Development of a Rapid Method for Identifying Carryover Contamination of Positive Control DNA, Using a Chimeric Positive Control and Restriction Enzyme for the Diagnosis of White Spot Syndrome Virus by Nested PCR

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Abstract Chimeric positive plasmids have been developed to minimize false-positive reactions caused by polymerase chain reaction (PCR) contamination. Here, we developed a rapid method for identifying false-positive results while detecting white spot syndrome virus (WSSV) by nested PCR, using chimeric positive plasmids. The results of PCRs using WSSV diagnostic primer sets showed PCR products of a similar size (WSSV 1st PCR product, 1,447 bp; WSSV 2nd PCR product, 941 bp) using WSSV chimeric plasmids or DNA from shrimp infected with WSSV. The PCR products were digested with *DraI* for 1 h at 37 °C. The digested chimeric DNA separated into two DNA bands; however, the WSSV-infected shrimp DNA did not separate. Thus, chimeric plasmid DNA may be used as positive control DNA instead of DNA from WSSV-infected shrimp, in order to prevent PCR contamination. Thus, the use of restriction enzyme digestion allowed us to rapidly distinguish between WSSV DNA and WSSV chimeric plasmid DNA.

Keywords White spot syndrome virus · PCR · Chimeric positive control · Restriction enzyme · Identifying carryover contamination

Introduction

White spot syndrome virus (WSSV), a member of the genus *Whispovirus* of the family Nimaviridae, is the causative agent of white spot disease (WSD), one of the most serious diseases affecting decapod crustaceans in global culture systems [1, 2]. WSSV has been found to infect a variety of wild and cultured crustaceans and arthropods and is considered a major pathogen of *Litopenaeus vannamei*, that is, primary farm-raised shrimp [3–6]. Thus, WSD has been listed as a notifiable disease by the World Organization of Animal Health (Office International des Epizooties, OIE) since 1997. The OIE Manual of Diagnostic Tests for Aquatic Animals [7] provides a standardized approach for the diagnosis of the diseases listed in the aquatic code, and the nested polymerase chain reaction (PCR) is considered the standard for detecting WSSV [4]. According to the protocol, in the first-step PCR, 1,447 bp of the WSSV *SalI* DNA fragment is amplified with the WSSV 146F1 and R1 primer set. The first-step PCR product is then used as a template for amplification of a 941 bp second-step product using the WSSV 146F2 and R2 primer set [4, 7].

Although PCR is a very efficient method, it is prone to errors resulting from DNA contamination introduced into the PCR. DNA that confounds the results is most often introduced from other test samples; experimental materials, such as recombinant clones used as positive controls; or previous PCR amplification of the same target sequence [8]. The contaminated DNA is very difficult to distinguish from the original pathogen gene or contamination from positive-control clones, containing the original pathogen gene. Recently, positive-control plasmids were developed to minimize false-positive reactions due to PCR contamination during detection of spring viremia of carp virus (SVCV) by semi-nested reverse-transcription PCR [9].

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In the present study, chimeric plasmids were developed to minimize the likelihood of false-positive results due to PCR contamination during WSSV detection by nested PCR. In addition, a rapid identification method, minimizing false-positive reactions, was also developed using chimeric plasmids and a restriction enzyme.

Materials and Methods

Detection of WSSV DNA

Shrimp tissues infected with WSSV were kindly provided by Prof. K. H. Kim (Department of Aquatic Medicine, Pukyong National University, Korea) [10]. Genomic DNA was extracted from the gills and cuticular epithelia of two diseased shrimp using a genomic DNA extraction kit (Qiagen, Germantown, MD, USA). All PCR procedures were performed using an iCycler thermal cycler (Bio-Rad, Hercules, CA, USA). The DNA was amplified using an initial primer set (WSSV 146F1 and 146R1) that encompassed the sequence encoding the WSSV gene [7]. The genomic DNA was used in a 25- μ L PCR mixture containing 10 pmol of each primer, Tris–EDTA (TE) buffer, and 1.25 U of Ex-*Taq* DNA polymerase (TaKaRa, Tokyo, Japan). The PCR procedure included one cycle at 94 °C for 4 min, 55 °C for 1 min, and 72 °C for 2 min; followed by 39 cycles at 94 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min; and a final extension step lasting 5 min at 72 °C.

Nested PCR amplifications were carried out using the first-step PCR product and the primer sets WSSV 146F2

and 146R2. The first-step PCR product (1 μ L) was added to a 25- μ L PCR mixture containing 1.25 U of Ex-*Taq* DNA polymerase (TaKaRa, Tokyo, Japan), 0.2 mM dNTP, 2 mM MgCl₂ and 10 pM of each primer. For the second-step PCR procedure, the thermocycling procedure described in the first-step PCR was used.

Amplified DNA was separated on 1.5 % agarose gels containing ethidium bromide (0.5 μ g mL⁻¹; Sigma, St. Louis, MO, USA) and visualized using a Gel Doc UV trans-illuminator (Bio-Rad).

