3.1. Materials

3.1.1 Animal

3.1.1.1 Cell culture

In an attempt to obtain tissues from normal uninfected fish, juvenile grouper, *Epinephelus merra* fish were caught using traditional traps (Figure 3.1) from Gulf of Mannar, Keelakarai (9° 14' N/78° 50' E) and Mandapam (9° 17' 0" N / 79° 7' 0" E), Tamil Nadu, India. The fishes were transported alive to the laboratory in oxygenated plastic bags within overnight after they caught from the wild. Fifty two juvenile fish (of similar size, measuring approximately 30 cm in fork length) were randomly selected for cell culture, and appropriate tissues were removed aseptically while at sea and placed into transport medium on ice. Tissues were transported to the Laboratory and further processed, approximately 6 hours after collection.
Materials and Methods

Figure. 3.1. Traditional traps used to capture the *E. merra* at Keelakarai, Tamilnadu.

Figure. 3.2. *Epinephelus merra* Honey Comb Grouper caught at Gulf of Mannar

3.1.1.2. Specimen collection for virus isolation

Groupers were sampled monthly for 10 months from wild from Gulf of Mannar, Keelakarai (9° 14' N/78° 50' E) and Mandapam (9° 17' 0" N / 79° 7' 0" E), Tamil Nadu, India. A total of 93 *E. merra* (Figure. 3.2) collected during April to November 2009 were fry to juvenile groupers (1-3 inches) and young adult to adult stages (Table. 3.1.). Specimens were sacrificed, separated into four parts, and treated differently for virus isolation, total RNA extraction and DNA extraction.
Table. 3.1. Grouper samples collected for virus isolation, PCR and RT-PCR diagnosis during January - December 2009.

<table>
<thead>
<tr>
<th>Sample code</th>
<th>Date</th>
<th>Number of samples</th>
</tr>
</thead>
<tbody>
<tr>
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<td></td>
<td>Mandapam</td>
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<tr>
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</tr>
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<td>EM2</td>
<td>April</td>
<td>8</td>
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<td>EM3</td>
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<tr>
<td>EM5</td>
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<td>10</td>
</tr>
<tr>
<td>EM6</td>
<td>December</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td></td>
<td>54</td>
</tr>
</tbody>
</table>

3.1.2. Equipment (The number given in the superscript denotes the supplier of the item)
- Centrifuge, model 3K30.1
- Laminar-flow biological safety cabinet, Class II Type A/B3.2
- Liquid nitrogen storage tank, XLC-1110.3
- Microscope, inverted, model TE 2000S.4
- Mini gel electrophoresis.5
- Refrigerated incubators, model LBI-250M.6
- Ultra low freezer, U41085.7
- Vetri™ 96-well thermal cycler.8
- Waterbath, model Z652903-1EA.9

3.1.3. Culture media, solutions, and chemicals (The number given in the superscript denotes the supplier of the item)
- 2-propanol, 4 l, No. A416-4.10
- Amphotericin B, 250 µg/ml, No. A-2942.11
- Chloroform:Isoamyl Alcohol (CHCl3/IAA) 24:1,100 ml, No. C0549.11
- Colcemid, 1.0 mg, D6165.11
- Dimethyl sulfoxide (DMSO), 100 ml, No.D-2650.11
Materials and Methods

- Dulbecco’s phosphate buffered saline (PBS), No. D-8537.\(^{11}\)
- EDTA disodium salt, No. E-5134.\(^{11}\)
- Ethanol, anhydrous, 4 l, No. A405P-4.\(^{10}\)
- Fetal bovine serum (FBS), 500 ml, No. 7D2078.\(^{11}\)
- Fibroblast growth factor (FGF), 25 µg, No. F5542.\(^{11}\)
- Gentamicin sulfate, 5 g, No. G1264.\(^{11}\)
- L-15 Leibovitz medium, No. L4386.\(^{11}\)
- Phenol red, 25 g, No. P-3532.\(^{11}\)
- Phenol: Chloroform: Isoamyl Alcohol (phenol/CHCl\(_3\)/IAA) 25:24:1 solution, 100 ml, No. P2069.\(^{11}\)
- Potassium chloride, KCl, No. P-9541.\(^{11}\)
- Potassium phosphate, KH\(_2\)PO\(_4\), No. P-8416.\(^{11}\)
- Sodium Bicarbonate, 1 kg, No. S-6014.\(^{11}\)
- Sodium chloride, NaCl, No. S271-3.\(^{10}\)
- Sodium phosphate, Na\(_2\)HPO\(_4\), No. S-5136.\(^{11}\)
- Trypan blue, 20 ml, No. T-8154.\(^{11}\)
- Trypsin, 10x, No. T-4549.\(^{11}\)

