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A TaqMan Real-Time PCR Assay for Quantifying Type III Hepatopancreatic Parvovirus Infections in Wild Broodstocks and Hatchery-Reared Postlarvae of *Fenneropenaeus chinensis* in Korea

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A highly sensitive and specific TaqMan real-time PCR was used to quantify hepatopancreatic parvovirus (HPV) type III infections in wild broodstocks and hatchery-reared postlarvae (PL) of *Fenneropenaeus chinensis*. Totals of 159 and 162 wild brooders from three locations were captured, and 140 and 180 PL were obtained from seven and six commercial hatcheries in 2007 and 2008, respectively. Among the three wild broodstock groups from 2007, only 1 group showed HPV infection and 3.2% of 159 brooders were positive for HPV infection. In 2008, HPV infections were observed from all three wild broodstock groups with 1.93×10^4 copies/mg tissue of pleopods. Of 162 brooders, 26.6% were positive for HPV infection. No PL from the two hatcheries collected in 2007 showed HPV infection, and PL from the rest of the five hatcheries had up to 1.74×10^6 copies/ng of DNA, and PL from three hatcheries showed HPV infections with over 1,000 copies/ng of DNA. The PL from all seven hatcheries collected in 2008 showed up to 2.10×10^5 HPV copies/ng of DNA. PL from two hatcheries showed less than 100 copies/ng of DNA, but PL from the rest of the hatcheries showed HPV infections with over 1,000 copies/ng of DNA. These results show that HPV type III is widely distributed in Korea in addition to previously reported HPV type I, and they can be effectively detected by type-specific real-time PCR.

Keywords: Hepatopancreatic parvovirus, genotype III, *Fenneropenaeus chinensis,* wild broodstock, hatchery, real-time PCR

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Shrimp comprise an economically important aquaculture species worldwide, but production of cultured shrimp can be influenced by several pathogens, including many viruses. Of more than 22 viruses reported in shrimp, white spot syndrome virus (WSSV) and hepatopancreatic parvovirus (HPV) are major threats to shrimp aquaculture around the world [9]. Although, unlike WSSV, HPV does not cause a total loss of shrimp, both are responsible for severe growth retardation and even significant mortality when they infect during the early stages of shrimp growth [8, 14, 24]. HPV is a small, nonenveloped, icosahedral, 22–24 nm virus containing a single-stranded DNA genome of about 6 kb that belongs to the Parvoviridae family [2, 11, 25]. The genome of HPV has hairpin structures at its 5' and 3' ends, and contains three open reading frames (ORFs) called the left, mid, and right ORF, which encode nonstructural protein 2 (NS2), NS1, and viral capsid protein, respectively [12, 25, 28]. HPV was first reported in 1982 in cultured *Penaeus merguiensis* in Singapore [5], and its distribution has been confirmed in the Indo-Pacific area, the Middle East, Africa, and the Americas in *P. monodon*, *P. esculentus*, *P. indicus*, *P. penicillatus*, *Fenneropenaeus chinensis*, and the freshwater prawn *Macrobrachium rosenbergii* [1, 13, 14].

In addition to the worldwide distribution of HPV, sequence analysis of their genome has shown variations among HPV isolates from different origins. Phylogenetic analysis of seven geographic isolates using the right ORF, which encodes the major capsid protein and shows the greatest distance (24% compared with 13% and 7% for the left and mid ORFs, respectively), revealed three types of HPV. The isolates from Korea, Tanzania, and Madagascar are type I; the isolates from Thailand and Indonesia are type 2; and isolates from Australia and New Caledonia are type 3 [28].

To date, no treatment or vaccination against shrimp viral diseases has been available, and the best control method has been to avoid the use of infected stock. However, the absence of specific signs of HPV infection and possible masking by other pathogens make diagnosing the disease by simple observation difficult [4, 18]. Therefore, several detection methods have been developed. Traditional detection methods for HPV infection include histological observation of intranuclear inclusion bodies [15, 17], *in situ* hybridization [21], and the use of gene probes [16, 20]. In addition, nondestructive detection methods, including polymerase chain reaction (PCR) [22, 23, 27], nested PCR Manjanaik *et al*. [19], and PCR–enzyme linked immunosorbent assay (PCR–ELISA) [26], have been used for the detection of HPV. More recently, a TaqMan real-time PCR assay showed that 1 copy of a cloned gene or 10 copies of the HPV genome could be detected [11, 29].

