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Short Communication

Analysis of repeated compound units in ORF94 of white spot syndrome virus isolated from *Litopenaeus vannamei* from outbreak and non-outbreak shrimp farms in Sonora, Mexico

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ABSTRACT. White spot syndrome virus (WSSV) is the viral pathogen with the most negative impact on shrimp farming. In Sonora, *Litopenaeus vannamei* culture has decreased 50% during 2010-2012 due to WSSV outbreaks. The ORF94 has proven to be most useful for the analysis of WSSV variability. Several studies have suggested a correlation between its Repeat Units (RUs) and WSSV virulence as follows: the fewer RUs (<9) the higher mortality rate. In order to support this, we analyzed shrimps from eight farm periods and identified the WSSV-variety present in each one. In outbreaks, the presence of <8 RUs with a dominance of 3 RUs was notable in the last four years. Although it is still not clear how host-virus interactions and pond´s environment affect the transition of the infection just from the presence of the virus in shrimps to an outbreak, these results are a step forward in understanding the pond status and ways of predicting the likelihood of a WSSV infection becoming an outbreak.

Keywords: *Litopenaeus vannamei*, shrimp farming, WSSV, ORF94, repeat unit, outbreak, Mexico.

Análisis de las unidades repetidas compuestas en el ORF94 del virus del síndrome de la mancha blanca, aislado de *Litopenaeus vannamei* en granjas camaroneras con brotes y sin brotes en Sonora, México

RESUMEN. El virus del síndrome de la mancha blanca (VSMB) es un patógeno viral que impacta negativamente la producción de camarón de cultivo. En Sonora, el cultivo de camarón *Litopenaeus vannamei* disminuyó un 50% durante el período 2010-2012 debido a las mortalidades ocasionadas por la presencia del VSMB. El ORF94 es una región minisatélite muy usada para determinar la variabilidad genómica del virus. Varios estudios han sugerido una correlación entre sus unidades repetidas (UR) y la virulencia de VSMB: a menor UR (<9) mayor frecuencia de mortalidad. Para soportar esta hipótesis, se analizaron camarones de ocho ciclos de cultivo y se identificaron variantes del VSMB presente en cada uno de los ciclos. En ciclos con mortalidades masivas se notó la presencia de UR <8 con una dominancia de 3 UR en los últimos cuatro años. Aunque no está muy claro, la interacción VSMB-hospedero y como afectan los parámetros ambientales de las granjas en la transición de la sola presencia del VSMB a causar mortalidades masivas, estos resultados son solo un avance en el entendimiento de como una infección por el virus puede ocasionar un brote de la enfermedad con mortalidades masivas.

Palabras clave: *Litopenaeus vannamei*, camaronicultura, WSSV, ORF94, unidad repetida, brote, México.

Presently, the white spot syndrome virus (WSSV) infects a wide variety of families of marine brackish and freshwater crustaceans (Molina-Garza *et al*., 2008). Worldwide, is the viral pathogen with the most negative impact on shrimp farming, causing mass mortality resulting in large economic losses. In Sonora, Mexico, *Litopenaeus vannamei* farming, during the seasons/ periods of 2006-2009, had reached an annual average

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production of 82,000 ton. However, the production has decreased 50% during the period 2010-2012 (Galaviz & Molina, 2014) due to outbreaks of WSSV.

WSSV has a circular double-stranded DNA genome with a length of ~300 kb (Vlak *et al*., 2005), encoding 184 open reading frames (ORF), of which the majority have an unknown function. Only 11 ORFs translations have similarity to annotated proteins in public databases. The products of these genes are related to biological processes such as nucleotide metabolism, DNA replication and protein modification (Van Hulten *et al*., 2001). Except for some variable *loci*, the four WSSV genomes deposited in GenBank (WSSV-Taiwan accession AF440570, WSSV-China accession AF332093, WSSV-Thailand accession AF369029, WSSV-Korea accession JX515788) share a high degree of similarity (>99%) (Chai *et al*., 2013).The difference between genome sizes among the four isolates are principally due to a deletion of ORFs 23/24 and 14/15 and the presence of variable numbers of tandem repeats (VNTR) in ORFs 75, 94 and 125. These variables *loci* have been considered as important molecular markers for WSSV genotyping. The single nucleotide mutations, including deletions, insertions, and single nucleotide polymorphisms (SNP) have been suggested as markers for genetic diversity studies of WSSV (John *et al*., 2010; Hoa *et al.*, 2011; González-Galaviz *et al*., 2013).