3.1.4. Glassware and plastic ware (The number given in the superscript denotes the supplier of the item)

- Aerosal barrier universal tips racked sterile. TF-1000 R-S.\(^{12}\)
- Centrifuge tubes, 15 ml, No. 352097.\(^{12}\)
- Centrifuge tubes, 50 ml, No. 352098.\(^{12}\)
- Cryogenic controlled-rate freezing container, No.15350-50.\(^{12}\)
- Disposable sterilization filter units, 150 ml, No.09740-1A.\(^{12}\)
- Disposable sterilization filter units, 250 ml, No.09740-2A.\(^{12}\)
- Disposable sterilization filter units, 500 ml, No.09740-25A.\(^{12}\)
Materials and Methods

- Microscope slides, 75 X 25 mm, No. 12544-7.\textsuperscript{12}
- Non-latex exam gloves, No. 6005PF.\textsuperscript{12}
- NUNC CryoTube vials, 1.8 ml, No.12565-171N.\textsuperscript{12}
- Premium cover glasses, 24 X 45 mm, No.12548-5M.\textsuperscript{12}
- Serological pipettes, 10 ml, No. 13678-11E.\textsuperscript{12}
- Tissue culture flasks, 25 cm, No. 10-126-39.\textsuperscript{12}
- Tissue culture flasks, 75 cm, No. 10-126-41.\textsuperscript{12}

3.1.5. Other reagents (The number given in the superscript denotes the supplier of the item)

3.1.5.1. DNA Extraction Chemicals

- EDTA, 0.5 M No. 105439\textsuperscript{13}
- Proteinase K No. 10597\textsuperscript{13}
- Tris-Cl, 1.0 M pH-8, No. 105443\textsuperscript{13}

3.1.5.2. Electrophoresis Chemicals

- Agarose No. 0144162\textsuperscript{14}
- Boric Acid No. 0249133\textsuperscript{14}
- Bromo phenol Blue No. 0240168\textsuperscript{14}
- EDTA No. 054448\textsuperscript{14}
- Ethidium Bromide No. 054817\textsuperscript{14}
- Tris Buffer No. 204982\textsuperscript{14}

3.1.5.3. Polymerase Chain Reaction Chemicals

- 10X Assay Buffer, 10X, No. 105876\textsuperscript{13}
- dNTP Mix, 10mM each, No. R0191\textsuperscript{15}
- MgCl\textsubscript{2}, 25mM, No. METB5\textsuperscript{13}
- Taq DNA Polymerase  5u/µl, No. EP042\textsuperscript{15}
3.1.5.4. Sequencing

- 5X Sequencing Buffer, No. 433669716
- Big Dye Terminator v3.1 cycle sequencing kit, No. 433691716

**Suppliers of the Equipment, Culture media, solutions, chemicals,**
**Glassware, plastic ware and other reagents**

1. Sigma Laborzentrifugeh GmbH, Postfach, 1713, D. 37507 Osterode am Harz, Germany.
2. Becton Dickinson Labware, 1 Becton Drive, Franklin Lakes, NJ 07417, USA.
3. IBP Company Ltd., B.G. (Cryogenics), A-4 MIDC Industrial Area, Ambad, Nasik, Maharashtra – 422010. India.
5. Bio-rad laboratories, Life Science Group Div., 2000 Alfred Nobel Dr., Hercules, CA 94547, USA.
7. New Brunswick Scientific Co., Inc., P.O. Box 4005, 44 Talmadge Road, Edison, NJ 08818-4005 USA.
8. Applied Biosystems, Foster City, CA, USA.
10. Fisher Scientific Co., 200 Park Lane Drive, Pittsburgh, PA 15275, USA.
11. Sigma Chemical Co., St. Louis, MO 63178, USA.
12. Nalge Nunc International, International department, 75 Panorama Creek Drive, Rochester, NY 14625, USA.
14. Sisco Research laboratories Pvt Ltd., 26, Navketan Industrial Premises Co-op Society Ltd., Shanti Nagar, Mahakali Caves Road, Andheri (E), Mumbai, India.

