Chandipura Encephalitis

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5 Chandipura Encephalitis

- Analysis of host immune response against Chandipura virus in murine model
- Functional analysis of Chandipura virus recombinant proteins (M&N) and its utility in diagnostics and vaccine
- Chandipura virus encephalitis: The role of virus interaction with toll like receptor 4
- Cloning and expression of G-gene of Chandipura virus and evaluation of immunogenicity in mice
- Evaluation of siRNA in curing Chandipura virus infection *in-vitro* and *in-vivo*
- HLA Phenotypes and outcomes of Chandipura virus infection in Andhra Pradesh
- In-silico sequence and structure-based antigenic analysis of the G, N and M proteins of Chandipura virus

Analysis of host immune response against Chandipura virus in murine model

B Anukumar and AC Mishra

Age dependent susceptibility to Chandipura Virus (CHPV) was noticed in mice through all routes except intra cranial route of inoculation. Pathogenesis in adult mice through intra cranial inoculation was studied in this project.

Objective

- To study the immuno pathogenesis of CHPV in murine model

Work done

Pathogenesis in adult mice

Three groups of mice were inoculated with CHPV Blood Brain Barrier (BBB) through intracranial, intra nasal and intra venous (BBB Damage) route respectively. Mice were followed up to 96h PI and blood and brain was collected every 24h interval up to 96h PI. Virus titer in blood and brain, IgM, IgG kinetics, mortality pattern and level of CD4+, CD8+ and CD19+ cells in blood are the parameters studied. Detectable level of virus titer only noticed in blood at 24h PI in all the groups with various levels. Continuous replication of virus noticed in brain from 24h PI onwards invariably in all groups. Seroconversion noticed from 48h PI onwards but none of the mice switched into IgG type. All the mice died in intracranial as well as BBB damaged mice at 96h PI. No significant level of difference noticed in CD4+, CD8+ and CD19+ cell populations in these mice in all the PI hours tested. This experiment concluded that entry of virus in the brain is essential for CHP pathogenesis. Even though the virus reaching the brain through intra nasal route, the virus replication was restricted in some part of brain and no mortality observed.

Mechanism of reduction of immune cells in Chandipura infection

Reduction of CD4+ and CD8+ cells were noticed in Chandipura virus infection in both *in vivo* and *in vitro*. In this study role of Fas/Fas L in reduction was studied. *In vitro* infected RAW cells upregulated significant level of Fas L simultaneously down regulated Fas expression. Similarly splenocytes co cultured along with infected RAW cells also significantly upregulated the Fas/Fas L expression. Significant level of reduction in CD4+ cells noticed when the splenocytes cultured with supernatant from virus infected RAW cells. The supernatant contained significant level of TNFα, MCP-1 and IL-10. *In vivo*, the CD4+, CD8+ and CD14+ cells from CHP infected mice upregulated the Fas/ Fas L expression. The plasma from infected mice contains significant level of all pro inflammatory cytokines including TNFα. MCP-1, IL-10, IL-6, IL-12 and IFNγ. Purified CD4+ and CD8+ cells from infected mice were highly positive for Annexin V staining. Overall these experiments indicated that the reduction of CD4+ and CD8+ cells are regulated by expression of Fas/Fas L and secretion of proinflammatory cytokines

Immune status of CHP infected susceptible mice

Immune status of mice during infection was studied by lymphocyte proliferation assay using specific (inactivated antigen) and non specific (Con A and LPS) stimulators and IFNγ secretion assay. Antigen specific proliferation was only noticed in 48h PI, similarly LPS mediated proliferation was also noticed in 48h PI. Results based on Con A stimulation indicated that suppression of proliferation T cells in all post infective days. Significant level of IFNγ secreting CD4+ and CD8+ cells observed in 24h PI. At 48h PI only significant amount of CD4+ cells secreted IFNγ.

These experiments indicated that during infection mice undergone immune suppression due to various factors.

Functional analysis of Chandipura virus recombinant proteins (M&N) and its utility in diagnostics and vaccine

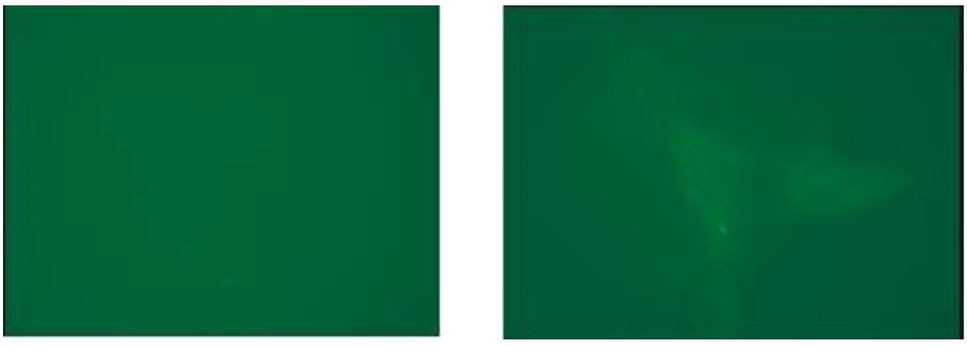
B Anukumar and AC Mishra

It was observed in our earlier study that expression of N gene in Vero cells induced cell lysis. The cells were also positive for TUNEL when employed TUNEL assay. Analyzing the expression of caspase-3 is one of the methods to confirm the involvement of apoptosis.

Work done

N gene mammalian expression

N gene transfected Vero cell was stained with anti caspase-3 antibody conjugated with FITC. It was observed that these cells are positive for caspase-3. This experiment indicated that expression of N gene in Vero cells induced apoptosis through caspase-3 pathway.



Mock transfected cells (100X) N gene transfected cells (100X)

Fig.1: Staining of caspase-3 in N gene transfected Vero E6 cells

Expression and purification of glycoprotein (G) of Chandipura virus in prokaryotic expression system

Full length G gene was cloned into pET 32b vector. The positive clone was transformed into BL-21 host. The protein was expressed as inclusion body. The inclusion body was isolated and the G protein was purified. The purified protein was refolded using sucrose. The refolded protein was further purified by ion exchange chromatography. Purity was checked by SDS-PAGE and western blot.

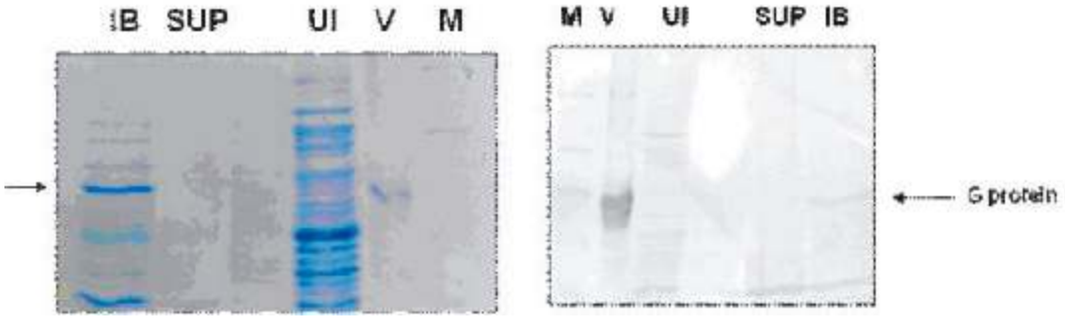


Fig.2: G protein expression in *E.coli* system. IB-Inclusion body, SUP-supernatant, UI- un induced, V- virus, M- marker