Sequence analysis of a Korean HPV isolate showed that this was a type I isolate, along with isolates from Tanzania and Madagascar [28]. However, our recent survey revealed that variants showing sequence homology to type III were also present (unpublished data). In this study, we utilized TaqMan real-time PCR to detect type III HPV in cultured and wild broodstocks of *F. chinensis*.

Materials and Methods

Sampling

In total, 159 and 162 female wild *F. chinensis* brooders were captured from the west and south coasts of the Korean peninsula from April to June in 2007 and 2008, respectively. Another 140 and 180 postlarvae (PL) individuals were collected from hatcheries (20 and 30 individuals from each of 7 and 6 hatcheries, respectively) located in Chungnam Province from May to June in 2007 and 2008, respectively (Table 1). All live animals were transported to the West Sea Mariculture Research Center of the National Fisheries Research and Development Institute (NFRDI) and measured. The samples were stored at −80°C for brooders or in 70% alcohol for PL until analyzed.

DNA Extraction

Total genomic DNA and associated HPV viral DNA were extracted using the DNeasy Kit (Qiagen, Valencia, CA, USA). For broodstock samples, about 25 mg of pleopods was removed from the abdomen of each individual. The whole body of each individual PL was used. The DNA concentration and quality were determined by measuring the optical density at OD_{260} nm with the spectrophotometer Genesys 5 (Spectronic Instruments, Inc., Rochester, NY, USA).

Real-Time PCR Primers and Probes

Primer 3 software was used to design primers and TaqMan probes with the Genotype III hepatopancreatic parvovirus (*Pmerg*DNV)

Table 1. Summary of sample information on wild broodstocks and postlarvae of *F. chinensis.*

Sources of shrimps[a]		Sampling date	Location	Number of specimens	Body weight	Body length or larval stage
Broodstocks	B_1	April 20, 2007	Haenam	57	52.06±8.97 g	166.57±9.31 mm
	B_2	May 15, 2007	Youngkwang	70	53.16±7.48 g	164.88±7.57 mm
	B_3	June 6, 2007	Taean	32	55.08±8.69 g	167.34±12.28 mm
		Mean			53.43±8.38 g	166.26±9.72 mm
	B_4	April 15, 2008	Narodo	51	94.37±14.30 g	198.07±9.05 mm
	B_5	May 12, 2008	Youngkwang	81	74.49±8.38 g	184.62±6.65 mm
	B_6	June 2, 2008	Taean	30	74.33±8.98 g	184.17±6.25 mm
		Mean			80.73±10.55 g	188.95±7.32 mm
Hatcheries	H_1	May 31, 2007	Taean-1	20	1.33 mg	PL_7
	H_2	May 31, 2007	Taean-2	20	2.44 mg	PL_7
	H_3	June 8, 2007	Taean-3	20	1.22 mg	PL_4
	H_4	June 8, 2007	Seocheon-1	20	1.47 mg	PL_{5-6}
	H_5	June 12, 2007	Seocheon-2	20	0.38 mg	PL_{2-3}
	H_6	June 12, 2007	Hongseong-1	20	0.70 mg	PL_{1-3}
	H_7	June 14, 2007	Hongseong-2	20	1.95 mg	PL_{7-8}
		Mean			1.36±0.65 mg	
	H_8	May 07, 2008	Chungnam-FRI	30	0.031±0.01 mg	Mysis (I)
	H_9	May 27, 2008	Haenam	30	1.84±0.79 mg	PL_{10}
	H_{10}	June 4, 2008	Taean-1	30	1.32±0.50 mg	PL_{10-11}
	H_{11}	June 12, 2008	Taean-2	30	0.88 ±0.49 mg	PL_{11-12}
	H_{12}	June 16, 2008	Hongseong	30	4.38±2.13 mg	PL_{11}
	H_{13}	June 17, 2008	Seocheon	30	4.26±1.89 mg	PL_{10}
		Mean			2.11± 0.97 mg	

