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COMMUNICATION

The Endemic Copepod *Calanus pacificus californicus* as a Potential Vector of White Spot Syndrome Virus

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Abstract

The susceptibility of the endemic copepod *Calanus pacificus californicus* to white spot syndrome virus (WSSV) was established by the temporal analysis of WSSV VP28 transcripts by quantitative real-time PCR (qRT-PCR). The copepods were collected from a shrimp pond located in Bahia de Kino Sonora, Mexico, and challenged per os with WSSV by a virus–phytoplankton adhesion route. Samples were collected at 0, 24, 48 and 84 h postinoculation (hpi). The VP28 transcripts were not detected at early stages (0 and 24 hpi); however, some transcript accumulation was observed at 48 hpi and gradually increased until 84 hpi. Thus, these results clearly show that the copepod *C. pacificus californicus* is susceptible to WSSV infection and that it may be a potential vector for the dispersal of WSSV. However, further studies are still needed to correlate the epidemiological outbreaks of WSSV with the presence of copepods in shrimp ponds.

Aquaculture is a valuable source of food, an especially important economic activity, and provides employment to a significant number of people in several countries around the world. One of the challenges in contemporary aquaculture is to produce organisms successfully at the lowest cost; however, the shrimp farming industry has been severely affected by the emergence of viral diseases, causing significant economic losses (Lightner 1996). The white spot syndrome virus (WSSV) is considered to be the most devastating and virulent viral agent threatening the penaeid shrimp culture industry (Moser et al. 2012), and under farming conditions, mortalities can reach 100% within 3–10 d after the onset of clinical signs (Chou et al. 1995). To date, more than 90 species of arthropods, including shrimp, lobsters, crayfish, and crabs, and a number of nonarthropod species as rotifers, polychaete worms, and mollusks, have been reported to be potential carriers or hosts of WSSV (Sánchez-Paz 2010).

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Furthermore, some of these organisms are commonly used as live feed in the culture of penaeid shrimp larvae (Reymond and Lagardère 1990). Thus, the identification of susceptible species to infection with WSSV should be considered an essential topic for the use of sanitary programs and management strategies to reduce the negative impact of this disease in shrimp farms. Copepods, rotifers, and *Artemia* may act as mechanical vectors for WSSV dispersion, but only few reports have demonstrated their susceptibility to this lethal virus (Zhang et al. 2006, 2008, 2010; Chang et al. 2011). Copepods are a diverse group of crustaceans, and over 12,000 marine species have been described. They constitute the biggest source of animal protein in the oceans and provide food for many fishery species (Olsen 2007). Some calanoid copepod species are regularly used as aquaculture feeds, and a number of species are highly abundant in the estuarine coastal waters surrounding shrimp grow-out ponds in Mexico, where they are an important part of the natural diet of penaeid shrimp species, at least in their postlarval and juvenile phases (Martínez-Córdova et al. 2011). Thus, the aim of this study was to investigate the susceptibility of the endemic copepod *Calanus pacificus californicus* to the WSSV.

METHODS

Sample collection.—In April 2012, copepods samples were collected from a shrimp pond located in Bahia de Kino Sonora, Mexico, (Figure 1) using a 100-μm-mesh plankton net. The specimens were reared in 60-L plastic containers containing 35‰ seawater, at 28°C with continuous aeration, and were fed daily with three species of algae (*Chaetoceros* sp., *Dunaliella* sp., and *Tetraselmis* sp.). Partial nucleotide sequences of the mitochondrial 16S rRNA were used for identification of the organisms according to the method described by Lindeque et al. (1999). The primers used were 16SAR (5′-CGCCTGTTTAACAAAAACAT-3′) y 16SBR (5′-ATTCAACATCGAGGTCACAAAC-3′), and the PCR thermocycling conditions were set as follows: 95°C for 5 min, 35 cycles of 95°C for 1 min, 55°C for 30 s, 72°C for 1 min, and a final extension step at 72°C for 10 min. The purified PCR amplicons were directly sequenced using a Perkin Elmer/Applied Biosystems automatic sequencer, model 3730, at the facilities of the Instituto de Biotecnología, Universidad Nacional Autónoma de México. The obtained 16S rRNA gene sequence was compared with sequences previously submitted to GenBank by others by using the program BLASTN with a minimum expect-value threshold of 1×10^{-10}.