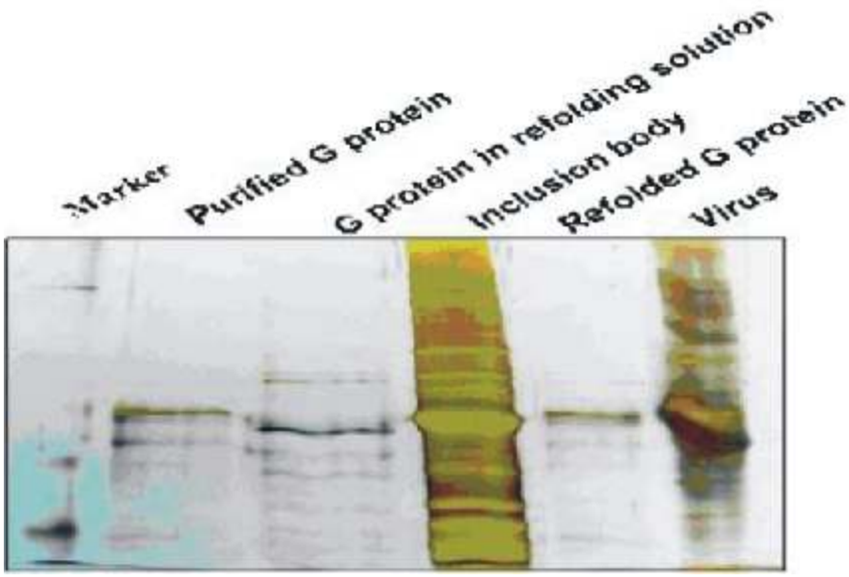


Fig.3: Refolding of expressed G protein

Chandipura virus encephalitis: The role of virus interaction with toll like receptor 4

B Anukumar and AC Mishra

The cytokines expression is negatively regulated by different SOCS proteins using different pathways. Some viruses exploit this mechanism for their replication by inhibiting Type I interferons signaling. In this study the phenomenon was checked in RAW cells.

Work done

(a) Role of suppressor of cytokine signaling (SOCS) protein in CHP pathogenesis

CHP infection of RAW cells induced the expression SOCS1, 2, 3 and CIS protein. This cell also expressed IFNα/β and IFNα/β receptor. The induction level was quantitated in transcript level by semi quantitative RT-PCR. These SOCSs proteins might suppress the Type I IFN activity **(Fig.4, 5)**

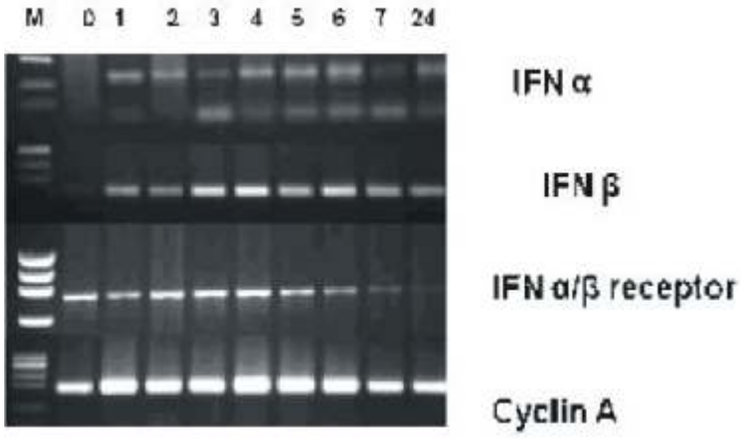


Fig.4: Transcript level of Interferon a/í and Interferon a/í receptor expression in Chandipura virus infected RAW cells at different hours post infection

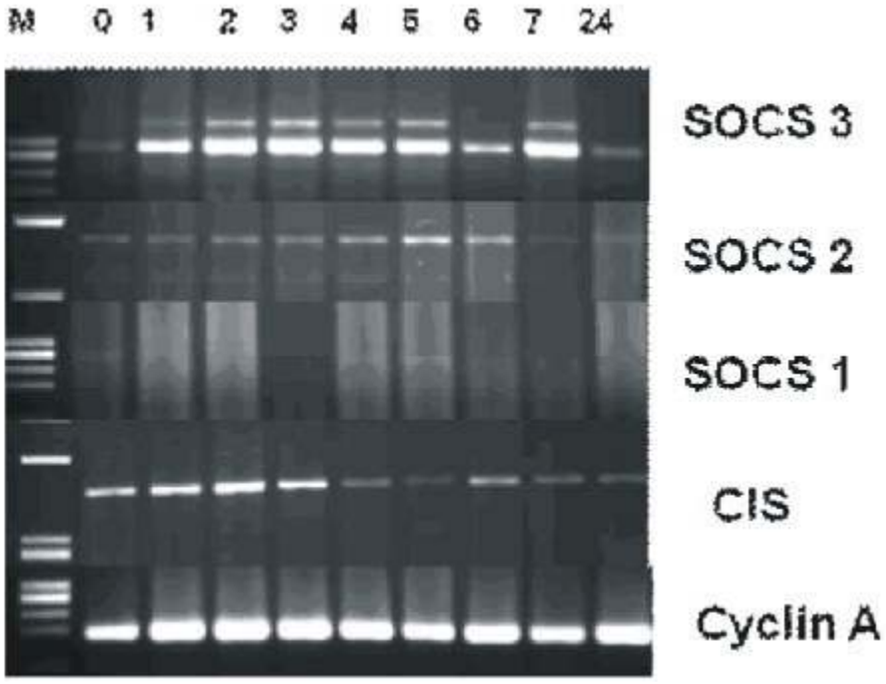


Fig. 5 : Transcript level of SOCS and CIS expression in Chandipura virus infected RAW cells at different hours post infection

(b) Role of TNFa in CHP pathogenesis

In vitro it was observed that compare to the unprimed RAW cells the TNFa primed RAW cells synthesis more virus particles. Similarly inhibition of TNFa either by small molecule TNFa inhibitor or neutralizing antibody reduced the virus replication. This indicates that TNFa, suppress the host cell defense mechanism through SOCS pathway **(Fig.6)**.

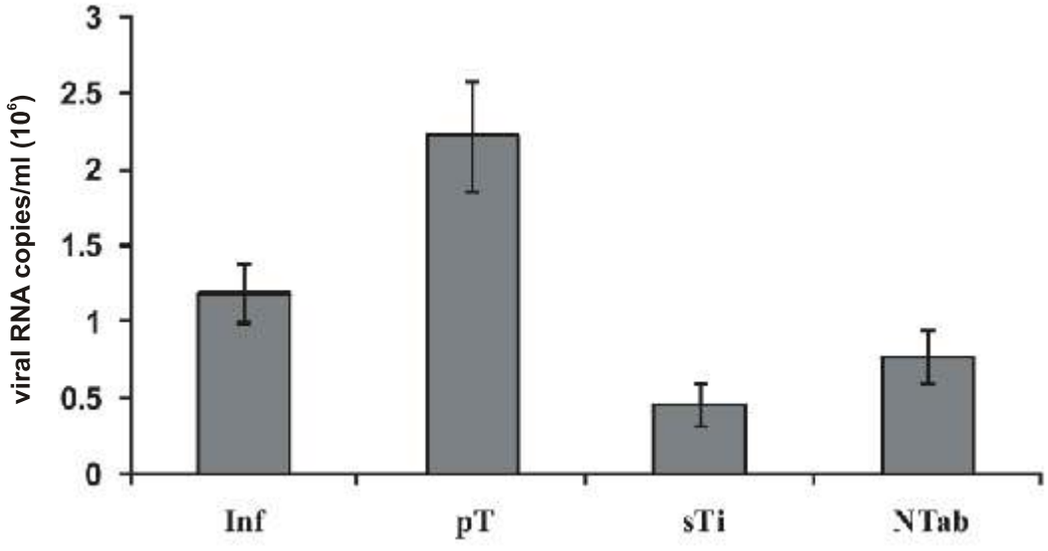


Fig.6 : Virus titer in supernatant of RAW cells infected with virus by real time RT-PCR.

