INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.
Isolation and characterization of a rhabdovirus isolated from cultured penaeid shrimps

Lu, Yuanan, Ph.D.

University of Hawaii, 1992
ISOLATION AND CHARACTERIZATION OF A RHABDOVIRUS ISOLATED FROM CULTURED PENAEID SHRIMP

A DISSERTATION SUBMITTED TO THE GRADUATE DIVISION OF THE UNIVERSITY OF HAWAII IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN MICROBIOLOGY
August 1992

BY

YUANAN LU

Dissertation Committee
Philip C. Loh, Chairman
Leslie R. Berger
James A. Brock
Roger S. Fujioka
Fred I. Kamemoto
ACKNOWLEDGEMENTS

I would like to express my deep thanks and great appreciation to many persons whose support and assistance made this work possible.

First and foremost, I would like to thank Dr. Philip C. Loh, my major professor and my committee chairman, for providing me the opportunity to work in his laboratory, for his endless supporting, consistent assistance and helpful suggestions in the conduct of this work, and for his friendship, his enthusiasm, and his great consideration.

I am especially grateful to Dr. Fred I. Kamemoto for his willingness to be my outside committee member. Thanks to Drs. Leslie R. Berger and Roger S. Fujioka for serving as the members of my graduate committee and for their advice and friendship. Appreciation is also expressed to Dr. Jame A. Brock for providing me the infected animal samples, for his assistance in carrying out all the bioassay experiments, and for his critical suggestions.

My special appreciation is extended to Dr. Elpidio Cesar B. Nadala Jr. for assistance with this manuscript and his friendship over the years.

Thanks to the staff and faculty of the Department of Microbiology. Especially to the Department office, Margaret Morimoto and Betty Ueda, for their kind help and consideration during these years.
Appreciation to Tina Carvalho and Dr. Marylyn Dunlap for letting me use the TEM and for their technical assistance, and to Dr. Susan Ayin, Nancy Liao, Sam, and Leonard Pollard for various forms of assistance rendered during this study.

Finally I would like to express my great thanks to my family. A special thank to my parents-in-law and to my brother and sister-in-law, Daan and Xiulan, for their love encouragement, support, and patience. My warmest thanks to my wife, Chunmei Wu, for her endless love and care, her understanding and encouragement, and for being there whenever I needed her.
ABSTRACT

Viral diseases have resulted in great losses of cultured penaeid shrimp. Little is known about the diseases and the causative agents. The present study describes the isolation of a new virus from two species of penaeid shrimp collected from Hawaii and Ecuador. The virus has a bullet-shaped structure measuring 65-77 x 115-138 nm and is identified to be a member of the family, Rhabdoviridae. The virus was named Rhabdovirus of Penaeid Shrimp (RPS).

The virus was found to replicate in an established heteroploid fish cell line, epithelioma papillosum cyprini (EPC), originated from the carp. In the cell line the virus produced a marked cytopathic effect characterized by focal areas of cellular destruction surrounded by rounded-refractile cells. The virus was sensitive to low pH, lipid solvents, and high temperatures and was unstable to freezing-thawing and low temperature storage. Optimal temperature for virus replication was 20 °C. The non-interference of viral replication by the halogenated nucleic acid antagonist 5-bromo-2'-deoxyuridine (BUDR) indicated an RNA genome.

A plaque assay protocol was developed for viral titration and the optimum conditions for the plaque assay were determined. Efficiency of plating (EOP) was 30 viral particles per infectious unit in EPC cells. The density of the intact virus in sucrose gradients was 1.19 g/cc. SDS-polyacrylamide gel electrophoresis of viral polypeptides
revealed four major virus proteins with molecular weights of 165,000 (L), 67,000 (G), 44,500 (N), and 28,000 (M) daltons. Analysis of viral RNA on polyacrylamide-agarose gel indicated a non-segmented single-stranded RNA genome with a molecular weight of $3.6 \times 10^6$ daltons. Serological studies employing neutralization kinetics, plaque reduction and Western blot procedures indicated that the RPS could be distinguished from three fish rhabdoviruses.

A nitrocellulose-enzyme immunoassay for the detection of RPS antigen was developed and revealed a sensitivity of detection of 1 nanogram of RPS protein or an equivalent of $7 \times 10^4$ virus particles.

In vivo studies revealed that the virus infection occurred only in the Oka organ which consequently resulted in cytopathic changes. However, under the present experimental conditions pathogenicity for penaeid shrimp is limited and the infection did not directly result in mortality.
# TABLE OF CONTENTS

ACKNOWLEDGEMENTS ............................................. iii  

ABSTRACT .................................................................. v  

LIST OF TABLES ....................................................... xii  

LIST OF ILLUSTRATIONS ........................................ xiv  

I. INTRODUCTION ...................................................... 1  

II. LITERATURE REVIEW ........................................... 4  
   A. Shrimp Aquaculture ............................................. 4  
   B. Viral Diseases of Penaeid Shrimp ......................... 10  
   C. Rhabdoviruses of Crustacean ............................... 27  

III. MATERIALS AND METHODS .................................... 33  
   A. Cell Culture Containers and Media ....................... 33  
   B. Cell Lines Used in Study ..................................... 35  
   C. Virus Extraction and Concentration from Infected Animals ........................................... 37  
   D. Virus Isolation and Purification ......................... 38  
   E. Preparation of Virus Stocks ............................... 40  
   F. Other Viruses Used in This Study ......................... 40  
   G. Staining and Microscopy .................................... 43  
      1. Staining of Infected Cells ............................... 43  
      2. Light Microscopy and Dissecting Microscopy ......... 45  
      3. Electron Microscopy ..................................... 45  
   H. Titration of Virus ............................................ 47  
      1. Development of Plaque Assay Technique ............ 47  
      2. Comparison of Plaque Assay and TCID\(_{50}\) Assay ......................................................... 48  
      3. Comparison of Plaque Assay Overlays ............... 49  
      4. Comparison of Plaque Forming Efficiency in Selected Cell Culture Wares .................. 50  
      5. Comparison of Plaque Forming Efficiency in Selected Fish Cell Lines .................. 51
6. Relationship between Volume of Inoculum and Number of Plaques..........................51
7. Relationship between Adsorption Times and Virus Titer.....................................52
8. Relationship between Selected Postincubation Times and Virus Titer....................53
9. Efficiency of Plating (EOP) in EPC Cells..........................................................53

I. Propagation of Virus..................................................58
   1. Single-Cycle Growth Curve in EPC Cells.........................................................58
   2. Cell-Associated and Released Virus.................................................................58
   3. Virus Replication in Selected Fish Cell Lines....................................................59
   4. Virus Replication at Selected Temperatures.......................................................60
   5. Virus Replication in Three Growth Media ..........................................................60
   6. Relationship between Multiplicities of Infections (MOI) and Virus Yields.................61

J. Serological Experiments..................................................62
   1. Preparation of Anti-RPS serum .....................................................................62
   2. Neutralization Experiments..............................................................................63
   3. Fluorescent Antibody Staining...........................................................................65
   4. Nitrocellulose-Enzyme Immunoassay (NC-EIA).....................................................66

K. Biochemical and Biophysical Techniques..................................................70
   1. Stability of RPS to Freezing-Thawing.................................................................70
   2. Stability of RPS Stored at -10 and -70 °C............................................................71
   3. Stability of RPS Incubated at 4, 20, 37 and 56 °C..............................................72
   4. Stability at Low pH (pH 3)..................................................................................72
   5. Stability to Lipid Solvents....................................................................................73
   6. Replication of the RPS in the Presence of 5-bromo-2'-deoxyuridine..........................74
   7. Density of RPS in Sucrose Gradient.......................................................................75
   8. Protein Analysis in Polyacrylamide Gels...............................................................76
   9. Western Blot Experiment......................................................................................79
  10. RNA Analysis in Polyacrylamide-Agarose Gels.....................................................81

L. In Vivo Studies.................................................................83
   1. Pathogenicity of RPS for Penaeid Shrimp.............................................................83
2. Virus Replication in Different Organs and Tissues of Penaeid Shrimp.........................84
3. Pathology in Virus Infected Penaeid Shrimp.85
4. Immunofluorescent Staining of the lymphoid organs from Infected Penaeid Shrimp...........86

IV. RESULTS.........................................................87

A. Isolation of Rhabdovirus of Penaeid Shrimp......87
B. Staining and Microscopy.........................92
1. Staining of Infected EPC Cells..................92
2. Morphology of the Virus.........................92
3. Electron Microscopy of Infected Cells...........98
C. Titration of Virus.................................101
1. Plaque Assay Technique...........................101
2. Comparison of Plaque Assay and TCID50 Assay.....................................101
3. Comparison of Plaque Assay Overlays.........104
4. Comparison of Plaque Forming Efficiency of Virus in Selected Cell Culture Containers.........................106
5. Comparison of Plaque Forming Efficiency of Virus in Selected Fish Cell Lines...........108
6. Relationship between Volume of Inoculum and Plaque Formation.................................113
7. Relationship between Adsorption Times and Plaque Production.................................115
8. Relationship between Postinfection Time and Virus Titer........................................118
9. Efficiency of Plating (EOP) in EPC Cells..120
D. Propagation of virus.................................124
1. Single-Cycle Growth Curve in EPC Cells...124
2. Comparison of Cell-Associated and Released Virus.................................................124
3. Comparison of Virus Replication in Different Fish Cell Lines.................................130
4. Virus Replication at Selected Temperatures........................................................132
5. Comparison of Virus Replication in Three Growth Media.........................................135
6. Relationship between Multiplicities of Infection and Virus Yield..................135

E. Serological Experiments.................................138
1. Preparation of Immune Serum in Rabbits......138
2. Neutralization Experiments.........................138
3. Fluorescent Antibody Staining of Infected Cells.................................142
4. Nitrocellulose-Enzyme Immunoassay (NC-EIA).................................147

F. Biochemical and Biophysical Properties of the Virus..........................157
1. Stability to Freezing-Thawing.......................157
2. Stability of Virus Stored at -10 °C and -70 °C.................................159
3. Stability at 4, 20, 37, and 56 °C......................161
4. Stability at Low pH.....................................161
5. Stability to Lipid Solvents.............................165
6. Replication of Virus in the Presence of 5-bromo-2'-deoxyuridine (BUDR).........169
7. Buoyant Density of Virus in Sucrose Gradients.................................171
8. Protein Analysis in Polyacrylamide Gels....171
9. Western Blot.............................................174
10. Viral RNA Analysis in Polyacrylamide-Agarose Slab Gels..................179

G. In Vivo Experiments........................................182
1. Pathogenicity of Virus for Penaeid Shrimp..................................182
2. Virus Replication in Different Organs and Tissues of Penaeid Shrimp.........182
3. Pathology in Virus Infected Penaeid Shrimp..................................183
4. Immunofluorescent Staining of Lymphoid Organs Prepared from Infected Penaeid Shrimp.............................................188

H. Characteristics of Rhabdovirus of Penaeid Shrimp.............................191
V. DISCUSSION ......................................................... 192
VI. SUMMARY AND CONCLUSION ................................. 209
    LITERATURE CITED .............................................. 213
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>12</td>
</tr>
<tr>
<td>2</td>
<td>34</td>
</tr>
<tr>
<td>3</td>
<td>36</td>
</tr>
<tr>
<td>4</td>
<td>42</td>
</tr>
<tr>
<td>5</td>
<td>105</td>
</tr>
<tr>
<td>6</td>
<td>107</td>
</tr>
<tr>
<td>7</td>
<td>114</td>
</tr>
<tr>
<td>8</td>
<td>119</td>
</tr>
<tr>
<td>9</td>
<td>123</td>
</tr>
<tr>
<td>10</td>
<td>131</td>
</tr>
<tr>
<td>11</td>
<td>136</td>
</tr>
<tr>
<td>12</td>
<td>137</td>
</tr>
<tr>
<td>13</td>
<td>139</td>
</tr>
</tbody>
</table>
14 Binding curve for rabbit IgG and rhabdovirus of penaeid shrimp.................................148

15 Binding curve for secondary antibody to rabbit IgG......................................................151

16 Endpoint titration of rhabdovirus of penaeid shrimp using NC-EIA....................................154

17 Stability of rhabdovirus of penaeid shrimp to freezing and thawing...............................158

18 Stability of rhabdovirus of penaeid shrimp to storage at cold temperature.........................160

19 Stability of rhabdovirus of penaeid shrimp, rhabdovirus carpio and poliovirus type 1 following incubation at pH 3 for 3 h at 22 °C........164

20 Titers of rhabdovirus of penaeid shrimp, rhabdovirus carpio and poliovirus type 1 following treatments with ethyl ether and chloroform at 4 °C for 24 h.............................168

21 Comparison of the effect of 5-bromo-2'-deoxyuridine on the replication of rhabdovirus of penaeid shrimp, rhabdovirus carpio, channel catfish virus, vaccinia, and poliovirus type 1..............170
# LIST OF ILLUSTRATIONS

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Air-fuge ultracentrifuge employed in determination of efficiency of plating.</td>
</tr>
<tr>
<td>2</td>
<td>Hybri-slot manifold used in the nitrocellulose-enzyme immunoassay (NC-EIA).</td>
</tr>
<tr>
<td>3</td>
<td>Phase contrast photographs of EPC and GCSB cells infected with MEM-0 and with rhabdovirus of penaeid shrimp at 3 days p.i. at 20 °C. (A) GCSB control, (B) GCSB infected with RPS, (C) EPC control, and (D) EPC infected with RPS.</td>
</tr>
<tr>
<td>4</td>
<td>Phase contrast photomicrographs of EPC cells infected with rhabdovirus of penaeid shrimp at 3 days (A) and 5 days (B and C) p.i. at 20 °C.</td>
</tr>
<tr>
<td>5</td>
<td>Electron micrographs of rhabdovirus of penaeid shrimp negatively stained with 2% uranyl acetate. (A) Virus particles purified by sucrose density gradient centrifugation. (B) A series of transverse striations along entire length of some virus particles.</td>
</tr>
<tr>
<td>6</td>
<td>Electron micrographs of a negatively stained rhabdovirus of penaeid shrimp. (A) Showing some viral peplomers still intact. (B) A highly magnified transverse section of virus showing the helical nucleocapsid, dark staining envelope, and the knoblike peplomers.</td>
</tr>
<tr>
<td>7</td>
<td>Electron micrographs of thin sections of EPC cells infected with rhabdovirus of penaeid shrimp. (A) Viruses are present outside of the cytoplasmic membrane. An arrow showing a virus particle budding through the cytoplasmic membrane. (B) Viruses localized within a cytoplasmic vesicle.</td>
</tr>
<tr>
<td>8</td>
<td>Linear relationship between virus dilutions and the plaque titers of rhabdovirus of penaeid shrimp established in EPC cells at 20 °C.</td>
</tr>
<tr>
<td>9</td>
<td>Plaque forming efficiency of rhabdovirus of penaeid shrimp in different fish cell lines.</td>
</tr>
<tr>
<td>10</td>
<td>The plaques of rhabdovirus of penaeid shrimp formed in EPC cells (A) and GCSB cells (B) stained with 1% crystal violet.</td>
</tr>
</tbody>
</table>
11 Rate of adsorption of rhabdovirus of penaeid shrimp in EPC cells in six-well plates .............. 116

12 Electron micrographs of 2% of uranyl acetate stained highly purified rhabdovirus of penaeid shrimp. (A) Even distribution of virus in a field under a TEM prepared for viral particle counting. (B) High magnification of the virus ....... 121

13 Single cycle growth curve of rhabdovirus of penaeid shrimp in EPC cells incubated at 20 ºC for 2 days ........................................ 125

14 Cell-associated and released virus in EPC cells at 20 ºC ........................................... 128

15 Production of rhabdovirus of penaeid shrimp in EPC cells at different incubation temperatures ........................................ 133

16 Percentage of plaque reduction of rhabdovirus of penaeid shrimp and rhabdovirus carpio by anti-RPS serum ........................................ 140

17 Rates of neutralization of rhabdovirus of penaeid shrimp, rhabdovirus carpio, infectious hematopoietic necrosis virus, and viral hemorrhagic septicemia virus by anti-RPS serum as measured by reduction in numbers of plaques by serum-virus mixtures inoculated in monolayer cultures of EPC cells ........................................ 143

18 Immunofluorescent antibody staining of EPC cells infected with rhabdovirus of penaeid shrimp for 48 h at 20 ºC ........................................ 145

19 Binding curve of rabbit IgG to rhabdovirus of penaeid shrimp ........................................ 149

20 Binding curve of goat IgG against rabbit serum ...... 152

21 Photograph of NC-EIA endpoint titration of rhabdovirus of penaeid shrimp using rabbit IgG .... 155

22 Stability of rhabdovirus of penaeid shrimp suspended in MEM-0 to incubation at 4, 20, 37 and 56 ºC ........................................ 162
23 Electron micrographs of negatively stained rhabdovirus of penaeid shrimp particles (A) after exposed to pH 3 for 3 h at room temperature and (B) following the incubation with ethyl ether for 24 h at 4 °C..................................................166

24 Density of rhabdovirus of penaeid shrimp in sucrose gradients......................................172

25 Electropherograms of viral proteins after electrophoresis for 3.5 hr at 200 v in a 10% polyacrylamide slab gel. Stained with silver stain.................................................................175

26 Western blot analysis of viral structural proteins which were transferred to nitrocellulose membranes and reacted with anti-RPS serum..................177

27 Electropherograms of acrylamide-agarose gels stained with silver nitrate after electrophoresis of the viral RNAs extracted from rhabdovirus of penaeid shrimp (RPS) and vesicular stomatitis virus (VSV)......................................................180

28 Microphotographs of sections of the Oka organ stained with hematoxylin and eosin (mag. 100x). (A) RPS infected and (B) uninfected control...........184

29 Microphotographs of sections of the Oka organ stained with hematoxylin and eosin (mag. 200x). (A) RPS infected and (B) uninfected control...........186

30 Photographs of impression smears of the Oka organs harvested from Penaeus stylirostris stained with immunofluorescent antibody against rhabdovirus of penaeid shrimp. (A) The RPS-infected Oka organ smear. (B) Control lymphoid organ smear..............189
I. INTRODUCTION

The high demand for shrimp and the limited supply of shrimp in the world market has stimulated tremendous developments in shrimp aquaculture in many countries around the world. However, the rapid growth of the penaeid shrimp culture industry has been accompanied by an increased awareness of the negative impact of diseases on the industry, particularly in the area of viral diseases.

In the last 10 years, seven viruses (2 baculoviruses, 3 parvo-like viruses, 1 reo-like virus, and 1 rhabdovirus) have been recognized in the cultured species of penaeid shrimp. Some of these shrimp viruses were reported to cause significant epizootics in the population of penaeid shrimp and resulted in great losses of the animals. Little is known concerning the properties of these viruses and only a very few have been extensively characterized.

Although the viral diseases of penaeid shrimp have received considerable attention in recent years, the progress in studying these viral diseases has been very slow. The major problem has been the lack of simple and adequate methodologies and technologies for the study of these viral agents. Thus, there is no simple procedure established for detecting the presence of the viruses in cultured shrimp populations and for the isolation of the viral agents from the infected animals. All the reported shrimp viruses have not been found to replicate in established cell cultures nor
are they well characterized. There are no serological or immunological methods presently developed for the routine use in identification of viral antigens or the intact virus.

Three methods including direct microscopical (wet-mount) examination, histopathology, and bioassay are currently used for screening penaeid shrimp stocks for virus infections. Problems associated with these protocols are that they are time consuming, tedious, not cost-effective, and not sufficiently sensitive. Therefore, there is need for the development of simpler, more rapid and highly sensitive methods to study the shrimp viruses.

In order to solve these problems, four approaches have been adopted in the present study:

The objective of the first approach was the development of a relatively simple protocol for the consistent recovery and isolation of RPS from infected shrimp. A simple, reproducible and effective procedure would also facilitate the isolation of other shrimp viruses.

The objective of the second approach was to look for an established cell line which would support the replication of RPS. Such a cell line would provide a sensitive and convenient cell culture system for the primary isolation and detection of other shrimp viruses. Also, this cell culture system will very importantly contribute to the efforts to verify "virus-free" shrimp brood stock which is vital for successful shrimp aquaculture.
The objective of the third approach was to characterize the viral isolate in terms of its biological, biochemical and physical properties. This approach would provide invaluable information to understanding the viral agent itself and the associated disease if any.

The objective of the last approach was the development of safe, simple, rapid, highly sensitive, and reproducible immunologically-based assay which would be invaluable for laboratory research and for the early detection of viral infections in the cultured shrimp populations.
II. LITERATURE REVIEW

A. Shrimp Aquaculture

Human beings have been constantly searching for new means of food production in the face of a rapidly expanding world population and a developing world food crisis. Fish have been an important protein source since ancient times. Today, the prospects for increased availability of marine fish is dim since wild fishery production has been at maximum capacity in the past few years and is on the decline due to overfishing and increasing operating costs (Aquacop, 1985; Trust, 1986). Crustaceans are a favorite animal food source and they have become another very important protein source for the United States and other developed countries.

Marine shrimp farming is the commercial production of marine shrimp in impoundments, ponds, raceways and tanks. It is a century-old practice which traces its origins to Southeast Asia where farmers raised incidental crops of wild shrimp in tidal fish ponds. The culture technique in traditional shrimp farming was entirely dependent on wild-caught postlarvae or gravid females present in the surrounding waters. Wild shrimp fry either enter during tidal water exchange or are intentionally gathered from the wild and stocked directly in ponds. Production is totally dependent on two factors: the seasonal availability of wild fry which fluctuates widely from year to year and natural
pond fertility since fertilizers and feeds are not generally used. Over the years, some improvements in the traditional methods of culturing shrimp have gradually evolved. However, large scale development of the industry has not been reached because the supply of seed from the wild is often inconsistent and insufficient. Until 20 years ago, this commodity was still generally considered a secondary crop in traditional fish farming practice.

Modern shrimp farming did not begin until the 1930's when hatchery work started with Kuruma shrimp (P. japonicus) in Japan. In 1934, Fujinaga successfully spawned and partially reared larvae of P. japonicus (Hudinaga, 1935) The success in larval rearing and the subsequent grow-out of the shrimp had brought the art to a point where mass culture was possible. This technique led to the mass production of postlarvae from wild-caught gravid females and even from wild-caught adults (Lumare, 1979) induced to mature and mate in captivity. Broodstock could be maintained in captivity all year around and through successive generations. The technique was later adopted in other countries for their local species. A total of 24 penaeid species have matured in captivity, 14 of which have successfully spawned. A constant supply of large quantities of shrimp seeds and fry became available and completely eliminated dependence on the wild in most advanced shrimp farms. At present, shrimp farming technique has advanced to a point where the whole shrimp
culture processes can be controlled. This includes broodstock in captivity through successive generations, massive production of postlarvae, pregrowing of high quality juveniles, and water quality and feeding management in the relatively high-density growing phase. The yield has increased from 0.1-0.3 tons per hectare (ha) per year (yr) in traditional culture to 5-10 tons/ha/yr in the present semi-intensive culture (Kungvankij, 1976; Liao, 1977).

In shrimp farming, a complete production system consists of five major steps. These include broodstock in captivity and reproduction (hatchery), larval rearing and pregrowing (nursery), and growth-out. The first two are the key steps in shrimp farming since commercial shrimp farms rely on the availability of seed. In fact, insufficient availability of postlarvae is a common limiting factor for shrimp farms.

The grow-out phase where stocked juvenile shrimp grow up to commercial size (15-45 gram) takes from 3 to 6 months. It is the most important step from an economic point of view because it includes the major costs of labor and feeding. Based on the shape and size of the water body used to cultivate the shrimp, grow-out operations can be characterized as extensive, semi-intensive, or intensive (Wickins, 1976).

Extensive culture is done at low density (less than 1 shrimp/m²) and is common only where large areas are available. In general, the shrimp are reared in natural
bodies of water with little or no management, and the yields are small and operating costs low. Semi-intensive culture is done at medium density (5-15 animals/m$^2$). The shrimps are raised in ponds, cages, or tanks with some sort of management, i.e. introduction of a nursery phase, pond feeding and diesel pumping. Semi-intensive culture is the most common production technique. Intensive culture is done at high density (several hundred animals per square meter). The shrimp are raised in tanks and raceways and are intensively managed, i.e. strong aeration, huge renewal of water, frequently high quality feeding, and waste removal. As the culture density increases, the farms get smaller, the technology gets more sophisticated, the capital costs go up and the production per unit area increases dramatically.

Penaeid shrimp are the major animal species for shrimp aquaculture in the world. There are a total of 318 species belonging to the shrimp family, Penaeidae. Of these some 109 species have present or potential commercial value (Holthuis, 1980) and are reared in grow-out ponds and tanks. There are only nine species (P. merguiensis, P. japonicus, P. monodon, P. semisulcatus, P. vannamei, P. orientalis (chinesis), P. setiferus, P. kerathurus, and P. stylirostris) on which practical commercialized propagation has been carried out on a large scale.

Penaeid shrimp are the most valuable seafood product. There is a high demand for the shrimp in Japan, the United
States, and some European countries. Americans consumed over 500 million pounds of shrimp in the early 1980s'. In 1983, Japan imported 148,589 tons of frozen prawn and shrimp (Tan, 1984). In the same year, the United States consumed 216,400 tons of tropical shrimp, of which 155,800 tons were imported since the US shrimp fishery was unable to meet domestic demand. Therefore, the total animal market demand for frozen prawn and shrimp in the USA and Japan alone was about 300,000 tons (Apud, 1985). However, during the last few years the total imports in these two countries have increased to over 500,000 tons (227,000 tons for the US (Anon, 1991a) and 283,448 tons for Japan) in 1990 (Anon, 1991b).

Constant increases in shrimp demand and their limited supply in the world market have stimulated tremendous development in shrimp aquaculture in the last fifteen years. The high market demand and export price for shrimp, growing opportunities in shrimp farming, and generation of employment and foreign exchange earnings have encouraged many countries rich in aquatic resources in the region to place high priority on the development of the shrimp culture industry.