[a]Samples B1-B3 and H1-H7 were collected in 2007 and B4-B6 and H8-H13 were collected in 2008.

whole sequence (GenBank: DQ458781). Primers RT-HPV-F_{119}, 5'-CTA GCA TGG GAG CAG TCG TA-3', at positions 3,310–3,329 in DQ458781, and RT-HPV-R_{119}, 5'-GAC CTG AAC AGT CTC TGC CA -3', at positions 3,428–3,409 in DQ458781, produced a fragment of 119 bp after amplification. The TaqMan probe RT-HPV-TP_{119}, 5'-CCA CGA AAG CTG CTT CCA CGA C-3', at positions 3,408–3,378 in DQ458781, was labeled by Operon Co. (Gimpo, Korea) with the fluorescent dyes 5-carboxyfluorescein (FAM) on the 5' end and Black Hole Quencher (BHQ) on the 3' end.

Clinical Specificity of the RT-PCR Assay

The clinical specificity of the RT-PCR assay for detecting the Genotype III hepatopancreatic isolate from *F. chinensis* was determined by performing the assay on other crustacean viruses, including WSSV and HPV-chin (type I), which had been confirmed by PCR using the type I-specific PCR primers 1120F/1120R [22].

Construction of Positive Control Vectors and Standards for Quantification

A HPV fragment containing a 119 bp target amplicon for real-time PCR was ligated into the pGEM-T-Easy Vector (Promega, Madison, WI, USA) and cloned into *Escherichia coli* (DH5α). The target segment in the recombinant plasmid, pHPV-119, was confirmed using an automated DNA sequencer (ABI 3730XL; Applied Biosystems, Foster, CA, USA). The copy number of the target amplicon in the plasmid was estimated, and 10 serial dilutions were made to use as absolute standards for quantification.

Real-Time PCR Amplification

Real-time PCR was performed with Perfect Real Time premix (RR039A; Takara Bio, Otsu, Japan) containing high-performance Taq polymerase (Takara Ex Taq HS), for hot start real-time PCR. A sample of 40–200 ng total DNA was added to a PCR mixture containing 0.25 μM of each primer and 0.12 μM TaqMan probe in a final reaction volume of 20 μl. The amplification program consisted of 30 s at 95°C for initial denaturation, followed by 45 cycles of 5 s denaturation and 20 s annealing/extension at 60°C. Thermal cycling was performed on the Rotor Gene 6000 (Corbett Research Inc., Sydney, NSW, Australia).

Data Analysis

The threshold was automatically detected, and the preliminary data were analyzed by Rotor Gene Operating Software version 1.7.61, resulting in a fractional cycle number (Ct value) assigned to each individual sample. A set of standard dilutions (from 4.7×10^0 to 4.7×10^6 viral copies μl^{-1}) was created from a plasmid containing the target amplicon and run simultaneously with DNA of the specimens. All samples were run in duplicate for PL and in triplicate for brooders. Regression of the log of the viral copy number and Ct value were used to determine a standard curve for determining viral load. Viral copy number was normalized per nanogram of genomic DNA, per milligram of tissue, and per individual (for PL only). For each new run, at least two non-template controls (NTCs) were used as negative controls.

RESULTS

Specificity of Primers and Probe

A 119 bp amplicon was produced using the set of HPV119 primers (data not shown). A BLAST comparison of the 119 bp product had no significant matches against any organism, but it had a 100% sequence identity with the targeted sequence from the genome of HPV type III [GenBank: DQ458781].

