

Development and applications of the real-time polymerase chain reaction method for rapid quantitative detection of the newly emerged novel genotype of *Decapod hepanadenovirus 1* in *Penaeus vannamei*

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ABSTRACT

Decapod hepanadenovirus 1 (decapod hepanhamaparvovirus [DHPV]) is a member of the family *Parvoviridae*. The natural host of DHPV includes various captured or cultured penaeid shrimp species. The virus infects the host's hepatopancreas, leading to disturbance in digestion and reduced shrimp yields, causing substantial economic losses. Recently, the emergence of a new DHPV genotype (nDHPV) in *Penaeus vannamei* has been reported, and considering that the shrimp is one of the most extensively cultivated species in the global aquaculture industry, the development of diagnostic methods for the virus is urgent. To address the need for accurate detection and diagnosis of nDHPV, this study presents a diagnostic quantitative polymerase chain reaction method designed to detect the virus currently prevalent in *P. vannamei*. The assay was applied to various nDHPV-positive samples to confirm its practical utility and quantify the prevalence of nDHPV. Moreover, the analytical specificity of this assay to other several shrimp pathogens was also addressed. To the best of our knowledge, this study represents the first report of a quantitative method based on nDHPV identified from *P. vannamei*. The findings provide valuable insights into the pathogenesis and epidemiology of nDHPV in this economically significant species.

1. Introduction

Decapod hepanadenovirus 1 (decapod hepanhamaparvovirus [DHPV]) (Srisala et al., 2021) is a small (22–24 nm in size), icosahedral-shaped, non-enveloped virus that harbors a single-stranded DNA genome approximately 6 kb in size (La Fauce et al., 2007a; Tang et al., 2008). Based on its characteristics, DHPV is placed within the genus *Hepadenovirus*, subfamily *Densovirinae*, family *Parvoviridae*.

The natural host of DHPV includes various captured or cultured penaeid shrimp species such as *P. merguensis*, *P. semisulcatus*, *P. chinensis*, *P. esculentus*, *P. monodon*, *P. indicus*, *P. penicillatus*, *P. japonicus*, *P. stylirostris*, and *P. vannamei* (Lightner and Redman, 1985; Lightner, 1996; Roubal et al., 1989). The virus is known to infect the

hepatopancreas of the host, resulting in disturbed digestion. This results in lower harvests, causing significant economic losses to shrimp farms. Additionally, given that *P. vannamei* is one of the most widely cultivated species in shrimp aquaculture (FAO, 2022), the detection of DHPV infection in this species highlights the need for careful attention and appropriate action. Previous studies have reported that DHPV is also susceptible to *P. vannamei* and also widely spread among the shrimp-culturing farms in the global shrimp industry (Dhar et al., 2019). The emergence of a novel genotype of DHPV (nDHPV) in multiple *P. vannamei*-culturing farms was recently reported in Korea (Lee et al., 2023). These findings are consistent with those of some previous studies, including the detection of DHPV in the Indian (Singaravel et al., 2021) and nDHPV in the Taiwan shrimp farms (Lee et al., 2022). The

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emergence and prevalence of nDHPV in *P. vannamei* across different regions underscore the necessity for establishing a diagnostic method to identify and manage the new virus from this important culturing shrimp species.

Nucleic acid-based molecular diagnostic methods, such as the quantitative polymerase chain reaction (qPCR), are easy, rapid, and nondestructive compared to other methods, such as histology (Safeena et al., 2012). Thus, several molecular diagnostic methods have been developed for detecting DHPVs in various penaeid shrimp species, such as *P. merguensis*, *P. monodon*, and *P. chinensis* (La Fauce et al., 2007b; Sukhumsirichart et al., 1999; Phromjai et al., 2001). However, the genomic sequence of DHPVs varies among different hosts and geographical locations, and the knowledge of DHPV infection in *P. vannamei* remains limited compared to other culturing species despite its dominance in the global shrimp-culturing industry. Moreover, a notable disparity is observed in genome sequences between nDHPVs (Lee et al., 2022; Lee et al., 2023) and the previously reported DHPVs currently available in the GenBank database (NC_014357, AY008257, JN082231, EU247528, MT980830, EU588991, NC_011545, NC_007218, and DQ458781). Although the wide prevalence and possible transmissibility of nDHPV has been confirmed, a definitive diagnostic method for the accurate and selective detection of the virus in *P. vannamei* is yet to be established.

