3. Materials and Methods

3.1. Virus source:

The virus was first isolated from the throat swab of an encephalitis patient, collected from Karimnagar District of a southern Indian state, Andhra Pradesh during an outbreak in 2003 (Rao et al. 2004). The G gene sequence (1594 nucleotides of the complete gene sequence) was submitted in Genbank with Accession No: AY382603, strain: CIN0327M (Arankalle at al. 2005). The G-gene of this particular virus was used in the present study.

3.1.1. Determining the virus titer:

Initially this virus was isolated in MDCK cell line (Rao et al. 2004). The culture supernatant was used for the infection of Vero E6 cells. The supernatant of Vero E6 cells harvested at 48 hours post infection was serially diluted in DMEM (Dulbecco’s Modified Eagle’s Medium) with 10% Fetal Bovine Serum. Mono layered Vero E6 cell line, in 96 well plates was infected with 100ul / well of the above serially diluted medium containing the virus. The plates were incubated in CO$_2$ incubator for 48 hours and stained with amido-black. The TCID$_{50}$ was measured by observing the infection in the 96 well plates. The reciprocal dilution at which 50% cells were infected was assigned as the TCID$_{50}$ titer of the virus.

3.2. PCR amplification of the complete G-gene:

3.2.1. Primer designing:

Two primers were designed for RT-PCR to amplify the complete G-gene of CHPV based on strain: CIN034627M, Genbank Accession No: AY382603 using primer express software tool (Applied biosystems, USA). Following primers were synthesized by Integrated DNA Technologies, Inc. USA.

1) Forward primer (GF-1):

5’ ATG ACT TCT TCA GTG ACA ATT AGT GTG ATCC 3’.

2) Reverse primer (GR-8 (STOP)):

5’ TCA TAC TCT GGC TCT CAT GTT GAA GGG CTT 3’.
Underlined codons are start and stop codons respectively. The melting temperatures (Tm) of the primers were 52°C and 54°C respectively.

3.2.2. **RNA extraction from the supernatant of Vero E6 cells:**

Total RNA was extracted from 100µl of supernatant of *Vero E6* cell line harvested at 48 hours of post infection using TRIzol LS reagent (Invitrogen) as follows,

- 100µl aliquot of the supernatant of *Vero E6* cells (stored at −70°C) was thawed on ice for 30 minutes.
- 300µl of TRIzol LS was added to the above sample, mixed by inverting the tube for 30-40 times and incubated at RT for 30 minutes.
- 80µl of chloroform was added, mixed by inverting the tube for 15-20 seconds, and incubated at RT for 15 minutes.
- The tube was centrifuged at 16,000Xg (Plastocraft, India) for 5 minutes at 4°C.
- The upper aqueous phase was transferred to a fresh tube containing 200µl of isopropanol.
- The tube contents were mixed by inverting the tube 40-50 times, covered with aluminum foil and kept at RT for 30 minutes.
- The tube was centrifuged at 16,000Xg (Plastocraft, India) for 20 minutes at 4°C.
- The supernatant was poured off and the remaining liquid was removed with the help of a micropipette.
- 500µl of chilled 80% ethanol was added to the tube and the tube was flicked 3-5 times.
- The tube was centrifuged at 16,000Xg (Plastocraft, India) for 20 minutes at 4°C.
- The supernatant was poured off and the tube was re-centrifuged at 16,000Xg (Plastocraft, India) for 5 minutes at 4°C to remove the remaining ethanol.
- The RNA pellet was dried in an incubator at 37°C for 5-10 minutes.
3.2.3. Reverse transcription of complete G-gene:

- Dried RNA was suspended in 11.5µl mixture containing 10µl nuclease free deionized water (Bangalore Genei, Bangalore, India), 1µl of Forward primer (10μM; GF-1) and 0.5µl RNAsin, an RNAse inhibitor (40U/µl) (Promega, U.S.A.). The tube was flicked, spun briefly and incubated at 65°C for 5 minutes followed by a quick chill on ice.
- Following quick spin, 8.5µl RT mixture containing 4µl of 5X first strand buffer, 2µl 0.1M DTT, 0.5µl RNAsin (40U/µl), 1µl 10mM dNTP mix (Invitrogen, U.S.A.) and 1µl Superscript™II RNase H Reverse transcriptase (Invitrogen, U.S.A.) was added to the suspended RNA, and the tube was incubated at 42°C for 60 minutes and then at 70°C for 5 minutes to inactivate the enzyme. The tube was immediately kept on ice for 2-3 minutes followed by a quick spin. This cDNA was used for PCR amplification.

3.2.4. RT-PCR of complete G-gene:

- 90µl PCR mix was prepared containing 5µl 10X PCR amplification buffer, 5µl 10X PCR enhancer solution, 1.5µl 10mM dNTP mix (Invitrogen, U.S.A.), 5µl each of Forward and Reverse primers (10µM, GF-1 and GR-8 (STOP) respectively), 1µl 50mM MgSO₄, 0.5µl (2.5U/µl) Platinum Pfx DNA polymerase (Invitrogen, U.S.A.) and 66.5µl nuclease free deionized water.
- 10µl of cDNA was added and the contents were mixed followed by a quick spin.
- Thirty-five cycles of amplification were performed using an automated thermal cycler (GeneAMP PCR system 2400, Applied Biosystems, U.S.A.) with an initial hold step at 94°C for 5 minutes followed by denaturation at 94°C for 1 minute, annealing at 50°C for 1 minute and amplification at 68°C for 3 minutes and final extension at 68°C for 7 minutes.
3.2.5. Analysis of PCR product:

The completed RT-PCR reaction was resolved on 1% agarose gel (Invitrogen, U.S.A.) in 1X TAE buffer containing Ethidium bromide (Sigma, U.S.A.), electrophoresed at 45V for 45 minutes. GeneRuler™ 1Kb DNA ladder (500ng) (Fermentas Canada Inc, Canada) was used as DNA size marker for estimation of size of the PCR products. The bands were visualized under UV Transilluminator (SYNGENE, U.K.).

3.3. Sequencing of the PCR product:

Prior to sequencing, the RT-PCR product was purified using QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) as per the manufacturer’s instructions. Both the strands were sequenced using ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (version 3.1, Applied Biosystems, Foster city, CA) employing an automated sequencer (ABI 3130xl, Applied Biosystems, Foster city, CA). The primers described below were used for sequencing (Table 4).

