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ORIGINAL ARTICLE

Effect of processing treatments on the white spot syndrome virus DNA in farmed shrimps (*Penaeus monodon*)

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Abstract

Aims: To investigate the effect of processing treatments on the destruction of white spot syndrome virus (WSSV) DNA in WSSV-infected farmed shrimps (*Penaeus monodon*).

Methods and Results: The presence of WSSV was tested by single step and nested polymerase chain reaction (PCR). The primers 1s5 & 1a16 and IK1 & IK2 were used for the single step PCR and primers IK1 & IK2–IK3 & IK4 were used for the nested PCR. Various processing treatments such as icing, freezing, cooking, cooking followed by slow freezing, cooking followed by quick freezing, canning, and cold storage were employed to destroy the WSSV DNA. Of the processing treatments given, cooking followed by quick freezing was efficient in destroying WSSV DNA in WSSV-infected shrimp products. Canning, and cooking followed by slow freezing process had some destructive effect on the WSSV DNA, as WSSV DNA in such processed shrimp products was detected only by nested PCR. Icing, slow freezing, quick freezing, and cooking processes had no effect on the destruction of WSSV DNA. A gradual increase in the destruction of WSSV DNA was observed as the cold storage period increased.

Conclusion: The results indicated that cooking followed by quick freezing process destroy the WSSV DNA.

Significance and Impact of the Study: WSSV can be destroyed by cooking followed by quick freezing and this combined process can reduce the disease transmission risks from commodity shrimps to native shrimps.

Introduction

Aquaculture has become the world's fastest growing food producing sector with an annual growth rate of 10% since 1984. Globally, the total shrimp produced through farming was 3164 384 tonnes, in which, the share of India was 14 2967 tonnes (FAO 2008). The worldwide shrimp culture suffered significant economic losses due to disease outbreak during the last decades. Incidence of shrimp diseases has been recognized as a potential biological threat to the shrimp farming industry since 1992–1993 (Lightner and Redman 1998).

The appearance of white spot syndrome in Asia from 1993 caused significant economic losses for the shrimp farming industry (Lightner 1996; Heidarieh *et al.* 2010). About 100% mortality can occur in shrimps in 3–10 days after the onset of white spots in the exoskeleton and epidermis (Stentiford *et al.* 2009). Fresh/frozen shrimp products for human consumption imported into Australia have been subjected to mandatory testing since October 2007 using polymerase chain reaction (PCR) technology for three major shrimp viruses viz., white spot syndrome virus (WSSV), yellow head virus (YHV) at the level of 5% prevalence. Batches that tested positive have to be destroyed in an approved facility (Biosecurity Australia 2007). A series of experiments conducted by Durand *et al.* (2000) on the WSSV found that the virus was still surviving in the freezing process and cold storage. Since there are no reports on the effect of processing treatments other than the freezing and cold storage on the WSSV DNA in shrimps, the present study was undertaken.

Materials and methods

Samples

WSSV-infected farmed shrimps (*Penaeus monodon*), having an average weight of 24 g, were obtained from seafood processing plants located in Tuticorin, Tamil Nadu, India. The samples on reaching the laboratory were repacked in small quantities, in order to avoid repeated thawing and freezing. They were labelled and stored in an Ultrafreezer (Sanyo Gallenkamp PLC, Leics, UK) at −80°C until taken for analysis.

Positive control

A clear WSSV-infected shrimp sample (*P. monodon*) collected from a shrimp farm located in Nellore, Andhra Pradesh, India based on the typical clinical symptoms associated with the WSSV was selected as a positive control. This positive control sample was initially used for the standardization of polymerase chain reaction (PCR) for the detection of WSSV from farmed shrimp. The samples, that were highly positive, were chosen for further study to investigate the effect of processing treatments on the survival of the WSSV in shrimps.

Processing treatments

The WSSV-positive shrimp samples were divided into seven lots to examine the effect of icing, freezing, cooking, cooking and freezing, canning, and cold storage. The first lot was treated as control (raw) and designated as 'R'. The other lots were subjected to different processing treatments. The experiments were conducted in triplicates.

