White spot syndrome virus (WSSV) genome stability maintained over six passages through three different penaeid shrimp species

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ABSTRACT: White spot syndrome virus (WSSV) replicates rapidly, can be extremely pathogenic and is a common cause of mass mortality in cultured shrimp. Variable number tandem repeat (VNTR) sequences present in the open reading frame (ORF)94, ORF125 and ORF75 regions of the WSSV genome have been used widely as genetic markers in epidemiological studies. However, reports that VNTRs might evolve rapidly following even a single transmission through penaeid shrimp or other crustacean hosts have created confusion as to how VNTR data is interpreted. To examine VNTR stability again, 2 WSSV strains (PmTN4RU and LvAP11RU) with differing ORF94 tandem repeat numbers and slight differences in apparent virulence were passaged sequentially 6 times through black tiger shrimp *Penaeus monodon*, Indian white shrimp *Feneropenaeus indicus* or Pacific white leg shrimp *Litopenaeus vannamei*. PCR analyses to genotype the ORF94, ORF125 and ORF75 VNTRs did not identify any differences from either of the 2 parental WSSV strains after multiple passages through any of the shrimp species. These data were confirmed by sequence analysis and indicate that the stability of the genome regions containing these VNTRs is quite high at least for the WSSV strains, hosts and number of passages examined and that the VNTR sequences thus represent useful genetic markers for studying WSSV epidemiology.

KEY WORDS: White spot syndrome virus · WSSV · Genotyping · Variable-number tandem repeat · VNTR · Multiple passage · Host variation

INTRODUCTION

White spot syndrome virus (WSSV) (genus *Whispovirus*, family *Nimaviridae*) forms ellipsoid-shaped enveloped virions containing a ~300 kb circular double-stranded DNA genome and, over the past 2 decades, has been a major cause of mass mortalities and economic losses in shrimp farms in most regions of the world (Nakano et al. 1994, Wongteerasupaya et al. 1995, Karunasagar et al. 1997). As WSSV can infect many crustacean species (Flegel 1997, Chakraborty et al. 2002) and as free virus particles can remain infectious for considerable time periods in both soil and water (Satheesh Kumar et al. 2013), strict hatch-ery and farm biosecurity measures are required to prevent disease occurring during culture.

The genome sequence of different WSSV strains has been found to be generally highly conserved (>98% nucleotide sequence identity) except in 3 loci (open reading frame [ORF]75, ORF94 and ORF125) containing variable-number tandem repeat (VNTR) sequences and 2 loci (ORF14/15 and ORF23/24) containing indels (van Hulten et al. 2001, Yang et al. 2001, Marks et al. 2004). Determining VNTR numbers/types and ORF14/15 and ORF23/24 indel types of WSSV strains has provided insights into WSSV epidemiology (Dieu et al. 2004, Pradeep et al. 2008, Walker et al. 2011a,b). Of the 3 VNTR loci, ORF94

has proved to be the most informative single genetic marker for delineating WSSV strains (Wongteerasupaya et al. 2003).

Little is known about what host or environmental selective pressures drive genome variation in WSSV (Sablok et al. 2012). While it has been reported that a single passage of various WSSV strains in a different crustacean host can promote a shift in VNTR type (Waikhom et al. 2006), a very recent study found the ORF94 VNTR to be unaltered following several serial passages through 3 diverse crustacean host species (Gudkovs et al. 2014). As stable VNTR types have been identified among susceptible crustaceans sampled from aquaculture ponds containing WSSV-infected shrimp (Hoa et al. 2011), it appears likely that the rapid shifts in VNTR type reported by Waikhom et al. (2006) were due to host selection of a WSSV genetic variant present in low levels and not detected in the parental tissue used for challenge, or to the detection of a WSSV type pre-existing at low levels and/or prevalence in the less-susceptible host species challenged. Thus, to examine more closely what potential impacts host and passage number might impart upon VNTR stability, and to reaffirm how VNTR marker data can be interpreted in epidemiological studies, 2 WSSV strains with different VNTR genotypes were passaged sequentially through 3 different penaeid shrimp species.

