



RESEARCH ARTICLE

Screening human brain targets for Japanese encephalitis virus NS5 via yeast two-hybrid assay

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ABSTRACT

Japanese encephalitis (JE) is the most important vaccine-preventable acute meningoencephalitis in tropical and subtropical Asia with no known antiviral therapeutics. Available vaccines offer limited protection against emerging Japanese encephalitis virus (JEV) genotypes, posing a persistent public health threat. Disruption of the interactions between JEV non-structural protein 5 (NS5) protein and human brain cellular proteins could be explored to develop flavivirus druggable host targets. This study aimed to identify the human brain cellular proteins that interact with the JEV NS5 protein. A high-throughput yeast two-hybrid (Y2H) screen of a normalized human brain cDNA library was carried out to identify human brain proteins that physically interacted with the JEV NS5 protein. Colony polymerase chain reaction (colony PCR), sequencing and BLAST analysis were carried out to identify and confirm the positive clones. Of the 32 selected large blue colonies on the double dropout media (DDO), only 20 (62.5%) survived on the more stringent selection media (QDO) and retained their blue coloration. Colony PCR, sequencing and BLAST analysis showed that these colonies on a more stringent media carried human brain cDNAs including one uncharacterized cDNA as potential interactors of JEV NS5. Gene Ontology (GO) enrichment analysis identified chemical carcinogenesis-reactive oxygen species, Parkinson's disease, and necroptosis as important biological pathways for JEV neuropathogenesis. The identified JEV NS5-interacting human brain cDNAs highlight the role of NS5 protein and biological pathways such as oxidative stress, Parkinson's disease and necroptosis in JEV neuropathogenesis. These pathways represent potential targets for neuropathologic flavivirus infection.

Keywords: Human brain protein; Japanese encephalitis virus (JEV); Neuropathogenesis; non-structural protein 5 (NS5); Yeast two-hybrid (Y2H) assay.

INTRODUCTION

Japanese encephalitis (JE) is an endemic and vaccine-preventable disease that poses a high risk to public health primarily in tropical, and subtropical regions with 50% of the world population at risk of Japanese encephalitis virus (JEV) infection. JEV is an enveloped, single-stranded positive-sense RNA virus of the genus *Flavivirus* in the family *Flaviviridae* (Lindenbach & Rice, 2003). JEV infections in humans are either asymptomatic or cause febrile illness with mild flu-like symptoms lasting for 5–15 days. JE is associated with various clinical presentations following viral entry into the central nervous system (CNS), resulting neurological signs such as increased intracranial pressure, neck stiffness, disorientation, seizure, polio-

like flaccid paralysis, coma, and in severe cases death (Salimi *et al.*, 2016; Solomon *et al.*, 2003).

The tropical Southeast Asia region is an important region for emerging and re-emerging zoonotic pathogens transmitted by arthropods (Saba Villarroel *et al.*, 2023). Genetic studies suggest that JEV evolved from an African ancestral virus, which subsequently spread to the Indonesia-Malaysia region and diverged to all five genotypes (GI–GV) of JEV found circulating in this region (Solomon *et al.*, 2003). However, distribution and geographical shifts of different JEV genotypes were observed due to its emergence in non-traditional endemic tropical regions, suggesting its evolving nature as an emerging infectious disease (Gao *et al.*, 2019). More importantly, genotype I (GI) appears to have replaced genotype III

(GIII) as the dominant cause of JE, which used to be responsible for most human cases of JE in Asia in the '90s (Pan *et al.*, 2011; van den Hurk *et al.*, 2009). Despite vaccination efforts, JE remains a public health burden due to the non-protection of existing vaccines against newly emerging genotypes, its sporadic outbreaks, and the unavailability of specific antiviral therapeutics. However, treatment for JE has been only supportive with limited clinical trials on antiviral therapeutics conducted.