Previous to the above mentioned procedure, copepods were manually separated from the bulk sample and tested for WSSV detection by quantitative PCR (qPCR) according to the method described by Mendoza-Cano and Sánchez-Paz (2013) using iQ SYBR Green Supermix (Bio-Rad) and the primers VP28-140Fw (5′-AGGTGTGGAACAACACATCAAG-3′) and VP28-140Rv (5′-TGCCAACTTCATCCTCATCA-3′) by using the following protocol: 95°C for 5 min, 30 cycles of 95°C for 30 s, 61°C for 30 s, and 72°C for 30 s (signal acquisition). For WSSV detection DNA was isolated from copepods following the GeneClean Spin protocol (MP Biomedicals).

FIGURE 1. The arrow represents the location of the shrimp pond located in Bahia de Kino Sonora, Mexico, where samples were collected.

Inoculum preparation and infection assay.—Virus inoculum was prepared from WSSV-infected shrimp tissue according to the method described by Escobedo-Bonilla et al. (2005) with some modifications. The tissue was homogenized in sterile phosphate-buffered saline (PBS), pH 7.4 (1:6 w/v), and clarified by centrifugation at 3,000 $\times$ *g* for 20 min at 4°C. The supernatant was then removed and centrifuged once more at 15,000 $\times$ *g* for 20 min. The recovered supernatant was then filtered through a 0.45-μm membrane (Millipore) and used for experimental assays. For the susceptibility study on the copepod *C. pacificus californicus*, organisms were challenged with freshly prepared WSSV inoculum according to the methodology (virus–phytoplankton adhesion route) proposed by Zhang et al. (2008). Accordingly, 5 mL of the inoculum were mixed for 30 min with 50 mL of a mixed algal culture of *Chaetoceros* sp., *Dunaliella* sp., and *Tetraselmis* sp., and subsequently the copepods were fed once with this mix. About 30 specimens of each treatment were sampled at 0, 24, 48, and 84 h postinoculation (hpi) and preserved in 96% ethyl alcohol for further analysis. The control

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C.pacificus     GGCGGCCAGACCGNCACCCATACGCTCCTGCACATATGTGAGTATTTAATTCAACATCGA 60
AF295333        GGCGGCCAGACCGTCACCCATACGCTCCTGCACATATGTGAGTATTTAATTCAACATCGA 60
                ************ ************************************************

C.pacificus     GGTCACAAACACCTATTCTGATGAGAACTCTTTTAGGTATAATGCTGTTATCCCTAGAGG 120
AF295333        GGTCACAAACACCTATTCTGATGAGAACTCTTTTAGGTATAATGCTGTTATCCCTAGAGG 120
                ************************************************************

C.pacificus     AGCTTCTTCATATTTCCCAGAGGATACGATTAAGAAATTTAATACTAATACTTTTATTAA 180
AF295333        AGCTTCTTCATATTTCCCAGAGGATACGATTAAGAAAATTAATACTAATACTTTTATTAA 180
                ************************************ ********************

C.pacificus     ATTTTACCCCAAAAAATAAGCCTGCAACTATGAAATAATTATCTTTTTGGCCNAGCAGCT 240
AF295333        ATTTTACCCCAAAAAATAAGCCTGCAACTATGAAATAATTATCTTTTTGGCCTAGCAGCT 240
                ************************************************ *******

C.pacificus     TCATAGGGTCTTCTCGTCTAAATATATCTGCTGAGTATTTTCACTCAGAATAAAATTTCG 300
AF295333        TCATAGGGTCTTCTCGTCTAAATATATCTGCTGAGTATTTTCACTCAGAATAAAATTTCG 300
                ************************************************************