Inf: infected cells, **pT**: TNFα primed cells, **sTi**: small molecule TNFα inhibitor treated cells

NTab: TNFα neutralizing antibody treated cells.

Most important finding

How the Chandipura Virus overcomes the protective innate mechanism of cell was ruled out in this study. It was observed that Chandipura virus stimulate the TLR4 receptor in RAW cells. Stimulation of TLR4 leads to the secretion of proinflammatory cytokines especially the higher level of TNFa. Sensitization of these cytokines by cells inherently induce the synthesis of suppressor of cytokine signaling protein to prevent the damage of host cells by excess secretion of proinflammatory cytokines. These proteins bind to the cytokine receptor to prevent the downward signaling. These signaling affect invariably most of the cytokine signaling including Type I interferons, which protect the cell from virus infection. So the cells lose the protective role and allow the virus to replicate inside the cells. This is the overall mechanism by which Chandipura virus infects the cells and replicate inside the cells.

Cloning and expression of G-gene of Chandipura virus and evaluation of immunogenicity in mice

Venakateswarlu.Ch, VA Arankalle

Introduction

Encephalitis caused by Chandipura virus (CHPV) has emerged as an important pediatric health problem in India as evidenced by the epidemics of the disease with high mortality in 2003 in the state of Andhra Pradesh (183/329, 55%), in 2004 in the state of Gujrat (20/26, 78.4%) and in 2007 in Maharashtra and Andhra Pradesh states (our unpublished observations). These fatalities occur within 48 hours of appearance of clinical symptoms, majority being within 24 hours. No specific treatment or vaccine is available. Since the problem is geographically focussed at this time, an effective vaccine is desirable for this dreadful disease.

Objectives

- To develop recombinant G-protein-based candidate vaccine for Chandipura infection.

Work done

Expression of G-protein (rGp) and its evaluation as an antigen in the development of IgM/IgG-anti-CHP ELISA was reported last year. This year evaluation of rGp candidate vaccine was pursued.

Purification of rGp

Fig. 7 depicts elution profile of the serum-free concentrate of the rGp positive SF9 cell culture supernatant loaded on gel filtration HPLC column. All the fractions were subjected to ELISA for rGp detection. Among these, only fractions corresponding to a single peak (Peak: 13.61) were scored as reactive. Similar to culture supernatant, this rGp peak was confirmed by SDS-PAGE and Immunoblotting to be a single protein of approximately 60-kDa (expected size of rGp). This protein was used for mice immunization.

Humoral Immunity

Both pre and post-immunization mice sera were subjected to ELISA and NT for the detection of anti-CHPV antibodies. Both assays detected antibodies as early as two weeks after 1st dose **(Table-1)**. Pre-immunization sera were scored negative by both the tests. As compared to NT, ELISA detected seroconversion earlier when the dose

of the immunogen was low (100 ng). Antibody response was immunogen concentration dependent, increasing gradually with dose from 100ng-1µg. Both 1 and 2 µg gave comparable response. Percentage sero-conversion increased after each dose and the maximum (90%) sero-conversion was observed at 10th week of post immunization i.e. 2nd week of the last dose, either 1 or 2 µg.

Fig. 8 compares anti-CHP-antibody titers by ELISA and NT at 2nd week after the last dose. Similar to seroconversion rates, the antibody titers were function of the concentration of the immunogen used, 1 and 2-µg rGp producing comparable titers. The maximum titers in NT and ELISA were 1:320 and 1:1200 respectively. Neutralizing antibody titers were consistently lower than the ELISA titers. As evident from figure 4, irrespective of the dose of rGp used for immunization, the anti-CHP-antibody titers remained almost constant during the observation period of six months.

To assess the efficacy of the antibodies generated by immunizing with the 2003 isolate-derived rGp against the viruses isolated in 1965, 2004 and 2007 neutralization tests were performed employing different virus isolates **(Table 2)**. Serum samples from five immunized mice were screened in NT with the homologus as well as heterologus CHPV isolates. An excellent cross protection was recorded; NT titers employing different isolates did not differ significantly (p=0.423 to 0.510).

Cell Mediated Immunity

To assess the T cell-response, spleens from 10 mice from each group harvested at 2-3 week after the last immunization were used. The optimum concentration of rGp for the stimulation of spleen cell was found to be 20µg/ml. Table 3 records stimulation indices for the mice belonging to different groups. Similar to humoral immune response, lymphocyte proliferative response increased in terms of stimulation index, with the dose of the immunogen. Sixty percent of mice immunized with 1 or 2 ug rGp responded with high SI values.

Intra-cerebral virus challenge experiments:

An intracerebral challenge of 100LD_{50} of the homologus strain yielded satisfactory results. Survival was directly proportional to the immunogen dose, 20 and 40% mice surviving with 100 and 500ng doses respectively. Both 1 and 2 ug doses gave 90% protection. Mortality in control, un immunized group was 100%. As evident from the table 4, an ELISA titer of 1:40 and NT titer of 1:20 was predicting protection against the intracerebral challenge. Overall, 1ug purified recombinant G protein expressed employing Baculovirus Expression System was shown to be a promising candidate vaccine.

Table-1 Percent seroconversion in mice immunized with three doses of different concentrations of rGp at 0th, 4th, 8th and 10th weeks as documented by ELISA and NT.

	% Sero conversion at 0 th week I 4th week I 8th week I 10th week							
	ELISA	NT	ELISA	NT	ELISA	NT	ELISA	NT
100 ng	0	0	20	10	50	40	40	40
500 ng	0	0	30	20	50	50	60	50
1 ug	0	0	40	40	70	70	90	90
2 ug	0	0	40	40	80	80	90	90

Table 2: NT titers of serum samples from immunized mice employing homologous and heterologus CHPV isolates.

Mice No.	NT Titer of CHPV isolate 034627	NT Titer of CHPV isolate 653514	NT Titer of CHPV isolate 2004	NT Titer of CHPV isolate 076324
1	80	40	40	20
2	320	160	160	320
3	20	40	20	40
4	160	80	80	160
5	320	160	160	80

Table 3: T cell proliferation in response to rGp as measured by stimulation Indices in different mice groups

Mice group (n=10)	S.I. value range (Mean ± Std error)	No of responder mice (Percentage)
100ng	0.95 to 8.57 (2.17 ± 0.68)	2 (20)
500ng	1.09 to 11.45 (3.50 ± 1.02)	3 (30)
1ug	1.15 to 15.82 (7.73 ± 1.69)	6 (60)
2ug	1.21 to 16.26 (8.35 ± 1.78)	6 (60)
$AlPO_4$	1.26 to .2.56 (2.21 ± 0.07)	0

Table 4: Comparison of survival following intracerebral challenge of CHPV with antibody titers as estimated by ELISA and NT

rGp Conc.	No. of mice showing ELISA titers of 1:40, (n=10)	No. of mice showing NT titers of 1:20, (n=10)	Survival status (100 LD_{50}), (n=10)
100ng	3	2	2
500ng	5	4	4
1µg	9	9	9
2µg	9	9	9
$AlPO_4$ alone	0	0	0