Non-structural protein 5 (NS5) of JEV is the most conserved region of the virus and has a vital role in virus replication, host immune response modulation, contains multifunctional enzymatic domains crucial for viral replication and immune evasion (He *et al.*, 2018; Klema *et al.*, 2015; Wang *et al.*, 2018). However, the information on the emerging JEV NS5-targeting proteins in the human brain and their impact on virus replication and neuropathology is still limited. To explore the human brain interacting proteins, we used a high-throughput Y2H assay to screen a normalized human brain cDNA library to identify the host proteins that interact with JEV NS5. Taken together, the findings from this study demonstrate that the NS5 protein interacts with human brain proteins and play significant role in enhancing preventive and therapeutic strategies for JEV infections affecting CNS in the future.

MATERIALS AND METHODS

JEV NS5 gene retrieval and *in silico* insertion cloning into the bait vector

The nucleotide coding sequence of the JEV NS5 gene (accession number HM596272) was retrieved from the National Centre for Biotechnology Information (NCBI) GenBank and codon-optimized using Integrated DNA Technologies (IDT) Codon Optimization Tool for *Saccharomyces cerevisiae* (*S. cerevisiae*) yeast. The optimized viral bait (NS5) coding sequence was recombined into a pGBKT7 (Takara Bio, California, USA) expression vector in fusion downstream of the binding domain of GAL4-BD using EcoRI and Sall restriction sites. The construct (pGBKT7-NS5) was verified by *in silico* translation using the Expasy web tool to confirm the open reading frame (ORF) (Supplementary Figure 1).

Autoactivation and toxicity tests

The recombinant pGBKT7-JEV NS5 and the empty pGBKT7 plasmids were transformed into the Y2HGold yeast strain using a high-efficiency polyethylene glycol (PEG)/LiAc-based method (Clontech, USA) and spread on the following plates; minimal yeast medium without tryptophan (SD/-Trp), SD/-Trp supplemented with 40 µg/mL X-alpha-Gal (SD/-Trp/X), and SD/-Trp supplemented with 40 µg/mL X-alpha-Gal and 125 ng/mL Aureobasidin A (SD/-Trp/X/A) and incubated for 3-5 days.

Preparation of human brain cDNA library strain

Human brain prey coding sequences were recombined into pGADT7 (Takara Bio, California, USA) to be expressed in fusion downstream of the activation domain of Gal4 (Gal4-AD) and transformed into prey Y187 yeast strains (Paiano *et al.*, 2019).

Recombinant gene synthesis and laboratory confirmation

The synthesis of the bait construct (pGBKT7-NS5) was synthesized by a commercial provider (Integrated DNA Technologies, Singapore) and confirmed via PCR using specific primers (Table 1) and MyTaq™ Red Mix reagent (Meridian Bioscience, London, United Kingdom). Thermal cycling was performed in an Eppendorf Mastercycler Nexus Gradient Thermal Cyclers (Eppendorf SE, Germany) as follows: initial denaturation at 95°C for 1 minute, followed by 30 cycles of denaturation at 95°C for 15 seconds, annealing at 47°C for 15 seconds, and extension at 72°C for 45, 30 and 15 seconds, depending on the fragment size of the DNA, respectively with a cycle of the final extension at 72°C for 5 minutes. PCR products were resolved on 2% agarose gels, visualized on a gel imager (Azure Biosystems, California, USA), sequenced, and analyzed using the BioEdit and NCBI BLAST tool.

Yeast two-hybrid (Y2H) screening of human brain cDNA library

The Y2H screening was carried out to generate and identify the human brain cellular cDNAs that may physically interact with JEV NS5. Using standard microbiological techniques, a sterilized microbiological loop was used to streak the yeast peptone dextrose agar (YPDA) plate with the yeast stock and incubated in an inverted position at 30°C until the colonies appeared in 3-5 days. A single colony from the YPDA agar plate was used to prepare competent yeast cells for transformation. Lithium acetate (LiAc)-mediated transformation (Paiano *et al.*, 2019) was used to transfer the recombinant plasmid pGBKT7-NS5 into the Y2HGold strain of *S. cerevisiae* using Yeastmaker™ Yeast Transformation System 2 protocol (Takara Bio, California, USA). The transformed yeast cells were spread onto 100 mm agar plates containing synthetic/single dropout (SD) selective medium lacking tryptophan (SD/-Trp) for the growth of transformed yeast cells (Y2H Gold [pGBKT7-NS5]). Colonies were confirmed by colony PCR and prepared for yeast mating and two-hybrid screening.