Standard Curve and Detection Limitation

Strong linear correlations ($R^2 = 0.99920–0.99777$) were obtained between threshold cycles (Ct) with the target plasmid standard ranging from 4.7×10^0 to 4.7×10^6 HPV copies in PCR with a high reaction efficiency (E = 0.97–1.01) and proper slope (M = −3.393 to −3.308) (Fig. 1A and 1B), indicating that the assay had a large dynamic range. To determine the reproducibility, intra- and inter-assays were compared from 4.7×10^0 to 4.7×10^6 HPV copies of four independent standard curves. Small correlations of variation (0.32%–1.21%) indicated that the assays had little variation between runs. With an optimal PCR mixture, we could detect from 2 to 10^9 HPV copies with specimen genomic DNA in one reaction. Negative controls made no amplification.

HPV Type III Quantification in *F. chinensis* Broodstocks

Among the three groups of wild broodstock from 2007, only 1 group (B_1) showed HPV (*Pmerg*DNV) infection in

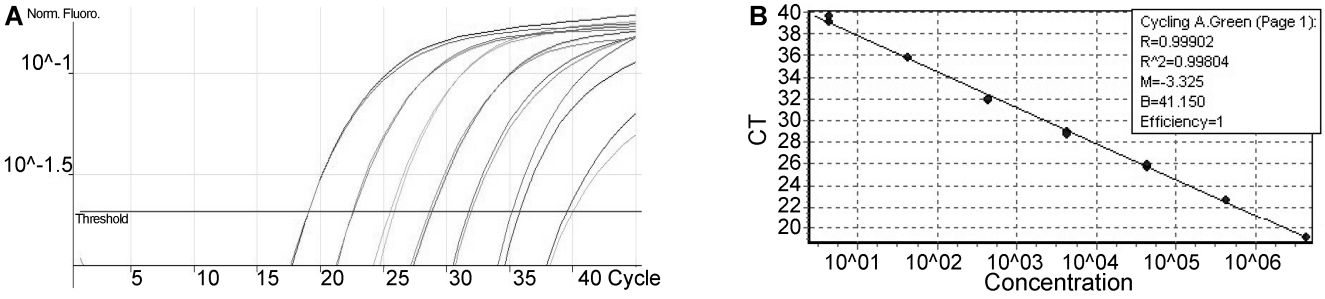

Fig. 1. Sensitivity of real-time PCR for the HPV type III detection.
Real-time PCR analysis of serial 10-fold dilution standard DNA was conducted to generate a fluorescence curve (**A**) and a standard curve with threshold cycles (CT) plotted against template DNA concentration (**B**). R, correlation coefficient; M, slope; B, intercept; Efficiency, PCR amplification efficiency.

Table 2. The mean, standard deviation, and range of HPV copies in wild *F. chinensis* broodstocks of years 2007 (B_1–B_3) and 2008 (B_4–B_6).

Broodstock	HPV copy/ng DNA		HPV copy/mg tissue	
	Mean	Range	Mean	Range
B_1	$2.58\times10^{-2}\pm9.83\times10^{-2}$	$0-4.82\times10^{-1}$	$3.14\times10^{1}\pm1.22\times10^{2}$	$0-7.56\times10^{2}$
B_2	0	0	0	0
B_3	0	0	0	0
Mean	$8.60\times10^{-3}\pm3.13\times10^{-2}$	$0-1.61\times10^{-1}$	$1.05\times10^{1}\pm4.08\times10^{1}$	$0-2.50\times10^{2}$
B_4	1.79±4.13	0–19.00	$1.88\times10^{3}\pm4.39\times10^{3}$	$0-1.93\times10^{4}$
B_5	0.01±0.05	0–0.30	14.04±65.91	$0-3.93\times10^{2}$
B_6	0.13±0.25	0–1.10	$1.73\times10^{2}\pm4.17\times10^{2}$	$0-1.93\times10^{3}$
Mean	0.64±1.48	0–6.80	$6.89\times10^{2}\pm1.63\times10^{3}$	$0-7.21\times10^{3}$

the range of 0–0.48 copies/ng of DNA and $0–7.56\times10^{2}$ copies/mg tissue of pleopods. Of 159 brooders, 154 (96.85%) were negative and 5 (3.15%) were positive for HPV type III infection (Table 2, Fig. 2).