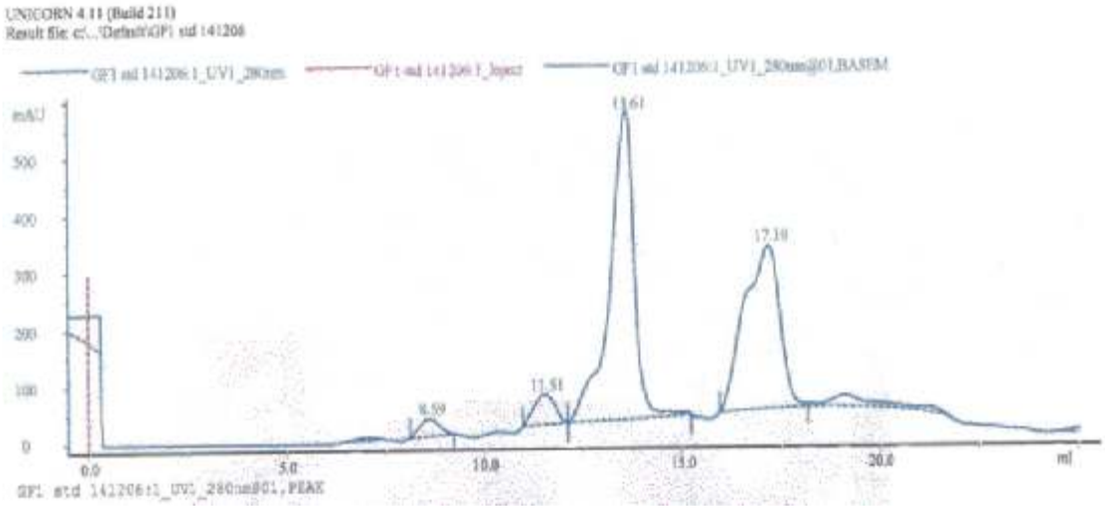


Fig. 7: Elution profile of the serum-free concentrate of rGp positive SF9 culture supernatant loaded on gel filtration HPLC column. Peak: 13.61 corresponded to rGp.

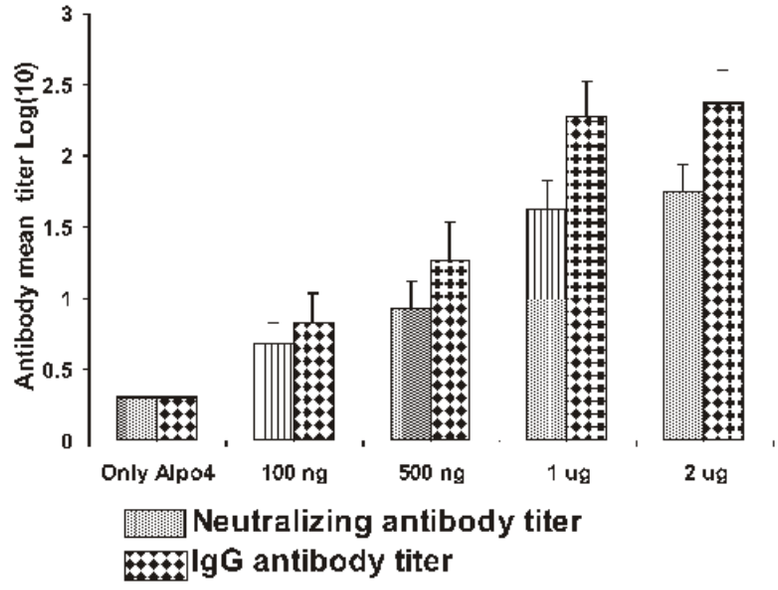


Fig. 8 : The Geometric mean of reciprocal anti-CHPV antibody titers (Log10) at 10 weeks i.e., 2 weeks after the third dose of rGp. Bar represents standard error.

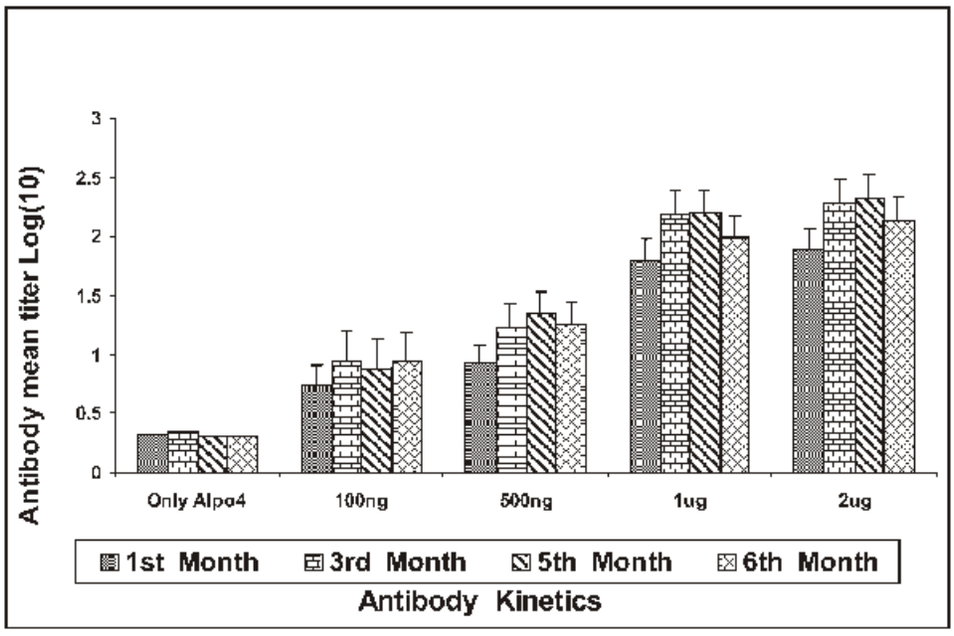


Fig. 9 : The Geometric mean of reciprocal IgG-anti-CHPV antibody titers (Log10) as determined by ELISA. Antibody titers were determined 1, 3, 5 and 6 months after the third dose of rGp. Bar represents standard error.

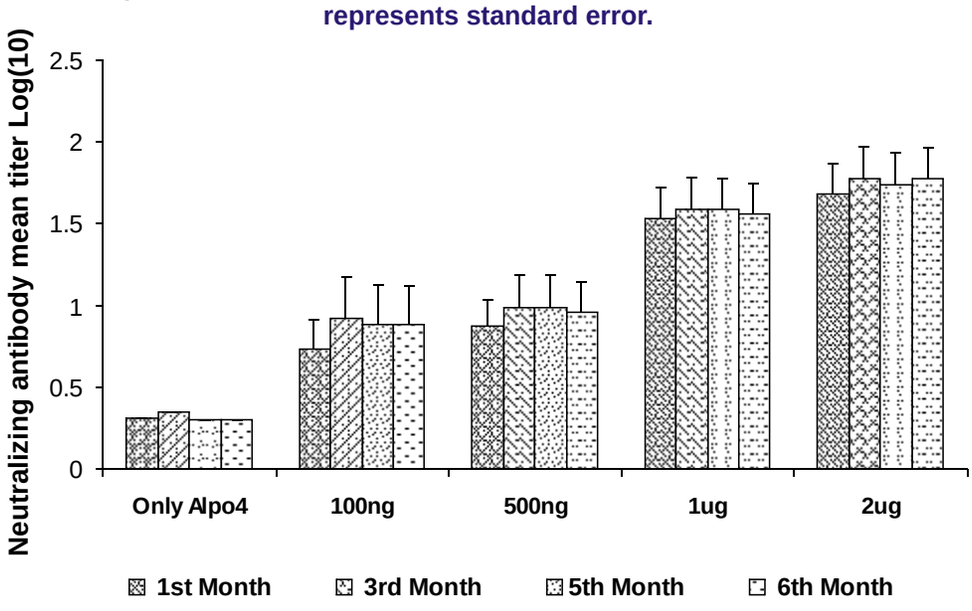


Fig. 10 : The Geometric mean of reciprocal anti-CHPV neutralizing antibody titers (Log10) of as determined by in-vitro Neutralization test. Antibody titers were determined 1, 3, 5 and 6 months after the third rGp immunization. Bar represents standard error.