Today, shrimp aquaculture has become an established industry in many areas of the world including both developing and developed countries. Over forty countries have shrimp farms. In China, Ecuador, Indonesia, Thailand and Taiwan, farm revenues range from $300 to $600 million a year. Malaysia, India and the Philippines also have
active shrimp industries.

Shrimp farming in the United States has come a long way in the last fifteen years. At present, the United States has a relatively small shrimp farming industry. There are a total of 25 private sector shrimp farms and 8 hatcheries mainly in Puerto Rico, Hawaii, Texas and South Carolina (Anon, 1991c).

The increase in cultivation activity of marine shrimp has dramatically increased production of this valuable seafood. In 1980, the world's shrimp farmers produced only about 2% of the world's shrimp needs. In 1990, for the tenth year in a row, the world's shrimp farmers produced a record crop of 663,000 metric tons of whole shrimp. This record is up 17% from the harvest of 565,000 metric tons in 1989 (Anon, 1991d). Shrimp farmers now produce 25% of the shrimp placed on world markets (fishermen 75% out of a total market of 2.6 million metric tons).

There are still some problems facing the marine shrimp industry. In addition to water quality control and management, diseases have become a very serious problem both in hatcheries and rearing ponds. Diseases that are caused by infectious agents often result in great losses of postlarvae and juveniles. Treatment for bacterial infection is very difficult and expensive, and viral diseases are untreatable. Use of antibiotic therapy or prophylaxis in the farms has been discouraged because of the potential toxic effect on the
consumer. New technological developments including improved diets and improved intensive culture system have had a positive effect on the development of the industry. However, these developments have been very slow and are unable to meet the needs of the rapidly growing industry.

Marine shrimp farming technology has not been optimized even though commercial operations are already profitable. Research is urgently needed to solve existing problems and to speed up major improvements in shrimp culture techniques. This will lead to increased productivity with a corresponding decrease in costs.

As the world's demand for shrimp continue to increase, the production of farm-raised shrimp will continue to expand at the current rate. It has been predicted that the production of farmed-raised shrimp will capture 50% of the total market by the year 2000 (Anon, 1991). At that time, shrimp prices will stabilize at lower levels, and the world's consumers will have a new, moderately priced alternative to fish, red meat and chicken.

B. Viral Diseases of Penaeid Shrimp

Viral diseases are major economic problems in the shrimp aquaculture industry. Research investigations on the viral diseases of shrimp have been stimulated by the requirement of the knowledge concerning the viral diseases of the shrimp and the etiological agents themselves and by the world-wide
interest in shrimp aquaculture. Knowledge of the diseases of penaeid shrimps and prawns has been reviewed in a number of papers during the last 17 years (Aquacop, 1977; Brock et al., 1983; Brock and Lightner, 1990; Couch, 1981; Lightner, 1985, 1988; Lightner and Redman, 1991, 1992; Johnson, 1975, 1978; Overstreet, 1978, 1990; Sindermann, 1974). It is presently known that there are more than 30 known viruses affecting crustacean (Lightner et al., 1985), of which at least eight viral diseases are presently recognized in cultured penaeid shrimp (Table 1). All but one of these eight have been detected in cultured penaeids in the Americas (North, Central, and South America, Hawaii, and the Caribbean Islands). Each of these eight penaeid viral diseases may be caused by a multitude of individual strains. Some of the viruses including baculovirus penaei, monodon-type baculovirus, baculoviral midgut gland necrosis, and infectious hypodermal and hematopoietic necrosis virus, are highly pathogenic to some species of penaeid shrimp and have a significant effect on the development of shrimp aquaculture industry. However, other viral diseases are of only little significance to the industry and are reported only regionally.

**Baculovirus penaei (BP)** Baculovirus penaei was the first virus recognized from cultured penaeid shrimp, and was the first occurrence of nuclear polyhedrosis virus reported in animals other than insects and mites (Couch, 1974a). In 1974, Couch reported his discovery of a virus from cultured
Table 1. Summary of Known Penaeid Viruses

<table>
<thead>
<tr>
<th>Virus</th>
<th>Shape, Size nm</th>
<th>NA</th>
<th>Classification</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>BP</td>
<td>Rod, 75x300</td>
<td>dsDNA</td>
<td>Baculoviridae</td>
<td>Couch, 1974</td>
</tr>
<tr>
<td>BMN</td>
<td>Rod, 75x300</td>
<td>dsDNA</td>
<td>Baculoviridae</td>
<td>Lightner et al., 1981</td>
</tr>
<tr>
<td>MBV</td>
<td>Rod, 75x300</td>
<td>dsDNA</td>
<td>Baculoviridae</td>
<td>Sano et al., 1981</td>
</tr>
<tr>
<td>IHHNV</td>
<td>Icosahedron, 17-20 ssRNA</td>
<td>?</td>
<td>Lightner, 1983b; Lu et al., 1990</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Icosahedron, 22-25 ssDNA</td>
<td>Parvoviridae</td>
<td>Bonami et al., 1990.</td>
<td></td>
</tr>
<tr>
<td>HPV</td>
<td>Icosahedron, 22 ssDNA</td>
<td>Parvoviridae</td>
<td>Lightner and Redman, 1985</td>
<td></td>
</tr>
<tr>
<td>REO</td>
<td>Icosahedron, 60 dsRNA</td>
<td>Reoviridae</td>
<td>Tsing and Bonami, 1987</td>
<td></td>
</tr>
<tr>
<td>RPS</td>
<td>Bullet, 65-70x115-138 ssRNA</td>
<td>Rhabdoviridae</td>
<td>Lu et al., 1991</td>
<td></td>
</tr>
</tbody>
</table>

NA - Nucleic acid  
BP - baculovirus penaei  
BMN - baculoviral midgut gland necrosis virus  
MBV - P. monodon-type baculovirus  
IHHNV - infectious hypodermal hematopoietic necrosis virus  
HPV - hepatopancreatic parvo-like virus  
REO - reo-like virus  
RPS - rhabdovirus of penaeid shrimp  
LPV - lymphoidal paroviruse-like
pink shrimp (*Penaeus duorarum*). It looked similar to baculovirus, and localized in hepatopancreatic cells. Numerous tetrahedral crystalloid inclusion bodies were also recognized in nuclei of hepatopancreatic cells using light microscopy. Couch was able to demonstrate rod-shaped free (non-occluded) and occluded virions using electron microscopy. The viruses filled the nuclei of the affected cells. They are enveloped and rod-shaped with an average size of 288 nm long by 75 nm in diameter. This shrimp virus was named *Baculovirus penaei*. After its discovery, BP has been thoroughly studied in terms of its geographic distribution and host range (Couch, 1974b, 1978, and 1981; Couch et al., 1975; Overstreet, 1978; Lightner et al., 1983a).

*Baculovirus penaei* has been reported to be the cause of a serious hatchery epizootic disease in several penaeid species cultured in the northern coast of the gulf of Mexico and the Pacific Coast of Central America (Couch, 1978; Lightner et al. 1983a). Infection by the BP virus has historically affected penaeid shrimp production in both hatcheries and ponds. In larvae and post-larvae, BP epizootics are typically acute. Cumulative losses of the affected populations can approach 100% within 24-48 hours when infection becomes first apparent (Couch, 1978; Lightner, 1988). *Baculovirus penaei* may also cause disease and mortalities in juvenile and subadult animals of certain penaeid species (Couch, 1981). In addition to *P. duorarum*, *P.*
marginatus was found to be another penaeid species infected by BP in Hawaii (Brock et al. (1986). Both light and electron microscopic examination of juvenile P. marginatus indicated BP infection. The characteristics of the inclusion body induced in P. marginatus were similar to those reported for BP in P. duorarum except that the viruses found in P. marginatus were slightly smaller, 286.0 nm (270.8-296.1) in length by 54.4 nm (54.2-59.2) in diameter. It was concluded that the dissimilarity in size could indicate that the baculovirus identified from P. marginatus in Hawaii may be a heterologous strain of BP.

Baculovirus penaei infection is diagnosed by the demonstration of the tetrahedral inclusion bodies in the greatly hypertrophied nuclei of affected cells (Couch, 1978). In 1977, Summers described a serological technique which was feasible for direct identification of BP viral antigens. In 1988, McGuine et al. reported the growth and maintenance of shrimp hepatopancreatic cells from P. vannamei in vitro and the use of these primary cultured cells to replicate BP. During the same period, another research group (Overstreet et al., 1988) established a protocol for experimental infections with BP and indicated the system is very useful for detecting and assaying the infective agent in the white shrimp, P. vannamei.

Other studies involving BP were the transmission of BP in feral shrimp populations carried out by Couch (1978) and
transmission electron microscopic studies of BP-infected populations (Lightner et al. 1985). The latter indicated similar cytopathology and virus morphology in the three penaeid species, but there were small differences in the size of BP particles. In 1977, Summers published his studies on the selected biochemical and structural characteristics of BP. Recently, LeBlance and Overstreet (1991a) have tested the stability of BP to desiccation, pH, heat, ultraviolet irradiation (uv), and sea water (32 ppt). The bioassay tests were carried out by using larval *P. vannamei* and the results revealed that the virus is sensitive to all these treatments. *B. penaei* was completely inactivated after exposures to pH 3 for 30 min, 60-69 °C for 10-min, UV for 40 min, 32 ppt sea water for 7 d and desiccation for 48 h. In the same year, they did a similar study and found that infectivity of BP was completely inactivated after exposing the virus to calcium hypochlorite for a short time (800 mg/l for 1 h or 1,600 mg/l for 20 sec) (LeBlanc and Overstreet, 1991b). The first approach to purify shrimp baculovirus particles and then to extract viral DNA from the purified BP was recently reported by Bruce and his associates (1991).

**Monodon-type baculovirus (MBV)** Monodon baculovirus was the second virus reported from penaeid shrimp. It was the first reported virus from *P. monodon*. The first recognition of MBV was in 1977-1978 by Lightner and Redman (1981) from laboratory reared *P. monodon* adults which originated from
Taiwan. The virus was considered to be a serious pathogen for postlarval farms of *P. monodon* in high density tank and raceway cultures (Lightner and Redman, 1981, Lightner et al. 1983a).

Infection by MBV has been diagnosed in *P. monodon* from culture facilities or shrimp farms in Taiwan (Lightner et al., 1987), the Philippines, French Polynesia, Malaysia (Lightner, et al. 1983a), and Israel (Colorni et al., 1987). However, in the Americas, the virus has only been observed in populations of *P. monodon* imported from Indo-Pacific penaeid culture facilities into Hawaii (Lightner, 1988). A baculovirus, similar to MBV was found to infect postlarval and juvenile *P. plebejus* in Australia (Lester et al., 1987). However, light and electron microscopical studies indicated this isolate, Plebejus Baculovirus (PBV) is different from MBV in many aspects. The envelope of PBV has 2 electron-dense zones while MBV has only one. Furthermore, PBV bodies are Gram negative and strongly eosinophilic compared to MBV bodies which are Gram positive and very weakly eosinophilic.

Monodon baculovirus has also been found to infect cultured *P. semisulcatus* (Lightner et al., 1987) and *P. merguiensis* from other sites in Asia and the Middle East (Brock et al., 1983), and possibly *P. kerathurus* in Italy (Lightner et al., 1985). In 1986, Lightner et al. conducted an investigation of the disease problems affecting cultured shrimp in Southern Taiwan and revealed that MBV was the most
common pathogen in cultured stocks of P. monodon. Three years later, Chen et al. (1989) studied virogenesis and cytopathology of MBV using the two prawns, P. monodon (giant tiger) and P. penicillatus (red tail). Chen and his co-workers found virogenesis of MBV infection induced formation nucleic acid, capsid substance, envelopment and occlusion body in hepatopancreatocytes and the main cytopathologic changes of MBV infection involved in nuclear hypotrophy.

Several papers have been published on the geographic distribution, virogenesis, pathogenesis and pathogenicity of MBV (Lightner and Redman, 1981; Lightner et al., 1983a; Chen et al. 1989; Chen and Kou, 1989). An important breakthrough on MBV studies was made by Chen and Kou (1989). They successfully prepared primary cell cultures from the lymphoid organ of P. monodon in vitro. This primary monolayer cell culture was found to be susceptible to MBV. However, such cultures did not survive for the second passage.

The morphological studies on MBV infections were mainly contributed by Lightner et al. (1981 and 1983a). The investigators examined affected hepatopancreatic epithelial cells using both light- and electron-microscopy and revealed the presence of the conspicuous intranuclear multiple inclusion bodies within the cells. The inclusion bodies were recognized to be associated with rod-shaped virus. Viral particles were visualized to be either free in the nucleoplasm or occluded within the inclusion bodies. The
viral particles possessed a relatively thin multilaminar envelope and were 275 ± 41 nm in length with a nucleocapsid diameter of 69 ± 8 nm (Lightner and Redman, 1981). In 1983, Lightner et al. performed additional morphological studies on MBV and concluded that the virus was consistent with members of the genus Baculovirus, subgroup A.

Acute MBV infection can be easily diagnosed by demonstration of its characteristic occlusion bodies (Lightner et al., 1983a). Diagnostic methods for MBV are similar to those described for BP (Couch, 1974 and 1981) and BMNV (Sano et al., 1981).

**Baculoviral mid-gut gland necrosis (BMN)** Baculoviral mid-gut necrosis disease was originally reported from shrimp culture farms in Southern Japan (Sano et al., 1981). It was recognized to be a serious and fairly common disease at facilities culturing *P. japonicus* only within Japan. Two years later, the disease was found to have spread throughout Japan (Cowan, 1983). However, this viral disease has only been reported in Japan so far.

Baculoviral mid-gut necrosis disease was first recognized in 1971 in some shrimp farms in the Kyushu and Chugoku areas of Japan. Extremely high mortality and fully advanced necrosis of the mid-gut gland were observed in affected kuruma shrimp larvae, *P. japonicus* (Sano et al., 1981). Through the mid-1980s, the disease was reported to occur almost every year during the growing season from May to
September. The disease was initially named mid-gut gland cloudy disease and white turbidity disease of kuruma shrimp in the early 1970's. At that time, the etiologic agent of the disease was not known. In 1981, Sano et al. proposed the designation of Baculoviral Mid-Gut Gland Necrosis (BMN) for this shrimp epizootic after finding the viral agent of the disease.

Sano et al. (1981) initiated their studies on BMN in terms of pathological observation, electron microscopic examination and infectivity tests and noted that the infection induced remarkable cellular necrosis and collapse in the mid-gut gland. The disease was transmitted by either oral feeding with infected mid-gut gland or water-borne infection of bacteria-free filtrates prepared from diseased shrimp. This indicated the epizootic to be a viral infection. Further evidence supporting this view was the identification of viral particles in the nuclei of the affected mid-gut gland cells. The size of the virus averaged 310 nm in length by 72 nm in diameter.

In 1989, Momoyama tested the stability of the BMN virus to ultraviolet (UV), sunlight, heating and drying. These infectivity trials were carried out by employing water-borne infection and using larval and postlarval kuruma shrimp as indicator animals (Momoyama, 1989a). He found that the virus was completely inactivated by U.V. irradiation (15 W) within 20 min and by Summer sunlight (30 °C) for 3 h. Heating or
drying can also resulted in the rapid inactivation of the virus (Momoyama, 1989a).

In the same year, Momoyama also determined the sensitivity of the BMN virus to ether, selected NaCl concentrations and different pH values (Momoyama, 1989b). He found that BMNV was very sensitive to all the treatments. BMNV was completely inactivated at 25% NaCl within 10 h, pH 3-4 within 3 h, or 18 h treatment with ether. The virus was also demonstrated to be unstable when suspended in seawater, but very stable in frozen tissue when stored at low temperature (-80 °C) (Momoyama, 1989c).

**Hepatopancreatic parvo-like virus (HPV)** A disease of penaeid shrimp termed hepatopancreatic parvo-like virus (HPV) disease was first recognized in cultured *P. merguiensis* in Singapore and Malaysia by Lightner and Redman (1985) in 1983. Subsequently this disease was recognized in wild *P. escalentus* in Australia (Paynter et al., 1985). It was also reported in three additional penaeid species in both wild and cultivated shrimps in Asia (Lightner and Redman, 1985). Hepatopancreatic parvo-like virus has not been observed in the Americas either in captive wild populations or in cultured populations (Lightner, 1988).

The principal lesion of the disease is characterized by the necrosis and atrophy of the hepatopancreas, which is common to all the species (Lightner, 1988). Large prominent basophilic, PAS-negative, Fuelgen-positive (Luna, 1968)
intranuclear inclusion bodies were often observed in affected hepatopancreatic tubule epithelium. In certain affected populations, cumulative mortality rates in epizootics were reported to be as high as 50-100% within 4-8 weeks of the disease outset (Lightner and Redman, 1985). The disease was presumed to be a viral infection probably by a parvo-like virus. This conclusion was based mainly on the discovery of virus-like particles, approximately 22-24 nm in diameter, from the thin section of the hepatopancreatocytes of the infected animals. The histo-cytopathology examination of the affected tissue also indicated the presence of specific intranuclear inclusion bodies. However, the virus-like particles have not been isolated from the infected animals yet. In addition, the transmission of HPV has not been demonstrated experimentally. In Taiwan, a case of dual infections by HPV and MBV was found in postlarval and the infections resulted serious losses of the animals (Lightner and Redman, 1991).

Reo-like virus (REO) Reo-like virus is a recently described virus in penaeid shrimp. This virus was first recognized in tank-reared *P. japonicus* in the South of France by Tsing and Bonami (1984). Two years later, Nash and Nash (1988) described a reo-like virus, or a closely related form which was associated with a serious disease syndrome in pond-cultured *P. monodon* in Southeast Asia.

Tsing and Bonami (1987) isolated and purified the virus
from infected animals. The purified viruses were found to be non-enveloped, icosahedral, and they measured about 60 nm in diameter. They were able to demonstrate the infectious nature of the disease experimentally by inoculation of healthy *P. japonicus* with the purified virus and by feeding new hosts with the pieces of infected animal tissues. Histopathological examination revealed the cytoplasmic location of the virus in affected F-cells and R-cells (Johnson, 1980) of the hepatopancreatocytes (Tsing and Bonami, 1987). The progress of the disease was very slow (infection taking about 45 days) and secondary infections often occurred in the REO-infected shrimp. Tsing and Bonami (1987) tentatively termed the viral agent as reo-like virus based on their electron microscopic studies.

Lightner et al. (1984 and 1985) documented the presence of a similar agent in virtually every culture population of *P. japonicus* in Hawaii. Another paper published recently (Krol et al., 1990) reported the discovery of a reo-like virus which was found in white shrimp larval *P. vannamei* experimentally infected with BP. Both viruses were found to occur in the same tissues and occasionally within the same cell. This is the second report on mixed viral infections occurring in cultured penaeid shrimp. However, nothing is known concerning their disease-causing capacity.

**Infectious hypodermal and hematopoietic necrosis virus (IHHNV)** Infectious hypodermal and hematopoietic
necrosis virus was the fourth viral disease reported from cultured penaeid shrimp. The name of the IHHN disease was derived from the principal lesions observed in infected animals. IHHN disease was first recognized in the early 1980s in Hawaii. Lightner et al. (1983b) found the population of the experimentally cultured blue shrimp *P. stylirostris* imported from a number of commercial penaeid hatcheries in South and Central America was severely infected by a highly acute and lethal disease. Since its discovery in Hawaii, IHHN disease has been reported worldwide in cultured shrimp populations (Lightner 1983; Bell and Lightner 1984; Lightner and Redman, 1991). Electron microscopic examination of the target cells in the infected animals revealed small icosahedral viral particles, approximately 17-27 nm in size (Lightner et al., 1983b).

Experimental transmission of the disease to previously unexposed *P. stylirostris* and asymptomatic *P. vannamei* was conducted by Lightner et al. (1983c) using an intramuscular injection of a 0.45 um filtered tissue homogenate prepared from IHHNV-infected animals (*P. stylirostris*). Based on cell pathology and morphology and the small size of the virus-like particles, Lightner (1983c) suggested the virus to be either a picornavirus or a parvovirus. In 1989, Lu et al. (1989) reported their successful isolation of IHHN virus from the infected animals. The purified virus particles visualized under an electron microscope had an icosahedral morphology.
The virus measured $19 \pm 1$ nm in diameter and was naked. Colorimetric analysis (Shatkin, 1969) of its nucleic acid type indicated the causative agent of the IHHN disease to be an RNA-containing virus. However, a recent report by Bonami et al. (1991) suggests that a parvo-like virus is the agent responsible for the IHHN disease. This result is directly contradictory to that obtained by Lu et al. (1989). There is the plausibility that IHHN disease can be induced by more than one agent since the disease was experimentally demonstrated in susceptible animals by inoculating either of the agents obtained by the two groups. Thus far six species of penaeid shrimp have been found to be susceptible to IHHNV infection (Bell and Lightner 1984, Brock et al., 1983, Lightner et al. 1983c). Epizootics occur only in the younger populations of *P. stylirostris* within a size range of 0.05 to 4.0 gram. Viral infection often results in massive mortalities (exceeding 80-90% of the original population within 2 weeks of onset). Older and larger animals of this species may contract the disease, but it does not result in high mortality. The highly acute epizootics of the disease was not observed in the other penaeid shrimp species. In 1984, Bell and Lightner conducted an investigation on infectivity and pathogenicity of IHHNV in *P. stylirostris* and *P. vannamei* and reconfirmed that IHHN disease is virus-caused. The effect of shrimp size on IHHN disease expression was studied by Bell and Lightner (1987). Five different

24
sizes of *P. stylirostris* were experimentally exposed to IHHNV per os and the result indicated that the smaller size shrimp were more sensitive to the virus than the large shrimp.

The histopathology of acute and subacute IHHN disease in *P. stylirostris* was initially carried out by Lightner et al. (1983b). They determined that the target tissues of the infection were those primarily derived from the ectodermal origin (epidermis, hypodermal epithelium of fore and hindgut, nerve cord and nerve ganglia) and mesodermal origin (hematopoietic organs, antennal gland, gonads, mandibular organ, connective tissue, and striated muscle). Conspicuous eosinophilic Cowdry type A intranuclear inclusion bodies (Cowdry, 1934) were present in the affected tissues. This unique type of inclusion bodies were subsequently employed as the principal criterion for the diagnosis of the IHHN disease. A presumptive diagnosis of IHHNV depends on the gross signs, behavior, mortality rate and pattern within a population with acute or subacute epizootic disease. However IHHNV infection has to be confirmed by histopathological examination to demonstrate the occurrence of the specific type of the inclusion bodies in the affected animal tissues. Lightner et al. (1983b) described three different methods for the confirmed diagnosis of the IHHN disease and for the detection of asymptomatic carriers of IHHNV. The natural host(s) and geographic distribution of IHHNV are unknown. In shrimp culture facilities in the Americas, IHHNV has been
detected in Hawaii, Florida, Texas, Cayman islands, Panama, Costa Rica, Belize and Ecuador. IHHNV has also been diagnosed in Guam, Tahiti, Singapore, Israel and the Philippines. Recently IHHNV has been detected in Taiwan, Malaysia, and Mexican (Lightner et al., 1987; Lightner, 1991). This extensive geographical range of IHHNV in cultured shrimp populations is thought to have resulted from dissemination of IHHNV in shipments of IHHNV-contaminated hatchery-reared live shrimp (*P. stylirostris* and *P. vannamei*) stocks which directly or indirectly originated from shrimp hatcheries or farms located in South or Central Americas countries (Bell and Lightner, 1984). The IHHNV was not demonstrated from those shrimp originated from Mexican shrimp hatcheries for many years (Bell and Lightner, 1984. and Brock et al., 1983). However, Very recent studies (Moore, 1991; Lightner et al., 1991; Lightner, 1991) revealed the occurrence of IHHN disease in Mexican shrimp hatcheries.

**Lymphoidal parvovirus-like (LPV)**

Lymphoidal parvovirus-like (LPV) is the newest shrimp virus, and it was recognized only very recently in Australia (Owens et al., 1991). Three farmed penaeid species, *P. monodon*, *P. merguiensis* and *P. esculentus*, were found to be infected by this virus. The Lymphoid organ was identified to be the major target for the viral infection. The formation of multinucleated giant cells was the most distinguishing manifestation occurring in the hypertrophied lymphoid organs.
following the infection. Basophilic inclusions were commonly observed within the nucleus which stained Feulgen positive. Electron microscopical examination revealed the presence of virus-like particles associated with inclusion bodies. The virus measured 18 to 20 nm in diameter. Attempts to isolate the virus from infected animals were unsuccessful. No virus particles were visualized by the negative staining technique.

C. Rhabdoviruses of Crustacean

Members of Rhabdoviridae are found in plants and vertebrates, and also found in invertebrates (arthropods). Many animal rhabdoviruses are bullet-shaped and relative large (70 x 170 nm) while plant rhabdoviruses are usually bacilliform-shaped, and with smaller diameter.

At present, four rhabdoviruses are known in crustacean and are exclusively found in crabs. All these viruses except for rhabdo-like virus A (RhVA) were detected only by electron microscopy and information on their biological properties, host range, host susceptible stage, geographic distribution, and transmission is lacking. The crustacean rhabdoviruses, unlike most vertebrate rhabdoviruses, are not pathogenic and thus are not significant pathogens of their crab hosts.

Two review papers were published in the early of 1980s (Johnson, 1983, 1984). Recently, a comprehensive review summarized most of the current available knowledge regarding the crustacean rhabdoviruses (Brock and Lightner, 1990).
**Y-organ virus** This virus represents the first rhabdovirus among the crustacean rhabdoviruses (Brock and Lightner, 1990) of crustacean. It was initially reported from European shore crab, *Carcinus maenas* in 1975 (Chassard-Bouchaud and Hubert, 1975). Chassard-Bouchaud and Hubert observed virus-like particles were located within and beneath the basal lamina of the infected Y-organs (ecdysial glands) during electron microscopic study of Y-organs of crabs.

