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Molecular Identification of Ribonucleotide Reductase Subunit Genes of White Spot Syndrome Virus Isolates from Saudi Arabia

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Abstract: White Spot syndrome virus is a lethal pathogen which causes significant mortality to penaeid shrimps worldwide including Kingdom of Saudi Arabia (KSA). In the present study, in an attempt made to obtain the virulence related genes encoding the large (rr1) and small (rr2) subunit of ribonucleotide reductase (RNR) from WSSV isolates of KSA. *Litopenaeus vannamei* and *Fenneropenaeus indicus* samples were collected from Jazan region and stored in -80°C for the analysis. The WSSV infection was confirmed by OIE protocol. Amplification and sequencing of rr1 (2550bp) and rr2 (1242 bp) genes were done using gene specific primers. Subunits of rr1 and rr2 fragments were identified KSA isolates for the first time. The rr1 and rr2 sequences had significant identity (98-100%) with other known sequences in BLASTX and the sequences were matched with conserved domain family of ribonucleotide reductase. The phylogenetic analysis revealed that sequences had greater homology with other isolates of WSSV ribonucleotide reductase family and it was formed as monophyletic group. The obtained information so for from the sequences of rr1 and rr2, supports that the family of RNR was highly conserved among WSSV isolates. The present study will helpful to reveal the genetic linkage between the different isolates. In future, from this information we try to get the full-length sequences of rr1 and rr2 subunits to understand the epidemiological relevance and transcriptional regulations.

Key words: White Spot Syndrome Virus · Litopenaeus Vannamei · Fenneropenaeus Indicus · Kingdom Of Saudi Arabia And Ribonucleotide Reductase

INTRODUCTION

The culture and production of large-scale finfish and shrimps has assumed global significance in recent years in the Kingdom of Saudi Arabia and it's considered to be the major source of food security of the Saudi population. The major cultivated species in Saudi Arabia were *Fenneropenaeus indicus* and *Litopenaeus vannamei* that introduced 1990s & 2010. Shrimp aquaculture production was increased gradually from 6000 tonnes in 2000 to around 26000 tonnes in 2009 and 2010 in Saudi Arabia [1]. Shrimp aquaculture industry was affected due to a disease which causes economic losses in many ways; to date more than twenty viral diseases have been reported to affect shrimp, of these white spot syndrome virus (WSSV) is the major and most serious pathogen [2]. In KSA it was also a major problem for the shrimp aquaculture industry causing a huge economic loss after WSSV outbreak in 2009 [3, 4].

Ribonucleotide reductase (RNR) involved in nucleotide metabolism and reduces ribonucleotides into deoxyribonucleotides as immediate precursors of DNA in every living cell [5, 6]. The large and small subunits (rr1& rr2) of RNR, is an essential enzyme required for DNA replication and DNA repair. The large and the small subunits of rr1 and rr2 identified from ORF of WSSV rr1 (2547 bp) and rr2 (1242 bp) [7]. Temporal expression analysis suggest that the rr1 and rr2 was expressed in 4-6 hours of post WSSV infection, it might be the indication of these both genes are essential for early part of infective

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stage [8]. Subunits of rr1 and rr2 of WSSV has been reported to be functionally involved during infection [9]. WSSV rr1 gene was directed for silencing viral multiplication by RNAi and the results showed that rr1 can controls the viral multiplication in *Penaeus monodon* [10]. Upon functional importance of the nonstructural proteins we focused on the identification and functional characterization of rr1 and rr1 genes as an approach to identify from the Saudi Arabia strain of WSSV. Identification and understanding of sequence relationships is to be important for native strain that can pave the way to know the virus mechanisms and anti-WSSV strategies.

MARTIALS AND METHODS

Sample Collection: WSSV infected animals (*F. indicus* and *L. vannamei*), were collected from Red Sea coast, Jazan, Saudi Arabia during epizootics in October 2014 and August 2015. At the time of sample collection, *F. indicus* exhibited visible macroscopic white spots in the carapace and red pigmentation of the body. The shrimp pleopod samples were dissected at the farm site, fixed in 95% ethanol, transported to Jeddah Fisheries Research Center, Ministry of Environment, Water and Agriculture and stored at -80°C for further analysis.

DNA Extraction: The ethanol fixed pleopod samples were dried and incised into small pieces and DNA was extracted using DNeasy Blood & Tissue Kit (Qiagen, Germantown, MD) following the manufacturer instructions. The eluted DNA was assessed by spectrophotometer (Nanodrop 2000, Thermo Scientific) and its quality and quantity were determined.

