MAPPING INTERACTIONS OF CHIKUNGUNYA VIRUS STRUCTURAL PROTEINS

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DOCTOR OF PHILOSOPHY

By

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BACKGROUND AND RATIONALE

Chikungunya disease is an acute febrile illness caused by the arbovirus Chikungunya virus (CHIKV). This virus is transmitted primarily by *Aedes* mosquito species in a sylvatic cycle. CHIKV was first isolated from Makonde plateau of Tanzania in 1952 [1, 2]. Earlier it was confined to the developing countries of Africa and Asia, but since its re-emergence in 2005, it has spread to more than 40 countries across the globe including the developed countries of Europe and Americas. As a result, in 2008, the National Institute of Allergy and Infectious Diseases (NIAID) listed CHIKV as a category C pathogen [3, 4]. Infection associated with CHIKV is manifested with high fever, rash, photophobia and incapacitating arthralgia which may last for several months [5-7]. The recent outbreaks of this virus demonstrate its ability to spread and infect large proportions of the population. Despite the gravity of infection potency, no vaccine or antiviral treatment is available for chikungunya disease [8].

CHIKV (Family: *Togaviridae*, genus: *Alphavirus*) is an enveloped virus with a positive sense, single stranded RNA genome of ~11.8 kb. The viral genome consist of two open reading frames (ORF)- the first ORF encodes the nonstructural polyproteins (nsP1-nsP4) which represents two-thirds of the viral genome in the 5′ terminal region and is responsible for viral replication. The second is transcribed by the subgenomic RNA and codes for five structural proteins namely, capsid (C), E2 and E1 and small proteins (6K and E3). Envelope proteins E1 and E2 are arranged as triplets of heterodimeric spikes on the surface of the viral membrane which encapsulates the capsid containing the genomic RNA. The viral spike proteins are critical for successful infection as they facilitate attachment to cell surfaces and virus entry.

In the absence of herd immunity and acquisition of mutations in the viral genome in recent past it has become imperative to characterize CHIKV strains currently circulating across globe to identify molecular features that have led to increased infectivity. Further, to understand the molecular pathogenesis of virus, it is important to generate a systemic interaction screen of both viral-viral and viral-host proteins. Such studies will enable the identification of potential interactions that assist virus to hijack host machinery and facilitate successful infection. These potential interactions may be targeted to develop therapeutic interventions against this disease.

OBJECTIVES OF THE THESIS

The current study aims to generate and characterize reagents for CHIKV research. The reagents thus available could be used for interaction analysis of CHIKV proteins. The thesis objectives are defined as follows:

1. Cloning and characterization of CHIKV genes from Indian isolate of 2006 outbreak.
2. Expression and characterization of CHIKV Structural Proteins
3. Interaction analysis of structural proteins of CHIKV.
4. Identification of cellular partners of envelope protein E1 and E2 of CHIKV.

THESIS CHAPTERS

The research work required to achieve the stated objectives has been divided into the following chapters:

CHAPTER 1: **Introduction** explains the background of the topic, a brief review of current knowledge, rationale behind the study and outlines the objectives of the study.

CHAPTER 2: **Review of literature** focuses on the work that has been carried out for CHIKV till date.

CHAPTER 3: **Material and methods** details the various reagents and experimental protocols used to achieve the objectives.

CHAPTER 4: **Cloning and characterization of genes from IND-06-Guj, a 2006 CHIKV isolate** focuses on cloning and characterization of CHIKV genes from an Indian isolate(IND-06-Guj) followed by phylogenetic analysis of the characterized sequence.

CHAPTER 5: **Expression, solubilisation and characterization of CHIKV structural proteins** describes the optimization of expression and solublisation of structural proteins in bacterial expression vectors, followed by their purification.

CHAPTER 6: **Mapping of interaction among structural proteins of CHIKV** involves the generation of intraviral interaction network for structural proteins of CHIKV using yeast two-hybrid and GST pulldown assay.

CHAPTER 7: **Identification of cellular interactors of Envelope proteins (E1 and E2) of CHIKV** deals with identification of cellular interactors of E1 and E2 proteins using yeast two-

hybrid screening of human fetal brain cDNA library followed by validation of selected host proteins by pull down and protein interaction ELISA.

