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, Yogananthandhan 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 philargius, Charybdis annulata, C. lucifera, Doclea hybrida, Grapsus albolineatus, Halimede ochtodes, Liagorae rubronaculata, Lithodes maja, Matuta miersi, Paradorippe granulata, Parthenope prensor, Philyra syndactyla, Podopthalmus vigil, Portunus sanguinolentus, Scylla serrata and Thalamita danae*) were susceptible and 4 (*Atergatis integrissimus, Charybdis natator, Demania splendidia or Menippe rumphi*) 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 cephalothoracic tissue. The 4 WSSV-refractive species represent potential reservoirs or carriers of WSSV.

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, Liagorae rubronaculata, Lithodes maja, Matuta miersi, Paradorippe granulata, Parthenope prensor, Philyra syndactyla, Podopthalmus vigil, Portunus sanguinolentus, Scylla serrata and Thalamita danae*) were susceptible and 4 (*Atergatis integrissimus, Charybdis natator, Demania splendidia or Menippe rumphi*) 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 cephalothoracic 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, Yogananthandhan et al. 2003).

(Paratelphusa hydrodomous and Paratelphusa pulvinata), 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, Halimedea ochtodes, Liagore rubronaculata, Lithodes maja, Matuta miersi, Menippe rumphii, Paradorippe granulata, Parthenope presnori, Philyra syndactyla, Podophthalmus vigil, Portunus sanguinolentus, 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 50 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 × g (20 min, 4°C). The supernatant was removed and centrifuged at 8000 × g (20 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, cephalothoracic 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 × 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 TCAG TCC-3' and 5'-AAT TCG TGG AGA GAG TCC 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

<table>
<thead>
<tr>
<th>Species</th>
<th>Days to 100% mortality by injection</th>
<th>% mortality by oral route (30 d)</th>
<th>PCR test</th>
<th>G</th>
<th>HT</th>
<th>H</th>
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<tr>
<td>Thalamita danae</td>
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G: gill, HT: head soft-tissue, H: heart, M: muscle, E: eyestalk  
++: WSSV-positive by single-step PCR, +: WSSV-positive by nested PCR

**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 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 sanquinoletus 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 hyper-trophied nuclei (i.e. Calappa philargius, Charybdis annulata, Charybdis lucifera, Grapsus albolineatus, Lithodes maja, Matuta mieri, Paradorippe granulata, Parthenope prorsor, Podopthalmus 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. Docilea 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 integerimirus, Charybdis natator, Demania splendid and Menippe rumphii). These different responses suggest that WSSV is species-specific, and other studies have also shown differences in WSSV pathogenicity. For example, Macrobachium idella and M. lamarrae are susceptible, whereas M. rosenbergi 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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