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Original Article

Multiplex-PCR protocol development for rapid screening of white spot syndrome virus (WSSV) in shrimp

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Abstract

This study was aimed to develop a faster single step multiplex PCR protocol for the simultaneous detection of white spot syndrome virus (WSSV) with its host (i.e. shrimp) as internal positive control. To do so, four combinations of primer were tested (I. 16S rRNA+Lo F1R1; II. 16S rRNA+Lo F2R2; III. 16S rRNA+Lo F1R2; IV. 16S rRNA+Lo F2R1) which were selected based on two pairs of WSSV specific primer (Lo F1R1 and Lo F2R2) and one pair of shrimp specific primer (16S rRNA). DNA extracted from WSSV infected shrimp were amplified by PCR in a single tube using each of the primer combinations and the thermal cycling conditions as well as reagent compositions were optimized. All the primer combinations yielded their expected band sizes with stronger band resolution intensity that indicated the development of four multiplex PCR protocols. The developed multiplex protocols reduced the chance of cross contamination and these were found to be faster, single step and unique with less effort and resource use. Considering sensitivity and specificity, among the protocols, we suggested the protocols based on 16S rRNA+Lo F1R1 and/or 16S rRNA+Lo F2R2 primer combinations for rapid and routine screening of WSSV in shrimp PL, juvenile and adult.

Keywords: faster; multiplex PCR; shrimp; simultaneous detection; single step; WSSV

1 | INTRODUCTION

The sustainability of shrimp sector largely depends on addressing a number of challenges among which, the occurrence of white spot disease (WSD) caused by white spot syndrome virus (WSSV) in black tiger shrimp (*Penaeus monodon*) has been considered the most crucial one (Karunasagar *et al.* 1997; Hossain *et al.* 2001; Karim *et al.* 2011). The virus is by far the most devastating pathogen threatening the shrimp industry worldwide (Ganjoor 2015) after the earliest epidemic report from shrimp farms in Taiwan in 1992 (Chou *et al.* 1995). It is capable of transmitting both vertically and horizontally (Kanchanaphum *et al.* 1998; Chakraborty and Ghosh 2014) that causes 90 – 100% mortality within 3 – 10 days of post-infection (Vaseeharan *et al.* 2003; Sanchez-Martinez *et al.* 2007; Ayub *et al.* 2008). Since complete mortality of shrimp occurs within a few days following the appearance of first clinical sign, stocking of virus free healthy PL and

screening the virus repeatedly during the culture period, as the preventive measure of vertical and horizontal transmission, could be the available alternatives to hand for minimizing the WSSV propagation due to the lack of medication against its infection (Islam *et al.* 2007; Karim *et al.* 2011).

Therefore, a rapid and efficient diagnosis method is a prerequisite for the proper screening of the virus/disease. Of the classical diagnostic methods, PCR has attracted widespread applicability due to its higher sensitivity limits (Maeda *et al.* 1998; Sahul Hameed *et al.* 2002; Chakraborty and Ghosh 2014) and PCR-based analysis are now used in a number of countries as the preferred method for the detection of shrimp viruses including WSSV (Kim *et al.* 1998; Withyachumnarnkul 1999; Ganjoor 2015). The PCR-based approach, developed by Lo *et al.* (1996) is considered the reference standard by the renowned screening laboratories worldwide for diagnosing the virus (Nunan and Lightner 2011). In addition to Lo *et al.* (1996), to date, researchers have developed many primers based on WSSV genome and developed different variations of PCR based detection protocols (Otta *et al.* 1999; Tang and Lightner 2000; Nunan and Lightner 2011). However, those have some disadvantages, as for example; the PCR protocols developed so far usually take extended time (3 – 5 hours with at least 40 – 60 thermal cycles of amplification) and large volume of reagents (20 – 25 µl) in a single tube to operate a single run and are potentially costly (Yang 2006). Moreover, conventional monoplex protocols only can confirm WSSV presence in a single tube without the presence of host which requires another tube reaction. The protocols also have high risk of cross-contamination during nested PCR due to opening of the first PCR tube (Sritunyalucksana *et al.* 2006; OIE 2009; Nunan and Lightner 2011).

