In-Vivo Comparative Virulence of Different White Spot Syndrome Virus Isolates In *Penaeus Vannamei* And Whole Genome Comparison Analysis

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Research Article

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Abstract

White Spot Syndrome Virus (WSSV) infects several economically important aquacultural species, causing significant losses to the industry. This virus belongs to the *Nimaviridae* family, and has a dsDNA genome ranging from 257 to 309 kb (more than 20 isolate genomes fully sequenced and published to date). Multiple routes of infection could be the cause of the high virulence and mortality rates detected in shrimp species. In particular, *Penaeus vannamei*, differences in isolate virulence have been observed, along with controversy over whether deletions or insertions are associated with gain or loss of virulence.

The pathogenicity of three isolates from three locations in Mexico (two from Sinaloa: 'CIAD', and 'Angostura', and one from Sonora: 'Sonora') was evaluated *in vivo* in white shrimp (*P. vannamei*) infection assays. Differences were observed in the mortality rate of shrimp among the three isolates, with the Sonora isolate being the most virulent. Subsequently, the complete WSSV genomes were sequenced in depth from the tissues of infected shrimp, and assembled in reference to the genome of isolate CN01 (KT995472), identifying genome sizes for Angostura and Sonora of 289,350 bp and 288,995 bp, respectively.

Where three deletion zones were identified compared to CN01 comprising 15 genes, including three envelope proteins VP41A, VP52A and VP41B, one non-structural protein ICP35 and 11 others encoding proteins whose function is currently unknown. In addition, five genes (wsv129, wsv178, wsv204, wsv249 and wsv497) show a modified number of repeat motifs. The main implications and possible effects on viral infection of these modifications are discussed.

Introduction

The white spot syndrome virus (WSSV) is a highly contagious pathogen. With a replication cycle of approximately 20 hours at 25 °C, it is capable of causing a 100% mortality rate in white shrimp (*Penaeus vannamei*) within a period of 3 to 10 days [1, 2], generating massive economic losses in the industry [3]. The first massive losses due to WSSV occurred in China and Taiwan in the years 1991–1992 especially in Kuruma shrimp (*Marsopeneaus japonicus*), subsequently the massive mortalities due to WSSV spread throughout the rest of Asia and the world [4].

WSSV appeared in aquaculture farms in Mexico in 1999 [5] and since then, it has been persistent with regional epidemics [6, 7]. Transmission of the virus can be horizontal, through cannibalism or by ingesting water containing WSSV particles, by a vertical transovarial route [8], with ballast water from ships, birds acting as vectors, and live or frozen imported products [9].

WSSV belong to the *Nimaviridae* family, genus Whispovirus (ICTVdb.org) and recently other Nimaviruses were identified in crabs [10] which they can also infect in aquaculture settings [11]. WSSV is enveloped, has an elliptical shape with an approximate size of 250–380 nm in length by 80–120 nm in width [12] and has an filamentous appendage. Its genome is a double-stranded circular DNA [13]. The largest published genome (CN01 with 309,286 kb) was isolated from China in 1994 (KT995472.1) [14]. To date,

> 20 WSSV genomes have been published (<https://www.ncbi.nlm.nih.gov/>), with the latest isolate MG432482 from Mexico [7](257,675 kb).

Two opposite hypotheses have been postulated to explain differences in genome size: 1) the reduction of the genome increases virulence [15], and 2) the reduction of the genome reduces virulence [16]. These discrepancies cannot not be fully resolved, because of differences in experimental designs, host species and molecular techniques used. To date a direct cause-effect between genome size and virulence has not been identified [17]. It has been hypothesized that the pathogenicity depends on the presence of “factors” associated with virulence, which are absent or have lost function in less virulent isolates [14]. Deletions, mutations in open reading frames (ORFs) are believed to increase virulence rates in their hosts [8], such as ORFs 75, 94, and 125 involved in receptor recognition which potentially increases tropism. WSSV gene characterization identified some gene functions potentially involved in pathogenicity and pathogen-host interaction [18–23]. However, these analysis in *M. japonicus* did not include *in vivo* comparison of virus strains, viral load, and genome analyses altogether.