This study presents a diagnostic qPCR method targeting nDHPV that recently emerged at *P. vannamei* in the global shrimp industry. The practical usability of the newly developed assay was evaluated using various nDHPV-positive samples to detect and quantify the nDHPV prevalence. To the best of our knowledge, this is the first report of a diagnostic method established based on nDHPV isolated from *P. vannamei*, and this assay may provide valuable insights into the pathogenesis and epidemiology of nDHPV in this economically important species.

2. Materials and methods

2.1. Primer set and probe for the qPCR assay

The qPCR primers and TaqMan probe were designed based on a partial sequence of nDHPV identified from *P. vannamei* in Korea (Accession No. ON015651) (Lee et al., 2023) using the Primer Express 3.0.1 software (Life Technologies). The forward (HPVqF1: 5'-AGG AGA ATC AGC TGG CTA TGG T-3') and reverse (HPVqR1: 5'-TGA GGT CAA TCG GCT TAT TGC-3') primers were able to amplify 74 base-pair (bp) DNA fragment. The TaqMan probe (5'-AAA CCC CAG TGA TGC ATT TCA GCG C-3') is synthesized and labeled with 6-carboxyfluorescein on the 5' end and N, N, N', N'-tetramethyl-6-carboxyrhododamine on the 3' end.

For the qPCR assay, the AccuPower® 2× DualStar™ qPCR Master Mix (Bioneer) was used. Samples of the extracted DNA were added to a qPCR mixture containing 0.3 μM of each primer and 0.1 μM TaqMan probe to a final volume of 20 μL. The qPCR profile consisted of 5 min at 95 °C, followed by 40 cycles of 20 s at 95 °C and 30 s at 60 °C. Amplification detection and data analysis for qPCR assays were performed using the StepOnePlus real-time PCR system (ThermoFisher).

2.2. Validation of primers and probe

A positive control plasmid, the pBHA vector, containing the target sequence (74 bp) of nDHPV was synthesized (Bioneer), and the recombinant plasmid was designated as pqHPV3, 2649 bp in size. To determine the analytical sensitivity of the qPCR, 10-fold dilutions of purified pqHPV3 plasmid ranging from 10² to 10⁸ copies/μL are used.

The analytical qPCR specificity test includes the DNA of a total of six samples: one sample for each crayfish infected with infectious hypodermal and hematopoietic necrosis virus (IHHNV), white spot syndrome virus (WSSV), acute hepatopancreatic necrosis disease-pathogenic *Vibrio parahaemolyticus* (Vp_{AHPND}), and *Enterocytozoon hepatopenaei* (EHP),

and two samples of uninfected *P. vannamei* shrimp that were free of all the aforementioned four pathogens (IHHNV, WSSV, Vp_{AHPND}, and EHP) and nDHPV.

Moreover, the primer and probe sequences were compared with nine previously reported aforementioned DHPVs currently available in the GenBank database.

2.3. Field sample collection and nDHPV quantification

For field samples, 48 *P. vannamei* shrimp, two pond water, two feces, 3 barnacles, and six crabs were collected from 14 shrimp farms across in Korea from April to November 2021 (Table 1). For DNA extraction, the hepatopancreas (average 30 mg) of the shrimp was pooled from three representative shrimps from each of the sampled rearing ponds. The DNA was extracted using a DNeasy Blood & Tissue kit (Qiagen Korea Ltd., Seoul, Korea) according to the manufacturer's instructions. Similarly, pond water (300 μL), shrimp feces (30 mg), barnacles (30 mg), and crab hepatopancreas (30 mg) samples were used for DNA extraction. The conventional PCR was previously performed to detect nDHPV from DNA samples used in this study (Lee et al., 2023). These DNA samples were further used for the qPCR assay.

2.4. Determining the tissue preference of nDHPV

To determine the tissue tropism of nDHPV, four types of tissues, including the hepatopancreas, gill, body muscle, and cuticle, were aseptically collected from DHPV-infected *P. vannamei* shrimp (*N* = 6, Sample No. 21-084B1-S1, 21-084B1-S2, 21-084B1-S3, 23-084B1-S1, 21-084B2-S2, and 21-084B2-S3) obtained from the shrimp farm located in Seosan-si and Chungcheongnam-do, Korea. The DNA was extracted from 30 mg of each tissue using the aforementioned method.