A concentrated overnight culture of the bait strain (Y2HGold [pGBKT7-NS5]) was prepared for yeast mating and two-hybrid screening of the human brain cDNA library. The bait strain culture (OD₆₀₀ = 0.8) was combined with the human brain cDNA prey library in a 2X YPDA medium with kanamycin (Takara Bio, California, USA). Mating cultures were incubated at 30°C with gentle shaking at 40 rpm until zygotes formed (Supplementary Figure 2). Mated cultures were spread on different selective agar plates: Single dropout (-Trp) SD/-Trp, Single dropout (-Leu) SD/-Leu, Double dropout (DDO), and Double dropout containing X-alpha-Gal and Aureobasidin A (DDO/X/A) and incubated at 30°C for 5 days. Relatively big blue yeast colonies indicating yeast mating were marked and selected on the double dropout agar plates (DDO/X/A) (Figure 1A). The blue yeast colonies on the DDO/X/A were patched onto more stringent quadruple dropout agar plates (QDO/X/A) (Figure 1B), to further confirm the expression and interaction of both bait and prey proteins.

Table 1. List of primers, plasmid and yeast used for Y2H assay

Plasmid	Yeast Strain	Transcription factor domain	Primer Name	Sequence (5' – 3')	Target Size (bp)
pGBKT7-NS5 (Bait)	Y2HGold (<i>S. cerevisiae</i>)	Gal4-DNA-BD	pGBKT7-NS5-1-F	TCA TCA TCG GAA GAG AGT AGT	3095
			pGBKT7-NS5-R	TGA CCT ACA GGA AAG AGT TAC	
			pGBKT7-NS5-2-F	TTT ATC CAG GAA CTC CAA TCA	2332
			pGBKT7-NS5-3-F	CCA TTT GAG AGG TGA GTG TC	1633
pGADT7-P (Prey)	Y187 (<i>S. cerevisiae</i>)	Gal4-AD	pGADT7-P-F pGADT7-P-R	CTA TTC GAT GAA GAT ACC CCA CCA GTG AAC TTG CGG GGT TTT TCA GTA TCT	Different amplicon sizes (1000 – 2500 bp)