We aimed to compare experimentally (*in vivo*) the virulence of three WSSV isolates from shrimp farms in México isolated during outbreaks in 2008 and 2013, and to analyze specific genomic regions that could be associated with higher virulence by comparing the isolate genomes with each other and with available sequences from public databases.

Materials And Methods

Origin of the isolates

The bioassay was carried with three isolates from different geographical origins and epidemics. One isolate was obtained by Dr. Jorge de la Rosa Vélez[†] during an outbreak that occurred in Sonora in 2008, called the Sonora isolate. The second isolate was provided by Dr. Leobardo Montoya from the Food and Development Research Center (CIAD) located in Mazatlán Sinaloa, from an outbreak in Sonora in 2013 designated CIAD. The third isolate was provided by MC Joel Lizárraga Valdez from the company Aquapacific S.A. de C.V. obtained from an outbreak in the Angostura, Sinaloa area in 2013.

Preparation of WSSV viral inocula

Inocula of each isolate were prepared from 25 mg of tissue of four cryopreserved dead shrimps, placed in a 1.5 mL Eppendorf tube, supplemented with 500 µL of TN buffer (Tris/HCl 10mM. pH 7.4, NaCl 3M, passed through 0.2 µm microfilter) and homogenized with a pistil, followed by another 400 µL of TN buffer [24]. The samples were centrifuged at 1000 × for 5 min, 3000 × for 5 min, 10,000 × for 5 min and 14,000 × for 15 min. The supernatant was recovered with a 3 mL syringe and filtered again with a 0.45 µm microfilter. The filtrate was placed in a new 1.5 mL eppendorf tube and was kept at -4°C until use, for less than 2 hours. This procedure was repeated every time shrimp were infected.

Verification of the viability of isolates

White shrimps from the ACUAMOS aquaculture farm (located in San Felipe, Mexicali B.C) were infected with the viral inoculum. Juveniles were maintained at $28 \pm 1^\circ\text{C}$, a salinity of 34 ppt, and constant aeration until reaching 15.7 ± 2 g and 14 ± 0.6 cm. For each isolation ($n = 5$), two replicates were used). 20 μL of inoculum was injected into the fifth abdominal segment of the shrimp. Moribund shrimps were cryopreserved in liquid nitrogen and stored at -80°C . The virus replication was verified by end-point PCR, with a set of primer targeting the structural protein VP664 [25].

Challenge experiment to compare the isolates virulence

White shrimps from the Mahar aquaculture farm (Pichilingue, La Paz, Baja California Sur, Mexico) weighing 4.4 ± 0.9 g and 8.4 ± 0.6 g, were infected with the different viral inocula. A PCR test was done to corroborate animals were wssv-free [2]. For each isolate challenges of shrimps ($n = 7$) were performed in triplicate and placed in 40 L tanks, with a temperature of $28 \pm 1^\circ\text{C}$, a salinity of 34 ppt, constant aeration, and a biological filter (activated carbon). Three other replicas were used for the non-infected shrimps.

DNA extraction to quantify viral load

Once bioassays were completed, DNA was extracted from all samples ($N = 63$). 100 mg of tissue was taken from the 5th abdominal segment and DNA was extracted using the Phenol: Chloroform: Isoamyl alcohol [25:24:1] standard protocol [26]. DNA concentration (260 nm) and quality (ratio 260/280) was verified with a Nanodrop LITE (Thermo brand). Additionally, the DNA quality was assessed in a 1% agarose gel electrophoresis in 40 mL of 1X TAE (0.04M Tris-acetate, 1mM EDTA) and visualized by ethidium bromide [10 mg/mL].