Table 4: Details of the primers used for sequencing

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Primer ID</th>
<th>Sequence 5’→3’</th>
<th>Polarity</th>
<th>Oligonucleotide size (mer)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CHP-GF-1</td>
<td>ATGACTTCTTCAGTGACAATTAGGTGATCC</td>
<td>Sense</td>
<td>31</td>
</tr>
<tr>
<td>2</td>
<td>CHP-GF-2</td>
<td>GTCTTGTGGTTATGCTTCTGT</td>
<td>Sense</td>
<td>21</td>
</tr>
<tr>
<td>3</td>
<td>CHP-GF-3</td>
<td>TGTGTCCGACCGGGATCAGAGGT</td>
<td>Sense</td>
<td>23</td>
</tr>
<tr>
<td>4</td>
<td>CHP-GF-4</td>
<td>GACAATGAACTACACGAGCT</td>
<td>Sense</td>
<td>20</td>
</tr>
<tr>
<td>5</td>
<td>CHP-GR-1</td>
<td>TCATCCACCGGTTGAGATCCAT</td>
<td>Anti-sense</td>
<td>23</td>
</tr>
<tr>
<td>6</td>
<td>CHP-GR-2</td>
<td>TGAGCATGAGGTAGCTGTGGAT</td>
<td>Anti-sense</td>
<td>22</td>
</tr>
<tr>
<td>7</td>
<td>CHP-GR-3</td>
<td>TCCTCTGAATCTCTGAGGTC</td>
<td>Anti-sense</td>
<td>20</td>
</tr>
<tr>
<td>8</td>
<td>CHP-GR-4</td>
<td>TGATTACCAAGAACTCAGAGT</td>
<td>Anti-sense</td>
<td>21</td>
</tr>
<tr>
<td>9</td>
<td>CHP-GR-8(STOP)</td>
<td>TCATACTCTGGCTCTCATGGTGAAGGCTT</td>
<td>Anti-sense</td>
<td>30</td>
</tr>
</tbody>
</table>

3.3.1. Purification of PCR product:

The procedure for Qiagen column purification of PCR product, described by the manufacturer (Qiagen, Hilden, Germany) was strictly...
followed. DNA adsorbs to silica-gel membrane of spin column in the presence of high salt in the buffer, while contaminants like unused primers, EtBr etc. flow through the column. Adsorbed DNA is subsequently eluted in nucleases free deionized water. We eluted DNA in 20µl of the deionized water.

3.3.2. Cycle sequencing:

The purified PCR product was subjected to cycle sequencing. Sequencing reaction for each primer used was prepared in separate 0.5ml sterile thin walled microcentrifuge tubes (Tarsons, India) as follows,

| TRRM       | 4.0µl |
| Primer (10µM) | 0.5µl |
| Purified PCR product (~1.6kb) | 1.0µl |
| Nucleases free deionized water | 4.5µl |
| Total       | 10.0µl |

The cycle sequencing was carried out on GeneAMP PCR system 2400 (Applied Biosystem, U.S.A.). The cycling conditions were 94°C for 5 minutes (hold step) followed by 25 cycles of 96°C 10s, 50°C 5s and 60°C 4 min. The cycle-sequenced reactions were purified using Qiagen kit before loading on to an automated sequencer (ABI 3130 xl) as follows,

- Master Mix I: 10µl MilliQ water+2µl 125mM EDTA.
- Master Mix II: 2µl 3M NaOAc (pH 4.6) + 50µl Ethanol.
- Add 12µl Master Mix I to 10µl of cycle sequenced mixture and mix content properly.
- Add 52µl of Master Mix II to each of the above reaction and mix few times by inverting the tubes.
- Incubate at R.T. for 15 minutes.
- Spin 3000Xg for 30 minutes at R.T.
- Discard supernatant and spin up to 185Xg to remove the residual supernatant.
- Add 100ul of 70% ethanol and spin 3000Xg for 5min at R.T. Repeat once.
- Air dry and add 20ul of HiDi Formamide, denature at 94°C for 2 minutes, snap chill, vortex and spin.
- Transfer to the sequencing tubes.
From the chromatogram, nucleotide sequence was read and compared with available sequence (strain: CIN034627) employing NCBI Blast tool and MEGA 4 program.

3.4. **TA Cloning of complete G-gene in pGEM®-T Easy Vector system:**

3.4.1. **Addition of deoxyadenosine overhangs at 3’ prime ends of the PCR product:**

Following sequence confirmation of the desired PCR product, the amplification of the product was repeated for subsequent TA-cloning. To add deoxyadenosine overhangs at 3’ prime ends, the completed RT-PCR reaction was supplemented with 0.5µl AmpliTaq® DNA polymerase (5U/µl) (Perkin Elmer, Applied Biosystems), 1µl of 25mM dATPs and incubated at 72°C for 20 minutes in a thermal cycler. Subsequently, the completed reaction (100µl) was resolved on 1% preparative agarose gel and stained with EtBr. The DNA band of the desired size (~1.6kb) was cut from the gel with a sterile blade and was purified using QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) as per the manufacturer’s instructions.

3.4.2. **TA cloning of the G-gene in pGEM®-T Easy Vector:**

After gel purification, the RT-PCR product was TA-cloned in pGEM®-T Easy Vector, 3015bp (Promega Corporation, Madison, USA). A ligation reaction was set as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>2X Rapid Ligation Buffer, T4 DNA Ligase</td>
<td>5.0µl</td>
</tr>
<tr>
<td>pGEM®-T Easy Vector (50ng)</td>
<td>1.0µl</td>
</tr>
<tr>
<td>PCR product</td>
<td>3.0µl</td>
</tr>
<tr>
<td>T4 DNA Ligase</td>
<td>1.0µl</td>
</tr>
<tr>
<td>Total</td>
<td>10.0µl</td>
</tr>
</tbody>
</table>

The reaction was incubated at 4°C O/N.

3.4.3. **Transformation of JM109 *Escherichia coli* cells:**

For this, the protocol provided with the kit (Promega Corporation, Madison, USA) was followed.
25μl of competent cells were transformed with 4μl of ligation reaction, gently flicked and left on ice for 15 minutes.

The cells were given heat shock at 42°C for 45 seconds and quickly cooled on ice for 2 minutes.

500μl of SOC medium was added to the tube and incubated for 1 hour, at 225rpm on orbital shaker incubator (Thermo forma, U.S.A.).

50μl and 100μl quantities of transformation mix were plated on LB agar plates containing 100μg/ml ampicillin, 500μM IPTG and 80μg/ml X-Gal using sterile polystyrene beads.

The plates were incubated at 37°C for 18 hours and then shifted to 4°C for development of white and blue colonies. Bacteria carrying recombinant plasmid formed white colonies.

Minimum 10 white, separate colonies were selected for plasmid isolation.

### 3.4.4. Isolation of plasmid DNA by alkaline lysis (plasmid miniprep):

10 white colonies were picked with sterile microtips and inoculated in 5ml LB broth (in sterile 50ml centrifuge tubes) separately containing ampicillin (100μg/ml) and incubated for ~16 hours at 37°C and 225rpm in an orbital shaker incubator (Thermo forma, U.S.A.).

1ml aliquot (taken in sterile 1.5ml microcentrifuge tubes) from each 50ml tube was centrifuged at 6000rpm (Rotina 35 R, Hettich, Germany) for 5 minutes at RT.

The pellet was resuspended by vortexing in 100μl solution I (GTE buffer) containing 100μg/ml RNase A (Boehringer Mannheim, Germany).