15. Fermentas Inc, 798 Cromwell Park Drive, Glen Burnie, MD 21061, USA.

16. Applied Biosystems, 850 Lincoln Centre Drive, Foster City, CA 94404, USA.

3.1.6. Procurement of positive nucleic acid controls for fish pathogens

The different nucleic acids of exotic fish pathogens to be used as positive controls in PCR have been obtained from various reference laboratories, which are as follows:

3.1.6.1. Nucleic acid of viral pathogens

- **Infectious hematopoietic necrosis virus (IHN):** The cDNA of IHN has been obtained from Dr Peter Enzmann, Research Centre for Virus Diseases of Animals in Tubingen, Germany.
- **Viral hemorrhagic septicaemia (VHS):** The cDNA of VHS has been obtained from Dr Michael *Snow*, FRS Marine Laboratory, Aberdeen, Scotland for use as reference.
- **Infectious Pancreatic necrosis virus (IPN):** The cDNA of IPN has been obtained from Dr A E Ellis, Marine Laboratory, Victoria Road, Aberden, Scotland, UK for use as reference.
- **Koi Herpes Virus (KHV):** Ronald P. Hedrick. Department of Medicine and Epidemiology, School of Veterinary Medicine, University of California, Davis, CA 95616, USA.
- **Betanodavirus genotype RGNNV and Iridovirus:** Dr. Somkiat Kanchanakhan, Aquatic Animal Health Research Institute (AAHRI), Department of Fisheries, Bangkok 10900, Thailand
3.1.6.2. Nucleic acid of bacterial pathogens

- **Bacterial Kidney disease (BKD):** Dr. Ronald J Pascho, Geological Survey, Biological Resources Division, Western Fisheries Research Centre, 6505 NE 65th Street, Seattle. Washington 98115, USA.
- **Aeromonas salmonicida:** Rocco C. Cipriano U.S. Geological Survey/Leetown Science Center National Fish Health Research Laboratory, 1700 Leetown Road, Kearneysville, West Virginia 25430.
- **Yersinia ruckeri:** Toranzo AE. Departamento de Microbiología y Parasitología, Facultad de Biología, Universidad de Santiago de Compostela, Spain.

3.2. Methods

3.2.1. Aliquoting of Fetal bovine serum (FBS)

Divide 500 ml bottle into 10 X 50 ml aliquots in 50ml sterile conical tubes. Heat inactivate at 56°C for 30 min in water bath. Store at 30°C.

3.2.2. Preparation of media

3.2.2.1. Leibovitz L-15 medium with additives

- L-15 powder 14.8 g
- Amphotericin B, 250 µg/ml 10 ml
- Gentamicin, 50 mg/ml 1 ml
- Sodium bicarbonate 1.5 g
- Double distilled water (ddH₂O) to 900 ml

Dissolve L-15 powder in about 800 ml of ddH₂O. Add sodium bicarbonate and other additives. Dissolve and sterile, glass serum bottles and sterilize by autoclaving at 121°C (250°F) at 15 psi for 15 min with validation. Store at 4°C.
3.2.2.2. Preparation of 2X freezing medium

- L-15 medium with additives 6.0 ml
- FBS 2.0 ml
- Dimethyl sulfoxide 2.0 ml

Mix ingredients together aseptically and prepare fresh mixture for freezing cells. All the above media were sterilized by either autoclaving or filtering through a 0.22 μm filter before use.

3.2.2.3. Preparation of Antibiotic-incubation medium (AIM) /Transport medium

- L-15 medium (2X) 100 ml
- Amphotericin B (250 μg/ml) 10 ml
- Gentamicin (50 mg/ml) 1.0 ml
- Double distilled water (ddH₂O) to 200 ml

Aseptically add ingredients together and mix thoroughly. Store at 4°C.

3.2.2.4. Washing medium

Phosphate buffered saline, PBSA pH 7.4 (without Ca²⁺ and Mg²⁺); supplemented with 50 μg/ml gentamycin sulphate and 5 μg/ml amphotericin B was used to rinse the tissues to remove excess blood.

3.2.2.5. Trypsinization medium

0.25% (w/v) trypsin in PBS without Ca²⁺ and Mg²⁺, adjusted to pH 7.2 with 1 N sodium hydroxide and filtered through 22 μm filter was sterility checked at 37°C prior to being stored frozen (−20°C). The trypsin solution was used to treat tissues in attempts to obtain single cell suspensions of the various pilchard tissues for subsequent culture.
3.2.3. Preparation of solutions

3.2.3.1. Cell stain-fixative

- Crystal violet 18 g
- Formaldehyde (37%) 1.8 l
- Double distilled water (ddH₂O) to 3.0 l

Mix 3 components thoroughly in a 4-l brown-colored glass bottle and store at room temperature.