Viral load quantification

Viral load was determined by real-time PCR (Q-PCR) absolute quantification with standard curve (1:10 dilution factor, with dilutions 10^0 to 10^7) according to Durand and Lightner, 2002 (OIE procedure). The 64 bp fragment of *vp664* was inserted on TOPO TA plasmid (Invitrogen, United States) as previously described [2].

DNA extraction and purification for sequencing of the WSSV genome

For deep sequencing of the whole genome of the viruses from the bioassay, the DNA of 4 shrimps tested positive for WSSV by Q-PCR and of two free-wssv were extracted and purified. The extraction was done with the 5 Prime Archive Pure DNA blood kit (# 2900258 5prime GmbH, Germany) with Econo Spin columns (Epoch # 1910 – 250, Epoch Life Science Inc, United States). The column was washed with a Type 1 wash buffer (GE28-9031-70, Gelifesciences, United Kingdom). DNA was eluted with 50 μL of water.

Preparation of samples for deep sequencing

Samples were stabilized with DNASTable Plus® (Biomatrica, UK) following the manufacturer's instructions and were sent for sequencing by regular mail service at the Institute of Aquaculture

(University of Stirling in Scotland, UK) with an Illumina MiSeq, V3 75 bp, Paired-End.

Deep sequencing data cleaning and mapping

The quality of the sequencing raw reads was checked with FastQC [27]. Quality scores below 30 along with Illumina adapters were removed with Trimmomatic v.0.39 [28]. The cleaned reads were mapped against the reference genome China CN GCF_000848075.2 (KT995472.1), using the BWA aligner v.0.7.15 [29] and SAMtools/BCFtools v1.11 (<http://www.htslib.org>).

Statistical analysis

The survival probability was evaluated by the Kaplan-Meier approach, using the Greenwood formula to estimate the variance, to stabilize the variance, the confidence intervals were calculated by the log (-log) transformation. The comparison between the curves was made with the long-rank test, using the survival package version 3.2-7 for survival analysis. An adjustment of generalized additive models was also carried out, by means of the local regression method, to evaluate the correlation of hours post inoculation (HPI) and viral load (VL) with a confidence interval of 0.95, using the Gam package in R studio version 1.3.959.

Genome alignment and phylogenetic analysis

The alignment of the isolated genomes from Angostura and Sonora was performed with MAFFT v7,017 [30] with score matrix of 200PAM/K = 2. GSP open penalty of 1.53 and offset value of 0.123 and automated sequence addressing, together with the sequences KR083866, MG432478, MG432474, AF440570, AF332093, AF369029, KT995470, KY827813, KT995471, JX515788, KT995472, KU216744, MG432479, MG432475, MG432477, MG432482, MG432476, MG432481 and MG432480, extracted from GENBANK and using the genome GCF_000848075.2 corresponding to the sequence CN01_KT995472 isolated from China as a reference since it is the longest WSSV genome Sequence. Sonora and Angostura isolates were also included: ERR5659803, ERR5659804 respectively). The phylogenetic tree was inferred from this alignment with 1,000 bootstrap repeats using RAxML 7.2.8 method, GTR GAMMA nucleotide model, Bootstrapping algorithm, genetic distance in annotated as a scale bar.

A multiple genome comparison with circular architecture was made with BLAST with the BLAST Ring Image Generator (BRIG) version 0.95 [31], comparing the all the above listed genomes, using the CN01 isolate as a reference. A multiple genomic alignment to observe genomic conservation and rearrangements was performed with MAUVE version 2.4.0. Repeated motifs (RM) in specific genes were detected with The MEME Suite 5.3.3 (Multiple EM for Motif Elicitation) [32] on line version <https://meme-suite.org/meme/tools/meme>. A motif is an approximate sequence pattern that occurs repeatedly in a group of related sequences that usually mediate a common function. Motif function was inferred by comparing motif to motifs databases with Tomtom motif comparison Tool version 5.3.3 [33] available in the same link.