200μl freshly prepared solution II (lysis buffer) (Sigma, U.S.A.) was added and mixed gently; the tube was incubated at RT for 5 minutes.

150μl of solution III (3M-potassium acetate, pH 5.5) (Sigma, U.S.A.) was added to the tube, mixed and centrifuged at 10,000 rpm for 10 minutes at 4°C.

250μl of isopropanol was added to the clear supernatant and mixed in a fresh tube to precipitate DNA.

The tubes were centrifuged at 10,000 rpm for 15 minutes at 4°C.
The DNA pellet was washed with 70% ethanol, air-dried and dissolved in 30-40µl of nuclease free deionized water (Bangalore Genei, Bangalore, India).

To confirm the cloning and orientation of the insert, plasmid DNA from each tube was subjected to restriction digestion analysis with Sac I restriction enzyme (Promega, U.S.A.).

A 10µl digestion reaction was set for each clone (taken in sterile 1.5ml microcentrifuge tube) by addition of 1µl restriction enzyme, 2µl buffer H (Promega, U.S.A.), 0.5µl BSA acetylated (Promega, U.S.A.), 4µl plasmid DNA and 2.5µl nuclease free deionized water. The tubes were incubated at 37°C for 2 hours. The digested and undigested plasmid clones were analyzed on 1% agarose gel with GeneRuler™ 1Kb DNA ladder (500ng) (Fermentas Canada Inc, Canada) as size marker (Promega, U.S.A.).

Sequencing was done using sense T7 promoter primer and another sense GF-4 primer as described earlier to confirm the start and stop codons respectively.

The confirmed clones (both sense and anti-sense) were cryo-preserved at −70°C in 10% (v/v) glycerol.

After confirming the complete G-gene in Sense orientation in pGEM®-T Easy Vector, the bacterial culture containing plasmids with the recombinant G gene was inoculated in 50ml LB broth containing ampicillin (100µg/ml), and incubated for 16 hours at 37°C and 225rpm in an orbital shaker (Thermo forma). The plasmid DNA was isolated (midiprep) using QIAfilter Plasmid Midi Kit (Qiagen) as per the instructions provided, subjected to restriction digestion analysis, sequenced as described above and stored at −20°C for further use. The protocol for plasmid midiprep is rapid, simple and based on the alkaline lysis of the bacteria, followed by binding of plasmid DNA to Qiagen anion-exchange resin under appropriate low salt and pH conditions. Plasmid DNA is eluted in high salted buffer and then desalted by isopropanol precipitation.

3.5. Recombinant G-protein (rGp) expression using baculovirus expression system:
The Bac-to-Bac® Baculovirus Expression System (Invitrogen) facilitates rapid and efficient generation of recombinant baculoviruses to express heterologous genes in cultured insect cells and insect larvae. The heterologous genes are expressed under the transcriptional control of the strong polyhedrin promoter of the *Autographa californica* nuclear polyhedrosis virus (AcNPV, 130kb circular dsDNA) during the late stages of infection. The gene of interest is cloned into pFastBac1 vector, 4775bp (donor plasmid). The mini-Tn7 in pFastBac1 vector contains an expression cassette consisting of a Gm' gene, the polyhedrin promoter from AcNPV, a multiple cloning site and an SV40 poly (A) signal inserted between the right and left arms of Tn7. The recombinant plasmid is transformed into DH10Bac *Escherichia coli* competent cells which contain the bacmid (bMON14272) with mini-att Tn7 site at the N-terminus of the lacZα gene (derived from pUC based cloning vector), a low copy number mini-F replicon, a kanamycin resistance marker and the helper plasmid (pMON7124). The Tn7 transposition proteins are provided *in trans* by the helper plasmid. The mini-Tn7 on pFastBac1 vector is transposed to the mini-att Tn7 target site on the bacmid. Colonies containing recombinant bacmids are identified by disruption of the lacZα gene. High molecular weight mini-prep DNA is prepared from selected *E.coli* clones (white colonies) containing the recombinant bacmid and these DNA are used to transfect insect cells.

### 3.5.1. Cloning of complete G-gene in pFastBac1 vector:

Restriction digestion of recombinant pGEM®-T Easy Vector + G-gene plasmid construct and pFastBac1 vector was done separately using *EcoR I* restriction enzyme (Promega, U.S.A.) as follows,

1. pGEM®-T Easy Vector + G-gene plasmid construct 15.0µl
   10X Buffer H 3.0µl
   *EcoR I* (10 U/ml) 2.0µl
   BSA (10mg/ml) 0.5µl
   Nucleases free deionized water 19.5µl
<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>40.0µl</td>
</tr>
<tr>
<td>1. pFastBac1 vector</td>
<td>10.0µl</td>
</tr>
<tr>
<td>10X Buffer H</td>
<td>3.0µl</td>
</tr>
<tr>
<td>EcoRI (10 U/ml)</td>
<td>1.5µl</td>
</tr>
<tr>
<td>BSA (10mg/ml)</td>
<td>0.5µl</td>
</tr>
<tr>
<td>Nucleases free deionized water</td>
<td>5.0µl</td>
</tr>
<tr>
<td>Total</td>
<td>20.0µl</td>
</tr>
</tbody>
</table>

The above 2 digestion reactions were incubated at 37°C for 2 hours and resolved on preparative gel (1% agarose) along with GeneRuler™ 1Kb DNA ladder (Fermentas Canada Inc, Canada) as size marker. A ~1.6kb band of complete G-gene and linearized pFastBac1 plasmid were cut from the gel and purified using QIAquick Gel Extraction Kit (Qiagen). The DNA was eluted in 15µl nuclease free deionized water.

The purified linear pFastBac1 vector (5µl) and G-gene (15µl) were added to a Ready-To-Go™ ligation mixture containing T4 DNA ligase (Amersham Biosciences, U.S.A.), incubated at RT for 5 minutes, mixed gently, spun briefly and incubated overnight (16-18 hours) at 4°C.

25µl JM109 competent cells were transformed with 2µl of above purified ligation reaction (using Qiaquick mini elute column, as described earlier). 25µl, 50µl and 150µl quantities of transformation mix were plated on LB agar plates containing ampicillin (100µg/ml).

The plates were incubated at 37°C for 18 hours. Bacteria carrying recombinant plasmid formed white colonies.

Minimum 10 white, separate colonies were selected for plasmid isolation as described earlier.

To confirm the cloning and orientation of the insert, plasmid DNA from each tube was subjected to restriction analysis with Xho I restriction enzyme (Promega, U.S.A.). A 10µl digestion reaction for each clone was set (taken in sterile 1.5ml microcentrifuge tube) by addition of 1µl each restriction enzyme, 2µl buffer D (Promega, U.S.A.), 0.5µl BSA acetylated (Promega, U.S.A.), 2.5µl plasmid DNA and 4µl nuclease free deionized water. The tubes were incubated at 37°C for 2 hours. The digested and undigested plasmid clones were analyzed on 1% agarose gel.
agarose gel with GeneRuler™ 1Kb DNA ladder as size marker (Fermentas Canada Inc, Canada).

