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NOTE

Blue crabs *Callinectes sapidus* as potential biological reservoirs for white spot syndrome virus (WSSV)

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ABSTRACT: White spot syndrome virus (WSSV) is a virulent pathogen of cultured shrimp and was first detected in farms in South Carolina (USA) in 1997 and subsequently in wild shrimp in 1999. We screened groups of 1808 wild Atlantic white shrimp *Litopenaeus setiferus* and 300 blue crabs *Callinectes sapidus* collected from South Carolina, Georgia, and Florida for the presence of WSSV using the Shrimple® immunoassay-strip test, with all positives and random subsets of negatives tested by TaqMan real-time PCR and in infectivity bioassays. Of 87 shrimp and 11 crabs that tested positive using the Shrimple® test, only a single *C. sapidus* was confirmed to be infected with WSSV by PCR and the infectivity bioassay. The data indicate that the prevalence of WSSV in these species is low in these southeastern US regions, but that *C. sapidus* may serve as a biological reservoir.

KEY WORDS: Atlantic white shrimp · *Litopenaeus setiferus* · Penaeid · Decapoda · Bioassay · WSSV · Real-time PCR · Shrimple® · False positive

INTRODUCTION

White spot syndrome virus (WSSV), an extremely virulent virus that emerged in shrimp farms in Thailand in the early 1990s, can result in 100% mortality of shrimp in aquaculture ponds within 3 to 7 d of the disease first being observed, and now impacts most shrimp-farming regions globally (Flegel 1996, Salehi 2010). WSSV has a very broad crustacean host range, with essentially all cultivated penaeid shrimp species susceptible to disease (Lo et al. 1996, Flegel 1997, OIE 2012). Many crab species carry WSSV (Lo et al. 1997, Sahul Hameed et al. 2003), including blue crabs *Callinectes sapidus* that inhabit coastal regions of the USA (Chang et al. 2001). Such potential reservoirs for WSSV can represent a source of WSSV infection in farmed shrimp as well as in commercially significant wild shrimp fisheries (Kanchanaphum et al. 1998).

While various PCR tests have been developed to detect WSSV with high sensitivity (Durand & Lightner 2002), simple immuno-strip tests such as the Shrimple® WSSV test kit (EnBioTec Laboratories), based on antibody-capture of WSSV protein, have

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also been commercialized for rapid pond-side diagnosis of WSSV infection (Powell et al. 2006, Sithigorngul et al. 2006).

To investigate potential origins and outcomes of WSSV disease outbreak in shrimp aquaculture facilities in South Carolina (USA) in 1997, a group of 1150 shrimp collected from the South Atlantic Bight region in 1999 was surveyed by PCR for the presence of WSSV (Chapman et al. 2004). WSSV DNA was detected at 4.7% prevalence among 586 Atlantic white shrimp *Litopenaeus setiferus* tested, but only 2 of the 28 PCR-positive shrimp could be confirmed as positive in 3 different WSSV PCR tests, with only 1 of these shrimp showing evidence of WSSV infection by histology and *in situ* hybridization (Chapman et al. 2004).

Reported here are WSSV prevalence data on a further 1808 *L. setiferus* and 300 blue crabs collected in 2004 from across South Carolina, Georgia, and Florida regions of the southern USA, with crabs included to identify whether they might represent a reservoir for the virus.

MATERIALS AND METHODS

Samples

Wild Atlantic white shrimp (n = 1808) and blue crabs (n = 300) were collected from commercial fisheries in Charleston, South Carolina (n = 991 shrimp, 176 crabs), Brunswick, Georgia (n = 556 shrimp, 124 crabs), and Jacksonville, Florida (n = 261 shrimp), during 2004. Shrimp were collected between March and October and crabs between April and July. As was done for the shrimp tested in 1999 (Chapman et al. 2004), the specimens represented random representatives of commercial catches not selected based on signs of morbidity or disease. Specimens were frozen (–20°C) immediately upon collection and transported on dry ice to the Hollings Marine Laboratory (Charleston, SC) for testing.

WSSV protein immuno-test

Upon receipt at the laboratory, shrimp pleopods and crab gills were sampled from partially thawed specimens for testing using the Shrimple® WSSV protein detection kit (EnBioTec Laboratories) according to the manufacturer's protocol. Results were scored as either positive or negative with due care to avoid scoring false positives or false negatives (Powell et al. 2006). While not evaluated previously using *Litopenaeus setiferus* or *Callinectes sapidus*, the Shrimple® WSSV protein immuno-strip test method is most effective in detecting higher-level WSSV infection (Sithigorngul et al. 2006).