2.5. Detection of nDHPV in laboratory bioassay

The infection experiment aimed to understand the mode of transmission of DHPV infections. Furthermore, it was carried out to determine the effectiveness of the developed qPCR method for the diagnosis. The nDHPV stock (chopped shrimp whole body, 5.26 × 10⁶ copies/mg) was previously acquired from a local shrimp farm in 2021. The stock was tested and established to be negative for four major shrimp pathogens (IHHNV, WSSV, Vp_{AHPND}, and EHP) using PCR assays (Tang et al., 2007; Nunan and Lightner, 2011; Han et al., 2015; Jaroenlak et al., 2016). nDHPV was detected and quantified from the stock with the qPCR assay presented in this study. The obtained stock was stored in a −80 °C deep freezer until further use.

For nDHPV laboratory infection, healthy *P. vannamei* (*N* = 40, mean weight: 5 g) was used. The shrimps were divided into two groups: the negative control group, and the infection group. The experiment was conducted in duplicates, with two tanks per group, each tank containing 10 shrimps. The negative control group was fed only the conventional feed for 6 days, whereas the infection group was fed conventional feed mixed with nDHPV stocks for 3 days, followed by conventional feed for 3 days. The feed-stock mixture administered to the infection group consisted of the conventional feed mixed in a one-to-one weight ratio with the nDHPV stock. Each tank was fed three times a day with a total daily feed weight equivalent to 5 % of their initial body weight. The tanks were set up with 8 L of 25 ppt seawater, and the temperature was maintained at 28 °C. The shrimps were observed for gross signs and mortality every 6 h during the experimental period. Representative shrimps from each tank were sampled 24 (day 4) and 60 h (day 6) after 3 days of experimental feeding. The DNA was extracted from their hepatopancreas and was analyzed using the newly developed qPCR method.

Table 1

History and qPCR results of collected samples utilized in this study.

Aquafarm	Sample ID	Month / Year	Province/area	Sample types (numbers)	Ct value (copy no. per mg)
Farm 1	21-022A1	Apr/2021	Chungcheongnam-do/Taeon-gun	Pond water (<i>N</i> = 1)	23.26 ± 0.30 (5.79 × 10 ⁶ –8.75 × 10 ⁹)
	21-022B1	Apr/2021		Shrimp (<i>N</i> = 3)	25.03 ± 0.26 (1.72 × 10 ⁶ –2.40 × 10 ⁹)
	21-023F1	Apr/2021		Feces (<i>N</i> = 2)	22.34 ± 0.10 (1.32 × 10 ⁷ –1.50 × 10 ⁷)
	21-055B2	Aug/2021		Shrimp (<i>N</i> = 3)	31.36 ± 0.11 (2.30 × 10 ⁴ –2.71 × 10 ⁴)
Farm 2	21-042B	Jun/2021	Chungcheongnam-do/Taeon-gun	Shrimp (<i>N</i> = 3)	23.33 ± 0.11 (6.61 × 10 ⁶ –7.61 × 10 ⁶)
					18.83 ± 1.42 (7.72 × 10 ⁷ –5.04 × 10 ⁸)
Farm 3	21-044B1	Jun/2021	Incheon/Ganghwa-gun	Shrimp (<i>N</i> = 3)	22.32 ± 0.13 (4.04 × 10 ⁸ –5.67 × 10 ⁸)
Farm 4	21-046B	Jul/2021	Incheon/Ganghwa-gun	Shrimp (<i>N</i> = 3)	24.52 ± 0.38 (2.22 × 10 ⁶ –3.55 × 10 ⁶)
					27.33 ± 0.09 (3.99 × 10 ⁵ –4.52 × 10 ⁵)
Farm 7	21-058B2	Aug/2021	Jeollanam-do/Sinan-gun	Shrimp (<i>N</i> = 3)	35.37 ± 1.00 (6.77 × 10 ² –2.31 × 10 ³)
					32.84 ± 0.19 (7.90 × 10 ³ –1.03 × 10 ⁴)
Farm 8	21-060B2	Aug/2021	Incheon/Ganghwa-gun	Shrimp (<i>N</i> = 3)	30.54 ± 0.16 (4.13 × 10 ⁴ –5.09 × 10 ⁴)
	21-098I1	Nov/2021		Barnacle (<i>N</i> = 3)	11.39 ± 1.26 (1.37 × 10 ¹⁰ –7.67 × 10 ¹⁰)
Farm 9	21-063B1	Aug/2021	Jeollanam-do/Sinan-gun	Shrimp (<i>N</i> = 3)	30.55 ± 0.12 (4.01 × 10 ⁴ –4.68 × 10 ⁴)
					28.62 ± 0.29 (1.35 × 10 ⁴ –1.97 × 10 ⁵)
Farm 10	21-028A3	Apr/2021	Chungcheongnam-do/Seosan-si	Pond water (<i>N</i> = 1)	14.95 ± 1.69 (1.17 × 10 ⁹ –9.62 × 10 ⁹)
	21-028B1	Apr/2021		Shrimp (<i>N</i> = 3)	22.32 ± 0.13 (1.27 × 10 ⁷ –1.48 × 10 ⁷)
	21-064B4	Aug/2021		Shrimp (<i>N</i> = 3)	31.20 ± 0.07 (2.67 × 10 ⁴ –2.93 × 10 ⁴)
Farm 11	21-073B	Aug/2021	Chungcheongnam-do/Seosan-si	Shrimp (<i>N</i> = 3)	29.52 ± 0.10 (8.43 × 10 ⁴ –9.70 × 10 ⁴)
					32.31 ± 0.02 (1.27 × 10 ⁴ –1.30 × 10 ⁴)