Results

Challenge experiment to compare the virulence of WSSV isolates

Shrimp infection with the CIAD isolate produced a survival rate of 19% 10 days after challenge, with a first drop in survival at 29 h 40 min post infection (PI) and a final drop at 145 h 50 min PI. On the other hand, the infections carried out with the Angostura and Sonora isolates produced a mortality rate of 100% at 168h 40min PI, with a first drop in survival at 35 HPI and 27 HPI respectively (Fig. 1A).

Comparison of the long-range analysis for the three isolates (Chisq 0.4, 2 degrees of freedom or d, p 0.81) and the Kaplan-Meier approach indicates a 50% survival for the CIAD isolate at 131 HPI, Angostura at 150 HPI and Sonora at 99 HPI (Fig. 1A).

Viral load quantification of the three treatments

Viral loads or viral genomes / mg (VL) were compared between treatments by vp664 qPCR on all infected shrimp (Fig. 1B). VL ranged from 8.48×10^7 (CIAD), 1.26×10^8 (Sonora) to 1.91×10^8 (Angostura). The variance analysis indicates no correlation between HPI and VL for all isolates (F 0.002459, p 0.001, $R^2 < 0.29$).

Comparison of WSSV genomes and identification of genes with variations

Due to concentration and sample quality problems, it was not possible to sequence the CIAD isolate. However, we obtained a total of 1,717,195 and 1,657,796 reads with a length of 76 bp that were assembled to 155,993 and 105,993 reads for the Angostura and Sonora genomes respectively (KT995472.1). The genome size of the Angostura and Sonora isolates is 289,350 bp and 288,995 bp, respectively (S1), with an overall GC percentage of 41.1%. The Sonora isolate has a slightly shorter genome (355 bp) than the Angostura isolate. The data for this study have been deposited in the European Nucleotide Archive (ENA) at EMBL-EBI with the accession number PRJEB44096 (<https://www.ebi.ac.uk/ena/browser/view/PRJEB44096>).

Nucleotide alignment with identity % among all genomes ranges from 78.3 % (LG vs GVE05 isolates) to 99.2 % (ACF vs DVI isolates). There is about 99.7% similarity between the Sonora and Angostura isolates (S1, S4). Phylogenetic analysis indicates that Sonora and Angostura isolates are closely related to the Mex2008 isolate and have a 96.4% similarity to isolate CN02 from China. Another clade groups the isolate from Taiwan, AC1 and GVE. The most recent branch contains isolates JP, ACF2, DVI, LG, ACF4, LC1 and LC10.

The circular alignment of all genomes with BRIG clearly identified 3 deletion areas with specific genes (shown in red) when compared the CN01 isolate (Figs. 3A, B) in both Angostura and Sonora genomes (Table 1). The genes *wsv463a*, *wsv463b*, *wsv463c*, *wsv463d* are absent in both genomes when compared to CN01 (Fig. 3A and B) and genes *wsv489*, *wsv490*, *wsv492*, *wsv493* and *wsv495* had a < 50% similarity among isolates. The only exception is the Taiwan isolate which has the complete sequences for the

mentioned genes, followed by the isolates from China (Mj) and Eg3 containing a deletion of *wsv495* (Fig. 3A). The genes that code for proteins VP41B, VP52A and VP41A presented deletions or a similarity of less than 50% with respect to CN01, in all the Mexican genomes, with the exception of Mex2008 (Fig. 3B). Particularly these genes coding are absent in the genome of isolate CN03, while *wsv234* has a low similarity (Fig. 3A). On the other hand, CN04 presented only the deletions of VP52A, VP41A and *wsv234*.

Table 1

Deletions identified in the isolates from Angostura and Sonora when compared to CN01 (KT995472). *wsv* nomenclature corresponds to isolate from China_MJ (or CN) (AF332093) used in this work for annotations, and *wssv* corresponds to equivalent genes in Taiwan_Pm (AF440570). * Protein name not defined. In parentheses is the name according to Taiwan based studies.