WSSV PCR test

At the same time tissues were sampled for Shrimple® testing, 2 pleopods from each shrimp were preserved in sarcosyl-urea solution (1% sarcosyl, 8 M urea, 20 mM sodium phosphate, 1 mM EDTA, pH 6.8) for PCR analysis (Chapman et al. 2004). These tissues were chosen for PCR due to pleopods and uropods being identified to contain up to 10-fold more WSSV DNA per mass compared to whole heads or tails of shrimp (Lightner et al. 2001). For all specimens that tested WSSV-positive by Shrimple® as well as a random subsample that tested WSSV-negative, the pleopod or gill samples were lysed for 72 h at 37°C, and DNA was extracted using the Sprint Prep magnetic bead kit (Agencourt Bioscience) according to the manufacturer's protocol. DNA extractions were undertaken within 1 to 3 mo of sample collection. The quality and quantity of DNA extracted from representative preserved samples were evaluated by electrophoresis in 0.8% agarose gels containing ethidium bromide, by determining the absorbance at 260/280 nm, and by PCR amplification of a conserved crustacean β-actin sequence (data not shown).

WSSV DNA was detected by TaqMan real-time PCR using the primers WSS1011F and WSS1079R, QuantiTect Probe PCR Kit reagents (Qiagen), and an ABI 7500 Sequence Detection System (Applied Biosystems) as described previously (Durand & Lightner 2002). PCR included testing of a dilution series of WSSV plasmid DNA (ranging from 10^2 to 10^6 DNA copies) as positive controls.

WSSV infectivity

Upon receipt of shrimp and crab specimens at the laboratory, samples were prepared for infectivity bioassays. Shrimp cephalothorax tissues were removed and stored at –80°C. Hepatopancreas, heart, gill, eyestalk, stomach, and reproductive organ tissues from crabs were pooled and stored at –80°C. Clarified tissue homogenates to be used as inocula were prepared as described previously (Prior et al. 2003). Inocula were prepared for all individuals tested by PCR which included all Shrimple® test-positive

shrimp/crabs, 23 representative Shrimple® test-negative shrimp, and 13 representative Shrimple® test-negative crabs. Bioassays were conducted in January 2006 using specific pathogen-free (SPF) *L. vannamei* obtained from the Oceanic Institute, Hawaii (USA), as post-larvae and reared to 1.0–1.5 g in an indoor biosecure environment-controlled facility. Shrimp (n = 15) were maintained in 19 l polypropylene aquaria filled with UV-sterilized recirculating artificial seawater (27°C) in a facility employing a 12 h L:12 h D photoperiod. Tail muscle of shrimp was injected with inoculum at a dose of 0.02 ml g^{-1} shrimp weight. Shrimp in 2 aquaria (n = 30) were injected with each inoculum or with positive-control or mock inocula, and mortality was recorded daily. Each group of bioassays was terminated between 10 and 14 d post-injection, i.e. at a time double that at which 100% shrimp mortality occurred in positive control aquaria (typically 5 to 7 d). The positive-control inoculum consisted of a cell-free filtrate of a cephalothorax homogenate prepared from WSSV-infected shrimp harvested during a confirmed WSSV outbreak in South Carolina (Prior et al. 2003). A cell-free filtrate prepared similarly from SPF *L. vannamei* was used as a mock inoculum.

Bioassays were conducted in blocks of 25 aquaria, with 2 each used for 10 sample homogenates, 2 each used as positive and negative controls, and 1 aquarium used as a non-injection 'water-quality' control. Either when 100% mortality occurred or at 10 d post-injection, 2 shrimp from each sample group were sacrificed to collect tissue for Shrimple® WSSV protein testing as well as 2 pleopods for WSSV PCR analysis (Durand & Lightner 2002).

Differences in cumulative mortality between bioassay cohorts were assessed statistically using Kaplan-Meier analysis in GraphPad Prism 3.00 for Windows (GraphPad Software).

RESULTS

Using Shrimple® immuno-strip tests to screen for WSSV protein, tissue samples collected from ~4.8% *Litopenaeus setiferus* (87/1808) and ~3.8% *Callinectes sapidus* (11/300) were scored as WSSV-positive. Of these, however, only 1 mature female *C. sapidus* (SK040167-1) collected near Charleston, South Carolina, was confirmed as WSSV-positive by TaqMan real-time PCR and infectivity bioassay as well as in a conventional nested PCR test (Lo et al. 1996, data not shown). The WSSV real-time PCR cycle threshold (C_T) value obtained with this crab was comparable to those obtained with moribund *L. vannamei* either collected from a WSSV disease outbreak or generated experimentally. In the 440 bp genome region (position 224 757–225 196, GenBank accession number AF332093; Yang et al. 2001) amplified by WSSV primers 146F and 146R (Lo et al. 1996), the WSSV strain detected in crab SK040167-1 was identical to that detected in *L. vannamei* inoculated with the SK040167-1 tissue homogenate as well as in 2 *L. vannamei* injected with WSSV positive-control inoculum.

