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ORIGINAL ARTICLE

Development of a real-time multiplex PCR assay for detection of viral pathogens of penaeid shrimp

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Abstract A real-time multiplex polymerase chain reaction (rtm-PCR) assay was developed and optimized to simultaneously detect three viral pathogens of shrimp in one reaction. Three sets of specific oligonucleotide primers for white spot syndrome virus (WSSV), infectious hypodermal and haematopoietic necrosis virus (IHHNV) and Taura syndrome virus (TSV), along with three TaqMan probes specific for each virus were used in the assay. The rtm-PCR results were detected and analyzed using the Light Cycler 2.0 system. Forty-five PCR-positive samples and four negative samples were used to confirm the sensitivity and specificity of the rtm-PCR. The rtm-PCR identified and differentiated the three pathogens. With one viral infection of shrimp, a specific amplified standard curve was displayed. When samples from shrimp infected with two or three pathogens were analyzed, two or three specific standard curves were displayed. The sensitivity of the rtm-PCR assay was 2,000, 20, and 2,000 template copies for WSSV, IHHNV and TSV, respectively. No positive results (standard curves) were displayed when nucleic acid from *Vibro* spp., and *Streptococcus* spp. DNA were used as PCR templates. The results indicate that real-time multiplex PCR is able to detect the presence of and differentiate each pathogen in infected shrimp. This real-time multiplex PCR assay is a quick, sensitive, and specific test for detection of WSSV, IHHNV and TSV and will be useful for the control of these viruses in shrimp.

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Introduction

White spot syndrome virus (WSSV), infectious hypodermal and haematopoietic necrosis virus (IHHNV) and Taura syndrome virus (TSV) are three major viral pathogens that infect penaeid shrimp [2–4, 6, 8]. Mixed infections with these viruses have been described [12] and can cause high mortality, leading to economic losses that are detrimental to the shrimp farming industry [9, 16, 21].

Multiple diagnostic methods such as histologic examination, electron microscopy and *in situ* hybridization are required to detect and differentiate these viral pathogens [8, 9, 16, 18]. However, these methods are time consuming and labor intensive. Molecular assays, such as DNA probes [13, 15] and PCR methods, have been used for rapid and sensitive detection of these viruses [7, 10, 14, 19, 20]. Recently we developed multiplex reverse transcription-PCR for the simultaneous differentiation of three viral pathogens of penaeid shrimp [22]. Real-time PCR is preferred over conventional PCR in clinical laboratories because there is no need for post-amplification handling, leading to faster analysis and reduced risk of amplicon contamination [11, 17]. Real-time PCR can also provide an estimate of pathogen titer [1]. Recently, real-time PCR

assays have been developed for the separate detection of WSSV, TSV and IHHNV [5, 23]. This study describes a real-time multiplex PCR assay for simultaneous detection of these three viruses.

Materials and methods

Clinical samples and plasmids

WSSV-, IHHNV- and TSV-infected clinical tissue samples and positive controls consisted of recombinant plasmids containing specific and conserved genes of the three viruses (TSV-pMD18-T, WSSV-pMD18-T, IHHNV-pMD18-T). These three plasmids were cloned using the specific primers described previously [22] and are listed in Table 3.

Isolation of nucleic acids from clinical tissues samples

RNA and DNA extractions from WSSV, IHHNV, and TVS isolates were carried out using Trizol according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). Total nucleic acid was extracted from clinical tissue samples from shrimp infected with WSSV, IHHNV, and TVS as well as from disease-free white shrimp and *Penaeus orientalis* Kishinouye according to a method described previously [22]. Extracted DNA of *Vibro* spp. and *Streptococcus* spp. were kindly provided by the China Institute of Veterinary Drug Control, Beijing.

Oligonucleotide primers and DNA probes

Three sets of primers and DNA probes, listed in Table 1, were designed to amplify highly conserved gene sequences of WSSV (AF369029), IHHNV (AF218226) and TSV (NC003005) (GenBank sequence data). All three sets of primers and probes were synthesized at TaKaRa, Dalian, China.

Real-time multiplex PCR assay

Amplification reactions were performed in volumes of 20 μl with TaKaRa premix, 40 μM MLV reverse transcriptasease, 16U RNase inhibitor, 0.6 μM primer for WSSV and TSV or 0.4 μM primer for IHHNV, 0.4 μM probe for WSSV and TSV or 0.2 μM probe for IHHNV, and 2 μl of the DNA/RNA sample. The PCR amplification consisted of 2 min at 95°C, followed by 40 cycles of 10 s at 95°C and 30 s at 60°C. Amplification, detection, and data analysis were performed with the Light Cycler 2.0 system (Roche Molecular Biochemicals, Mannheim, Germany).