In the following year, Chassard-Bouchaud et al. (1976) reported the virus as enveloped, ovoid to elongate, measured to be 70–90 nm in diameter and 150–170 nm in length. The envelope is 9 nm thick and covered externally with spicules (7–10 nm long). They observed that Y-organ virus buds through the plasma membrane to an extracellular space and suggested it to be related to *Rhabdoviridae*. The virus was only found in one of 16 crabs collected from Roscoff, France. The infected crab did not exhibit any disease effect. The viral infection was only limited to the Y-organ and its distribution in other organs and tissues of the crabs or in other crab populations was not known.

**Rhabdolike Virus A (RhVA)** The second reported crustacean rhabdovirus is Rhabolike virus A which was originally described by Jahromi (1977) from blue crab, *Callinectes sapidus*, and named by Johnson (1983). Jahromi observed RhVA during the course of studying neuromuscular junctions of the gastric mill muscles of the blue crab (1977). The virus...
particles were found to be rod-shaped with two rounded ends, measured approximately 30 x 115 nm, and present in many different tissues such as in glial (Schwann) cells of nerves, blood-vessel endothelia, presumptive fibroblasts and hemocytes (Jahromi, 1977). A year later, Yudin and Clark (1978, 1979) found a similar rhabdo-like virus, along with another rhabdo-like particle in blue crab while studying on ultrastructure of the "ecdysial gland", which actually was the mandibular gland (Johnson, 1983). The virus particles had a diameter of 25-30 nm and a length of 100-170 nm, and were seen in the cells of the blue-crab mandibular organ (Yudin and Clark, 1978). They initially named this virus as "ecdysial gland virus 2" (EGV-2) and the other as "ecdysial gland virus 1" (EGV-1). However, since the viruses were not really found in the ecdysial gland cells, Johnson (1983) renamed these two viruses as rhabdo-like virus A (RhVA) and rhabdo-like virus B (RhVB) for EGV-2 and EGV-1, respectively. In 1983, Johnson also found RhVA particles but in other organs and tissues including hematopoietic tissues, connective-tissue cells, epidermis, bladder epithelium, epicardial tissue, and the CNS (Johnson, 1983). The particles were either bacilliform measuring 20-30 nm x 110-170 nm, or long and flexuous (up to 600 nm long). RhVA was not seen either within axons and in muscle cells (Jahromi, 1977) or in epithelia of gut and antennal gland, striated muscle and the cells of Y-organ (Johnson, 1983).
RhVA particles were found to bud from both the endoplasmic reticulum and the inner nuclear membrane (Johnson, 1984) and observed in many different tissues (Jahromi, 1977, Johnson, 1983). RhVA particles are often stacked either parallel or perpendicular to the nuclear surface.

The first isolation of RhVA was carried by Yudin and Clark (1979) who demonstrated the purified RhVA was bacilliform-shaped by both positive and negative stains. At the same year, Yudin and Clark (1979) demonstrated the proliferation activity of RhVA in ablated crabs to be transitory. The virus proliferation occurred within the nuclear envelope and tubular endoplasmic reticulum during days 3, 4, and 5 following eyestalk removal.

Pathogenicity of RhVA to its crab hosts is very limited. Jahromi (1977) indicated RhVA primarily infected the Schwann cells causing no cytopathic effects. Yudin and Clark (1978) noted the crab possessing such "infected" glands appeared physically normal and showed no signs of abnormal behavior. However, some stressors such as concurrent-infection by other viral agents, bilateral eyestalk ablation, and transport were found to be able to mediate the manifestation of RhVA infection in host cells (Jahromi, 1977; Yudin and Clark, 1978, 1979; Johnson, 1983, 1984). Johnson (1988) documented a fatal disease (RLV-RhVA disease) of blue crab which was due to such a factor, concurrent infection by RhVA and RLV.
**Rhabdo-like virus B (RhVB)** RhVB is the third crustacean rhabdovirus and is known only from the report by Yudin and Clark (1978). During an electron microscopical study of mandibular organ of blue crab, they discovered ovoid-shaped virus particles with a diameter of 50-70 nm and a length of 100-170 nm. The virus particles were observed to contain an envelope membrane with surface projections, and were extracellularly beneath the basal lamina of the mandibular organ. Based on morphological properties, Yudin and Clark (1978, 1979) grouped this virus into *rhabdoviridae* and called it EGV-1 (Ecdysial gland virus 1). Later, it was redesignated RhVB (Rhabdo-like virus B) by Johnson (1983).

Yudin and Clark (1978) found RhVB to be consistently associated with the calyx material that coated the hemolymph sinuses of the mandibular gland but was not seen within the cytoplasm of parenchymal cells. Also, RhVB was not observed to bud through the plasma membrane (Yudin and Clark, 1978, 1979).

Occurrence of RhVB in crabs seems limited to those originally from the Gulf of Mexico (Johnson, 1983). RhVB was only found in 2 of 60 (3%) mandibular organs examined by electron microscopy, and in one case RhVB was found to be associated with another rhabdovirus, RhVA. However, crabs infected with RhVB did not show any pathogenic alteration and signs of abnormal behavior.
**Enveloped helical virus (EHV)** The fourth crustacean rhabdovirus was also found from blue crab, *Callinectes sapidus*. (Johnson and Farley, 1980). This enveloped helical virus was initially discovered by Johnson and Farley (1980) in tissues of blue crabs collected from the Tred Avon River, Maryland and Tangier Sound, Virginia.

EHV is similar in shape to RhVB, but larger. EHV particles are membrane bound, either ovoid or rod-like shaped and measure 105 x 194-300 nm (Johnson and Farley, 1980, Johnson, 1984). The enveloped membrane has external indistinct spikes. The EHV particles mature by budding through the plasma membrane of hemocytes and hemopoietic-tissue cells and were observed only extracellularly (Johnson and Farley, 1980, Johnson, 1983). Based on the similarity of development, Johnson (1983, 1984) concluded EHV was closely related to Y-organ virus.

EHV was initially considered to be a paramyxovirus (Johnson and Farley, 1980, Johnson, 1983) since its development and morphology were similar to those of Paramyxoviridae except the smaller size. Soon after, Johnson reassigned EHV to the Rhabdoviridae (Johnson, 1984, 1988).

EHV infections are always associated with one or more other viruses, i.e., Baculo-B, CBV, RhVA, or RLV, and its effect on the crab hosts is unknown (Johnson, 1983, 1984). Also nothing is known concerning the occurrence of EHV in natural crab populations.
III. MATERIALS AND METHODS

A. Cell Culture Containers and Media

Cell culture containers used for supporting cell growth in this study are listed in Table 2. The epithelioma papillosum cyprini (EPC) cells and other fish cell lines were maintained in 150 cm² and 75 cm² plastic tissue culture flasks (Corning Glass Works). The viral experimental studies were carried out in 25 cm² flasks (Falcon) and 24-well plates (Falcon). Plaque assay was performed mainly in 24-well plates, and in 6-well plates (Falcon) and 25 cm² flasks as well. The 50% tissue culture infectious dose (TCID₅₀) assay was conducted in 96-well plates. Plastic tissue culture flasks (150 cm²) were employed for preparation of virus stocks.

Cells were grown and maintained in Eagle's minimal essential medium (MEM) with Earle's salt (Automod, Sigma Chemical Co., St.Louis, Missouri) supplemented with fetal bovine serum (Hyclone Laboratories, Inc., Logan, Utah) at a concentration of either 10% (MEM-10) for cell growth or 5% (MEM-5) for cell maintenance, virus propagation and virus tests. Non-serum containing MEM (MEM-0) was used for making virus dilutions and the washing of cell monolayers. Tissue culture medium 199 (Sigma Chemical Co., St.Louis, Missouri) and Leibovitz L-15 (Sigma Chemical Co., St.Louis, Missouri) were also used in this study to compare their
Table 2. Cell culture containers used for the propagation and titration of viruses.

<table>
<thead>
<tr>
<th>Containers</th>
<th>Abbreviation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>150 cm$^2$ plastic tissue culture flask</td>
<td>150 cm$^2$ flask</td>
<td>Corning</td>
</tr>
<tr>
<td>75 cm$^2$ plastic tissue culture flask</td>
<td>75 cm$^2$ flask</td>
<td>Corning</td>
</tr>
<tr>
<td>25 cm$^2$ plastic tissue culture flask</td>
<td>25 cm$^2$ flask</td>
<td>Corning &amp; Falcon</td>
</tr>
<tr>
<td>6-well plastic tissue culture plate</td>
<td>6-well-plate</td>
<td>Falcon</td>
</tr>
<tr>
<td>24-well plastic tissue culture plate</td>
<td>24-well-plate</td>
<td>Falcon</td>
</tr>
<tr>
<td>96-well plastic tissue culture plate</td>
<td>96-well-plate</td>
<td>Falcon</td>
</tr>
</tbody>
</table>
support in viral production.

B. Cell Lines Used in this Study

Epithelioma papillomus cyprini (EPC) cells, derived from common carp, *Cyprinus carpio*, was the main source of cells employed in the isolation and propagation of virus for this study. Other fish cell lines and mammalian cell lines involved in this study are listed in table 3. Monolayer cultures of EPC cells in 25 cm$^2$ flasks were used during the virological examination of shrimp samples from shrimp farms in Hawaii and other places. The rhabdovirus of penaeid shrimp (RPS) was isolated and routinely propagated in EPC cell line. Stocks of RPS were prepared in the EPC cell line. Virus replication and plaque forming efficiency studies were conducted in EPC cells as well as in brown bullhead (BB), grass carp fins (GCF), grass carp snout-2 (GCS-2), grass carp swimming bladder (GCSB), fathead minnow (FHM), Chinook salmon embryo (CHSE-214), and rainbow trout gonad (RTG-2) cells. A mammalian cell line, buffalo green monkey kidney (BGMK) cells, was employed in this study for the propagation of mammalian viruses, poliovirus type-1, vaccinia virus, and vesicular stomatitis virus (VSV). The routine cultivation of fish cells was performed by the method described in 1976 (Wolf and Quimby). All the fish cell lines were grown at 20°C while the BGMK cell line was incubated at 37°C.
Table 3. Established cell lines used for the isolation, propagation and characterization of viruses

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Abbreviation</th>
<th>Species of Origin</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epithelioma papillosum cyprini</td>
<td>EPC</td>
<td>Cyprinus carpiní</td>
<td>Tomasec and Fijan, 1971</td>
</tr>
<tr>
<td>Brown bullhead</td>
<td>BB</td>
<td>Ictalurus nebulosis</td>
<td>Wolf and Quimby, 1969</td>
</tr>
<tr>
<td>Chinook salmon embryo</td>
<td>CHSE-214</td>
<td>Oncorhynchus tshawytscha</td>
<td>Nims, Fryer and Pilcher, 1970</td>
</tr>
<tr>
<td>Grass carp fins</td>
<td>GCF</td>
<td>Ctenopharyngodon idella</td>
<td>Lu et al., 1990</td>
</tr>
<tr>
<td>Grass carp swim bladder</td>
<td>GCSB</td>
<td>Ctenopharyngodon idella</td>
<td>Lu et al., 1990</td>
</tr>
<tr>
<td>Grass carp snout-2</td>
<td>GCS-2</td>
<td>Ctenopharyngodon idella</td>
<td>Lu et al., 1990</td>
</tr>
<tr>
<td>Fathead minnow</td>
<td>FHM</td>
<td>Pimephales promelas</td>
<td>Gravel and Malsburger 1965</td>
</tr>
<tr>
<td>Rainbow trout gonad</td>
<td>RTG-2</td>
<td>Salmo gairdneri</td>
<td>Wolf and Quimby, 1962</td>
</tr>
<tr>
<td>Buffalo green monkey kidney</td>
<td>BGMK</td>
<td>Cercopithecus aethiops</td>
<td>Barron, Olshevsky and Cohen, 1970</td>
</tr>
</tbody>
</table>
C. Virus Extraction and Concentration from Infected Animals

The infected white shrimp, *P. vannamei*, and blue shrimp, *P. stylirostris* were collected from both Hawaiian shrimp farms and a farm in Ecuador. The general protocol for virus extraction from these infected animals was as follows: after removing hepatopancreas and stomach, the other internal organs and tissues of the head were harvested and a 10-30% homogenized suspension in TNE buffer (0.05 M tris, 0.1 M NaCl, 0.001 M EDTA, pH 7.4) was prepared. The homogenate was then frozen and thawed 3 times. After slow speed centrifugation at 500 x g (IEC Centra-8R, International Equipment Company) for 10 min at 4 °C, the supernatant was harvested and further clarified at 3,000 x g for 15 min. The cleared supernatant was treated with equal volume of trifluorotrichloroethane (genetron 113), and the aqueous supernatant was collected and precipitated by adding polyethylene glycol (PEG) and sodium chloride (8% PEG in 0.125 M NaCl). The precipitate was pelleted at 3,000 x g 30 min at 4 °C, and resuspended in 1/10 volume of TNE buffer. The suspension was then concentrated by centrifugation at 243,000 x g (L7-65 Ultracentrifuge, Spinoco Division of Beckman Instruments, Inc., Palo Alto, California) for 30 min at 4 °C onto a 60% sucrose cushion. The pellet was collected by side puncture, diluted in TNE and stored at -70 °C.
D. Virus Isolation and Purification

Monolayer cells of EPC grown in MEM supplemented with 10% FBS (MEM-10) in 25 cm² flasks at 20 °C were inoculated with 0.5 ml of diluted and undiluted aliquots of the various suspensions prepared from infected shrimp tissue. After adsorption for 60 min at room temperature (rt), experimental medium consisting of MEM 5% FBS was added and the infected cultures were incubated at 20 °C and examined daily for cytopathology (CP) using an inverted microscope (Nikon, NIPPON KAGAKU). Uninfected EPC cells inoculated with 0.5 ml TNE buffer served as controls. After the discovery of rhabdovirus of penaeid shrimp (RPS), the preparation, purification and concentration of virus stocks was conducted as follows: monolayers of EPC cells grown in 150 cm² flasks were infected with RPS at a low multiplicity of infection (MOI) of approximately 0.01–0.001 plaque forming units (PFU) per cell. The cells were incubated with 20 ml MEM-5 for 3–4 days at 20 °C until the cytopathic effects had destroyed the entire cell monolayer. The flasks were then frozen at −70 °C, subsequently thawed, and the cells removed by vigorous shaking of the thawing flasks. The cultures were pooled and centrifuged at 3000 x g (IEC Centra-8R centrifuge, International Equipment Company) for 15 min. The resulting supernatant was treated with 8% PEG (w/v) and 0.1 N NaCl, and stirred for 3 hours (h) or overnight at 4 °C. The
precipitate was pelleted at 3000 x g for 30 min at 4 °C and resuspended in 1/10 volume of the TNE buffer. After removing PEG by centrifugation for 15 min at a speed of 3,000 x g, the virus particles in the supernatant were pelleted onto a 60% sucrose cushion by centrifuging at 243,000 x g for 30 min at 4 °C. The pellet was harvested either by side puncture using a 1 cc tuberculin syringe or by drainage from the bottom. The pelleted virus was diluted in TNE buffer and layered on top of an ultra-clear centrifuge tube (Beckman) containing a discontinuous density gradient composed of 50% (1 ml), 35% (1.5 ml), and 20% (1.5 ml) sucrose (w/w) prepared in the TNE buffer. This gradient was centrifuged at 243,000 x g for 60 min in an SW50.1 rotor (L7-65 ultracentrifuge, Beckman).

After centrifugation, a opalescent-bluish band located in the middle of the tube (20/35% interface) was collected by side puncture, diluted 1:10 in TNE buffer, and pelleted by centrifugation at the speed of 243,000 x g for 30 min at 4 °C. The pellet was resuspended in a small amount (0.5-1 ml) of TNE buffer and the virus was centrifuged at 243,000 x g for 60 min through a 10 to 50% (w/w) continuous sucrose gradient. The band containing the purified virus was harvested by side puncture, diluted in TNE buffer, and pelleted onto a 60% sucrose cushion by centrifugation (243,000 x g for 30 min at 4 °C). Finally, the pelleted virus was resuspended in a small volume of buffer and stored at -70 °C and used for characterization of the virus. For viral
protein and viral RNA analysis, the virus was further purified by passing it through both discontinuous and continuous sucrose density gradients once more.

E. Preparation of Virus Stocks

After the initial discovery of rhabdovirus of penaeid shrimp, virus isolations were made in 25 cm² flasks containing EPC cells. Briefly, a 0.5 ml of sample suspension suspected to contain the RPS was inoculated into a monolayer of EPC. After 3 to 7 days incubation at 20 ºC, the culture exhibiting cytopathic changes was frozen at -70 ºC. Subsequently, the flask was thawed in 25 ºC waterbath, and clarified by a low speed centrifugation, 1000 x g for 20 min. The supernatant was passed through a filter, 0.22 um pore size. The filtrate was then reinoculated into a 75 cm² flask of EPC cells and incubated at 20 ºC until cytopathic effects (CPE) developed over the entire cell monolayer. One ml aliquots of the culture fluid from this second passage was dispensed into 1.0 ml plastic vials (NUNC), frozen at -70 ºC, and used as a master stock. All experiments with RPS were conducted with third to seventh passage virus.

F. Other Viruses Used in this Study

Several viruses of aquatic animals were used as controls in certain experiments (Table 4): Channel catfish virus (CCV), a fish herpesvirus, and three fish rhabdoviruses:
spring viremia of carp virus (SVCV) or rhabdovirus carpio (RC), infectious hemorrhagic necrosis virus (IHNV) (gifts of Dr. John L. Fryer and Dr. J.C. Leong, Oregon State University, Corvallis, Oregon), and viral hemorrhagic septicemia virus (VHSV) (from Dr. J.R. Winton, National Fisheries Research Center, Seattle). Spring viremia of carp virus was propagated in EPC cells 20 °C and CCV was prepared in BB cells at 25 °C, while IHNV and VHSV were replicated in CHSE-214 and EPC cells at 16 and 18 °C, respectively. Three mammalian viruses were also used in this study. A mammalian rhabdovirus, vesicular stomatitis virus (VSV), Indiana strain, was received from Dr. Sandra Horakami, University of Florida, Gainesville, Florida). The others were poliovirus type-1 and vaccinia virus from this laboratory. These mammalian viruses were propagated in the BGMK cell line at 37 °C.
<table>
<thead>
<tr>
<th>Virus</th>
<th>Abbreviation</th>
<th>Family</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infectious hemotopoietic necrosis virus</td>
<td>IHNV</td>
<td>Rhabdoviridae</td>
<td>Amend et al., (1969)</td>
</tr>
<tr>
<td>Viral hemorrhagic septicemia virus</td>
<td>VHSV</td>
<td>Rhabdoviridae</td>
<td>Brunson et al. (1989)</td>
</tr>
<tr>
<td>Rhabdovirus carpio</td>
<td>RC</td>
<td>Rhabdoviridae</td>
<td>Fijan et al. (1971)</td>
</tr>
<tr>
<td>Vesicular stomatitis Virus</td>
<td>VSV</td>
<td>Rhabdoviridae</td>
<td>Cotton, 1926</td>
</tr>
<tr>
<td>Poliovirus type 1</td>
<td>Polio-1</td>
<td>Picornaviridae</td>
<td>Loh, Hawaii</td>
</tr>
<tr>
<td>Channel catfish virus</td>
<td>CCV</td>
<td>Herpesviridae</td>
<td>Wolf and Darlington (1971)</td>
</tr>
</tbody>
</table>
G. Staining and Microscopy

1. Staining of infected cells

Glass coverslips, 15 mm in diameter (Fisher Scientific Allied Co.), were rinsed well with distilled water, dried and autoclaved. The sterile coverslips were then placed in a 24-well flat-bottom tissue culture plate, one coverslip per well along with 0.2 ml of MEM-5. To each well, 0.5 ml of EPC cell suspension (1 x 10^5 cells/ml) was added. The 24-well plates were incubated at 20 °C overnight to allow the cell monolayer to form. The medium was decanted, the cells were rinsed twice with MEM-0 and then infected with a concentration of 30-50 PFU of RPS in 0.2 ml of MEM-0, and controls received only MEM-0. After an adsorption period of 60 min at rt, 0.5 ml of maintenance medium was added into each well. The infected cultures were then incubated at 20 °C. At each of the selected-postinfection times 1, 2 and 3 days, 24 coverslips (12 infected and 12 controls) were carefully removed from the wells for fixation and staining with crystal violet and acridine orange (AO).

For crystal violet staining, both the control and infected cultures were treated with 0.2 ml of 0.6% fixative-stain solution (6 g crystal violet, 600 ml formaldehyde, and 400 ml distilled water). The cultures were allowed to fix-stain for 60 min and then rinsed with tap water. Subsequently, the coverslips were removed from the wells and
The infected cells were examined with a phase-contrast microscopy and photographed using Kodak high-speed Ektachrome film.

The acridine orange staining of the infected cultures and the controls were conducted by using the method described by Hsiung (1982). The cultures on the coverslips were rinsed 2-3 times with PBS, and fixed for 2 min in freshly prepared Carnoy's fluid (6 ml absolute ethanol, 3 ml chloroform and 1 ml glacial acetic acid). The fixed cells were hydrated rapidly by immersing the coverslips successively in 95%, 80%, 70%, and 50% ethanol briefly, rinsed for 1 min in 0.002 M MgSO₄ and then 2 min in PBS containing 0.067 M Na₂HPO₄ and KH₂PO₄ (pH 6.0). Then the infected cells were stained in 0.01% acridine orange solution for 3 min and rinsed in the same phosphate buffer (pH 6.0) for 2-5 min. Finally the coverslips were immersed in 0.1 M CaCl₂ containing 0.002 M MgSO₄ for 2 min, briefly rinsed in PBS (pH 6.0), then mounted onto a microscopic slide (Fisher Brand, Fisher Scientific). The prepared slides were examined in a dark-field microscope with a UV light source (Nikon Corporation).

Viral plaques formed in monolayer cells of EPC and other fish cell lines were fixed and stained by adding the fixative-Crystal violet. At 1 to 3 days postinfection (p.i.), counting of the plaques formed was facilitated by the use of a dissecting microscope because the focal lesions were small. At later times because the plaques were sufficiently large,
they were counted by the naked eyes.

2. **Light microscopy and dissection microscopy**

   An inverted microscope, DIAPHOT-TMD, (Nikon, Nippon Kogaku K.K.) was used throughout this study for routine observation of growing cells and cytopathic change, and for taking pictures of normal growing cells and viral infected cell cultures. A dissecting microscope (Bausch and Lomb) was employed mainly for an accurate determination of the number of viral plaques formed in grass carp cell cultures.

3. **Electron microscopy**

   Negatively stained virus were prepared by the method of Christie et al. (1987). A length of double-coated cellophane tape was attached to a microscope slide, parallel to the long axis of the slide and overlapping one side (the right side) by 2-3 mm. Then formvar-coated carbon-stabilized 200-mesh copper grids were positioned along the length of the tape, slightly overlapping the bottom surface (the shining side of the grids) of each grid on the upper surface of the tape. To mount the virus particles on the grid, a droplet of purified virus suspension was applied to the grid and allowed to stand for approximately 5 min. The droplet was then removed from the specimen grid with a folded corner of a piece of blotting paper (Fisher Brand Bibulous Paper, Fisher Scientific Allied Co.) by touching the grid rim where it was attached to the
tape folded in half. The grid was washed immediately with 3-5 drops of distilled water using a pasteur pipette. As the washing proceeded, the blotting paper was withdrawn by pulling it away from the grid and across the tape for a distance approximately 6-8 mm, meanwhile maintaining contact between the blotting paper and the tape surface, and changing fresh blotting paper as often as necessary to maintain a fast flow. The grid was then stained with 1 drop of 2% uranyl acetate solution for 15 to 30 seconds and the stain was simultaneously removed by using a dry corner of the blotting paper. One drop of triple-distilled water was added directly on the grid to insure a thin and even distribution of the stain, and the excess fluid was drained away with the blotting paper. The grids were allowed to air dry and then removed from the tape and stored in a grid box. The box containing the grids was placed in a desiccator until the grids were examined with a Zeiss 10/A electron microscope.

Thin-section electron microscopy was performed by infecting monolayers of EPC cells growing in 35 x 10 mm tissue culture dishes (Falcon) with virus at a multiplicity of infection (MOI) of 0.001. After adsorption for 60 min at room temperature, maintenance medium (MEM-5) was added and the infected cultures incubated at 20°C. Following approximately 24 h p.i., the medium was decanted from the dish and immediately replaced with freshly-prepared 3% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.0) for
30 min. After removal of the glutaraldehyde, the cells were rinsed 4 times with the buffer and postfixed with 2% osmium tetroxide in the buffer for 30 min at rt. The infected cells were rinsed again 4 times with the buffer following postfixation, and dehydrated in ethanol from 10% to 100% by gradually increasing the ethanol concentration. The dehydrated cultures were then immersed in a mixture containing 100% ethanol/propylene oxide (1:1) for 30 min. Then, 30 min in propylene oxide, 8 h in propylene oxide/Spurrs (1:1), and lastly embedded in Spurrs and polymerized at 70 °C oven for 8 hr or overnight. Thin sections of 700 to 1000 Å thickness (silver sections) were prepared using an ultramicrotome and placed on formvar-coated carbon-stabilized 200-mesh copper grids. The specimens were stained in 2% uranyl acetate for 45 min on a warm tray (35 °C) under darkness and then in a drop of lead citrate for 8 min. The grids were examined in a Zeiss 10/A electron microscope. The preparations were photographed on Kodak electron image plates.

H. Titration of Virus

1. Development of plaque assay technique for the titration of the virus

A suspension of rhabdovirus of penaeid shrimp harvested from the infected EPC cell culture was frozen at -70 °C and then thawed. Following low speed centrifugation, the
recovered supernatant containing the virus was diluted to different concentrations in MEM-0. For each of these concentrations, a further serial 10-fold dilution was made in the same buffer. A 0.1 ml aliquot of each dilution was added in triplicate wells of 24-well plates containing monolayers of EPC cells. One plate was inoculated for each concentration. The cultures were placed on a rocking platform for 60 min at rt to allow the virus to be evenly distributed. Then 0.5 ml of overlay medium consisting of 0.75% methyl cellulose and MEM-4 was added into each well. The infected cultures were incubated for 5 days at 20 °C. The plates were then removed from the incubator and were fixed and stained by depositing in each of the wells 0.5 ml of the fixative-staining solution. After approximately 60 min, the plates were washed with tap water and air dried. The number of plaques formed in each well was counted. The relationship between virus dilution and the number of plaques formed was determined.