Table 1 (continued)

Aquafarm	Sample ID	Month / Year	Province/area	Sample types (numbers)	Ct value (copy no. per mg)
Farm 12	21-077B1	Aug/2021	Chungcheongnam-do/Taeon-gun	Shrimp (<i>N</i> = 3)	22.32 ± 0.13 (1.27 × 10 ⁷ –1.48 × 10 ⁷)
					31.20 ± 0.07 (2.67 × 10 ⁴ –2.93 × 10 ⁴)
Farm 13	21-078B2	Aug/2021	Jeollanam-do/hampyeong-gun	Shrimp (<i>N</i> = 3)	29.52 ± 0.10 (8.43 × 10 ⁴ –9.70 × 10 ⁴)
					32.31 ± 0.02 (1.27 × 10 ⁴ –1.30 × 10 ⁴)
Farm 14	21-080	Sep/2021	Chungcheongnam-do/Taeon-gun	Crab (<i>N</i> = 3)	0.07 (2.67 × 10 ⁴ –2.93 × 10 ⁴)
	21-086	Sep/2021		Crab (<i>N</i> = 3)	0.10 (8.43 × 10 ⁴ –9.70 × 10 ⁴)
	21-090B	Sep/2021		Shrimp (<i>N</i> = 3)	0.02 (1.27 × 10 ⁴ –1.30 × 10 ⁴)

Note: A previous report (Lee et al., 2023) has detected DHPV in all the field samples used in this study.

2.6. Statistical analysis

The data was expressed as mean ± standard deviation (SD) and analyzed using repeated measures of variance. The Tukey test was used to test for differences among means for which the analysis of variance (ANOVA) indicated a significant ($p \leq 0.05$) *F*-ratio.

3. Results

3.1. Analytical sensitivity and specificity of the qPCR assay

To determine the analytical sensitivity of the qPCR, a plasmid pQHPV3 is used as the positive control. The standard curve (10² to 10⁸ copies of pQHPV3 plasmid) is depicted in Fig. 1. The resulting Ct values are plotted against the logarithm of their respective copy number.

The analytical specificity of the qPCR assay was evaluated by analyzing the DNA of the hepatopancreas of a pathogen-free *P. vannamei* and DNAs of the four major shrimp pathogens: IHNV, WSSV, VpAHPND, and EHP. The results showed no cross-reaction of the primer with host genomes or other tested shrimp pathogens (data not shown).