In 2008, HPV type III infections were observed from all three wild broodstock groups with a range of $0–1.90\times10^{1}$ copies/ng of DNA and 1.93×10^{4} copies/mg tissue of pleopods (Table 2). The mean copy number was 6.41×10^{-1}/ng of DNA. The lowest mean HPV copy number was detected in broodstock B_5, which showed 1.0×10^{-2} copies/ng of DNA and 1.40×10^{1} copies/mg of tissue. Broodstocks B_4 and B_6, respectively, showed slightly higher infections with mean values of 1.79×10^{0} and 1.3×10^{-1} copies/ng of DNA and 1.88×10^{3} and 6.89×10^{2} copies/mg of tissue (Table 2).

HPV Type III Quantification in PL of Hatcheries

No PL from the two hatcheries, H_1 and H_2, collected in 2007 showed HPV infection, and PL from the rest of the five hatcheries had a range of $0–1.74\times10^{6}$ (mean, 6.3×10^{4}) copies/ng of DNA. This was equivalent to 0 to 2.35×10^{9} (mean, 8.2×10^{7}) copies/mg tissue, and to $0–2.85\times10^{10}$ (mean, 8.01×10^{8}) copies/individual PL (Table 3). Only 10% of the PL from hatchery H_5 showed HPV infection, but the copy number was less than 10 copies ng^{-1} of DNA. With the exception of 5% of hatchery H_7 showing 100–1,000 copies/ng of DNA, the rest of the PL from hatchery H_7 and all PL from hatcheries H_3, H_4, and H_6 showed HPV infections with over 1,000 copies/ng of DNA (Fig. 3).

The PL from all seven hatcheries collected in 2008 showed a range of $0–2.10\times10^{5}$ (mean, 4.38×10^{3}) HPV copies/ng of DNA. This was equivalent to $0–2.13\times10^{9}$ (mean, 2.88×10^{7}) copies/mg tissue, and to $0–8.60\times10^{8}$ (mean, 2.96×10^{7}) copies/individual (Table 3). PL from hatcheries H_8 and H_{10} showed less than 100 copies/ng of DNA, but PL from the rest of the hatcheries had high numbers of HPV infection (Fig. 3).

DICUSSION

The worldwide distribution of HPV, including that in Pacific Asia, is a big threat to shrimp aquaculture in Korea. Because of heavy infection and severe economic loss by WSSV, HPV has not been of importance in Korea until recently. However, because of its effect on shrimp growth and high mortality *via* infection of early larval stages [8, 14, 24], more careful monitoring of this virus is necessary.

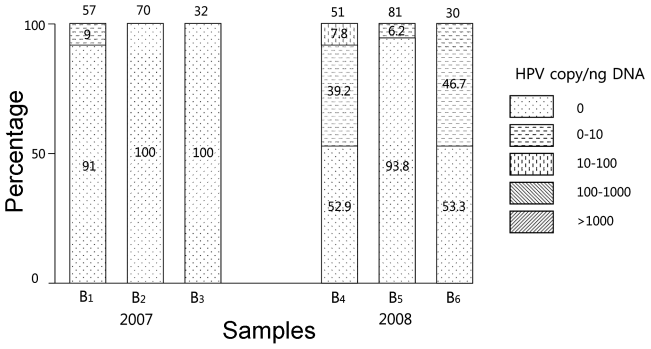

Fig. 2. Percentage of shrimps with different range of HPV copies in wild *F. chinensis* broodstocks of years 2007 and 2008.
The number above each bar indicates the number of analyzed shrimp.

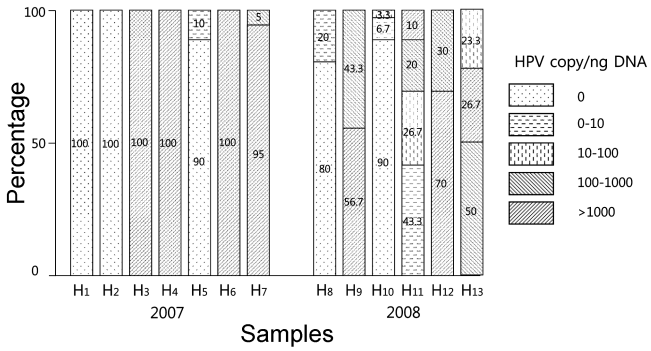

Fig. 3. Percentage of postlarvae with different ranges of HPV copies in seven *F. chinensis* hatcheries in years 2007 and 2008.
Twenty and 30 shrimps were analyzed from each of the hatcheries in 2007 and 2008, respectively.