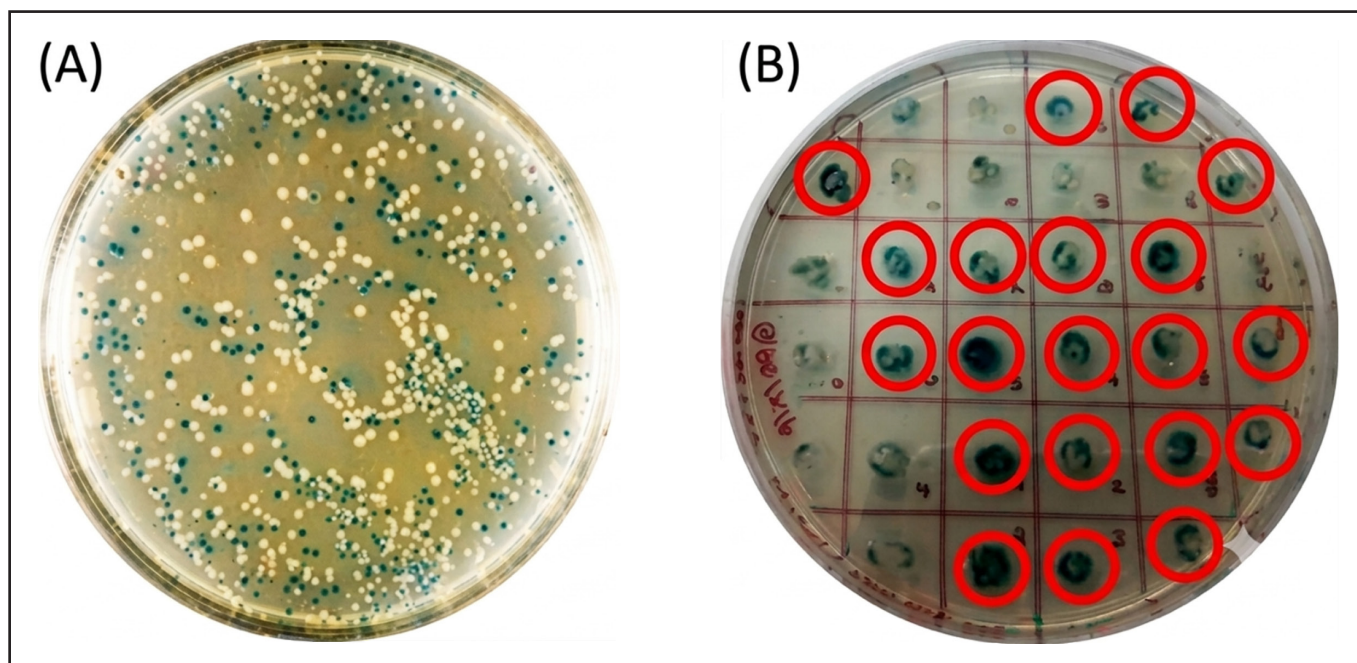


Figure 1. Y2H screening of human brain cDNA library: (A) Selection of mating yeasts (blue) due to interaction of bait and prey protein on DDO/X/A. (B) Representative positive colonies (red circles) selected for downstream confirmation on QDO/X/A medium. All surviving positive colonies were subsequently analysed by colony PCR and sequencing.

Detection of bait and prey genes from the positive yeast colonies for protein-protein interactions

All blue yeast colonies that survived and retained their blue colour on the quadruple dropout (QDO/X/A) agar plate (Figure 1B) were subjected to colony PCR using Terra™ PCR Direct Red Dye Premix (Takara Bio, California, USA) with primers flanking the bait insert in the pGBKT7 vector (Table 1). Thermal cycling was done in Eppendorf Mastercycler Nexus Gradient Thermal Cycler (Eppendorf SE, Germany) as follows: initial denaturation at 98°C for 2 minutes, followed by 30 cycles of denaturation at 98°C for 10 seconds, annealing at 47°C for 15 seconds, and extension at 68°C for 3 minutes. The final 1 cycle of the extension was set at 68°C for 5 minutes. The amplicon was resolved in 2% agarose gels and viewed using a gel imager (Azure Biosystems, California, USA).

Plasmid isolation from the positive blue yeast colonies on QDO/X/A agar plate was performed using the Easy Yeast Plasmid Isolation Kit (Takara Bio, California, USA). High-fidelity PCR targeting the prey gene was conducted using CloneAmp™ HiFi PCR Premix (Takara Bio, California, USA), with the primer that hybridizes within pGADT7 region flanking cDNA inserts (Table 1) targeting 1000 – 2500 bp producing multiple sizes of PCR amplicons. The PCR cycling condition was set as follows: 1 cycle of initial denaturation at 98°C for 30 seconds, followed by 30 cycles of denaturation at 98°C for 10 seconds, annealing at 47°C for 10 seconds, and extension at 72°C for 2 minutes with the final extension step set at 72°C for 5 minutes. The amplicons were resolved in 2% agarose gels and viewed using a gel imager (Azure Biosystems, California, USA).

Identification of prey proteins interacting with bait protein (NS5 protein)

High-fidelity amplicons from positive yeast colonies were Sanger sequenced using pGADT7-P-F and pGADT7-P-R primers (Table 1). Sequence identities were analyzed using MEGA software and

NCBI BLAST Tool. The identified genes from the cDNA inserts were cross-referenced using GeneCards® (<https://www.genecards.org/>) and UniProt databases (<https://www.uniprot.org/uniprot/>) and subsequently enriched using STRING (version 12.0) for the various Gene Ontology (GO) categories. We also compared our data with previously published works (Kovanich *et al.*, 2019; Le Breton *et al.*, 2011) on JEV-NS5 interacting proteins. Furthermore, pathway enrichment analysis was conducted using the ShinyGO 0.80 web tool (<http://bioinformatics.sdstate.edu/go/>) to identify top biological pathways associated with the interacting proteins.