CHAPTER 8: **Conclusion and future direction** summarizes the findings of the study and discusses its future prospects.

OVERVIEW OF THE RESEARCH

Genomic RNA from the re-emergent strain of CHIKV (GenBank ID: JF274082), isolated from 2006 Indian outbreak (IND-06-Guj) was used to characterize all the genes. Gene specific primers were used to synthesize the cDNA of each CHIKV gene [nonstructural: nsP1- nsP4; structural: capsid (C), E3, E2, 6K and E1] and cloned in TOPO sequencing vector. The CHIKV-TOPO recombinants were sequenced using M13 forward and reverse primers. Nucleotide sequences obtained for each gene were assembled in contig to generate nonstructural [nsP1-4: GenBank ID: JF272473-JF272476] and structural [C-E3-E2-6K-E1; JF272477-JF272481] ORFs, respectively. The deduced polyprotein sequence of the assembled nonstructural and structural ORFs of IND-06-Guj strain were compared with the respective polyprotein sequences of the CHIKV-S27 prototype strain and 41 other isolates from South Asia by multiple sequence alignment using the NCBI BLAST suite of tools (http://www.ncbi.nlm.nih.gov/BLAST). Out of the 41 isolates, 38 strains were reported from South Asia between 2005 and 2008. Phylogenetic relationship of IND-06-Guj with South Asian isolates was assessed by constructing neighbor-joining tree based on distance estimations by the Kimura 2-parameter model of nucleotide substitution. The phylogenetic tree and multiple sequence analysis revealed that IND-06-Guj belongs to the East, Central and South African (ECSA) genotype which is responsible for resurgent CHIKV outbreak. Moreover, South Asian isolates displayed higher sequence similarity with IND-06-Guj than S27.Considering the observed sequence differences between South Asian and CHIKV-S27 strain, the study suggests that IND-06-Guj strain of CHIKV could be considered as prototype strain instead of S27 in South Asian context [10].

Structural proteins of CHIKV play a significant role in viral life cycle, like recognition of host receptor, fusion of viral and host cell membrane and viral maturation. Therefore, studying the association of these proteins with each other is significant for understanding the virus

biology. CHIKV structural proteins were cloned, expressed and solubilised using prokaryotic expression system, to serve as reagents for protein interaction studies. Structural genes of CHIKV were PCR amplified and cloned in pGEX-4T3 (GST tag), pCAK (Strep tag) [11] and pLTA (His tag) [11] bacterial expression vectors. The recombinant clones were transformed in *E. coli* BL21 cells and expressed as GST, Strep and His fusion tags. Expression and solubilisation profile for each protein was optimized by varying conditions of temperature, induction time and inducer concentration. Small proteins (E3 and 6K) could only be expressed as GST fusions and were found soluble on cell lysis. C, E2 and E1 were expressed with all three tags but were found to be insoluble. Treatment of induced cell pellet with detergent cocktail (Tween-20, CHAPS, Triton X-100; 0.2% each) along with 5 mM β-mercaptoethanol resulted in solubilisation of C, however, E1 and E2 still remained insoluble and were eventually solubilised by 10% sarkosyl. Hydrophobic transmembrane regions are known to render proteins insoluble; therefore ectodomains of envelope proteins (TrE1 and TrE2) were cloned and expressed as N-terminal Strep fusion. Both proteins appeared in supernatant on cell lysis, indicating the deletion of transmembrane region of E1 and E2 proteins made them soluble. This was followed by purification of CHIKV structural proteins by one-step affinity chromatography. Strep tag has been reported to generate functionally active proteins, is biochemically inert and does not interfere with protein folding, therefore C, E1, E2, TrE1 and TrE2 were purified using Strep-Tactin resin. Since, 6K and E3 could only be expressed as GST fusion; they were purified using glutathione resin.