Table 3. HPV quantification result in *F. chinensis* postlarvae from hatcheries of years 2007 (H_1–H_7) and 2008 (H_8–H_{13}).

Hatchery	Copy/ng DNA	Copy/mg tissue	Copy/individuals
H_1	0	0	0
H_2	0	0	0
H_3	$2.14\times10^{5}\pm3.87\times10^{5}$	$2.71\times10^{8}\pm5.38\times10^{8}$	$3.30\times10^{9}\pm6.54\times10^{9}$
H_4	$1.81\times10^{5}\pm2.81\times10^{5}$	$1.71\times10^{8}\pm2.44\times10^{8}$	$2.51\times10^{8}\pm3.58\times10^{8}$
H_5	$1.62\times10^{-2}\pm5.10\times10^{-2}$	$6.13\times10^{1}\pm2.01\times10^{2}$	$2.33\times10^{1}\pm7.63\times10^{1}$
H_6	$1.25\times10^{4}\pm8.24\times10^{3}$	$2.78\times10^{7}\pm1.58\times10^{7}$	$1.93\times10^{7}\pm1.10\times10^{7}$
H_7	$3.32\times10^{4}\pm5.11\times10^{4}$	$1.04\times10^{8}\pm1.95\times10^{8}$	$2.03\times10^{9}\pm3.80\times10^{9}$
Mean	$6.30\times10^{4}\pm1.04\times10^{5}$	$8.20\times10^{7}\pm1.42\times10^{8}$	$8.01\times10^{8}\pm1.53\times10^{9}$
H_8	$3.40\times10^{-1}\pm8.46\times10^{-1}$	$1.99\times10^{4}\pm5.32\times10^{4}$	$6.61\times10^{2}\pm1.91\times10^{3}$
H_9	$4.82\times10^{3}\pm7.14\times10^{3}$	$3.96\times10^{7}\pm6.59\times10^{7}$	$5.31\times10^{7}\pm7.86\times10^{7}$
H_{10}	1.13±6.18	$4.97\times10^{3}\pm2.71\times10^{4}$	$1.10\times10^{4}\pm6.02\times10^{4}$
H_{11}	$1.29\times10^{4}\pm4.89\times10^{4}$	$1.17\times10^{8}\pm4.53\times10^{8}$	$5.39\times10^{7}\pm2.02\times10^{8}$
H_{12}	$7.45\times10^{3}\pm1.33\times10^{4}$	$1.09\times10^{7}\pm1.80\times10^{7}$	$4.65\times10^{7}\pm7.91\times10^{7}$
H_{13}	$1.10\times10^{3}\pm1.92\times10^{3}$	$5.57\times10^{6}\pm1.10\times10^{7}$	$2.44\times10^{7}\pm4.50\times10^{7}$
Mean	$4.38\times10^{3}\pm1.19\times10^{4}$	$2.88\times10^{7}\pm9.12\times10^{7}$	$2.96\times10^{7}\pm6.75\times10^{7}$

Recent real-time PCR surveys on shrimp tissue and seawater from culture ponds of *F. chinensis* and *Litopenaeus vannamei* showed the presence of HPV type I in the tissues from both species and from the culture pond of *F. chinensis* [10]. To date, three types of HPV have been reported, suggesting their genetic difference is based on the host or geographical region from which the viruses were isolated [28]. The HPV isolated in Korea was determined to be type I based on *in situ* hybridization and the nucleotide sequence analysis of its genome [22, 23]. However, recent PCR analysis of shrimp showing HPV infection did not produce any PCR product, by using a type I-specific 1120F/1120R primer set [10, 22]. Further analysis of these shrimp showed that they were infected with HPV that showed sequence homology to *Pmerg*DNV, a type III isolate [10], whose prevalence in *F. chinensis* broodstock and PL was analyzed by real-time PCR.