Sensitivity and specificity of rtm-PCR

The sensitivity of the rtm-PCR was determined using ten-fold dilutions of template of each specific plasmid containing specific genes of viruses (WSSV-pMD18-T, IHHNV-pMD18-T and TSV-pMD18-T). The results of the rtm-PCR assay were compared to those of our previously developed PCR assay [22]. To determine the specificity of the rtm-PCR assay, specific DNA fragments from WSSV, IHHNV and TSV were amplified and cloned in pMD18-T cloning vector according to the manufacturer's protocol (Takara Dalian, China). These three recombinant plasmids were sequenced, and sequence data were analyzed using Dnastar software and compared with the corresponding sequence data in GenBank. For negative controls, DNA from *Vibrio* and *Streptococcus* and distilled water were included.

Interference assay and reproducibility

Various concentrations of plasmid containing WSSV, IHHNV and TSV genes (10^4, 10^8 and 10^8 copies of each; or 10^8, 10^1 and 10^8 copies of each; or 10^8, 10^8 and 10^4 copies of each) were mixed together and subjected to rtm-PCR. The copy numbers of the genes were calculated

Table 1 Sequences of primers and TaqMan probes

Primer	Sequence	Size
Ihhnv24	5-AAACTGAACACTGGCCTAGTAACAA-3	77 bp
Ihhnv100	5-TAGGACTTTCCGATGAGGTTTTG-3	
Ihhnv50T	5-FAM-AACAGGAGACTCAAACACCTTCCATCT-ECLIPSE-3	
Wssv270	5-ACCATGGAGAAGATATGTACAAGCA-3	76 bp
Wssv345	5-GGCATGGACAGTCAGGTCTTT-3	
Wssv296T	5-ROX-TTACAGTGATGGAATTTCGTTTATC-ECLIPSE-3	
Tsv20F	5-GCTTGCGTGGTGGGACTAAAT-3	76 bp
Tsv95R	5-CCTCCACTGGTTGTTGTATCAAAA-3	
Tsv42T	5-HEX-AATGCCTGCTAACCCAGTCGAAATT-ECLIPSE-3	

according to the following formula: (copies/μl = (The plasmid's concentration × 6×10^{14})/(The plasmid's size in base pairs × 324.5).

Detection of clinical samples

DNA from 15 clinical samples each from WSSV, IHHNV and TSV that were known to be positive by routine PCR were also subjected to real-time multiplex PCR.

Results

The real-time multiplex PCR assay for detection of three viral pathogens of penaeid shrimp was designed as a multiplex assay for simultaneous detection of WSSV, IHHNV and TSV. Since the presence of other oligonucleotides and fluorescent probe could alter the efficiency of PCR amplification, each set of primers and probe was tested in an individual format as well as in a multiplex format, using different concentrations of the three viruses. The result of these experiments indicates that there was no systematic deviation in the amplification curves when comparing the multiplex assay with the single-target assays. No difference in amplification efficiency was observed between the singleplex and multiplex formats, as measured by the slopes of amplification curves during the exponential phase and the cycle threshold (CT) values obtained with individual samples. Furthermore, the detection limits for the multiplex and individual assay formats were nearly identical, since even the most diluted samples were detected in both types of assay.

Detection limit

The limit of detection for the real-time multiplex assay was determined with TSV-pMD18-T, WSSV-pMD18-T and IHHNV-pMD18-T that were serially diluted tenfold. The sensitivity of the real-time multiplex PCR assay was 20,000 for WSSV, 20 for IHHNV and 20,000 for TSV template copies, respectively (Fig. 1a–c), and its sensitivity was 10, 1,000 and ten times higher than that of the routine PCR. Standard curves are shown in Fig. 2a–c. Different concentrations of WSSV, IHHNV and TSV, when mixed together, still could be identified by this assay, which implies that the rtm-PCR assay can be used for simultaneous detection of infection with the three viruses.

