PCR based DNA marker analysis for WSSV resistant populations of Giant Black Tiger Shrimp, *Penaeus monodon* Fabricius in selected shrimp farming areas in Sri Lanka

Y.S.L. Kumaranayake[1], N.S. Kottearachchi[1*] and J.A. Athula[2]

1. Department of Biotechnology, Faculty of Agriculture and Plantation Management, Wayamba University, Makandura, Gonawila (NWP), Sri Lanka

2. National Aquaculture Development Authority (NAQDA), No 1/41, Parliament Road, Pelawatta, Battaramulla, Sri Lanka

Abstract

White Spot Syndrome Virus (WSSV) is the causative agent of White Spot Disease and it creates a major loss to shrimp aquaculture industry throughout the world. Selective breeding of resistant shrimp cultures would be a cost effective strategy for long term solution to this problem. This can be achieved by identification of WSSV resistant cultures using DNA markers linked with genes responsible for underlying mechanism of disease resistance. Therefore, this research was conducted aiming at developing a suitable DNA marker for the identification of WSSV resistant *Penaeus monodon.* DNA samples of disease infected and healthy *P. monodon,* giant black tiger shrimp originated in Sri Lanka were amplified using previously reported markers in Asian region, namely, 457 bp SCAR marker, KM 19/20 and RS0622 that were linked with WSSV resistance. Successful amplification was detected in shrimp samples with the DNA markers of 457 bp SCAR and KM 19/20 corresponding to the reported alleles. However genetic diversity at each of the three marker loci among the shrimp samples were not detected and therefore, any association between marker genotype and the infected phenotype were not observed in this study. This implies that although 457 bp SCAR marker, KM 19/20 and RS0622 markers are useful as a tool for the investigation of genetic diversity or resistant genotype among the *P. monodon* cultures in some countries, no such relationship could be explained with the cultures originated in Sri Lanka. This could probably be due to different genetic background of *P. monodon* cultures and therefore, further studies on more DNA markers with diversified culture samples are necessary for the detection of resistant populations of giant black tiger shrimp in Sri Lanka.

Keywords: DNA markers, *Penaeus monodon*, Sri Lanka, WSSV

[*]Corresponding author: – Email: kottearachchins@yahoo.com

Introduction

Giant black tiger shrimp, *Penaeus monodon* Fabricius, is the most prominent farmed crustacean product in international trade and has driven a significant expansion in aquaculture in many developing countries in Asia (FAO, 2014). Shrimp farming was commenced in mid 1980's as an export oriented activity, first in the Eastern Province and later, it was expanded to the Northwestern Province of Sri Lanka. The quantity of shrimp exports remarkably increased with the development of shrimp aquaculture in Sri Lanka; but subsequently indicated a drastic drop in both quantity and value due to frequent outbreaks of diseases.

Sri Lanka experienced three major shrimp disease outbreaks in 1988-1990, 1996 and in 1998, which resulted in a severe collapse of the industry (Wimalasena and de Mel, 2010). The second major disease outbreak was reported in 1996 and confirmed as white spot disease (WSD) and the disease was first detected in Uddappuwa and Karukupane areas at Arachchikattuwa in the Northwestern Province. The disease was subsequently spread throughout the shrimp farming area in the Northwestern Province. The total loss in foreign exchange earnings due to the disease was estimated as Sri Lankan Rs. 1,000 million and around 85 percent of total farm areas became non-functional due to this outbreak (Krishnaswamy, 2012).

White Spot Syndrome Virus (WSSV), the causative agent of WSD is an ellipsoidal, enveloped, dsDNA virus of 305107 bp length, having a bacilliform nucleocapsid belongs to the genus *Whispovirus* of the family *Nimaviridae* (Witteveldt *et al.*, 2004; Durand *et al.*, 1997). WSSV infects nauplii to adult shrimps with transmission ability by both vertically and horizontally and the severity of infection is very high in *Penaeus monodon*. The obvious characteristic sign is the presence of white spots on the cuticle and the infection can result mortality up to 100% within 3 to 7 days (Chang *et al.*, 1996; Zhan *et al.*, 1998).

The disease, WSSV can be prevented if good household practices are maintained. If the disease is detected, destruction of all *P. monodon* stocks and creation of a quarantine buffer zone surrounding the farming area would be some measures to modulate this disease (Dejager *et al.*, 2014). But developing of any permanent remedy has been

unsuccessful for WSSV infection (Witteveldt *et al.*, 2004; Rameshthangam and Ramasamy, 2007). As an alternative approach, enrichment of disease resistant brood stock and maintenance of their genetic sustainability through selective breeding using DNA markers hold high potential in several species of fish and shellfish (Houston *et al.*, 2008). It will help to overcome severe breakdown of the industry and also for the future brood stock development programs aiming the establishment of sustainable industry (Dejager *et al.*, 2014).