No.	WSSV gene		Protein name	Sub-category function	Reference
1	<i>wsv234</i>		*	Hypothetical Protein	
2	<i>wsv237</i>	<i>wssv293</i>	VP41A	Envelope protein	Huang <i>et al.</i> (2013)
3	<i>wsv238</i>	<i>wssv294</i>	VP52A (VP51A)		Chang <i>et al.</i> (2008)
4	<i>wsv242</i>	<i>wssv298</i>	VP41B		Zuo <i>et al.</i> (2011)
5	<i>wsv244</i>		*	Hypothetical Protein	
6	<i>wsv247</i>		*		
7	<i>wsv463 a</i>		*		
8	<i>wsv463 b</i>		*		
9	<i>wsv463 c</i>		*		
10	<i>wsv463 d</i>		*		
11	<i>wsv489</i>		*		
12	<i>wsv490</i>		*		
13	<i>wsv492</i>		*		
14	<i>wsv493</i>	<i>wssv019</i>	ICP35 (VP35)	Nonstructural Protein	Kang <i>et al.</i> (2013)
15	<i>wsv495</i>		*	Hypothetical Protein	

An additional multiple MAUVE alignment between Angostura and Sonora genomes in reference to isolate CN01 corroborates the deletion of *wsv247*, *wsv244*, VP41B, VP52A, VP41A and *wsv234* in both Mexican isolates in reference to the genome of isolate CN01 (Fig. 4; S2, orange region in CN01 at 140kb). This analysis showed the deletions of *wsv463a*, *b*, *c* and *d* in the 180 kb region of the Angostura genome (S2, red region in Angostura at 180 kb), and deletions corresponding to *wsv489*, *wsv490*, *wsv492*, *wsv493* and *wsv495* in a terminal region close to the 3-prime end of the genome (S2, yellow region in Angostura at 260 kb).

A difference of < 30% at nucleotide level was observed in *wsv129*, *wsv178*, *wsv204*, *wsv249* and *wsv497* genes between the genomes of the Sonora and Angostura isolates (S3). The Sonora isolate *wsv129* has lost several Repeated Motifs (RM) like RM1 and RM2 but gained RM3 (S3, A), *wsv178* has lost five RM1 (S3, B), *wsv204* gene has acquired one RM2 (S3, C), while *wsv249* has lost one RM1 in the Sonora isolate (S3, D, while *wsv497* is similar in both isolates, with 1 more RM2 compared to CN01, (S3, E). RM is a repeated motif, and the number makes reference to the motif number for each case.

Discussion

Three WSSV isolates from Mexican shrimp farms were characterized by bioassay and through whole genome sequencing. WSSV isolates tested in bioassays generally induce the following clinical signs in infected white shrimp; lethargy, decreased food intake, discoloration of the hepatopancreas, cuticular loosening and red uropods [12]. Apart from these general clinical signs Shrimp infected with the CIAD isolate in the challenge bioassay showed 19% survival at 10 days post infection similar to the percentage obtained by Sekar, with 100% mortality until day 20 [34]. In contrast the, isolates from Angostura and Sonora induced 100% mortality between 3- and 10-days post-infection [35, 2].

The Angostura isolate produced the highest viral load of the three isolates (Fig. 1B). The differences between isolates however were in the range of published data reporting the viral load of WSSV in three *Penaeus* species (*P. vannamei*, *P. stylirostris* and *P. monodon*) obtained by real-time PCR in the range of $2 \times 10^4 - 2 \times 10^9$ WSSV copies μg^{-1} of total DNA, in infected shrimp [25]. A review to evaluate the risk associated for the shrimp trade, data on WSSV concentrations obtained from tissues of different species, including *P. vannamei*, indicated high viral load discharges at the beginning of mortality, with concentrations of $1 \times 10^9 - 1 \times 10^{10}$ [36].