The aforementioned potential disadvantages could be avoided by optimizing a simple PCR method for simultaneous detection of the virus with the host (i.e. shrimp) in a single tube reaction which can conventionally be defined as multiplex PCR. The multiplex PCR is a variant of PCR by which two or more loci are simultaneously amplified in a single tube reaction by using multiple primer sets where each of the primer pairs has to be optimized so that all primer pairs can work at the same annealing temperature (Chamberlain *et al.* 1988). Application of multiplex PCR analysis has the ability to provide faster detection and it can reduce the reagent cost as well labor (Yang 2006; Lightner 1996). Therefore, this study was aimed at optimizing a single step multiplex PCR protocol that can be conveniently used for simultaneous and rapid screening of WSSV presence in shrimp thereby reducing the overall reaction time, reagent amount and chance of carry over contamination.

2 | METHODOLOGY

2.1 Study period and location

The study was conducted during July 2014 to June 2015 at Biochemistry and Molecular Genetics (BMG) laboratory of Fisheries and Marine Resource Technology (FMRT) Discipline of Khulna University, Bangladesh.

2.2 Sample collection and processing

Fifty juvenile/adult tiger shrimp (*Penaeus monodon*) having apparently gross external symptoms of white spot disease were collected by preserving in 70% ethyl alcohol (Hossain *et al.* 2004) from the shrimp ponds located at Satkhira district, a coastal district of Bangladesh, devoted to shrimp viral diseases endlessly (Islam *et al.* 2007). About 25 – 30 mg tissue from the pleopods of collected shrimps were dissected separately with sterilized surgical blades to avoid cross contamination and placed into a 1.5 ml sterile micro-centrifuge tube.

2.3 Total genomic DNA extraction

Sodium dodecyl sulfate (SDS) based DNA extraction protocol previously developed by our laboratory was followed for the extraction of total genomic DNA from tissue samples. Briefly, samples were homogenized and lysed by 500 µl of 1% SDS based DNA extraction buffer (10 mM Tris-HCl (pH 8), 25 mM EDTA, 100 mM Nacl, 1% SDS) which followed a centrifugation at 12000 rpm for supernatant collection (~350 µl). DNA of the supernatant was precipitated by adding 700 µl (two volumes of the supernatant) 100% ethanol and 75% ethanol was used to wash the precipitated DNA. Finally, the ethanol was removed by pipetting/decanting carefully. The DNA pellets into the tubes were air dried immediately by keeping tubes open at room temperature for 10 – 15 min. The extracted DNA inside the tube was solubilized with 100 µl TE buffer (10 mM Tris-HCl (pH 8), 1 mM EDTA) by slowly passing the pellet through a pipette tip. DNA quantity and quality was measured spectrophotometrically according to Strauss (1998) where DNA quantity was measured photometrically at 260 nm wavelength (OD260) and quality was determined by measuring the ratio of one more photometric value at 260 nm wavelength (OD260/ OD280).

2.4 Primer Selection and combination

One pair of shrimp specific (host / internal positive control) primer and two pairs of WSSV specific primer were taken into consideration. The WSSV specific primers correspond to a cloned, 1461 bp SalI-digested WSSV genome fragment as described by Lo *et al.* (1996) where, Lo F1R1 and Lo F2R2 act as the outer primer and inner primer respectively. Using both of the WSSV specific primer, two more primer pairs were also theoretical obtained in factorial design. The shrimp specific primer was retrieved from its ribosomal RNA (16S rRNA) gene described by Dieu *et*

al. (2004) (Table 1). To develop the multiplex protocol, four primer combinations were finally selected using the WSSV and shrimp specific primer pairs as depicted in Table 1.

2.5 Testing of the major primers by PCR

Prior to the development of multiplex PCR protocol, the three major primer pairs were tested separately in separate tube employing single step PCR. A 10 µl reaction mixtures for each specific tube was prepared with a combination of appropriately diluted approximately 10 – 25 ng sample tissue DNA as the templates with 2 µM of each primer (F / R) and 5 µl of premix (1X PCR buffer, 2 mM $MgCl_2$, 200 µM dNTP's and 0.25 unit Taq polymerase enzyme; TAKARA BIO INC., Japan) in 0.2 ml thin-walled PCR tubes. PCR thermal cycling conditions were empirically determined based on the previous studies (Lo *et al.* 1996; Islam *et al.* 2007). Thermal cycling conditions that were tested ranged 25 – 30 sec at 95°C for the first round followed by 30 cycles of denaturation for 30 – 40 sec at 94°C, 25 – 30 sec of annealing at 54 – 62°C and 40 – 45 sec of extension at 72°C. The last extension step at 72°C was extended for 5 min.