2. Comparison of plaque assay and TCID<sub>50</sub> assay

A virus stock sample containing 10<sup>8</sup> PFU/ml of RPS was thawed and serial 10-fold dilutions were made in MEM-0. The plaque assay was conducted by using 6-well plates of EPC monolayers. An aliquot of 0.2 ml of each diluted solution was deposited into duplicate wells. The virus was allowed to adsorb for 1.5 h at rt. Two milliliters of 0.75% methyl
The infected cultures were incubated at 20 °C for 5 days, then fixed and stained as described above. The plaques were counted from the wells having 10-105 individual plaques. For the 50% tissue culture infectious dose (TCID₅₀) assay, 96-well flat-bottom tissue culture plates containing monolayers of EPC cells were inoculated with 0.1 ml from each dilution of the same preparation of the virus concentrations used for plaque assay. The plates were sealed with pressure sensitive film (Falcon) and placed in a 20 °C incubator. At 4, 7, 14, and 21 days after infection, the wells were read for any Cytopathic changes. The TCID₅₀ titer was calculated according to the method of Reed and Muench (1938).

3. **Comparison of plaque assay overlays**

The methyl cellulose overlay medium used for plaque assay in this study was prepared by mixing equal volume of double strength (2x) MEM-4 and 1.5% methyl cellulose (4000 cps, Sigma Chemical Co., St.Louis, Missouri), supplemented with appropriate amounts of L-glutamine and antibiotics. The methylcellulose solution was prepared in 1,000 ml of triple-distilled water, autoclaved, and then magnetic-stirred overnight at rt. Another overlay, the agarose overlay medium, was prepared by mixing equal amounts of 2x Earle's medium MEM-4 heated to 45 °C and 3% agarose (Seakem Inc.)
autoclaved and cooled to 45 °C in a sterile serum bottle. In this experiment, a series of 10-fold dilutions of stock virus was made in MEM-0, and 0.2 ml of each dilution was inoculated onto one-day old EPC monolayer cell cultures grown in 6-well plates. The virus was allowed to adsorb for 90 min at rt and 2 ml of each overlay medium per well was added. Following 5 days incubation at 20 °C, the cultures were fixed and stained by adding 2 ml of the fixative-staining solution per well. The plates were then rinsed, air dried and the plaques were counted.

4. Comparison of plaque forming efficiency in different cell culture containers

A number of cell culture containers were compared to determine which would be most efficient for plaque assay of RPS. In this test, 24-well plates, 6-well plates and 25 cm² flasks were seeded with EPC cells and incubated overnight at 25 °C to allow the cell monolayer to form. A series of 10-fold dilutions of the stock virus was prepared in MEM-0, and 0.5 ml of each dilution was inoculated into either each well or each flask in quadruplicate. The virus was allowed to adsorb for 1.5 h at rt and then overlay medium was added as follows: 0.5 ml/well for 24-well plates, 2 ml/well for 6-well plates, and 5 ml/25 cm² flask. After incubation at 20 °C for 5 days, 0.5 ml, 2.0 ml or 4.0 ml of fixative-staining solution was added to each well or flask, respectively. The cultures were allowed to fix and stain for 60 min at rt. The
plates and the flasks were rinsed with tap water, air dried, and the plaques that formed were counted.

5. Comparison of plaque forming efficiency in selected fish cell lines

The plaque forming efficiency of RPS was compared in the following fish cell lines: EPC, BB, GCF, GCS-2, GCSB cells, CHSE-214, RTG-2, and FHM. Four 6-well plates seeded with cells from each cell line were inoculated with 0.2 ml of various 10-fold dilutions of the virus made up in MEM-0. The virus was allowed to adsorb for 1.5 h at rt. Five milliliters of 0.75% methyl cellulose-MEM-4 was added to each flask and the cultures were incubated at 20 °C for 5 days. The cultures were then fixed and stained by adding 2 ml of the fixative-stain solution and the plaques in each well were counted and the mean computed. The plaque titers of the virus obtained from these assays were calculated. The sensitivity of the EPC cell line to the virus was arbitrarily set at 100%, and the results were computed as an estimate of the relative ability of each cell line to produce plaques.

6. Relationship between volume of inoculum and number of plaques

To determine the optimal inoculum for plaque assay performed in 6-well plates, nine different volumes of inocula beginning from 0.1/well to 1.0 ml per well were used. The 6-well plates seeded with EPC cells were placed at 25 °C to
allow the cell monolayer to form. A virus stock diluted in MEM-0 to yield a final concentration of approximately 300 PFU per milliliter was used as inocula. The virus was allowed to adsorb for 1.5 h at rt and subsequently the overlay medium was added into each well as described previously. The plates were then incubated at 20 °C to let the viral plaques develop. At day 5 p.i., the infected cultures were fixed and stained, and the number of plaques formed in each well was counted. The average number of plaques produced in each of the plates was compared for the different volumes of inocula.

7. Relationship between adsorption times and virus titers

The time required for maximal RPS adsorption to EPC cells was determined by plaque assay. Serial 10-fold dilutions of a stock virus were made in MEM-0, and 0.2 ml of each dilution was inoculated into each well of a 6-well plate. One plate was used for each adsorption period. After 0.5, 1, 1.5, 2, 3, and 4 h incubation at rt, the unadsorbed virus was removed from the monolayer cells by rinsing twice with MEM-0. Half a milliliter of the methyl cellulose overlay medium was added into each well of the plates and the cultures were incubated for 5 days at 20 °C. Infected cultures were fixed and stained for 60 min with the fixative-stain solution. The plaques were counted and the titers for each adsorption period were determined. A 2 h adsorption period was assigned a value of 100% and the percentage of
virus adsorbed at other periods was calculated.

8. Relationship between selected postinfection periods and plaque yield

Eight 24-well plates were seeded with EPC cells and incubated overnight at 25 °C to allow the formation of cell monolayers. Ten-fold dilutions of a virus stock containing $10^6.61$ PFU/ml were prepared with MEM-0. An inoculum of 0.1 ml sample from each dilution was applied into each of four individual wells. Two wells receiving 0.1 ml MEM-0 were used for negative controls. After 1.5 h adsorption at rt, the overlay medium was added and the plates were incubated at 20 °C. At every 24 h period for 7 successive days, one of the plates was removed daily from the incubator, fixed-stained by adding 1 ml fixative-stain solution to each well, and the plaques formed counted. The plaque titers for each of the selected postinfection periods was compared.

9. Efficiency of plating (EOP) in EPC cells

The purpose of this experiment was to determine the ratio of the number of viral particles per infectious unit. To do this test, RPS isolated from *P. stylirostris* was propagated in EPC cells (MOI = 0.01). The virus produced from the cell culture was concentrated and highly purified by the method described earlier except that two cycles of sucrose gradients were employed rather than one. The final purified virus was then employed for both viral plaque assay
and viral particle counting.

**Plaque assay titer** Plaque assay for the virus was conducted in EPC cells growing in 6-well plates as described previously.

**Viral particle counting** The total number of viral particles contained in the same virus sample employed in the plaque assay was determined by a method developed in this laboratory (Nadala and Loh, 1991) Formvar-coated carbon-stabilized 200-mesh copper grids were placed in the 3 mm sector core chambers of a Beckman EM-90 rotor (Beckman Instruments Incorporated, Palo Alto, California), standing with the coated side inwards. Exactly 45 ul of the virus solution was loaded in each of the 6 chambers using an eppendorf micropipette. The gasket was lightly coated with a thin layer of Apiezon grease (Apiezon Products Limited, London) and placed over the sector core to insure no leaking of the virus solution during centrifugation. The EM-90 rotor was assembled and centrifuged in a Beckman airfuge (Beckman Instruments Incorporated, Palo Alto, California) (Figure 1) for approximately 20 min at an air pressure of 30 psi which was equivalent to a terminal speed of approximately 95,000 rpm. After the run was completed, the grids were removed and immediately stained with 1% uranyl acetate for 30 sec as described previously except that there was no washing before and after staining. The grids were examined using the Zeiss
EM10 electron microscope.

The number of viral particles per milliliter of the virus solution was calculated as follows:

\[
\text{Viral particles/ml} = \frac{\text{virus counted} \times \text{dilution factor}}{\text{column height (cm)} \times \text{area (cm}^2\text{)}}
\]

Efficiency of plating of the virus in EPC cells was determined as follows:

\[
\text{EOP} = \frac{\text{number of counted viral particles/ml}}{\text{number of plaques formed/ml}}
\]
Figure 1. Air-fuge ultracentrifuge employed in the determination of the efficiency of plating.
I. Propagation of Virus

1. Single-step growth curve in EPC cells

The single-step growth cycle of the RPS was carried out in EPC cells at 20 °C. Monolayers of cells formed in sixteen 25 cm² flasks were infected with 0.5 ml of RPS virus at a multiplicity of infection (MOI) of 0.1. Virus was allowed to adsorb for 90 min at rt with intermittent rocking every 10 to 15 min. The infected monolayers were washed twice with MEM-0, then 5 ml of maintenance medium (MEM-5) was added to each flask and incubated at 20 °C. Two flasks were removed from the incubator and frozen (-70 °C) at each of the following selected intervals of 0, 0.5, 3, 6, 12, 24, 36, 48, 72 and 96 h p.i.. For virus assay, the two flasks from each time interval were thawed and then were pooled into a 15 ml plastic tube (Falcon). The virus yield in the cell-free culture fluid for each selected time was titrated by the TCID₅₀ method as described previously.

2. Production of cell-associated and released virus

Monolayers of EPC cells in sixteen 25 cm² flasks were infected with 0.5 ml of a virus stock containing 1.03 x 10⁴ PFU/ml. After incubation for 1.5 h at rt, the infected cultures were rinsed twice with MEM-0, filled with 5 ml MEM-5/flask, and then incubated at 20 °C. At each incubation period of 1.5, 3, 6, 9, 12, 24, 30 and 48 h, two flasks were
removed from the incubator. The culture fluids were pooled and stored at -70 °C. The residual cell monolayer inside the flasks were carefully rinsed twice with MEM-0 and then the flasks were filled with 5 ml of MEM-5. The flasks were then frozen at -70 °C. Following one cycle of freezing and thawing, the cell suspensions from the two flasks were pooled and clarified by centrifugation at 1000 g for 5 min. Serial 10-fold dilutions were made in MEM-0 for both the pooled culture fluid and the pooled cell supernatant and the titer of each was determined by plaque assay performed in 6-well plates.

3. Virus replication in selected fish cell lines

Epithelioma papillomus cyprini (EPC) cells were initially used for the isolation, replication and titration of RPS. In this experiment, seven fish cell lines available in this laboratory were tested for their ability to support the replication of the virus. Monolayers of GCF, GCS-2, GCSB, FHM, RTG-2, BB, CHSE-214, and EPC cells were grown in 25 cm² flasks in MEM-10 at rt. The various cell cultures were infected with 0.5 ml of a virus solution containing 10⁵ PFU of virus. After an adsorption period of 1.5 h at rt, the infected cultures were rinsed 2x with MEM-0 and 5 ml of MEM-5 was added to each flask which has incubated at 20 °C. At each of the desired incubation period of 2, 4, 6, 8, and 10 days, 0.5 ml of infected culture fluid was removed from two
flasks (0.25 ml/flask) of each cell line. The virus yield in each cell line at various times was measured by the plaque assay in EPC cells.

4. Virus replication at selected temperatures

The experiment was carried out to determine the optimal temperature for the replication of RPS in EPC cells. Monolayers of EPC cells in ten 25 cm² flasks were infected with RPS at an 0.001 MOI. After 90 min adsorption at rt, the inoculum was removed and the monolayers rinsed twice with MEM-0. Subsequently, 10 ml of MEM-5 were added to each of the flasks. Duplicate flasks were incubated at the following temperatures, 10, 15, 20, 25 and 30 °C. At each 48-hour interval for a total period of 10 days, one milliliter of the culture fluid was removed from two flasks at the same temperature (0.5 ml/flask), and the virus yield determined by the plaque assay as described previously.

5. Virus replication in three kinds of growth media

Medium 199 (Sigma Chemical CO., St.Louis, Missouri) supplemented with 5% fetal bovine serum (199-5), Leibovitz L-15 (Sigma Chemical Co, St.Louis, Missouri) medium containing 5% fetal bovine serum (L15-5) and MEM-5 were compared for their ability to support the replication of RPS. Monolayer cultures of EPC cells in six 25 cm² flasks were inoculated with 0.5 ml of a virus stock (10⁵.0 PFU/ml). The virus was
allowed to absorb for 90 min at rt. Following rinsing twice with MEM-0, duplicate flasks received 5 ml of medium 199-5, L15-5 or MEM-5. After 2 days incubation at 20 °C, the flasks were frozen at -70 °C, and then thawed. The infected cultures containing the same medium were pooled and the virus yield determined by plaque assay conducted in 6-well plates of EPC cells.

6. Relationship between different multiplicities of infection (MOI) and virus yields

Various multiplicities of infection (MOI) were compared to determine the optimum MOI which produces the highest virus yield. This experiment was carried out in EPC cells grown in 25 cm² flasks. Each duplicate flask was infected with 0.5 ml of virus at an MOI of 0.00001, 0.0001, 0.001, 0.01, 0.1, 1 and 3 prepared in MEM-0. After 90 min adsorption at rt, the unadsorbed residual virus was removed and the cell monolayer was rinsed twice with MEM-0. The flasks received 10 ml of MEM-5 and then were incubated at 20 °C. At day 1, 2, 3, 4, 5, 6 and 7 p.i., 0.4 ml of the infected cell culture fluid was removed from duplicate flasks and the virus yield determined by the plaque assay method.
J. Serological Experiments

1. Preparation of anti-RPS serum

Two adult New Zealand white rabbits (2.5 kg/each), named rabbit-a (Ra) and rabbit-b (Rb) were used to produce polyclonal antibody against RPS. Three milliliters of preimmune sera were obtained from each of the two rabbits before they were immunized with the purified RPS. The preimmune sera were stored at -70 °C for later determination of their neutralization activity to RPS. The inoculum for the primary immunization was a 1:1 mixture of purified RPS and Freund's Complete Adjuvant (FCA) (Difco Laboratories). Rabbit-a was injected with 0.15 ml of this inoculum into each hind foot pad and 1.0 ml was injected subcutaneously in 12 different sites on the back. Rabbit-b received the same amount of the inoculum using the same method. The rabbits were boosted with a 1:1 mixture emulsion of the purified RPS and Freund's Incomplete Adjuvant (FIA) (Difco Laboratories) subcutaneously on the back 10 days after primary immunization. The second booster was conducted after 20 days following the primary immunization. Rabbit-a was subcutaneously inoculated with 0.7 ml RPS in FIA (10 sites), and rabbit-b was intravenously inoculated with 0.8 ml RPS. For the third, fourth and fifth boosters, 0.5 ml RPS was inoculated intravenously per animal at days 41, 62, and 77, respectively. The rabbits were bled at days 20, 33, 48, and
following the primary immunization, and each serum sample from the animals as titered for neutralizing antibody to RPS. The rabbits were bled to death by day 87 following primary immunization. The sera were titered for neutralizing antibody and aliquots of 10 ml were stored at -70 °C.

2. Neutralization experiments

**Neutralization titer** The neutralization test was carried out by using the constant-virus and varying-antiserum method. The serum titer was determined as the Neutralization Titer (NT). Serial 2-fold dilutions of a stock serum were made in test tubes containing 0.5 ml MEM-O, and each serum dilution was mixed with an equal amount (0.5 ml) of the stock virus suspension. The mixtures were incubated for 60 min at rt. Each virus-serum mixture was assayed for its infectivity by inoculating 0.1 ml of each sample into each of four wells of a 24-well plate of EPC cells. After 1.5 h adsorption at rt, 0.5 ml of the overlay medium was added to each well and the plates incubated at 20 °C. Incubation was continued for 5 days, after which the plates were fixed and stained, and the plaques counted. The neutralization titer was expressed as the highest serum dilution that reduces viral plaque formation by 50%.

**Neutralization kinetics** The neutralization procedure employed was essentially that described by McBride (1959), with slight modifications. Briefly, it was as follows. Stock
viruses to be tested were diluted with TNE buffer (pH 7.4) to contain \(10^5\) to \(10^6\) PFU per ml as determined by preliminary experiment. The 50 x dilution of the antiserum in TNE used in the actual experiments was previously determined to be the concentration which inactivated homologous virus by approximately 2-3 logs within 10 min at rt.

Ten seconds prior to time zero, 0.5 ml of the prediluted virus was added to 0.5 ml of the prediluted serum in a 13 x 100 mm test tube covered with an aluminum cap. The tube was gently agitated for 10 to 15 seconds to mix the contents, and exactly at time zero and three other intervals of 3, 6, and 9 min following the combination, a 0.1 ml sample was removed from the tube and dispersed into 9.9 ml of MEM-O contained in a 250 ml flask at 4 °C, producing a 1:100 dilution. This was further diluted to 1:1,000 by placing 1 ml taken from the 1:100 mixture into 9 ml of MEM-O at 4 °C. Then 0.1 ml samples collected at 5 and 10 min, respectively, were added to 9.9 ml TNE at 4 °C, producing 1:100 dilutions of each sample. Each diluted sample was agitated gently to mix the contents and assayed for residual virus by inoculation into duplicate wells of 6-well plates containing confluent monolayers of EPC cells. The results of the assays of surviving virus of the neutralization were plotted on a logarithmic scale against time on a linear scale and a straight line was drawn through the points. Subsequently, K values for the antiserum with each of the viruses were calculated and compared with each
The calculation of the K values were based on an equation described by McBride (1959):

\[ K = \frac{D}{t} \times 2.3 \times \log \frac{V_0}{V_t} \]

where \( K \) = the neutralization rate constant, \( D = \frac{1}{C} \) = the dilution of antiserum, \( V_0 \) = active virus at time zero and \( V_t \) = concentration of active virus at time \( t \).

3. **Fluorescent antibody staining of the infected cells**

Monolayers of EPC cells were grown on sterile glass cover-slips, 15 mm in diameter, and placed in 24-well plates. The growth media were decanted and the cultures were infected with various dilutions of a virus suspension made in MEM-O (0.1 ml/well). Control cultures were inoculated with 0.1 ml of MEM-O. The infected cultures and the controls were incubated at 20 °C for 24-36 hr. The medium was carefully removed, the cultures were briefly rinsed with PBS (pH 7.4), and then fixed with 80% cold acetone for 5 min. The coverslips were then removed from the wells of the plates very carefully, air dried and stored at 4 °C. For the immunofluorescent antibody (IFA) staining, the coverslips were immersed in 5% nonfat milk (Carnation Company) in PBS (pH 7.4) solution for 60 min at rt to block the non-specific binding sites. Then the coverslips were washed 2 twice with 0.05% Tween-20/PBS and once with PBS only, at 5 min per wash. The cultures were flooded with primary antibody (rabbit anti-RPS serum at 1:500 in 1% nonfat milk/PBS) for 60 min at rt.
The cultures were then rinsed three times as described above. The cover-slips were soaked in secondary antibody (goat anti-rabbit IgG antibody conjugated with fluorescence isothiocyanate (FITC) (Kirkeggard & Perry Laboratories Inc.) at 1:100 dilution in 1% nonfat milk/PBS) for 60 min at rt, then rinsed 3 times as described above and the IFA stained cultures were examined under a UV microscope (Nikon Corporation).

4. Nitrocellulose-enzyme immunoassay (NC-EIA)

With the production of anti-RPS antiserum of high neutralization titer to RPS, attempts were directed to developing a solid-phase immunologically-based enzyme assay procedure, the nitrocellulose-enzyme immunoassay (NC-EIA) for the rapid and specific detection of RPS. These studies involved three major steps. The first step in the development of the NC-EIA method for RPS was to determine the optimum concentration of the secondary antibody, goat anti-rabbit IgG antibody conjugated to horseradish peroxidase (HRP), for the assay. Nitrocellulose paper (Schleicher & Schuell BA 83, 0.2 um, Keene, N.H.) was cut into the proper size for a Hybri-Slot Manifold apparatus (Bethesda Research Laboratories, Life Technologies, Inc., Gaithersburg, Maryland) (Figure 2), soaked in distilled H2O for approximately 5 min, and then placed on top of the filter pads both being there sandwiched
Figure 2. Hybri-slot manifold used in the nitrocellulose-enzymeimmunoabsorbent assay
between the top and bottom templates of the manifold. The highly purified virus antigen used was diluted in TNE buffer (40 ul or 50 ul) and applied to each of the 24 wells. The samples were allowed to soak and then filtered through the NC membrane by gentle vacuum. The membrane was then removed from the template, air dried and cut into strips. Each of the strips was soaked with 5% nonfat dry milk/PBS solution and gently rocked at rt for at least 60 min on a Belly Dancer rocking platform (Stovall Life Science, Inc., Greensboro, North Carolina) to block the remaining protein binding sites of the membrane. The strips were washed 3 times as described previously and then transferred into a container holding antibody, rabbit anti-RPS serum at a concentration of either 1:100 or 1:1,000 diluted in 1% nonfat dry milk/PBS and incubated for 60 min at rt. Following the incubation, the strips were washed 3 times as before, and then transferred to different dilutions (2-fold dilution from 1:100 to 1:1,000,000 in 1% nonfat dry milk/PBS) of secondary antibody, goat anti-rabbit IgG conjugated horseradish peroxidase (HRP). After incubation for another 60 min, the NC membrane strips were washed another 3 times, and then flooded with DAB substrate (0.5 mg/ml 3,3'-diaminobenzidine tetrahydrochloride with 0.02% H2O2 in PBS). Following a period of 90 to 120 seconds, reaction of the enzyme with DAB substrate was halted by picking up the strip from the substrate solution and placing it into distilled water. The strips were air-dried,
and reflectance densitometer readings were carried out in a GS 300 transmittance/Reflectance Scanning Densitometer (Hoefer Scientific Instruments, San Francisco, California).

The second step was to determine the optimum concentration of the first antibody for the assay. This test was carried out by using the same procedure as described above for determining the optimal concentration of second antibody except that in this experiment, various dilutions of the first antibody (2-fold diluted from 1:1,000 to 1:16,000 in 1% nonfat dry milk/PBS) were tested for maximal reaction with the optimal concentration of the second antibody.

The last step was to determine the minimum amount of virus antigen necessary for a positive reaction in the NC-EIA assay. In this experiment, highly purified virus was diluted from 1:10 to 1:500,000 in PBS and the test was carried out by the methodology described above. All the procedures were performed at rt and the incubation and washings were carried out in 20 x 150 mm screw-capped test tubes placed on the Belly Dancer rocking platform (Stovall Life Science, Inc., Greensboro, North Carolina).

K. Biochemical and Biophysical Techniques

1. Stability of RPS to freezing-thawing stability

The stability of RPS to repeated freezing and thawing was tested by titering virus suspensions in TNE buffer before and after freeze-thaw cycles. The virus titers were
determined by plaque assay carried out in 6-well plates with EPC cells. A virus stock was prepared in 2.0 ml of the buffer. A 0.5 ml of the suspension was used to determine the viral titer of the solution before freezing (time 0), the rest of the suspension (1.5 ml) was transferred into a screw-capped plastic vial (NUNC) and frozen at -70 °C. The vial was removed from the freezer and thawed at 25 °C water bath after 1 day and 0.5 ml of the solution was removed for the determination of residual viral infectivity using plaque assay. The vial was then placed again at -70 °C. Using this protocol, the second and third freeze-thaw cycles were conducted at days 7 and 30 after initially freezing, and 0.5 ml of the suspension was removed each time. Employing this method, other cell culture buffers, i.e. Hank's balanced salt solution (HBSS), MEM-0, and MEM-4 were also tested for their ability to protect the virus during freezing and thawing.

2. Stability of the virus stored at -10 °C and -70 °C

The stability of RPS to -10 °C and -70 °C was tested by storing suspensions of the virus at these temperatures and removing aliquots for plaque assay at selected intervals. A portion of a stock of the virus prepared in TNE buffer was titered at the beginning of the storage period. Aliquots (0.5 ml) of the remaining stock solution were dispensed into 1.0 ml plastic screw-capped vials (NUNC). These vials were stored at -10 °C and -70 °C freezers, respectively.
Following a storage period of 1, 7 and 30 days, vials containing the virus suspension were removed from the freezers and assayed for surviving virus using the plaque assay. In this experiment, other buffers i.e. HBSS, MEM-0, and MEM-4 were also tested using the same method to compare their effect on the stability of RPS when stored at -10 °C and -70 °C.

3. **Stability of RPS incubated at 4, 20, 37, and 56 °C**

   The stability of the RPS at 4, 20, 37, and 56 °C was examined. A virus stock containing $10^7$ PFU/ml was prepared in MEM-0, and 5 ml of the virus suspension was dispensed into each of 4 screw-capped tubes. One was placed at each selected temperature of incubation. At 0, 0.13, 0.5, 1, 3, 6, 12 and 24 h, aliquots (0.5 ml) of the suspension were removed from each of the 4 tubes and residual infectivity was determined by TCID$_{50}$ assay as described previously.

4. **Stability at low pH (pH 3)**

   The stability of RPS at low pH (pH 3) was determined by employing the method of Ketler et al (1962). A 0.2 ml of virus suspension was mixed with 1.8 ml of MEM-0 and adjusted to pH 3.0 by addition of 0.1 N HCl. A 1 : 10 dilution of the virus solution in MEM-0 at pH 7.2 served as control. Both the treated sample and controls were incubated at room temperature (23 °C ± 1) for 3 h, and the residual infectious virus was
titered using the plaque assay technique. The treated sample and control were also negatively stained with 2% uranyl acetate and then examined under a Zeiss 10/A° electron microscope to determine any morphological change due to the treatment. Poliovirus type 1, a naked isometric virus stable at pH 3, and vaccinia, a complex enveloped virus unstable at pH 3 were included as both negative and positive controls in this test.