The second lot was packed with flake ice at a ratio of 1 : 1 as recommended by Lima dos Santos *et al.* (1981). The iced shrimps was stored for 1, 3, 6, 9, and 12 h and designated as I-1, I-2, I-3, I-4 and I-5, respectively. The third lot was further divided into three sub-lots viz. whole (W), headless (HL) and peeled and undevenied (PUD). Each sub-lot was further divided into two parts. First part of each sub-lot was subjected to quick freezing (QF) in an Ultrafreezer at −40°C and the second part of each sub-lot was subjected to slow freezing (SF) in a deep freezer at −20°C as per the protocol given by Jeyasekaran *et al.* (2002). They were designated as QFW, QFHL, QFPUD, SFW, SFHL and SFPUD, respectively. The fourth lot was subjected to cooking at 100°C in boiling water for 10, 15, 20, 25 and 30 min and designated as C-1, C-2, C-3, C-4 and C-5, respectively.

The fifth lot, after dividing into three sub-lots viz. whole, HL and PUD, was subjected to cooking in boiling water at 100°C for 15 min. Each sub-lot was again divided into two parts. First part was subjected to slow freezing (SF) at −20°C and the second part was subjected to quick freezing (QF) at −40°C. They were designated as CSFW, CSFHL, CSFPUD, CQFW, CQFHL and CQFPUD, respectively. The sixth lot was subjected to canning by following the standard procedure as described by Saralaya (1978) with slight modification in 6 0z. TFS cans at 121°C for 40 min. The shrimps were canned in brine pack, as this pack of canned shrimps is the most sought pack in the International market. The seventh lot was subdivided into two sub-lots viz. whole (W) and peeled and undevenied (PUD). They were frozen at −40°C and then stored in a cold store at −18°C for 7 months. Samples were taken for analysis after 1, 2, 5 and 7 months of storage and designated as S-1, S-2, S-5 and S-7, respectively.

DNA extraction

The WSSV DNA for PCR was extracted using standard phenol extraction procedure and ethanol precipitation (Jeyasekaran 2000). Briefly, about 500 mg of homogenized tissue of shrimp was taken in a 2·0 ml sterile microfuge tube. Then, 0·5 ml molecular grade water (Sartorius Stedim Biotech, Gottingen, Germany) was added to each tube and incubated for 15 min at −20°C. To each tube, 0·5 ml of buffered phenol (which was prepared by mixing 500 ml of phenol with 50 ml of 0·1 mol l^{-1} Tris (pH 8·0) and 100 μl of β-mercaptoethanol) was added. The tissue sample was then centrifuged at 13 000 ***g*** for 15 min at 4°C in a refrigerated microfuge (Eppendorf AG, Hamburg, Germany) and the supernatant was transferred to another sterile microfuge tube and 0·5 ml of buffered phenol was added and centrifuged. This process was repeated until the supernatant became clear. The supernatant was then transferred to another sterile microfuge tube and 0·5 ml of diethyl ether was added and centrifuged at 13 000 ***g*** for 10 min at 4°C. The supernatant was discarded and 50 μl of 3 mol l^{-1} sodium acetate and 1·5 ml of 100% ethanol were added and stored at −20°C overnight (or) at −80°C for 2 h. The extract was centrifuged at 13 000 ***g*** for 15 min at 4°C and the supernatant was discarded. The pellet was washed with 70% ethanol and dried at room temperature. The pellet was finally dissolved in 20 μl molecular grade water and stored at −20°C until further use.

Amplification of WSSV DNA

Oligonucleotide primers (1s5–1a16, IK1–IK2, IK3–IK4) chosen for the amplification of WSSV DNA fragments were based on the earlier reports of East *et al.* (2005) and

Table 1 Primers used for the detection of WSSV in farmed shrimp product samples

Primer specific for	Primer name	Sequence (5′–3′)	Product size (bp)
WSSV single step	1s5	CACTCTGGCAGAATCAGACCAGACCCCTGAC	198
	1a16	TTCCAGATATCTGGAGAGGAAATTCC	
WSSV (first step)	IK1	TGGCATGACAACGGCAGGAG	486
	IK2	GGCTTCTGAGATGAGGACGG	
WSSV (second step/nested)	IK3	TGTCATCGCCAGCACGTGTGC	310
	IK4	AGAGGTCGTCAGAGCCTAGTC	

Pradeep *et al.* (2009) for single step and nested PCR, respectively (Table 1). The primers produced an amplicon size of 486 bp (for IK1–IK2), 310 bp (for IK3–IK4) and 198 bp (for 1s5–1a16).