Evaluation of siRNA in curing Chandipura virus infection *in-vitro* and *in-vivo*

Satyendra Kumar, VA Arankalle

Introduction

Chandipura virus (CHPV) has emerged as an important encephalitis-causing pathogen in India. Outbreaks of the disease have been reported among children from the states of Andhra Pradesh, Gujarat and Maharashtra with mortality varying from 55-75%. There is no vaccine as well as therapy against CHPV infection. It was considered worthwhile evaluating the siRNA- based strategies to control the virus replication.

Objectives

- To assess the role of siRNA in controlling / inhibiting CHP virus infection.

Work done

P gene of Chandipura Virus isolate 034627 was amplified by RT-PCR by using PFloF1 and GFPPR1 primers and then both the vector and the insert were digested with the KpnI and SacI and ligated. After transformation colonies were screened and positive colonies were further sequenced to confirm the presence of P gene. To confirm the fusion protein western blot analysis was also done **(Fig. 11).**

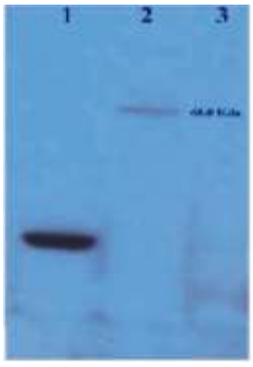


Fig. 11 : Western blot of P gene using GFP antibodies

P gene siRNA validation studies in 293 cells

Cells were transfected with both P gene siRNA and pAcGFP1N1-CHPV-P gene, which codes P protein with GFP as a fusion protein. Twenty-four hours after transfection cells were harvested and FACS and real time PCR was done. This data shows that out of four siRNAs (P1 to P4), P-2 is the best.

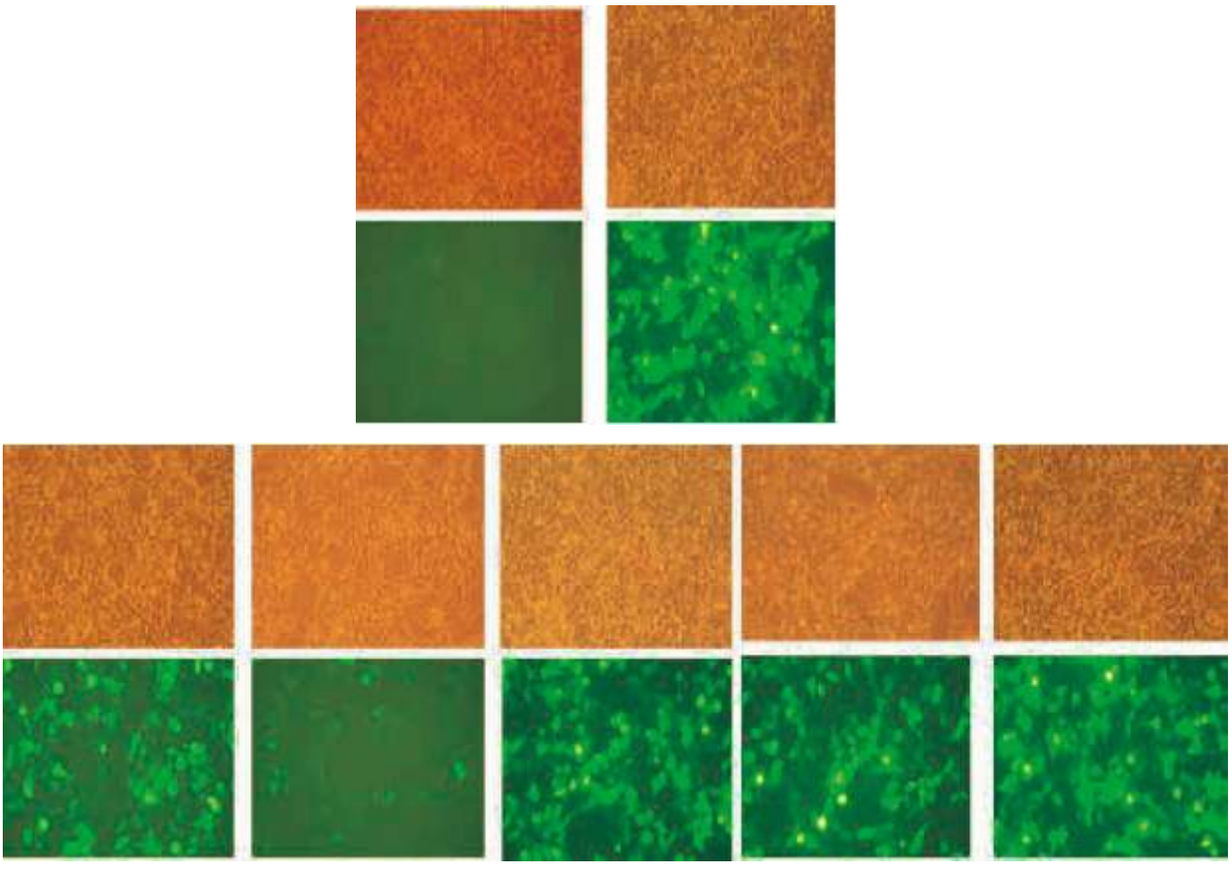
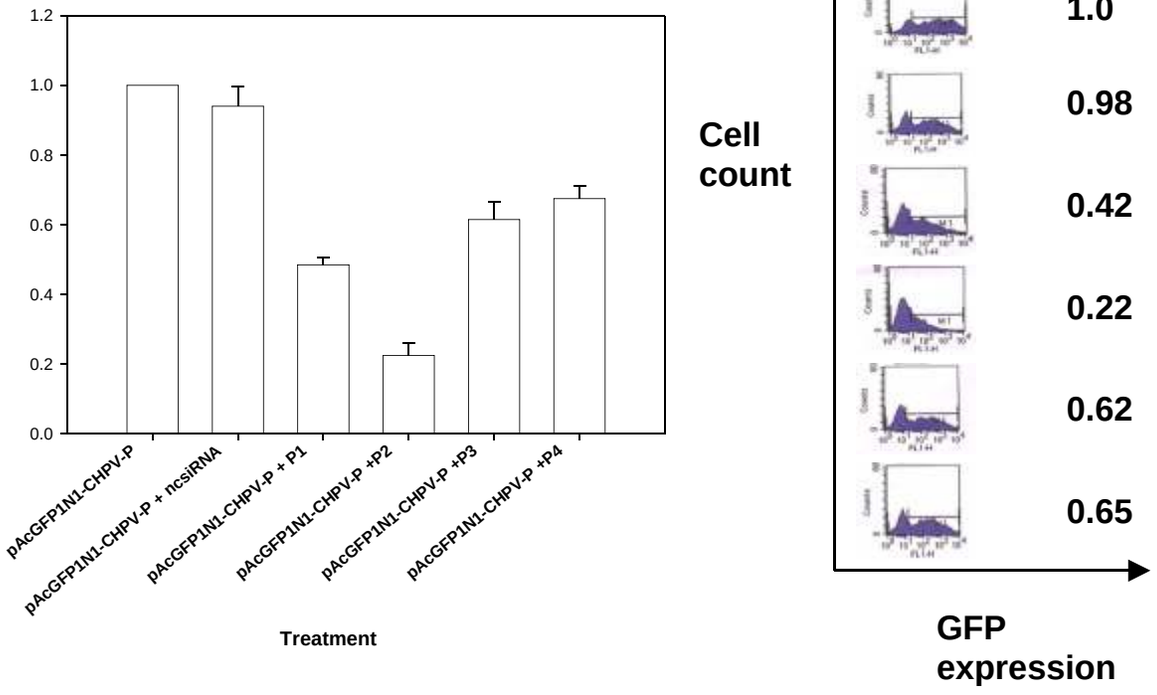
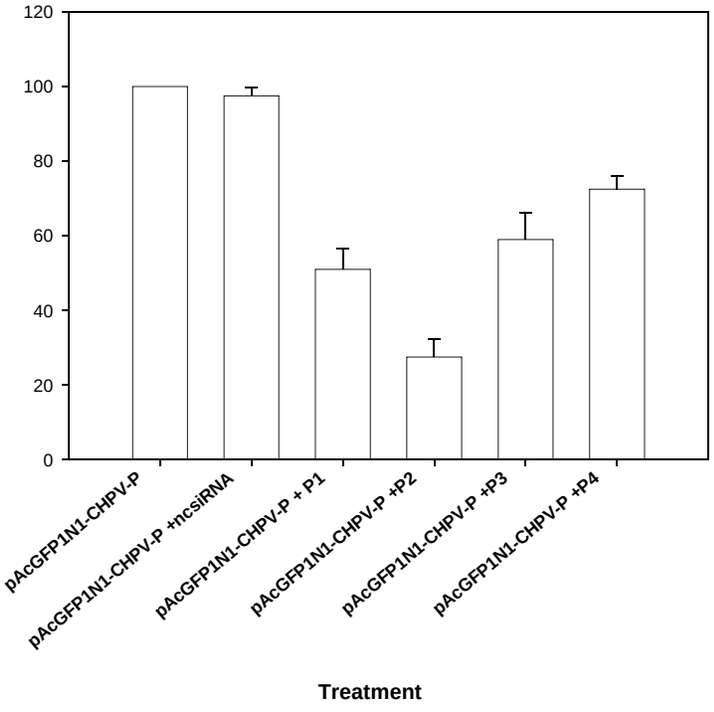