5. Stability to lipid solvents

The resistance of RPS to treatment with lipid solvents was determined by two methods. Poliovirus type 1, a naked isometric virus and vaccinia virus, a complex enveloped virus were employed as both negative and positive controls in this study. Ether sensitivity was tested according to the method described by Andrewes and Horstmann (1949). One ml of a virus stock was placed into each of two screw-capped tubes of which one contains 1.0 ml anhydrous diethyl ether and the other contains 1.0 ml sterile 0.85% saline as control. The tubes were shaken to mix thoroughly and then incubated at 4 °C for 24 h. Then the mixtures in the tubes were poured into an open sterile glass-petri dish to allow the ether to evaporate. The virus in the remaining aqueous suspension and in the control was titered by plaque assay. A morphological examination of the ether treated sample was conducted under transmission electron microscopy. Chloroform sensitivity was
examined by the method of Feldman and Wang (1961). One ml of chloroform (HPLC) (Fisher Scientific Allied Co. Pittsburgh, PA) was added to 1 ml of virus suspension at rt. A control tube received 1 ml of sterile 0.85% saline. The mixtures were shaken every 10 min during a 60 min period at rt, and the tubes were then transferred to a 4 °C incubator for an additional 21 h. The tubes were centrifuged at 1,000 x g for 10 min to separate the chloroform from the aqueous phase in the treated sample and infectious virus in the aqueous phase of the treated sample and in the saline control was titered by TCID$_{50}$ assay.

6. Replication of RPS in the presence of 5-bromo-2'-deoxyuridine (BUDR)

The sensitivity of RPS to the halogenated pyrimidine 5-bromo-2'-deoxyuridine (BUDR) was determined by the method of Revozzo and Burke (1973). Poliovirus type-1, an RNA-containing virus which is insensitive to BUDR, and vaccinia virus, a DNA-containing virus which is sensitive to BUDR, were included in this test as negative and positive controls. The test was carried out in 4 steps. 1) Preparation of cell cultures. Epithelioma papulosum cyprini cells were seeded into 6-well plates and incubated at 20 °C while BGMK cells seeded in 6-well plates were incubated at 37 °C. 2) Virus preparation and inoculation. A virus stock solution was diluted to 10$^0$ and 10$^{-1}$ in MEM-0 and 0.2 ml of each of the dilutions was inoculated into each of the two wells. 3)
Incubation in the presence of BUDR. After 60 min adsorption at rt, the liquid was decanted from the wells of the plates and the monolayer cells were rinsed twice with MEM-0. Two and a half ml of MEM-5 containing either 20 ug BUDR/ml or 40 ug BUDR/ml was added to each well. The cultures receiving 2.5 ml of MEM-5 per well as controls. Incubation was carried out at 20 °C for the RPS-EPC cultures and at 37 °C for the poliovirus- and vaccinia virus-BGMK cultures, respectively.

4) Virus titration. After 72 h p.i., 0.5 ml of the culture fluid was harvested from each well and the virus yields produced from each treatment were determined by TCID₅₀ assay.

7. Density of RPS in sucrose gradient

Virus was purified through alternate discontinuous and continuous sucrose gradients. The blue-whitish viral band was collected from the gradient by side-puncture hole or by bottom-puncture hole. The banded virus was diluted in TNE buffer and pelleted on to a 60% sucrose cushion by centrifugation at 243,000 x g for 30 min in a SW 50.1 rotor (Spinco Division of Beckman Instruments, Inc., Palo Alto, California). The virus pellet was resuspended in TNE buffer. To determine the buoyant density of the virus in sucrose gradient, 1.0 ml of the virus suspension was layered on top of a step gradient of 0.5 ml 50% w/w, 1.0 ml 40% w/w, 1.0 ml 30% w/w, 1.0 ml 20% w/w, and 0.5 ml 10% w/w sucrose. The gradient was centrifuged for 24 h at 243,000 g in the SW 50.1
rotor. Following the completion of the run, eight-drop fractions (200-250 ul) were collected from the gradient by puncturing a hole in the the bottom of the tube. The density of the different fractions was determined by taking 30 ul of each of the fractions and reading the refractive index with an ABBE refractometer (Scientific Instrument Division of American Optical Corporation, Buffalo, New York). The readings at 25 °C were corrected for the TNE buffer by substraction of the refractive index of the TNE buffer. The density of the fractions were measured by the international critical formula:

\[
D = (10.8601 \times RI) - 13.4974
\]

\(D = \text{density, } RI = \text{refractive Index}\)

The fractions were also assayed for infectivity by TCID50 assay.

8. **Protein analysis in polyacrylamide gels**

The virus was purified through sucrose gradients. One discontinuous and two continuous sucrose gradients were used. The viral band was collected either by using a 1 cc tuberculin syringe from the side or by puncturing from the bottom of the centrifuge tube then collecting the band. The purified virus was degraded by adding equal volume of 2x denaturing buffer (0.125 M Tris, pH 6.8, 4% SDS, 20% glycerol, and 10% mercaptoethanol) and then heated (in boiling water for 1.5-2 min. The dissociated proteins and
high and low molecular weight protein markers (Biorad Laboratories. Richmond, California) were run simultaneously on SDS gels prepared according to the Laemmli system (Laemmli, 1970b).

Electrophoresis was carried out in 1.5 mm thick, 14 cm x 16 cm polyacrylamide gels using a discontinuous buffer system where two different layers of gel were poured. The lower layer is the resolving or separating gel and the upper layer is the stacking gel. A 10% separation gel was prepared by combining the following: 10 ml 30% acrylamide (acrylamide : Bisacrylamide = 36.5 : 1), 7.5 ml 1.5 M tris-HCl (resolving gel buffer), pH 8.8, 0.3 ml 10% w/v SDS, 12 ml triple-distilled water, 0.15 ml 10% (w/v, freshly prepared) ammonium persulfate (initiator), and 0.01 ml TEMED (polymerization catalyst). Except for the polymerization catalyst and the initiator, the reagents were added into a 50 ml flask and the mixture was deaerated under vacuum for about 1 min, then the TEMED and the initiator were added to initiate polymerization. This mixture was poured into the electrophoresis unit, and a few drops of distilled water was layered above the mixture. The gels were allowed to polymerized for 1 to 1.5 h at rt. The 4% stacking gel was prepared by mixing 1.33 ml 30% acrylamide (acrylamide : Bisacrylamide = 36.5 : 1), 2.5 ml 0.5 M Tris-HCl buffer, pH 6.8 (stacking gel buffer), 0.1 ml 10% SDS, and 6.1 ml triple-distilled water. The mixture was deaerated and then treated
with 0.05 ml ammonium persulfate, and 0.005 ml TEMED. The stacking gel monomer solution was pipetted into the glass plate sandwich over the separating gel to a level of a few mm below the top of the glass plates. A well-forming comb was inserted into the stacking gel to form sample wells. The gel was allowed to polymerize at rt for 0.5 to 1 h. After polymerization of the stacking gel, the comb was carefully removed from the top of the gel sandwich. The sample wells were rinsed with running buffer prepared by mixing 12 grams Tris, 57.6 grams glycine, 40 ml 10% SDS, and triple-distilled water to 4 liters.

The samples for electrophoresis were prepared by mixing an equal volume of the sample and 2x buffer (2.5 ml 0.5 M tris-HCl, pH 6.8; 4.0 ml 10% SDS; 2.0 ml glycerol; 1.0 ml 2-mercaptoethanol; and 0.5 ml triple-distilled water) The treated samples were heated in boiling water for 1.5 min, then chilled in ice before electrophoresis. A 10 to 50 ul of the sample was then loaded into each well of the stacking gel using a 100 ul-micropipette. Electrophoresis was run at constant current of 30 ma (200 v) for 3.5-4 h at 20 °C until the bromophenol blue tracking dye had reached within 1 cm from the bottom of the gel.

The gels were removed from the unit by using a plastic spacer and placed in 400 ml fixative solution (160 ml methanol, 40 ml glacial acetic acid, and 200 ml triple-distilled water) for 60 min or overnight. Then the gels were
fixed in 400 ml solution (10% ethanol/5% glacial acetic acid) for 30 min with 1 x change of the solution. After fixation, the gel was immersed in 200 ml of 10% oxidizer (Biorad Laboratories. Richmond, California) for 10 min, followed by rinsing 3 times in 400 ml of triple-distilled water. Finally, the gel was exposed to 200 ml of silver staining reagent (10% V/V) for 30 min. The gel was briefly rinsed in 400 ml of distilled water and then placed in the developer until the bands appeared while the background remained clear. The developer was stopped by soaking the gel in 400 ml of 5% glacial acetic acid (v/v). The gel was dried on a filter paper in the Gel Slab Dryer (Biorad Laboratories). The molecular weights of unknown viral proteins were determined from a standard curve generated by plotting the molecular weight of the low range molecular weight standards run on the gel (12% SDS polyacrylamide gel) vs the distance migrated from the interface of the stacking and separating gels in centimeters.

9. Western blotting experiment

In addition to the viral protein studies, a western blotting experiment was carried out to identify the specific viral proteins of RPS. Three other rhabdoviruses (IHNV, RC, and VHSV), all isolated from fish, were also employed in this comparison test. The viruses were purified by sucrose density gradient centrifugation and the protein
concentrations were determined for each of the virus preparations. Electrophoresis was carried out exactly as described above. The viral samples were treated as described previously and the same amount of viral antigen from each of the viral preparations was loaded into the same gel and run together. After the electrophoresis was completed, the gel was removed from the unit, placed in blotting buffer (methanol 200 ml, Tris 3 grams, glycine 14.4 grams, and distilled H₂O 800 ml) and shaken for approximately 10 min to remove the SDS. The banded proteins were immediately transferred to nitrocellulose membranes (Biorad Laboratories, Richmond, California) in methanol-Tris glycine buffer using the method described by Towbin (1979). Transfer was accomplished in a home-made electrophoretic transfer apparatus at 150 ma for 1.5 h. The nitrocellulose membrane was soaked in 5% nonfat milk/PBS for 60 min or overnight at 4 °C. The membrane was rinsed twice in PBS and then incubated for 60 min with rabbit anti-RPS IgG diluted 1:5,000 in 1% nonfat milk in PBS. After being washed twice with 0.5% Tween-20/PBS and once with PBS, the nitrocellulose membrane was incubated for 60 min with goat anti-rabbit IgG conjugated HPR diluted 1:100 with 1% nonfat milk in PBS. The membrane was washed 3X as previously described and incubated with the freshly prepared DAB substrate solution for 1.5 min. The color reaction was stopped by washing the membrane in distilled water, and the protein profiles for each the
viruses were documented by photography.

10. **Viral RNA analysis in polyacrylamide-agarose gels**

Extraction of RNA from rhabdovirus of penaeid shrimp for electrophoresis was conducted using the pronase-SDS-urea method described by Kelly (1972). Briefly, the rhabdovirus of penaeid shrimp was purified through sucrose gradients and the virus band was harvested by side-puncture using a 1 cc tuberculin syringe. The banded virus was pelleted and resuspended in very small amount of TNE buffer. Virus suspension was mixed with pronase E (Sigma) at a final concentration of 50 ug/ml (81 ul of virus/9 ul of 500 ug pronase/ml) in a 1.5-ml microcentrifuge tube (Fisher Brand, Fisher Scientific Allied Co.). The mixture was incubated for 60 min at 37 °C. Ten ul of 10% SDS solution and reagent grade urea crystals (0.024 grams) were then added into the tube at a final concentration of 1% and 4 M, respectively. The mixture was incubated for 10 min at 37 °C. The SDS was removed by precipitation with 11 ul of 20% potassium acetate in the cold (2% final concentration) for 20 min, followed by low speed centrifugation (1,000 x g, 10 min) at 4 °C. After centrifugation, the aqueous phase (100 ul) containing RNA was transferred to a fresh tube, mixed with 3 volumes of cold 100% ethanol, and the viral RNA was precipitated overnight at -20 °C. The RNA was sedimented at 10,000 x g for 10 min at 4 °C and the recovered RNA pellet was resuspended in 20-30 ul
of electrophoresis running buffer and stored at -20°C until use. Electrophoresis of RNA was carried out in slab gels (1.5 mm thick, 14 cm x 16 cm) consisting of 2% polyacrylamide (0.1% bisacrylamide) and 0.6% agarose. Vertical slab unit SE400 (Hoefer Scientific Instrument, San Francisco, California), an apparatus routinely used for protein analysis, was adopted for the purpose of analyzing the genomic RNA of RPS. The gels were prepared by mixing 12.5 ml 8% acrylamide (acrylamide : bisacrylamide =20 :1), 25 ml of 2x Tris-acetate-EDTA buffer (0.08 M Tris, 0.04 M Na-acetate, and 0.004 M di-Na EDTA), and 12.5 ml 2.4% agarose (Ultrapure Agarose, electrophoresis grade, Bethesda Research Laboratories, Gaithersburg, Maryland). The slab gels were allowed to polymerize overnight. After polymerization of the gel, the comb was removed and the sample wells were rinsed twice with running buffer (1x Tris, Acetate, and EDTA buffer, pH 7.8). The RNA samples for electrophoresis were prepared by mixing an equal volume of the sample and gel-dye solution (0.1% Bromophenol blue, 50% sucrose, and 0.1 M EDTA, pH 6.8). A 20-30 ul of RNA sample was loaded into each well. Both vesicular stomatitis virus RNA and RNA molecular-weight marker II (molecular Biology Boehringer Mannheim, Germany) were used as known molecular weight markers. Electrophoresis was carried out in 1x buffer for 2.5-3 h at 150 V, 68 ± 1 mA/gel. After the completion of the electrophoresis, the gels were carefully removed, fixed in 400 ml of 40%
methanol/10% acetic acid (v/v) for 60 min, and followed in 400 ml of 10% ethanol/5% acetic acid (v/v). The gel was then immersed in 200 ml of 10% oxidizer. After 10 min, the gel was rinsed 3x in 400 ml of triple-distilled water and flooded in 200 ml of silver reagent (10% v/v) to stain for 30 min, then briefly rinsed with the distilled water, and finally immersed in 200 ml of developer. After approximately 20 seconds, the yellow-brown solution was poured off and fresh developer was added and shaken gently until the RNA bands appeared (3-5 min). Development was stopped by flooding the gel in 5% of acetic acid solution. The gel was photographed and then dried onto filter paper in the Gel Slab Dryer (Biorad Laboratories, Richmond, California). To determine the molecular weight of the rhabdovirus of penaeid shrimp RNA segments, a standard curve was plotted using the relative mobilities of the vesicular stomatitis virus RNA versus molecular weight as described by Shatkin et al. (1968).

L. In Vivo Studies

1. Pathogenicity of RPS for penaeid shrimp

The pathogenicity of the virus for penaeid shrimp was tested in blue shrimp (Penaeus stylirostris). Shrimp weighing 1-4 grams were injected intramuscularly (i.m.) with either 10⁵ PFU of virus in TNE buffer or 0.05-0.1 ml TNE buffer. The shrimps were held in 15 L water aquaria at rt (20-24 °C) for 3 weeks and observed daily for mortality.
Shrimp dying during the experiment were frozen and kept at -70°C until they were titered for virus by plaque assay.

2. Virus replication in different organs and tissues of penaeid shrimp

The amount of virus produced in different organs of the blue shrimp was determined by inoculating 6 shrimp (approximately 15 grams each) i.m. with each 0.05-0.1 ml of TNE containing 10^7 PFU of highly purified sucrose-banded virus. The control group consisting of 6 shrimp that received the same amount of TNE buffer only. At days 6, 10 and, 20, a single shrimp was removed from each of the test and control groups and dissected. The oka organ, heart, gills, nerve cord, body muscle, intestine, hepatopancreas, and head soft tissue were recovered separately. A 10% (w/v) homogenate in TNE containing antibiotics was prepared by grinding each of the organs in a mortar and pestle with some sterile sand. The homogenates were centrifuged at 1000 x g for 20 min to remove cellular materials. The supernatant was then mixed with an equal amount of antibiotic incubation mixture (AIM) and incubated for approximately 30 min at 4 °C. The sample was diluted in MEM-O and assayed for viral infectivity in 6-well plates of EPC cells.

After identifying the Oka organ to be the primary target organ for RPS, the Oka organs from both RPS-infected and from uninfected shrimps were collected and weighed individually. The weights were then averaged and compared.
3. Pathology in virus infected penaeid shrimp

Histological changes in RPS infected blue shrimp were determined following the i.m. injection of 0.05-0.1 ml virus solution (10^6 PFU/ml) into each 5-6 g shrimp. Shrimp for histopathology examination were killed by injection of Davidson's fixative (95% ethyl alcohol:formalin:glacial acetic acid:distilled water = 3:2:1:3) (Humason, 1979) directly into head and body tissues of the animals. Then the cuticle for the length of the shrimp was opened along the lateral midline using dissecting scissors, and the whole animal was immersed into the fixative. Shrimp tissues for light microscopy were processed using routine standard histopathology techniques (Humason, 1979), and the tissue sections were stained with hematoxylin and eosin (Luna, 1968). The number and approximate size of lymphoid organ proliferative or reactive centers (PCs) were assessed microscopically. The PC frequency was measured in five saline control and five RPS-infected shrimp by counting the number of PCs in two 200x microscopical field per lymphoid organ per shrimp (10 PCs per group). The approximate size of lymphoid organ PCs in the RPS vs the control group was determined by ocular micrometer measurement. For the evaluation of lesion size, the diameter of five PC lesions from each of five shrimp per group was made. Thus, a total of 50 lesions (25 per each group) were compared. A paired t-Test was used to assess the PC frequency and microscopic size
between the RPS-infected and the non-exposed animals.

4. Immunofluorescent staining of the Oka organs from infected penaeid shrimp

After the discovery of the Oka organ as the target of RPS infection, an experiment was carried out to detect RPS infection in shrimp by use of the fluorescent antibody technique. In this test, both blue shrimp and white shrimp were evaluated. The test group of shrimp were inoculated with purified RPS while the control group only received the buffer. At days 6 and 21, Oka organs from both infected and uninfected shrimp were identified and carefully removed, then crushed and spread out to form a thin layer on microscope slides. The slides were air dried and fixed with 100% acetone for 5 min. For immunofluorescent antibody (IFA) staining, the slides were soaked in 5% nonfat milk/PBS for 60 min at rt for blocking, then washed twice with PBS, and subsequently incubated with primary antibody (1:100 rabbit anti-RPS IgG in 1% nonfat milk/PBS) for 120 min at rt. The slides were washed twice with PBS and then soaked in secondary antibody (1:200 goat anti-rabbit IgG FITC conjugate in 1% nonfat milk/PBS) for 60 min at rt. The slides were washed another two times with 0.05% Tween 20/PBS and then examined using the fluorescent microscopy. Photographs of the preparation were taken with Kodak High Speed Ektachrome film.
IV. RESULTS

A. Isolation of Rhabdovirus of Penaeid Shrimp

Sucrose gradient banded samples from the entire internal organs and tissues of the head (except for hepatopancreas and stomach) of IHNV-infected shrimps prepared as described were inoculated into monolayer cultures of EPC, GCF, GCS-2, and GCSB cells. The infected cultures were incubated at 20 °C for EPC and 25 °C for the three GC cell lines. The infectivity of RPS in the different cell lines is shown in Figure 3. Focal areas of cytopathology (CP) consisting of rounded cells were observed in the EPC cultures infected in the undiluted virus sample as early as 2 days after infection. These areas consisted of a clear central zone surrounded by rounded-refractive cells. These foci progressively increased in size with incubation time. By day 5, almost the entire cell monolayer was destroyed (Figure 4). Cultures infected with higher dilutions of virus did not show CPE until later. Infectivity titers as measured by the 50% tissue culture infectious dose end point (TCID50) assay were approximately $10^6$ TCID50/ml. No cytopathogenic effect (CPE) was observed in the GC cell cultures infected with the same samples. The uninfected GC and EPC control cultures did not show any CPE during the period of the observation. Virus harvested from the infected culture
Figure 3. Phase contrast photographs of EPC and GCSB cells inoculated with MEM-0 and with rhabdovirus of penaeid shrimp at 3 days post-infection at 20 °C. (A) GCSB control, (B) GCSB infected with RPS, (C) EPC control, and (D) EPC infected with RPS.
Figure 4. Phase contrast photomicrographs of EPC cells infected with rhabdovirus of penaeid shrimp for 3 days (A) and 5 days (B and C) p.i. at 20 °C.
was filtered through a 0.22 μm membrane filter and the filtrate was diluted 1:10 in MEM-0 and then reinoculated into fresh EPC cells at 20 °C. A similar CPE reappeared within 3-4 days. Virus was then successfully passed 3 more times in EPC cells yielding each time the characteristic CPE.

**B. Staining and Microscopy**

1. **Staining of infected cells**

   The crystal violet stain was routinely used to visualize any cytopathic alterations in infected cell cultures. Since the stain was dissolved in fixative solvent, the fixation and staining was completed in a single step. This staining rendered the foci of CPE easily discernible.

   Acridine orange staining of uninfected and infected EPC cells revealed no specific characteristic staining changes associated with RPS-infected cell cultures.

2. **Morphology of the virus**

   Electron microscopical examination of negatively stained sucrose banded virus particles showed bullet-shaped structures (B). The virus is cylindrical with one rounded and one planar end (Figure 5). Surrounding the internal transversely arranged helical nucleocapsid is an envelope from which emanated regularly spaced projections (10-12 nm). Each of the projections has a knoblike structure at the
distal end, covering the surface of the virus. However, these structures were difficult to visualize and could only be seen occasionally (Figure 6). The particles measured 65-77 nm in diameter and 115-138 nm in length. A few shortened or truncated structures (T) were also observed in the virus preparations (Figure 5A). These particles had a mean diameter and length of 65-77 nm by 90-95 nm. Like the B particles, the T particles were surrounded by regularly spaced projections with knoblikes structures at the distal end. Several particles permitted partial or complete penetration of the negative stain (Figure 5B). The morphology of this virus isolate from penaeid shrimp is characteristic of the viruses of the family *Rhabdoviridae*. 
Figure 5. Electron micrographs of rhabdovirus of penaeid shrimp negatively stained with 2% uranyl acetate. 
(A) Virus particles purified by sucrose density gradient centrifugation (magnification: x 180,000). 
(B) the series of transverse striations along entire length of some virus particles. 
(magnification: x 368,000)
Figure 6. Electron micrographs of a negatively stained rhabdovirus of penaeid shrimp showing some peplomers still intact (A) (magnification: x 650,000). (B) A highly magnified transverse section of the virus showing the helical nucleocapsid, dark staining envelope, and the knoblike peplomers (magnification: 992,000).
3. Electron microscopy of infected cells

Examination of thin-sections of RPS-infected EPC cells revealed bullet-shaped particles both inside and outside the cells which are characteristic of members of the rhabdovirus family (Figure 7). When present inside the cells, the particles were primarily localized in membrane-bound vesicles in the cytoplasm. The nuclei of the cells did not appear to be involved in viral replication. A few particles were observed budding at the cell's surface (Figure 7A). Also present in thin sections were several circular profiles representing transverse sections of the cylindrical virus. These particles had the appearance of a broken ring encircled by an envelope (Figure 7B). The envelope was composed of two dense lines separated by a translucent zone. The broken ring structure represents the internal transversely arranged helical nucleocapsid which is seen as a dense beaded layer immediately beneath the envelope. Such particles have a lucent center space. Around the peripheries of the virus particles were regularly spaced surface projections. The individual projections were not well resolved.
Figure 7. Electron micrographs of thin sections of EPC cells infected rhabdovirus of penaeid shrimp. (A) Virus particles are present outside of the plasma membrane. An arrow showing a virus particle budding through the cytoplasmic membrane (magnification: x 89,000). (B) Virus particles localized within a cytoplasmic vesicle (magnification: x 132,000).
C. Titration of Virus

1. Relationship between virus dilution and virus titer

The quantitative relationship was determined between virus concentration and the number of plaques formed. The results, which are summarized in Figure 8, showed that the number of viral plaques formed is linearly related to the dilution of the virus preparation. This linear relationship was reproducible within ±12%.

2. Comparison of plaque assay and TCID50 titer

The enumerative plaque assay and the quantal 50% tissue culture infectious dose (TCID50) are the two principal methods routinely used to determine virus titers. In this experiment, the two methods were compared to determine which was superior for the titration of RPS in EPC cells. This study also allowed comparison of virus titers obtained from different experiments employing the two different assays. For the virus preparation, the maximal plaque assay titer obtained was 5.75 x 10^6 PFU/ml at day 5 p.i. For the same virus preparation, the TCID50 titer was 1.58 x 10^7.0 TCID50/ml on day 4, and increased to 3.98 x 10^7.0 TCID50/ml on day 7. No further changes in TCID50 titer was obtained by day 14 and day 21. The 7 day incubation period was found to be adequate for the TCID50 assay. If RPS titer determined by
Figure 8. Linear relationship between the dilutions and the plaque titers of rhabdovirus of penaeid shrimp established in EPC cells at 20 °C.
TCID\textsubscript{50} method was assigned a value of 100%, then the plaque assay would represent a 14% reading of the virus titer. The correlation between the two methods for measuring the same virus sample was that one PFU unit is equivalent to 7 TCID\textsubscript{50} units.

3. Comparison of plaque assay overlays

When the same suspension of virus was assayed using different overlay media, the results revealed marked differences in viral titers (Table 5). The 1.5% agarose overlay was found to be unsatisfactory for use in the plaque assay. The lack of plaques formed suggested that the warm agarose might have inactivated the virus and also damaged the cell monolayer. The cells under this overlay appeared in very poor condition and were often destroyed following the deposition of the overlay. The liquid overlay MEM-4 was also found to be inadequate for use in the plaque assay because no localized plaques were formed. The 0.75% methyl-cellulose plus MEM-4 mixture was found to be most satisfactory for the plaque assay. When the 0.75% methyl-cellulose plus MEM-4 overlay was used, the cell monolayer was maintained in very good condition and the resulting plaques were distinct and well defined. The 0.75% methyl-cellulose plus MEM-4 was adopted as the overlay medium for the plaque assay in this study.
Table 5. Comparison of different overlay media for plaque assay.

