

# **INTRAVIRAL PROTEIN INTERACTIONS OF CHANDIPURA VIRUS**

## **SYNOPSIS**

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## **DOCTOR OF PHILOSOPHY**

by

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## **BACKGROUND AND RATIONALE OF CURRENT STUDY**

Viral encephalitis is the inflammation of the brain as a result of viral infection. Viral encephalitis has gained worldwide importance in recent times due to the continued emergence of neurotropic viruses such as West Nile, Monkey pox, Japanese Encephalitis and Chandipura viruses in both the developed and developing countries [1, 2]. The most common viral encephalitis in India is caused by Japanese encephalitis virus (JEV), Rabies virus (RV) and Chandipura virus (CHPV). Unlike global reach of JEV and RV, CHPV encephalitis is endemic to India [3, 4, 5]. Although, CHPV was isolated in 1965 during an outbreak of Dengue and Chikungunya viruses [6, 7], it gained much attention only when it was directly associated with human infections for the first time in 2003 in Andhra Pradesh [3]. Since then, repeated outbreaks are being reported almost every year with high mortality rate from different parts of central India, the most recent one being in July, 2012. The virus has marked features of high fatality rate, rapid pathogenesis leading to deaths within 48-72 hours and characteristic ability to affect only children between the age of 9 months to 15 years [3, 4].

CHPV is an arbovirus belonging to the genus *Vesiculovirus* and family *Rhabdoviridae* [6]. It is closely related to Vesicular Stomatitis Virus (VSV), the prototype virus of the genus, in terms of nucleic acid and protein composition [8] and to Rabies virus (RV, yet another rhabdovirus), with respect to its pathogenesis. CHPV and RV are the only two viruses in the entire family which inflict human encephalitis. The genetic material of Chandipura virus is a single stranded negative sense RNA of approximately 11 kb. Transcription of genomic RNA results in five capped and polyadenylated mRNAs which encode five proteins - Nucleocapsid (N), Phosphoprotein (P), Matrix (M), Glycoprotein (G) and Large polymerase subunit (L) in a sequential manner [9]. The role of these proteins in CHPV pathogenesis has been deduced and speculated through existing research data on VSV.

Starting from infection, replication to viral budding, viral proteins play major role through interactions with factors of viral and host origins. Thus the important initial step in understanding CHPV pathogenesis could be the optimization of protocols for obtaining purified proteins and understanding the interactions among them. These interactions can also be used for antiviral therapeutic intervention using small peptides or inhibitors in the absence of any vaccine or therapy. Relentless CHPV outbreaks and short window period necessitates

the need to generate molecular reagents and elucidate the molecular mechanisms of viral replication and mode of pathogenesis.

## **OBJECTIVES AND STRUCTURE OF THE THESIS**

The objective of the present research was to generate an unbiased data of protein-protein interactions among CHPV viral proteins. As a prerequisite for protein interaction studies, all proteins were cloned, expressed and solubilised for the first time using prokaryotic expression system. Later, the main objective was achieved by using yeast two hybrid system (Y2H) for the screening of interactions among viral proteins. This was followed by further studies of interactions using GST pull down and ELISA. Lastly, the key viral interaction was subjected for domain analysis and the association was mapped to domain level.

The research work carried out for the achievement of the specified objectives is presented in the form of six chapters in the thesis:

Chapter-1: INTRODUCTION deals with the identification of the research target and the rationale for carrying out the present research.

Chapter-2: LITERATURE REVIEW covers the detailed background of the existing literature.

Chapter-3: CLONING, EXPRESSION AND CHARACTERISATION OF CHANDIPURA VIRAL PROTEINS describes the generation of reagents in the form of recombinant clones, standardisation of expression and solubilisation protocols for purification of proteins to be used for further work.

Chapter-4: IDENTIFICATION OF CHPV INTRAVIRAL INTERACTIONS BY YEAST TWO HYBRID SYSTEM involves the screening of protein interactions among CHPV N, P, M and G proteins using Gal4 based yeast two hybrid system.

Chapter-5: ANALYSIS OF CHPV PROTEIN-PROTEIN INTERACTIONS BY PULL DOWN AND ELISA ASSAYS explains the analysis of interactions among CHPV proteins by two more independent methods, i.e. GST pull down assay and ELISA.

Chapter-6: DOMAIN MAPPING OF CHPV NUCLEOCAPSID PROTEIN follows the observation of previous chapters and deals with the mapping of CHPV N protein domains involved in its interactions with other four viral proteins.