The source of type III HPV in Korea is not clear at this moment. The HPV type I Korean strain was originally isolated from *P. chinensis*, which is now *F. chinensis* [2]. The other two type I strains from Tanzania and Madagascar were isolated from *P. monodon*. HPV type II, isolated from Thailand and Indonesia, was also from *P. monodon* [28]. In contrast, the host for HPV type III isolated from Australia and New Caledonia was *P. merguiensis* [12, 28]. *P. merguiensis* is widely distributed from the Persian Gulf and Pakistan through the Malay Archipelago and South China Sea to Australia, and part of this area overlaps with the overwintering area of *F. chinensis*. Therefore, the type III strain may have been transmitted from *P. merguiensis* to this area. Further analysis of the HPV population and sequence analysis of its entire genome will reveal the origin of this genotype.

Among the three broodstock groups tested in 2007, only one was positive for HPV type III, and the copy number was less than 10 copies/ng of DNA (Table 2, Fig. 2). In contrast, all three broodstock groups tested in 2008 were positive, and the copy numbers reached up to 100 copies/ng of DNA in the two groups (Table 2, Fig. 2). Although samples were collected from only three sampling sites and the locations differed in the 2 years, these data indicate that HPV type III is widely distributed in Korea and that the prevalence is increasing.

The results from broodstocks are somewhat related to the data from the hatcheries. In 2007, samples from two of seven hatcheries were negative for HPV (Table 9). However, all samples from the seven hatcheries were positive in 2008 (Fig. 3). The origin of the broodstocks in each hatchery is unknown, but the increase in the prevalence can be seen in the data. In contrast, the viral load in shrimp tissues was not positively related to the prevalence of the virus. In 2007, most of the infected shrimp showed over 1,000 copies of viral genome/ng of DNA (Fig. 3), but the HPV copy numbers/ng of DNA in 2008 were different in each sample (Fig. 3). The differences in viral load were probably not related to the developmental stage or progress of virus infection because the developmental stages for the samples in 2008 were later than those in 2007 (Tables 1). Horizontal transmission of HPV was demonstrated by feeding *P. monodon* with HPV-infected PL [3]. Although vertical transmission of HPV has not been experimentally validated, vertical transmission of other penaeid shrimp viruses, such as WSSV and gill-associated virus, has been confirmed [6, 7]. Therefore, the differences in viral copy numbers in PL observed in 2007 and 2008 could have been related to the viral load of broodstocks from which the larvae were obtained.

Sensitivity and specificity are major requirements in the development of virus detection methods. In the early stages of HPV detection method development, the detection limit by conventional PCR was 300 virus particles [22], and up to 3 copies of HPV could be detected by PCR–ELISA [26]. Recent development of TaqMan real-time PCR made it possible to detect three copies of virus particles or a single copy of cloned DNA [11, 29]. In our experiment, we could detect as few as two copies of viral genome with an optimal PCR mixture, which is comparable to the detection limit by TaqMan real-time PCR. We tested our primers for specificity using samples positive for WSSV and HPV type I. No positive result was obtained from samples confirmed positive for HPV type I by PCR using the type I-specific PCR primers 1120F/1120R [22], which indicated that the primers used in this experiment were specific to HPV type III.

Because of heavy economic loss by WSSV, the major aquaculture shrimp species *F. chinensis* has been replaced with the Pacific white shrimp *L. vannamei* since 2003 [10]. Although this species was selected because of its resistance to WSSV and HPV, a recent survey by real-time PCR indicated that HPV was present in the pond water of *F. chinensis* farms and tissues of *L. vannamei*. At this moment, the major known pathology of HPV is retardation of shrimp growth [8, 14, 24]. However, because of its wide geographical distribution, wide host range, and the presence of different genotypes, this virus can cause mass mortality of shrimp. Continuous monitoring for emergencies and the development of new strains of this virus are necessary.

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