<table>
<thead>
<tr>
<th>Overlay media</th>
<th>No. of Plaques/ml</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEM-4</td>
<td>0</td>
<td>no localized plaques</td>
</tr>
<tr>
<td>1.5% ionagar-MEM-4</td>
<td>0</td>
<td>toxic to cell monolayer</td>
</tr>
<tr>
<td>0.75% methylcellulose-MEM-4</td>
<td>$10^6.8$</td>
<td>focal areas of cellular changes &amp; forming distinct plaques</td>
</tr>
</tbody>
</table>
4. Comparison of plaque forming efficiency of RPS in selected cell culture containers

Three kinds of cell culture containers (6-well plates, 24-well plates and 25 cm$^2$ flasks) commonly employed for cell culture were tested for their efficiency to produce viral plaques. When the same amount (0.5 ml) of the 10-fold diluted virus suspension made in MEM-0 was inoculated into EPC cell monolayers formed in these containers, the number of plaques produced were found to be different. The plaque titer of 2.37 x 10$^6$ PFU/ml obtained from 25 cm$^2$ flask was much higher than the titers of 4.95 x 10$^5$ and 9.0 x 10$^5$ PFU/ml produced in 24-well plates and 6-well plates, respectively, (Table 6). Thus, the higher number of viral plaques produced in the 25 cm$^2$ flasks makes it superior to the plates for plaque assay. The result indicates that because of its large cell surface area as compared to the 6-well and 24-well plates, the 25 cm flask promotes more efficient virus-cell contact and consequently yielding a greater number of plaques. Although the number of plaques produced in 25 cm$^2$ flasks was higher than that formed in 6-well plates, the latter plates were selected to perform plaque assay because they were simple, convenient, and economical to use.
Table 6. Comparison of plaque forming efficiency of rhabdovirus of penaeid shrimp on the selected cell culture containers

<table>
<thead>
<tr>
<th>Virus Dilution</th>
<th>Plates</th>
<th></th>
<th>Flasks</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24-Well</td>
<td>6-Well</td>
<td>25 cm²</td>
<td></td>
</tr>
<tr>
<td>$10^{-1}$</td>
<td>TNTC, TNTC</td>
<td>TNTC, TNTC</td>
<td>TNTC, TNTC</td>
<td></td>
</tr>
<tr>
<td>$10^{-2}$</td>
<td>TNTC, TNTC</td>
<td>TNTC, TNTC</td>
<td>TNTC, TNTC</td>
<td></td>
</tr>
<tr>
<td>$10^{-3}$</td>
<td>48,50</td>
<td>78,102</td>
<td>176,298</td>
<td></td>
</tr>
<tr>
<td>$10^{-4}$</td>
<td>3,7</td>
<td>6,10</td>
<td>16,20</td>
<td></td>
</tr>
<tr>
<td>$10^{-5}$</td>
<td>0,0</td>
<td>0,1</td>
<td>1,3</td>
<td></td>
</tr>
<tr>
<td>$10^{-6}$</td>
<td>0,0</td>
<td>0,0</td>
<td>0,1</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0,0</td>
<td>0,0</td>
<td>0,0</td>
<td></td>
</tr>
</tbody>
</table>

| Titer (PFU/ml) | 495,000 | 900,000 | 2,370,000 |
| PFE (%)        | 21      | 38      | 100       |
5. Comparison of plaque forming efficiency of virus in selected fish cell lines

The plaque forming efficiency of the RPS in different fish cell lines using 0.75% methylcellulose-MEM-4 overlay medium was determined in 6-well plates. Figure 9 shows the results of this experiment. As expected, the efficiency of plaque production reflected the ability of the cell line to support RPS replication as previously determined. More plaques were produced in EPC cells which makes this cell line superior to the others in terms of plaque forming efficiency. Although plaques were also formed in the three GC cells and BB cell line, the plaque forming efficiencies of these four cell lines were comparatively low. In the fathead minnow cell line, which did support the replication of the virus and produced a significant titer, no plaques were formed. No plaques were also produced in RTG-2 and CHSE-214 cell lines.

Compared to the plaques formed in the three GC cell lines, those produced in EPC cells were large, circular, clear and mostly uniform in size (Figure 10A). In contrast, the plaques formed in the three GC cells were small, irregular, not distinct and with fuzzy edges (Figure 10B). In such cell lines, the counting of these plaques was difficult and a dissecting microscope was always needed.
Figure 9. Plaque forming efficiency of rhabdovirus of penaeid shrimp in different fish cell lines.

EPC = epithelioma papillosum cyprini
BB = brown bullhead
GCF = grass carp fins
GCS-2 = grass carp snout-2
GCSB = grass carp swim bladder
RTG-2 = rainbow trout gonad
FHM = fathead minnow
CHSE-214 = chinook salmon embryo
Figure 10. The plaques of rhabdovirus of penaeid shrimp formed in EPC cell (A) and GCSB cells (B) stained with 1% crystal violet.
6. Relationship between volume of inoculum and plaque formation

Inoculum volume is one of the important factors which contribute to the accuracy and efficiency of the plaque assay for a virus. Since the 6-well plates were chosen for plaque assay in this study, the effect of inoculum volume was evaluated on plaque production. Nine different inocula volumes were employed in this test. Following adsorption for 1.5 h at rt and an infection period of 5 days, the viral plaques were counted and compared with the number of plaques expected for each inoculum size. The results (Table 7) showed that the largest number of plaques produced was from the inoculum volume of 0.2 ml per well. The observed number was 58, which was very close to the theoretical number of plaques (60) expected and represented a recovery rate of approximately 97%. The actual numbers of plaques produced from the other inocula were considerably smaller than the corresponding theoretical number of plaques expected. The results indicated that the optimal inoculum volume for plaque assay conducted in 6-well plates was 0.2 ml per well.
Table 7. Comparison of the number of viral plaques produced in 6-well plates with different inoculum volumes.

<table>
<thead>
<tr>
<th>Inoculum (ml)</th>
<th>Expected Plaques</th>
<th>Counted Plaques*</th>
<th>Plaques/ml</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>30</td>
<td>25</td>
<td>250</td>
<td>83</td>
</tr>
<tr>
<td>0.2</td>
<td>60</td>
<td>58</td>
<td>290</td>
<td>97</td>
</tr>
<tr>
<td>0.3</td>
<td>90</td>
<td>66</td>
<td>220</td>
<td>73</td>
</tr>
<tr>
<td>0.4</td>
<td>120</td>
<td>83</td>
<td>207</td>
<td>69</td>
</tr>
<tr>
<td>0.5</td>
<td>150</td>
<td>103</td>
<td>206</td>
<td>69</td>
</tr>
<tr>
<td>0.6</td>
<td>180</td>
<td>122</td>
<td>204</td>
<td>68</td>
</tr>
<tr>
<td>0.7</td>
<td>210</td>
<td>138</td>
<td>197</td>
<td>66</td>
</tr>
<tr>
<td>0.8</td>
<td>240</td>
<td>147</td>
<td>184</td>
<td>61</td>
</tr>
<tr>
<td>1.0</td>
<td>300</td>
<td>146</td>
<td>146</td>
<td>49</td>
</tr>
</tbody>
</table>

* Infection period was 5 days at 20 °C.
7. Relationship between adsorption time and plaque production

In the present experiment the optimal adsorption time of RPS to EPC cells was determined. Monolayers of EPC cells were infected with serial 10-fold dilutions of RPS for the following adsorption periods 0.5, 1, 1.5, 2, 3, and 4 h. Figure 11 shows that the rate of adsorption of RPS to EPC cells was linear up to 1 h p.i.. The rate then increased slowly until 2 h p.i., at which time adsorption was maximal. The results indicated that the maximal rate of adsorption of RPS to EPC cells was 1.5 h at rt. This adsorption period was adopted for all the experimental infections in this study.
Figure 11. Rate of adsorption of RPS in EPC cells in 6-well plates
8. Relationship between postinfection time and virus titers

This experiment was designed to determine the optimal postinfection period required for the maximal production of plaques by RPS in EPC cells. The results shown in Table 8 indicated that no plaques were visible until 3 days p.i. At this time the plaques were 0.1 - 0.2 mm in diameter and the virus titer was $9.3 \times 10^5$ PFU/ml. As the postinfection period increased, the plaque size and the titer also increased. At day 4 and 5 p.i., the plaque titers were $3.87 \times 10^6$ PFU/ml (0.4 mm) and $4.0 \times 10^6$ PFU/ml (0.65 mm), respectively. After 5-day p.i., the plaque titer did not significantly increase. The plaque titers determined at days 6, 7 and 10 p.i. were $4.1 \times 10^6$, $3.93 \times 10^6$ and $3.68 \times 10^6$ PFU/ml, respectively. Although there existed a difference among the plaque titers obtained from 4 to 10 days p.i., the changes were not significant (standard deviation = 0.157). Based on the plaque titer and plaque size, a post-infection period of 5 days was optimal for the plaque assay in 6-well plates.
Table 8. Comparison of plaque titers of rhabdovirus of penaeid shrimp determined at different postinfection times at 20 °C.

<table>
<thead>
<tr>
<th>Postinfection Time (day)</th>
<th>Plaque Titer (PFU/ml)</th>
<th>Plaquing Efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>$9.3 \times 10^5$</td>
<td>23</td>
</tr>
<tr>
<td>4</td>
<td>$3.87 \times 10^6$</td>
<td>94</td>
</tr>
<tr>
<td>5</td>
<td>$4.0 \times 10^6$</td>
<td>98</td>
</tr>
<tr>
<td>6</td>
<td>$4.1 \times 10^6$</td>
<td>100</td>
</tr>
<tr>
<td>7</td>
<td>$3.93 \times 10^6$</td>
<td>96</td>
</tr>
<tr>
<td>10</td>
<td>$3.68 \times 10^6$</td>
<td>90</td>
</tr>
</tbody>
</table>
9. Efficiency of plating (EOP) in EPC cells

The number of viral plaques to the number of infectious unit ratio was determined according to the method described under Materials and Methods. Briefly, highly purified RPS was prepared by differential centrifugation, and two cycles of discontinuous and continuous sucrose density gradients. The viral band having a density of 1.18-1.20 g/cm$^3$ was harvested and shown to contain only complete virus particles by electron microscopy.

The plaque titer of the virus sample in EPC cells was $6.0 \times 10^9$ PFU/ml. Electron microscopical examination of the negatively stained virus samples prepared for particle counting revealed that the virus particles were evenly distributed in the grids and contained very few or no aggregates (Figure 12). To determine the precision of the counting method, several counts were made in the same RPS sample and the maximum deviation in ten counts was 18% with an average deviation of 7.2% (Table 9). The counts shown in the table refer to the number of virus particles found in an area of $4.3 \times 10^{-8}$ cm$^2$. Since the column height was 0.6 cm, the dilution of the sample was 100, and the average count was 46.5, the average number of virus particles was calculated to be $1.8 \times 10^{11}$ particles /ml.

Since the virus sample counted above had an infectivity titer of $6.0 \times 10^9$ PFU/ml, the efficiency of plating (EOP) was calculated to be 30 particles per infectious unit.
Figure 12. Electron micrographs of 2% of uranyl acetate stained highly purified rhabdovirus of penaeid shrimp. (A) Even distribution of the virus in a field under a TEM prepared for viral particle counting (magnification: x 25,000). (B) High magnification of the virus (magnification: x 100,000)
Table 9. Precision of RPS particle counting.

<table>
<thead>
<tr>
<th>Replicas&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Virions /unit Area&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Deviation&lt;sup&gt;c&lt;/sup&gt; (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>40</td>
<td>14</td>
</tr>
<tr>
<td>2</td>
<td>49</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>51</td>
<td>10</td>
</tr>
<tr>
<td>4</td>
<td>45</td>
<td>3</td>
</tr>
<tr>
<td>5</td>
<td>46</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>49</td>
<td>5</td>
</tr>
<tr>
<td>7</td>
<td>47</td>
<td>1</td>
</tr>
<tr>
<td>8</td>
<td>38</td>
<td>18</td>
</tr>
<tr>
<td>9</td>
<td>53</td>
<td>14</td>
</tr>
<tr>
<td>10</td>
<td>47</td>
<td>1</td>
</tr>
<tr>
<td>Average</td>
<td>46.5</td>
<td>7.2</td>
</tr>
</tbody>
</table>

<sup>a</sup>Replicate counts taken from the different area of the same grid by transmission electron microscopy

<sup>b</sup>Numbers of virus particles counted from an area of 4.3 x 10^-8 cm^2

<sup>c</sup>Deviation from average was determined as follows:

\[
\frac{\text{Actual counted number} - \text{average number}}{\text{Average number}} \times 100\%
\]
D. Propagation of Virus

1. Single-cycle growth curve in EPC cells

The one-step growth cycle of the virus in EPC cells revealed that there was an eclipse period of approximately 3 h during which time, no virus was detectable by the TCID$_{50}$ assay. This was followed by a period of exponential growth in which virus titer increased dramatically. This period was completed by 48 hours postinfection and the total virus produced at this time was $10^{8.3}$ TCID$_{50}$ (Figure 13).

2. Comparison of cell-associated and released virus

The proportion of virus produced by infected cells which is liberated into the culture fluid may vary considerably depending on the type of virus. With some of the naked viruses, such as poliovirus there is a burst-like release of progeny virus from the infected cell. With several of the enveloped viruses including the rhabdoviruses release is continuous with no retention of the progeny in the infected cells. The present results indicated that 9 h p.i. at 20°C approximately 25% of the infectious RPS was liberated into the culture fluid of infected EPC cultures. The titer of the virus released in the fluid at this time was $1.03 \times 10^3$ PFU/ml (Figure 14). After 48 h p.i., the amount of virus released in the culture medium (extracellular virus) was $2.67 \times 10^7$ PFU/ml, and represented about 99.5% of the total virus.
Figure 13. Single-cycle growth curve of rhabdovirus of penaeid shrimp in EPC cells incubated at 20 °C for 2 days.
Figure 14 showed that a large amount of virus was cell-associated during the early period of the infection cycle. This is consistent with the electron micrographs (Figure 7B) obtained from thin-section electron microscopy which indicated that numerous viral particles were aggregated within the cytoplasm of the infected EPC cells. The accumulated data suggested freezing and thawing of the virus infected cell cultures was necessary in order to recover all the virus produced.
Figure 14. Cell-associated and released virus in EPC cells at 20 °C.
3. Comparison of virus replication in different fish cell lines

Several fish cell lines available in this laboratory were evaluated for their capacity of primary isolation of RPS from infected animals. The results obtained indicated that only the EPC cell line was useful for the primary recovery of the virus. Other fish cell lines were also tested for their ability to support replication of RPS. It was found that most of the cell lines were susceptible to the virus only after initial passage of the virus for 1-2 times in EPC cells. Cytopathology was observed 3-5 days following inoculation of the cell lines. However, the cell lines varied considerably in their capacity to support virus replication (Table 10).

The EPC cell line was the most susceptible cell line to RPS and had the highest yield of the virus. The entire EPC monolayer was destroyed within 2-days p.i. and the virus titer had increased more than 1000 times (10^{8.3} PFU/ml). In contrast, RPS replication in the other fish cell lines occurred at a slower rate. No apparent CPE was observed in these infected cells following 2 days p.i., except that some cytopathic change occurred in BB cells. At 4 days p.i., the virus yields produced in BB, GCF, GCSB, GCS-2, and FHM cell lines were all less than 10% of that produced in EPC cells. Virus production from these cell lines did not significantly increase at days 6, 8, and 10 p.i. Two of the fish cell
Table 10. Comparison of RPS production in eight fish cell lines

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Abbreviation</th>
<th>Titer (TCID$_{50}$/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epithelimo papulosum cyprini</td>
<td>EPC</td>
<td>10^8.33</td>
</tr>
<tr>
<td>Brown bluegill</td>
<td>BB</td>
<td>10^7.0</td>
</tr>
<tr>
<td>Grass carp fin</td>
<td>GCF</td>
<td>10^6.67</td>
</tr>
<tr>
<td>Grass carp swim bladder</td>
<td>GCSB</td>
<td>10^7.0</td>
</tr>
<tr>
<td>Grass carp snout-2</td>
<td>GCS-2</td>
<td>10^6.3</td>
</tr>
<tr>
<td>Chinook salmon embryo</td>
<td>CHSE-214</td>
<td>0</td>
</tr>
<tr>
<td>Rainbow trout gonads</td>
<td>RTG-2</td>
<td>0</td>
</tr>
<tr>
<td>Fathead minnow</td>
<td>FHM</td>
<td>10^5.8</td>
</tr>
</tbody>
</table>

Infection period was 2 days at 20 °C with 0.01 MOI of rhabdovirus of penaeid shrimp.
lines, CHSE-214 and RTG-2 did not support the replication of RPS. Cytopathic alterations were not observed in either of these two cell lines. These results provided important information as to the selection of a cell line for initial isolation and propagation of large quantities of RPS virus. The data also indicated the host range of the virus.

4. Virus replication at selected temperatures

The replication of RPS at five different temperatures was carried out to determine the optimum temperature for virus replication. The EPC cells were used for this experiment because it supported RPS growth and was capable of tolerating a wide range of temperatures (5-35 °C). The results revealed that the RPS multiplied at all the selected temperatures from 10 to 30 °C. The growth pattern was similar except at 10 °C (Figure 15). The optimal temperature for RPS replication was determined to be 20°C and the highest yields of virus were attained by 4 days p.i. (10\(^7\).5 PFU/ml). Virus production was delayed at lower temperatures. At 10 °C, virus replication occurred at a very slow rate, and maximum virus yield (10\(^6.8\) PFU/ml) similar to that obtained at 20 °C and 25 °C was not reached until 10 days p.i.. In contrast, at 30 °C, the virus titer approached its peak on day 2 but the viral titer was very low (10 \(^6.2\) PFU /ml), and the production of virus progressively declined soon after.
Figure 15. Production of rhabdovirus of penaeid shrimp in EPC cells at different incubation temperatures.
5. **Comparison of virus replication in three growth media**

The effect of three kinds of growth media was examined on RPS replication in EPC cells. The results (Table 11) shows that virus production in MEM-5 was highest \((2.4 \times 10^7\) PFU/ml) followed by medium 199-5 \((1.27 \times 10^7\) PFU/ml). The amount of virus produced in medium L15-5 was lowest \((6.5 \times 10^6\) PFU/ml). Medium MEM-5 was employed for all viral infection experiments in this study.

6. **Relationship between multiplicity of infection (MOI) and virus yield**

Multiplicity of infection (MOI) is one of the important factors which may affect virus production and also the physical properties of the virus particles produced. In this experiment the effect of seven different MOIs on RPS production in EPC cells at 20 °C was examined. The results revealed that the highest virus yield was produced at a MOI of 1.0 (Table 12). Viral production decreased markedly as the inoculum MOI was decreased. Virus yield at the higher MOI of 3 was also reduced.
Table 11. Effect of experimental media on production of rhabdovirus of penaeid shrimp in EPC cells.

<table>
<thead>
<tr>
<th>Experimental Medium</th>
<th>Virus Yield (PFU/ml)</th>
<th>Efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minimum essential medium (MEM-5)</td>
<td>2.4 x 10^7.0</td>
<td>100</td>
</tr>
<tr>
<td>Medium 199 (M199-5)</td>
<td>1.27 x 10^7.0</td>
<td>53</td>
</tr>
<tr>
<td>Leibovitz L-15 (L15-5)</td>
<td>6.5 x 10^6.0</td>
<td>27</td>
</tr>
</tbody>
</table>

Incubation at 20 °C for 2 days.
Table 12. Comparison of rhabdovirus of penaeid shrimp production from the EPC cells infected with different multiplicities of infection\(^a\).

<table>
<thead>
<tr>
<th>Multiplicity of Infection (MOI)</th>
<th>Virus Yields ((x 10^5) PFU/ml)</th>
<th>Efficiency ((%))</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.0</td>
<td>690 ND(^c)</td>
<td>77</td>
</tr>
<tr>
<td>1.0</td>
<td>900 692</td>
<td>100</td>
</tr>
<tr>
<td>0.1</td>
<td>570 630</td>
<td>70</td>
</tr>
<tr>
<td>0.01</td>
<td>358 570</td>
<td>63</td>
</tr>
<tr>
<td>0.001</td>
<td>270 293</td>
<td>33</td>
</tr>
<tr>
<td>0.0001</td>
<td>41 178</td>
<td>18</td>
</tr>
<tr>
<td>0.00001</td>
<td>1.2 148</td>
<td>16</td>
</tr>
</tbody>
</table>

\(^a\) Incubation at 20 °C.

\(^b\) Percentage of virus production for each of the MOIs was calculated by assigning a value of 100% to the virus yield obtained at MOI of 1.0.

\(^c\) ND=not done.
E. Serological Experiments

1. Preparation of immune serum in rabbits

The preimmune sera from both rabbit-a and rabbit-b showed no neutralizing antibodies to RPS. At 33 days after primary immunization, rabbit-a had no neutralizing antibody to RPS while rabbit-b had a titer of 80. At 48 days after immunization rabbit-a had a titer of 20 and rabbit-b a neutralizing antibody titer of 320. By eighty-seven days after primary immunization the neutralizing antibody titers for rabbit-a and rabbit-b rose to 80 and 6400, respectively (Table 13).

2. Neutralization experiments

Neutralization titer determination  The 50% plaque reduction neutralization titer of RPS by rabbit-b antiserum was found to be approximately 6,400 (Figure 16). In contrast, the neutralization titer of the anti-RPS serum against RC, a rhabdovirus isolated from carp was found to be approximately 2,000. These results indicated that there are common antigens shared between these two viruses which can be detected by this neutralization assay. A slight cross neutralization of the anti-RPS serum with another fish rhabdovirus, infectious hematopoietic necrosis virus (IHNV), was also observed but not with viral hemorrhagic septicemia virus (VHSV), another fish rhabdovirus of salmonid.
Table 13. Neutralizing antibody development in two rabbits following immunization with purified RPS.

<table>
<thead>
<tr>
<th>No. of Immunization</th>
<th>No. of Bleeding</th>
<th>Neutralizing Antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Rabbit-a</td>
</tr>
<tr>
<td>1 (day 1)</td>
<td>Pre-immune sera</td>
<td>0</td>
</tr>
<tr>
<td>2 (day 10)</td>
<td>1 (day 20)</td>
<td>0</td>
</tr>
<tr>
<td>3 (day 20)</td>
<td>2 (day 33)</td>
<td>0</td>
</tr>
<tr>
<td>4 (day 41)</td>
<td>3 (day 48)</td>
<td>20</td>
</tr>
<tr>
<td>5 (day 62)</td>
<td>4 (day 68)</td>
<td>20</td>
</tr>
<tr>
<td>6 (day 77)</td>
<td>5 (day 87)</td>
<td>80</td>
</tr>
</tbody>
</table>
Figure 16. Percentage of plaque reduction of rhabdovirus of penaeid shrimp and rhabdovirus carpio by anti-RPS serum.
Neutralization kinetics The serological relationship between RPS and three other rhabdoviruses of fishes were examined by neutralization kinetics. When the amount of surviving infectious virus was plotted on a logarithmic scale against time on a linear scale, a straight line was obtained (Figure 17). The neutralization rate constants were calculated from the slopes indicated that the $K$ values for RPS, RC, IHNV, and VHSV were 52.4, 39.2, 3.5, and 0.5, respectively. Repeated tests exhibited similar results. These results indicated that although the RPS antiserum neutralized the infectivity of 3 of the viruses tested, RPS, RC, and IHNV, the serum gave a higher $K$ value with its homologous virus (RPS) than it did with the other heterologous rhabdoviruses, RC, IHNV, and VHSV. Furthermore, the neutralization activity for RPS occurred very rapidly within 6 min.

3. Fluorescent antibody staining of the infected cells

When RPS-infected EPC cultures and control incubated for 48 hr at 20 °C were stained by the fluorescent antibody technique, specific fluorescence was only observed in the infected cultures and was seen in the areas immediately surrounding the foci of cell destruction (Figure 18). No fluorescence was observed in the uninfected cells surrounding these focal areas of cytopathic changes and also
Figure 17. Rates of neutralization of Rhabdovirus of penaeid shrimp, rhabdovirus carpio, infectious hematopoietic necrosis virus, and viral hemorrhagic septicemia virus by anti-RPS serum, as measured by reduction in numbers of plaques by serum virus mixtures inoculated in monolayer cultures of EPC cells.
Figure 18. Immunofluorescent staining of EPC cells infected with rhabdovirus of penaeid shrimp for 48 h at 20 °C.
in the control cultures.

4. Nitrocellulose-enzyme immunoassay (NC-EIA)

The optimal dilutions of the primary rabbit anti-RPS serum and the second antibody, the goat anti-rabbit IgG HRP conjugate, were determined by checkerboard titration experiments. The optimal dilution for primary serum was 1:5,000 (Table 14 and Figure 19) and for the second antibody was 1:5,000 (Table 15 and Figure 20). Employing the above conditions the endpoint dilution of RPS to produce a positive reaction detected by densitometric reading was 5,000 which represented a viral concentration of 1 ng (Table 16 and Figure 21). This amount of viral antigen is equivalent to $7 \times 10^4$ viral particles. However, by visual inspection, the endpoint dilution of viral antigen for a positive reaction revealed even a smaller amount of the virus antigen. As shown in the Figure 21, a positive reaction detectable through visual inspection increased to a virus dilution of 10,000, which is equivalent to approximately 0.5 ng of viral protein.
Table 14. Binding Curve for rabbit IgG and rhabdovirus of penaeid shrimp.