- Sequencing was done using Polyhedrin promoter primer and GF-4 as described earlier to confirm the start and stop codons respectively.
- The confirmed clones were cryo-preserved at –70°C in 10% (v/v) glycerol.
- One of the confirmed clones with sense orientation was inoculated in 50ml LB broth containing ampicillin (100µg/ml), and incubated for 16 hours at 37°C and 225 rpm in an orbital shaker (Thermo forma). The plasmid DNA was isolated (midiprep) using QIAfilter Plasmid Midi Kit (Qiagen) as per the provided instructions, subjected to restriction analysis and sequencing as mentioned above and stored at –20°C for further use.

3.5.2 Transformation of DH10Bac competent cells to generate recombinant bacmids:

- Recombinant pFastBac1+G-gene plasmid construct was briefly centrifuged and placed on ice.
- One vial (100µl) of MAX Efficiency DH10Bac competent cells (Invitrogen, U.S.A.) was thawed on ice.
- 5µl recombinant plasmid construct was mixed with cells, with gentle tapping.
- The vial was incubated on ice for 30 minutes.
- Heat shock was given for 45 seconds at 42°C in a water bath and the tube was placed on ice immediately.
- SOC medium (1ml) was added to the vial.
- The vial was incubated at 37°C in a shaker incubator for 4 hours at 225 rpm.
- 750µl SOC buffer was removed after quick centrifugation at 1000rpm for 3 minutes.
- 25µl, 50µl and 100 µl of transformation mix was plated on two LB agar plates containing kanamycin (50µg/ml), gentamycin (7µg/ml), tetracycline (10µg/ml), Bluo-Gal (100µg/ml) and IPTG (40µg/ml) (all antibiotics, substrate and inducer from Invitrogen, U.S.A.).
The plates were incubated at 37°C for at least 48 hours and then shifted to 4°C for development of white and blue colonies.

10 white colonies were picked up by sterile microtips and inoculated into 5ml LB broth containing kanamycin (50µg/ml), gentamycin (7µg/ml) and tetracycline (10µg/ml) and incubated overnight at 37°C in an orbital shaker with 225 rpm.

The recombinant bacmid (rBac) was isolated by alkaline lysis method as described earlier.

All 10 rBacs were resolved on 1% agarose gel to check their integrity.

The bacmid DNA pellet was washed with 70% ethanol, air-dried and dissolved in 40µl nuclease free deionized water with gentle tapping.

The complete G-gene in rBac was sequenced by utilizing Polyhedrine promoter primer, GF-2, GF-3, GF-4, GR-2 and GR-8 (STOP) primers and the protocol as described earlier.

Total 5 confirmed rBacs were stored at 4°C for transfection of insect cells.

3.5.2. Culture and maintenance of Sf9 insect cells:

Cells derived from ovaries of Spodoptera frugiperda insect, clone 9, Sf9 cells (Invitrogen, U.S.A.) were maintained as monolayer culture in supplemented 1X unsupplemented Grace’s insect medium (Invitrogen, U.S.A.) with 10% FBS (Invitrogen, U.S.A.) and antibiotics, 50 units/ml penicillin and 50µg/ml streptomycin (Invitrogen, U.S.A.) (It is now referred to as complete Grace’s Insect Medium) up to 30 passages in polystyrene tissue culture flasks (T-25, T-75 and T-225; figures denote the surface area of flasks in cm²) (Corning Costar). The cells were incubated at 27°C (Newtronics, India) and cryopreserved in medium containing 60% Grace’s medium, 30% FBS and 10% DMSO (Dimethyl sulfoxide, Sigma, U.S.A.) in liquid nitrogen for further use.

3.5.3. Transfection of Sf9 insect cells with recombinant bacmids:

Six-well tissue culture plate (Nunc, Denmark) was seeded with 9X10⁵ log phase Sf9 cells in 2ml complete Grace’s Insect Medium (Invitrogen, U.S.A.) with antibiotics.

The cells were allowed to attach at 27°C for 60 minutes.
The transfection mix for each bacmid to be evaluated was prepared separately as follows,

**Solution A**

5µl bacmid DNA with 100µl unsupplemented Grace’s Insect Medium medium without FBS and antibiotic.

**Solution B**

6µl liposomes, Cellfectin (1mg/ml) (Invitrogen, U.S.A.) with 100µl unsupplemented Grace’s Insect Medium medium without FBS and antibiotic.

The total contents of solutions A and B were mixed gently and incubated at RT for 45 minutes.

While DNA-lipid complex was incubating, the medium from Sf9 cells was removed and cells were washed with 2ml unsupplemented Grace’s Insect Medium without FBS and antibiotic.

DNA-lipid complex was diluted to 1ml with unsupplemented Grace’s Insect Medium without FBS and antibiotic. One ml of each bacmid was added to separate wells containing Sf9 cells. One well was kept as untransfected cells control.

The cells with DNA-lipid complex were incubated at 27°C in an incubator for 5 hours; the DNA-lipid complex was removed and 2ml complete Grace’s Insect Medium with antibiotics was added to each well.

The cells were incubated at 27°C in an incubator for 96 hours.

Every day, the cells were visually inspected under an inverted microscope for the signs of viral infection.

At the end of 96 hours the cells from each well were harvested. The medium containing cells was collected in sterile 1.5ml microcentrifuge tubes and centrifuged (Rotina 35 R, Hettich, Germany) at 500Xg for 5 minutes.

The cleare supernatants and cell pellets were stored separately at −80°C. The cleare supernatants were designated as P1 recombinant baculoviral stock.

**3.5.4. Detection of G-protein by ELISA:**
Cell pellets and supernatants from transfection experiment including untransfected cells control were harvested and the pellets were kept on ice. The pellet was disrupted with 50µl lysis buffer (0.1% NP 40 in 0.01M PBS pH 7.2) (Sigma, U.S.A. and Qualigens, India, respectively). Immediately, protease inhibitors (PMSF 100µg/ml, Leupeptin 0.5µg/ml, Aprotinin 0.5µg/ml and Pepstatin A 1µg/ml) (Sigma, U.S.A.) were added to both the pellet and supernatant.

The cell lysates were diluted in 500µl (1:10 dilution) Carbonate/bicarbonate buffer (0.05M, pH 9.5) and the microtiter wells of the Maxisorp (Nunc) strips were coated with the diluted cell lysates (100µl/well). The cell supernatant was diluted to 1:10 in Carbonate/bicarbonate buffer and 100µl/well was added.

The strips were incubated at 37°C for 2 hours; post coated for 30 minutes at 37°C with the diluent containing 0.01M PBS pH 7.2 with 0.5% gelatin (both Qualigens, India), 0.5% Tween 20 (Sigma, U.S.A.), 10% DCS (Invitrogen, U.S.A.). The above diluent was also used for sample and conjugate dilution. The wells were washed 5 times with 0.01M PBS pH 7.2 containing 0.5% Tween 20 (wash buffer) using an automatic ELISA plate washer (Bio-TEK instruments INC, U.S.A.).