Within the 3 minisatellites mentioned above, ORF94 is located between two subunits of ribonucleotide reductase (rr1 and rr2) and has proven to be most useful for analysis of WSSV variability. This region shows more variation, with a wide range of compound repeat units (RUs) between the WSSV isolates originating from farming and wild hosts (Wongteerasupaya *et al*., 2003; Dieu *et al*., 2004; Musthaq *et al*., 2006; Pradeep *et al*., 2008; John *et al*., 2010; González-Galaviz *et al*., 2013). Several studies have suggested a correlation between the RUs of ORF94 and WSSV virulence as follows: the fewer RUs (<9) the higher mortality rate in shrimps (Waikhom *et al*., 2006; Pradeep *et al*., 2008; Hoa *et al*., 2012). However, there is little evidence in the analysis of samples from non-outbreak ponds (Wongteerasupaya *et al*., 2003; Hoa *et al*., 2005; Musthaq *et al*., 2006). Thus, the aim of this study was to characterize WSSV with ORF94, from infected tissue stored from previous farming seasons (outbreaks and non-outbreaks) and to correlate them with mortalities that have occurred in each period.

We analyzed 428 *Litopenaeus vannamei* organisms infected with WSSV, that were collected during the periods of 2005-2013 (except 2007). A total of 66 shrimp samples were obtained during the non-outbreak periods of 2006-2009, whereas 362 samples were obtained during the outbreak period of 2005, 2010 to 2013. From each 30-50 mg shrimp tissue (pleopods) DNA was extracted using Genomic DNA PureLink™ Kits (Invitrogen, CA), according to the manufacturer's instructions. ORF94 analysis was performed using PCR reactions by adding 10 ng of DNA and 30 pmol of primers ORF94-F (5'-TCTCGAACTGGAGACGG TGAC-3') and ORF94-R (5'-AGGAGCTCATGT GTAATTCACTG-3') in reaction volume of 50 µL according to the incubation protocol described by Muller *et al*. (2010). The presence of VNTR were calculated as [amplicon size - (171+12)]/54 (Pradeep *et al*., 2008; Muller *et al*., 2010).

The samples were grouped in outbreak and non-outbreak periods, and then the frequency of RUs in each group was calculated in percentage.

From the 428 samples analyzed, amplicons ranging from 217 to 900 bp belonging to the ORF94 region were amplified. VNTR analysis of ORF94 showed 11 variants with a range of 1-13 RUs. No isolate presented amplicons with 11 and 12 tandem repeats (Table 1). We observed a higher prevalence of genotypes with a low number of RUs in the outbreak periods than in the non-outbreak periods (Fig. 1). It is notable the prevalence of 3 RU and 5 RU in the last outbreak periods, in fact, any of these genotypes was found in the non-outbreak events. On the other hand, for non-outbreak the 8 RU, 9 RU was the dominant, followed by 7 RU, 10 RU and 13 RU with similar frequency.

Previous studies related to ORF94 from WSSV have demonstrated the variability of this genomic region, suggesting that genotypes with fewer RUs in this region are associated with virulent disease. A study in Thailand revealed the presence of different genotypes with a range of 6-20RUs, where 8 RUs was the most frequent (Wongteerasupaya *et al*., 2003). In two ORF94 studies in Vietnam, the number of RUs varied

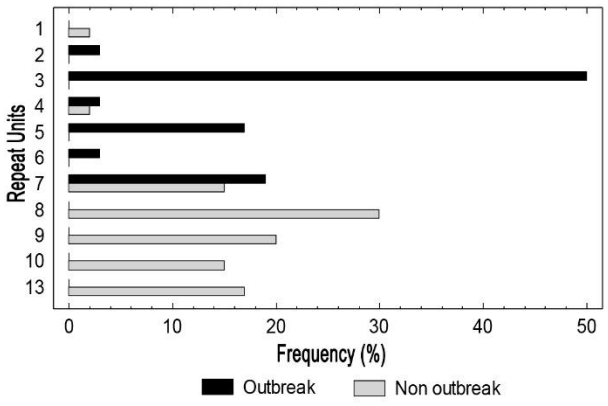

Figure 1. Frequency of white spot syndrome virus genotypes from outbreak and non-outbreak periods in shrimp farms (Sonora, Mexico).

Table 1. Date, origin, number of white spot syndrome virus infected shrimp, and number of tandem repeat units found for ORF94 region.