3.2. Quantification of nDHPV in field samples

The primary aim was to verify the practical utility of the qPCR assay and confirm the prevalence of nDHPV in shrimp farms. The new qPCR assay was able to detect nDHPV from all analyzed samples. Specifically, Ct values in the shrimp samples exhibited a wide range, spanning from 11.39 to 35.37. The Ct values observed in the pond water samples ranged from 23.26 to 30.54, and a single feces sample displayed a Ct value of 22.34. The Ct value of the barnacle samples was 35.37, and Ct values in crab samples ranged from 29.52 to 31.20. The results are presented in Table 1.

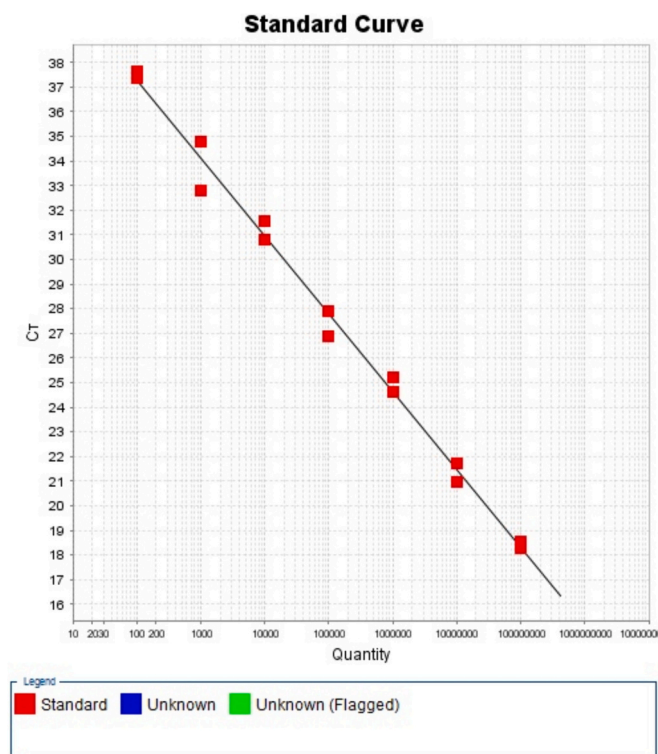


Fig. 1. The standard curve (10^2 to 10^8 copies of pHPV3 plasmid). The resulting Ct values are plotted against the logarithm of their respective copy number.

3.3. Quantification of nDHPV from different tissues

To analyze differences in the viral status among four different tissues, the hepatopancreas, gill, body muscle, and cuticle of six individual shrimps (Sample No. 21-084B1-S1, 21-084B1-S2, 21-084B1-S3, 23-084B1-S1, 21-084B2-S2, and 21-084B2-S3) were used. The qPCR assay detected nDHPV from all shrimp tissues (Table 2). Notably, the Ct value of the hepatopancreas was comparatively lower than that of the other tissues. The statistical comparison indicated a significant difference,

Table 2
Result of qPCR on four different shrimp tissues.

