APPENDIX- A
A.1 Reagents, Buffers and Chemicals

The solutions used for various experiments were prepared with analytical grade chemicals. Solutions were prepared using either Milli-Q or distilled water. Sterilisation was done by filtration and/or autoclaving at 121 °C, 15 psi for 20 minutes.

A 30% Acrylamide (100 ml)

29 g Acrylamide
1 g Bis-acrylamide
60 ml water
Dissolve by stirring and make up the volume to 100 ml with water. Filter with Whatman filter paper grade 1 and store at 4 °C.

1% Agarose gel (100 ml)

1 g Agarose
100 ml 1X TAE buffer
Dissolve the agarose in 1X TAE buffer by heating in a microwave oven and add Ethidium Bromide (EtBr) to a final concentration of 0.5 µg/ml

Alkaline Lysis Solution I (Resuspension Buffer)

25 mM Tris-Cl (pH 8.0)
10 mM EDTA (pH 8.0)
50 mM Glucose
Autoclave and store the solution at 4 °C.
Add 100 µg/ml RNase A and 4 mg/ml lysozyme prior to use

Alkaline Lysis Solution II (Lysis Buffer)

0.2 N NaOH (freshly diluted from a 1 N stock)
1% (w/v) SDS
Alkaline Lysis Solution III (Neutralisation Buffer)

- 60 ml 5 M potassium acetate
- 11.5 ml Glacial acetic acid
- 28.5 ml distilled water
- Store the solution at 4°C

10% APS (Ammonium persulphate; 1ml)

- 0.1 g APS
- In 1 ml water

Anti-GST antibody (primary antibody; diluted 1:10k; 10ml)

- 1 µl anti GST antibody
- In 10 ml 1% BSA in PBS

Anti-His antibody (primary antibody; diluted 1:3k; 10ml)

- 3.3 µl anti His antibody
- In 10 ml 1% BSA in PBS

Anti-Strep antibody (primary antibody; HRP conjugated; diluted 1:4k; 10ml)

- 2.5 µl anti Strep antibody
- And 10 µl biotin blocking buffer
- In 10 ml PBST

Anti-HA antibody (primary antibody; diluted 1:1k; 10ml)

- 10 µl anti HA antibody
- In 10 ml PBST

Anti-c-myc antibody (primary antibody; diluted 1:2k; 10ml)

- 5 µl anti c-myc antibody
- In 10 ml PBST
Anti-mouse antibody (secondary antibody; HRP conjugated; diluted 1:2k; 10ml)
5 µl anti-mouse antibody
In 10 ml PBST

20% Arabinose (10ml)
2 g arabinose
In 10 ml water

Anhydrotetracycline (2mg/ml) (10ml)
20 mg anhydrotetracycline
In 10 ml 70% Ethanol

B
5% BSA (Bovine Serum Albumin; 20ml)
1 g BSA in 20 ml of 1X PBS

Binding Buffer for ELISA
25 mM Tris-HCl
2 mM EDTA
140 mM NaCl
Adjust the pH to 7.6 and sterilize by filtration.

C
1M Calcium chloride (1L)
14.7 g Calcium chloride
In 1 litre of Milli-Q water
Cracking Buffer Stock (100 ml)

8 M Urea (48 g)
5% w/v SDS (5 g)
40 mM Tris-HCl [pH6.8] (4 ml of 1M stock solution)
0.1 mM EDTA (20 μl of 0.5 M stock solution)
0.4 mg/ml Bromophenol blue (40 mg)
Make up the volume to 100 ml with distilled water.

Cracking Buffer Complete (1.13 ml /protein extract)

Cracking buffer stock solution (1 ml)
β-mercaptoethanol (10 μl)
Protease inhibitor cocktail (70 μl)
PMSF (500 μl of 100X stock solution)

DNA Loading Dye (6X)

40% (v/v) Glycerol
0.25% (w/v) Bromophenol Blue
Mix and store at 4 °C.

Destaining Solution (100 ml)

30 ml Methanol
10 ml acetic acid
60 ml water

DAB (Diaminobenzidine; 20 ml)

0.05% DAB (10 mg)
In 20 ml PBS
Add 60 μl of 30% H₂O₂ just before adding to blot
Ethidium Bromide (10 mg/ml; 10ml)
0.1 g Ethidium Bromide
In 10 ml water
Stir on a magnetic stirrer for several hours and transfer the solution to a dark bottle and store at room temperature.