Reproducibility and specificity

The samples were examined repeatedly using the rtm-PCR (Table 2), and the results indicated that the rtm-PCR was reproducible. The rtm-PCR results of different samples

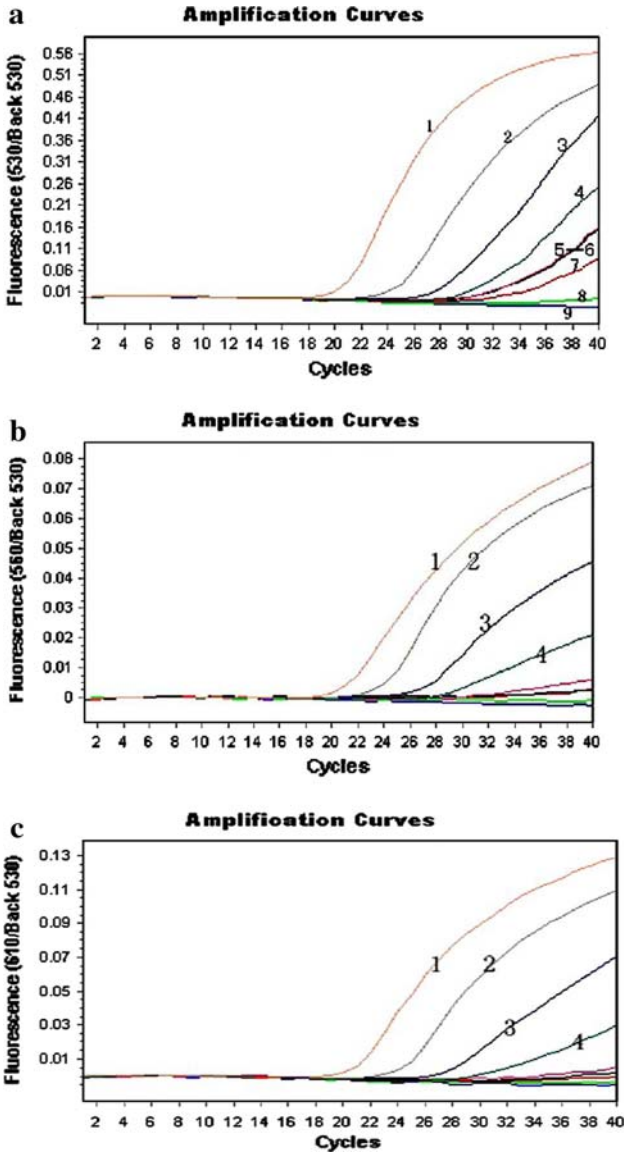

Fig. 1 **a** Sensitivity of the real-time multiplex PCR for IHHNV. *1* 2×10^7 copies/μl; *2* 2×10^6 copies/μl; *3* 2×10^5 copies/μl; *4* 2×10^4 copies/μl; *5* 2×10^3 copies/μl; *6* 2×10^2 copies/μl; *7* 2×10^1 copies/μl; *8* 2×10^0 copies/μl; *9* Negative control. **b** Sensitivity of the real-time multiplex PCR for TSV; *1* 2×10^7 copies/μl; *2* 2×10^6 copies/μl; *3* 2×10^5 copies/μl; *4* 2×10^4 copies/μl. **c** Sensitivity of the real-time multiplex PCR for WSSV. *1* 2×10^7 copies/μl; *2* 2×10^6 copies/μl; *3* 2×10^5 copies/μl; *4* 2×10^4 copies/μl

showed that one specific amplification curve was displayed when shrimp were infected by only one of these three viral pathogens, whereas two or three specific amplification curves were displayed when shrimp were infected by two or three viral pathogens, and no amplification curves were displayed for samples containing *Streptococcus, Vibrio* and water (Figs. 2, 3a–c). The results indicate that rtm-PCR was able to detect and differentiate the presence of each pathogen in clinically infected shrimp.