Sample	Tissue	Ct value (Copy no. per mg)
21-084B1-S1	Hepatopancreas	$17.17 \pm 0.10 (1.47 \times 10^8 - 1.63 \times 10^8)$
	Gill	$20.38 \pm 0.27 (1.94 \times 10^7 - 2.55 \times 10^7)$
	Muscle	$21.82 \pm 0.14 (7.26 \times 10^6 - 8.37 \times 10^6)$
	Cuticle	$19.85 \pm 0.09 (3.11 \times 10^7 - 3.40 \times 10^7)$
21-084B1-S2	Hepatopancreas	$16.49 \pm 0.13 (3.60 \times 10^8 - 4.12 \times 10^8)$
	Gill	$22.86 \pm 0.33 (3.08 \times 10^6 - 4.31 \times 10^6)$
	Muscle	$22.43 \pm 0.17 (4.57 \times 10^6 - 5.44 \times 10^6)$
	Cuticle	$22.79 \pm 0.01 (3.53 \times 10^6 - 3.85 \times 10^6)$
21-084B1-S3	Hepatopancreas	$17.49 \pm 0.28 (1.58 \times 10^8 - 2.10 \times 10^8)$
	Gill	$24.10 \pm 0.25 (1.30 \times 10^7 - 1.68 \times 10^7)$
	Muscle	$20.45 \pm 0.05 (2.05 \times 10^8 - 2.16 \times 10^8)$
	Cuticle	$19.70 \pm 0.15 (3.38 \times 10^8 - 3.95 \times 10^8)$
21-084B2-S1	Hepatopancreas	$22.05 \pm 0.04 (6.42 \times 10^7 - 6.70 \times 10^7)$
	Gill	$20.91 \pm 0.00 (1.50 \times 10^8 - 1.51 \times 10^8)$
	Muscle	$26.49 \pm 0.15 (2.39 \times 10^6 - 2.80 \times 10^6)$
	Cuticle	$22.38 \pm 0.01 (5.12 \times 10^7 - 5.18 \times 10^7)$
21-084B2-S2	Hepatopancreas	$20.79 \pm 0.13 (1.53 \times 10^8 - 1.76 \times 10^8)$
	Gill	$22.87 \pm 0.15 (3.33 \times 10^7 - 3.90 \times 10^7)$
	Muscle	$30.16 \pm 0.29 (1.54 \times 10^5 - 2.07 \times 10^5)$
	Cuticle	$27.87 \pm 0.05 (9.21 \times 10^5 - 9.68 \times 10^5)$
21-084B2-S3	Hepatopancreas	$16.97 \pm 0.18 (2.43 \times 10^9 - 2.91 \times 10^9)$
	Gill	$22.65 \pm 0.08 (4.00 \times 10^8 - 4.35 \times 10^8)$
	Muscle	$22.59 \pm 0.08 (4.25 \times 10^7 - 4.62 \times 10^7)$
	Cuticle	$27.83 \pm 0.25 (8.55 \times 10^5 - 1.10 \times 10^6)$

with the mean Ct value of the hepatopancreas in six individual shrimp being notably lower than those observed in the other organs of the same six shrimps, correlated with the result (Data not shown).

3.4. nDHPV detection in laboratory bioassay

In the laboratory bioassay, healthy shrimps were exposed to nDHPV by *per os* feeding of infected shrimp whole body for three consecutive days. All individual shrimps lacked any gross signs of the virus and appeared healthy during the experimental period, and no mortality occurred throughout the bioassay. The qPCR assay detected the presence of nDHPV at the 24-h point and its persistence at the 60-h point. Ct values of the control group were not determined. The results of the laboratory bioassay are shown in Table 3.

4. Discussion

The DHPV genome comprises three major open reading frames (ORFs), and among these, the viral capsid protein (VP) region exhibits the most significant sequence variation (Tang et al., 2008). Due to its high sequence difference, the VP region sequence is currently employed to define the genotype of DHPV and remains a meaningful tool for genotype-specific studies. The qPCR assay in the present study is developed based on the VP region sequence.

Previous studies have demonstrated a notable negative correlation between the DHPV severity index and the body length of the shrimp (Flegel et al., 1999; Phuthaworn et al., 2016). This inverse relationship is due to the fact that DHPV infection primarily targets the hepatopancreas, resulting in disrupted digestion and subsequent impaired growth of the affected shrimp. The present study compared Ct values of the qPCR assay among four different tissues, the hepatopancreas, gill, body muscle, and cuticle, to determine the tissue preference of DHPV. The results revealed that five out of six hepatopancreas samples exhibited lower Ct values (ranging from 16.49 to 22.05) than that of other tissues from the same shrimp (ranging from 19.70 to 30.16). The finding supports that the hepatopancreas is the primary organ targeted by the virus, corresponding to that of previous studies (Lightner and Redman, 1985; Safeena et al., 2012).

In *P. vannamei*, it has been demonstrated that DHPV specifically targets the rapidly dividing E cell in the hepatopancreas (Lee et al., 2022), and since it is the main target organ of the virus, the hepatopancreas is a preferred tissue sample for detecting DHPV (Lightner and Redman, 1985). However, obtaining the hepatopancreas for analysis requires sacrificing the shrimp; therefore, developing alternative noninvasive methods is important. Like other pathogens targeting the enteric organs, such as AHPND or EHP (Han et al., 2017; Tang et al., 2016), Pantoja and Lightner (2000), has successfully identified DHPV

Table 3
Result of qPCR on the laboratory transmission bioassay.