Polymerase chain reaction

First/single step PCR. The volume of reaction mixture for the first/single step PCR was 25 μl containing 18 μl molecular grade water, 2·50 μl reaction buffer (100 mmol l^{-1} Tris with 15 mmol l^{-1} $MgCl_2$), 1 μl each of forward and reverse primer (1s5 and 1a16; IK1 and IK2), 0·25 μl dNTPs (deoxyribonucleotide triphosphates) mix, 0·25 μl Taq DNA polymerase, 2 μl crude DNA extract (template DNA). The PCR reaction was conducted in the Thermalcycler (GeneAmp 9700, ABI Systems, Rotkreuz, Switzerland). The PCR protocol comprised of 35 cycles of 60 s at 94°C, 60 s at 55°C and 90 s at 72°C. The programme included an initial delay of 4 min at 94°C and final extension of 5 min at 72°C before and after 35 cycles, respectively.

Second step/nested PCR. In this case, an aliquot of 2 μl from the first step PCR product was used as the DNA template together with the nested primer pair, IK3 and IK4. The rest of the PCR mixtures were the same as described above.

Electrophoresis. After completion of PCR, 5 μl of PCR product was taken and mixed with 1 μl of six times loading buffer and subjected to electrophoresis (GE Healthcare Biosciences, Kowloon, Hong Kong) in 2% agarose gel containing ethidium bromide at a concentration of 0·5 μl ml^{-1} in one time Tris–acetate–EDTA (TAE) buffer and the gel was analysed under UV trans-illumination and photographed using Gel Documentation System (Alpha Innotech, Cell Biosciences, Santa Clara, CA, USA).

Results

The WSSV-infected shrimp samples of second lot that were iced at 0°C, third lot that were subjected to slow and quick freezing at −20 and −40°C, respectively, and fourth lot that were cooked at 100°C showed positive results by the single step PCR for the primers 1s5 &1a16 (Figs 1–3), but not with IK1 & IK2 primers. The PCR product yield of shrimps iced for various durations was found to be almost same. The PCR product yield was,

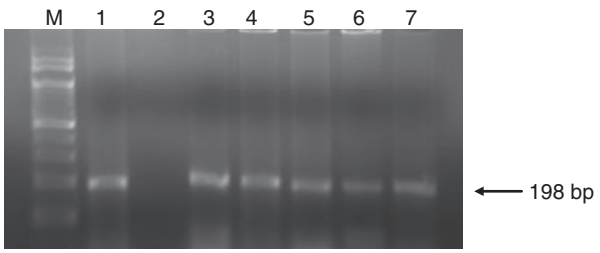

Figure 1 Detection of WSSV in iced shrimp product samples with 1s5 & 1a16 primers in the single step PCR.

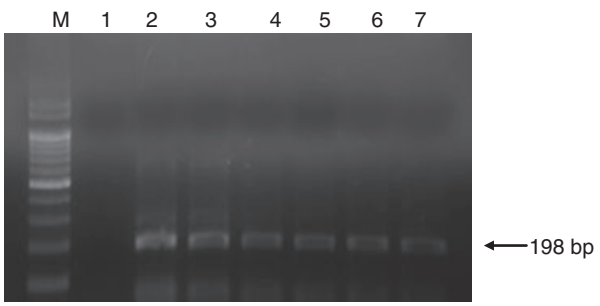

Figure 2 Detection of WSSV in slow and quick frozen shrimp product samples with the primers 1s5 & 1a16 in the single step PCR.

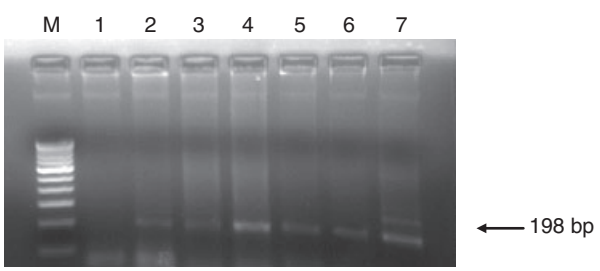

Figure 3 Detection of WSSV in cooked shrimp product samples with the primers 1s5 & 1a16 in the single step PCR.

however, lesser in slow frozen shrimp products. In quick frozen products, the PUD shrimps showed lower product yield than the whole and HL shrimps. The PCR product yield was found to be same in the cooked shrimps that were cooked for different duration. However, all these samples were also positive by the nested PCR for the primers IK3 & IK4 (Table 2).