RESULTS

Gene retrieval, codon optimization, and *in silico* insertion cloning

JEV NS5 coding sequence with accession number HM596272 was successfully retrieved from the NCBI database and prepared for expression in *S. cerevisiae*. The NS5 gene was successfully recombined into the pGBKT7 vector in frame with Gal4-DNA-BD and ExPASy translation of the recombined sequence confirmed and validated the complete open reading frame (ORF) in frame with Gal4-DNA-BD.

Confirmation of recombinant bait construct using PCR amplification

PCR amplification using three different forward primers with the same reverse primer (Table 1) yielded clear, distinct bands on the 2% agarose gel, with the expected sizes of 3095 bp, 2332 bp, and 1633 bp corresponding to the three different primer sets used (Figure 2). Sequenced amplicons were submitted to online NCBI BLAST analysis, which confirmed 100% identity with the JEV NS5 coding sequence. This further validated the correct insertion and orientation of the bait gene in the plasmid construct.

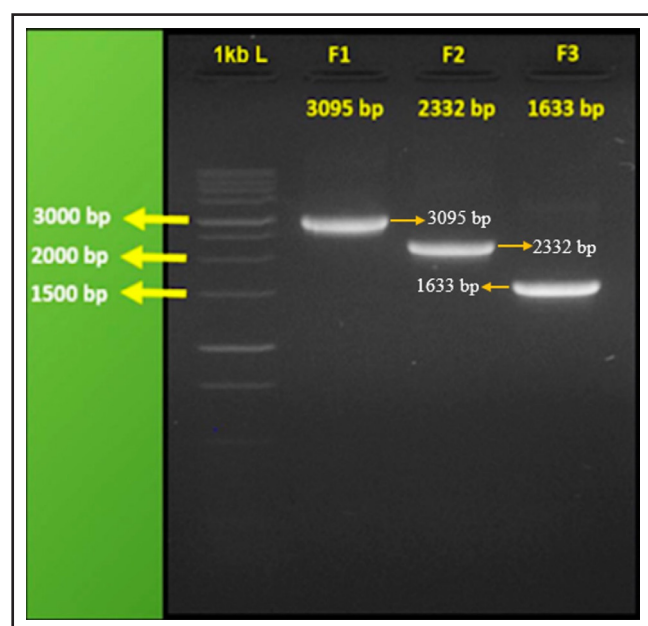


Figure 2. PCR detection of NS5 gene in the bait plasmid construct using three different forward primers with the same reverse primer. F1, F2, and F3 are showing bands amplified by forward primers pGBKT7-NS5-1-F, pGBKT7-NS5-2-F, pGBKT7-NS5-3-F, and reverse primer pGBKT7-NS5-R respectively.

The growth of yeast colonies on SD/-Trp, SD/-Trp/X-alpha-Gal plates, and the absence of yeast colonies on SD/-Trp/X-alpha-Gal/AbA agar plates indicated that the bait plasmid was devoid of autoactivation. The transformed Y2HGold showed a relatively bigger size when compared to the empty Y2HGold yeast indicating that the plasmid construct was not toxic and suitable for yeast two-hybrid screening.

Screening for human brain cDNAs that interact with NS5 protein

Following screening of the normalized human brain cDNA library using yeast mating, a total of thirty-two (32) relatively big blue

yeast colonies on selective (DDO/X/A) agar plates were patched onto the stringent selective (QDO/X/A) agar plate. Of the 32 blue yeast colonies, only 20 survived and retained the blue color on the QDO/X/A agar plate representing a 62.5% positivity rate (Figure 1B). All surviving colonies were subjected to colony PCR, plasmid isolation, sequencing, and BLAST analysis. As these colonies produced consistent identification results, only one representative positive colony is shown in Figure 1 for illustrative purposes.