C.pacificus     CAATATGGGATAAATACTTTATTTTAGTGAAACCGTTCATTCTATTTTCCAATTAMAAAA 360
AF295333        CAATATGGGATAAATACTTTATTTTAGTGAAACCGTTCATTCTATTTTCCAATTAAAAAA 360
                ***************************************************** ****

C.pacificus     CTAATTACTATGCTACCTTAACACTAACGCGGCTGTTTAAATTTCACTGAACAGGTAAGA 420
AF295333        CTAATTACTATGCTACCTTAACACTAACGCGGCTGTTTAAATTTCACTGAACAGGTAAGA 420
                ************************************************************

C.pacificus     TAAATTTAATTTGGGC 436
AF295333        TAAATTTAATTTGGGC 436
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FIGURE 2. Nucleotide sequence alignment of samples collected from Bahia de Kino Sonora, Mexico, and that reported for *C. pacificus californicus* (AF295333) in GenBank. Letters in bold text represent nucleotide differences.

copepods were treated in the same manner as in the susceptibility study, except that the algal culture was first mixed with sterile PBS (0.9% NaCl) as a substitute for the WSSV inoculum.

Temporal analysis of WSSV VP28 transcription.—Total RNA was purified with TRIzol Reagent (Invitrogen) from copepods collected at each sampling time according to the manufacturer's instructions and then treated with DNase I (Invitrogen) to eliminate any residual DNA. The concentration of total RNA was calculated by measuring its optical density (OD) at 260 nm using a Nanodrop ND-2000 spectrophotometer. For monitoring WSSV temporal transcription in challenged specimens and the control treatment, gene fragments of the VP28 viral envelope protein were amplified from the RNA isolated with RT-PCR using iScript One-Step RT-PCR Kit With SYBR Green (Bio-Rad) following the methodology described by Mendoza-Cano and Sánchez-Paz (2013) with an initial cDNA synthesis of 10 min at 50°C and following the protocol described above. As quality control, DNA contamination of the RNA isolates was confirmed by PCR. Equal RNA concentrations (30 ng/μL) were used for molecular tests.

RESULTS AND DISCUSSION

Copepods are an important part of the penaeid shrimp diet and the most common and abundant zooplankton species found in shrimp culture ponds (Martínez-Córdova and Peña-Messina 2005). In Bahia de Kino, Sonora, copepods often represent >90% of the zooplankton abundance during spring and fall, while its relative abundance decreases progressively to an abundance of 39% throughout the ecosystem in summer and winter (Salas 2011).

Sequence analysis of the partial 16S rRNA gene obtained from the copepods samples (456 bp) showed a 99% similarity to the best-scoring reference sequence (*C. pacificus californicus*, GenBank accession number AF295333), confirming the identity of the sample as *C. pacificus californicus* (Figure 2).

It has been recently proposed that WSSV may be dispersed to neighboring ponds or farms through the water as viral particles suspended in water and/or by means of particulate fractions as viral particles carried by zooplankton or adhered to microalgae (Esparza-Leal et al. 2009). Copepods collected from the shrimp pond were tested for WSSV by qPCR and diagnosed as WSSV-free. However, this does not imply that these organisms could not be vectors of this disease. Very low level infections in crustaceans can occur, sometimes at undetectable levels, even by highly sensitive PCR procedures (Walker and Winton 2010). The temporal expression of VP28 in *C. pacificus californicus* after WSSV exposure was analyzed by real-time PCR (RT-PCR) (Figure 3). No amplification for VP28 transcripts was observed at 0 and 24 hpi; however, transcripts were continuously detected