## **OVERVIEW OF THE RESEARCH AND THESIS CHAPTERS**

Interactome analysis of CHPV proteins is crucial for understanding the mechanism of disease and its progression. Chapter 1 describes the rationale behind the present research, the lacunae in the existing knowledge about CHPV and the underlying need for the current study in the absence of research data. Introduction of the research target is followed by review of literature (Chapter 2) in which the detailed information of the viral epidemiology, infection, genome organisation, protein functions and life cycle is provided. Keeping in view the limited knowledge about the virus, some details have been addressed from related viruses. The importance of detecting protein-protein interactions and various techniques to identify them are also discussed in Chapter 2.

Chapter 3 describes the optimization of protocols for the generation of soluble and purified viral proteins which can find their usage in further experimental work. CHPV N, P, M and G gene clones were obtained as a kind gift from Prof. Dhrubajyoti Chattopadhyay (Kolkatta, India) as starting materials for the current study [10, 11]. These genes were PCR amplified and cloned in pGEX-4T3 (GST tag), pCAK (Strep tag) [12] and pLTA (His tag) [12] bacterial expression vectors as presented in Chapter 3. L gene was not considered in the present research due to its large size (~7 kb) and associated cloning and expression problems. A total of 12 clones were generated for four viral proteins with three different tags. The N, P, M and G proteins were expressed as GST, Strep and His fusions in BL21 strain of *E. coli*. The bacterial cultures were induced and the desired expression levels with maximum solubility were obtained for all four proteins by varying conditions of temperature, induction time and inducer concentration. N, P and M proteins were observed to be soluble i.e., present in the supernatant after cell lysis but G protein, irrespective of all variations in conditions and change of tags, was found insoluble. G protein was solubilised using sarkosyl according to the recently described protocol by Tao and co-workers [13]. The protocol was modified to prepare the protein for purification and the G protein as GST, His and Strep fusion was successfully solubilised. This was followed by purification of both M and G proteins using their soluble fractions for the first time by a bacterial expression system [14].

The major objective of this study i.e., the identification of interactions among viral proteins, was achieved by yeast two hybrid screening as described in Chapter 4. The viral genes (N, P, M and G) were cloned in 'bait' plasmid pGBKT7 and 'prey' plasmid pGADT7 of Y2H system as BD (binding domain) and AD (activation domain) fusions. These recombinant BD and AD vectors were transformed in Y187 and AH109 strains of yeast cells, respectively. Both Y187 and AH109 strains carrying respective CHPV-ORF-BD and CHPV-ORF-AD were mated and plated on SD/-Trp/-Leu media for a total of possible 16 interaction pairs accounting for 10 unique associations. The diploid yeast cells harbouring both the fusion plasmids were checked for the interaction on selective deficient triple dropout media (SD/-Trp/-Leu/-His). The growth of cells on this media indicated positive protein interactions. These putative protein pairs were also checked for another independent reporter gene *MEL1* by  $\alpha$ -galactosidase assay. Ten unique protein interactions possible among four genes were tested for their association and six positive interactions (NN, NP, NM, NG, MM and GG) were identified by Y2H system. NN and NP binding was already known for the virus [15, 16], the other four associations (NM, NG, MM and GG) are novel for CHPV. Interestingly, the NG interaction identified in this study has not been reported for the entire *Rhabdoviridae* family. PP interaction was also observed but due to the autoactivation of BD-P fusion protein, this association remained inconclusive by Y2H system.

The interaction of CHPV proteins was further subjected to analysis using separate methods such as GST pull down assay and ELISA (discussed in Chapter 5). All the ten possible pairs considered in Y2H were also checked by these two binding assays. The viral proteins expressed and solubilised as GST, Strep and His fusions in Chapter 3 were used for the pull down and ELISA. GST (GST-N, GST-P, GST-M and GST-G) and His (His-N, His-P, His-M and His-G) fusions were used for GST pull down. Both GST and His fusion lysates were mixed and allowed to bind to glutathione beads and the co-elution of two fusion proteins as complex was checked by western blotting using anti-His monoclonal antibodies which indicated positive interaction among two test proteins. Binding of only GST with His (His-N, His-P, His-M and His-G) fusion proteins was taken as experimental control. Seven interactions (NN, NP, NM, NG, PP, MG and GG) were confirmed to be positive by GST pull down assay.