0.5 M EDTA (Ethylenediaminetetraacetate; 1L)
186.1 g disodium EDTA.2H₂O
800 ml water
Dissolve by stirring on magnetic stirrer and adjust the pH 8.0 with NaOH.
Autoclave and store at room temperature

Elution Buffer (Reduced Glutathione)
Prepare 0.1 M reduced glutathione stock solution in water
Dilute reduced glutathione stock to 20 mM in 50mM Tris-Cl pH 9.0

Elution Buffer (Desthiobiotin)
100 mM Tris-Cl
150 mM NaCl
1 mM EDTA
2.5 mM Desthiobiotin

IPTG (isopropyl b-D-thiogalactopyranoside)
0.5 M IPTG
In Milli-Q water.
Store at -20°C.
10X Lithium Acetate

1 M Lithium acetate. Adjust the pH to 7.5 with dilute acetic acid and autoclave.

Lyticase

Resuspend 10 KU lyophilized lyticase in 1 ml of 1X TE.

1X PBS (Phosphate-buffer saline) (1L)

137 mM NaCl (8 g)
2.7 mM KCl (0.2 g)
2.0 mM KH₂PO₄ (0.24 g)
10 mM Na₂HPO₄ (1.44g)
800 ml water
Adjust the pH to 7.4 with HCl.
Make up the volume to 1L with water. Autoclave and store at room temperature.

PBST (Phosphate-Buffer Saline Tween-20; 100 ml)

0.05% Tween-20 (50 µl) in 1X PBS

PEG/Lithium Acetate (10 ml)

8ml of 50% PEG 3350
1ml of 10X TE Buffer
1ml of 10X lithium acetate
Prepare freshly prior to use.

Phenylmethyl-sulfonyl fluoride (PMSF) stock [100X]

0.1742 g PMSF in 10 ml isopropanol. Wrap the tube in aluminium foil and store at room temperature.
**R**

5X Running buffer (1L)

72 g glycine
15 g Tris
5 g SDS

Make up the volume to 1 litre with distilled water

**S**

4X Sample buffer (50ml)

1.4 ml 1M Tris-Cl pH 6.8
22.4 ml 10% glycerol
6 g SDS
10 ml β-mercaptoethanol
20 mg Bromophenol Blue

Make up the volume to 50 ml with water

10% SDS (Sodium dodecyl sulfate; 100 ml)

10 g SDS

In 100 ml water

Store at room temperature

20% SDS (Sodium dodecyl sulfate; 100 ml)

20 g SDS

In 100 ml water

Store at room temperature

Staining Solution (100 ml)

0.25 g Coomassie R-250
45 ml distilled water
Add 45 ml methanol

Make up the volume to 100 ml with acetic acid
1X Transfer buffer (1L)

- 25 mM Tris (3.03 g)
- 192 mM Glycine (14.4 g)
- 800 ml water
- 20% Methanol (200 ml)

Tris 1M (pH 6.8; 200 ml)

- 24.2 g Tris
- 100 ml water
- Adjusted the pH to 6.8 using 1N HCl
- Make up the volume to 200 ml
- Autoclave and store at room temperature.

Tris 1.5 M (pH 8.8; 200 ml)

- 36.3 g Tris
- 100 ml water
- Adjusted the pH to 8.8 using 1N HCl
- Make up the volume to 200 ml
- Autoclave and store at room temperature.

10X TAE (Tris-acetate-EDTA; 1L)

- 48.4 g of Tris base
- 11.2 ml glacial acetic acid
- 20 ml 0.5 M EDTA (pH 8.0)
- Make up the volume to 1 L with water

10X Tris EDTA (TE)

- 100 mM Tris-Cl (pH 8.0)
- 10 mM EDTA (pH 8.0)
- Autoclave and store at room temperature.
1X TE/LiAc (10 ml)

- 1 ml 10X TE
- 1 ml 10X LiAc
- 8 ml water

W

Wash Buffer (Streptactin Resin)

- 100 mM Tris-Cl
- 150 mM NaCl
- 1 mM EDTA

X

X-α-Gal solution

Dissolve 20 mg/ml of 5-Bromo-4-chloro-3-indoyl-α-D-galactopyranoside (X-α-Gal) in N, N-dimethylformamide (DMF). Store at -20 °C wrapped in aluminium foil.
A.2 Media

**LB broth (1L)**

- 10 g/L of peptone
- 5 g/L yeast extract
- 10 g/L NaCl
- Adjust the pH to 7.0 with NaOH and autoclave.