2.6 Optimization of the multiplex PCR protocol

Both WSSV DNA and shrimp DNA (host/internal positive control) were amplified simultaneously in a single tube using each pair of the four (04) combinations of primer (Table 01). All the PCR assays for the multiplex protocol were done with a 10 µl PCR cocktail in a single tube using a thermo cycler (BioRed C1000, Singapur). The multiplex PCR protocol was developed by manipulating the primer and template concentrations, thermal cycling conditions (temperature and time) and reagents compositions found for the separate primer optimization. If all the conditions were found to be optimum for the protocol, only annealing temperatures were empirically altered to obtain the best banding pattern with stronger intensity. Appropriate control reactions (reagent control and negative control) were run in parallel to rule out the possibility erroneous amplification or no amplification at all. The PCR mixture being all the reagents without primers was considered as reagent control and the PCR mixture being all the reagents with DNA of tilapia species replacing shrimp DNA was considered as negative control. All the amplified PCR products were run by electrophoresis in TAE buffer on 1.5% agarose gel mixed with ethidium bromide. The banding pattern, intensity and sizes were identified in comparison with the 100 bp DNA ladder by visualizing under UV trans-illuminator.

3 | RESULT AND DISCUSSION

The three major primers were tested separately to check the efficiency of the primer developed by Lo *et al.* (1996) and Dieu *et al.* (2004) and to manipulate the optimization conditions for developing the multiplex PCR protocol. It was found that each of the primers yielded expected size fragments (Figure 1A – 1C) at specific thermal cycling conditions and reagent compositions in separate tube reactions. The findings of the expected band size indicated that the tested primers were efficient to amplify shrimp and WSSV DNA.

TABLE 1 Type, name, sequence, expected band sizes and the four combinations of the selected primer.

Primer type	Primer name: nucleotide sequence (5'–3')	Band size	Combinations
Shrimp-specific: 1	16SF: GTGCGAAGGTAGCATAATC 16SR: CTGCTGCAACATAAGGATAC	414 bp	N/A
WSSV-specific: 1	LoF1: ACTACTAACTTCAGCCTATCTA LoR1: TAATGCGGGTGTAATGTTCTTA	1447 bp	Combination-1 16S rRNA+Lo F1R1
WSSV-specific: 2	LoF2: GTAACTGCCCCTTCCATCTCC LoR2: TACGGCAGCTGCTGCACCTTG	941 bp	Combination-2 16S rRNA+Lo F2R2
WSSV-specific: 3	LoF1: ACTACTAACTTCAGCCTATCTA LoR2: TACGGCAGCTGCTGCACCTTG	1192 bp	Combination-3 16S rRNA+Lo F1R2
WSSV-specific: 4	LoF2: GTAACTGCCCCTTCCATCTCC LoR1: TAATGCGGGTGTAATGTTCTTA	1196 bp	Combination-4 16S rRNA+Lo F2R1

Following separate primer testing, each of the selected theoretical primer combinations (Table 1) were assessed by manipulating the thermal reagent compositions and thermal cycling conditions. Initial denaturation at 95°C for 45 sec, denaturation at 95°C for 25 sec, extension at 72°C for 40 sec and a final extension at 72°C for 5 min were found to be optimum commonly for all the combinations. Based on the intensity and clarity of the products (bands), annealing temperature at 58°C for 30 sec yielded the best results for all the assays, except 16S rRNA+Lo F1R1 combination which annealed at 56°C for 30 sec. At least 30 cycles of PCR amplification was found to be optimum for the combination of 16S rRNA+Lo F1R1 primers whereas the rest of the combinations yielded the best banding pattern after carrying out at least 32 cycles PCR amplification. Time required for the assays ranged between ap-

proximately 1 h 18 min to 1 h 40 min. Results of the banding pattern for each of the combinations are shown in Figure 2A – 2C which indicated simultaneous detection of the virus in shrimp host visualized under UV and thus, four different single step multiplex PCR protocols were optimized using the selected theoretical primer combinations.