The WSSV-infected shrimp samples of fifth lot that were cooked at 100°C for 15 min and then subjected to slow freezing at −20°C, sixth lot that were subjected to canning at 121°C for 40 min, and seventh lot that were frozen at −40°C and held in the cold storage up to 7 months were found to be positive only by the nested PCR (Figs 4–6), and not by the single step PCR with either the primers 1s5 & 1a16 or IK1 & IK2. The PCR product yield of cooked and slow frozen shrimps was observed to be almost similar irrespective of the package style. However, the WSSV DNA was not detected by both the single step and nested PCR assays in shrimps that were cooked at 100°C for 15 min and subjected to quick freezing at −40°C (Table 2). The quick frozen shrimps stored in cold storage were positive for WSSV by the single step PCR with the primers 1s5 & 1a16 up to 5 months (Fig. 6).

Table 2 Comparison of various processing treatments on the destruction of WSSV DNA

	Detection of WSSV		
	Non-nested PCR		Nested PCR
Processing treatments	1s5&1a16	IK1&IK2	IK3&IK4
Icing	+	−	+
Slow freezing	+	−	+
Quick freezing	+	−	+
Cooking	+	−	+
Cooking and slow freezing	−	−	+
Cooking and quick freezing	−	−	−
Canning	−	−	+
Cold storage	+	−	+

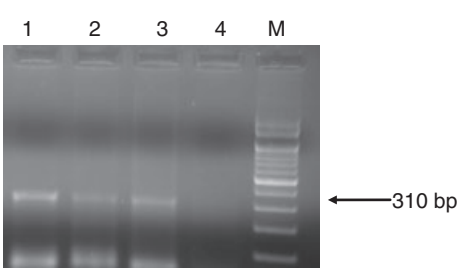

Figure 4 Detection of WSSV in cooked and slow frozen shrimp product samples with the primers IK3 & IK4 in the nested PCR.

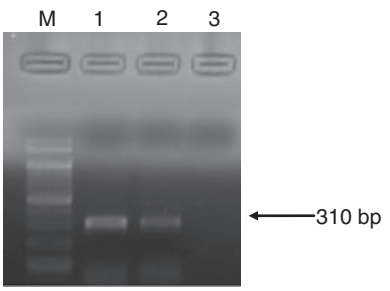

Figure 5 Detection of WSSV in canned shrimp product samples with the primers IK3 & IK4 in the nested PCR.

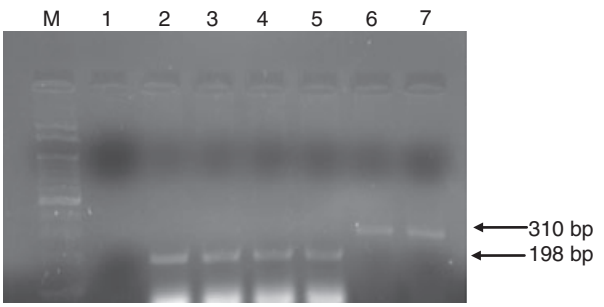

Figure 6 Detection of WSSV in cold stored shrimp product samples with the primers 1s5 & 1a16 in the single step PCR, and IK3 & IK4 in the nested PCR.

Discussion

The presence of WSSV in the iced farmed shrimp samples clearly indicated that the icing did not destroy the WSSV DNA (Fig. 1). Icing is the most common method of chilling shrimps immediately after harvest till they reach the seafood processing plants or markets. It is also considered as one of the best methods for the short term preservation of shrimps. Icing reduces the bacterial growth and activity (Lima dos Santos *et al.* 1981; Jeyasekaran *et al.* 2006). Ma *et al.* (2008) also reported that there was no significant reduction in viral load when the yellow head virus (YHV) was stored in a chilling temperature of 4°C even for 6 days. Sritunyalucksana *et al.* (2010) observed that fresh, whole, chilled shrimp would present a greater transmission risk of viruses to native stocks and reported that the whole shrimp is normally chilled on ice for 2 h after harvest before being sent for freezing in Thailand.

The WSSV was present in the farmed shrimps that had undergone slow, and quick freezing processes (Fig. 2). Gross clinical signs are not normally found in the headless (HL) and peeled and undeveined (PUD) and, hence, the shrimp processing plants process WSSV-infected whole shrimps either as HL or PUD. The present findings clearly indicated that freezing process did not

destroy the WSSV DNA in farmed shrimps. Momoyama *et al.* (1998) also found that the virus could survive in the cryopreservation process. However, Durand *et al.* (2000) reported that the WSSV DNA could be destroyed by block freezing process and also suggested that the virus might have lost its infectivity to indicator shrimp due to the ice crystal formation and resultant damage to the envelope or nucleocapsids during processing and storage or by repeated thawing and refreezing during marketing. Few workers have observed that the shrimp viruses particularly WSSV may remain viable in frozen shrimps (Nunan *et al.* 1998; Durand *et al.* 2000; McColl *et al.* 2004; Hasson *et al.* 2006). The experimental trials conducted by Sritunyalucksana *et al.* (2010) revealed that the viruses present in frozen shrimp may be viable.