For ELISA, lysates of His and Strep fusions of CHPV ORFs were prepared. Strep fusion proteins were allowed to bind to solid support Streptactin coated microtiter plate and the binding of His fusion proteins was checked. Results were revealed with the help of anti His monoclonal antibodies. One of the interactions, i.e. MG, which was negative in Y2H, appeared positive in both GST pull down and ELISA studies. Overall, the analysis by Y2H, GST pull down and ELISA detected a total of 8 interactions (NN, NP, NM, NG, PP, MM, MG and GG). Out of these 8 interactions, five novel interactions (NM, NG, MM, MG and GG) were observed for CHPV. Amongst the identified interactions, NG interaction was a novel finding for the family *Rhabdoviridae* [17].

Interestingly, it was observed in the interaction analysis that N protein binds to all four viral proteins. In an attempt to know that which regions of N protein are involved in its interactions with other proteins, domain mapping of nucleocapsid protein was carried out as detailed in Chapter 6. The structures of CHPV N and M proteins were generated by homology modeling using SWISS-MODEL workspace, G protein using I-TASSER and P protein using a modified *ab-initio* method as explained in Chapter 6. These predicted structures guided the division of N protein into three overlapping domains named as N1 (N-terminal, 30 aa), N2 (central domain, 278 aa) and N3 (C-terminal, 193 aa). ZDOCK and RDOCK based docking system was employed for the prediction of domains involved in its interactions with N, P, M and G proteins. For experimental validation, these domains were cloned as BD, AD and GST fusions and Y2H and ELISA based interaction screening was also carried out for the interactions of these domains with full length CHPV proteins. Some important observations were derived from this study; N1 domain, a 30 aa small region was shown to bind to all four proteins in a very specific manner, N2 domain bound to N and G proteins while N3 domain associated with N, P and M proteins. An appreciable percentage of the predicted dataset (75%) was confirmed experimentally and the data corroborated completely with the already known interactions in literature thus validating the research approach.

Overall the data in the present study provided valuable information regarding the interactions among CHPV viral proteins. This work was extended for determining the interacting domains of N protein. Taken together this study report purification of CHPV

proteins and provide critical interaction information which could be the basis for understanding CHPV pathogenesis and also for rationalization of future antiviral strategies.

### **SALIENT FEATURES OF THE PRESENT RESEARCH**

1. CHPV nucleocapsid (N), phosphoprotein (P), matrix (M) and glycoprotein (G) were cloned and expressed with different tags (GST, Strep and His). The protocols were optimized for successful solubilisation and purification. The CHPV matrix (M) and glycoprotein (G) have been successfully purified for the first time using bacterial expression system.
2. All possible interactions among four viral genes were screened by yeast two hybrid system. The interactions were analyzed by two different reporter systems (*HIS3* and *MEL1*) which utilized the ability of interactants to survive on histidine lacking media and blue coloration by  $\alpha$ -galactosidase assay.
3. The interacting as well as non-interacting pairs identified by Y2H were further checked by two independent binding assays (GST pull down and ELISA). A total of 8 interactions were identified (NN, NP, NM, NG, PP, MM, MG and GG). Five of these (NM, NG, MM, MG and GG) are being reported for the first time for CHPV.
4. The NG interaction identified has not been reported previously for any of the rhabdovirus.
5. Domain mapping of CHPV N protein identified a small N terminal region of 30 amino acids which interacts with N, P, M and G proteins and can be explored further as a potential antiviral target.

### **FUTURE PROSPECTS**

The interactions reported in this study could be considered as potential targets for peptide/small molecule inhibitors. In this regard, N terminal 30 aa domain of CHPV nucleocapsid protein which interacts with N, P, M and G proteins can be directly investigated for the ability to prevent viral replication in cells or be used as target for inhibitors.

The expression/purification protocols can be further optimized for purifying CHPV proteins on large scale for structural/functional studies.

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## LIST OF PUBLICATIONS DURING Ph. D.

1. **Kumar K.**, Rana J., Sreejith R., Gabrani R., Sharma S.K., Gupta A., Chaudhary V. K., Gupta S., “*Intraviral protein interactions of Chandipura Virus*”, Archives of Virology, vol. 157, pp. 1949-1957, 2012.
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## GENBANK SUBMISSIONS

Gupta, S., Dudha, N., **Kumar, K.**, Gabrani, R., Sharma, S.K., Gupta, A., Chaudhary, V.K. submitted the full length cloned sequences of the following Chikungunya virus (isolate IND-06-Guj, of 2006 outbreak) genes to Genbank on January 31, 2011. nsP1 gene (JF272473), nsP2 gene (JF272474), nsP3 gene (JF272475), nsP4 gene (JF272476), capsid gene (JF272477), E3 gene (JF272478), E2 gene (JF272479), E1 gene (JF272480) and 6K gene (JF272481).

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