Fig. 12: Fluorescent microscopy of cells transfected with pAcGFP1N1-CHPV-P plasmid and different siRNA (P1-P4)



(a) (b)



(c)

Fig. 13 (a) (b) and (c) P gene siRNA Validation by flow cytometry and real time one step RT-PCR.

Optimization of concentration of P-2

Different concentrations of P-2 siRNA were used in combination with pAcGFP1N 1-CHPV-P gene FACS and Real time analyses were done.

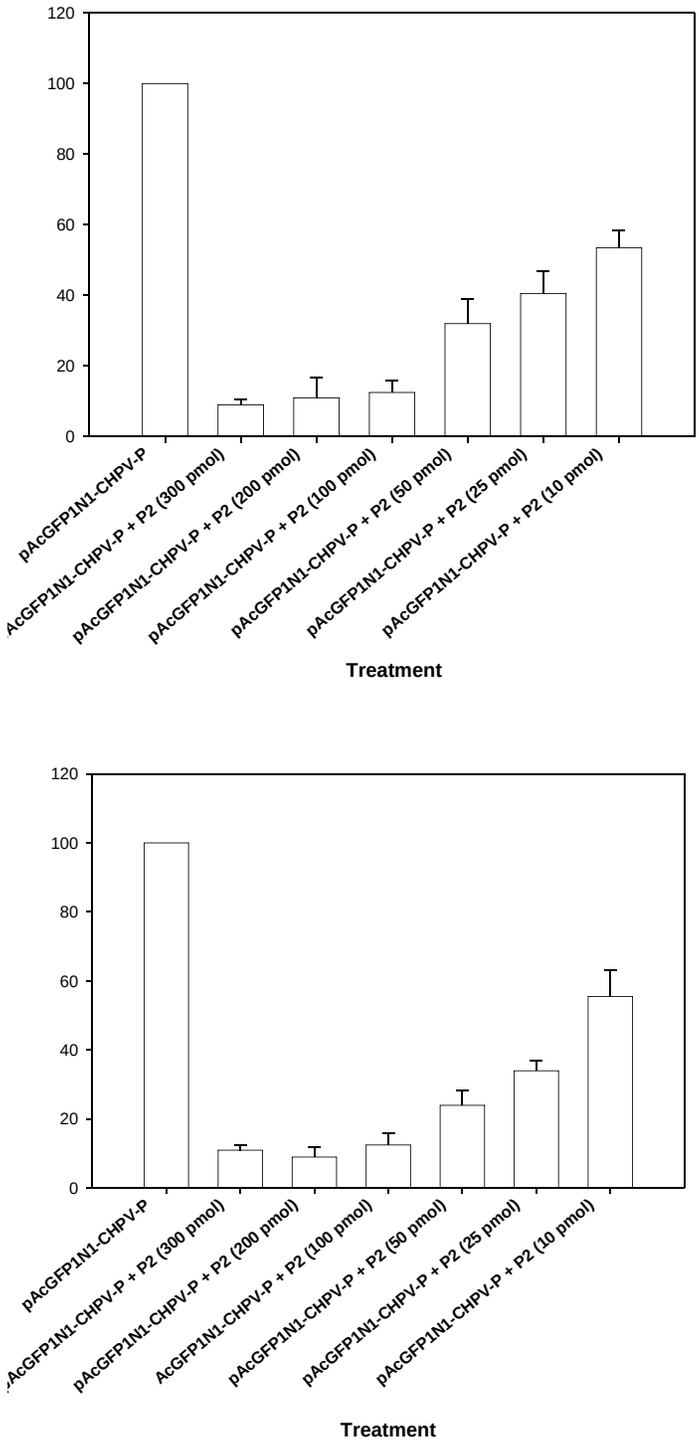
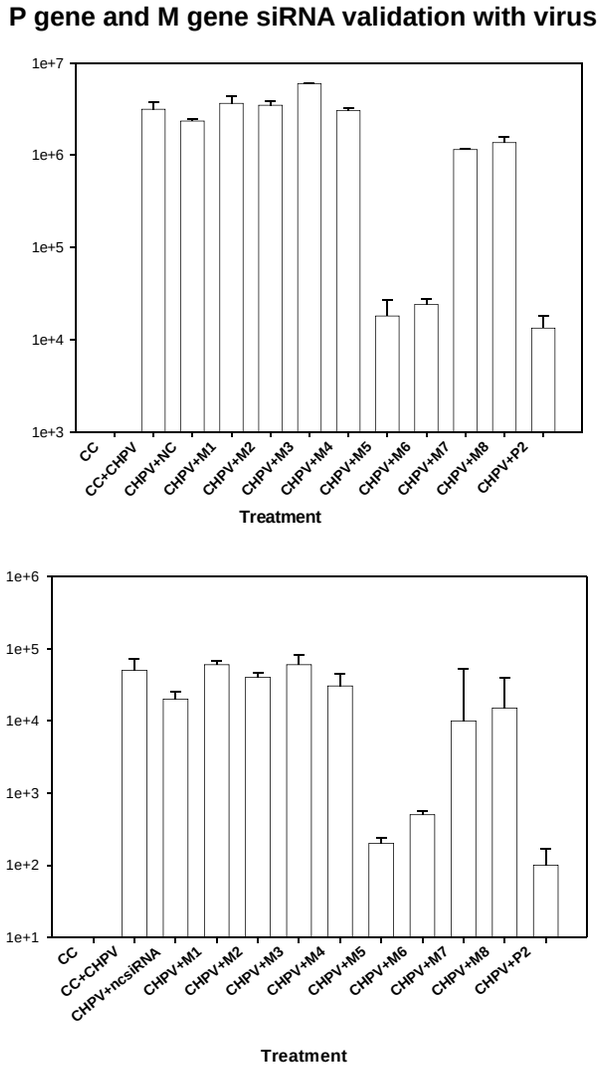
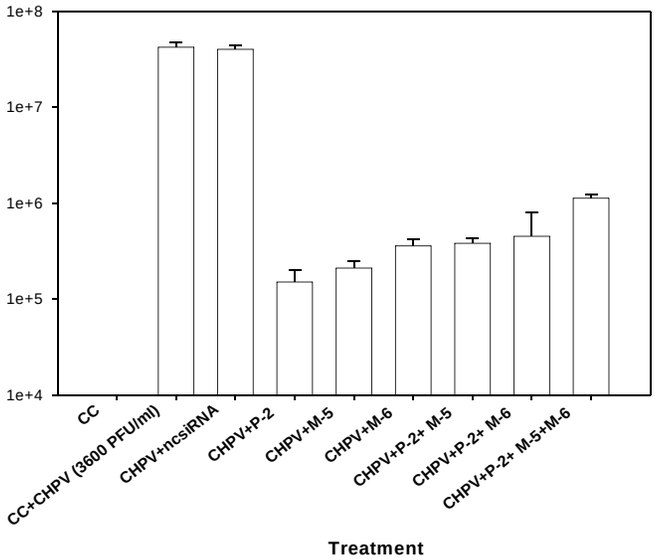