The presence of WSSV DNA in the cooked shrimps clearly showed that cooking process at 100°C even after 30 min did not destroy the WSSV DNA (Fig. 3). Sritunyalucksana *et al.* (2010) studied the effect of cooking of whole shrimps at 85°C on the YHV before freezing and reported that the cooking temperature given was the core temperature of the processed shrimp. Even though the present findings showed that cooking did not destroy the WSSV DNA, Hasson *et al.* (2006) reported that the shipments found to be WSSV positive should be cooked to inactivate the viral pathogens. Croci *et al.* (2005) studied the resistance of Hepatitis A virus (HAV) in mussels subjected to different domestic cookings and found that the virus was still present even after cooking for 9 min at boiling temperature. It is known that the environment may influence the virus sensitivity to thermal inactivation, particularly in a fat or protein environment, as in shellfish flesh. The virus is more resistant to the inactivation action of heat (Murphree and Tamplin 1995; Croci *et al.* 1999). The detection of WSSV in chilled, frozen, and cooked shrimps by the single step PCR with the primer pair of 1s5 & 1a16 showed that this primer set is suitable for the detection of degraded WSSV DNA, as reported by East *et al.* (2005).

The WSSV DNA was not detected in cooked and quick frozen shrimp samples. Destruction of WSSV DNA might be due to cooking at 100°C followed by immediate quick freezing at −40°C. However, the WSSV was detected in the cooked and slow frozen shrimps by nested PCR (Fig. 4), which showed that cooking at 100°C followed by slow freezing at −20°C did not destroy the WSSV DNA. Sritunyalucksana *et al.* (2010) also found that the cooking followed by quick freezing process destroyed the YHV and reduced the transmission risks from commodity shrimps. The WSSV DNA was detected in canned shrimp samples by nested PCR (Fig. 5), even though they were subjected to high temperature of 121°C for 40 min. followed by sudden cooling to room temperature (28 ± 2°C). Croci *et al.* (2005) also reported that the HAV in mussels was detectable even after grilling in the oven at a temperature of 250°C for 5 min. The virus is known to be inactivated by heat, which causes coagulation and breakdown of the virus protein coat, but the environment, in which virus is found, may influence its sensitivity to thermal inactivation (Millard *et al.* 1987; Croci *et al.* 1999). The lack of viral inactivation to high temperature like canning was probably due to the insulation because of the synergetic barrier effect of both the shrimp flesh and packing medium, which shows that the ingredients also play a role on the prevention of a complete decontamination of the product (Croci *et al.* 2005).

The WSSV DNA was detected in frozen cold stored shrimp samples (Fig. 6). It has been earlier reported that WSSV DNA was detected by PCR in frozen cold stored shrimp products (Nunan *et al.* 1998; Durand *et al.* 2000; McColl *et al.* 2004; Reville *et al.* 2005; Hasson *et al.* 2006; Reyes-López *et al.* 2009). The detection of WSSV DNA in cold stored shrimps by the single step PCR with the primer pair of 1s5 & 1a16 showed that this primer set is suitable for the detection of degraded WSSV DNA, as reported by East *et al.* (2005). The product yield reduced considerably as the cold storage period of the shrimps increased. Up to 5 months of storage, the WSSV DNA was detected by the single step PCR with the primers 1s5 & 1a16 and in the 7th month cold stored shrimps, the WSSV DNA was detected only by nested PCR and not by the single step PCR. These findings clearly indicated that cold storage did not completely destruct the WSSV DNA. Our earlier report on bio-inoculation studies of WSSV extracts obtained from infected frozen shrimp products on live healthy WSSV-free shrimps also showed that WSSV was viable (Reddy *et al.* 2010).

It can be inferred that the WSSV DNA was not destroyed by icing, freezing, cooking, and cooking followed by slow freezing, canning, and cold storage processes. The nested PCR was found to be more reliable in detecting WSSV DNA from shrimp products. The primer set of 1s5 & 1a16 in single step PCR was observed to be reliable in detecting degraded WSSV DNA from shrimps that had been subjected to processing treatments. It clearly showed that WSSV DNA could be destroyed by cooking followed by quick freezing process and thereby eliminating the transmission risk of WSSV from infected shrimp products to the native aquaculture systems and reducing the risk of economic losses to the aquaculture industries throughout the World.

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