100µl of known anti-CHPV IgG and IgM positive as well as anti-CHPV IgG and IgM negative human serum samples diluted (1:20) in above mentioned diluent were added and the wells were incubated for 30 minutes at 37°C.

The wells were washed 5 times with wash buffer. 100µl/well of Goat anti-human IgG or Goat anti-human IgM HRP conjugates (Sigma, U.S.A) diluted in diluent (1:10,000 dilution each) were subsequently added to respective wells and incubated at 37°C for 30 minutes.

The wells were washed 5 times with wash buffer.

200µl/well substrate (20mg OPD and 10mg Urea peroxide in 50ml of 0.15M Citrate phosphate buffer pH 4.5–5.1 (substrate from Sigma and buffer components from Qualigens) was added and the strips were kept in dark at RT for 10 minutes for colour development (golden yellow). The reaction was terminated by adding 100µl 4N H₂SO₄ and...
the absorbance was read at 492nm on an ELISA reader (Labsystems multiscan MS). Test samples giving OD values ≥ 3 times the mean of negatives were considered positive for recombinant G-protein (rGp) antigen. Reactivity of control Sf9 cells was similarly tested (Arankalle at al. 2007).

Following the confirmation of the expression of rGp by ELISA, it was subjected to SDS-PAGE and westernblot.

3.5.5. Preparation of high titer recombinant baculovirus stock:

✓ After the confirmation of expression of rGp protein, high titer recombinant baculoviral stock was prepared from P1 recombinant virus for further studies.
✓ One T-75cm² flask with 80-90% confluent log phase Sf9 cells (~1X10⁷ cells) in 12ml Grace’s Insect Medium (un supplemented) with antibiotics was used for infection.
✓ 1ml of P1 recombinant baculoviral stock was added to the flask and incubated at 27⁰C for 6 days.
✓ The cells were harvested and centrifuged at 500Xg for 5 minutes.
✓ The supernatant and cells were separated and stored as mentioned earlier
✓ This supernatant was called P2 recombinant baculoviral stock.
✓ 2ml from P2 recombinant baculovirus stock was inoculated to T-225cm² flasks containing log phase Sf9 insect cells (90% confluence) (~3X10⁷) in 30ml complete Grace’s insect medium with antibiotics and 10% FBS.
✓ The supernatant was harvested after 96hours of post infection as 1ml and 5ml stocks and stored at −80⁰C.
✓ This virus stock was designated as P3 viral stock and used in further infections.

3.5.6. Temporal kinetics of rGp expression:

✓ To determine the time point of maximum rGp expression, the Sf9 cells infected with recombinant baculovirus were harvested at different time points. The clear supernatants and cell pellets were assessed for the presence of rGp in ELISA. Six-well tissue culture plates (Nunc,
Denmark) were seeded with $9 \times 10^5$ log phase SF9 cells in 2ml complete Grace’s Insect medium with antibiotics.

- The cells were allowed to attach at 27°C for 60 minutes.
- 200µl P2 viral stock was added to each well containing SF9 cells. One well was kept as uninfected cells control.
- The cells were incubated at 27°C in an incubator.
- The cells were harvested at 12, 24, 36, 48, 60, 72, 84, 96, 108 and 120 hours post-infection.
- After Spinning at 500Xg for 5 minutes, 50µl lysis buffer (0.1% NP 40 in 0.01M PBS pH 7.2) (Sigma, U.S.A. and Qualigens, India respectively) was added to the pellet. Immediately, protease inhibitors (PMSF 100µg/ml, Leupeptin 0.5µg/ml, Aprotinin 0.5µg/ml and Pepstatin A 1µg/ml) (all Sigma, U.S.A.) were added to the pellet as well as supernatant.
- The above lysed cell pellets (50µl lysis buffer) and clear supernatants were assessed for the presence of rGp in ELISA (procedure described earlier).
- For ELISA, dilutions of 1:10 (Ag), 1:25 (serum) and 1:10000 of HRP conjugate were found to be optimum and used for all the subsequent ELISAs.

3.5.7. SDS-PAGE:

- 40µl of the cell lysates at 48hours post infection and supernatants including their respective controls were added to 20µl 3X SDS reducing buffer and samples were kept in boiling water bath for 5 minutes.
- The samples were centrifuged at 10,000rpm (Rotina 35 R,Hettich, Germany) for 3 minutes.
- A protein molecular weight marker (#SM0431, range 14.4-116 kDa; Fermentas Canada Inc, Canada) was similarly treated.
- Two 10% acrylamide gels were prepared using Mini-PROTEAN 3 system (Bio-Rad Laboratories, U.S.A.). 20µl of each sample of Sf9 cell lysate as well as supernatants harvested at 48hours of post infection; control cells and protein molecular weight marker (6µg/well) were loaded in separate wells of the gels.
Electrophoresis was carried out in tank buffer for 90 minutes at constant voltage 90V (3A Power Supply unit from Bio-Rad Laboratories, U.S.A.).

Two gels were electrophoresed simultaneously with different samples loaded in identical sequence.

3.5.7.1. Staining of SDS-PAGE gels:

Staining and destaining of SDS-PAGE gels were done with CBBR stain.

The two gels were taken out separately from the gel run apparatus, rinsed into CBBR and incubated in CBBR de-stainer for 2-4 hours on rocker incubator for complete de-staining.

It was observed that the rGp and BSA were present at close proximity.

Henceforth all Sf9 cells were infected in serum free medium and harvested at 48 hours of post infection.

3.5.8. Western blot:

Two gels were electrophoresed simultaneously with different samples loaded in identical sequence.

The electrophoresed proteins on both the gels were transferred using transfer buffer onto separate nitrocellulose membranes (Invitrogen, U.S.A.) in Bio-Rad’s semi-wet transfer unit (Bio-Rad Laboratories, U.S.A.).

A constant current voltage of 15V was passed for 20 minutes for effective transfer.

The transfer of the electrophoresed proteins on nitrocellulose membranes was confirmed by staining with Ponceau stain (Sigma, U.S.A.).

After transfer, nitrocellulose membranes were incubated overnight at 4°C in blocking solution (0.01M PBS pH 7.2 containing 5% skimmed milk powder) (Sagar, India).

The membranes were incubated at RT for 2 hours separately with 5ml each of anti-CHPV IgG positive human serum samples diluted (1:20) in 0.01M PBS pH 7.2 containing 1% milk powder.

The membranes were washed thrice with wash buffer (0.01M PBS pH 7.2 containing 0.5% Tween 20)
The CHPV specific IgG antibodies were probed with Goat anti-human IgG HRP conjugate. 5ml of each conjugate at 1:5000 dilution in 0.01M PBS pH 7.2 containing 1% milk powder was added over respective membranes and incubated at RT for 45 minutes.