Purification of structural proteins was followed by intraviral interaction analysis of CHIKV structural proteins using yeast two-hybrid (Y2H) and GST pull down assays. For this, five structural proteins (C, E3, E2, 6K and E1) were cloned in yeast expression plasmids namely, bait plasmid pGBKT7 (containing the Gal4 binding domain) and prey plasmid pGADT7 (containing Gal4 activation domain). The recombinant bait (BD) and prey (AD) plasmids were transformed in *Saccharomyces cerevisiae* strains Y187 and AH109, respectively. Transformants were selected on SD media lacking amino acids tryptophan (selection marker for bait) and leucine (selection marker for prey). Each bait transformant was mated with each prey transformant and diploid cells containing both plasmids were selected on SD media lacking tryptophan and leucine (SD/-Trp/-Leu). CHIKV structural proteins were screened for interaction

on SD/-Trp/-Leu/-His media. Positive interaction among test protein pairs results in expression of reporter gene *HIS3* and hence appearance of colonies on SD/-Trp/-Leu/-His media. Initial screening revealed that E3, E2 and E1 as BD fusion and C, E2, 6K and E1 as AD fusion interacted with control plasmids, respectively. Conformation of bait/prey constructs in yeast was perhaps responsible for their interaction with control. In order to study the viral-viral interaction of CHIKV, ectodomains of envelope proteins (TrE1 and TrE2) were cloned as BD and AD fusion and transformed in Y187 and AH109 cells of yeast, respectively. Ectodomain of E1 as BD/AD fusion did not interact with corresponding empty vector (only AD/BD vector) whereas ectodomain of E2 as AD fusion interacted with corresponding only BD vector. There have been several reports where transmembrane region of glycoprotein have been deleted to study their interaction in Y2H system. However, in the current study it was observed that deleted regions alone were not responsible for this behavior, as AD-TrE2 construct still interacted with only BD. Out of the fifteen unique pairs tested,Y2H assay identified twelve positive (E1-E1, E1-E2, E1-E3, E1-6K, E1-C, E2-E3, E2-6K, E3-E3, E3-6K, C-E3, C-6K and C-C) and one non interacting pair (C-E2) interaction. Two interaction pairs (E2-E2, 6K-6K) were inconclusive as TrE2 and 6K as AD fusion interacted with empty BD vector.

The interaction data obtained was independently validated by GST pulldown assay using GST-CHIKV fusion proteins as bait and Strep-CHIKV fusion proteins as prey. The complex was allowed to bind with glutathione resin followed by elution with 20 mM reduced glutathione. The eluted fractions were analysed by Western blotting using anti-GST antibody and Strep-Tactin HRP conjugate to detect the presence of bait and prey proteins, respectively. Among protein test pairs, interaction was confirmed by presence of corresponding protein specific band in both the blots while absence of immunoreactive band in Strep blot indicated no interaction. Nine out of twelve interactions observed in Y2H assay (C-C, C-E3, C-6K, C-E1, E3-E1, E2-6K, E2-E1, 6K-E1 and E1-E1) were confirmed positive by GST pull down. Three interactions involving E3 and 6K (E3-E3, E3-6K and 6K-6K) could not be validated as these proteins could not be expressed as Strep/His fusions by virtue of their size. Y2H positive interaction between E2 and E3 was shown to be negative by GST pull down and hence was inconclusive. The known interaction of C and E2 proteins (positive in GST pull down) was negative in Y2H because the ectodomain of E2, used in the study, lacked C-terminal cytoplasmic tail which is responsible for this interaction

(13). Apart from the six known interactions (C-C, E2-E1, 6K-E1 and E1-E1, E2-6K and C-E2), four novel interactions for genus *Alphavirus* (C-E3, C-6K, C-E1 and E3-E1) were identified among CHIKV structural proteins.

The intraviral interaction analysis of structural proteins was followed by identification of cellular binders of CHIKV envelope proteins E1 and E2. For this purpose, a human brain cDNA library cloned in pGADT7-Rec vector and pretransformed in Y187 yeast cells were screened with ectodomains of E1 and E2 (TrE1 and TrE2). AH109 yeast cells transformed with either TrE1 or TrE2 as BD fusion were mated with the cDNA library followed by selection of interacting pairs on SD/-Trp/-Leu/-His supplemented with X-α-gal for blue white screening. The blue colonies were further selected on SD/-Trp/-Leu/-His/-Ade supplemented with X-α-gal to significantly eliminate false positive interactions. The colonies obtained were then tested for the presence of library inserts by colony PCR using vector specific, T7 and 3' AD, primers. Prey plasmids were isolated from positive clones and transformed in *E. coli* DH5α strains. Plasmid DNA was purified from bacterial cells containing library inserts and sequenced using 3' AD sequencing primer and T7 sequencing primer. Host proteins were identified by comparing the sequences obtained with those in GenBank using nucleotide BLAST. The sequencing analysis identified sixteen human proteins interacting with E1 and seventeen human proteins interacting with E2, respectively.