**OR**

- 20 g/L LB broth powder

**LB Agar (1L)**

- 10 g/L of peptone
- 5 g/L yeast extract
- 10 g/L NaCl
- 15g/L agar
- Adjust the pH to 7.0 with NaOH and autoclave.

**OR**

- 20 g/L LB broth powder
- 15 g/L agar

**2X YT Broth**

- 20 g/L of peptone
- 10 g/L yeast extract
- 20 g/L NaCl
- Adjust the pH to 7.0 with NaOH and autoclave.

**Ampicillin Stock Solution (100 mg/ml)**

- 100 mg ampicillin
- In 1 ml water
- Store at -20°C.
Kanamycin Stock Solution (100 mg/ml)

100 mg kanamycin
In 1 ml water
Store at -20°C.

YPDA broth (1L)

20 g/L of peptone
10 g/L of yeast extract
0.2% of adenine hemisulphate
Add 950 ml of water and adjust pH to 6.5. Autoclave the media and cool to 55°C.
Add 2% dextrose (50 ml of 40% stock dextrose solution)

2X YPDA broth (1L)

40 g/L of peptone
20 g/L of yeast extract
0.4% of adenine hemisulphate
Add 950 ml of water and adjust pH to 6.5. Autoclave the media and cool to 55°C.
Add 4% dextrose (100 ml of 40% stock dextrose solution)

0.5X YPDA broth (1L)

10 g/L of peptone
5 g/L of yeast extract
0.1% of adenine hemisulphate
Add 950 ml of water and adjust pH to 6.5. Autoclave the media and cool to 55°C.
Add 1% dextrose (25 ml of 40% stock dextrose solution)
YPDA Agar (1L)

- 20 g/L of peptone
- 10 g/L of yeast extract
- 0.2% of adenine hemisulphate
- 20 g/L of agar
Add 950 ml of sterile water. Adjust pH to 6.5. Autoclave the media and cool to 55 °C.

Add 2% dextrose (50 ml of 40% stock dextrose solution)

SD broth (1L)

- 26.7 g/L SD minimal base
- 900 ml sterile water
Autoclave and add 100 ml of appropriate 10X Dropout solution.

10X Dropout Solution (100 ml)

Dissolve 620 mg of appropriate DO supplement in 100 ml water.
Autoclave and store at 4 °C.
A.3 ENZYMES

Taq DNA Polymerase (Fermentas)

Stock Concentration- 5 U/μL

10X Reaction Buffer

The enzyme is supplied with Reaction Buffer. 1X concentration of this contains 10 mM Tris-HCl (pH 9), 1.5 mM MgCl₂, 50 mM KCl and 0.01% gelatin. The buffer is supplied in 10X concentration.

Pfu DNA Polymerase (Promega)

Stock Concentration- 3 U/μL

10X Reaction Buffer

The enzyme is supplied with Reaction Buffer. 1X concentration of this contains 20 mM Tris-HCl (pH 8.8 at 25°C), 10 mM KCl, 10 mM (NH₄)₂SO₄, 2 mM MgSO₄, 0.1% Triton X-100 and 0.1 mg/ml nuclease-free BSA. The buffer is supplied in 10X concentration.

T4 DNA Ligase (Fermentas)

Stock Concentration- 5 U/μL

10X T4 DNA Ligase Buffer

The enzyme is supplied with 10X T4 DNA Ligase buffer. 1X concentration of this contains 40 mM Tris HCl, 10 mM MgCl₂, 0.5 mM ATP pH is 7.8 and 10 mM DTT.