Primer Design and PCR for the Construction of Chimeric Plasmids

The chimeric plasmids were constructed using a method similar to that used by Kim [9], with the following modifications: Primer sets were designed based on the sequence of the pGEM-T Easy vector (Promega, Madison, WI, USA) (Fig. 1); the primer sequences are shown in Table 1. The WSSV chimeric plasmid F1 primer contained the WSSV 146F1 sequence and nucleotides 1,351–1,371 of the ampicillin resistance (AR) gene in the pGEM-T Easy vector. The WSSV chimeric plasmid R1 primer included the WSSV 146R1 sequence and nucleotides 2,730–2,749 of the phage f1 gene. The WSSV chimeric plasmid F2 primer contained the WSSV 146F2 sequence and nucleotides 1,351–1,371 of the AR gene in the pGEM-T Easy vector. The WSSV chimeric plasmid R2 primer included the WSSV 146R2 sequence and nucleotides 2,223–2,247 between the AR gene and phage f1 region gene in the pGEM-T Easy vector (Fig. 1).

Fig. 1 Schematic representation of construction of the WSSV chimeric plasmid DNA positive controls AR and phage f1 genes are truncated with the WSSV 146F1 and R1 primer sets and with the WSSV 146F2 and 146R2 primer sets and cloned into a pGEM-T Easy vector

Table 1 Primer sequences used in this study

Primer name	Primer sequence (5' → 3')	References
WSSV 146F1	ACTACTAACTTCAGCCTATCTAG	OIE [7]
WSSV 146R1	TAATGCGGGTGTAATGTTCTTACGA	
WSSV 146F2	GTAAGTGGCCCTTCCATCTCCA	
WSSV 146F2	TACGGCAGCTGCTGCACCTTGT	
WSSV chimeric plasmid F1	<u>ACTACTAACTTCAGCCTATCTAG</u> CAGTGAGGCACCTATCTCAGC	Present study
WSSV chimeric plasmid R1	<u>TAATGCGGGTGTAATGTTCTTACG</u> ACGCTCCTTTCGCTTTCTTCC	
WSSV chimeric plasmid F2	<u>GTAAGTGGCCCTTCCATCTCCAC</u> AGTGAGGCACCTATCTCAGC	
WSSV chimeric plasmid R2	<u>TACGGCAGCTGCTGCACCTTGT</u> ATGAGACAATAACCCTGATAAATGC	

Underlines in the primer sequences indicate the WSSV primer sequences

The PCR was designed to generate a 1,447 bp PCR product in the first-step PCR using the WSSV chimeric plasmid F1 and R1 primers and a 941 bp product using nested PCR with the WSSV chimeric plasmid F2 and R2 primers (Fig. 1). These amplifications were performed in a 25- μ L PCR mixture containing 10 pmol of each primer (for plasmid WSSV chimeric plasmid-1, WSSV chimeric plasmid F1 and R1 primers; for plasmid WSSV chimeric plasmid-2, WSSV chimeric plasmid F2 and R2 primers), TE buffer, and 1.25 U of *Ex-Taq* DNA polymerase. The PCR procedure included an initial 10 min step at 95 °C; 35 cycles for 1 min at 95 °C, 1 min at 55 °C, and 1 min at 72 °C; and a final 10 min extension step at 72 °C.

Cloning and Nucleotide Sequence Analysis

Amplified PCR products were purified using a spin-column PCR purification kit (Promega) and cloned into the pGEM-T Easy vector. Plasmid DNA from the recombinant clones was extracted using a plasmid DNA extraction kit (Qia-gen), and the correct clones were selected using PCR, as described above. Inserts were sequenced using an ABI377 sequencer (Applied Biosystems, Foster City, CA, USA) with T7 and SP6 primers. The resulting sequences were

compared using BLAST (National Center for Biotechnology Information).

Treatment with Restriction Enzyme

Restriction digests were carried out in a 10- μ L reaction mixture containing 5 μ L PCR product, 1 μ L of 10 $\times$ enzyme reaction buffer, Tris–EDTA (TE) buffer, and 1 U of *Dra*I (Bioneer, Daejeon, Korea). All of the PCR products were digested with the restriction enzyme for 1 h at 37 °C. The products were separated on 1.5 % agarose gels, containing ethidium bromide (0.5 μ g mL⁻¹; Sigma), and visualized using a Gel Doc UV trans-illuminator (Bio-Rad).

