NOTE

Experimental infection of twenty species of Indian marine crabs with white spot syndrome virus (WSSV)

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ABSTRACT: Twenty species of Indian marine crabs were experimentally infected with white spot syndrome virus (WSSV), via the oral route and intramuscular injection, to determine their viral susceptibility. We determined that 16 species (*Calappa philargius*, *Charybdis annulata*, *C. lucifera*, *Doclea hybrida*, *Grapsus albolineatus*, *Halimede ochtodes*, *Liagore rubronaculata*, *Lithodes maja*, *Matuta miersi*, *Paradorippe granulata*, *Parthenope prensor*, *Philyra syndactyla*, *Podophthalmus vigil*, *Portunus sanquinolentus*, *Scylla serrata* and *Thalamita danae*) were susceptible and 4 (*Atergatis integerrimus*, *Charybdis natator*, *Demania splendida* or *Menippe rumphii*) were refractive at 50 d post-infection (p.i.). The presence of WSSV in these crabs was confirmed by PCR tests, histology and bioassay. WSSV was found in the gill, heart, eyestalks, striated muscle and cephalothoraxic tissue. The 4 WSSV-refractive species represent potential reservoirs or carriers of WSSV.

KEY WORDS: WSSV · Penaeid shrimp · Marine crabs · PCR · Histology

INTRODUCTION

White spot disease (WSD) is a serious viral disease of marine shrimp characterized by white spots in the exoskeleton of infected *Peneaus monodon* (Chou et al. 1995, Wang et al. 1995, Lightner 1996). The causative agent is white spot syndrome virus (WSSV), which is responsible for high mortalities and severe losses to global shrimp aquaculture (Lightner 1996). The losses in India alone have been estimated at several million dollars per year (Anonymous 1996). WSSV has been isolated and characterized from 2 species of shrimp found in India, *P. indicus* and *P. monodon* (Sahul Hameed et al. 1998). WSSV targets organs originating from embryonic mesoderm and ectoderm, producing hypertrophied nuclei with intranuclear inclusion bodies (Wang et al. 1995, Chang et al. 1996, Yoganandhan et al. 2003).

WSSV has a wide host range that includes more than 40 species of crabs, copepods and other arthropods (Cai et al. 1995, Chang et al. 1998, Lightner et al. 1998, Wang et al. 1998). It naturally infects all the major species of cultivated penaeid shrimp (Wongteerasupaya et al. 1995, Lo et al. 1996, Flegel 1997, Lightner et al. 1997, Nunan et al. 1998, Sahul Hameed et al. 1998). Both natural and experimental infections have been reported in caridean shrimp (*Exopalaemon orientalis*, *Macrobrachium rosenbergii*, *M. idella* and *M. lamerrae*; Chang et al. 1998, Peng et al. 1998, Wang et al. 1998, Sahul Hameed et al. 2000), crayfish (*Cambarus clarki* and *Pacifastacus leniusculus*; Huang et al. 2001, Jiravanichpaisal et al. 2001), wild crabs (*Calappa lophos*, *Portunus sanguinolentus*, *Portunus pelagicus*, *Charybdis* sp., *Helice tridens* and *Scylla serrata*; Chang et al. 1998, Kanchanaphum et al. 1998, Chen et al. 2000, Corbel et al. 2001), wild lobsters (*Scyllarus arctus* and *Panulirus* sp.; Chang et al. 1998), and planktonic copepods and pupae of an *Ephydridae* insect (Wang et al. 1998). Experimental WSSV infection has been demonstrated in freshwater crabs

(*Paratelphusa hydrodomous* and *Paratelphusa pulvi-nata*), and *Artemia* sp. (Sahul Hameed et al. 2001, 2002). The aim of this study was to investigate the effects of experimentally induced WSSV infection (per os and intramuscular injection) on 20 species of Indian marine crabs.