<table>
<thead>
<tr>
<th>Well number&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Antibody dilution&lt;sup&gt;b&lt;/sup&gt;</th>
<th>NC-EIA reading&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100</td>
<td>182</td>
</tr>
<tr>
<td>2</td>
<td>500</td>
<td>178</td>
</tr>
<tr>
<td>3</td>
<td>1000</td>
<td>156</td>
</tr>
<tr>
<td>4</td>
<td>5000</td>
<td>147</td>
</tr>
<tr>
<td>5</td>
<td>10,000</td>
<td>117</td>
</tr>
<tr>
<td>6</td>
<td>25,000</td>
<td>97</td>
</tr>
<tr>
<td>7</td>
<td>50,000</td>
<td>77</td>
</tr>
<tr>
<td>8</td>
<td>100,000</td>
<td>56</td>
</tr>
<tr>
<td>9</td>
<td>250,000</td>
<td>23</td>
</tr>
<tr>
<td>10</td>
<td>500,000</td>
<td>5</td>
</tr>
<tr>
<td>11</td>
<td>1000,000</td>
<td>0</td>
</tr>
<tr>
<td>12</td>
<td>PBS</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>a</sup> 25 microgram of RPS antigen was used per well.

<sup>b</sup>Purified IgG fraction was diluted in 1% nonfat dry milk/PBS

<sup>c</sup>Reflectance densitometric readings of NC-EIA color development.
Figure 19. Binding curve of rabbit IgG to rhabdovirus of penaeid shrimp
Table 15. Binding curve for secondary antibody to rabbit IgG

<table>
<thead>
<tr>
<th>Well number&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Antibody dilution&lt;sup&gt;b&lt;/sup&gt;</th>
<th>NC-EIA readings&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100</td>
<td>166</td>
</tr>
<tr>
<td>2</td>
<td>500</td>
<td>152</td>
</tr>
<tr>
<td>3</td>
<td>1000</td>
<td>152</td>
</tr>
<tr>
<td>4</td>
<td>5000</td>
<td>140</td>
</tr>
<tr>
<td>5</td>
<td>10,000</td>
<td>108</td>
</tr>
<tr>
<td>6</td>
<td>25,000</td>
<td>90</td>
</tr>
<tr>
<td>7</td>
<td>50,000</td>
<td>49</td>
</tr>
<tr>
<td>8</td>
<td>100,000</td>
<td>34</td>
</tr>
<tr>
<td>9</td>
<td>250,000</td>
<td>13</td>
</tr>
<tr>
<td>10</td>
<td>500,000</td>
<td>3</td>
</tr>
<tr>
<td>11</td>
<td>1000,000</td>
<td>0</td>
</tr>
<tr>
<td>12</td>
<td>PBS</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>a</sup> 25 nanogram of RPS antigen was applied per well.

<sup>b</sup> Purified IgG fraction was diluted in 1% nonfat dry milk/PBS

<sup>c</sup> Reflectance densitometric readings of NC-EIA color development.
Figure 20. Binding curve of goat IgG to rabbit serum.
Table 16. Endpoint titration of rhabdovirus of penaeid shrimp using NC-EIA

<table>
<thead>
<tr>
<th>Well Number</th>
<th>Viral Antigen&lt;sup&gt;a&lt;/sup&gt;</th>
<th>NC-EIA Reading&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>500 ng</td>
<td>246</td>
</tr>
<tr>
<td>2</td>
<td>500 ng</td>
<td>248</td>
</tr>
<tr>
<td>3</td>
<td>50 ng</td>
<td>185</td>
</tr>
<tr>
<td>4</td>
<td>50 ng</td>
<td>192</td>
</tr>
<tr>
<td>5</td>
<td>10 ng</td>
<td>37</td>
</tr>
<tr>
<td>6</td>
<td>10 ng</td>
<td>38</td>
</tr>
<tr>
<td>7</td>
<td>5 ng</td>
<td>31</td>
</tr>
<tr>
<td>8</td>
<td>5 ng</td>
<td>27</td>
</tr>
<tr>
<td>9</td>
<td>1 ng</td>
<td>25</td>
</tr>
<tr>
<td>10</td>
<td>1 ng</td>
<td>22</td>
</tr>
<tr>
<td>11</td>
<td>PBS</td>
<td>0</td>
</tr>
<tr>
<td>12</td>
<td>PBS</td>
<td>0</td>
</tr>
<tr>
<td>13</td>
<td>500 pg</td>
<td>0&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>14</td>
<td>500 pg</td>
<td>0&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>15</td>
<td>250 pg</td>
<td>0</td>
</tr>
<tr>
<td>16</td>
<td>250 pg</td>
<td>0</td>
</tr>
<tr>
<td>17</td>
<td>100 pg</td>
<td>0</td>
</tr>
<tr>
<td>18</td>
<td>100 pg</td>
<td>0</td>
</tr>
<tr>
<td>19</td>
<td>50 pg</td>
<td>0</td>
</tr>
<tr>
<td>20</td>
<td>50 pg</td>
<td>0</td>
</tr>
<tr>
<td>21</td>
<td>10 pg</td>
<td>0</td>
</tr>
<tr>
<td>22</td>
<td>10 pg</td>
<td>0</td>
</tr>
<tr>
<td>23</td>
<td>PBS</td>
<td>0</td>
</tr>
<tr>
<td>24</td>
<td>PBS</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>a</sup> A 45 ul of diluted viral antigen was loaded per well and duplicate wells were used per each antigen dilution.

<sup>b</sup> Reflectance densitometric readings of NC-EIA color development.

<sup>c</sup> The reading was zero in the densitometer but was visually positive.
Figure 21. Photograph of the NC-EIA endpoint titration of rhabdovirus of penaeid shrimp using rabbit IgG.
<table>
<thead>
<tr>
<th>Well Number</th>
<th>1-2</th>
<th>3-4</th>
<th>5-6</th>
<th>7-8</th>
<th>9-10</th>
<th>11-12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antigen Dilutions</td>
<td>10</td>
<td>100</td>
<td>500</td>
<td>1000</td>
<td>5000</td>
<td>PBS</td>
</tr>
</tbody>
</table>

| Antigen Dilutions | 10K | 20K | 50K | 100K| 500K | PBS   |
1. Stability to freezing and thawing

The RPS was found to be very sensitive to repeated cycles of freezing and thawing. The original virus solution suspended in HBSS had a titer of $4.6 \times 10^7$ PFU/ml, but after one freezing-thawing cycle, the titer fell to $4.2 \times 10^7$ PFU/ml. Following two additional freezing and thawing cycles, the titer was reduced to $8.6 \times 10^6$ PFU/ml. Over 80% of the viral infectivity of the original virus in the solution was lost after three cycles of freeze-thaw. Comparison of three buffers used to suspend the virus indicated that both HBSS and TNE were superior to MEM-0 for viral protection during the freezing and thawing cycles. As shown in Table 17, a greater preservation of the virus during freeze-thaw was achieved when 4% FBS was added to MEM-0.
Table 17. Stability of rhabdovirus of penaeid shrimp to freezing and thawing

<table>
<thead>
<tr>
<th>Buffers</th>
<th># of Freeze-Thaw*</th>
<th>Plaque Titer (10^5 PFU)</th>
<th>% Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBSS</td>
<td>0</td>
<td>460</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>420</td>
<td>91</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>260</td>
<td>57</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>86</td>
<td>19</td>
</tr>
<tr>
<td>TNE</td>
<td>0</td>
<td>370</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>370</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>290</td>
<td>78</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>120</td>
<td>32</td>
</tr>
<tr>
<td>MEM-0</td>
<td>0</td>
<td>410</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>400</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>86</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>32</td>
<td>8</td>
</tr>
<tr>
<td>MEM-4</td>
<td>0</td>
<td>590</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>550</td>
<td>93</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>400</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>320</td>
<td>54</td>
</tr>
</tbody>
</table>

* Freezing and thawing were done in a -70 °C freezer and a 25 °C water bath, respectively.
2. Stability of the virus stored at \(-10\) °C and \(-70\) °C

The stability of RPS to cold temperatures was measured by storing a virus suspension at \(-10\) °C and \(-70\) °C and then removing aliquots for titration at selected times. These titers were compared with the titer obtained for the virus before freezing. The results revealed that the virus was sensitive to cold temperature (Table 18). As shown in the table, the titer of the virus decreased following storage at \(-10\) °C and \(-70\) °C. However, virus was slightly more stable at \(-70\) °C for 24 h. The decline in virus titer continued as the freezing time increased. At \(-10\) °C the original virus solution suspended in TNE buffer containing \(3.7 \times 10^7\) PFU/ml (100% infectivity) declined to \(1.4 \times 10^6\) PFU/ml (4%) in 30 days. In contrast, the virus was more stable when stored at \(-70\) °C than at \(-10\) °C. The titer remained at \(1.8 \times 10^7\) PFU/ml (49%) when stored at \(-70\) °C for 30 days. Of the three buffers (HBSS, TNE and MEM-0) used for virus suspension, Hank's balanced salt solution was the best, TNE was intermediate and the MEM-0 was poorest. However, if a 2-4% fetal bovine serum was added to MEM-0, the virus remained very stable. Sixty-eight percent of the original virus infectivity in MEM-4 remained after 30 days at \(-70\) °C.
Table 18. Stability of rhabdovirus of penaeid shrimp to storage at cold temperature

<table>
<thead>
<tr>
<th>Buffers</th>
<th>Temperature (°C)</th>
<th>Virus Titer (x $10^5$ PFU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Freezing Time (days)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>HBSS</td>
<td>-10</td>
<td>460</td>
</tr>
<tr>
<td></td>
<td>-70</td>
<td>420</td>
</tr>
<tr>
<td>TNE</td>
<td>-10</td>
<td>370</td>
</tr>
<tr>
<td></td>
<td>-70</td>
<td>370</td>
</tr>
<tr>
<td>MEM-0</td>
<td>-10</td>
<td>410</td>
</tr>
<tr>
<td></td>
<td>-70</td>
<td>400</td>
</tr>
<tr>
<td>MEM-4</td>
<td>-10</td>
<td>590</td>
</tr>
<tr>
<td></td>
<td>-70</td>
<td>550</td>
</tr>
</tbody>
</table>
3. Stability at 4, 20, 37 and 56 °C

The rate of inactivation of the RPS suspended in MEM-O at different temperatures was examined. Viral infectivity was stable at 4 °C and 20 °C for as long as the duration of the experiment (24 h). In contrast, at 37 °C, complete inactivation of the virus occurred by 12 h. At 56 °C, the virus was rapidly inactivated within 10 min (Figure 22).

4. Stability to low pH

Incubation of RPS in MEM-O adjusted to pH 3 for 3-hour of treatment at rt resulted in a complete lost of viral infectivity. (Table 19). A similar result was obtained for the fish rhabdovirus, RC. In contrast, poliovirus type 1 was resistant to the low pH treatment. Both the pH 7.2 treated poliovirus control and the pH 3 treated samples had similar titers.

Treatment of the viruses at low pH resulted in the destruction of the virus particles. Electron micrographs of the virus preparations exposed to pH 3 demonstrated the absence of intact virus particles. Many virus particles were observed to be disrupted either partly at one end or completely at both ends. Some of the remaining viral particles had become spherical (Figure 23A) and had broken open to release a ribbon-like internal nucleoprotein.
Figure 22. Stability of rhabdovirus of penaeid shrimp suspended in MEM-0 at incubation of 4, 20, 37, and 56 °C.
Table 19. Titers of rhabdovirus of penaeid shrimp, rhabdovirus carpio, and poliovirus type 1 following incubation at pH 3 for 3 hours at 22 °C

<table>
<thead>
<tr>
<th>Virus</th>
<th>treatment</th>
<th>Titer PFU/ml</th>
<th>% Inactivation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhabdovirus of penaeid shrimp</td>
<td>MEM-0 (pH 7.2)</td>
<td>$5.2 \times 10^4$</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>MEM-0 (pH 3.0)</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Poliovirus type 1</td>
<td>MEM-0 (pH7.2)</td>
<td>$2.3 \times 10^6$</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>MEM-0 (pH 3.0)</td>
<td>$2.3 \times 10^6$</td>
<td>0</td>
</tr>
<tr>
<td>Rhabdovirus carpio</td>
<td>MEM-0 (pH 7.2)</td>
<td>$2.0 \times 10^5$</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>MEM-0 (pH 3.0)</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>

a Virus was suspended in MEM-0 adjusted to pH 3 and pH 7.2.
Preparations of the control virus maintained at pH 7.2 appeared normal. Viral particles were typical bullet-shaped with one end rounded and the other flattened.

5. **Stability to lipid solvents**

   a. Ethyl ether treatment. There appears to be a definitive correlation between the presence of an envelope and susceptibility of viruses to inactivation by lipid solvents (Rovozzo and Burke, 1973). The loss of infectivity after treatment with lipid solvents indicates that a virus possesses an essential lipid-containing envelope surrounding the infectious agent (Joklik et al., 1980). Both the RPS, the enveloped RC, and naked poliovirus were tested for their sensitivity to treatment with 20% ethyl ether. The RPS and RC were inactivated by ether treatment (Table 20). The MEM-O treated RPS had a titer of $2.75 \times 10^5$ PFU/ml while the titer of the ether treated sample was reduced to $9.0 \times 10^2$ PFU/ml, and represented a 99.5% loss of viral infectivity following treatment. Similarly, the enveloped RC was almost completely inactivated by exposure to ethyl ether. In contrast, the naked poliovirus control exhibited no loss of viral infectivity.

   Electron micrographs of the virus preparation exposed to ethyl ether revealed the destruction of the virus particles (Figure 23B). The morphological and structural alterations
Figure 23. Electron micrographs of negatively stained rhabdovirus of penaeid shrimp particles (A) after exposure to pH 3 for 3 hours at room temperature and (B) following the incubation with ethyl ether for 24 hours at 4° C.
Table 20  Titers of rhabdovirus of penaeid shrimp, rhabdovirus carpio, and poliovirus type 1 following treatments with ethyl ether and chloroform for 24 hours at 4 °C.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Treatment</th>
<th>Titer PFU/ml</th>
<th>Treatment</th>
<th>Titer TCID₅₀/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhabdovirus of penaeid shrimp</td>
<td>ethyl ether</td>
<td>9.0 x 10²</td>
<td>chloroform</td>
<td>1.2 x 10²</td>
</tr>
<tr>
<td></td>
<td>MEM-0</td>
<td>2.7 x 10⁵</td>
<td>MEM-0</td>
<td>10⁶.5</td>
</tr>
<tr>
<td>Poliovirus type-1</td>
<td>ethyl ether</td>
<td>2.7 x 10⁷</td>
<td>chloroform</td>
<td>2.7 x 10⁷</td>
</tr>
<tr>
<td></td>
<td>MEM-0</td>
<td>2.7 x 10⁷</td>
<td>MEM-0</td>
<td>2.7 x 10⁷</td>
</tr>
<tr>
<td>Rhabdovirus carpio</td>
<td>ethyl ether</td>
<td>1.5 x 10⁴</td>
<td>chloroform</td>
<td>5.2 x 10³</td>
</tr>
<tr>
<td></td>
<td>MEM-0</td>
<td>2.8 x 10⁶</td>
<td>MEM-0</td>
<td>1.8 x 10⁶</td>
</tr>
</tbody>
</table>
observed were similar to those seen in the low pH treated virus preparations.

b. Chloroform treatment. Rhabdovirus of penaeid shrimp, poliovirus type 1 and rhabdovirus carpio (RC) were also tested for their stability to chloroform, another lipid solvent. Both RPS and RC were completely inactivated by chloroform, but the naked poliovirus type 1 showed no loss of infectivity.

6. Replication of virus in the presence of 5-bromo-2’-deoxyuridine (BUDR)

Viruses with an RNA genome are usually resistant to the action of 5-bromo-2’-deoxyuridine (BUDR), a DNA antagonist. The RNA type of nucleic acid contained in RPS was reaffirmed by the use of the nucleic acid analogue, BUDR (Table 21). At a concentration of 20 ug/ml, the halogenated pyrimidine, did not inhibit the replication of RPS in EPC cells. In contrast, the inhibitor did interfere with the replication of the DNA-containing vaccinia virus. A similar selective inhibitory result was also obtained when the fish viruses, spring viremia of carp virus (RC), and the channel catfish virus (CCV) were used. The halogenated pyrimidine interfered with the replication of the DNA-containing CCV, but not with the RNA-containing RC.
Table 21. Comparison of the effect of 5-bromo-2'-deoxyuridine on the replication of rhabdovirus of penaeid shrimp, rhabdovirus carpio, channel catfish virus, vaccinia, and poliovirus type 1

<table>
<thead>
<tr>
<th>Virus</th>
<th>BUDR 20ug/ml</th>
<th>Virus yield TCID50/ml</th>
<th>%inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPS</td>
<td>-</td>
<td>10^7.7</td>
<td>0</td>
</tr>
<tr>
<td>RPS</td>
<td>+</td>
<td>10^7.7</td>
<td>0</td>
</tr>
<tr>
<td>Poliovirus (1)</td>
<td>-</td>
<td>10^8.5</td>
<td>0</td>
</tr>
<tr>
<td>Poliovirus (1)</td>
<td>+</td>
<td>10^8.5</td>
<td>0</td>
</tr>
<tr>
<td>Vaccinia</td>
<td>-</td>
<td>10^7.2</td>
<td>0</td>
</tr>
<tr>
<td>Vaccinia</td>
<td>+</td>
<td>10^6.0</td>
<td>93.7</td>
</tr>
<tr>
<td>RC</td>
<td>-</td>
<td>10^8.2</td>
<td>0</td>
</tr>
<tr>
<td>RC</td>
<td>+</td>
<td>10^8.2</td>
<td>0</td>
</tr>
<tr>
<td>CCV</td>
<td>-</td>
<td>10^6.5</td>
<td>0</td>
</tr>
<tr>
<td>CCV</td>
<td>+</td>
<td>10^5.0</td>
<td>98</td>
</tr>
</tbody>
</table>
7. **Buoyant density of virus in sucrose gradients**

Isopycnic centrifugation in sucrose density gradients was employed to determine the buoyant density of RPS. Refractive index measurements revealed that a linear isopycnic gradient was formed after the centrifugation. A characteristic opalescent-bluish band containing complete virus particles of RPS formed in the upper portion (fraction number 16) of the centrifuge tube. Twenty fractions (0.20–0.25 ml/fraction) were collected from bottom of the tube and aliquots (30 ul) were taken from each fraction for sucrose density determination and infectivity assay. The density of the virus was determined to be 1.19 g/cm$^3$ (Figure 24). This experiment was repeated twice with the same result. Although this density was little bit high it was still within the reported buoyant density ranges of 1.17 to 1.19 g/cm$^3$ for the rhabdoviruses in sucrose gradient (Matthew, 1982; Nishizawa et al., 1991). A peak infectivity titer ($10^{9.3}$ TCID$_{50}$/ml) was only found in fraction 16 which had the buoyant density of 1.19 g/cm$^3$.

8. **Protein analysis in polyacrylamide gels**

Electrophoresis of RPS proteins in SDS-polyacrylamide gels revealed four major viral proteins (Figure 25). Routinely at least seven polypeptides thought to be viral proteins, could be resolved in many preparations. When
Figure 24. Density of rhabdovirus of penaeid shrimp in sucrose gradients.
compared to the molecular weight markers used, the molecular weights of the four major viral proteins were 165,000, 67,000, 44,500, and 28,000 daltons. The other three minor protein bands had molecular weights of 43,000, 25,000 and 23,700 daltons, respectively. The extensive purification steps used (2 cycles of sucrose gradients for some preparations) suggested that the additional bands probably either be of viral origin (minor capsid proteins) or be fragments of the major viral structural proteins. The pattern resulting from the protein analysis indicated that the four major polypeptides corresponded to the L (Large protein), G (Glycoprotein), N (Nucleoprotein), and M (Matrix Protein) which are equivalent to those of prototype rhabdovirus, vesicular stomatitis virus (Genus Vesiculovirus). As shown in the Figure 25, the viral protein pattern of RPS was very similar to that of RC, but different from those of IHNV and VHSV.

9. Western blot

Western blot analysis of the proteins separated by polyacrylamide gel electrophoresis with anti-RPS serum indicated the presence of the four major designated L, G, N, and M (Figure 26). An extra viral protein with a molecular weight of 38,000 daltons which was not observed in SDS–polyacrylamide gel electrophoresis was now detected. Based on
Figure 25. Electropherograms of viral proteins after electrophoresis for 3.5 h at constant volts 200 in a 10% polyacrylamide slab gel. Stained with silver stain.
Figure 26. Western blot analysis of viral structural proteins which were transferred to nitrocellulose membranes and reacted with antiserum against RPS.
the mw, this polypeptide was presumed to be non-structure (NS) protein. Antiserum against RPS showed various patterns of reactivity with the structural proteins of RC, IHNV, VHSV, and VSV. Although cross-reactions of the antiserum were observed with all the structural proteins of RC, the staining for RC was weaker than that observed for RPS. Furthermore, the extra viral protein (38,000 d) detected in RPS was barely visible in RC. The antiserum cross-reacted with the G protein of both IHNV and VHSV, and also with M protein of VSV. The antiserum did not cross-react with the other structural proteins of IHNV, VHSV, and VSV to any detectable extent.

10. Viral RNA analysis in acrylamide-agarose slab gels

Viral RNAs extracted from purified RPS and VSV preparations by pronase-SDS-urea treatment were analyzed by acrylamide-agarose gel electrophoresis in slab gels followed by silver staining. The electrophoresis showed consistently single bands of RPS- and VSV-RNAs (Figure 27). Under the present conditions, the mobility of RPS-RNA was nearly the same as that of VSV-RNA. Based on RNA molecular-weight marker II and the molecular weight of VSV RNA which is 3.8 x 10^6 daltons (11 Kb) as determined by polyacrylamide gel electrophoresis (Bishop and Roy, 1971), the molecular weight of RPS-RNA is calculated to be approximately 3.6 x 10^6 daltons (10.4 Kb).
Figure 27. Electropherograms of acrylamide-agarose slab gel stained with silver nitrate after electrophoresis of the viral RNAs extract from rhabdovirus of penaeid shrimp (RPS) and vesicular stomatitis virus (VSV).
SRNA rps VSV
G. In Vivo Experiments

1. Pathogenicity of virus for penaeid shrimp

The pathogenicity of RPS was tested for two commercially important species of penaeid shrimp, *P. stylirostris* and *P. vannamei*. Although a few deaths occurred in both the control and infected animals as a result of water quality and cannibalism, none of the deaths could be directly attributed to infection by RPS. Visual examination of both groups of surviving animals did not reveal any gross cytopathic changes. Virus was recovered from the majority of infected animals after 21 to 30 days p.i..

2. Virus replication in different organs and tissues of penaeid shrimp

The different organs and tissues of infected penaeid shrimp were examined at different intervals p.i. for RPS replication. The RPS was only recovered from the Oka organ, but not isolated from the other organs and tissues. The data clearly indicate that the Oka organ in the infected animal solely support the replication of RPS while all the other organs and tissues of the shrimp are refractory to the virus.

The amount of virus recovered from the infected animals increased gradually. At day 6 p.i., the virus titer was measured at $2.7 \times 10^3$ PFU/animal. This titer increased to $1.1 \times 10^4$ PFU/animal and $6.4 \times 10^4$ PFU/animal at days 10 and
21, respectively. The viral titer did not significantly alter at the end of 30-day p.i..

Gross examination of the Oka organs harvested from the 21-day RPS-infected shrimp indicated that the organs were markedly larger than those from the shrimp inoculated with saline buffer. The average weight of the Oka organs was 110 ± 10 micrograms (ug) for the RPS-infected shrimp in comparison to the weight of 15 ± 5 ug for uninfected shrimp.

3. Pathology in virus-infected penaeid shrimp

Histological examination of *P. stylirostris* infected with RPS revealed the presence of pathological changes primarily involving the lymphoid organ. Focal necrosis was also seen in the epidermis of gills and hematopoietic tissues of infected shrimps.

Abundant and large proliferative centers (focal sites of cellular hyperplasia) (PC) were observed in the Oka organs of RPS infected shrimp. The predominant cells within these foci had a hypertrophic nucleus, dense chromatin along the nuclear membrane and prominent nucleoli. Also a majority of cells showed lightly stained basophilic cytoplasm with an indistinct cell membrane. Necrosis was often present in the central areas of the PCs and large and clear cytoplasmic vacuoles were commonly observed (Figures 28A and 29A). Variable sized, basophilic to non-basophilic, Feulgen positive and Feulgen negative cytoplasmic inclusions were
Figure 28. Photomicrographs of the sections of Oka organ stained with hematoxylin and eosin (magnification 100x). (A) RPS infected and (B) uninfected control.
Figure 29. Photomicrographs of the sections of Oka organ stained with hematoxylin and eosin (magnification 200x). (A) RPS infected and (B) uninfected control.
also observed in some PC cells. Although proliferative centers of the Oka organ were also observed in the control shrimp, but no cytological changes were minimal as compared to RPS infected shrimps were observed (Figures 28B and 29B).

The size of the PCs in the lymphoid organs of both control and RPS-infected shrimp was measured and statistically evaluated. The results revealed that the mean and standard deviation of proliferative center lesion diameter was 133.5 ± 34.5 microns for the RPS-infected shrimp and 32.8 ± 12.7 microns for the control animal group. The different PC sizes between the RPS-infected and control groups of shrimp was statistically significant (P<0.001).

4. Immunofluorescent staining of the Oka organs prepared from infected penaeid shrimp

Impression smears were prepared from the Oka organs and the control shrimp at 6 and 21 days p.i. were stained with IFA. Virus-specific immunofluorescence was seen only in impression smears prepared from the Oka organs of RPS-infected shrimps (Figure 30A) but not seen in the organs prepared from the control animals (Figure 30B).
Figure 30. Photograph of impression smears of lymphoid (Oka) organ harvested from *Penaeus stylirostris* and stained with immunofluorescent antiserum against RPS. (A) The RPS-infected lymphoid organ smear. (B) Control lymphoid organ smear.
H. Characteristics of Rhabdovirus of Penaeid Shrimp

Isolated from Juvenile and adult *P. stylirostris* and *P. vannamei*, January, 1989, Honolulu, Hawaii

Target organ Oka (lymphoid organ)

Morphology of virus Bullet-shaped with one rounded end and one planar end

Diameter of virus $65-77 \times 115-138$ nm

Viral genome/size ss RNA (−)/$3.6 \times 10^6$ daltons (10.4 Kb).