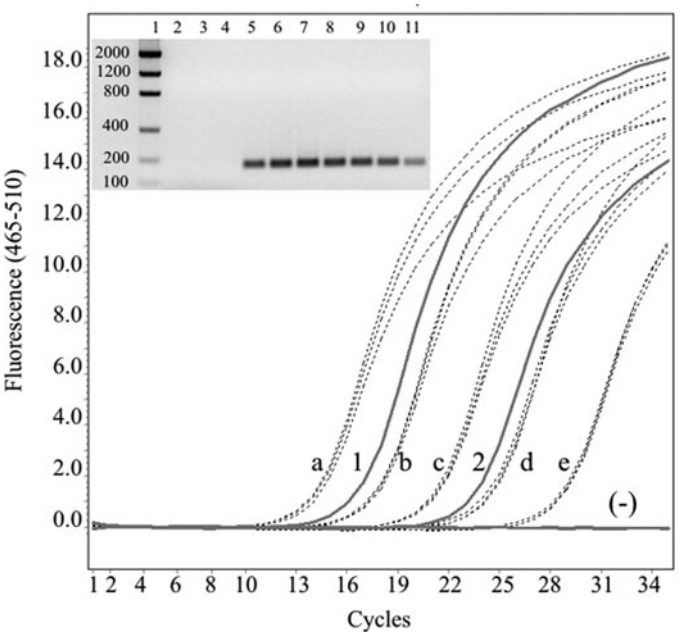

FIGURE 3. Detection and quantification of WSSV VP28 transcripts by qPCR in experimentally infected *C. pacificus californicus*. Standard curves for RT-PCR are shown based on 10-fold serial dilutions of a 141-bp WSSV VP28 DNA fragment. The letters a, b, c, d, and e represent 6.57×10^5, 6.57×10^4, 6.57×10^3, 6.57×10^2, and 657 copies/ng of total DNA, respectively. The solid lines represented by the numbers 1 and 2 are WSSV VP28 transcripts at 84 hpi and 48 hpi, respectively. The shaded box represents the end-point amplification of VP28 as follows: lane 1: low mass DNA ladder; lane 2: negative control; lane 3: 0 hpi; lane 4: 24 hpi; lane 5: 48 hpi; lane 6: 84 hpi; lanes 7–11: 10-fold serial dilutions (from 6.57×10^5 to 657 copies/ng) of total DNA.

from 48 to 84 hpi. Based on the quantification cycle (C_q) (Bustin et al. 2009), at 48 hpi 1.25×10^3 viral copies/ng of DNA were detected, and this amount increased to 1.13×10^5 copies/ng of DNA at 84 hpi. These results are in agreement with those reported by Chang et al. (2011) in a different copepod species (*Apocyclops royi*) in which transcripts of the viral protein were detected. Differences between this study and that of Chang et al. (2011) must be noted, as transcripts for VP28 were detected in *A. royi* at 24 hpi, while in *C. pacificus californicus* collected in Bahía de Kino, transcripts were not detected until 48 hpi. This difference in the detection of transcripts of VP28 may be due either to the susceptibility of both species to WSSV or to the total number of genomic copies inoculated to each species. Reports in penaeid shrimp indicate that expression of this protein (VP28) occurs in the first 6 hpi (Zhang et al. 2002). This virus has been reported in at least 93 WSSV vectors or hosts, including penaeid shrimps, lobsters, crabs, crayfish, shrimp, polychaetes, and different components of the zooplankton as brine shrimp, cladocerans, rotifers, and copepods (Sánchez-Paz 2010).

It is important to note that a positive detection of WSSV by PCR does not imply that the copepod is susceptible to WSSV because this technique is based on the presence or absence of fragments of the viral genome that could be localized intracellularly, on the surface, or in the organism's intestinal contents.

To confirm viral infection, several techniques, such as histology, in situ hybridization, or transmission electron microscopy, could be used. In this case, the gene expression profile of WSSV using RT-PCR may be considered a useful tool to define whether the virus is in an active (infective) or an inactive (noninfective) form, and it is applicable not only to WSSV, but also to other viruses (Chang et al. 2011).

Water may be a major pathway for WSSV dispersion into a shrimp farm (Lotz and Lightner 1999), and the virus can be also carried by zooplankton or can be attached to microalgae (Esparza-Leal et al. 2009). Thus, WSSV outbreaks have been related to ocean currents across the Gulf of California.The results obtained in the present study clearly show that the copepod *C. pacificus californicus* is susceptible to WSSV and that it is likely virus dispersion may occur through infected copepods. However, more tests are needed to correlate the epidemiological outbreaks of white spot syndrome and the presence of copepods in shrimp ponds.

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