The membranes were again washed thrice with wash buffer (0.01M PBS pH 7.2 containing 0.5% Tween 20)

The membranes were developed at RT in substrate containing 4mg DAB and 2mg Urea peroxide in 10ml 0.01M PBS pH 7.2. The reaction was terminated by placing the membranes in water.

3.5.9. Large-scale rGp preparation:

T-225cm² flasks containing log phase Sf9 insect cells (90% confluence) (~3x10^7) in 30ml Grace's insect medium with antibiotics and without FBS were infected with 6ml of P3 high titer recombinant baculoviral stock. The supernatant was harvested after 48hours post infection; aliquots of 0.5ml and 10ml infected cell suspensions were centrifuged in sterile 1.5ml and 15ml centrifuge tubes respectively. These supernatants were stored at −70°C. The supernatant in 1.5ml tubes were directly used as source of antigen in ELISA as described earlier. The cells in 15ml tubes were subjected to Gel filtration on HPLC for rGp purification to be used as immunogen in vaccine development.

3.6. Standardization of IgG ELISA:

Sf9 cells (2x10^6) infected with rGp recombinant baculovirus (1x10^7 pfu/ml) were harvested at 48h PI. Infected Sf9 supernatant containing rGp neat and diluted at 1:10, 1:20, and 1:50 in 50mM carbonated buffer (pH 9.5) and coated on Maxisorp microtitre 96-well plate (Nunc, Rosekilde, Denmark) as 100µl/well. Following coating at 37°C for 2h, blocking solution (10% donor calf serum, 0.5% Tween 20, 0.5% gelatin) was added to each well and incubated at 37°C for 30 min. After washing with wash solution (0.01M PBS, 0.5% Tween 20), serum samples diluted at 1:10, 1:20, 1:25, 1:50, and 1:100 in blocking solution were added (100µl/well) to different wells according to the
protocol. A 1:25 dilution of Neutralization test (NT) negative sera served as negative controls. Incubation was continued at the same temperature for 30 min. All the wells were washed thrice with the wash solution. Each well was added with, horseradish peroxidase (HRP) conjugated goat anti-Human/Swine IgG antibodies (Sigma chemicals, St. Louis, MO, USA) at 1:1000, 1:5000, 1:10000, and 1:20000 dilutions and incubated at 37°C for 30 min. Initially HRP conjugated goat anti-Human IgM antibodies were also tried. Unbound conjugate was removed by washing 4 times with the wash solution. The enzyme substrate (O-phenylenediamine and urea peroxide) was added (200µl/well) and allowed to incubate at RT in dark for 8-10 minutes. The reaction was stopped by the addition of 4N H₂SO₄ and the absorbance was measured at 492nm (Labsystems Multiskan MS, MTX Lab Systems Inc, Virginia, USA). A serum sample was considered to be reactive when the optical density (OD) value was ≥ cut-off value. The cut-off value for the anti-rGp IgG antibodies was calculated as mean OD values for the three negative controls multiplied by 3.

3.6.1. Serum samples used in the ELISA:

Serum samples were used from NIV virus repository, collected from patients as well as healthy people from various CHPV epidemics. They were all earlier screened for virus neutralizing antibodies. A panel of 490 human and 248 swine serum samples were tested for anti-CHPV IgG antibodies.

- 400 human serum samples used in the study were collected from Warangal district of Andhra Pradesh, India, during and after the 2003 epidemic of CHPV.
- 90 Human samples were collected for antibody sero-prevalence study in 2007 from school children in India.
- 248 swine serum samples were collected during 2003 epidemic of CHPV from Warangal district area of Andhra Pradesh, India.

3.7. Purification of rGp on HPLC:

Attempts to precipitate the rGp in the supernatant of infected Sf9 cell culture using Polyethylene Glycol 1500 (PEG 1500) in combination with NaCl were unsuccessful.
The 15ml supernatant of Sf9 cells infected with P3 recombinant baculovirus was concentrated to 5 ml by using low protein binding Amicon filters (Millipore Corporation, MA 01821, USA) with the cut off value 10kDa. The rGp was purified using HPLC (AKTA BASIC 100) system (Amersham Biosciences) and HiPrep 16/60 Sephacryl S-100 High Resolution column (Amersham Biosciences). The 2.5ml of above concentrated Sf9 cell supernatant was loaded on HiPrep 16/60 sepharose Gel-filtration column (AKTA BASIC 100 HPLC system, Amersham Pharmacia, USA). Fractions of 3.0ml were collected and coated at 1:10 dilution and screened for the presence of rGp by using anti-CHP-IgG positive and negative sera for each fraction. The fractions with high (≥0.815 at 492nm) OD values in ELISA were combined and concentrated. The protein in these concentrated fractions was estimated by Lowry’s method (BSA as standard protein). The concentrated fractions were reassessed in ELISA.

3.7.1. Sample preparation:

✓ The 15ml cell supernatant was loaded on Amicon filter column and centrifuged at 5000rpm at 4°C for 10 minutes (Rotina 35 R, Hettich, Germany).

✓ The eluted 5ml supernatant was retained at 4°C. 200µl of this supernatant was used for protein estimation and the remaining quantity was kept at 4°C until loaded onto the column.

3.7.2. Column preparation and purification:

The HPLC system was checked for proper functioning according to the instructions of the manufacturer. The flow rate, pressure over the column, molarity and pH of the buffers used were in accordance with those recommended for the column. All the purification procedures including column preparation was carried out at RT unless otherwise mentioned. Briefly,

✓ After washing all the pipes, the column was connected to the HPLC system and washed with filtered (through 0.2µ filter), degassed, deionized water according to instructions given in the manual.
The column was equilibrated with buffer A (0.05M sodium phosphate, 0.15M NaCl, pH 7.2) at a flow rate of 1ml/minute with two column volumes.

The 1.2ml of concentrated supernatant was loaded on to the column at a flow rate of 0.5ml/minute.

The column was again washed with 2 column volumes of buffer A to remove unbound proteins at a flow rate of 1.2ml/minute.

Total twenty one 3ml flow through fractions were collected in pre-chilled, sterile16/100 round bottom glass tubes kept on ice in an automatic fraction collector. Appropriate amount of protease inhibitor cocktail (protease inhibitor cocktail prepared by adding 1 EDTA free tablet in nuclease free deionized water, 25X stock; Complete, Roche) was added to the flow through fractions

ELISA was carried out for all the fractions as per the protocol described earlier.

The fractions showing ELISA positivity were pooled.

Protein concentration in the pooled fractions was estimated by Lowry’s method using BSA as protein standard.

ELISA, SDS-PAGE, Western blot and protein estimation was finally done for the HPLC purified fractions. The HPLC purified protein was aliquoted, stored at −70°C and used at required concentrations as immunogen in mice experiments. This protein was also used for in-vitro stimulation of mouse splenocytes.