Detection of bait and prey constructs in positive yeast colonies

Yeast colony PCR using Terra™ PCR Direct Red Dye Premix for detection of the bait gene (Figure 3A) in the survived blue colonies on stringent selective (QDO/X/A) plates (Figure 1B) indicated that all the colonies were positive for the bait gene. Following plasmid isolation from these colonies for the presence of human brain cDNAs by high-fidelity PCR assay, these colonies (20) were positive for the presence of interacting prey cDNAs confirming the interaction with the bait gene (NS5) (Figure 3B).

Yeast colony PCR amplification and detection of bait and prey genes (cDNAs) from the blue yeast colonies confirmed and validated the presence of both interacting partners. The prey amplicon sizes ranged from 1000 to 2500 bp, corresponding to different interacting prey proteins (Figure 3B). This successfully confirmed the interaction between the NS5 bait protein and human brain proteins.

Identification of human brain cDNAs (prey) interacting with JEV NS5 protein (bait)

Sequencing of the PCR amplicons from positive yeast colonies identified multiple human brain proteins interacting with the JEV NS5 protein. The BLAST analysis of the nucleotide sequences revealed several known protein-protein interactions, and these results were further validated through GeneCards and UniProt databases, which provided functional insights into the identified prey proteins (Table 2). Comparison with previous screening studies in the literature showed only CBR1 as the only human prey brain protein that had been previously reported (Figure 4).

Gene Ontology (GO) categories and pathway enrichment analysis

Independent Gene Ontology analysis using STRING (version 12.0) revealed no significant enrichment detected in the biological

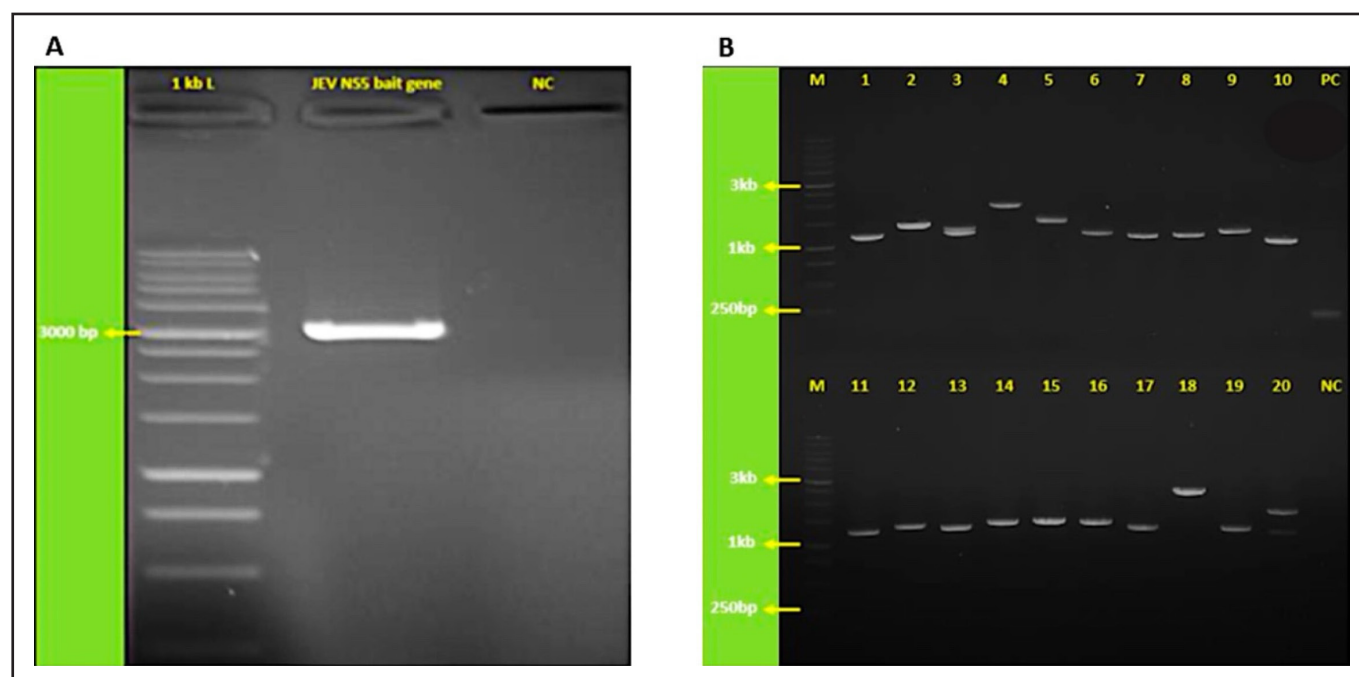
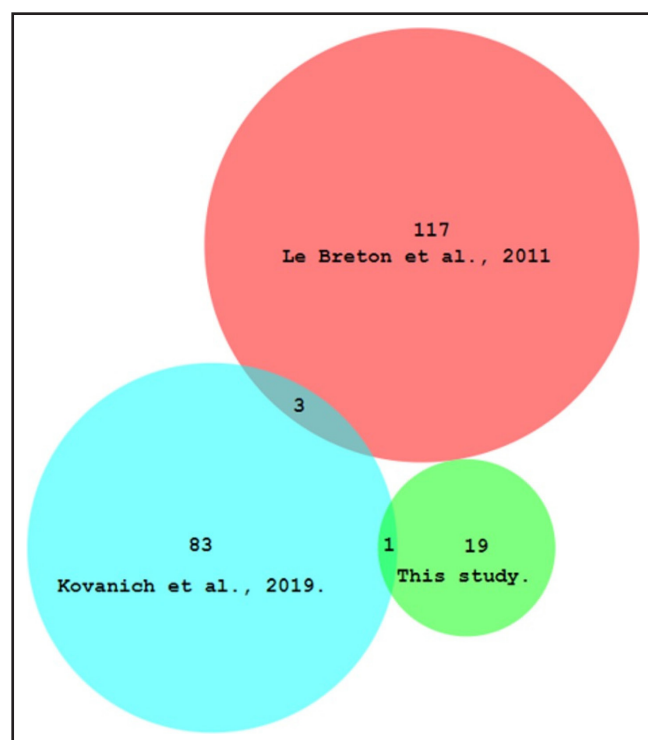