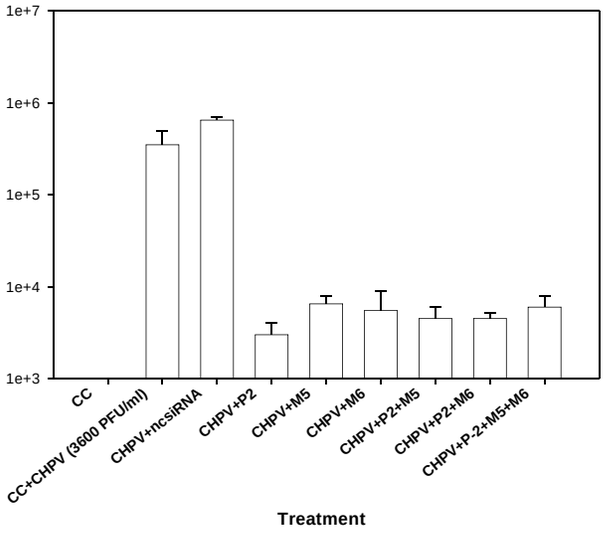
Fig. 14 : Optimization of concentration of P-2 by flow cytometry and real time one step RT-PCR



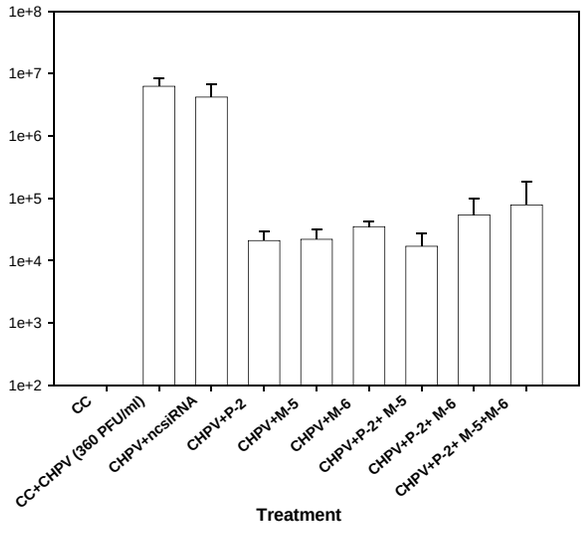
P gene siRNA, P2 and all the M gene siRNA were used for the transfection and 24 hour after transfection cells were infected with 360 pfu of CHPV. 24-hour PI cells were harvested and real time was done.



(a)

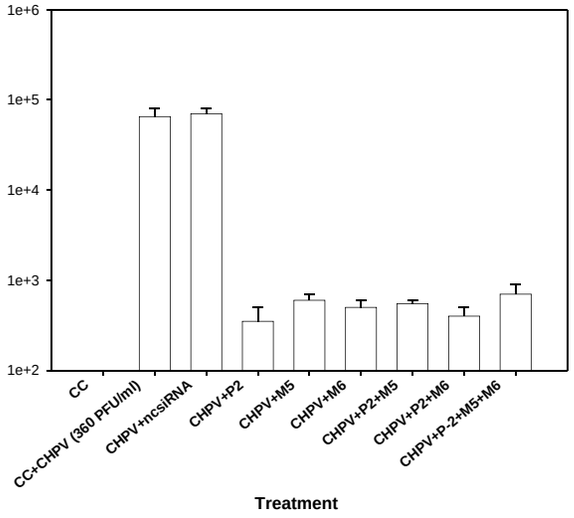


(b)

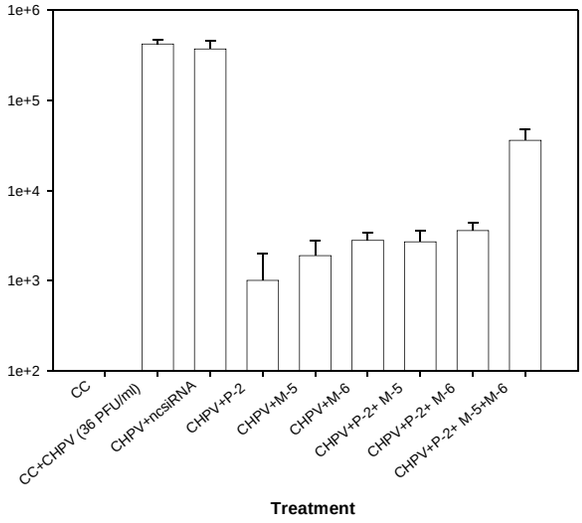


(c)

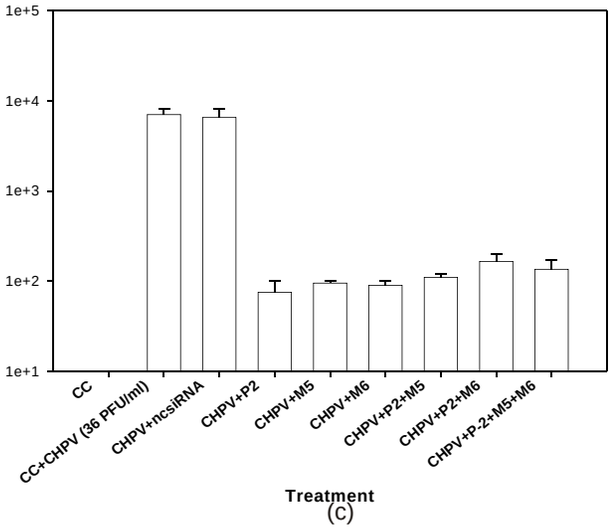
Fig. 15 : Different M gene and P-2 siRNA validation using CHPV



(a)



(b)



(c)

Fig. 16 : Effect of different virus concentration and different combination of siRNA on the replication of CHPV (a), (b) and (c)

Conclusion

- [illegible]
- [illegible]

HLA Phenotypes and outcomes of Chandipura virus infection in Andhra Pradesh

[illegible]

Introduction

[illegible]

Objective

- [illegible]

Work done

[illegible]

Future Plan

[illegible]

In-silico sequence and structure-based antigenic analysis of the G, N and M proteins of Chandipura virus

[illegible]

The Chandipura virus (CHPV) is emerging as a fatal infectious disease with very high mortality rate. Hence it would be apt to use in-silico sequence and structure based approaches to determine epitope driven vaccine candidates. Among (CHPV) structural proteins, glycoprotein G, the spike protein protrudes externally from the outer membrane of the virus particle and elicits antibody response thus acting as a major antigenic determinant (Neumann et al. 2002). Matrix protein M lies in the inner surface of the virion to tether the nucleocapsid to the membrane. L and P are packaged within the mature virion and remain associated with the core nucleocapsid particle.