3.8. Recombinant Glycoprotein vaccine candidate:

The HPLC purified rGp was used for immunization of mice (10 mice/group, Swiss albino, Female, 6-8 weeks old). Initially four concentrations of rGp were used for immunization, i.e., 100ng, 500ng, 1µg and 2µg. One µg of rGp was adsorbed on 3.25 µg of AlPO₄ (Sigma chemicals, St. Louis, MO, USA) diluted to the required rGp dose at the time of immunization. For further experiments, a total of 3 doses of rGp (1µg/mice), 4 weeks apart, were used for immunization. Serum samples were collected periodically by retro-orbital plexus bleeding and subjected to ELISA for IgG-anti-CHP detection /
quantitation as well as tissue culture based in vitro virus Neutralization test (NT).

3.8.1. **Adsorption of rGp to AlPO₄:**

Recombinant G-protein expressed in baculovirus system, purified on AKTA BASIC 100 HPLC system (Amersham Pharmacia, U.S.A.) as described earlier was adsorbed to AlPO₄ as follows,

- 1µg of rGp was adsorbed on 3.25µg of AlPO₄ (Sigma) for 1 hour at RT on a vortex mixer followed by centrifugation at 5000rpm for 5 minutes at RT. The supernatant was preserved for ELISA and pellet was resuspended in 0.01M PBS, pH 7.2. The suspension was refrigerated and diluted to the required dose at the time of immunization.

3.8.2. **Immunization of mice:**

Six to eight weeks old female Swiss albino mice were used for immunizations with rGp. Mice were bled by retro orbital bleeding before immunization for pre immune sera and at regular intervals, 2 weeks apart, after receiving the recombinant protein doses. Swiss albino mice were immunized at 0, 4 and 8 weeks interval with rGp adsorbed to AlPO₄ as an adjuvant as showed in the (Table 5).
### Table 5: Details of mice groups used for rGp immunization.

<table>
<thead>
<tr>
<th>Group of mice (n=10)</th>
<th>Mice details</th>
<th>Conc. Of rGp/mice</th>
<th>Volume of Inoculum</th>
<th>Route of Inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>Swiss Albino, 6-8 Weeks old, Female.</td>
<td>100ng</td>
<td>50µl/mice</td>
<td>Subcutaneous</td>
</tr>
<tr>
<td>Group 2</td>
<td>Swiss Albino, 6-8 Weeks old, Female.</td>
<td>500ng</td>
<td>50µl/mice</td>
<td>Subcutaneous</td>
</tr>
<tr>
<td>Group 3</td>
<td>Swiss Albino, 6-8 Weeks old, Female.</td>
<td>1µg</td>
<td>50µl/mice</td>
<td>Subcutaneous</td>
</tr>
<tr>
<td>Group 4</td>
<td>Swiss Albino, 6-8 Weeks old, Female.</td>
<td>2µg</td>
<td>50µl/mice</td>
<td>Subcutaneous</td>
</tr>
<tr>
<td>Group 5</td>
<td>Swiss Albino, 6-8 Weeks old, Female.</td>
<td>Adjuvant (AlPO₄)</td>
<td>50µl/mice</td>
<td>Subcutaneous</td>
</tr>
</tbody>
</table>

All protocols were approved by the Institutional Ethical Committee for the use of animals for experimentation. All mice were bred at the National Institute of Virology, Pune; housing and care met or exceeded all the requirements.

#### 3.8.3. ELISA for assaying anti-rGp antibodies in mice sera:

Mice sera were screened for the presence of anti-rGp antibodies in ELISA employing crude Sf9 cells supernatant harvested at 48 hours post infection with recombinant baculovirus as a coating antigen according to the method described earlier (Deshmukh et al. 2007). Briefly, Sf9 cell supernatant containing rGp was diluted in 50mM carbonate buffer (pH 9.5) and used for coating (100µl/ well) 96 well micro-titer plate (Maxisorp, Nunc; Denmark). Following coating at 37°C for 2 hours the wells were blocked with blocking solution (0.01M PBS pH 7.2 with 0.5% gelatin) at same temperature for half an hour. After washing, mice sera were added to wells at 1:20 dilution (100µl/well) in blocking solution. Incubation continued at same temperature for half an hour. A 1:20 dilution of pre-immune sera served as negative control. Horseradish peroxidase (HRP) conjugated Goat anti-mouse IgG (Sigma chemicals, St. Louis, MO) was used as detector antibody. The enzymatic reaction with substrate (O-phenylenediamine and urea peroxide) was stopped.
by the addition of 4N H$_2$SO$_4$ and the absorbance measured at 492nm. A serum sample was considered to be reactive when the optical density (OD) value was greater than or equal to thrice mean OD value (ELISA cut-off) of pre-immune sera (negative control).

3.8.3.1. Quantitation of anti-rGp antibodies by ELISA:
The mice sera were diluted 2-fold in the blocking solution / diluent and subjected to the ELISA protocol described above. The CHPV-specific IgG antibody titers were determined for individual mouse from each group. The reciprocal of the highest dilution that had an absorbance greater than or equal to the ELISA cut-off was taken as the CHPV-specific antibody titer. All the analyses were carried out on the geometric mean titers (GMTs) and log-transformed antibody titers with standard errors. Antibody negatives were included in the analysis.

3.8.3.2. Anti-CHPV IgG isotype analysis:
For detecting IgG isotypes, rGp was used as coating antigen as described above. The reaction with test serum (37° C, 30 min) was followed by incubation at 37°C for half an hour with goat anti-mouse IgG isotype antibodies (IgG1, IgG2a, and IgG2b) (Sigma chemicals, St. Louis, MO). HRP-conjugated rabbit anti-goat IgG (Sigma chemicals, St. Louis, MO) was used as detector antibody. The CHPV-specific titers for IgG isotypes namely IgG1, IgG2a and IgG2b were determined for individual mouse from each group using two-fold dilutions. The reciprocal of the highest dilution that had an absorbance greater than or equal to the ELISA cut-off was taken as the CHPV-specific antibody titer. All the analyses were carried out on the log-transformed antibody titers with standard errors.

3.8.4. In-vitro Neutralization test (NT) for measuring neutralizing antibodies:

Tissue culture based In vitro Neutralization test was carried out for all serum samples of all individual mice immunized with rGp. The protocol for this assay was as follows,

✓ Initially, Tissue culture infectious dose (TCID$_{50}$) of CHPV stock (CIN034627 strain), propagated in Vero E6 cell line was determined.
Equal volumes of mice serum and 100 TCID<sub>50</sub> CHPV stock were mixed and incubated for 1h at 37°C.

The 100µl of the above mixture was added on monolayer of Vero E6 cells incubated at 37°C and observed periodically for 48 hours for cytopathic effects (CPE).

Antibody negative and positive controls and virus alone were included for every test.

Based on the CPE in virus control wells, approximately at 35 - 48hours, the wells were stained with amido-black.

A sample was considered positive for neutralizing antibodies when no CPE observed.

The reciprocal of the highest dilution showing no CPE in at least two wells out of four used was taken as the anti-CHPV neutralizing antibody titer (NT titer).