MATERIALS AND METHODS

WSSV samples

Shrimp (*Penaeus monodon* and *Litopenaeus vannamei*) displaying gross signs of WSSV were collected from farms in different regions of South India. Once received at the laboratory, pleopods of moribund shrimp were preserved in 90% ethanol for PCR analysis, and the remainder of each shrimp was snap frozen on dry ice and stored at −80°C as a WSSV inoculum source.

DNA extraction

DNA was extracted from 30 to 50 mg pleopod tissue disrupted in a buffer containing 6 M guanidine hydrochloride and precipitated under cold ethanol as described by Otta et al. (2003). After washing in 70% ethanol, DNA pellets recovered by centrifugation were air dried, dissolved in 100 µl TE buffer and stored at −20°C.

WSSV nested PCR

To confirm WSSV infection, DNA was amplified using the PCR and nested PCR primers and thermal cycling conditions described by Kimura et al. (1996). In brief, each PCR (25 µl volume) was prepared using 2× Red PCR Master Mix (STRATEC Biomedical) and 0.5 µl (10 pmol) each forward and reverse primer, with 1 µl DNA used in the PCR and 1 µl PCR used in the nested PCR. A portion (5 µl) of each reaction was electrophoresed in a 1.2% agarose-Tris-acetate-EDTA (TAE) gel containing 0.5 µg ml^{-1} ethidium bromide, and DNA bands were detected and photographed using a Gel Doc 2000 UV transilluminator (BioRad).

WSSV VNTR genotyping PCRs

WSSV-positive shrimp samples were genotyped by PCR using the thermal cycling conditions and primer pairs described previously by Pradeep et al. (2008) for amplifying VNTR sequences in ORF94 (ORF94-F/ORF94-R), ORF125 (ORF125-F/ORF125-R), ORF75 (ORF75-F/ORF75-R) and the ORF75 flank region (ORF75-FLANK-F/ORF75-FLANK-R). The number of repeat units (RUs) comprising each VNTR was determined from amplicon sizes estimated by agarose gel electrophoresis. RU numbers present in ORF94 (54 bp RU) and ORF125 (69 bp RU) were calculated using the equations RU_{ORF94} = [amplicon size − (171 + 12)]/54 and RU_{ORF125} = [amplicon size + (35 − 92)]/69 to account for flanking sequence lengths in the PCR amplicons (Pradeep et al. 2008). To determine the number and arrangement of the composite ORF75 RU comprising 45 and 57 bp RU components, DNA products amplified using the ORF75 and ORF75-FLANK primer pairs were cloned and sequenced. DNA products amplified from ORF94 and ORF125 were also sequenced to verify RU numbers calculated from amplicon lengths.

DNA cloning and sequence analysis

PCR products were purified using Gel and PCR Extraction System spin columns (Bio Basic) and cloned into the pTZ57R/T vector using an InsTAclone PCR Cloning Kit (Thermo Scientific) as described in the manufacturer's protocol. Positive clones confirmed by PCR were used to prepare plasmid DNA for sequence analysis.

WSSV inoculum preparation and shrimp challenge

Based on ORF94 RU data, shrimp infected with WSSV types containing either the lowest or the highest RU number were selected to prepare inoculums. For each WSSV inoculum, gill and pleopod tissues pooled from 3 shrimp stored at −80°C were homogenized in TN buffer (0.02 M Tris-HCl pH 7.4, 0.4 M NaCl) at a ratio of 1:10 (w/v) and centrifuged at 3000 × g (20 min, 4°C). Each supernatant was recovered, clarified by centrifugation again at 10 000 × g (10 min, 4°C), passed through a 0.4 µm membrane filter (Millipore) and stored in aliquots at −80°C.

Juvenile *P. monodon*, *L. vannamei* and *Feneropenaeus indicus* (6 to 8 g weight) determined to be WSSV-free by nested PCR were collected from the Muthukadu Experimental Station, Central Institute of Brackishwater Aquaculture, Chennai, India. Shrimp were acclimated for 5 d in 500 l fibreglass-reinforced plastic (FRP) tanks filled with aerated 28 to 30 ppt salinity seawater at 30 to 32°C and fed commercial feed pellets. For WSSV challenge, muscle at the 3rd abdominal segment was injected with 50 µl of either the Low or High ORF94 RU inoculum. In total, 30 individuals of each species held in separate 500 l tanks were injected with each inoculum. Shrimp were observed at 8 h intervals, at which times any moribund or dead shrimp were stored at −80°C.