The CIAD isolate presented a mortality risk percentage between 95% and 19% with a probability of 50% at 131 h and an overall survival rate of 19%. Isolate Angostura presented a constant 65% mortality risk percentage between 50 and 150 h, for a 50% survival at 150 h (Fig. 1A). For isolate Sonora, three drops in survival were observed, with risks of death between 64% and 43% from 50 to 150 h, and a probability of survival of 50% at 99 h. This is a possible sign of a faster progressing replication and therefore increased virulence of the isolate Sonora compared to isolate Angostura. Both isolates Angostura and Sonora induced 100% mortality observed at 168 HPI. Therefore, we tentatively classified the isolates as low virulence (CIAD), moderate virulence (Angostura) and high virulence (Sonora) from the mean post-infection lethal time according to [37].

The genomes of the isolates from Angostura and Sonora, presented a length within the estimated range for Mexican isolates from localities between Sinaloa and Nayarit (257,675 – 290,879 bp) [7]. To assess genomic differences of the isolates we were able to identify specific sites with differences to isolates from foreign and domestic outbreaks (Fig. 3A, B). Deletions in the WSSV genome are considered as enhancements of the adaptive response by WSSV as it is reasoned that deleted regions, do not encode proteins vital for WSSV pathogenicity or virulence [38]. The differences are possibly caused by

recombination events mediated by transposons causing insertions or deletions [39]. An inversion is apparent among the 2008 Sonora and 2013 Angostura isolate as spotted in the MAUVE alignment of motives (Fig S2).

Since the isolate with the shortest sequence was the most virulent (Sonora), we analyzed the deletions. In our comparative analysis between the genome of the isolates of the present study, with sequences of international and national isolates, a group of deleted genes stands out: *wsv237*(VP41A) [40], *wsv238*(VP52A-VP51A) [41]. *wsv242*(VP41B) [42] and *wsv493*(ICP35-VP35) [43].

The VP41A envelope protein is known to interact with VP26, VP56 and VP28 envelope proteins [40, 44] and with the viral envelope protein VP51A (VP52A for AF332093) [45]. It has been suggested that VP26 is anchored to the envelope with its N-terminal hydrophobic region, as a C-terminal region binds to the nucleocapsid [46], while VP51A is a type II transmembrane protein, with a transmembrane domain highly hydrophobic at its N-terminus and an exposed C-terminus on the virion surface [47]. On the other hand, VP41A has only one transmembrane region at its C-terminal end, and this envelope protein is part of a group of 11 proteins that interacts with the chitin-binding protein of *P. monodon* (PmCBP) [16] which are known to participate in shrimp immune response [48]. It also interacts with preitrophin-like protein in *L. vannamei* (LvPT). LvPT is involved in the formation of the peritrophic membrane [49] and contains a chitin-binding domain facilitating binding of WSSV to the intestinal epithelium. It has been shown to promote infection, as silencing of LvPT decreased the viral load in pleopods, stomach and intestines in previously WSSV infected shrimp [50].

Therefore, the absence of the envelope proteins VP41A and VP51A could modify the usual conformation of the protein complex composed of VP26, VP28 and VP56, as well as the interactions of VP26 with VP51A reducing viral recognition by chitin-binding proteins such as PmCBP and LvPT. The observed deletions in Sonora and Angostura isolates could contribute to changes in the WSSV viral envelope and enhance virulence causing high mortality.

VP38 acts as a repressor of *Penaeus japonicus* caspase (Pjcaspase) transcription in *M. japonicus*, while VP41B activates its expression, regulating the apoptosis mechanism used by shrimp to control viral load [42]. Elevated Pjcaspase gene expression of *M. japonicus* was observed in shrimp survival challenges while siRNA silencing of Pjcaspase increased post-infection viral load [51]. So, deletion of VP41B would not allow timely induction of apoptosis of infected cells allowing the virus to replicate without limitation and thus having a major impact on the viral load control mechanism employed by WSSV.