Nested PCR Assay for the Confirmation of WSSV: The extracted DNA was analyzed by nested PCR to confirmed the WSSV infection with the modified PCR program based on OIE recommendation [11, 12]. The 25 µL of PCR reaction mixture was prepared according to the manufacturer instructions (ThermoFisher Scientific, USA). The reaction mixture comprised the following reagents; 10X Reaction Buffer 2.5 µL, dNTPs (10mM each dNTP) 2.5 µL, Forward Primer (10 µM) 1.0 µL, Reverse Primer (10 µM) 1.0 µL, Nuclease-Free water 16.6 µL, Dream Taq DNA polymerase (5U/ µL) 0.4 µL and Template DNA (~100ng/ µL) 1.0 µL. Thermal cycler conditions for the PCR reaction were as follows, initial denaturation at 94°C for 3 min, followed by 39 cycles of denaturation at 94°C for 20 sec, annealing at 62°C for 1 minute, extension at 72°C for 1 minute and a final extension step at 72°C for 3 min. The product from the first step PCR was used as template for second step PCR. To verify the extracted DNA quality decapod-specific primers used as control reactions [13]. PCR products were analyzed by 1.2% agarose gel electrophoresis and the gel was visualized under UV trans-illuminator. Amplified PCR products were recovered and purified using DNA Gel extraction kit (Norgen Biotek corp, CA) according to the manufacturer's instructions. The purified PCR products sent to Apical Scientific SdnBhd, Selangor, Malaysia for sequencing. The primer sequence details are listed in the Table 1.

Primer Designing and Amplification of rr1 and rr2: To amplify the full length and partial rr1 and rr2 from WSSV infected *F.indicus* and *L.vannamei* primers were designed based on database searches using gene or gene product names from National Centre for Biotechnology (NCBI) and the primers were designed using Primer3Plus; (http://www.bioinformatics.nl/cgi bin/primer3plus/primer3plus.cgi) Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) NCBI Primer blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Available sequences from NCBI data base (GenBank accession number) such as AF132669, AF099142 for rr1, AF267731, FJ756461, AF144620 for RR2, JX444994, DQ013883, AY249443, AY249440 were selected and these sequences are subjected to BLAST analysis. Based on the conserved regions primers were designed. The designed primers respected genes and their product sizes were mentioned in the Table 1.

PCR Assay for rr1 and rr2: The concentration of primer and annealing temperatures were optimized. Initially the primers were set up and diluted with nuclease free water (Ambion, USA) 10µM for the final working concentration. To avoid nonspecific secondary bands, Gradient PCR (Veriti Thermal Cycler, Thermo Fisher Scientific) technique was used to optimize the primer annealing temperature. The selection of the annealing temperature is possibly the most critical component for optimizing the specificity of a PCR reaction. Based on the primer melting point (Tm) the annealing temperatures were set from 50°C to 70°C with 2°C difference for rr1 and rr2 primers and annealing temperature were optimized with the known positive control (Provided by Arizona University, Tucson, USA). All PCR reaction was performed with known positive control, Non template control along with the samples to understand and evaluate the PCR results. Based on the gradient PCR it was found that 64°C was optimum forrr1

Table 1: Primers used for PCR analysis for WSSV confirmation, rr1 and, rr2 amplification

PCR/name of the gene	Primer	Sequences 5' to 3'	Product Size	Reference
WSSV 1st step	146F1	ACT ACT AAC TTC AGC CTA TCTA		
	146R1	TAA TGC GGG TGT AAT GTT CTT ACG A	1447bp	OIE 2016
WSSV nested step	146F2	GTA ACT GCC CCT TCC ATC TCC A		
	146R2	TAC GGC AGC TGC TGC ACC TTG T	941bp	OIE 2016
Decapod-specific primers	143F	TGC CTT ATC AGC TNT CGA TTG TAG		
	145R	TTC AGN TTT GCA ACC ATA CTT CCC	848 bp	OIE 2016
rr1	2550F	ATG GGT TCT AAC CAG CAA CAA TCA		
	2550R	TGG CTA GGA AGA ACA CAT TTC ACA	2550bp	this study
rr2	1242F	ATG GAG TCA ATC AAA CTG TTC		
	1242R	CTA AAA ATC GTC ATA ACT GAT G	1242bp	this study