Results and Discussion

Using genomic DNA from WSSV-infected tissue as a template, the first-step PCR amplified 1,447 bp of the WSSV gene with the WSSV 146 F1 and R1 primer set (Fig. 2a, Lane 1). Subsequently, the nested PCR step amplified 941 bp of the WSSV gene with the WSSV 146 F2 and R2 primer set (Fig. 2b, Lane 4). These results

Fig. 2 Agarose gel electrophoresis of PCR products. **a** The 1,447 bp PCR products from WSSV genomic DNA or the chimeric AR and phage f1 genes. **b** The 941 bp PCR products from WSSV genomic DNA or the chimeric AR gene. *L1*, *L4* PCR product from WSSV genomic DNA; *L2*, *L5* PCR products from the chimeric AR and phage f1 gene; *L3*, *L6* negative control; *M* DNA marker (Bioneer, Daejeon, Korea)

confirmed that the expected fragments were amplified from the WSSV gene, using the PCR methods recommended by OIE [7]. With a template comprised of pGEM-T Easy vector DNA, the first-step PCR amplified a 1,447 bp fragment from the pGEM-T easy vector DNA with the WSSV chimeric plasmid F1 and R1 primer set (Fig. 2a, Lane 2). The nested PCR amplified a 941 bp fragment with the WSSV chimeric plasmid F2 and R2 primer set (Fig. 2b, Lane 5). These results provided evidence that the PCR products, amplified by the designed primer sets from pGEM-T Easy vector DNA, were the same size as those amplified using PCR methods recommended by OIE [7]. Thus, it was found that chimeric plasmid DNA can be used as a positive control instead of DNA from shrimp infected with WSSV.

The four PCR products (1,447 and 941 bp) obtained from either the WSSV gene or the WSSV chimeric plasmid were inserted into the pGEM-T Easy vector, and then the plasmids were cloned. Excluding the primer sequences, a sequence analysis revealed 100 % identity with the WSSV gene (GenBank Accession: AF332093) in plasmids containing the WSSV-based inserts. However, the sequences from the WSSV chimeric plasmid templates did not demonstrate identity with the WSSV gene, but were 100 % identical to the pGEM-T Easy vector gene.

The 941 bp fragment was amplified by nested PCR using the WSSV first-step plasmid DNA or WSSV first-step PCR products, according to the OIE method (data not shown). However, the 941 bp PCR product was not amplified using the 1,447 bp WSSV chimeric plasmid-1 PCR product or the WSSV chimeric plasmid-1 when the OIE method was used (data not shown). These results confirmed that the use of the WSSV chimeric plasmid-1 eliminated the risk of false positives arising from contamination during nested PCR with WSSV chimeric plasmid-1. In addition, a 941 bp PCR product could be amplified from the WSSV chimeric plasmid-2 using the WSSV 146 F2-R2 primer set for nested PCR, but sequencing of this PCR product did not reveal identity with the WSSV gene. Thus, the use of WSSV chimeric plasmid-1 and chimeric plasmid-2 clones can ensure a correct diagnosis, without any PCR contamination.

Other researchers have developed chimeric plasmid DNA as a positive control to prevent PCR contamination, without using viral nucleic acid or pathogen-infected tissues [9]. Separating the original pathogen gene from contamination from the positive-control DNA clones, which contain the original pathogen gene, is a difficult task. The amplified PCR products indicate the presence or absence of the pathogen; therefore, accurate, error-free results are critical for preventing the spread of the infectious agent. Our results provide evidence that the contamination of PCR products, using only chimeric plasmid DNA, could be

(A) 1st PCR products

(B) 2nd PCR products

Fig. 3 Schematic representation of the cleavage site by the restriction enzyme. **a** *DraI* restriction site in the 1st PCR products of the WSSV *SalI* DNA fragment or AR and phage f1 region genes. **b** *DraI* restriction site in the 2nd PCR products of WSSV *SalI* DNA fragment or AR region genes

identified using nucleotide sequencing analysis. In this study, we investigated restriction enzyme treatment of PCR products to determine the possibility of contamination during PCRs using a WSSV gene and a WSSV chimeric plasmid (Fig. 3). The restriction enzyme site was designed based on the sequence of the amplified WSSV gene or the constructed WSSV chimeric plasmid DNA that included an AR gene and phage f1 region (Fig. 3). The *DraI* (5'-TTT↓AAA-3') restriction enzyme was selected because only one target site existed in the WSSV chimeric plasmid DNA (Fig. 3). *DraI* restriction sites were not observed in the amplified 1,447 bp of the WSSV gene, nor were they apparent in any of the searches of the BLAST database in other WSSV genes (data not shown). Upon digestion with *DraI*, the amplified 1,447 and 941 bp from the DNA of WSSV-infected shrimp were not cleaved, but the amplified products from the chimeric plasmid DNA were observed to have two digested DNA fragments (788 and 659 bp from the first-step PCR; 658 and 283 bp from the nested-step PCR) (Fig. 4). These results confirmed the utility of a restriction enzyme to rapidly identify WSSV DNA or WSSV chimeric plasmid DNA. Finally, the results showed that use of a chimeric plasmid and a restriction enzyme facilitated the ability to correctly diagnose whether the presence of actual WSSV DNA related to infection or a false-positive from positive control DNA.

Fig. 4 Agarose gel electrophoresis of digested DNA by restriction enzyme digestion products of WSSV DNA fragment PCR products (1st and 2nd) and WSSV chimeric plasmid PCR products after *DraI* treatment. *L1*, *L3* PCR products from WSSV DNA fragment; *L2*, *L4* PCR products from WSSV chimeric AR and phage *f1* gene

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