WSSV strain virulence

To determine the virulence potential of the Low and High ORF94 RU WSSV types examined, groups of 50 WSSV-free juvenile *P. monodon*, *F. indicus* or *L. vannamei* were challenged orally with meat of moribund shrimp challenged with either of these WSSV strains. Shrimp in the 6 challenge tanks were fed sufficient WSSV-infected shrimp meat to replace the commercial pellets they would have consumed during a morning feed. After 3 h, any remaining meat was removed, the tank seawater replaced, and feeding on commercial feed pellets was resumed. Each day post-challenge, excess feed was removed 3 h after the afternoon feed and 50% of the seawater was replaced. Tanks were inspected at 8 h intervals, at which times any dead shrimp observed were removed. Each bioassay was terminated when mortality reached 100%.

WSSV passage to assess VNTR stability

Using the same general tank maintenance and oral challenge systems described in the previous sections, WSSV-free juvenile *P. monodon*, *F. indicus* and *L. vannamei* were challenged similarly using meat of dead shrimp stored from the virulence bioassay (Passage 2). Meat from each of the 3 shrimp species was then fed to the same species as well as the other 2 species (Passage 3), resulting in 9 experimental groups. Meat from newly dead shrimp stored from each of the 9 groups comprising Passage 3 was then fed only to the same species (Passage 4), and this process repeated in Passages 5 and 6. WSSV infection in each experimental group was verified by PCR from Passage 3 onwards. WSSV genotypes (ORF94, ORF125 and ORF75) were also verified by PCR from Passage 3 onwards. ORF94, ORF125 and ORF75 genotypes determined by PCR for representative Passage 6 shrimp were also confirmed by cloning and sequence analysis.

RESULTS AND DISCUSSION

Genotyping of WSSV strains

All moribund *Penaeus monodon* and *Litopenaeus vannamei* tested from farms experiencing disease outbreaks were 1-step PCR-positive (982 bp amplicon) for WSSV (Fig. 1A). This was indicative of acute WSSV disease, and for *P. monodon* farmed in India, such disease occurs commonly due to the use of infected seedstock arising as a result of WSSV being prevalent among the wild broodstock used at hatcheries (Remany et al. 2012). Acute infection in the *L. vannamei* was also not unexpected due to the specific pathogen-free *L. vannamei* being farmed in India remaining highly susceptible to WSSV infection and disease (Otta et al. 2014). WSSV disease problems in India are also exacerbated by inadequate pond preparation and biosecurity measures (Satheesh Kumar et al. 2013).