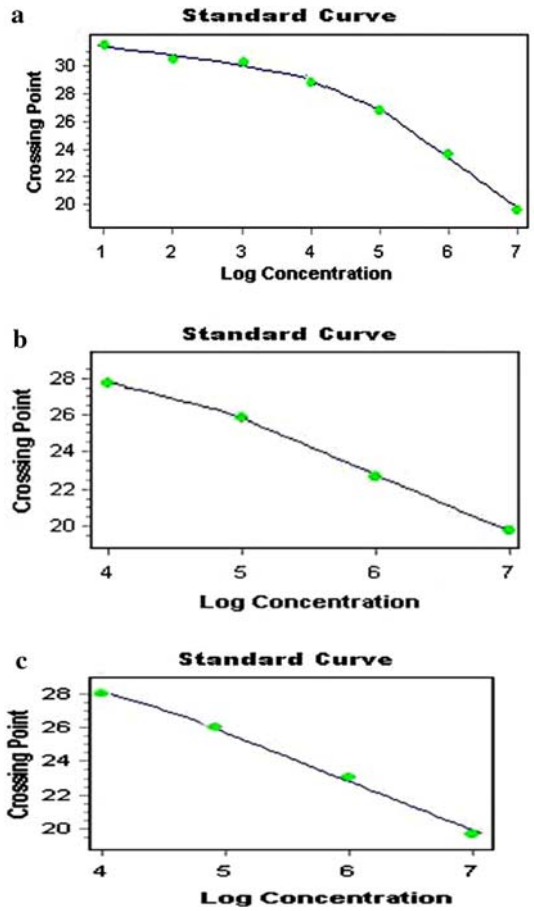

Fig. 2 **a** Standard curve of IHHNV. **b** Standard curve of TSV. **c** Standard curve of WSSV

Table 2 Real-time multiplex PCR results from three repeated detections

Sample	Ct value/copy number of the same sample at different times				
	First day	Fourth day	Seventh day	SD	CV (%)
WSSV	25.66/ 1×10^6	25.98/ 1×10^6	26.01/ 1×10^6	0.158	0.61
IHHNV	25.32/ 1×10^6	25.62/ 1×10^6	25.99/ 1×10^6	0.336	1.31
TSV	25.01/ 1×10^6	26.01/ 1×10^6	26.32/ 1×10^6	0.658	2.65

SD standard deviation, *Ct* cycle threshold, *CV* coefficient of variation

Clinical samples

Forty-five samples that were positive for each single virus by routine PCR were detected by real-time multiplex PCR (Table 3). The results showed that 1.82×10^7–3.34×10^5 copies/μl of WSSV were detected from 15 WSSV samples, and one sample was WSSV and IHHNV positive (7.72×10^6 and 2.10×10^2 copies/μl); 2.13×10^7–8.49×10^3 copies/μl of IHHNV were detected from 15 IHHNV samples, and one sample was both IHHNV and WSSV positive (6.78×10^6 and 1.12×10^4 copies/μl); 1.16×10^7–5.49×10^5 copies/μl of TSV were detected from 15 TSV samples, and none were IHHNV or WSSV positive.