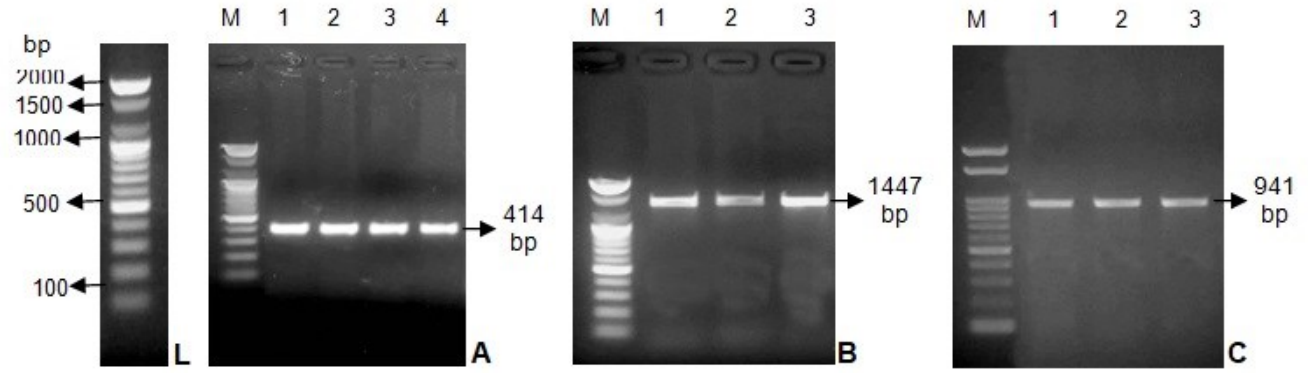

FIGURE 1 Electrophoretic banding patterns of PCR products in separate single tube using the primers of *(A)* 16S rRNA with shrimp DNA amplification; amplicon size 414 bp; Lane: 1 – 4 *(B)* LoF1R1 with WSSV DNA amplification; amplicon size 1447 bp; Lane: 1 – 3 and *(C)* LoF2R2 with WSSV DNA amplification; amplicon size 941 bp; Lane: 1 – 3 *(L)* Scale for showing the banding pattern size of the marker. Lane M in A, B and C: 100 bp DNA marker.

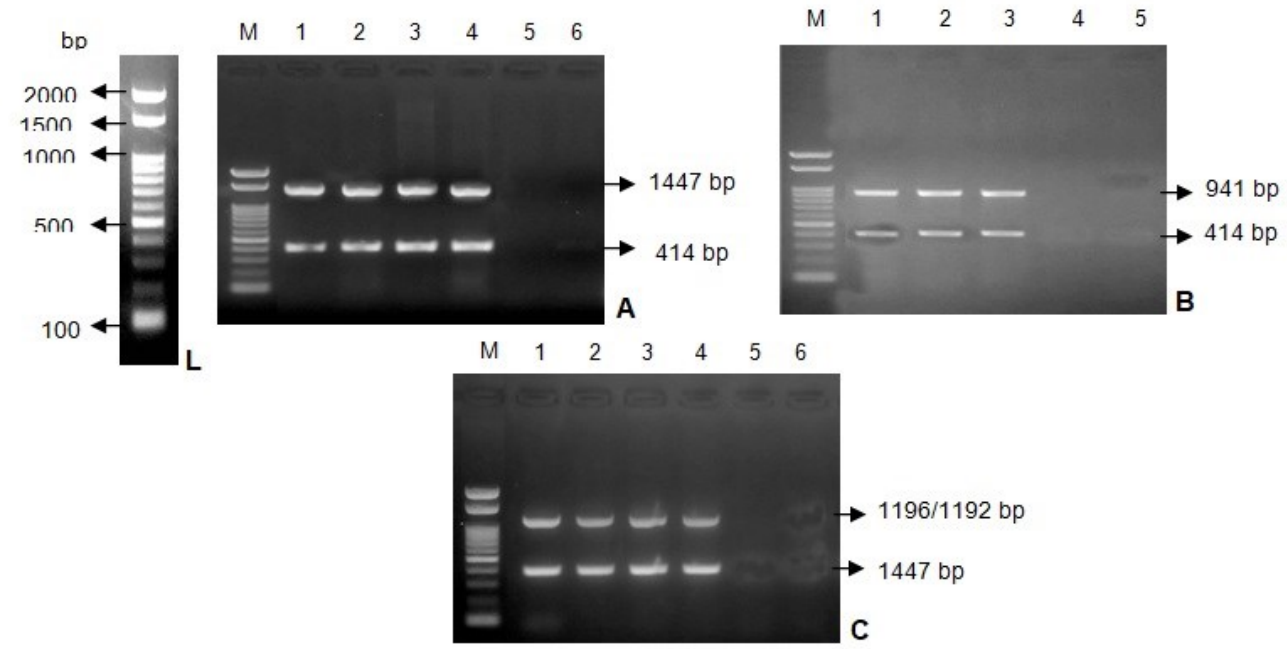

FIGURE 2 Electrophoretic banding pattern of single step multiplex PCR products for the simultaneous detection of WSSV DNA and shrimp DNA in a single tube using the primer combinations of *(A)* Lo F1R1 and 16S rRNA where required 30 cycles thermal cycling with 56°C annealing temperature *(B)* Lo F2R2 and 16S rRNA where required 32 cycles thermal cycling with 58°C annealing temperature and *(C)* Lo F1R2 or/and Lo F2R1 and 16S rRNA where required 32 cycles thermal cycling with 58°C annealing temperature. Lane 1 – 4 of A and C and 1 – 3 of B: simultaneous detection of WSSV and shrimp DNA, WSSV amplicon size 1447/941/1196/1192 bp and shrimp amplicon size 414 bp; Lane 5 of A and C and 4 of B: negative control; Lane 6 of A and C and 5 of B: reagent control. Lane M: 100 bp DNA marker; *(L)* Scale for showing the banding pattern size of the marker.