Among 4 batches of *P. monodon* and 4 batches of *L. vannamei* for which WSSV VNTR genotypes were determined by PCR, 5 different ORF94 VNTR types were identified, comprising between 4 and 11 RUs, and 3 different ORF125 VNTR types were identified, comprising 4, 5 or 6 RUs (Fig. 1B−E, Table 1). ORF94 and ORF125 VNTR types have been used widely to distinguish WSSV strains (Wongteerasupaya et al. 2003, Hoa et al. 2005, 2011, 2012), and many WSSV
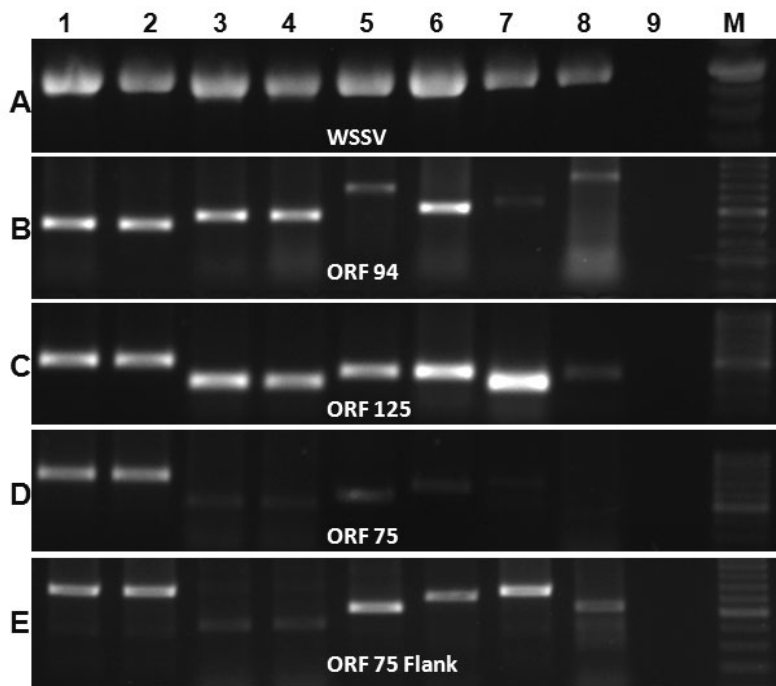
Fig. 1. Agarose gel images of DNA products amplified using PCR primers (A) used in the 1-step PCR to diagnose white spot syndrome virus (WSSV) infection or targeted to the variable-number tandem repeat (VNTR) regions (B) ORF94, (C) ORF125, (D) ORF75 and (E) ORF75-FLANK. DNA was amplified from WSSV strains existing in diseased *Penaeus monodon* (Lanes 1 to 4) and *Litopenaeus vannamei* (Lanes 5 to 8) collected from farms in different regions of south India. Shrimp originated from Nagapattinam (Lanes 1, 6) and Kalpakkam (Lanes 2, 5) in Tamil Nadu and from Bhimavaram (Lane 3), Nellore (Lanes 4, 8) and Gudur (Lane 7) in Andhra Pradesh. Negative control (Lane 9); M: 100 bp DNA ladder

strains have been associated with disease at shrimp farms in India (Syed Musthaq et al. 2006, Pradeep et al. 2008, John et al. 2010, Walker et al. 2011a,b). The VNTR variations detected were thus not unexpected considering the different species examined and the

fact that the diseased shrimp had been sourced from different farms in different regions of South India.

Genotype stability following repeated passage

WSSV strains detected among the *P. monodon* and *L. vannamei* that possessed ORF94 VNTRs with the lowest RU (4 RU = PmTN4RU) and highest RU (11 RU = LvAP11RU) numbers were selected to assess genotype stability. In initial virulence trials in *P. monodon*, *L. vannamei* and *Feneropenaeus indicus*, the inoculum containing the Pm-TN4RU strain appeared to be somewhat more potent in all 3 species compared to that containing the LvAP-11RU strain (Fig. 2A–C). For PmTN-4RU, 100% mortality occurred on Day 4 post-challenge in *P. monodon* and on Day 5 post-challenge in *L. vannamei* and *F. indicus*. For LvAP11RU, 100% mortality occurred on Day 7 post-challenge in all 3 shrimp species.

Differences in apparent virulence have been reported among WSSV strains originating from different geographical locations (Wang et al. 1999, Durand et al. 2000) However, whether VNTR numbers in ORF94, ORF125 and ORF75 influence virulence directly is not yet clear. Factors responsible for virulence are difficult to conclude from confounding reports of difficulties in correlating specific genotypes to shrimp mortality (John et al.

Table 1. Calculated repeat unit (RU) numbers in PCR products amplified across the ORF94, ORF125 and ORF75 variable-number tandem repeats (VNTRs) of white spot syndrome virus (WSSV) strains detected in diseased shrimp *Penaeus monodon* and *Litopenaeus vannamei* collected from various locations in South India. TN: Tamil Nadu, AP: Andhra Pradesh