T4 DNA Polymerase (Fermentas)

Stock Concentration- 5 U/μL

5X Reaction Buffer

The enzyme is supplied with 5X buffer. 1X concentration of this contains 33.5 mM Tris HCl (pH 8.8), 3.3 mM MgCl₂, 0.5 mM DTT and 8.4 mM (NH₄)₂SO₄.

DNase (IBA-GmbH)

Stock Concentration- 10 mg/ml
RNase (Fermentas)
Stock Concentration- 10 mg/ml

Lysozyme (IBA-GmBH)
Stock Concentration- 10 mg/ml

Protease Inhibitor Cocktail for Bacterial cell extracts (Clontech)
Complete EDTA-free protease inhibitor cocktail containing an optimized mixture of protease inhibitors—specially designed to protect proteins from being digested by endogenous proteases that can be released during protein extraction from cell lysates. The cocktail is a 100X stock solution in DMSO. It contains 0.8 µM Aprotinin, 50 µM Bestatin, 20 µM Leupeptin, 10 µM Pepstatin A and 1 mM PMSF,

Protease Inhibitor Cocktail for fungal and yeast extracts (Sigma Aldrich)
It is a mixture of protease inhibitors with broad specificity for the inhibition of serine, cysteine, aspartic and metallo-proteases. Contains 100 mM of AEBSF, 2.2 mM pepstatin A, 1.4 mM E-64 and 500 mM of 1,10-phenanthroline.
APPENDIX- B
B.1 MATERIALS

Strains, Plasmids and CDNA library:
E. coli (DH5α) and E. coli (BL21) strains were procured commercially. TOPO cloning vector was purchased from Invitrogen Life technologies, USA. Plasmids pGBKKT7 (bait or BD) and pGADT7 (prey or AD), control vectors and Saccharomyces cerevisiae strains (AH109 and Y187) were procured from Clontech, USA. pCAK (Strep Tag), pLTA (His Tag) bacterial expression vectors were a kind gift by Dr. Amita Gupta (Gupta, 2009). Vector pGEX-4T3 was purchased from Novagen Biosciences, USA. The fetal brain cDNA library (cloned in pGADT7-Rec plasmid) pre-transformed in Y187 yeast cells was procured from Clontech, USA.

CHEMICALS:

General Chemicals:
Deoxynucleotide triphosphates (dNTPs), Tris salt, Arabinose, Leucine, histidine, Tryptophan, Isopropyl β-D-thiogalactopyranoside (IPTG), Phenylmethyl-sulphonylfouride (PMSF), Lysozyme, Agarose, Polyethylene glycol (PEG-3350), Lithium acetate, Lyticase, Adenine hemisulphate, di-methyl sulphoxide (DMSO), NNN’N’- Trimethylethlenediamine (TEMED), Tween-20, Sarkosyl, Bovine serum albumin (BSA), 3, 3’-Diaminobenzadiene (DAB), Dimethy Formamide, Reduced Glutathione, CHAPS, Cellulose Dialysis tubing, Ethidium Bromide (EtBr), Glass beads 425-600µm, 3,3’,5,5’-Tetramethylbenzidine (TMB), Triton-X-100, Protease inhibitor cocktail for yeast were procured from Sigma Aldrich, USA.

1kb DNA ladder, 100bp DNA ladder, wide range molecular weight prestained and unstained protein markers from Fermentas, USA.

Calcium Chloride, Sodium dodecyl sulphate (SDS), EDTA, Acrylamide, Bis-acrylamide, β- mercaptoethanol, Ammonium persulphate (APS), Hydrochloric Acid, Glucose, Potassium acetate, Hydrogen peroxide, Glycine, sodium chloride (NaCl), Sodium hydroxide (NaOH), Zinc chloride (ZnCl₂), Sodium phosphate dibasic (Na₂HPO₄), Sodium di-hydrogen orthophosphate (NaH₂PO₄), Urea, Potassium dihydrogen phosphate (KH₂PO₄), Potassium chloride (KCl), Glacial acetic acid, Methanol and Isopropanol were from Fisher Scientific, USA (Qualigens).
Gluthathione sepharose beads, X-α-gal, Protease inhibitor cocktail for Bacterial cell extract and Herring testis DNA were from Clontech, USA.