and for rr2 was at 60°C. Once the primer and annealing temperature were optimized, PCR was done with selective primers such as to amplify the rr1 and rr2 genes. The amplification of rr1 and RR2 gene was done with the 10X dream taq green buffer (ThermoFisher Scientific, USA). The amplification was performed in 25µL reaction mixture. The reaction mixture comprised the following reagents; 10X Reaction Buffer 2.5 µL, dNTPs (10mM each dNTP) 2.5 µL, Forward Primer (10 µM) 1.0 µL, Reverse Primer (10 µM) 1.0 µL, Nuclease Free water 16.6 µL, Taq DNA polymerase (5U/ µL) 0.4 µL, Template DNA (~100ng/ µL)1.0 µL. The amplified products were electrophoresed through 1.2% agarose gel in TBE buffer at 100 V for 1 h. The gel was visualized under UV transillumination (GelDoc, BioRad). Amplified PCR products were recovered and purified using DNA Gel extraction kit (Norgen Biotek corp, CA) according to the manufacturer's instructions. The purified PCR products sent to Apical Scientific SdnBhd, Selangor, Malaysia for sequencing.

rr1 and rr2 homology to other sequences in GenBank were identified using BLAST program (http:// www.ncbi.nlm.nih.gov/ BLAST). Multiple sequence alignment of rr1 and rr1 were performed using ClustalW program [14]. Neighbor-Joining phylogenetic tree of rr1 and rr2 amino acid sequences were run using neighbor-joining algorithm by Mega 7 software [15].

RESULTS

Detection of WSSV by Nested PCR (OIE): The obtained samples of *F. indicus* and *L. vannamei* were shown WSSV positive for PCR amplification with theexpected size range using 146F2 and R2 primers (Figure.1).

Amplification of rr1 and rr2 Gene: A 2550 bp and 1242bp amplicon representing the WSSV large and small subunits of nucleotide reductase gene was amplified using the primers 2550F/2550R and 1242F/1242R from WSSV infected *F. indicus* and *L. vannamei* samples (Figure.2).

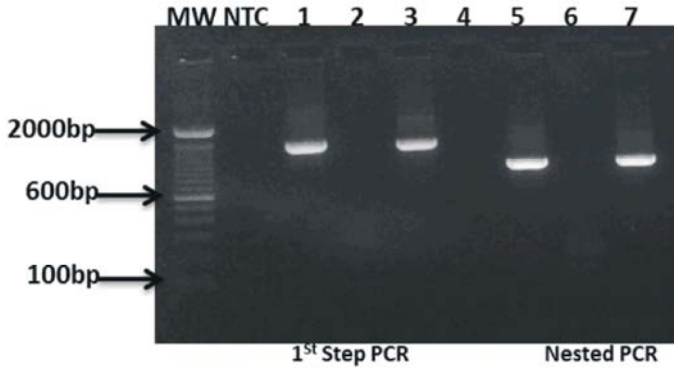

Fig. 1: First and second step PCR agarose gel electrophoresis for the confirmation of WSSV (MW: Molecular weight marker (100bp DNA ladder, Cat No; 15628019, Thermo Scientific, CA), NTC: Non template Control, Lane No: 1 & 5 : F.indicus WSSV positive, Lane No 2 & 6: Specific Pathogen free shrimp (SPF), Lane No 3 & 7: L.vannamei WSSV positive.

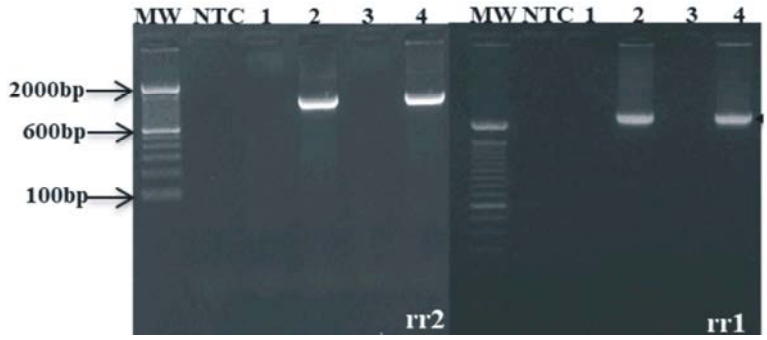

Fig. 2: Amplification of rr1 and rr2 genes. 1.2% agarose gel MW: Molecular weight marker (100bp DNA ladder, Cat No; 15628019, Thermo Scientific, CA). NTC: Non template control, Lane no 1: F. indicus control (Un-infected), Lane no 2: F. indicus WSSV infected, Lane no 3: L.vannamei SPF (Un-infected), Lane no 4: L.vannamei WSSV infected.