3.2.3.2. Phosphate buffered saline (PBS)

- Sterile-filtered Dulbecco’s PBS
- Without calcium chloride and magnesium chloride

3.2.3.3. Proteinase K buffer

- 5 M NaCl 15 ml
- 1 M Tris-HCl (pH 7.4) 5 ml
- 0.5 M EDTA (pH 8.0) 10 ml
- 10% SDS 4 ml
- double distilled water (ddH₂O) 466 ml

Combine all ingredients and mix thoroughly. Store at room temperature.

3.2.3.4. Proteinase K/RNase working solution

- Proteinase K buffer 10 ml
- Proteinase K stock solution (10 mg/ml) 0.5 ml
- RNase Stock solution (10 mg/ml) 50 µl
- Mix all ingredients well and prepare fresh (before use).

3.2.3.5. Tris–EDTA buffer (TE) (10 mM Tris-HCl, 1 mM EDTA)

- 0.5 M Tris–HCl, pH 8.0 10 ml
Materials and Methods

• 0.5 M EDTA, pH 8.0 1.0 ml
• double distilled water (ddH₂O) 489 ml

3.2.3.6. Trypsin stock 2.5%

• Trypsin solution (10X) 100 ml/bottle and store at −30°C.

3.2.3.7. Versene solution

• NaCl 40.0 g
• KH₂PO₄ 1.00 g
• KCl 1.00 g
• Na₂HPO₄ 5.75 g
• EDTA–disodium salt 1.00 g
• Phenol red 0.05 g
• double distilled water (ddH₂O) to 5 l

Set up a sterilized 5-l flask containing 3 l of ddH₂O and a magnetic bar. While gently stirring the water, add each of the chemical components into the flask. Stir until dissolved and mix thoroughly. Bring volume to 5 l with ddH₂O. Dispense into 500 ml sterile, glass serum bottles and sterilize by autoclaving at 121°C at 15 psi for 30 min with validation. Store sterilized versene solution at 4°C.

3.2.3.8. Trypsin-versene solution (TVS)

• Trypsin (2.5% Trypsin in 0.9% NaCl) 10 ml
• Sterilized Versene 90 ml
• Aseptically combine ingredients, mix, adjust pH to 7.5 with 7.5% sodium bicarbonate.

Store at 4°C.
Materials and Methods

3.2.3.9. Mitochondrial DNA primers

The origin of the cell line was authenticated by partial amplification and sequencing of 16SrRNA and COI regions of the *E. merra* using universal primers. The primer pair sequences of the 16SrRNA and COI are given in Table.3.2.

Table.3.2. List of mt DNA primers for the amplification of fragments of 16SrRNA and COI regions of the *E. merra*.

<table>
<thead>
<tr>
<th>Sl. No</th>
<th>Primers</th>
<th>Sequence 5′-3′</th>
<th>No. of bases</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>16SrRNA</td>
<td>L: CGCCTGTATTCGAAAAACAT</td>
<td>20</td>
<td>Palumbi et al. (1991)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H: CCGGCTGAAGACTCATCACGT</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>COI</td>
<td>F: TCAACCAACACAAAGACATTTGCA</td>
<td>26</td>
<td>Ward et al. (2005)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: TAGACTTCTGGGTGCGGAAGATCA</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: TAGACTTCTGGGTGCGGAAGATCA</td>
<td>26</td>
<td></td>
</tr>
</tbody>
</table>

3.2.4. Preparation of tissues for primary cell cultures

Fish were acclimatized in the laboratory for 2 weeks. The sterilization of surface of the fish was carried out by wiping the fish in 80% (v/v) ethanol. The fish was anaesthetized using 2 ml of the clove oil solution (9 parts ethanol (94%) + 1 part clove oil) into 5 l of water. Tissues such as caudal fin, heart, swim bladder, kidney and spleen were collected from the fish using sterile instruments and aseptic techniques and each tissue was processed individually (Figure 3.3). Containers were surface-disinfected with Betadine (10% Povidone Iodine) and all processes carried out in a laminar flow cabinet. Tissues were washed four times in Phosphate Buffer Saline (PBS) containing antibiotics (500 IU per ml penicillin, 500 μg per ml streptomycin and 2.5 μg per ml Fungizone) before preparing explants.
3.2.4.1. Tissue explants