Viral structural proteins Four major capsid proteins: 165,000 (L), 67,000 (G), 44,500 (N), and 28,000 (M).

Density in sucrose 1.19 $g/cm^3$

Stability

<table>
<thead>
<tr>
<th>Condition</th>
<th>Stability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frozen-thawed</td>
<td>-</td>
</tr>
<tr>
<td>37 and 56 °C</td>
<td>-</td>
</tr>
<tr>
<td>4 and 20 °C</td>
<td>+</td>
</tr>
<tr>
<td>-10 and -70 °C</td>
<td>-</td>
</tr>
<tr>
<td>Sea $H_2O$</td>
<td>-</td>
</tr>
</tbody>
</table>

Resistance to

<table>
<thead>
<tr>
<th>Condition</th>
<th>Resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 3</td>
<td>-</td>
</tr>
<tr>
<td>Lipid solvents</td>
<td>-</td>
</tr>
<tr>
<td>BUDR</td>
<td>+</td>
</tr>
</tbody>
</table>

Cell line for isolation EPC

Optimum temperature for replication 20-25 °C

Efficiency of Plating 30 virus particle/infectious unit.

Cytopathic effect Cell rounding and lysis. Plaque formation

Immunofluorescence Cytoplasmic fluorescence

Pathogenicity in vivo Primary organ affected is the lymphoid (Oka) organ which undergoes characteristic changes: marked enlargement of the organ involving abundantly large proliferative centers which contain large and clear cytoplasmic vacuoles with some cellular necrosis

191
V. DISCUSSION

The present studies describe the isolation and characterization of a new rhabdovirus from two species of penaeid shrimp. The physical and biological properties of the virus particle were characterized during the course of this study for the purpose of appropriate taxonomic placement. Also, the possible role played by this isolate in relation to IHHN disease and its pathogenicity for commercially important penaeid shrimps were examined. Of significance in this study was the discovery of the replication of a crustacean virus in an established fish cell line which to our knowledge has never been reported before.

Established susceptible cell lines are considered to be necessary for the isolation, propagation, and study of viruses. After extraction and purification of the virus from infected shrimps, the first experiment conducted was the screening of cell cultures for susceptibility to infection by the virus. Since there were no cell lines established from shrimp presently available, seven established fish cell lines carried in this laboratory were tested. Viral infectivity was demonstrated in one of these cell lines, EPC, an established heteroploid cell line derived from carp. Infected EPC cells showed marked cytopathic effect which was characterized by the formation of focal areas of cellular destruction. The discovery of a cell line capable of supporting the replication of the shrimp virus has provided a major step
towards the study of this viral agent of penaeid shrimp aquaculture. In particular, it facilitated characterization of the virus, reproducibility in biological investigations and eventually the development of sensitive and convenient serological procedures for the early detection of the virus in cultured group of shrimp.

Seven viral agents (2 baculoviruses, 3 parvoviruses, 1 reovirus, and 1 unclassified RNA-containing icosahedral virus) have been reported from cultured penaeid shrimp in the past decade (Lightner and Redman, 1981, Sano et al., 1981, Lightner and Redman, 1985, Tsing and Bonami, 1987, Lightner et al., 1983, Owens et al., 1991, Lu et al., 1991). The exception was baculovirus penaei which was recognized in the early 1970's. However, none of these shrimp viruses have been replicated in an established shrimp cell line or other established cell lines derived from lower vertebrates. Thus, the characteristics of these shrimp viruses have not been fully established. A recent publication by Chen and Kou (1989) reported the use of primary cell cultures prepared from the Oka organs of the penaeid shrimp for infectivity studies of the monodon-type baculovirus. However, several problems associated with this system in terms of studying the shrimp viruses were obviously inconsistency, difficulty, and cost. The need for established cell lines in shrimp virology is apparent. Therefore, the discovery of an established cell line susceptible to a shrimp virus will greatly
facilitate research on this isolated virus and possibly for other viruses of the penaeid shrimp.

The *Rhabdoviridae* is a well-defined family of viruses with a wide host range of vertebrates (including fishes), invertebrates, arthropods, and plants. It was reported that some rhabdoviruses that infect plants may also infect vertebrates (Johnson et al., 1969). Moreover, one member, vesicular stomatitis virus, aside from infecting several vertebrate hosts, will multiply in *Aedes* mosquitoes and in leafhoppers. The latter is the natural vector of maize mosaic virus, a plant rhabdovirus (Brown, 1987). All of this demonstrated the possibility of rhabdovirus replicating in a non-natural-host system or crossing phylogenetic species. Bussereau et al. have successfully demonstrated the replication of two fish rhabdoviruses, spring viremia of carp virus (SVCV) and Pike fry rhabdovirus (PFR) in a species of insect, *Drosophila melanogaster* (1975). The discovery of the replication of a shrimp virus, RPS, in a fish cell line is therefore not surprising and has confirmed the wide host range for this rhabdovirus.

After the discovery of the successful replication of the virus in EPC cells, a plaque assay protocol was developed for the titration of the virus using 0.75% methylcellulose, a solid overlay commonly used for the plaque assay of fish viruses (Burke and Mulcahy, 1980). The plaques formed in EPC cells were uniquely circular and distinct, and the number of
plaques formed in the cell culture was found to have a linear relationship with the virus dilution. This indicated the accuracy of the technique for quantitative determination of infectious virus. Even though the three GC cell lines, FHM and BB cell lines were capable of supporting the growth of virus, the high plaque forming efficiency of RPS was only observed in EPC cells. This indicated that the EPC cell is more susceptible to the RPS than any of the other cell lines and it also explains the fact that the successful primary isolation of the virus occurred only in EPC cells but not in GC cells.

The accuracy and reproducibility of viral assay procedures, such as plaque assay, are extremely important and are dependent on several factors, such as adsorption time, inoculum volume, incubation temperature, and cell line. Experiments were carried out to establish the optimal conditions for the plaque assay which would estimate more accurately the titer of RPS in EPC cells. As has been reported for several viral systems, both adsorption time and inoculum volume were found to have a marked effect on the plaque production of the virus (Rawls and Leung, 1979; Fendrick and Hallick, 1983; Loh et al., 1977). The optimal adsorption period for RPS was determined to be 1.5 hr and the optimal inoculum volume was 0.2 ml in 6-well plates. These results were very similar to those determined for IHNV by Burke and Mulcahy (1980).
The optimal infection period for plaque production of RPS was found to be 5 days after infection. The determination of the period for plaque production was mainly based on the plaque size formed. The importance of the infection period in plaque assays is obvious and it can directly affect plaque titer. It should also be stressed that the optimal period of the 5th day p.i. is only applicable for RPS in EPC cells under the conditions described. The period may vary widely with the cell line used, cell culture containers, overlay media and for different viruses under different plaque forming conditions. Burke and Mulcahy (1980) determined that the optimal plaque formation time of IHNV in EPC cells was 9 days p.i..

With the establishment of optimal plaque assay conditions for RPS, several experiments were carried out to determine the optimum conditions for the propagation of the virus. Although replication of RPS in EPC cells was found to occur between 10 to 30°C, the optimal temperature was 20-25 °C. This was in agreement with the growth temperature ranges (4-33 °C) reported for other rhabdoviruses isolated from aquatic animals, such as VHSV, IHNV, and RC (McAllister, 1979; Wolf & Mann, 1980; Ahne, 1981; Frerichs, 1989). Growth media was also demonstrated to play an important role in RPS production. Comparison of three growth media indicated that the MEM-5 was the best for RPS propagation. Since both medium 199 and L-15 were more
enriched than MEM, a possible explanation for the phenomenon was that the EPC cell had been grown and passed using medium MEM and was therefore more adapted to MEM-5. The one-step growth curve of RPS at 20 °C showed an eclipse period of approximately 3 h which was followed by exponential production of virus up to 48 hours. This differs from the data obtained by Ahne (1973) who demonstrated that the fish RC virus at 20–22 °C had an eclipse period of 4–6 h, and maximum viral production by 8–10 h p.i.

The efficiency of plating (EOP) of the RPS in EPC cells was determined to be 30 virus particles per plaque forming unit. This value was obtained through the modification of the technique originally developed to increase the detection of virus particles by high speed centrifugation onto electron microscopical grids (Jansons et al., 1985). The method was subsequently modified and adapted to quantitate virus particles by Nadala and Loh (1992). The procedure includes a centrifugation step which allowed the use of less concentrated virus preparations than those required by conventional droplet techniques. Electron microscopical examination of the grids revealed intact rhabdovirus particles, which were evenly distributed on the grids. Few or no viral aggregates were observed in most preparations. One limitation involved in this technique is the requirement for a highly purified preparation of virus samples. Crude preparations or partially purified virus suspensions were not
recommended since they could contain debris which would interfere with EM examination.

Much effort in this study went toward the characterization of the virus in terms of biological properties. The identification that RPS was a rhabdovirus was based on the morphological properties and biochemical characteristics. The first evidence that RPS was a rhabdovirus was the electron microscopical examination of negatively stained purified virus particles which exhibited typically bullet-shaped structures which measured 65-77 x 115-138 nm. This morphological structure is characteristic for most of the rhabdoviruses occurring in vertebrates and invertebrates. These viruses are bullet-shaped and consist of a helically wound ribonucleocapsid surrounded by a unit membrane envelope component. The envelope in turn contains evenly spaced projections (Brown, 1987).

The second evidence to indicate that RPS was a rhabdovirus was its sensitivity to acid pH (pH 3) and lipid solvents (20% ethyl ether and chloroform), instability to freezing-thawing, heating at 56 °C for 10 min, and storage at -10 °C. These experiments revealed the presence of an envelope associated with the virus. The third was the determination of the type of nucleic acid in the virus. The BUDR test indicated that the genome of RPS was composed of RNA only. Also, analysis by polyacrylamide-agarose gel electrophoresis of extracted viral RNA revealed a non-
segmented single stranded RNA genome \((3.6 \times 10^6\) daltons).

Rhabdoviruses have been reported to contain five separate polypeptides identifiable by polyacrylamide gel electrophoresis (Kang and Prevec, 1971; Mudd and Summers, 1970; Wagner et al., 1970). These rhabdovirus proteins were named large protein \((L)\), glycoprotein \((G)\), nucleoprotein \((N, 40-62K)\), nonstructural protein \((NS)\) and matrix protein \((M)\) (Wagner et al., 1972). Based on the patterns of the viral structural proteins, viruses belonging to the family, Rhabdoviridae, were classified into two major genera, lyssavirus and vesiculovirus (Mathews 1982, Wagner 1987). The former consists of \(L\) (150-190K), \(G\) (62-83K), \(N\) (40-62), and two viral matrix proteins \(M1\) (22-40K) and \(M2\) (19-36K), while the latter has \(L\) (145-190K), \(G\) (63-92K), two cores \(N\) (42-59K) and \(NS\) (38-45K) but one matrix protein \(M\) (19-29K) (McSharry, 1979). Analysis of the viral proteins of RPS in SDS-polyacrylamide gel showed that purified RPS was composed of 7 viral structural polypeptides. Four of these, with estimated molecular weights of 190 (\(L\)), 67 (\(G\)), 44.5 (\(N\)), and 28 (\(M\)) KDa, are similar to rhabdoviruses of the vesicular group. "Characterization of the rhabdovirus isolated from shrimp in the present study agrees with the data for rhabdoviruses gathered by McAllister (1979) and Wagner et al. (1970). The pattern of the viral proteins for RPS closely resembled that of the vesiculovirus group, such as the fish rhabdoviruses RC, PFR, EVEX, and EVA (Lenoir and Kinkelín,
1975; Nishizawa et al., 1991a). Therefore, the RPS belongs to the genus, *Vesiculovirus*. The other fish rhabdoviruses, VHSV, HRV and IHNV have structural protein patterns closely resembling that of rabies virus and belong to genus *Lyssavirus* (Nishizawa, 1991b).

The molecular weight of the RNA genome of RPS was determined by comparing its relative mobility in polyacrylamide-agarose gels with the viral RNA of VSV. Although the viral RNAs of some of the fish rhabdoviruses (Hill et al., 1975; Kurath et al., 1985; Kurath and Leong, 1985; Nishizawa, et al., 1991c) have been reported, VSV was selected in this study because it is the prototype of rhabdoviruses and has been widely used for biochemical studies (Banerjee, 1987). The RNA genome of VSV identified as early as in 1950's (Cooper, 1957a,b) was well defined. The RNA of VSV makes up 0.7 to 2% of the mass of the virus particle (Clewley and Bishop, 1979), and has a S value of 42S in sucrose gradients. The genome size of VSV RNA is 3.8 ± 0.14 x 10^6 as determined by Bishop and coworkers and is equivalent to some 11,000 or so nucleotides (Repik and Bishop, 1973).

The RNA genome of RPS was calculated to be approximately 3.6 x 10^6 daltons. The value was derived by coelectrophoresis with the RNA standards and the VSV RNA. The results indicated that the RNA genome of RPS fell within the genomic range (3.1 to 4.4 x 10^6 daltons) of other rhabdoviruses determined by a
variety of procedures including sedimentation analysis, electrophoresis, nuclease digestion, and length measurements of the viral nucleocapsid (Heyward et al., 1979). It can be concluded from the present studies that the viral RNA of RPS is single-stranded, and of negative polarity (Clewley and Bishop, 1979; Wagner, 1987). Also, there appears to be a single copy of the RNA in each virus particle.

The pronase-SDS-urea protocol employed in this study was found to a very successful method for extracting viral RNAs. The method was extremely simple, effective, and easily reproducible. By using the method, a 50 ul or less of purified virus suspension could provide enough of the viral RNA (approximately 5 µg) to form a visible band. A vertical slab unit SE400 apparatus routinely used for protein analysis in this laboratory was successfully adopted for the purpose of the RPS-RNA analysis. The electrophoresis was carried out in a unit composed of 2% polyacrylamide and 0.6% agarose and a single-stranded, non-segmented RNA genome was consistently observed after silver nitrate staining. Since this system excluded the use of radioactive labeling, the method was safe to perform. Also, the silver stained gels could be dried and be stored for a long time.

The relationship of the shrimp rhabdovirus to three rhabdoviruses isolated from other lower aquatic vertebrates (fish) was demonstrated by several experiments. The RPS is

(continued on next page)
readily differentiated from IHNV and VHS of the Lyssavirus group, either by the structural protein analysis, the antigenic profiles of structural proteins, or by the neutralization kinetics technique. Although cross-antigenic reactions were observed between the anti-RPS serum and the G protein of IHNV and VHSV, antigenic profiles for the structural polypeptides of RPS were clearly distinguishable from those of IHNV and VHSV. However, the pattern of RPS structural proteins formed in SDS-PAGE and antigenic profile of the structural proteins by western blot analysis showed great similarity to those of RC, the Vesiculovirus. The similar mobilities of the L, G, N, and M proteins and antigenic relatedness between RPS and RC indicated that RPS and RC are closely related.

However, the RPS is distinguishable from RC by neutralization techniques. Neutralization tests revealed the difference between RPS and RC and suggested that they are closely related, but unique viruses. The percent plaque reduction by neutralization is widely used for the determination of antigenic relationship between related viruses (McCain et al., 1971; Jorgensen, 1972; Bachman and Ahne, 1973). The difference in 50% plaque reduction titers by neutralization between RPS and RC is approximately 3 fold. The neutralization kinetics analysis also revealed the difference between these two vesiculoviruses. Neutralization kinetics test was initially developed by McBride in 1959 to
study poliovirus strains of the same type. He found close related strains could be uniquely specified by its homologous antiserum since heterologous strains were neutralized more slowly than was the homologous strain. Using this technique, Ashe and Scherp demonstrated the differences between serologically related strains of herpes simplex virus. They concluded that two strains should be considered to be serologically homologous if the ratio of their k values was 90% or more. Since the k value of RPS is 52.4 compared to 39.2 for RC, the ratio of the k values between RC and RPS is less than 75% indicating they are serologically heterologous.

Some other studies have also shown little or no differences when either the SDS-PAGE of viral proteins or the plaque reduction neutralization techniques were used to differentiate between some of the rhabdoviruses isolated from fish (Macain et al., 1971; Roy et al., 1975; Nishizawa et al., 1991b). Viral protein patterns of VHSV and IHNV, eel virus European X (EVEX) and eel virus of American (EVA), and pike fry rhabdovirus (PFR) and SVCV in the SDS-PAGE were very indistinguishable (Nishizawa et al., 1991b). McCain and associates using the plaque reduction test with the three salmonid rhabdovirus OSDV, SRCSDV, and IHHNV found that all three virus isolates are antigenically related to each other and that OSDV and IHNV were indistinguishable by the test. It was also shown by cross neutralization tests that SVCV and isolates obtained from carp swim bladder inflammation disease
were indistinguishable. Hill and collaborators (Hill et al., 1975) obtained some cross-neutralization between SVCV, PER, and IHN viruses.

The relative mobilities of structural proteins between IHNV and VHSV are indistinguishable in SDS-PAGE. The patterns of the structural proteins of IHNV and VHSV shown in this study are quite similar to those observed by other investigators except for M1 (Jorgensen et al., 1989, Kimura et al., 1989, Nishizawa et al., 1991b). The molecular weight of the M1 was determined to be 42 KDa in this study in comparison to 26 KDa obtained by Nishizawa et al. (1991b). The variation in the size of M1 protein may have resulted from either strain differences, sample treatment (heating time), or variations in the gel electrophoresis system.

Western blot analysis revealed an extra antigenic protein associated with RPS which was not visible on the SDS-PAGE. This extra protein was also observed with RC. However, this protein stained much stronger for RPS than for RC. This phenomenon could not be due to the variation in the amounts of viral proteins since the same amount of total viral protein was applied in these studies. The protein has a relative molecular weight of 38 KDa which is equivalent to the NS protein identified for the Vesiculoviruses. This observation has not been reported previously for RC and may suggest that there is an NS protein associated with RC which was often undetected in the SDS-PAGE due to
its low concentration.

Rhabdovirus of penaeid shrimp can also be differentiated from other known fish vesiculoviruses, pike fry rhabdovirus (PFRV) (de Kinkelin et al., 1973 and 1974) and eel rhabdoviruses (EVA and EVEX) (Hill et al., 1980; Castric & Chastle, 1980; Nishimura et al., 1981) either by its morphological length which is short or by its lack of replication in RTG-2 cells.

Preliminary studies of the pathogenicity of RPS for two commercially important species of penaeid shrimps was made. The purpose of this test was to assess the potential detrimental effect that this virus may pose on the shrimp industry. Two penaeid shrimp, the blue and white shrimp were examined for their susceptibility to RPS infection. The early results indicated that the virus was able to infect both species. The virus could be recovered from the infected animals 22-30 days after infection and replication of the virus was measured by the increase of virus production with time. Viral replication was localized only in the Oka organ which was significantly enlarged in the RPS-infected shrimp.

A parvovirus was recently reported to be the possible etiological agent which caused the IHHN disease in penaeid shrimp (Bonami et al, 1990). In earlier studies we had indicated that RPS may be associated with IHHN disease since the virus was isolated from 3 different sources of penaeid shrimps histopathologically diagnosed with IHHN disease.
However, subsequent infectivity experiments did not consistently show characteristic histopathological lesions indicative of IHHN disease. This suggested that RPS may not be directly associated with the disease because the virus did not appear to directly induce the formation of Cowdry type-A inclusions which are the current diagnostic indicators for IHHNV infection.

Very little is known regarding the role of RPS in causing disease in their penaeid shrimp host. Its capacity to infect and cause disease in the cultured penaeid shrimp remains to be comprehensively clarified. The preliminary studies described here indicated that RPS could infect two species of penaeid shrimp, *P. stylirostris* and *P. vannamei*, causing cytopathic alterations in their Oka organs. However, under the present experimental conditions the rate of replication of the virus was slow, and no apparent mortality was associated with the viral infection. Rhabdovirus of penaeid shrimp may be one of several viruses associated with the Oka organ of the wild and cultured shrimp. These viruses could conceivably affect the natural defense system of the animals and consequently impair the animals' ability to resist physical or chemical stresses, which in turn will render the animals more susceptible to infection by other biotic agents. Thus, the virus may be indirectly associated with mortality of the cultured shrimp even though RPS does not appear to be a highly virulent shrimp pathogen. The
possible role of RPS in either causing disease or in predisposing the affected animals to opportunistic infections should be considered.

Rhabdovirus of penaeid shrimp has been isolated from two species of penaeid shrimp collected from three different sources, including a shrimp farm in Ecuador, however, the infection caused by RPS in other penaeid shrimp and its geographical distribution is unknown. It is possible that this virus may exist widely in other species of penaeid shrimp around the world. Since there is no direct association between the virus infection and disease or animal mortality, the presence of RPS in penaeid shrimp could easily be overlooked. Therefore, a conclusion which should be drawn from this study is the necessity of a certification of the shrimp to be RPS-free before any shipment of the live penaeid shrimp stock be made. The IHHN disease which might be originally located in South and Central Americas (Bell and Lightner, 1983), has become a world-wide disease in penaeid shrimp because of shipment of live shrimp carrying IHHN virus.

One of the most important areas of future research is to examine Penaeus stylirostris and Penaeus vannamei and other species of penaeid shrimp among shrimp hatcheries or farms in the Asia and Americas for RPS infection. This would allow us to understand the host range and geographic distribution of the virus.
Another area of interest for future research should be the determination of the age of susceptibility of the penaeid shrimp to infection and disease expression by RPS. Because the present infectivity studies were only carried out in subadult shrimp, future infectivity studies should be conducted using shrimp at different stages of growth. This would certainly help to clarify the role of RPS in the causation of disease in penaeid shrimp.
VI. SUMMARY AND CONCLUSIONS

1. A protocol was established to isolate a new virus from two species of penaeid shrimp collected from different locations including Hawaii and Ecuador. This protocol is relatively simple, very effective, and reproducible.

2. Electron microscopy of the negatively stained purified virus showed a bullet-shaped structure which measured 65-77 x 115-138 nm in size. The virus was identified to be a member belonging to the rhabdovirus family and named Rhabdovirus of Penaeid Shrimp (RPS). Electron microscopical examination of RPS-infected EPC cells revealed that the virus particles are primarily localized in membrane-bound vesicles in the cytoplasm and the external space of the cells. No virus particles were present in the nuclei of the infected cells.

3. Virus was found to replicate in EPC cells, an established heteroploid fish cell line originating from the carp, and produced marked cytopathic effect characterized by the formation of a clear central zone surrounded by rounded-refractile cells.

4. The isolate also replicated in several other established fish cell lines but virus yields were poor. Optimal virus yield was produced in EPC cells.
5. Virus replication occurred between 10 to 30 °C and the optimum temperature of growth was 20-25 °C.

6. Single-step growth kinetics revealed that there is an eclipse period of 3 hours followed by virus production which is determined to be largely cell associated during the early phase of the virus growth cycle.

7. The virus was sensitive to treatments of low pH, lipid solvents, and to high temperatures (37 °C and 56 °C). The virus was unstable to freezing-thawing and low temperature storage (-10 and -70 °C). Virus replication was not inhibited by the halogenated pyrimidine, 5-bromo-2'-deoxyuridine (BUDR), a DNA antagonist.

8. A plaque assay protocol for the titration of infectious RPS was developed by using a semi-solid overlay medium consisting of minimal essential medium, 4% of fetal bovine serum, and 0.75% methylcellulose.

9. Several parameters important for the viral plaque assay were determined which included adsorption time, optimal period for plaque production, inoculation volume, and cell culture container.

10. For the determination of the efficiency of plating (EOP), a new technique developed in this laboratory was used for the direct determination of total virus particles in a virus preparation. The procedure was simple, direct,
effective, rapid and reproducible. The EOP for RPS in EPC cells was determined to be 30 viral particles per infectious unit.

11. The density of the intact virus in sucrose was 1.19 g/cm³. SDS-polyacrylamide gel electrophoresis of viral polypeptides revealed four major viral proteins with molecular weights of 165,000 (L), 67,000 (G), 44,500 (N), and 28,000 (M) daltons. Western blot analysis revealed a fifth protein, NS which has a molecular weight of 38,000.

12. The genome of the virus was composed of a non-segmented single-stranded RNA with a molecular weight of 3.6 x 10⁶ daltons.

13. An anti-RPS serum prepared in rabbits had a neutralization titer of approximately 6,400. This antiserum showed cross-neutralization with rhabdovirus carpio (RC) and infectious hematopoietic necrosis virus (IHNV), but did not react with viral hemorrhagic septisemia virus (VHSV).

14. A nitrocellulose-enzyme immunoassay for the detection of RPS antigen was developed and had a sensitivity to detect as little as 1 ng of RPS protein which is equivalent of 7 x 10⁴ virus particles.

15. In vivo studies revealed that RPS had limited pathogenicity for Penaeus stylirostris. Viral infection was localized in the Oka organ. The RPS infected Oka organs were
hypertrophied and showed gross cellular changes. Microscopically, the affected organs had abundant and large proliferative centers consisting cells with variable-sized and clear cytoplasmic vacuoles. Some cellular necrosis was also seen within the organ. However, RPS infection showed no direct association with mortality of the animals.

16. The role of RPS in causing disease in the penaeid shrimp is unknown. However, RPS infection may result in a greater susceptibility of shrimp to other biotic agents due to impairment of the lymphoid organ.


Anonymous. 1991d. Check out the shrimp. World Shrimp Farming 1990. pp.1


222


Momoyama, K. 1989a. Inactivation of baculoviral mid-gut gland necrosis (BMN) virus by ultraviolet irradiation, sunlight exposure, heating and drying. Fish Pathol. 24(2)115-118.


Momoyama, K. 1989c. Survival of baculoviral mid-gut gland necrosis virus (BMNV) in infected tissues and in sea water. Fish Pathol. 24(3)179-181.