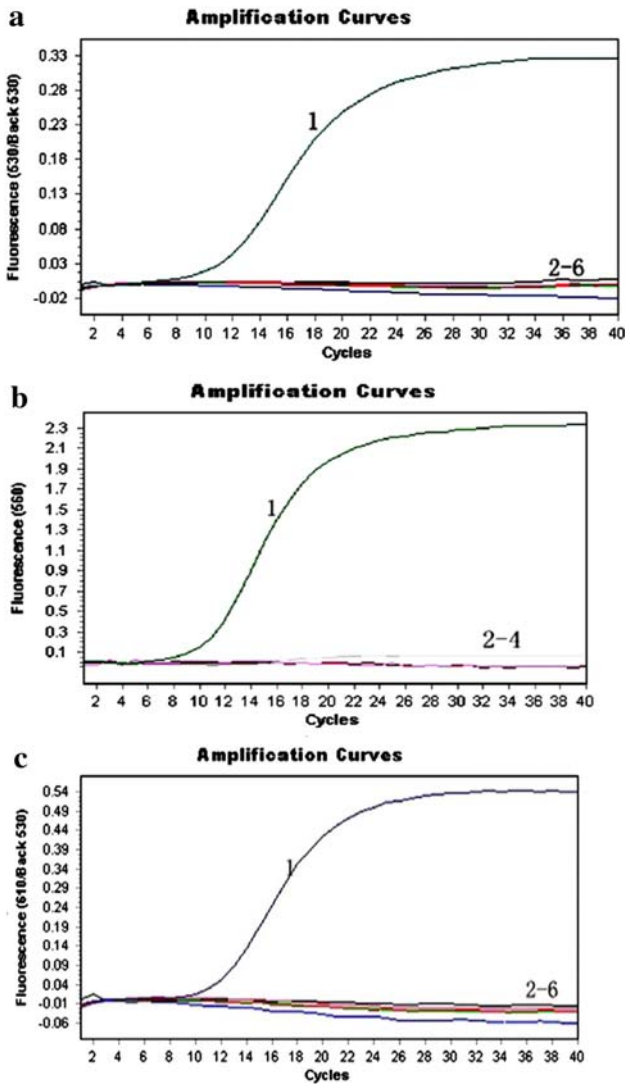

Fig. 3 **a** The specificity of IHHNV *1* IHHNV; *2* WSSV; *3* TSV; *4* Streptococcus; *5* Vibrio; *6* Negative. **b** The specificity of TSV. *1* TSV; *2* WSSV; *3* IHHNV; *6* Negative. **c** The specificity of WSSV. *1* WSSV; *2* IHHNV; *3* TSV; *4* Streptococcus; *5* Vibrio; *6* Negative

Discussion

The rtm-PCR assay described here uses PCR primers and TaqMan probes targeting conserved regions of WSSV, IHHNV and TSV genes. One main advantage of this assay compared to other available tests is that it is multiplex. By

Table 3 Results of real-time multiplex PCR of clinical samples

Species of shrimp	Shrimp pathogens/field samples	Origin/source	Results					
			Real-time multiplex PCR			Routine PCR		
			WSSV	IHHNV	TSV	WSSV	IHHNV	TSV
POK + white shrimp	WSSV 1 + IHHNV	Guangxi, China	+	+	−	+	−	−
POK	WSSV (GXPRC/1/06)	Beihai, Guangxi, China	+	−	−	+	−	−
POK	WSSV (GXPRC/2/06)	Beihai, Guangxi, China	+	−	−	+	−	−
POK	WSSV (GXPRC/3/06)	Beihai, Guangxi, China	+	−	−	+	−	−
POK	WSSV (GXPRC/4/06)	Beihai, Guangxi, China	+	−	−	+	−	−
POK	WSSV (GXPRC/5/06)	Beihai, Guangxi, China	+	−	−	+	−	−
White shrimp	WSSV (GXPRC/7/06)	Beihai, Guangxi, China	+	−	−	+	−	−
White shrimp	WSSV (GXPRC/8/06)	Beihai, Guangxi, China	+	−	−	+	−	−
POK	WSSV (GXPRC/1/06)	Hepu, Guangxi, China	+	−	−	+	−	−
POK	WSSV (GXPRC/2/06)	Hepu, Guangxi, China	+	−	−	+	−	−
POK	WSSV (GXPRC/3/06)	Hepu, Guangxi, China	+	−	−	+	−	−
POK	WSSV (GXPRC/4/06)	Hepu, Guangxi, China	+	−	−	+	−	−
White shrimp	WSSV (GXPRC/5/06)	Hepu, Guangxi, China	+	−	−	+	−	−
POK	WSSV (GXPRC/1/06)	Qinzhou, Guangxi, China	+	−	−	+	−	−
POK	WSSV (GXPRC/2/06)	Qinzhou, Guangxi, China	+	−	−	+	−	−
	WSSV-pMD18-T	GVRI	+	−	−	+	−	−
White shrimp + POK	IHHNV 1 + WSSV	Guangxi, China	+	+	−	−	+	−
POK	IHHNV (GXPRC/1/06)	Beihai, Guangxi, China	−	+	−	−	+	−
POK	IHHNV (GXPRC/2/06)	Beihai, Guangxi, China	−	+	−	−	+	−
POK	IHHNV (GXPRC/3/06)	Beihai, Guangxi, China	−	+	−	−	+	−
POK	IHHNV (GXPRC/4/06)	Beihai, Guangxi, China	−	+	−	−	+	−
White shrimp	IHHNV (GXPRC/5/06)	Beihai, Guangxi, China	−	+	−	−	+	−
POK	IHHNV (GXPRC/1/06)	Hepu, Guangxi, China	−	+	−	−	+	−
POK	IHHNV (GXPRC/2/06)	Hepu, Guangxi, China	−	+	−	−	+	−
POK	IHHNV (GXPRC/3/06)	Hepu, Guangxi, China	−	+	−	−	+	−
POK	IHHNV (GXPRC/4/06)	Hepu, Guangxi, China	−	+	−	−	+	−
POK	IHHNV (GXPRC/5/06)	Hepu, Guangxi, China	−	+	−	−	+	−
POK	IHHNV (GXPRC/6/06)	Hepu, Guangxi, China	−	+	−	−	+	−
POK	IHHNV (GXPRC/7/06)	Hepu, Guangxi, China	−	+	−	−	+	−
POK	IHHNV (GXPRC/1/06)	Qinzhou, Guangxi, China	−	+	−	−	+	−
POK	IHHNV (GXPRC/2/06)	Qinzhou, Guangxi, China	−	+	−	−	+	−
	IHHNV-pMD18-T	GVRI	−	+	−	−	+	−
POK	TSV (GXPRC/1/06)	Beihai, Guangxi, China	−	−	+	−	−	+
POK	TSV (GXPRC/2/06)	Beihai, Guangxi, China	−	−	+	−	−	+
POK	TSV (GXPRC/3/06)	Beihai, Guangxi, China	−	−	+	−	−	+
POK	TSV (GXPRC/4/06)	Beihai, Guangxi, China	−	−	+	−	−	+
POK	TSV (GXPRC/5/06)	Beihai, Guangxi, China	−	−	+	−	−	+
POK	TSV (GXPRC/6/06)	Beihai, Guangxi, China	−	−	+	−	−	+
POK	TSV (GXPRC/1/06)	Hepu, Guangxi, China	−	−	+	−	−	+
POK	TSV (GXPRC/2/06)	Hepu, Guangxi, China	−	−	+	−	−	+
POK	TSV (GXPRC/3/06)	Hepu, Guangxi, China	−	−	+	−	−	+
POK	TSV (GXPRC/4/06)	Hepu, Guangxi, China	−	−	+	−	−	+
POK	TSV (GXPRC/1/06)	Qinzhou, Guangxi, China	−	−	+	−	−	+
POK	TSV (GXPRC/2/06)	Qinzhou, Guangxi, China	−	−	+	−	−	+
POK	TSV (GXPRC/3/06)	Qinzhou, Guangxi, China	−	−	+	−	−	+
POK	TSV (GXPRC/4/06)	Qinzhou, Guangxi, China	−	−	+	−	−	+