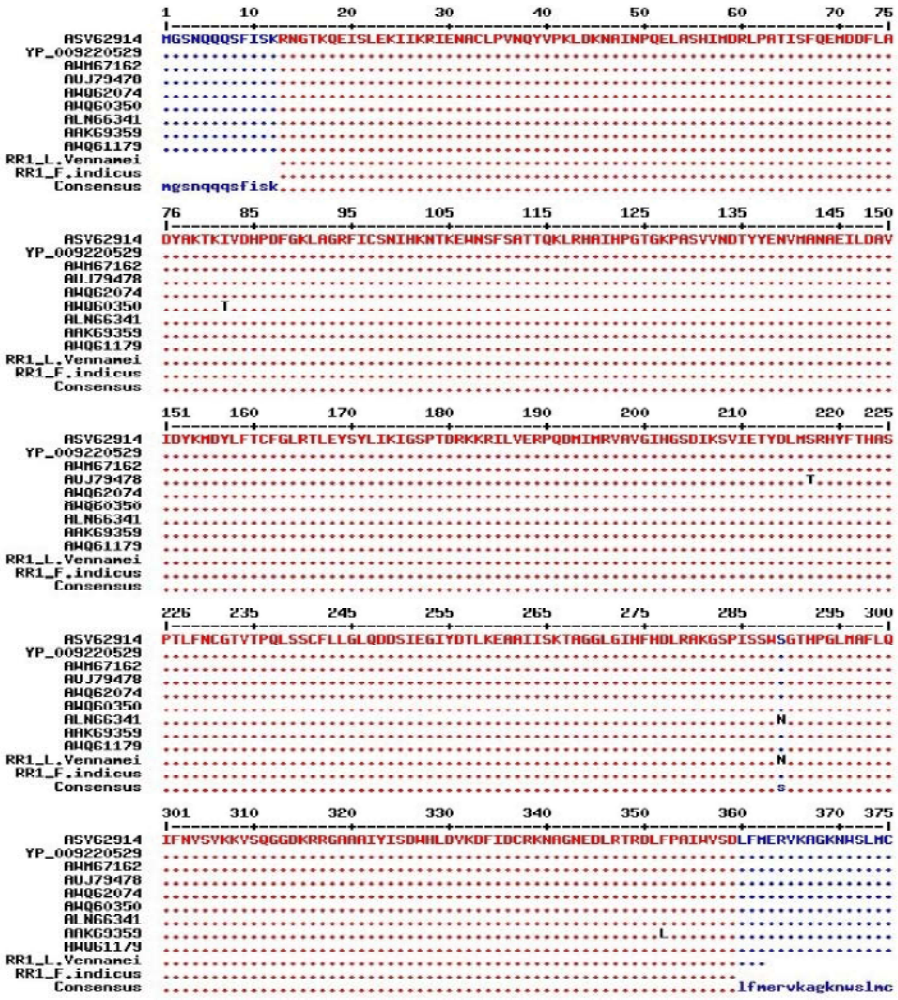

Fig. 3: Multiple sequence alignment of rr1 from WSSV infected F. indicus and L. vannamei in Saudi Arabia with other known isolates (ASV62914 China, YP_009220529 USA, AWQ62074 Mexico, AWM67162 Ecuador, AUJ79478 Brazil, AAK69359 Taiwan and ALN66341 P. R. China). Identical amino acids are indicated by dots and predicted dissimilarity sequences are indicated single-letter code. The sequences used were either published or present in GenBank.

