Infection of *Penaeus stylirostris* (Boone) with a Rhabdovirus Isolated from *Penaeus* spp.

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Infectivity and organ distribution studies for the rhabdovirus (rhabdovirus of penaeid shrimp-RPS) isolated from *Penaeus* spp. were conducted with subadult *Penaeus stylirostris*. Test shrimp were inoculated intramuscularly with different isolates of RPS from penaeid shrimp collected from different shrimp farms in Hawaii and Ecuador. Although the experimentally infected shrimp showed no clinical or gross manifestations of disease, the RPS was found to be infectious for the animals. Virus replication was demonstrated only in the lymphoid (Oka) organs of infected shrimp by virus assay and immunofluorescence. Also, the Oka organs of infected animals showed gross cellular changes and were significantly larger in size than the corresponding organs from uninfected shrimp. A possible role of the RPS in the causation of disease in penaeid shrimp is postulated.

Lu et al. (1991) recently reported the isolation of a rhabdovirus (RPS) from *Penaeus stylirostris* and *P. vannamei* sampled from populations histologically determined to be infected with infectious hypodermal and hematopoietic necrosis virus (IHHNV). However, the role of RPS in the causation of IHHN or of any disease in the penaeid shrimp was not elucidated. The present study was conducted to assess the role of the rhabdovirus isolate as a pathogen in subadult *P. stylirostris*.

Materials and Methods

**Shrimp Bioassay and Virus Exposure—Study 1**

Subadult, 5-6 gram *P. stylirostris* were used in the bioassay study to assess the pathogenicity of RPS. The experimental *P. stylirostris* were obtained from a closed population (2nd generation) of shrimp which were periodically monitored histologically and found to be free of both the IHHN virus and *Baculovirus penaei*.

The bioassay study was conducted in nine, 57 liter glass aquaria. Salinity of water was maintained at 20 to 25 parts per thousand (p.p.t.) and water temperature ranged 24–27°C during the study period. Water in aquaria was exchanged once or twice weekly to maintain acceptable water quality conditions. Shrimp were fed pelleted shrimp feed daily over the 21 day experimental period. At the end of the period the experimental animals were sacrificed for both virus isolation and histopathological examination.

For each test group, ten shrimp were individually injected into the abdominal musculature (0.05 to 0.10 ml/animal) with a RPS or PBS (phosphate buffered saline) inoculum. Eight test batches representing five separate isolations of the RPS agent and a PBS control were used to assess the infectivity and pathogenicity of RPS for subadult *P. stylirostris*. To identify the different preparations of RPS, we employed the following system of notation; PRS $x \cdot y \cdot y \cdot \cdots$, where $x$ is a specific isolation number and $y$ is a single passage in EPC (*epithelioma papulosum cyprini*) cells. Six of the RPS preparations (RPS 2.1.1.1, RPS 3.1.1.1, RPS 4.1.1.1, RPS 4.1.2.1, RPS 6.1.1, RPS 7.1.1.1) have been passed two or three times in EPC and were purified by sucrose density centrifugation. One inoculum (RPS 4) was a
crude extract from shrimp tissues and the other (RPS 4.1) was a crude preparation of the virus from its first passage in EPC cells.

Four of the RPS isolates (RPS 2, 3, 4 and 6) were recovered from shrimp collected from different shrimp farm locations in Hawaii. RPS 7 was isolated from shrimp specimens that originated from a shrimp pond in Ecuador. RPS 2, 4 and 6 were isolated from P. stylirostris while RPS 3 and 7 were isolated from P. vannamei. RPS 2 and 7 were recovered from frozen shrimp tissues, while RPS 3, 4 and 6 were isolated from freshly killed shrimp.

Shrimp Bioassay and Virus Exposure—Study 2

In Study 2, larger 15–16 gram P. stylirostris were used to determine the distribution of RPS in the organs and tissues of experimentally inoculated shrimp. The animals were maintained as described in Study 1. Twelve shrimp in the test group were inoculated intramuscularly with 0.10 ml of sucrose-banded RPS 4.1.1.1 (6.5 × 10^7 PFU/shrimp). Six shrimp served as the control group and were inoculated with PBS. The shrimp were sacrificed on day 6 (virus recovery and immunofluorescence), 10 (virus recovery) and 21 (virus recovery, immunofluorescence and histopathology). At each period one shrimp was sacrificed for each of the parameters evaluated.

Oka organs from two shrimp in the control group and two shrimp in the RPS-inoculated group were collected at 21 days post-inoculation, grossly examined and then weighed individually. The weights were then averaged and compared.