Sample No.	Sample	Ct value (Copy no. per mg)	
22-025A1	NC tank	Ctr group 1 24 h	Not determined
22-025A2	NC tank	Ctr group 2 24 h	Not determined
22-025A3	HPV tank	Exp group 1 24 h	28.76 ± 0.24 (5.28 × 10 ⁴ –7.24 × 10 ⁴)
22-025A4	HPV tank	Exp group 2 24 h	31.48 ± 0.11 (8.36 × 10 ³ –1.01 × 10 ⁴)
22-025B1	NC tank	Ctr group 1 60 h	Not determined
22-025B2	NC tank	Ctr group 2 60 h	Not determined
22-025B3	HPV tank	Exp group 1 60 h	35.03 ± 1.64 3.01 × 10 ² –1.68 × 10 ³)
22-025B4	HPV tank	Exp group 2 60 h	30.52 ± 0.14 (1.39 × 10 ⁴ –1.77 × 10 ⁴)

from shrimp feces. Noninvasive diagnosis methods, such as examining the presence of the pathogen through feces samples, are ideal for monitoring valuable brood stocks, and they may also not displease shrimp farmers. The results of the qPCR assay used in this study demonstrate that the assay can successfully detect nDHPV in fecal samples (Ct value of 22.34) collected from farms, and this holds a practical utility for effectively monitoring nDHPV in broodstock farms and local shrimp farms.

The transmission route of DHPV is not entirely understood, and this study does not provide any conclusive evidence regarding the transmission routes. Nonetheless, the detection of nDHPV in shrimp and various environmental samples and the significantly high copy numbers of environmental samples suggest that nDHPV spreads within the farmed shrimp population, likely facilitated by various factors, such as the marine environment and other organisms, and also emphasizes the significance of environmental factors in understanding the transmission patterns of the virus. Moreover, the detection of nDHPV in water and feces samples obtained from the field indicates that the virus is stable and persistent in the aquatic environment. Although PCR-based detection does not necessarily reflect infection and further studies regarding the infectivity are needed, however, the detection of the virus in environmental factors, which can act as a vehicle, signifies the virus's transmission capability. Viral pathogen transmission through the water, such as immersion or cohabitation, has been widely accepted and investigated in several studies regarding viral shedding and waterborne transmission. Durand and Lightner (2002) and Kumar et al. (2013) have reported an infection via viral immersion and determined the minimum infective dose of WSSV, a major pathogen affecting shrimps. Furthermore, several studies have explored the transmission mechanisms and shedding dynamics of other viral diseases, such as viral hemorrhagic septicemia virus, infectious hematopoietic necrosis virus, nervous necrosis virus, and red sea iridovirus (Hershberger et al., 2010; Garver et al., 2013; Krishnan et al., 2021; Kawato et al., 2021). Furthermore, the laboratory bioassay of the present study confirmed the possibility of horizontal transmission of nDHPV through cannibalism. Even though infected shrimps survived the laboratory bioassay without showing notable gross signs, they continue to carry a considerable amount of the virus, which could potentially act as a vector, leading to the spread of the disease and hindering the growth rates of shrimps in long-term farming practices. In addition, the gut passage time of juvenile *P. vannamei* typically ranges between 60 and 90 min (Beseres et al., 2006), and the virus was detected on both day 4 and day 6. This, coupled with the qPCR assay performed on the hepatopancreas rather than the stomach, provides a reasonable basis to assume that the source of nDHPV DNA detected in the qPCR assay is from infected virus rather than residual DNA fragment from ingestion.

The development of the nDHPV qPCR assay is an important step toward implementing appropriate measures to prevent the spread of nDHPV in the aquaculture industry. This assay can be used to screen shrimp populations for nDHPV infections, allowing the early detection and application of quarantine protocols to prevent the further spread within and between shrimp farms. Furthermore, the assay can be used to monitor the effectiveness of disease management strategies and to track the molecular epidemiology of nDHPV. However, to protect the sustainability of the aquaculture industry, further research is required to fully understand the potential implications of nDHPV spread in the shrimp-farming industry, and it is also essential to continue to develop effective nDHPV control and prevention measures.

CRediT authorship contribution statement

Bumkeun Kim: Writing – original draft. **Chorong Lee:** Formal analysis. **Hye Jin Jeon:** Formal analysis. **Patharapol Piamsomboon:** Writing – review & editing. **Ji Hyung Kim:** Supervision. **Jee Eun Han:** Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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