Following the identification of candidate host protein interactors by Y2H, eight interactors (five of E1 and three of E2) were selected to investigate their association with the viral proteins. These host proteins were predicted to be potential interactors of E1 and E2 proteins in an earlier published computational study, where interactors were identified using structural similarity approach [12]. Host proteins were cloned in pGEX-4T3 and expressed as GST fusion followed by solubilisation. All the eight host proteins [(E1: COMMD1, THBS1, DYNC1H1, ATP1B3, MAP1B); (E2: PTPN2, COL1A2 and ACTG1)] were found soluble on cell lysis and their association was tested using pull down and interaction ELISA. For pull down assay, Strep tagged viral proteins (TrE1/TrE2) immobilized on Strep-Tactin resin were used as bait to capture GST tagged host proteins. The eluted fractions were analysed by western blotting using Strep-Tactin HRP conjugate (for bait proteins) and anti-GST antibody (for prey proteins).

The interaction among test pairs was assessed by the presence of immunoreactive band in both GST and Strep blot. Lack of immunoreactive band in GST blot for corresponding Strep-tagged bait was indicative of negative interaction. Out of the five host proteins (COMMD1, THBS1, DYNC1H1, ATP1B3, MAP1B), only four (COMMD1, THBS1, DYNC1H1, ATP1B) were found to interact with E1; while E2 was found to interact with all the three host proteins (PTPN2, COL1A2 and ACTG1). These interactions were also validated by ELISA using Strep fusion TrE1 and TrE2 proteins as bait and GST fusion host proteins as prey on Strep-Tactin microtiter plates. The complex was detected with anti-GST antibodies and appearance of blue colour after addition of TMB as substrate indicated the presence of interaction while absence of colour meant no interaction. The results obtained in ELISA were similar to those obtained from pull down assay. In totality, eight viral host interactions were identified from high throughput Y2H assay and previously predicted virus-host interface. Pull down and interaction ELISA validated a total of seven interactions; four for E1 (COMMD1, THBS1, DYNC1H1, ATP1B) and three for E2 (PTPN2, COL1A2 and ACTG1) protein.

CONCLUSIONS FROM THE PRESENT RESEARCH

In the current study, multiple sequence alignment of CHIKV IND-06-Guj with 41 isolates reported from South Asia revealed that IND-06-Guj strain has higher sequence similarity with these isolates than the prototype S27 CHIKV strain. Further, phylogenetic analysis also placed these 41 isolates nearer to IND-06-Guj than S27. As a result, CHIKV IND-06-Guj instead of S27 was identified as a candidate prototype strain of ECSA genotype in South Asian context. Also, the availability of its cloned genes will considerably facilitate the research to understand the CHIKV biology during current outbreak in this region. Additionally, expression and small scale purification studies of the structural proteins of CHIKV have helped in development of protocols and recombinants for large scale purification of these proteins.

Mapping of intraviral interactions of CHIKV identified four novel interactions (E1-E3, C-E1, C-6K and C-E3) apart from six known interactions of the genus, among structural proteins of CHIKV. The interaction of C with E3 protein may be a result of the C-terminal protease activity of capsid that enables its autocatalytic cleavage from the structural polyprotein [C-p62

(E3-E2)-6K-E1] during maturation of viral proteins. E3 has been known to play a critical role in folding of p62, heterodimerization of E2 with E1 and its transport to the plasma membrane during spike assembly. At the time of viral assembly, C reaches the host plasma membrane where on interaction with C-terminus of E2 protein viral budding is triggered. Interactions obtained among structural proteins can be targeted for inhibiting viral entry and pathogenesis. Further as such E1 and C, which were found to interact with all other proteins, can be considered as potential targets for developing antiviral drugs.