The Sonora isolate has noticeable modifications on *wsv129*, *wsv178*, *wsv204* and *wsv249* as gain or loss of specific sequences equivalent to Repeated Motifs (RM) that strongly suggest a role in the infection process. A high variation from 3 to 20 in the number of repetitive sequences in WSV178 has been related to virulence [52, 53]. In contrast a lower mean number of repeated sequences was observed in outbreaks, where WSSV genotypes with 5, 6 and 7 of a 54 bp RM were those that predominated in semi-intensive shrimp crops [54]. In our study, we identified 4 and 3 repetitions of a 54 bp RM in WSV178 in the Angostura and Sonora genomes respectively. Previous studies identified *wsv178* between *rr1* and

rr2, coding for enzymes that catalyze the formation of deoxyribonucleotide precursors, for the DNA replication process [55–57]. A study conducted in outbreaks which occurred in Sonora during 2005 and between 2010–2013, indicated a higher frequency of genotypes with less than 8 RMs in WSV178. In addition, during mortality events in Tastiota and Atanasia regions in 2008, WSSV genomes were observed which showed between 1 and 4 RM in WSV178. However, the mortality of these events was low at about 1.5% [58]. In outbreaks on farms in Culiacán Sinaloa in 2000, WSSV genomes with 4, 8, 9 and 12 repetitions in WSV178 were identified. WSSV genomes with 4 repetitions detected on a particular farm in Cruz Blanca, suggested the emergence of WSSV genomes with 4 repetitions in Mexico [59]. A study analyzing possible variations in WSV178, suggested that VNTRs in the different genotypes are stable during multiple infection cycles in crustaceans [60]. Therefore, our observation of RM distribution in the Angostura and Sonora isolates in combination with the reported observations on shrimp farms in Mexico could suggest that if the frequency of WSSV genomes with varying and in particular lower number of repetitions in WSV178 reaches statistically significant frequencies in a given population, WSSV outbreaks may develop [58].

A similarity difference < 30% was observed in *wsv204* between the Sonora and Angostura isolates in comparison to CN01, with Angostura being distinctly different from Sonora and CN01. The gene *wsv204* was identified in isolates CN01 and CN03, but not in CN02 who were respectively shown to be high, low and moderate virulent in bioassays by Li, et al (2016) [14].

We compared the most variable regions at the protein level from the sequence of isolate CN01 (KT995472.1) [61], identifying that *wsv129*, *wsv178* and *wsv497* could be encoding structure- and packaging-related proteins, and appear to be involved in enhancing WSSV virulence by promoting viral replication.

A difference in similarity < 30% in *wsv204* was also observed between Sonora and Angostura isolates compared to CN01, with Angostura being clearly different from Sonora and CN01. *wsv204* gene was identified in CN01 and CN03 isolates, but not in CN02, which respectively proved to be of high, low and moderate virulence in bioassays performed by Li, et al (2016)[14]. The function of *wsv204*, which contains RMs, is currently unknown, but its function could be related to transcription factors, as the RMs were similar to those found in the SOX gene member (*wsv129*), the EST domain transcription factor (*wsv178*), the tryptophan cluster factors (*wsv129*, *wsv204* and *wsv 249*), the homeodomain motif (*wsv 249*) or the paired box factor motif (*wsv497*).

In eukaryotic organisms, sequences similar to these are found in transcriptional regulatory sites or factors that bind to DNA and control the activity of other genes. They are involved in the developmental process, as modulators or architectural components [62], in genome accessibility, DNA shape, and interact with many transcriptional partners, in activation of transcriptional programs [63], in neighbor cell communication, in cellular secretion of abrogation [64], and in tryptophan regulation. Tryptophan has a vital role in serotonin (5-HT) synthesis that regulates homeostasis, stress and cannibalism among other

vital functions in shrimp [65]. Therefore, it is not entirely clear how wsv204 might be involved in regulatory processes that have an influence on WSSV replication success in host cells.