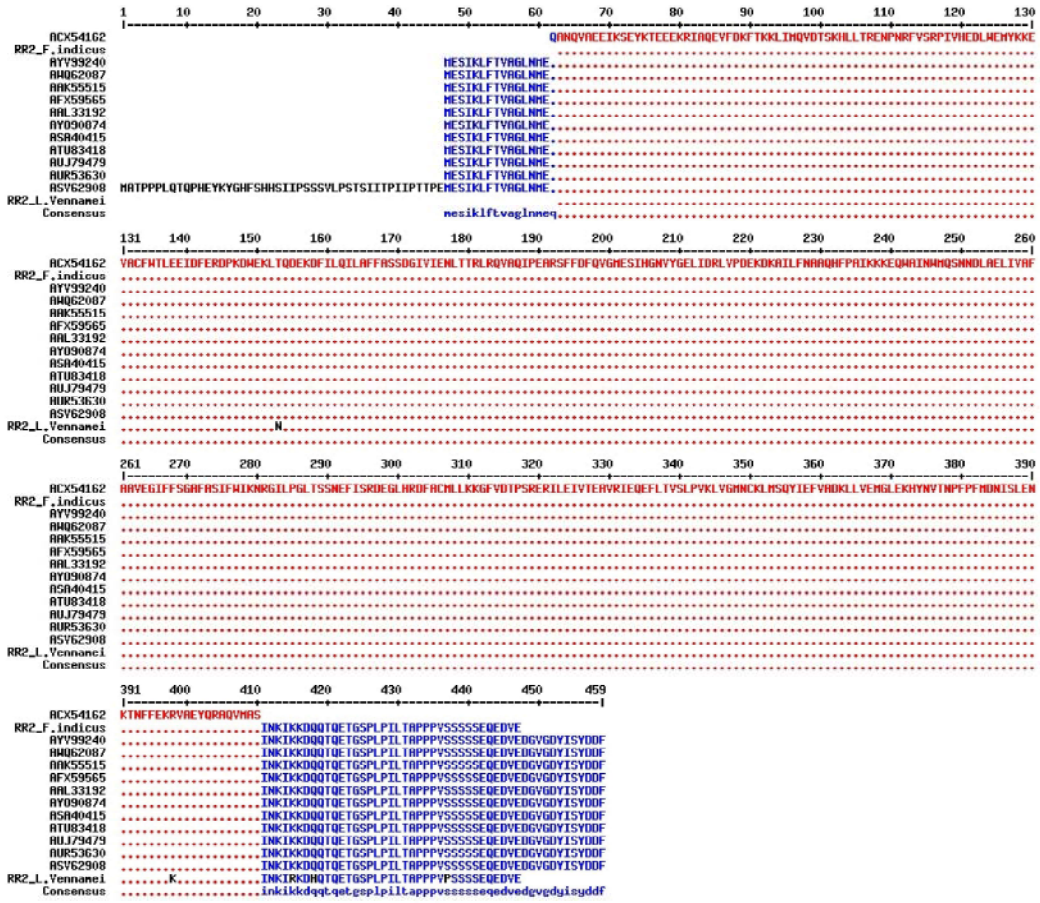

Fig. 4: Multiple sequence alignment of rr2 from WSSV infected F. indicus and L. vannamei in Saudi Arabia with other known isolates (AYV99240 India, AWQ62087 Mexico, ASV62908 China, AUR53630 Brazil, AUJ79479 Brazil and ATU83418 P. R. China). Identical amino acids are indicated by dots and predicted dissimilarity sequences are indicated single-letter code. The sequences used were either published or present in GenBank

After trimming incomplete ends that were unavailable from both ends, a standardized length was used for sequences analysis. BLASTN for Saudi Arabian isolates of WSSV rr1 and rr2 sequence showed 99-100% of similarity with other WSSV isolates and BLASTP results correspondence with the conserved domain of ribonucleotide reductase (RNR) super family.

The amino acid sequences of Saudi Arabia isolates of WSSV rr1 and rr2 were compared with those from other countries isolates of WSSV rr1 (Figure. 3) and rr2 (Figure. 4). An alignment of rr1 and rr2 made with CLUSTALW. The two alignments showed high degree of similarities; thereby it was most conserved among WSSV isolates. Neighbor-joining analyses of rr1 and rr2 from WSSV infected *F. indicus* and *L. vannamei* in Saudi Arabia with other known isolates relationships was assessed. rr1 was closely formed a monophyletic clade with the WSSV isolates of Mexico (AWQ62074, AWQ60350 and AWQ61179), Brazil (AUJ79478), China (ASV62914, Ecuador (AWM67162) P. R. China (ALN66341) USA (YP_009220529) and Taiwan (AAK69359) (Figure.5). rr2 of Saudi Arabia WSSV isolates formed a highly supported monophyletic group with the isolates of Taiwan (AAK55515), South Korea (AFX59565), P.R. China (AAL33192), Egypt (AYO90874), P. R. China (ASA40415), Australia (ATU83418), Brazil (AUJ79479) China (ASV62908), Mexico (AWQ62087), India (AYV99240) and Mexico (ACX54162, ALZ45753 and AWQ63704) (Figure.6). The phylogenetic tree of rr1 and rr2 resulted low numbers of bootstrap values and both trees resembled each other.