Nuclease free water (NFW) was from Ambion, USA and Coomasie brilliant blue-R250, Bromophenol blue and Glycerol, were from CDH, India.

**Oligos:**
Primers for cloning of genes in Yeast two-hybrid and bacterial expression vectors were synthesized by Sigma Aldrich, USA.

**Enzymes:**
_Taq_ DNA polymerase (Sigma Aldrich, USA), _Pfu_ DNA polymerase (Promega, USA), _Bsa_ I and CIP (New England Biolabs, UK). All Restriction enzymes, dTTP, T4 DNA polymerase and T4 DNA ligase were purchased from Fermentas, USA.

**Kits:**
PCR purification kit and Plasmid DNA isolation kit was from Qiagen, Germany and Gel extraction kit was purchased from Sigma Aldrich, USA, IBA bacterial lysis kit was procured from IBA-GmBH, Germany. All the kits were used according to manufacturer’s protocol.

**Antibodies:**
For identification of fusion proteins by western blotting and ELISA Monoclonal Mouse Anti-GST, Monoclonal Mouse Anti-His antibodies and Anti-c-Myc antibodies were purchased from Sigma Aldrich, USA. HRP-conjugated Anti-Strep Antibody was from IBA-GmBH, Germany and monoclonal mouse Anti-HA antibody was procured from Santa Cruz, USA. HRP labeled Goat Anti-mouse Secondary antibody was purchased from G Biosciences, India.
Media:
YPD media was from Titan Biotech, India. SD minimal base and dropout supplements for yeast strains and transformants were from Clontech, USA. Luria Bertani (LB) medium and agar were from Fisher scientific, USA (Qualigens).

Antibiotics:
Kanamycin sulphate, Ampicillin and anhydrotetracycline were purchased from Sigma Aldrich, USA.

Other materials:
Polyvinylidene fluoride (PVDF) membrane and Whatman filter paper for immunoblotting were purchased from Millipore, USA and Biorad, USA respectively. Streptactin coated 96-well plates were from IBA-GmbH technology, Germany. Ethyl alcohol was from Changshu Yangyuan Chemicals, China.
LIST OF AUTHOR’S PUBLICATIONS


ABSTRACTS

1. **J. Rana**, S. Rajasekharan and S. Gupta, “Elucidating the virus-host interactions of Chikungunya virus using a structural similarity based approach.” CHIKV-2013, Langkawi Island, Malaysia from 28\textsuperscript{th} -30\textsuperscript{th} Oct, 2013.

2. S. Rajasekharan, **J. Rana** and S. Gupta, “Intraviral protein interactions of Chikungunya virus non-structural proteins.” CHIKV-2013, Langkawi Island, Malaysia from 28\textsuperscript{th} -30\textsuperscript{th} Oct, 2013.

3. N. Dudha, **J. Rana**, S. Rajasekharan, K. Kumar and S. Gupta, “Genome wide protein interaction analysis of Chikungunya virus.” HIV Science-2014, Chennai, India from 30\textsuperscript{th} Jan-1\textsuperscript{st} Feb, 2014.
SYNOPSIS
MOLECULAR INTERACTIONS OF CHIKUNGUNYA VIRUS NON-STRUCTURAL PROTEINS

SYNOPSIS

submitted in fulfillment of the requirements for the Degree of

DOCTOR OF PHILOSOPHY

by

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BACKGROUND AND RATIONALE OF THE CURRENT STUDY

Chikungunya virus (CHIKV) is an arthritic old world alphavirus transmitted mainly by \textit{Aedes} species of mosquitoes [1, 2]. Its genome is composed of a single molecule of 11.8 kb positive sense single strand RNA which encodes two polyproteins i.e. non-structural and structural. The nonstructural polyprotein is cleaved into four mature non-structural proteins (nsP 1-4) by the viral protease nsP2 while the structural polyprotein produces five structural proteins (Capsid, E3, E2, 6k and E1). The febrile illness caused by this virus among humans is characterised by a triad of symptoms involving high fever, rash and chronic arthralgia [3]. Chikungunya disease gets its name from Kimakonde vernacular language which means “that contorts or bends up” [4, 5].