MATERIALS AND METHODS

Collection and maintenance of experimental animals. Twenty species of marine crab (*Atergatis integerrimus*, *Calappa philargius*, *Charybdis annulata*, *Charybdis lucifera*, *Charybdis natator*, *Demania splendida*, *Doclea hybrida*, *Grapsus albolineatus*, *Halimede ochtodes*, *Liagore rubronaculata*, *Lithodes maja*, *Matuta miersi*, *Menippe rumphii*, *Paradorippe granulata*, *Parthenope prensor*, *Philyra syndactyla*, *Podophthalmus vigil*, *Portunus sanquinolentus*, *Scylla serrata* and *Thalamita danae*) were collected from fish-landing centers at Chennai along the east coast of India. Taxonomic identification was based on the works of Alcock (1900) and Jayabaskaran et al. (2000). The crabs (20 to 50 g avg. weight) were transported to the laboratory and acclimated in separate 500 l aquaria (20 crabs of the same species per aquarium, 30 to 34 ppt, 27 to 30°C) for 5 d prior to the onset of study. They were fed minced fresh fish ad lib. Five of each species were randomly selected and screened for WSSV by PCR using the primers designed by Takahashi et al. (1996). Saltwater was pumped from the adjacent sea and allowed to sediment, thus removing sand and other particulate matter before use.

Preparation of viral inoculum. WSSV-infected *Penaeus monodon* with prominent white spots were collected from farms located near Nellore, India. Cephalothoraxic tissues (including gills) were homogenized in NaCl-Tris-EDTA (NTE) buffer (0.2 M NaCl, 0.02 M Tris-HCl and 0.02 EDTA, pH 7.4) (10% suspension) and centrifuged at $3000 \times g$ (20 min, 4°C). The supernatant was removed and centrifuged at $8000 \times g$ (30 min, 4°C). The resulting supernatant was then passed through a 0.4 µm filter and the filtrate stored at –20°C. Prior to storage, the total protein content of the filtrate was determined by the method of Lowry et al. (1951), and WSSV presence was confirmed by PCR using primers designed by Takahashi et al. (1996).

Infectivity experiments. The pathogenic effects of WSSV to the 20 crab species was tested by feeding them WSSV-infected shrimp tissue, or by intramuscular injection of the viral inoculum described above. For injection tests, each crab species (10 per tank) was challenged by injecting the viral inoculum (300 µg of total protein per crab, diluted to 50 µl with NTE buffer)

into the soft tissue at the base of the swimming legs. Negative-control crabs were injected with a WSSV-negative tissue inoculum (300 µg of total protein per crab) prepared from uninfected shrimp. For oral infection, 10 crabs per species were placed in a 100 l aquarium and starved for 24 h before being fed WSSV-infected shrimp tissue at a rate of 5% body weight d^{-1}. The daily ration was divided into 3 portions that were fed at 8 h intervals for 3 d, after which the crabs were fed minced fresh fish. The control group was fed uninfected shrimp tissue followed by minced fish. The experimental crabs were examined twice per day for gross signs of disease, and daily mortality was recorded. All of the negative-control and treatment-group experiments, either exposed per os or by injection, were done in replicate.