The fin tissues were minced in 10 ml PBS using two scalpel blades until a suspension of small tissue fragments was obtained. Each tissue suspension was then aspirated into a 10 ml pipette, placed in a 50 ml tube and centrifuged at 100 g for 5 min at 4°C. The supernatant of each tissue was removed and the pellet resuspended in 6 ml PBS. The tissue fragments were placed into 25 cm² cell culture flasks with Fetal Bovine Serum (FBS) for attachment of explants and kept overnight at 28°C. After ensuring the attachment of the explants 7 ml of complete growth medium (Leibovitz’s L-15 supplemented with 20% FBS and antibiotics (Penicillin, 100 IU/ml, Streptomycin, 100 μg/ml) and Fungizone (2.5 μg/ml) was added slowly. The flasks were incubated at 28°C in a normal atmosphere incubator and left undisturbed to allow optimum cell attachment, before examination by light microscopy. Half of the medium was changed every week. Nikon TE2000-S (Nikon Corporation, Japan) equipped with phase optics was used to observe and photograph living cell cultures every 3 d for primary cell cultures and subcultures.
3.2.4.2. Trypsinization of tissues

Caudal fin, heart, swim bladder, kidney and spleen tissues were slowly agitated in 5 ml trypsinization medium on a magnetic stirrer at room temperature (22–24 °C). Trypsinization times varied between tissues. After initial trypsinization, supernatants of each tissue were removed into sterile tubes, and 5% (v/v) foetal bovine serum added to protect the cells from the effects of further trypsinization. A second trypsinization treatment on the remaining tissues was undertaken. Again the supernatants were removed. The two supernatants of each tissue were combined and then aliquoted into six 25 cm² (Corning) tissue culture flasks, each containing 5 ml of one medium type. Cultures containing trypsinised tissue were incubated at 22°C and examined twice weekly using light microscopy, to determine cell attachment, cell growth, and occurrence of any microbial contamination.

3.2.5. Subculture, maintenance, storage and revival

When a confluent monolayer was formed in the primary culture, the cells were washed with Calcium- and Magnesium- free PBS (CMF-PBS) three times and the cells were harvested with 0.25% trypsin-EDTA solution (Invitrogen). The cells were subcultured at 1:2 to 1:3 ratios and maintained in the complete L-15 medium with 20% FBS, 200 IU/ml penicillin, 200 μg/ml streptomycin and 0.5 μg/ml amphotericin B. After 10th subculture, the concentration of FBS in medium was reduced to 10% (L-15-10), and antibiotics and antimycotics were reduced to the normal concentrations of 100 IU/ml Penicillin, 100 μg/ml Streptomycin and 0.25 μg/ml amphotericin B. The subcultures were stored in the liquid nitrogen (-196°C) in the freezing medium, which consisted of L-15 plus 50% FBS and 10% dimethyl sulphoxide (DMSO). Briefly, cells with 80% confluency were harvested as described earlier. These cells were resuspended to 2×10⁹ cells/ml and aliquoted to cryovials (1.5 ml cell suspension per vial). The cryovials were kept at −20°C for
2 h, −70°C overnight and then transferred to liquid nitrogen containers (−196°C). For revivals, a cryovial was thawed quickly in water bath at 37°C and centrifuged at 200 g for 5 min at room temperature. The cells were resuspended with L-15 medium supplemented with 10% FBS, 100 IU/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B, and seeded in a 25cm² tissue culture flask. The viability of the revival cells was estimated by trypan blue staining and the cells were counted on a hemocytometer.

3.2.6. Microscopic Examination

Flasks were observed under an inverted light microscope for attachment after fresh media was added. If no cell attachment was observed the flask was discarded. Contaminated cultures if any, were also discarded. Flasks which contained attached cells were examined weekly for growth and the culture media refreshed on a weekly basis. Morphology was observed and recorded using a Nikon digital camera (DS-Fi1). Fibroblastic-like cells were defined as cells which were bipolar, the length of which is usually twice its width. Generally epithelial-like cells are defined as a cell which is polygonal with regular dimensions when cells are confluent (Freshney, 2005). However, epithelial cells may appear to be more fibroblastic in shape when present in low numbers. It was presumed that a high harvest of viable cells gave rise to greater attachment. All cultures which were used for comparison tests were examined and graded with this scale after the initial 24-72 h attachment period.