Whole genome sequence data is available for CHPV isolates from febrile as well as encephalitis cases. As far as 3D structures are concerned, the homologous crystal structures available are that of the pre and post fusion forms of the Vesicular Stomatitis Virus glycoprotein G, matrix protein of the Vesicular Stomatitis Virus (VSV) and a recent crystal structure of the Rabies virus N protein complexed with RNA. The G and N proteins are known to have immunogenic characteristics. Hence the sequential B - cell epitopes as well as the T - cell epitopes on these proteins can be predicted. Peptides containing these antigenic regions may help in the design of subunit vaccines.

Objectives

- Modeling of the G, N and M proteins
- Prediction of T-cell and B-cell (linear and conformational) epitopes

Results

The complete genome sequences of CHPV of five isolates have been determined from febrile and encephalitis outbreak cases during the period from 1965 to 2007. The genome length of approximately 11,100 nt shows closest sequence homology with the genomes of VSV and Isfahan virus (ISFV). Pairwise comparison of the deduced amino acid sequences of the 5 isolates of CHPV showed that the M protein is the most conserved (99.9% aa identity).

Sequence alignment of the G protein sequences of viruses of the rhabdoviridae family including the sequences of the CHPV isolates available has been carried out ensuring the alignment of conserved cysteine residues. Mapping of the known antigenic sites of VSV and Rabies virus indicates that the specific mutations of CHPV isolates fall in the antigenic site G3. Mapping of these site have been done on a tertiary structure model of the CHPV G protein. Homology modeling approaches with Modeler in Discovery studio, using the VSV 'G' protein as a template would be done . Scan Prosite was used to locate functional motifs in the CHPV sequences of the G, M, N, P and L proteins for characterization of the genome.

Pathogenesis of Chandipura virus in laboratory rodents (mice & rats)

CG Raut

Chandipura virus causes acute encephalitis to the humans especially children. Because of the acute nature of the disease not much information is available on pathogenesis. Hence attempts were made to develop suitable animal model for the disease by mimicking the natural route of infection.

Objectives

- To find out the infectivity of CHPV in mice of 16 days old and distribution of virus in the body by subcutaneous route of infection.
- Behavioral and histological response of CHPV infected rats after subcutaneous route of inoculation.

Work done

Subcutaneous inoculation of CHPV was done in mice of age 16 days to mimic the natural infection. Every post-infection day animals were sacrificed humanely. There were no gross changes in all the organs. Initially sickness was transient. After 5th PID hindlimb weakness was noted and it was continued to 7-8 PID and further leads to the recovery. Histological changes were observed in gradation post infection day wise only in brain and spinal cord.

Brain Sections: Histopathological observations

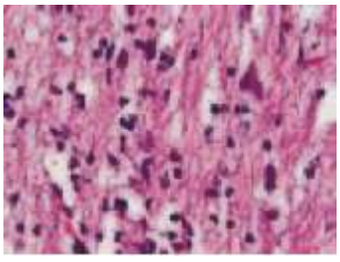


Fig.17 : 20X

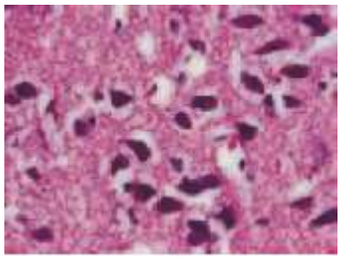


Fig. 18 : 60X

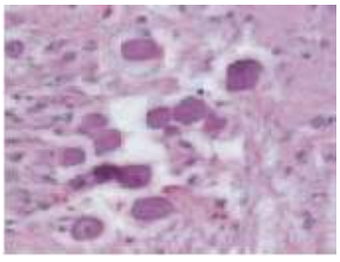


Fig. 19 : 40X

Fig.17 : Gliosis, degeneration of neurons
Fig. 18 : Shrinkage of neurons, vacuolation
Fig. 19 : Degeneration of neurons+++, Vacuolation, spongiosis.

Localization of antigen by immunohistochemistry in brain sections

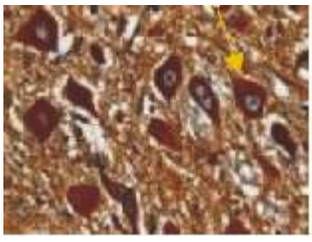


Fig. 20 : 40X

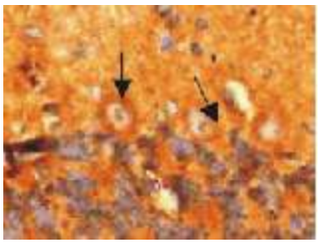


Fig. 21 : 60X

Fig. 22 : 20X

Fig.20: Antigen detection (dark brown color) in cytoplasm of neurons, chromatolysis of neurons.
Fig.21: Localization of antigen in Purkinje cells of neurons (dark brown).
Fig.22: Localization of antigen in choroid plexus.

Subcutaneous inoculation of CHPV was done in rats of ten days old to mimic the natural infection. Every post-infection day animals were sacrificed humanely. There were no gross changes in all the organs. Frank sickness was observed with ataxia, hyperasthesia, convulsions, quadriplegia and death. Marked histological changes were observed only in brain and spinal cord in gradation of post- infection period. Conclusively, rats of two weeks age could be suitable animals for studying the pathogenesis, host-virus interaction, drug development, etc for Chandipura virus.

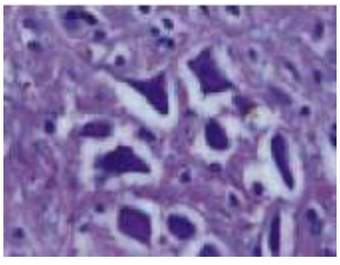


Fig. 23 : 40X

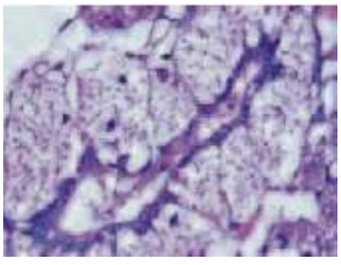


Fig. 24 : 60X

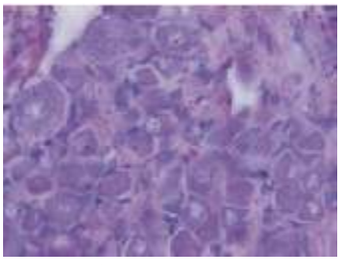


Fig. 25 : 20X

Fig. 23 Spinal cord : shrinkage of neurons leading to vacuolation
Fig. 24 Spinal cord : degeneration of nerve roots around the central canal
Fig. 25 Spinal cord : degenerative changes in the nerve roots.

Future plan

- To study in detail the progressive pathological changes in different parts of brain of the host (mice/rats/hamsters).
- Neuropharmacological studies of infected hosts to understand the changes in different neurohormones.
- Invivo imaging of infected animals to understand the progression of disease without sacrificing the animals.

Workshops / Conferences / Seminar / Meetings attended

- Anukumar, B and Mishra, AC, oral presentation entitled “Chandipura virus infected raw 264.7 cells regulates CD4+ and CD8+ levels by secretion of pro-inflammatory cytokines and expression of Fas ligand” in the, 34th Indian Immunological Society Conference, NARI, Pune December 16-18, 2007.
- Venkateshvaralu CH and VA Arankalle, oral presentation entitled 'Recombinant glycoprotein based vaccine for Chandipura virus' at '34th Indian Immunology Society Conference', at National AIDS Research Institute, Pune, during December 16-18, 2007.