CHIKV was first isolated in 1952 from the Makonde plateau in Tanzania, and since then has been responsible for a number of massive epidemic outbreaks posing major public health issues throughout the world. Although this virus is listed by National Institute of Allergy and Infectious diseases (NIAID) as a Priority C pathogen, that has been spreading throughout Asia, Africa and parts of Europe [6-8], there has been no commercially available vaccine or antiviral for this virus. The mechanism of infection and pathogenesis of CHIKV are poorly understood owing to limited scientific attention to this virus. The protein interaction interface (with host and self) of virus provides a means by which the virus invades and seizes control of their human host machinery to establish a successful infection. Identifying these interactions provide significant insight into mechanisms by which the virus manipulates the host and thereby provides several strategic targets for therapeutic interventions.

THESIS OBJECTIVES

CHIKV non-structural proteins are key players in viral life cycle as they form the replicase complex that synthesizes progeny viral RNA and are responsible for the cytopathic effects and host responses that are triggered by viral infection. In order to better understand them, the main objectives of this study are to map the interactions among the non-structural proteins of CHIKV and to identify the cellular interactors.
THESIS CHAPTERS

The research work carried out for the achievement of specified objectives is divided into following chapters:

CHAPTER 1: Introduction deals with the basic background about CHIKV and rationale of the study.

CHAPTER 2: Review of literature includes the existing literature about various aspects of CHIKV.

CHAPTER 3: Materials and methods describe the details of materials and various methods used in this study.

CHAPTER 4: Mapping interactions among the non-structural proteins of Chikungunya virus involves the analysis of intraviral protein interactions among nsPs using Y2H, GST pulldown and ELISA.

CHAPTER 5: Network mapping among the functional domains of Chikungunya virus non-structural proteins identifies the specific domains of nsPs involved in their associations and the generation of a structural model depicting the spatial arrangement of these proteins in viral replicase complex.

CHAPTER 6: Deciphering the host-pathogen protein interface in Chikungunya virus mediated sickness uses a protein structure similarity based approach for the identification of putative host interactors of CHIKV.


CHAPTER 8: Validation of nsP2-host interactions by involves the confirmation of Y2H identified interactions and domain mapping of nsP2 for these associations using pulldown and ELISA.
pCAK vector, expressed and solubilised. In vitro studies used for domain mapping revealed that CPNE6 and COX7A2 interacted with both nsP2 domains; CCDC130, EIF4A2, MAPK9 and POLR2C interacted with C-ter protease domain; while EEF1A1 and EIF3I interacted with N-ter helicase domain of the nsP2.

CONCLUSIONS FROM THE PRESENT RESEARCH

The present study has identified several intraviral and viral-host protein interactions for non-structural proteins of Chikungunya virus. The intraviral study identified six novel interactions among four non-structural proteins (nsP1-nsP1, nsP1-nsP2, nsP1-nsP3, nsP1-nsP4, nsP2-nsP4, nsP4-nsP4) which are achieved through ten interactions among the functional domains of these proteins (nsP1Mt-nsP1Mt, nsP1Mt-nsP2D1, nsP1Mb-nsP2D1, nsP1Cter-Macro, nsP1Cter-AUD, nsP1Mt-RdRp, nsP1Mb-RdRp, nsP2D1-RdRp, nsp2D2-RdRp and RdRp-RdRp). Among these, nsP1 has been found to interact with all other nsPs thus involved in keeping the replicase complex intact at the plasma membrane. Domain mapping of nsPs revealed that these interactions are based on functionality of proteins rather than the steric arrangement.

The identification of putative CHIKV interactors revealed that viral proteins mainly associate with the cellular hub proteins which are involved in multiple pathways of the cell thus hijacking the cellular machinery with only nine viral proteins. Also, this study identified nsP2 as an important candidate for screening of brain cDNA library using yeast two-hybrid (Y2H). Screening identified 27 host interactors of CHIKV nsP2 and from these 8 proteins (CCDC130, POLR2C, EEF1A1, EIF3I, CPNE6, EIF4A2, MAPK9 and COX7A2) were further validated for their interactions with the functional domains of nsP2. The identified proteins are involved in apoptosis, transcription and translational processes of host and nsp2 has previously been reported to be involved in these processes.