Confirmation of WSSV infection by PCR. WSSV-infection status of the crabs was confirmed by PCR analysis of ectodermal and mesodermal organs for the presence of lesions characteristic of WSD (Lightner 1996). Template DNA for PCR tests was extracted from eyestalks, heart, gills, cephalothoraxic tissue or muscle following the method of Lo et al. (1996). Organs (20 mg per organ) to be extracted were cut and homogenized separately in NTE buffer. Each homogenate was centrifuged at $3000 \times g$ (4°C) and the resulting supernatant (200 µl) then transferred to another centrifuge tube together with 600 µl of digestion buffer (100 mM NaCl, 10 mM Tris HCl, pH 8.0, 50 mM EDTA, pH 8.0, 0.5% sodium dodecyl sulfate, 0.1 mg ml^{-1} proteinase K). After a 2 h incubation (65°C), the digest was deproteinized by successive phenol/chloroform/isoamyl alcohol extractions, the DNA ethanol precipitated and dried. The dried DNA pellet was resuspended in 10 µl of TE buffer (Tris-HCl 100 mM, pH 8.0, 10 mM EDTA, pH 8.0). Single-step PCR and nested-PCR tests were used to confirm the WSSV-infection status. For single-step PCR, primers were designed to amplify a 426 bp sequence of WSSV DNA based upon the genomic sequence of van Hulten et al. (2001). The primer sequences were: 5′-TTC TTC TTG ATT TCG TCC-3′ and 5′-AAT TCG TGG AGA GAG GTC C-3′. The reaction mixture contained 2 µl of template DNA, 1 µM of each primer, 200 µM of deoxynucleotide triphosphate and 1.25 U of Taq DNA polymerase in PCR buffers supplied with a commercially available kit (Finnzymes). The PCR protocol comprised 35 cycles of 0.5 min at 95°C, 1 min at 55°C and 1 min at 72°C, with a final extension of 5 min at 72°C. For the nested PCR, the internal primers for a 200 bp fragment were 5′-ATC TCT ACC GTC ACA GCC C-3′ and 5′-GAA GAT TTA ATG TCC TTG CTC G-3′ (Yoganandhan et al. 2003). PCR products were analyzed by electrophoresis using 1% agarose gels stained with ethidium bromide and visualized by ultraviolet transillumination.

Table 1. Cumulative percent mortality of Indian crabs at different time intervals after inoculation (intramuscular injection or oral route) with white spot syndrome virus (WSSV), and results of PCR analysis. Histology was positive in all cases

Species	Days to 100% mortality by injection	% mortality by oral route (30 d)	PCR test				
			G	HT	H	M	E
Atergatis integerrimus	None (30)	0	++	++	++	+	+
Calappa philargius	4.3	70	++	++	+	+	+
Charybdis annulata	3.5	90	+	++	+	++	+
Charybdis lucifera	4.0	80	+	++	++	+	+
Charybdis natator	None (30)	0	++	++	+	+	+
Demania splendia	None (30)	0	++	++	++	+	+
Doclea hybrida	10.0	60	++	++	++	++	+
Grapsus albolineatus	2.0	90	++	++	++	++	+
Halimede ochtodes	10.0	70	++	++	++	++	++
Liagore rubronaculata	10.0	60	++	++	++	+	++
Lithodes maja	3.5	80	++	++	++	+	++
Matura miersi	4.0	80	++	++	++	+	++
Menippe rumphii	None (30)	0	+	+	+	+	++
Paradorippe granulata	4.3	70	++	++	++	+	++
Parthenope prensor	4.3	80	++	++	++	+	+
Philyra syndactyla	10.0	70	++	++	++	+	++
Podophthalmus vigil	4.0	80	++	++	++	+	+
Portunus sanquinolentus	5.0	70	++	++	++	++	++
Scylla serrata	4.0	90	++	++	++	++	++
Thalamita danae	6.0	70	++	++	++	++	++

G: gill, HT: head soft-tissue, H: heart, M: muscle, E: eyestalk
++: WSSV-positive by single-step PCR, +: WSSV-positive by nested PCR

Histology. Dissected organs were preserved in Davidson's fixative for 48 h and then transferred to 70% alcohol following the methods of Bell & Lightner (1988). Sections (4 to 5 μm thick) were stained with hematoxylin and eosin and observed by light microscopy.

RESULTS AND DISCUSSION

Cumulative percent mortality results (Table 1) revealed that WSSV was pathogenic to 16 of the 20 marine crab species, both through intramuscular injection and per os. A mortality of 100% was observed in these challenged groups at 10 d post-injection (p.i.), but the onset time and extent varied with species. Oral challenge caused mortality ranging from 60 to 90% at 30 d p.i. No mortality was observed in the negative control groups of both oral and intramuscular routes. To confirm WSSV infection, hemolymph from the experimentally infected crabs was intramuscularly injected into marine shrimp, which displayed similar gross signs of disease and severe mortality.