| Shrimp species	Origin	ORF94		ORF125		ORF75	ORF75-FLANK
		RU	Amplicon size (bp)	RU	Amplicon size (bp)	Amplicon size (bp)	Amplicon size (bp)
P. monodon	Nagapattinam, TN	4	399	4	370	660	676
	Kalpakkam, TN	4	399	4	370	660	676
	Bhimavaram, AP	6	507	5	402	525	542
	Nellore, AP	6	507	5	402	525	542
L. vannamei	Kalpakkam, TN	11	777	6	471	525	542
	Nagapattinam, TN	7	561	6	471	614	632
	Gudur, AP	8	615	5	402	610	677
	Nellore, AP	11	777	6	471	610	627
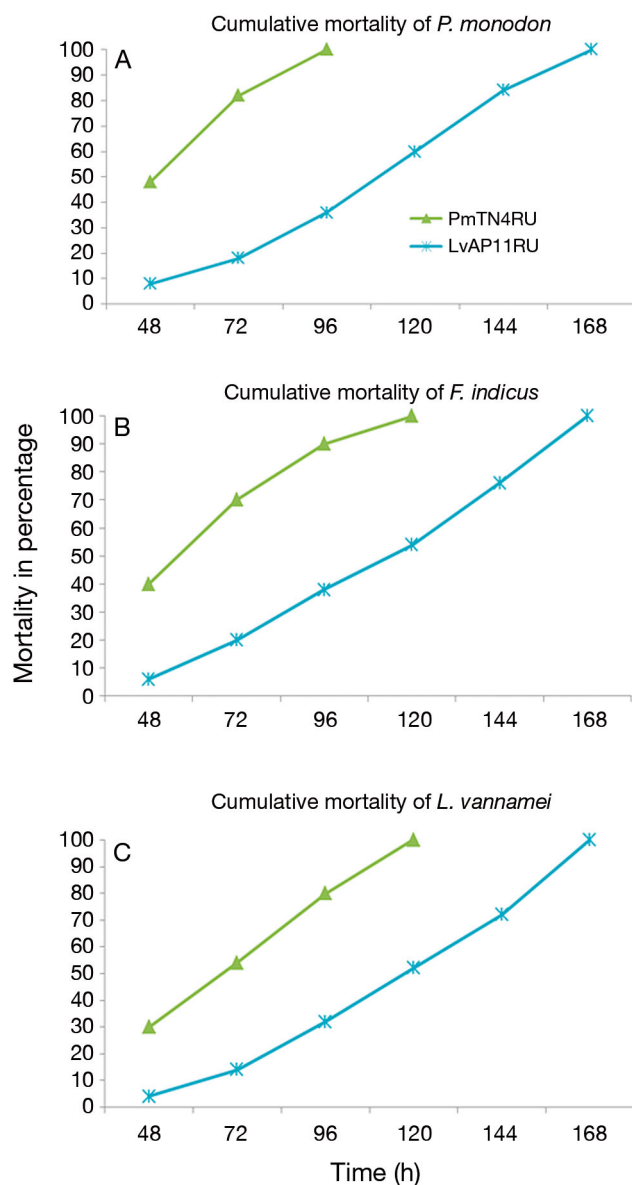
Fig. 2. Cumulative mortality over time in the penaeid shrimp species (A) *Penaeus monodon*, (B) *Feneropenaeus indicus* and (C) *Litopenaeus vannamei* challenged with white spot syndrome virus (WSSV) strains originating from *P. monodon* (PmTN4RU) and *L. vannamei* (LvAP11RU) that possessed low and high variable-number tandem repeat (VNTR) repeat unit (RU) numbers in ORF94

2010), different genotypes being associated with farm disease outbreaks (Walker et al. 2011a,b) or the presence of mixed WSSV genotypes being related inversely to shrimp mortality at farms (Hoa et al. 2011). In another study examining WSSV strains from the same geographic region, a WSSV strain containing a 19 kb genome deletion was found to be more virulent, possibly as a result of the smaller

genome providing the strain with some advantage in replication (Marks et al. 2005). While the PmTN4RU WSSV strain examined here similarly displayed slightly higher virulence compared to the LvAP11RU strain, comparisons of larger numbers of strains with characterized genome compositions will be needed to identify definitively what genome factors affect virulence.

While some studies have detected no substantial difference in the susceptibility of different penaeid shrimp species to different WSSV strains (Chou et al. (1995), in the challenge experiments reported here, mortalities accumulated slightly faster in *P. monodon* than in *L. vannamei* and *F. indicus* with both the PmTN4RU and LvAP11RU strains. Due to the myriad of factors that will vary between reported challenge experiments, conclusions are difficult to derive. However, to try to accommodate any WSSV virulence and host susceptibility influences on the propensity for VNTRs to evolve following repeated passaging through *P. monodon*, *L. vannamei* and *F. indicus*, both the PmTN4RU and LvAP11RU strains were examined. The virulence of strain PmTN4RU was maintained over 6 passages in all 3 shrimp species challenged (Fig. 3A in the top panel), and no variations in VNTR numbers arose in ORF94, ORF125 and ORF75 (Fig. 3B–D in the top panel). Similar findings were made with strain LvAP11RU (Fig. 3 bottom panel). Cloning and sequence analysis of the VNTRs showed that they remained unaltered to those present in the parental WSSV strains, confirming the data obtained from the PCR analyses (data not shown).