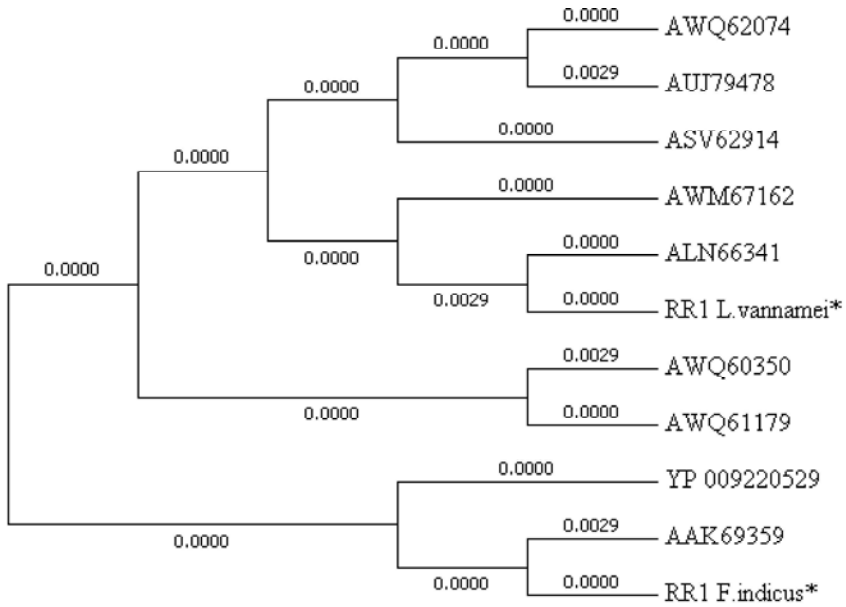

Fig. 5: Neighbor-joining analyses of rr1 from WSSV infected F. indicus and L. vannamei in Saudi Arabia with other known isolates (AWQ62074, AWQ60350, AWQ61179 Mexico, AUJ79478 Brazil, ASV62914 China, AWM67162 Ecuador, ALN66341 P. R. China, YP_009220529 USA and AAK69359 Taiwan). The numbers on the branches indicate bootstrap values after 1000 replicates. The optimal tree with the sum of branch length = 0.01155364 and, numbers at the branches indicate branch lengths. The sequences used were either published or present in GenBank.