Wei-Ji *et al.*, (2008) have reported two quantitative trait loci (QTLs) associated with WSSV resistance in fleshy prawn, *Fenneropenaeus chinensis* Osbeck using amplified fragment length polymorphism (AFLP) markers. In addition the microsatellite locus RS0622 was identified to be associated with WSSV resistance in *F. chinensis* (Dong *et al.*, 2008).

Another study conducted by Mukherjee & Mandal (2009) has identified a 71 bp DNA fragment which was amplified from the microsatellite marker, KM 19/20 as the indicator for disease resistant populations of *P. monodon*, and this marker was strongly confirmed as much closer to the resistant candidate gene due to low recombinant frequencies observed in their population. This phenomenon was further confirmed by challenging the shrimp by infecting WSSV (Dutta *et al.*, 2013a). Also the randomly amplified polymorphic DNA (RAPD) has been proven to be very useful for the development of DNA markers in penaeid shrimp particularly of those having limited genomic information (Phongdara *et al.*, 1999). Proving this fact, Dutta *et al.*, (2013b) have developed a sequence characterized amplified region (SCAR) marker based on previously identified RAPD marker that was linked with the resistance to the disease.

This research was aimed at identifying a suitable DNA marker for the detection of WSSV resistant/tolerant populations in giant black tiger shrimp, *P. monodon* Fabricius for generating specific pathogen resistant (SPR) brood stock for the development of disease-free shrimp aquaculture industry in Sri Lanka. As the first step of the study, here we describe the feasibility of use of previously reported makers, 457 bp SCAR marker,

KM 19/20 and RS0622 markers in *P. monodon* culture specimens from selected areas in Sri Lanka.

Materials and Methods

Experimental Location

The study was carried out at the Department of Biotechnology, Faculty of Agriculture and Plantation Management, Wayamba University of Sri Lanka and Shrimp Farm Monitoring and Extension Unit (SFMU), Battulu Oya under the National Aquaculture Development Authority of Sri Lanka (NAQDA), from March to July, 2014.

Sample Collection

Seventy one tissue samples of *P. monodon* brood shrimps (pleopods) and juveniles (pleopods or gills) were obtained from shrimp hatcheries and grow-outs situated in the Northwestern Province and brood shrimp collectors from Ambakandawilla, Hendala, Beruwala, Thoduwewa and Negombo areas during the period from February to July, 2014.

Genomic DNA Extraction

The genomic DNA was extracted from 71 samples of *P. monodon* brooders (pleopods), juveniles (gills) using DNA extraction reagents supplied in IQ2000^{TM} WSSV (Farming IntelliGene Technology Corporation, Taiwan) following the manufactures guidelines. Extracted DNA was stored in TE buffer (0.1 mM EDTA, 0.1 mM Tris-HCl pH 7.0) at -20°C.

WSSV Screening by Nested PCR

The genomic DNA extracted from each shrimp sample was examined for the infection of WSSV using a commercially available kit (IQ2000^{TM} WSSV).The "First PCR reaction" was carried out with 10 µl reaction mixture which contained "First PCR premix" 7.5 µl, 0.5 µl IQzyme DNA polymerase (2 U/µl) (IQ2000^{TM} WSSV) and 2 µl (20ng) of genomic DNA. The reaction mixture was placed in a thermocycler (A200; LongGene) for 5 repeated cycles at 94°C/30 s, 62°C/30 s, 72°C/30 s and then 15 repeated cycles at 94°C/15 s, 62°C/15 s, 72°C/20 s and PCR was followed by final incubation at 72°C/30 s.

In the Nested PCR step, 10 µl of first PCR product was used as the template DNA for the 25 µl reaction mixture which contained "Nested PCR Premix" 14 µl and 1 µl IQzyme DNA polymerase (2 U/µl) (IQ2000™ WSSV). The reaction mixture was amplified with 25 repeated cycles at 94°C/20 s, 62°C/20 s, 72°C/30 s and final extension at 72°C/30 s. The PCR products were electrophoresed in 0.7% agarose with 1 µg/ml ethidium bromide and visualized in gel documentation system (Gel doc 1001, VILBER LOURMAT). A negative control and quantified positive standard were run with the samples to ensure accuracy and reliability of results. Each shrimp sample from 71 samples, was categorized either as WSSV positive sample or WSSV negative sample based on the amplification pattern appeared in Fig. 1 and manufacture's specifications.