FUTURE PROSPECTS

The intraviral and viral-host interactions identified for CHIKV non-structural proteins can be targeted for designing molecules or peptides which could inhibit viral replication. The network of putative associations presented by the computational study puts forth a set of
CHAPTER 9: Conclusion and Future prospects describes overall findings of this research and their future prospects.

OVERVIEW OF THE RESEARCH

The interactions among non-structural proteins (nsPs) were analysed by three different assays viz. Yeast two-hybrid (Y2H), pull-down analysis and ELISA. The initial screening was performed by Y2H, for this all four nsP genes were cloned in two different yeast expression plasmids i.e. bait plasmid pGBK7T (containing binding domain) and prey plasmid pGAD7T (containing activation domain). These recombinant BD and AD vectors were transformed into Y187 and AH109 yeast cells and transformants were selected on SD/-Trp and SD/-Leu media, respectively. Each bait transformant was mated with each prey transformant constituting 16 interaction pairs and mated clones were selected on SD/-Trp/-Leu media. The diploid yeast cells harboring both the fusion plasmids were checked for the interaction on SD/-Trp/-Leu/-His, where interaction resulted in the expression of histidine. Appearance of colonies on triple drop out media indicated positive protein interactions. This analysis identified four positive interactions (nsP1-nsP3; nsP1-nsP4; nsP2-nsP4 and nsP4-nsP4) and two negative interactions (nsP2-nsP3 and nsP3-nsP4). Some of these interactions were obtained from one direction as nsP1 and nsP2 as AD fusion interacted with only BD whereas nsP3 as BD fusion interacted with only AD protein. All the pairs were further independently validated by GST pulldown and ELISA analysis for interactions.

For validation experiments, nsP genes were cloned in three bacterial expression vectors- pCAK (Strep tag), pLTA (His tag) and pGEX-4T3 (GST tag). The positive clones were transformed in E. coli BL21 cells and induced with suitable inducer (0.5% L-arabinose for pCAK; 50 ng tetracycline for pLTA; 1 mM IPTG for pGEX-4T3). The induced cultures were lysed and solubilisation profile of desired protein was analysed by SDS-PAGE followed by Western blotting using tag specific antibodies. Soluble fractions of the fusion proteins were used for pulldown and ELISA assays. Pulldown assay was performed using GST fusion proteins as bait and Strep fusion proteins as prey. The complex is allowed to bind with glutathione resin followed by elution of complex with 20 mM reduced glutathione. The eluted fractions were analysed by Western blotting using anti-GST (for control as presence of bait) and anti-Strep antibodies (for presence of prey proteins). Presence of corresponding protein specific band in both blots confirmed the interaction while absence of Strep fusion protein indicated no interaction among test pair proteins. The pulldown
assay confirmed five interactions among CHIKV nsPs (nsP1-nsP1, nsP1-nsP2, nsP1-nsP3, nsP1-nsP4 and nsP4-nsP4). Further, ELISA was also performed using Strep fusion protein as bait and His fusion as prey proteins on Streptactin microtitre plates. The complex was detected with anti-His antibodies and appearance of blue colour after addition of TMB as substrate indicated the presence of interaction while absence of colour meant no interaction. After integrating data from all experiments, six novel interactions were identified among CHIKV nsPs (nsP1-nsP1, nsP1-nsp2, nsp1-nsP3, nsP1-nsP4, nsP2-nsP4, nsP4-nsP4). Three of these interactions are novel for genus Alphavirus (nsP1-nsp1, nsp1-nsp2 and nsP4-nsp4).