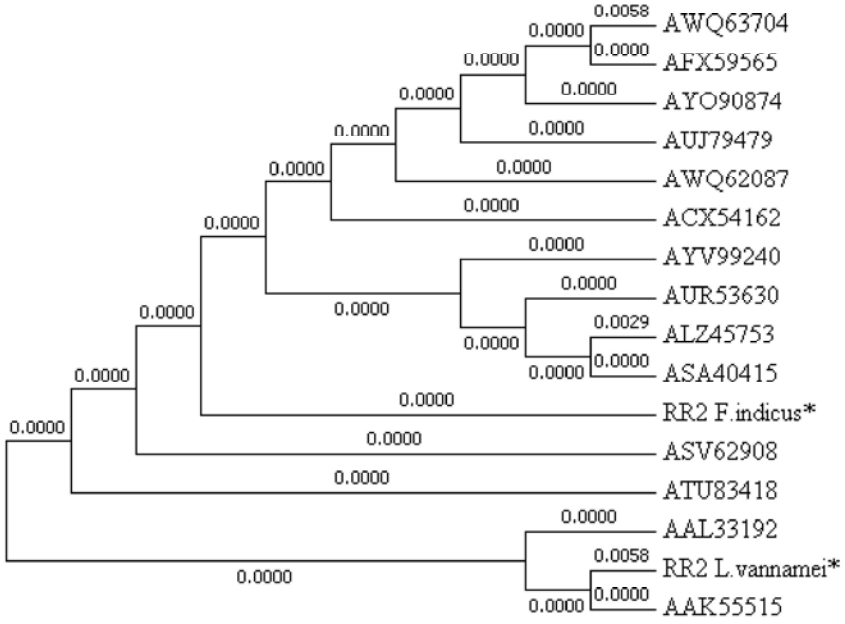

Fig. 5: Neighbor-joining analyses of rr1 from WSSV infected F.indicus and L.vannamei in Saudi Arabia with other known isolates (AWQ62074, AWQ60350, AWQ61179 Mexico, AUJ79478 Brazil, ASV62914 China, AWM67162 Ecuador, ALN66341 P. R. China, YP_009220529 USA and AAK69359 Taiwan). The numbers on the branches indicate bootstrap values after 1000 replicates. The optimal tree with the sum of branch length = 0.01155364 and, numbers at the branches indicate branch lengths. The sequences used were either published or present in GenBank.

DISCUSSION

Whit spot syndrome caused by white spot syndrome virus (WSSV) is one of the serious diseases in shrimp farming industry worldwide including Kingdom of Saudi Arabia. WSSV was assigned by the International Committee of Taxonomy of Viruses (ICTV) to new genus, Whispovrius, under the family Nimaviridae based on its unique morphological structure and genomic functionality [16 - 18].WSSV was identified from *F.indicus* and *L.vannamei* which caused significant losses in Saudi Arabian shrimp industry [19, 20]. Sequencing of WSSV isolates from different countries revealed that dsDNA genome is about 300kb in size and the nucleotide analysis shown that the WSSV nucleotide identity of 99.32% with diminutive dissimilarity [21, 22]. The disclosed WSSV genomic DNA sequence availability in database, now research focused on the functional analysis of particular gene and its gene products as example structural and nonstructural genes were elucidated [23 - 25].

The present study was aimed to identify and sequence analysis the virulence related genes encoding the large (rr1) and small (rr2) subunit of ribonucleotide reductase from WSSV isolates of KSA. Firstly, the WSSV infection was confirmed through OIE protocol with the PCR amplification of expected size for WSSV using primers 146F2/R2 and the same was confirmed by sequencing the PCR products followed by sequence analysis. Amplified sequences of Saudi Arabian samples were all identical (99-100% similarities) to each other and also identical to a large number of other or overseas strains which a consistent is with recent report by Knibb *et al.* [26]. Amplified products were sequenced and the sequence shown great similarity with other known rr1 and rr2 WSSV isolates found in databases (GenBank). Genes involved in the replication and transcription which are typically encodes the proteins are homologues and also determine the characteristic feature of baculovirus family [27]. Saudi Arabia isolate of WSSV rr1produced the specific hit in the superfamily of ribonucleotide reductase (RNR) of large subunits in Blastp analysis which is an indication of WSSV rr1 diverged from a common ancestor. *Aedes aegypti* rr1 shows high levels of identity to other known rr1 proteins and conserved its active site residues [28]. Saudi Arabia WSSV isolates of rr2 amino acid sequences had identity to ribonucleotide small chain with the conserved domain of ferritin likediiron-carboxylate family. The conversion of ribonucleotides to deoxyribonucleotides is carried out by these enzymes which are found in all multicellular and, many unicellular organisms and some viruses. Multiple sequence alignment shown that WSSV isolates of rr1 and rr2 amino acid sequences had resemblance to foreign country WSSV isolates. Similarly, the Thailand isolates of WSSV rr1 and rr2 amino acid sequences had 100% with two different geographical isolates [29].The highest similarity of the WSSV rr1 and rr2 proteins of Taiwan isolates was found to human and vaccinia virus Rrs [30]. Present study could concludes the WSSV isolates of rr1 and rr2 amino acid sequences are 99 to100% identical, it may undergoes similar features of genomic organization. The phylogenetic tree of rr1 and rr2 was observed in subsets with low bootstrap values. We found that diversity of rr1 and rr2 is uniform across different countries isolates of WSSV. It was evident that the uniformity of rr1 and rr2 with other WSSV isolates may undergo similar functionality from and share a common ancestor. Furthermore, the identical regions could serve as potential sites for new control strategies. For example, dsRNA targeting rr1 genes could be used for the potential target to protect shrimp against WSSV infection [31].

CONCLUSION

Saudi Arabian isolates of WSSV rr1 and rr2 nucleotide and amino acid sequences share high degree of similarity, thus it may have a similar role in pathogenesis or genomic organization. Furthermore, with the short piece of sequence information the full length sequence can be obtained and biological function as well as the significance of active domains can be determined.

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