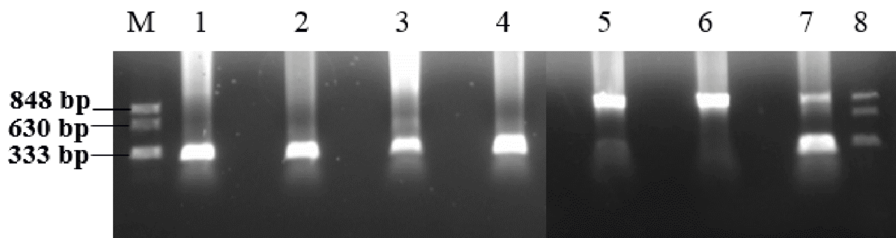

Fig. 1. Agarose gel electrophoresis of PCR products generated by IQ2000™ WSSV Detection and Prevention System

Lane M: Marker provided in IQ2000™ WSSV,

Lane 1, 2, 3, 4: Sample of light WSSV infection,

Lane 5, 6: WSSV negative samples.

Lane 7: Sample of very light WSSV infection,

Lane 8: WSSV P (+) standard

PCR amplification was conducted using primers of three selected markers. All 71 genomic DNA samples were amplified with SCAR marker while only 25 and 15 genomic DNA samples were amplified with KM 19/20 and RS0622 markers respectively (Table1).

Table 1. DNA sequence of the primers used in the study

Name of the primer	Annealing Temp.(°C)	Fragment Size (bp)	F/R	Sequence (5'- 3')	Reference
457bp SCAR primers	57	457	NM-F	ATCCTCTGGAGTGGAAAGCA	Dutta *et al.*, 2013b
			NM-R	CACCTGGGCTCACCTTACT	
KM 19/20	50	317 & 71	F	GATCATCATCATCGGCTG	Mukherje e& Mandal, 2009
			R	TATGTACGTTAGTCCAAG	
RS0622	55	494 & 482	F	TCAGTCCGTAGTTCATACTTGG	Dong *et al.*, 2008
			R	CACATGCCTTTGTGTGAAAACG	

PCR was repeated three times for the samples that produced null allele with SCAR primers. Amplified band of the RS0622 marker also was rechecked for the reproducibility by repeating PCR. PCR was carried out on BioRad (My Cycler™) Thermal cycler in a final volume of 12 µl mixture containing 5 µl of template DNA, 1.2 µl of 10X PCR buffers with 2.5 mM $MgCl_2$, 1.2 µl of 2.5 mM dNTPs (Promega Madison, WI U.S.A), 0.8 µl of primer (20 pmol/µl) (forward and reverse primers), and 0.16 µl of 5 U/µl *Taq* DNA polymerase (Dream Taq, Fermentas) in a thermal profile of initial denaturation (4 min at 94°C) followed by 30 cycles of denaturation (at 94°C for 45 sec), annealing (at 50-60°C for 30 sec, Table 1), extension (at 72°C for 1 min) and final extension at 72°C for 5 min.

The amplified PCR products were electrophoresed (Electrophoresis unit, MUPID-exu, England) on a 1% agarose gel for 457 bp SCAR marker and 3% agarose gel for KM 19/20 and RS0622 DNA markers followed by staining with ethidium bromide (0.5 µg/ml). After electrophoresis the gels were visualized and photographed using gel documentation apparatus (Quantum ST4) to identify WSSV resistant populations.

Results

Identification of WSSV positive and negative samples

Using IQ2000™ WSSV Detection and Prevention System, infected samples were differentiated into four different levels of infection; very light infection (333 bp band with 848 bp band), light infection (only 333 bp band), moderate infection (333 bp band

with 630 bp band) and severe infection (all three bands of 333 bp, 630 bp and 848 bp) as compatible with manufactures specification. They were considered as WSSV positive samples. The negative results (only 848 bp band) of this test indicate that the specimen is either not infected or infection level is lower than the detection limit (10-20 copies/reaction) of the test kit (Table 2, Fig.1).

Table 2. Infection Severity of the Samples Collected from Different Locations

Location	**No. of Severely Infected Samples**	**No. of Lightly Infected Samples**	**No. of Very Lightly Infected Samples**	**No. of Negative Samples**	**Total**
Ambakandawila	2	2	9	9	22
Hendala	2	_	4	5	11
Negombo	_	_	13	16	29
Beruwala	_	_	3	1	4
Toduwewa	1	_	2	2	5
Total	5	2	31	33	71

PCR amplification with the 457 bp SCAR marker

Results indicated a successful amplification with the SCAR marker and produced a 457 bp band as reported by Dutta *et al.*, (2013b). Accordingly, the most of the WSSV positive and negative samples generated the 457 bp band while only few samples did not amplify the band (Table 3, Fig. 2). There was no significant difference between WSSV positive and negative samples based on the frequency of 457 bp allele.

PCR amplification with the KM 19/20 microsatellite allele marker

In this study only one band with 317 bp was observed in 25 samples tested (Table 3, Fig. 2) regardless the WSSV infection. The expected band of 71 bp was not observed in any specimen.