Gross signs of WSSV infection in the crabs included lethargy, reduced feed consumption, an initial dark and pinkish color on the dorsal sides, and a pale appearance at the time of morbidity. White spots in the cuticle were not observed. Some species (*Charybdis*

lucifera, *Grapsus albolineatus*, and *Parthenope prensor*) displayed autotomy, detaching their legs segment by segment before reaching the moribund stage. No gross signs of disease were observed in *Atergatis integerrimus*, *Charybdis natator*, *Demania splendida* or *Menippe rumphii*.

All of the challenged crabs, including those without gross signs of WSD, were 1-step PCR-positive for WSSV, but not for all of the organs tested (Table 1). However, all organs were WSSV-positive by nested PCR (Table 1). No WSSV-positive results were obtained for the negative control groups by PCR.

Histological examination of the experimentally challenged crabs revealed virus-infected cells characterized by basophilic intranuclear inclusions in tissues of ectodermal and mesodermal origin (gill tissue, striated muscle, eyestalks, and heart). However, the number of cells with hypertrophied nuclei was lower, or they were absent, in tissues of crabs showing no signs of WSD (i.e. *Atergatis integerrimus*, *Charybdis natator*, *Demania splendida* and *Menippe rumphii*), when compared to those with WSD.

WSSV has a wide host-range and is a highly pathogenic virus that is transmitted to cultured shrimp via contaminated water and ingestion of WSSV-infected animals (Supamattaya & Boonyaratpalin 1996). Transmission is also possible through cohabitation of infected species with uninfected stocks in both the shrimp farming environment and the wild (Flegel 1997, Flegel & Alday-Sanz 1998). Ingestion of WSSV-infected tissue by shrimp and crabs is probably one of the principal infection routes of this virus, in both natural and farm environments.

The mortality data, together with PCR tests and histological observations, confirmed that all the marine crabs in this study may act as carriers of WSSV. Thus, they should not be used as raw feed materials for pond-reared shrimp or brood-stock. This coincides with a previous recommendation made by Lo & Kou (1998) to avoid using the marine crabs *Charybdis feriatus* and *Portunus sanguinolentu* as shrimp-feed additives or components.

Based on the present results, WSSV infection in marine crabs can be classified into 3 types. Type 1 is an acute infection with high mortality starting at 2 d p.i. and ending with 100% mortality at 5 d p.i. These crabs

contain large numbers of infected cells with hypertrophied nuclei (i.e. *Calappa philargius*, *Charybdis annulata*, *Charybdis lucifera*, *Grapsus albolineatus*, *Lithodes maja*, *Matuta miersi*, *Paradorippe granulata*, *Parthenope prensor*, *Podophthalmus vigil*, *Portunus sanquinolentus*, *Scylla serrata* and *Thalamita danae*). Type 2 is a subacute infection with mortality starting at 5 d p.i. and ending with 100 % mortality at 10 d p.i. These crabs display moderate numbers of infected cells (i.e. *Doclea hybrida*, *Halimede ochtodes*, *Liagore rubronaculata* and *Philyra syndactyla*). Type 3 infections are characterized by no mortality, but crabs are WSSV-PCR positive, with few or no infected cells seen by routine histology (i.e. *Atergatis integerrimus*, *Charybdis natator*, *Demania splendida* and *Menippe rumphii*). These different responses suggest that WSSV is species-specific, and other studies have also shown differences in WSSV pathogenicity. For example, *Macrobrachium idella* and *M. lamerrae* are susceptible, whereas *M. rosenbergii* is resistant (Sahul Hameed et al. 2000). Experimentally challenged *Cancer maenas* and *Cancer mediterraneus* show no signs of WSD (Shi 2000, Corbel et al. 2001), but Corbel et al. (2001) did not carry out tests to determine the presence of WSSV in survivors. In our experiments, the species with Type 3 infections survived for more than 50 d, even though PCR results, histopathology, and hemolymph bioassay confirmed the presence of WSSV. Further work should be carried out to determine the mechanism of their resistance to WSD.

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