Green I suffers from the disadvantage that it binds nonspecifically to any dsDNA and emits the fluorescent signal, including nonspecific PCR products and primer dimers; thus, the formation of primer dimers or nonspecific amplification must be monitored in the real-time PCR assay. Primer dimer formation and nonspecific amplification of PCR products can be determined by melting curve analysis of real-time PCR [28]. As shown in Fig. 3, no dimers or nonspecific PCR products were detected from our Green Star kit, indicating that this kit would not give rise to nonspecific signals that would result in false-positive results. Melting curve analysis cannot be used for the test of the Dual Star kit in which the fluorescent probe is degraded during PCR. However, gel electrophoresis of the PCR products showed no nonspecific amplification. In addition, use of a sequence-specific probe can enhance the specificity of the reaction.

The reaction component for SYBR Green-based detection or TaqMan assay using the probe can be prepared separately and mixed before reaction with the template. However, repeated handling of the component can result in false positives due to contamination, which may interfere with the original results [3]. Therefore, our two real-time PCR diagnostic kits, which are efficient, rapid, sensitive, and easy to use, will be useful for the detection of HPV isolates from Korea in the screening of shrimp larvae before introduction to culture ponds and for regular monitoring of HPV infection.

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References