Each of the optimized assay under this study needs only 10 µl PCR mixtures in a single step / tube reaction for less than 1.5 h of 30 – 32 cycles thermal cycling for the detection of the virus in shrimp simultaneously by performing a single run; this will save time and effort, reduce the use of the resource and ultimately the cost. The recommended single step multiplex PCR test minimizes cross-contamination due to the fact of not having to open an amplified PCR sample for use as template in the second nested step. Laboratory technicians can follow any of the four optimized protocols with specific primer combination for diagnosis of the virus with the shrimp as internal

positive control in a single tube. Since no size difference was visualized between the combinations of 16S rRNA+Lo F1R2 and 16S rRNA+Lo F2R1 because of only 4 bp sizes difference (Figure 2), therefore, both of the combinations were recommended to exclude from the study for avoiding any ambiguity in analysis.

Of the rest two combinations, one should choose the specific protocol based on the purpose of diagnosis. If the purpose of the PCR is to screen for early or light WSSV infection with a low virus load (not showing mortality), use of primers yielding smaller amplicons (band size) would give more accurate results (Hossain *et al.* 2004; Ayub *et al.* 2008). Therefore, we recommend the protocol using the combination of 16S rRNA+Lo F2R2 primers as first priority because of higher sensitivity of the inner (nested) primer being a smaller amplicon size, it may be useful in confirming the early stages of WSSV infection when the virus concentration is relatively low or before the manifestation of infection or onset of the disease. If sensitivity is not a major concern, we recommend the protocol using the primer combination of 16S rRNA+Lo F1R1 because if PCR is used for confirmatory diagnosis of overt disease, primers yielding larger fragments can be used (Hossain *et al.* 2004; Nunan and Lightner 2011). The combination of 16S rRNA+Lo F1R1 can also be recommended because of reduced cycling number, lower time consumption and good banding pattern with stronger intensity compare to the other. Specificity was found to be satisfied because the recommended protocols could detect the virus in every stage (PL, juvenile and adult) of shrimp (Figure 3). However, further study is suggested for determining the sensitivity of the optimized protocols.

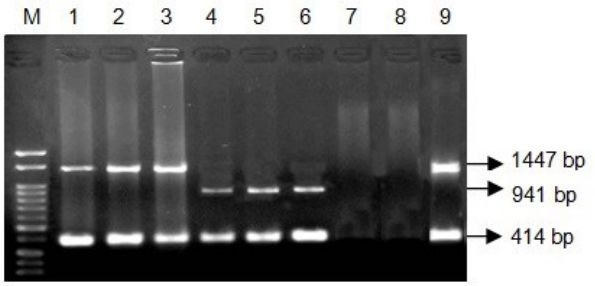

FIGURE 3 Multiplex PCR detection of WSSV DNA in shrimp PL, Juvenile and adult using the primer combination of 16S rRNA+Lo F1R1 and 16S rRNA+Lo F2R2. Lane M: 100 bp DNA marker; Lane 1 – 3: Multiplex PCR amplification using 16S rRNA+Lo F1R1 primer combination where templates were extracted from shrimp PL, juvenile and adult respectively; Lane 4 – 6: Multiplex PCR amplification using 16S rRNA, Lo F2R2 primer combination where templates were extracted from shrimp PL, juvenile and adult respectively; Lane 7: negative control; Lane 8: reagent control; Lane 9: known WSSV positive sample. (Templates / DNAs used in the reactions were personally taken from the PCR lab of WorldFish, Bangladesh).

4 | CONCLUSION

The study optimized four multiplex PCR protocols targeting both WSSV and shrimp DNA; each of which was optimized employing a single tube in a faster, non-stop single step PCR with less resource and time use. However, considering sensitivity and specificity, the protocols using the primer combination of 16S rRNA+Lo F1R1 and/or 16S rRNA+Lo F2R2 are suggested to follow for rapid and routine screening of WSSV in shrimp.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author.

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SA laboratory analysis;
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