The host-viral interactions identified in the current study shed light on the possible host machinery modulated by E1 and E2 proteins for successful viral infection. A total of thirty three host binders of CHIKV were identified by high throughput Y2H screening by E1 and E2 proteins. Comparative analysis of the interaction data with predicted interactors of CHIKV from structural similarity approach [12] revealed a total of eight proteins (five for E1 and three for E2) common to both datasets. Seven of these interactions were validated by pulldown and interaction ELISA. E1 was found to interact with COMMD1, THBS1, DYNC1H1 and ATP1B3 while E2 interacted with PTPN2, COL1A2 and ACTG1. Interaction of ATP1B with E1 is particularly interesting as it may be involved in facilitating fusion of viral envelope to host membrane at the time of infection by maintaining electrochemical gradient in the endosome. In addition, host proteins, ACTG1, PTPN2, COMMD1 and DYNC1H1, are integral part of cellular trafficking machinery and are probably involved in transportation of viral structural proteins in host cell through their interaction with envelope proteins of CHIKV. The identified host proteins have also been associated with the maintenance of cellular structure, cellular trafficking, and regulation of immune response, thus, indicative of utilization of these processes by envelope proteins for facilitating CHIKV propagation in hosts.

FUTURE PROSPECTS

The knowledge of how the structural proteins orchestrate the viral infection will help in identification of possible inhibitory and/or therapeutic targets for the virus. The viral-viral and viral-host interactions identified for CHIKV structural proteins here, present a comprehensive list of targets for generation of peptide inhibitors. Analysis of E1/E2-host interactions obtained from

this study, in mammalian system, will assist in understanding the significance of these interactions for successful infection and propagation of the virus in host.

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1. **Dudha, N.**, Rana, J., Rajasekharan, S., Gabrani, R., Gupta, A., Chaudhary, V.K. and Gupta, S., "Host-pathogen interactome analysis of Chikugunya virus envelope proteins E1 and E2." ***Virus Genes,*** doi: 10.1007/s11262-014-1161-x. 2014.[*Indexed in SCOPUS, Impact factor:* **1.837**]
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GENBANK SUBMISSIONS

1. Gupta, S., **Dudha, N.**, Kumar, K., Gabrani, R., Sharma, S.K., Gupta, A., and Chaudhary, V. K., submitted the following Genbank Submissions:
   a. "Full-length cloned sequence of the non-structural protein 1 (nsP1) gene of Chikungunya virus, isolate IND-06-Guj, of 2006 outbreak." Genbank Accession No. JF272473, Jan. 31, 2011.

b. “Full-length cloned sequence of the non-structural protein 2 (nsP2) gene of Chikungunya virus, isolate IND-06-Guj, of 2006 outbreak.” Genbank Accession No. JF272474, Jan. 31, 2011.
c. “Full-length cloned sequence of the non-structural protein 3 (nsP3) gene of Chikungunya virus, isolate IND-06-Guj, of 2006 outbreak. “ Genbank Accession No. JF272475, Jan. 31, 2011.
d. “Full-length cloned sequence of the non-structural protein 4 (nsP4) gene of Chikungunya virus, isolate IND-06-Guj, of 2006 outbreak.” Genbank Accession No. JF272476, Jan. 31, 2011.
e. “Full-length cloned sequence of the capsid protein gene of Chikungunya virus, isolate IND-06-Guj, of 2006 outbreak.” Genbank Accession No. JF272477, Jan. 31, 2011.
f. “Full-length cloned sequence of the Envelope protein 3 (E3) gene of Chikungunya virus, isolate IND-06-Guj, of 2006 outbreak.” Genbank Accession No. JF272478, Jan. 31, 2011.
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i. “Full-length cloned sequence of the 6K gene of Chikungunya virus, isolate IND-06-Guj, of 2006 outbreak.” Genbank Accession No. JF272481, Jan. 31, 2011.

ABSTRACTS

1. **Dudha, N.**, Rana, J., Rajasekharan, S., Kumar, K. and Gupta, S., "Genome wide protein interaction analysis of Chikungunya virus" in **2nd International Symposium on HIV and Infectious Diseases**, Chennai, 30th Jan - 1st Feb., 2014.
2. **Dudha, N**., Kumar K. and Gupta S., "Chikungunya virus protein-protein interaction among viral proteins" in **International Union of Biochemistry and Molecular Biology (IUBMB) Workshop** "**Human RNA Viruses**" ICGEB, New Delhi, 10th Feb - 12th Feb., 2010.

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