3.2.7. Effect of temperature and FBS on cell growth

To examine the effect of temperature and varied concentration of FBS on fin cell growth, the cells at the concentration of 5×10⁴ were inoculated into 25 cm² cell culture flasks and incubated at 28°C for 2 h. The
batches of culture flasks were then incubated at selected temperatures of 20, 26, 28, 30, and 37°C. Temperature experiments were performed using normal growing medium supplemented with 20% FBS. Every other day, duplicate flasks at each temperature regime were washed with CMF-PBS twice, after which 0.2 ml of 0.25% trypsin solution was added to each flask. When the cells rounded up, the cell density was measured microscopically by using a haemocytometer. The experiment was carried out for 5 d. The experiments were conducted in triplicates. The cell growth in different concentrations of FBS (5, 7.5, 10, 15 and 20%) was carried out using the same procedure as mentioned above at 28°C.

The comparative effect of FBS and New born calf serum (NBCS) on the growth of the PSF cells at 30th passage was studied by using FBS, NBCS and equal mixture of FBA and NBCS in L-15 medium at varying concentrations (5, 10 and 15%). The seeding amount of cells was $1 \times 10^5$ per ml and incubated at 28°C. The formation of monolayer of the cells and health status of the cells were examined daily. After 10 d, cells were trypsinized and then counted microscopically with a haemocytometer.

### 3.2.8. Cell-plating efficiency

Cell lines in passages >15 were used to determine the plating efficiencies of GFM and GFSB. Cell densities of 200, 500, and 1000 cells per flask were seeded in duplicate in 25 cm² tissue culture flasks at 25°C in L-15 medium with 10% FBS. After 12 d, the medium was discarded and the cells were fixed with 5 ml of crystal violet–formalin stain–fixative for 15 min, rinsed with tap water, and air-dried. The stained colonies were then counted under the microscope, and plating efficiency was calculated as described by Freshney (2005).
3.2.9. Chromosomal analysis

Standard procedure as described by Freshney (2005) with some modification was followed. Briefly, RTF cells at 25th passage were incubated in a 25 cm² tissue culture flasks until 80% confluence at 28°C. Colchicine solution (Invitrogen) was added to the cells at a final concentration of 0.2 µg per ml and then the cells were incubated overnight at 28°C. After gentle pipetting, detached cells were collected by centrifugation at 200 g for 5 min at 4°C, treated with a hypotonic solution of 0.65% KCl for 20 min and fixed in 1:3 of acetic acid: methanol for 5 min at room temperature. The fixed cells were collected by centrifugation at 200 g for 5 min at 4°C and resuspended in fixative solution. The cell suspension was dropped onto a clean slide glass. After air-dry, 5% Giemsa solution was added for 20 min at room temperature to stain chromosomes and one hundred cells at metaphase were counted under a light microscope.

3.2.10. Viral susceptibility and cytopathic effect (CPE)

Viral nervous necrosis virus (VNNV) infected RT-PCR positive tissue samples (the only virus reported from India) of Asian sea bass Lates calcarifer tissue samples were collected from hatcheries in Nagapattinam of TamilNadu, India. The virus was isolated from the infected tissues by homogenizing them in L-15 medium with antibiotics and without FBS. The homogenate was frozen and thawed three times before centrifuging at 13000 g for 1 h at 4°C and the supernatant was filtered by a 0.22 µm membrane and inoculated into HGF cells. After 1 h of adsorption at room temperature, the supernatant was discarded, and the cells were washed with phosphate buffer three times. Following this, L-15 medium with 5% FBS was added to the cells and incubated at 30°C and the cells were examined daily for the occurrence of CPE for 10 d. To ensure the viability of VNNV in
the tissue homogenate, the disease was reproduced in the juveniles of the Asian sea bass by exposing them to the filtrate of the infected tissue homogenate in a biosecure area.

3.2.11. Cytotoxicity test of bacterial extracellular products (ECP)

The cytotoxicity of bacterial ECP from *Vibrio cholerae* MTCC 3904 (type strain that was readily available in our laboratory) and *Aeromonas hydrophila* (isolated and characterized in our laboratory) to HGF cells was tested. The cellophane plate technique of Liu (1957) was used. Briefly, sterilized cellophane sheets were placed on the surface of Brain Heart Infusion Agar (Himedia Laboratories, Mumbai, India) plates and inoculated by spreading 0.5 ml of a 24 h old broth culture of *V. cholerae* with a sterile swab and incubated at 37°C. After incubation for 48 h, cells were washed off the cellophane with a minimum volume of PBS. All the cell suspensions were centrifuged at 10000 g for 30 min at 4°C. The supernatants were filtered through a 0.22 μm pore-size membrane (Millipore), freeze-dried and reconstituted in PBS to a final volume of 10 ml. All ECP samples were stored at –30°C until use. The cells were grown as a monolayer in 24-well plates at 28 °C using L-15 medium supplemented with 5% FBS. For the toxicity test, the cell line was inoculated with 0.1 ml serial dilutions of ECP. For negative controls, plates were inoculated with sterile saline. Plates were incubated at 28°C and the effects of ECP on the cells were observed after 12 h for 3 d.