Following the above protocol the In-vitro Neutralization test (NT) was performed for mice serum samples.

3.8.4.1. In-vitro Neutralization test (NT) for measuring neutralizing antibodies using heterologous isolates of CHPV:

To assess the efficacy of the neutralizing antibodies generated by immunizing with the 2003 isolate-derived rGp against the viruses isolated in 1965, 2004 and 2007, neutralization tests were performed. Serum samples from five immunized mice were screened in NT with the homologus as well as heterologus CHPV isolates.

3.8.5. Intracerebral challenge study of immunized mice with CHPV:

The homologous strain of CHPV (CIN034627) was used as a challenge virus. The lethal dose 50 (LD<sub>50</sub>) of the stock was determined in 16-18 wks old Swiss Albino female mice by Reed and Munch method (Reed and Muench, 1938). The immunized mice were bled and challenged intracerebrally at 2 weeks after the 3<sup>rd</sup> dose with 100 LD<sub>50</sub> of the virus.

3.8.5.1. Measuring the mouse lethal dose 50 (LD<sub>50</sub>):
The CHPV (Strain: CIN034627M) was serially 10-fold diluted in DMEM and 30ul from each dilution was inoculated through intra cerebral route.

Swiss albino, female 14-16 weeks old mice were used in this study.

Mice dying within 24h of inoculation were considered non-specific deaths and those dying after 24h of infection were recorded as CHPV-specific deaths.

The survival of the mice after infection was observed up to 14 days (2 weeks post infection).

The Data was analyzed according to the Reed and Muench method and the virus dilution, which caused 50% death, was considered as the mouse LD50 of CHPV in that particular viral stock.

3.8.5.2. Challenging the Immunized mice with CHPV:

After observing the maximum immunogenicity of rGp in mice by ELISA and NT (2nd or 10th week of post immunization), Immunized mice were challenged with 100LD50 of homologous CHPV (Strain: CIN034627M) at two weeks after the last immunization. In control mice, 100LD50, 10LD50, and 1LD50 of CHPV was given to un-immunized mice. The survival was observed and recorded on every day up to 14 days.

3.8.6. Lymphocyte proliferation assay:

To assess the cell mediated immune response, mice were immunized as described in Table 5 and sacrificed 2 to 3 weeks after the last dose to obtain spleen. The Splenocytes were cultured in vitro and stimulated with HPLC column purified rGp antigen. Briefly, 1X10^5 cells/ well were cultured in quadruplicates in 96-well flat bottom plate (Nunc, Denmark) in RPMI medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) at 37°C with 5% CO2 partial pressure. Cells were stimulated with T cell mitogen phytohaemagglutinin (PHA) (Sigma chemicals, St. Louis, MO) 5µg/ml as positive control. Twenty microgram /ml of rGp was added to cells for specific proliferation. Cells with medium alone were treated as negative control. Initially, dose-response experiment was carried out to decide optimum concentration of PHA and rGp to be used. Cells were pulsed at 96 hours with 1µCi of tritiated thymidine for 24 hours. Cells were harvested
onto GF/C filter (Whatman, UK) membrane and counts taken on β-counter (LKB Pharmacia, Sweden) using standard protocol. The stimulation index (SI) was determined as ratio of counts per minutes (CPM) in the presence of rGp / CPM in absence of rGp. Mice with SI value ≥3 were considered to be the responders (Deshmukh et al. 2007).

3.8.7. **Long-term antibody kinetics in immunized mice:**

To assess the persistence of anti-CHP antibodies, mice immunized according to the schedule described in table 5, were bled by retro-orbital plexus at every month from the 10th week of post immunization over a period of six months. The ELISA and NT tests were carried out for individual mouse samples. All the analyses were carried out on the geometric mean titers (GMTs) and log-transformed antibody titers with standard errors.

3.9. **Development of combination vaccine with DPT:**

3.9.1. **Vaccine components and Immunization:**

The animal ethical committee of the institute approved this study. Expression and purification of rGp was carried out as described earlier. Briefly, the G-gene of Chandipura virus was cloned and expressed using baculovirus expression system. The recombinant protein was purified employing HPLC HiPrep 16/60 Gel filtration column and characterized by ELISA and Immunoblot.

The DPT vaccine manufactured by the Serum Institute of India, Pune, India was purchased from the market and dose/mouse was calculated based on the average weight of the mice used for the experiments (25µl/mouse). Aluminium phosphate (AlPO₄) was used as adjuvant. 1µg of rGp was adsorbed on 3.25µg of AlPO₄ (Sigma chemicals, St. Louis, MO, USA) by vortexing at a low speed for 1 hr at RT followed by centrifugation at 5000 rpm for 5 min at RT. The pellet was re-suspended in 0.01M PBS, pH 7.2 and diluted to the required rGp dose at the time of immunization. This rGp (1µg or 2µg/dose) adsorbed on AlPO₄ was mixed with commercially available DPT vaccine (25µl/dose).

The rGp (1 and 2µg) with or without DPT vaccine was used for immunization (3 doses, intramuscular, 4 weeks apart) of mice (10mice/group,
Swiss albino, Female, 6-8 weeks old). DPT alone and adjuvant alone were administered to two groups of mice.

3.9.2. Evaluation of immune response:

Serum samples were collected periodically by retro-orbital plexus bleeding and subjected to ELISA for detection/quantitation of IgG-anti-CHP antibodies as well as tissue culture based in vitro virus Neutralization test (NT) according to the methods described earlier (Rao et al. 2004). Detection/quantitation of antibodies against each component of DPT vaccine was carried out by SERION ELISA classic test (Institut Virion\Serion GmbH, Germany) according to the manufacturer’s instructions except that Horseradish peroxidase (HRP) conjugated goat anti-mouse IgG (Sigma chemicals, St. Louis, MO, USA) replaced the anti-human-IgG-HRP conjugate. Antibody titers were determined for individual mouse from each group and the reciprocal of the highest dilution was taken as the titer against the respective component of the vaccine. The titers are presented as geometric mean titers (GMTs) and log-transformed antibody titers with standard error.

Lymphocyte proliferative response against rGp was determined in mice immunized with rGp alone or with DPT. The stimulation index (SI) was determined as ratio of counts per minutes (CPM) in the presence of rGp / CPM in absence of rGp. Mice with SI value ≥ 3 were considered to be the responders.

Mice lethal dose 50 (LD₅₀) of homologous CHPV strain (CIN034627) was determined in 16-18 weeks female Swiss albino mice. All immunized/control mice were challenged with 100LD₅₀ of CHPV at 2 weeks after the last immunization.

3.10. Statistical methods:

Statistical analysis was carried out using SPSS version 9.0. Concordance between the positivity/negativity as scored by the NT and ELISA were compared using Pearson’s correlation coefficient and Spearman’s rank correlation coefficient. Student t-test was done for comparison of the different groups. Survival analysis was done on mice infected with CHPV by generating Kaplan-Meier survival plots using SPSS 9.0 software.

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