Conclusions

The comparison of the virulence of WSSV isolates Angostura and Sonora in *in vivo* bioassays in *Penaeus vannamei* resulted in similar survival but differences in time of mortality Sonora being more lethal (99 HPI) than Angostura (150 HPI). Viral load indicated no correlation between HPI, so virulence may be associated with a different factor. In comparison to the Chinese CN01 isolate 15 genes are deleted from the genomes of these isolates and they may contribute to changes in the WSSV viral envelope attachment function, however the deletion of proteins associated with chitin binding sites does not appear to be related directly with the increase of virulence since several genomes share this characteristic. The deletion of VP41B may be related to the apoptotic viral load control mechanism employed by WSSV. Specific repeats lost or acquired in *wsv129*, *wsv178*, *wsv204*, *wsv249* and *wsv497* might interact and/or regulate expression of other proteins that may enhance the virulence of the Sonora isolate. Additional molecular epidemiological and transcriptional studies are needed to corroborate these findings. Altogether the study identified several interesting leads on the importance of the presence and absence of nucleocapsids, envelope proteins and proteins involved in immune evasion. Due to the complex interactions and the current state of knowledge on the function and expression of WSSV proteins detailed follow up studies are required to clarify the role of the implicated proteins.

Declarations

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Figures

Figure 1

In vivo WSSV infection of *Penaeus vannamei*. A, survival of shrimp from Ecuadorian line exposed to isolates with different virulence. Kaplan-Meier approach, using the Greenwood formula to estimate the variance, to stabilize the variance, the confidence intervals were calculated by the log (-log) transformation (3 replicates for each treatment, n = 21). B, Fitting of generalized additive models, using the local regression method, to evaluate the correlation of HPI and VL with a confidence interval of 0.95.

Figure 2

Complete genome analysis of available WSSV sequences. The phylogenetic tree was inferred from this alignment with 1,000 bootstrap repeats using RAxML 7.2.8 method, GTR GAMMA I nucleotide model, Bootstrapping algorithm, obtaining similarity percentages that are indicated in the branches of the phylogenetic tree. Angostura and Sonora isolates are included (arrow). Sequences used for the alignment: KR083866, MG432478, MG432474, AF440570, AF332093, AF369029, KT995470, KY827813,

KT995471, JX515788, KT995472, KU216744, ERR5659803, ERR5659804, MG432479, MG432475, MG432477, MG432482, MG432476, MG432481, MG432480.

Figure 3

BLAST genome comparison of WSSV genomes. A, Blast between the international isolates available to date on the NCBI data base; Taiwan (AF440570), WSSV-MX08 (KU216744), South Korea (JX515788), CN02 (KT995470), China Mj (AF332093), EG3 (KR083866), CN03 (KT995471), CN04 (KY827813), Thailand. B, Blast between Mexican isolates GVE05 (MG432478), ACF2 (MG432475), ACF4 (MG432476),

LC1 (MG432481), LC10 (MG432480), DVI (MG432477), AC1 (MG432474), JP (MG432479) and LG (MG432482). The isolate from China (CN01) was used as the reference genome (A and B). In red the genes deleted in Angostura and Sonora genomes. The gradient of identity by samples with respect to the reference ranges between 100% and 50%; with blanks representing percentages less than 50% or deletion.

Figure 4

BLAST genome comparison between the Angostura and Sonora isolates against CN01. Blast analyses indicates depth of coverage of reads in yellow with a scale from 0 to 30x with respect to CN01-CDS, the

blue bars indicate a coverage greater than 30x and the red bars the coverage between 0 to 10x. Genes with deletions identified in Angostura and Sonora were noted in red. Genes with 30% difference are noted in green. BRIG version 0.95 (Alikhan, et al 2011). Sequences used for the alignment: ERR5659803, ERR5659804 and KT995472.

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