The shrimp infectivity bioassay using the tissue homogenate prepared from crab SK040167-1 was the only one of the 134 performed that resulted in cumulative mortality significantly different (Kaplan-Meier, $p < 0.00$) than the negative control (Fig. 1). None of the other 133 homogenates resulted in significant mortality (p-value range 0.18–1.0). SPF *L. vannamei* injected with the crab SK040167-1 tissue homogenate were Shrimple® test-positive for WSSV protein as well as TaqMan real-time PCR test-positive for WSSV. Of the SPF *L. vannamei* tested from the other bioassays, none of the 240 was Shrimple® test-positive for WSSV and none of the 42 shrimp was real-time PCR test-positive for WSSV.

DISCUSSION

Of 1808 Atlantic white shrimp and 300 blue crabs collected from commercial fisheries in South Carolina, Georgia, and Florida in 2004, WSSV infection was confirmed in only a single crab by PCR and sequence analysis as well as by infectivity bioassay.

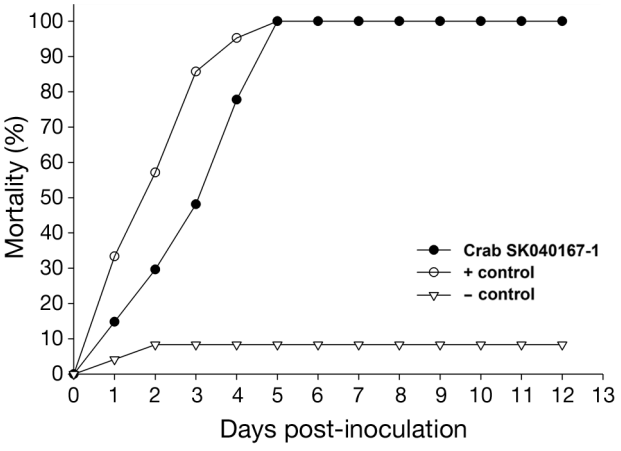

Fig. 1. Mortality among groups of specific pathogen-free (SPF) Atlantic white shrimp *Litopenaeus vannamei* (n = 30) injected with tissue homogenates of blue crab *Callinectes sapidus* SK040167-1 (●), WSSV-positive SPF shrimp (○), or WSSV-negative SPF shrimp (▽)

However, use of Shrimple® immuno-strip tests to screen for WSSV protein scored 4.8% (87/1808) of the shrimp and 3.8% (11/300) of the crabs as WSSV-positive, which was comparable to the prevalence of WSSV (4.7%) determined by 1-step PCR analysis of a group of 1150 specimens collected from the same regions in 1999 (Chapman et al. 2004).

The reasons for the discrepancies between the Shrimple® test positives that tested negative by real-time PCR and in infectivity bioassays are not clear. False positives have not been encountered during Shrimple® test development and field trials (EnBio-Tec Laboratories pers. comm.). However, despite it being a +/− test, shrimp infected more heavily with WSSV, thus possessing higher WSSV protein levels, result in qualitatively darker positive bands, while shrimp infected at low levels result in lighter and often faint positive bands. In support of the Shrimple® test having limited detection sensitivity, statistically significant differences have been detected between WSSV infection loads quantified by real-time PCR and Shrimple® test-negatives or low-level positives (Powell et al. 2006). Moreover, as part of the virus surveillance activities undertaken at the Marine Infectious Disease Laboratory, South Carolina Department of Natural Resources, 3 separate SPF populations of *Litopenaeus vannamei* screened regularly over a 2 yr period always tested negative using the WSSV Shrimple® test. Furthermore, no Shrimple® test false-positives for WSSV were detected among negative-control or uninfected SPF *L. vannamei* used in bioassays or in bioassay shrimp infected with viruses other than WSSV (Powell et al. 2006, Powell 2007). It is possible, therefore, that the Shrimple® test might be able to detect WSSV protein in the absence of productive infection, or alternatively that the antibodies used in the test can cross-react with unknown host proteins occasionally expressed in some crustaceans or with proteins of a related but genetically distinct virus such as the enveloped bacilliform nuclear virus B2 (Bonami & Zhang 2011). In support of these hypotheses, infectious WSSV was not detected in any of the *L. setiferus* or *Callinectes sapidus* specimens that were Shrimple® test-positive but real-time PCR test-negative.

Regardless of whether the Shrimple® test can generate false-positives that exaggerated WSSV prevalence, data from the various diagnostic tests applied here demonstrate that there has, at a minimum, been no increase in WSSV prevalence in wild *L. setiferus* since last being surveyed in 1999 (Chapman et al. 2004). The detection of infectious WSSV in an Atlantic blue crab warrants further investigation and possibly a broader survey of this species to identify whether it serves as a biological reservoir for WSSV in the southeastern USA, with potential to place the substantial commercial wild shrimp fisheries in this region at risk.

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