This interaction analysis was followed by domain mapping of nsPs for identification of functional domains involved in associations of nsPs to form late replicase complex. The objective was achieved by pulldown, ELISA and Y2H assays. Initially, functional domains of CHIKV nsPs were cloned in pGEX-4T3 vector followed by expression and solubilisation profile analysis of these proteins. All the domains were successfully expressed and solubilized except for N-terminal methyltransferase domain of nsP1 which couldn’t be expressed even after optimisation trials with different expression conditions. Pulldown was performed using GST fusion domains and Strep fusion nsPs while ELISA was performed using strep fusions as bait and His fusions as prey protein. These assays identified 10 interactions among full length nsPs and their domains. As methyltransferase domain could not be expressed, the interaction of nsP1 domains with other nsPs was identified using Y2H assay. All three domains were cloned in pGBK7T7 and pGADT7 plasmids as BD and AD fusions, respectively. Following transformation in yeast cells, each bait transformant was mated with nsP prey transformants and interacting pairs were selected on SD/-Trp/-Leu/-His media. Overall 14 interactions were identified among nsPs and their domains which in conclusion are responsible for 10 interactions among individual domains (nsP1Mt-nsP1Mt, nsP1Mt-nsP2D1, nsP1Mb-nsP2D1, nsP1Cter-Macro, nsP1Cter-AUD, nsP1Mt-RdRp, nsP1Mb-RdRp, nsP2D1-RdRp, nsp2D2-RdRp and RdRp-RdRp). Based on these interactions, a model for spatial arrangement of nsPs in late replicase complex has been proposed. For this, structures of full length nsPs were generated using I-TASSER and late replicase complex was constructed using rigid body docking in combination with rescoring and refinement. Initial sets of structures for refinement were produced by using nsp1-nsp2, nsP1-nsP3 and nsP1+nsP2-nsP4 as a complex [9].

Mapping of intraviral nsP interactions was followed by prediction of CHIKV cellular partners involved in viral life cycle. This study is based on the principle that if two proteins have similar structural motifs than their interaction dataset will be highly similar. CHIKV human and
Drosophila similar proteins were identified for each protein using Dali webserver and their interactors were obtained from HPRD and DroID database. For Aedes interactors of CHIKV, orthologs of Drosophila proteins were obtained from FlyBase. These interactors were then shortlisted on the basis of cellular compartmentalization using DAVID GO tool. The annotation of selected proteins for biological processes and molecular functions revealed that in humans, CHIKV mainly targets antigen processing, apoptosis, JAK-STAT, transcriptional and translational pathways while in Aedes vector, the viral proteins affect protein localization, modification and transport pathways. Also, the orthologs obtained among human and mosquito proteins are involved in human intracellular and nucleocytoplasmic transport while in Aedes these affect protein and macromolecule localization. In conclusion, more than 40% of the previously identified alphavirus-host interactions were also obtained in our analysis as CHIKV interactors which mainly involves receptors (CD209, laminin receptor and 60-kDa neural adhesion molecule), members of heterogeneous ribonucleoproteins (hnRNP) and 14-3-3 family. The members of hnRNPs mainly interacted with nsP2 and 14-3-3 family proteins were involved in association with nsP4. In addition, 15 cellular proteins were identified as partners of CHIKV nsP3 that were supported from the Sindbis virus (SINV) nsP3 interaction dataset.

The results of computational approach and previous studies suggested that nsP2 is the key player among CHIKV proteins which affect various host pathways to circumvent cellular responses against viral infection. Thus, nsP2 was selected for screening of human brain cDNA library cloned in pGADT7-Rec vector and transformed in Y187 cells. Mating of nsP2 as BD fusion transformed in AH109 cells with cDNA library was followed by selection of interacting pairs on SD/-Trp/-Leu/-His/-Ade supplemented with α-gal for blue white screening. The blue colonies were used for yeast colony PCR using T7 and AD primers for confirmation of presence of AD fusion in the selected clones. The positive clones were used for isolation of plasmid DNA and transformed in E. coli DH5α cells. Plasmids isolated from bacterial cells were sequenced and host proteins were identified using BLAST suite of tools. The sequencing analysis identified 27 human proteins as nsP2 interactors. Among these, 8 proteins were selected for further validation based on their functional relevance in viral life cycle. The validation study was performed by using in vitro assays like pulldown and ELISA. This objective was achieved by cloning of host genes in pGEX-4T3 vector, followed by expression and solubilisation analysis. The interaction of selected host protein was found positive after pulldown and ELISA, hence further analysis was performed to identify the domains of nsP2 responsible for association with these proteins. For domain mapping, nsP2 domains were cloned in

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potential interactions that are amenable to further experimental investigations to understand the viral pathogenesis. Further, the elucidated CHIKV nsP2-host interactions are of much importance both in understanding the virus biology and as potential targets for drug development.

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PUBLICATIONS


ABSTRACTS


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