3.2.12. PCR for confirmation of origin of cell lines

The origin of the HGF cell line was authenticated by partial amplification and sequencing of 16S rRNA and COI regions of the *E. merra*. Briefly, the samples were homogenized separately in NTE buffer (0.2 m NaCl, 0.02 m Tris–HCl, 0.02 m EDTA, pH 7.4) and centrifuged at 3000 g at 4°C, after which the supernatant fluids were placed in fresh centrifuge tubes together
with an appropriate amount of digestion buffer (100 mM NaCl, 10 mM Tris–
HCl, pH 8.0, 50 mM EDTA, pH 8.0, 0.5% sodium dodecyl sulphate, 0.1 mg/ml, 
proteinase K. After incubation at 65°C for 2 h, the digests were deproteinized 
by successive phenol/chloroform/isoamyl alcohol extraction and DNA was 
recovered by ethanol precipitation, drying and resuspension in TE buffer. The 
universal primer pair sequences of the 16SrRNA (Palumbi et al., 1991) and 
COI (Ward et al., 2005) were used in the PCR reactions. PCR was carried out 
in a VetriTM 96 well thermal cycler (Applied Biosystems, Singapore). Each 
PCR reaction was in a 25 µl volume containing both forward and reverse 
primers (10 µm, 0.5 µl each), MgCl₂ (25 mM, 1.5 µl) dNTPs (2 mM, 2.0 µl), 
PCR buffer (10X, 2.5 µl), Taq DNA polymerase (1U, 0.5 µl), template DNA 
(0.3–0.4 µg) and nucleic acid free water. PCR cycling conditions included an 
initial denaturation at 95°C for 5 min, followed by 30 cycles of 95°C for 45 s, 
annealing temperature of 50°C for 30 s, 72°C for 45 s and a final extension of 
5 min at 72°C. The PCR products were visualized on 1.2% agarose gels and 
the amplified products were selected for sequencing. The cleaned up PCR 
products were sequenced in Applied Biosystems AB 3730 XL capillary 
sequencer following manufacturer’s instructions at the sequencing facility. 
The raw DNA sequences were aligned against the sequences of wild caught 
samples from Gulf of Mannar and Vizhinjam (India) and known sequences 
from the National Center for Biotechnology Information (NCBI) database; 
and edited using BIOEDIT sequence alignment editor version 7.0.5.2. 

3.2.12.1. Sequencing PCR

The amplified PCR products were used as the template for 
sequencing PCR. The conditions for PCR were as follows; 25 cycles of 95°C for 
30 s, 50-55°C for 5 s and 60°C for 4 min and finally stored at 4°C. The cleaned 
up PCR products were sequenced in Applied Biosystems AB 3730 XL capillary 
sequencer at the sequencing facility.
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3.2.12.2. Analysis of Data

The raw and partial DNA sequences of mitochondrial sequences of *E. merra* were aligned and edited using BIOEDIT sequence alignment editor version 7.0.5.2 (Hall, 1999) and NJ tree constructed using MEGA 4.0 (Tamura *et al.*, 2007).

3.2.13. Virus isolation

3.2.13.1. Virus isolation on FHM and BF-2 cells

The Fathead minnow (FHM; Gravell and Malsberger 1965) Blue gill fry -2 (BF-2; Wolf and Quimby 1966) cell lines obtained from National Centre for Cell Science (NCCS), Pune, India, were grown in L-15 medium supplemented with 50 μg per ml gentamicin and 10% FBS using 25 cm² cell culture flasks. The cells were grown at 25°C. The cells were subcultured approximately every 10 d using trypsin-versene.

Cells grown to 60–80% confluency (3 d after last subculture) were inoculated with the tissue material prepared as described above. After removal of the growth medium, 1 ml of tissue supernatant diluted with serum-free L-15-medium (final tissue dilution 1%, w/v) was added to each flask. The sample filtrate was allowed to absorb for 3 h at 20°C before 4 ml fully supplemented L-15 medium was added. The cultures were thereafter incubated at 20°C and inspected daily for CPE. In some cases, the cell cultures were frozen and thawed once to ensure a high yield of virus in the supernatant.