Year	*Location*	*Number of shrimp*	*RUs*	*Status of farming cycle*
2005	Tobari	10	6	Outbreak
2005	Melagos	10	6	Outbreak
2005	Melagos	10	4	Outbreak
2006	Santa Barbara	10	9	Non-outbreak
2006	Siari	3	9	Non-outbreak
2006	Riito	10	8	Non-outbreak
2006	Siari	10	8	Non-outbreak
2008	Santa Barbara	2	13	Non-outbreak
2008	Aquiropo	10	10	Non-outbreak
2008	Riito	10	7	Non-outbreak
2008	Atanasia	1	4	Non-outbreak
2008	Tastiota	1	1	Non-outbreak
2009	Agiabampo	1	13	Non-outbreak
2009	Santa Barbara	8	13	Non-outbreak
2010	Riito	10	7	Outbreak
2010	Santa Barbara	20	7	Outbreak
2010	Riito	10	6	Outbreak
2010	Atanasia	10	3	Outbreak
2010	Melagos	10	3	Outbreak
2010	Siari	10	3	Outbreak
2010	Tobari	10	3	Outbreak
2010	Cardonal	10	2	Outbreak
2011	Atanasia	30	7	Outbreak
2011	Melagos	10	7	Outbreak
2011	Cardonal	50	3	Outbreak
2011	Atanasia	20	3	Outbreak
2011	Riito	40	3	Outbreak
2011	Aquiropo	20	3	Outbreak
2011	Kino	10	3	Outbreak
2012	Tastiota	30	5	Outbreak
2012	Siari	10	5	Outbreak
2012	Tobari	10	5	Outbreak
2012	Cardonal	10	5	Outbreak
2013	Tobari	2	3	Outbreak

from 7-17 RUs (Dieu *et al*., 2004) and 4-17 RUs (Dieu *et al*., 2010), the former was performed using samples from eight localities from central and southern regions, while the latter was performed using samples from nine localities in the northern, central and southern part of the country. In India¸ the genomic analysis included 513 samples from 13 localities, showing genotypes with a range of 2-16RUs, where genotypes with <8 RUs were predominant during the outbreak periods and genotypes with >9 RUs were more frequent in the non-outbreak periods (Pradeep *et al*., 2008). Hoa *et al*. (2012) showed 18 different genotypes with a range of 3 to 20 RUs, where the 5, 6 and 7 RUs dominant in outbreak ponds. Controversially, samples with 7 RUs were found in both outbreak and non-outbreak periods (Pradeep *et al*., 2008, Hoa *et al*., 2012). Similar to our results, however genotypes with 7 RUs had a higher prevalence in outbreak that non-outbreak. In general, our results show that there is a pattern of the repeat units with the dominance of fewer RUs during outbreak seasons, as suggested by the higher frequency of genotypes <8 RUs during the outbreak periods (2005, 2010-2013). In the 2006-2009 periods there were no records of WSSV outbreaks, however the locations of Tastiota and Atanasia in 2008 experienced mortality events by WSSV where genotypes with 1 RU and 4 RUs variants were found. Nevertheless, that was not considered an outbreak cycle because the mortality rate was not significant (COSAES, 2008) and the frequency of these two genotypes was only 1.5%.

Variability in VNTR RU number has been found useful as an application to study genotypic variation

and virulence in pathogenic bacteria *Haemophilus influenzae*, *Neisseria* spp., *Moxarella catarrhalis* and *Yersinia pestis*, (Peak *et al*., 1996; Klevytska *et al*., 2001). There are also viral epidemiology reports (Perdue *et al*., 1997). Hoa *et al*. (2012) suggesting a strong link between the ORF94 flanking regions and WSSV virulence. Since the ORF94 is located between the rr1 and rr2, this enzyme catalysis the formation of deoxyribonucleotide precursors, which are involved in DNA replication process. The ribonucleotide reductase has been implicated in the virulence of other viruses such as herpes and poxviruses (Lembo & Brune, 2009; Gammon *et al*., 2010). In conclusion, this study provides data from multiple farming periods (8 years) where different mortalities rates were reported, contributing the comprehension of viral activity. It is common to see ponds where despite having shrimps with no visible signs or symptoms of the disease are carriers of the WSSV. It is still not clear yet, how host-virus interactions and the pond environment affect the transition of the infection from just the presence of the virus in the population to an outbreak. Nevertheless, these results are a step forward in understanding the pond status and ways of predicting the like hood of a WSSV infection would become an outbreak. Further studies in susceptibility are needed, using infection challenges assays together with comparative genomics analysis of WSSV strains where the causes for the outbreak transitions can be understood.

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