The stability of ORF94, ORF125 and ORF75 VNTRs in the 2 WSSV strains examined here contrasts with the ORF94 instability reported previously among WSSV strains following a single passage of various strains in the same or different penaeid shrimp species, as well as other less susceptible crustacean hosts (Waikhom et al. 2006). In contrast, passaging of the WSSV PmTN4RU *P. monodon* strain and the LvAP11RU *L. vannamei* strain examined here in *P. monodon*, *L. vannamei* and *F. indicus* followed by multiple passages in the same species had no affect on their VNTR makeup. This suggests strongly that changes in these VNTRs evolve more gradually. Indeed, while this study was being undertaken, another assessing ORF94 VNTR stability during sequential and cross-species passages of WSSV strains in *P. vannamei* as well as a *Macrobrachium* sp. and a *Cherax* sp. reported findings comparable to those reported here (Gudkovs et al. 2014). The reasons for the VNTR instability reported by Waikhom et al.
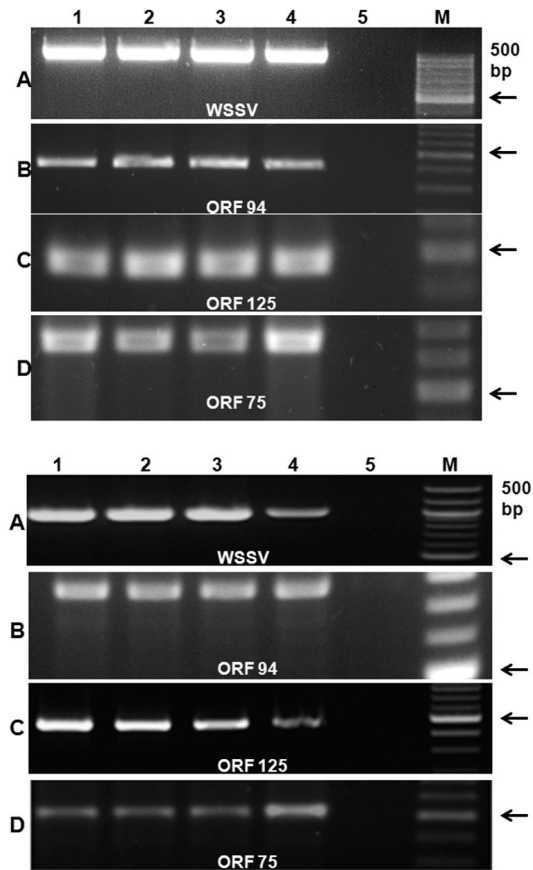
Fig. 3. Agarose gel images of DNA products amplified using PCR primers (A) used in the 1-step PCR to diagnose white spot syndrome virus (WSSV) infection or targeted to the variable-number tandem repeat (VNTR) regions (B) ORF94, (C) ORF125 and (D) ORF75 for different passages in *Penaeus monodon* using the WSSV challenge strains PmTN4RU (top panel) and LvAP11RU (bottom panel). WSSV passage numbers 3, 4, 5 and 6 (Lanes 1 to 4, respectively); negative control (Lane 5); M: 100 bp DNA ladder, arrows: 500 bp

(2006) remain unclear. However, it is possible that specific VNTR types were selected rapidly due to the nature of the WSSV strains examined, the challenge inoculums containing more than a single WSSV strain, or the host species challenged possessing subclinical WSSV infections that were detected preferentially post-challenge due to their low susceptibility to, or the low infectivity of, the challenge inoculums examined. While it is clear that larger numbers of WSSV genotypes and more sophisticated analyses will be needed to determine how and why different VNTR types arise, their relative stability in the WSSV strains examined here and by Gudkovs et al. (2014) support their continued use as epidemiological markers for investigating WSSV disease transmission and spread.

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