3.2.13.2. Virus isolation on HGF cells

Diseased grouper were collected from wild at Keelakarai and Mandapam in TamilNadu, India during 2008-2010 and transported to the laboratory in dry ice. Infected fresh organs (brain, eye, kidney, spleen and
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gill) from fish were dissected out and homogenized in L-15 medium without FBS. After centrifugation, the supernatant was filtered by a 0.22-μm membrane, and then inoculated into HGF cell line. After 1 h of adsorption at room temperature, the supernatant was discarded, and the cells were washed with phosphate buffer three times. Next, L-15 medium with 5% fetal bovine serum (FBS) was added to the cells and incubated at 23°C. After 15 d of incubation the cells were harvested, cells and culture supernatant were separated by centrifugation at 1000 g for 10 min. The supernatant was used for challenge test and to extract the viral nucleic acids for RT-PCR examination.

3.2.14. Detection of virus through nucleic acid amplification

3.2.14.1. Total RNA and DNA extraction for PCR / RT-PCR

Approximately 200-400 mg tissue of eyes, brain and internal organs were collected from the fish and treated immediately with Tri-reagent (Sigma). Total RNA and DNA extractions were conducted according to suggested protocols supplied with the reagent. A standard phenol-chloroform method was applied for DNA extraction. The total RNAs were used directly for reverse transcriptase-polymerase chain reaction (RT-PCR) detection using an OneStep RT-PCR kit from QIAGEN.

3.2.14.2. PCR and RT-PCR

Two sets of primers for betanodavirus detection were used as suggested by Nishizawa et al (1994). Primer set RGNNV770 was designed to amplify the coat protein gene at nucleotide sequence position 53 - 823 from the RGNNV nucleotide sequence from GenBank accession #D38636 (Nishizawa et al., 1995). PCR primers for red sea bream iridovirus and ranavirus detection were used according to the published literatures (Oshima et al., 1998; Mao et al., 1999). Sea bass iridovirus (SIV) DNA was
selected as a positive PCR control for marine fish iridovirus (Khongpradit and Kasornchandra, 1996). A known betanodavirus genotype RGNNV and Iridovirus were used for positive control in PCR amplification. The details of the primer used were given in Table. 3.3.

Table. 3.3. List of primers used in the PCR and RT-PCR detections from fish tissue

<table>
<thead>
<tr>
<th>Virus</th>
<th>Primer set code</th>
<th>Forward primer 5’-3’</th>
<th>Reverse primer 5’-3’</th>
<th>Exp. product (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Betanodavirus</td>
<td>SJNNV</td>
<td>CGTGTCAGTCAT GTGTCGCT</td>
<td>CGAGTCAACAC G GGTGAAGA</td>
<td>426</td>
<td>Nishizawa et al., 1994</td>
</tr>
<tr>
<td>Iridovirus</td>
<td>Mao</td>
<td>GTTTCATCGACT TGGCCACT</td>
<td>ATGTTGTGCATG GGGTTCTT</td>
<td>300</td>
<td>Mao et al., 1999</td>
</tr>
<tr>
<td></td>
<td>Osh</td>
<td>GCATGTATGCTG TTTAGAGA</td>
<td>GAGCATCAAGC AGGCATCT</td>
<td>187</td>
<td>Oshima et al., 1998</td>
</tr>
</tbody>
</table>

3.2.14.3. Screening of the grouper samples for other important fish pathogens

The collected grouper samples were also screened for the following other important pathogens viz., viral hemorrhagic septicemia (VHS) (Olesen, 1998), infectious hematopoietic necrosis virus (IHN) (Winton, 1991), infectious pancreatic necrosis virus (IPN) (Hill, 1982), bacterial kidney disease (BKD) (Pascho & Mulcahy 1987), Aeromonas salmonicida (Hiney et al., 1992), Yersinia ruckeri (Gibello et al., 1999) and koi herpes Virus (KHV) (Bercovier et al., 2005) and other bacterial pathogens such as Aeromonas hydrophila, (Chu and Lu, 2005), Edwardsiella tarda (Chen and Lai, 1998) and Vibrio choleare (Singh et al., 2002) by amplification of the targeted gene using standard procedures. A known respective pathogens obtained from different designated laboratories (section 3.1.6.1.) were used for positive control in PCR amplification in order to validate the test procedures.