Virus Recovery

Rhabdovirus was recovered from whole shrimp by the following method. Shrimp were suspended in TNE (Tris-NaCl-EDTA) buffer (10 ml per shrimp) and homogenized in a Brinkmann Polytron PT 3000 homogenizer and the homogenate was centrifuged at 1865 × g for 15 min, 3,000 × g for 30 min, and at 12,000 × g for 15 min, respectively. The final supernate was filter sterilized and then used to assay for RPS in EPC cells according to procedures described by Lu et al. (1991). The dilution and infection procedures inherent in the isolation and plaque assay allow only for a sensitivity limit of 200 or more plaque-forming units per shrimp. In Study 1, one shrimp each was used to recover RPS 2.1.1.1, RPS 3.1.1.1, RPS 4.1.2.1, RPS 6.1.1, and RPS 7.1.1.1. Three shrimp were used to recover RPS 4.1.1 and any virus in the buffer control. Four shrimp were used to recover RPS 4.1 and five shrimp were used to recover RPS 4. Nine shrimp were used for virus recovery from presample shrimp.

Virus recovery from various tissues and organs of the shrimp were carried out as follows. Organ tissues were removed by dissection from individual shrimp and grounded separately in a mortar and pestle with sterile sand and TNE buffer. The homogenates were centrifuged 2× at 1,300 × g for 5 min and incubated in an antibiotic fortified media for 30 min at 4°C. Dilutions of the sample were assayed for infectivity in EPC cells according to procedures described by Lu et al. (1991).

Immunofluorescence Assay

Impression smears of the Oka organs from both inoculated and control shrimp (days 6 and 21—Study 2) were made on glass microscope slides, air dried, and briefly fixed with acetone. The slides were soaked in 5% skim milk/PBS for one hour at room temperature (rt), washed 2× with PBS, and treated with primary antibody (1:500 rabbit anti-RPS IgG in 1% skim milk/PBS) for 2 hours at rt. After washing 2× with PBS, the slides were treated with secondary antibody (1:200 goat anti-rabbit IgG FITC conjugate in 1% skim milk/PBS) for 1 hour at rt, washed 2× with 0.05% Tween 20/PBS and examined under a fluorescent microscope.

Histopathology

Shrimp were sacrificed by injection and immersion in Davidson fixative (Humason, 1979). Shrimp tissues were processed by routine methods for histopathology and tissue sections were stained with hematoxylin and eosin or by a Feulgen method (Humason, 1979).

Results

Survival

The survival and RPS recovery results for Study 1 are summarized in Table 1. In the eight groups inoculated either with shrimp tissue extract or purified RPS the survival of shrimp ranged from 50 to 100% (average = 72.5%). In the PBS control
Infection of *Penaeus stylirostris* with RPS

In Study 2, the survival of shrimp was 67% in both the buffer control group and the experimental group.

**Oka Organ Wet-Weight**

The average weight of the Oka organs from two different shrimp at day 21 was 15 ± 5 mg in the control and 110 ± 10 mg in the RPS-inoculated shrimp. Thus, RPS infection resulted in gross enlargement of the Oka organs in the inoculated shrimp. Similar results were obtained when the experiment was repeated (107 ± 11 mg in the RPS-inoculated versus 17.8 ± 0.3 mg in the uninoculated control group).

**Histopathology**

In RPS-inoculated experimentals the Oka

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**Table 1. RPS inoculation and recovery results**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Dose&lt;sup&gt;a&lt;/sup&gt; (PFU/shrimp)</th>
<th>Survival</th>
<th>Yield&lt;sup&gt;b&lt;/sup&gt; (PFU/Shrimp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Presample</td>
<td>0</td>
<td>—</td>
<td>0</td>
</tr>
<tr>
<td>PBS Control</td>
<td>0</td>
<td>6/10</td>
<td>0</td>
</tr>
<tr>
<td>RPS 2.1.1.1</td>
<td>2.5 × 10⁶</td>
<td>5/10</td>
<td>0</td>
</tr>
<tr>
<td>RPS 3.1.1.1</td>
<td>5.9 × 10⁶</td>
<td>8/10</td>
<td>1.2 × 10⁶</td>
</tr>
<tr>
<td>RPS 4</td>
<td>N.D.</td>
<td>8/10</td>
<td>0</td>
</tr>
<tr>
<td>RPS 4.1</td>
<td>7.5 × 10⁶</td>
<td>7/10</td>
<td>1.3 × 10⁶</td>
</tr>
<tr>
<td>RPS 4.1.1</td>
<td>3.0 × 10⁷</td>
<td>6/10</td>
<td>4.2 × 10⁷</td>
</tr>
<tr>
<td>RPS 4.1.2.1</td>
<td>1.1 × 10⁷</td>
<td>7/10</td>
<td>3.8 × 10⁷</td>
</tr>
<tr>
<td>RPS 6.1.1</td>
<td>7.8 × 10⁶</td>
<td>10/10</td>
<td>1.0 × 10⁷</td>
</tr>
<tr>
<td>RPS 7.1.1.1</td>
<td>7.2 × 10⁶</td>
<td>7/10</td>
<td>8.0 × 10⁶</td>
</tr>
</tbody>
</table>

<sup>a</sup> Ten 5–6 gram juvenile shrimp were each inoculated intramuscularly (IM) with 0.1 ml of RPS. Control animals were inoculated with PBS. PFU = plaque forming units.