Table 3 continued

Species of shrimp	Shrimp pathogens/field samples	Origin/source	Results					
			Real-time multiplex PCR			Routine PCR		
			WSSV	IHHNV	TSV	WSSV	IHHNV	TSV
White shrimp	TSV (GXPRC/5/06)	Qinzhou, Guangxi, China	−	−	+	−	−	+
	TSV-pMD18-T	GVRI	−	−	+	−	−	+
	pWSSV + pIHHNV + pTSV	GVRI	+	+	+	+	+	+
	Vibrio (Extracted DNA)	CIVDC, Beijing, China	−	−	−	−	−	−
	Streptococcus (Extracted DNA)	CIVDC, Beijing, China	−	−	−	−	−	−
	Distilled water	Qinzhou, Guangxi, China	−	−	−	−	−	−
	White shrimp (Nucleic acid)	Qinzhou, Guangxi, China	−	−	−	−	−	−
	POK (Nucleic acid)		−	−	−	−	−	−

CIVDC China Institute of Veterinary Drug Control, *GVRI* Guangxi Veterinary Research Institute, *POK* Penaeus Orientalis Kishinouye

using this approach, it was possible to identify all three pathogens in the same reaction vessel. The simultaneous detection of WSSV, IHHNV and TSV is especially useful, because these viruses commonly cause mixed infection in shrimp [9, 12, 16, 21].

In addition to its use in clinical diagnostics, the multiplex assay may be of value for detection of pathogen-free shrimp in environmental samples. However, the multiplex feature of this assay is optional; if so preferred, the three components can be utilized as single-targeting assays or combined into duplex assays without impacting the quality of the results. This makes this assay adaptable to circumstances that may not require the simultaneous detection of all three for a diagnostic decision.

Another important aspect of this real-time PCR approach is the short turnaround time. The confirmatory result of a suspected WSSV, IHHNV and TSV infection was obtained within 5 h of receiving the sample in the laboratory. This included time for DNA extraction and multiplex real-time PCR assay. The current methods for laboratory diagnosis of these three viruses are labor-intensive and may lack the sensitivity and speed required to reveal the cause of infection before it is too late to take the appropriate measure. The real-time multiplex PCR assay presented here can therefore be extremely useful as a fast and sensitive complement to existing diagnostic methods. In addition, this real-time multiplex PCR does not require the unique expertise involved in morphology-based tests but can be performed in any laboratory with adequate infrastructure for real-time PCR testing.

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