<sup>b</sup> Total virus yield per animal determined at 21 days postinoculation. Plaque assay used to recover virus had a detection limit of 200 PFU per animal.

<sup>c</sup> Not done.

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**Fig. 1.** Photomicrograph of proliferative centers (PCs) and adjacent Oka organ tissue from a *Penaeus stylirostris* experimentally inoculated with RPS. A capsule of layered hemocytes surrounds the larger PC. Within this PC are hyperplastic cells of which some are necrotic; many have clear cytoplasmic vacuoles or variable-sized, single to multiple, cytoplasmic inclusions. Hematoxylin and Eosin stain; bar = 25 μm.
organ had numerous and extremely large hyperplastic nodules (so designated as proliferative centers or PCs [Fig. 1]). Cells in the PCs had one or more of the following changes: hypertrophic nuclei, cytoplasmic vacuolation, variable-sized basophilic, Feulgen-positive or negative cytoplasmic inclusions. Sites of cellular necrosis were commonly present in PCs from the RPS-exposure groups. While PCs were also observed in the control shrimp, the lesions were fewer and smaller. A more detailed description of the histopathology changes found is currently being prepared (manuscript in preparation).

Recovery and Localization of RPS in the Shrimp

Rhabdovirus was re-isolated from six of the eight groups exposed to RPS on day 22 following inoculation. Virus was not recovered from the shrimp inoculated with RPS 2.1.1.1 and RPS 4, the buffer control, and the presample control groups. Attempts to recover RPS from the different organ tissues of experimentally inoculated *P. stylirostris* are summarized in Table 2. RPS was reisolated from the Oka organs of these shrimp on day 6, 10 and 21 but not from the other organs.

Impression smears of the Oka organ from individual shrimp were stained with IFA on day 6 and 21 and virus-specific immunofluorescence

<table>
<thead>
<tr>
<th>Organ</th>
<th>PFU per Organ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 6</td>
</tr>
<tr>
<td>Oka</td>
<td>$2.7 \times 10^3$</td>
</tr>
<tr>
<td>Gill</td>
<td>0</td>
</tr>
<tr>
<td>Heart</td>
<td>0</td>
</tr>
<tr>
<td>Nerve Cord</td>
<td>N.D.</td>
</tr>
<tr>
<td>Hepatopancreas</td>
<td>N.D.</td>
</tr>
<tr>
<td>Intestine</td>
<td>N.D.</td>
</tr>
<tr>
<td>Stomach</td>
<td>N.D.</td>
</tr>
<tr>
<td>Soft Head Tissues</td>
<td>N.D.</td>
</tr>
<tr>
<td>Body Muscles</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

*P. stylirostris* were inoculated intramuscularly with $6.5 \times 10^7$ of RPS. At various periods post-infection one animal was sacrificed and the various organs and tissues processed and assayed for RPS in EPC cells. PFU = plaque-forming units.

a Not done.
Infection of *Penaeus stylirostris* with RPS (Fig. 2) was seen only in impression smears of the Oka organ of infected shrimp sampled on day 21.

**Discussion**

The low survival of shrimp in both the buffer control and the inoculated groups is attributed to probable variable water quality conditions within the bioassay tanks and the relatively long (21 days) period of maintenance of shrimp which encouraged cannibalism during molting.

The results presented above clearly showed that RPS was able to infect the *P. stylirostris* shrimp and virus could be recovered in increasing concentration up to 22 days post inoculation (p.i.), although at relatively low concentrations. We have no explanation at this point why virus could not be recovered from shrimp exposed to either RPS 2.1.1.1 or RPS 4.

Replication of the RPS was localized only in the Oka organ of *P. stylirostris* and was demonstrated both by virus reisolation and immunofluorescence staining. The significantly larger size of the Oka organ in the RPS-infected shrimp is consistent with an increased activity in the organ perhaps brought about by the virus infection.

Initial histological examination of the Oka organs showed an abundance of very large proliferative centers. The predominant cell type in these centers had a large nucleus with chromatin arranged along the nuclear membrane and a prominent central nucleolus (manuscript in preparation).

The present study does not clarify the role of RPS in the causation of disease in penaeid shrimp. Since the studies were conducted in preadult shrimp, additional studies must be conducted on shrimp at different stages of growth and under different supplementary conditions in order to determine susceptibility to infection and disease expression.

It was earlier believed that RPS may be associated with IHNN disease. However, the present results do not support a direct association of RPS in IHNN disease causation.

The predilection of RPS for the Oka organs strongly suggests that the virus could conceivably affect the natural defense system of the shrimp and consequently render the animals more susceptible to infection by other biotic agents. This phenomenon is currently under investigation.

**Acknowledgements**

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**References**