Figure 3. Yeast colony PCR detection of bait and prey cDNAs: (A) Bait detection of blue yeast colony on QDO/X/A media. (B) Prey detection of blue yeast colony on QDO/X/A media. PC: Positive control, NC: Negative control, M: DNA Ladder.

Table 2. List of human brain cDNAs that interact with JEV NS5 protein using Y2H assay

Protein Name	Gene Name	Function
Krueppel-like factor 9	KLF9	Transcription factor
Serine/threonine-protein kinase PINK1, mitochondrial	PINK1	Mitochondrial protection; cellular stress response
QKI, KH Domain Containing RNA Binding	QKI	Translation regulation
Synaptojanin 2	SYNJ2	Membrane trafficking and signal transduction pathways
Voltage Dependent Anion Channel 2	VDAC2	Ion channel activity
Ubiquinol-Cytochrome C Reductase Core Protein 2	UQCRC2	Catalyzes mitochondrial electron transport
Carbonyl Reductase 1	CBR1	Catalytic activity
Mitochondrial contact site and cristae organizing system (MICOS) subunit 10	MICOS10	Regulation of mitochondrial morphology and functions
F-box/SPRY domain-containing protein 1	FBXO45	Regulation of neurotransmission
BRICK1 Subunit of SCAR/WAVE Actin Nucleating Complex	BRK1	Regulation of actin and microtubule organization
Uncharacterized	DKFZp434H018	Protein trafficking
Integral Membrane Protein 2B	ITM2B	Regulatory role in the transport of molecules across cell membranes
Zinc Finger Protein 597	ZNF597	Transcriptional regulation
Leucine-rich Repeat-containing 59	LRRC59	Nuclear transport
Disks large homolog 2	DLG2	Regulation of neural synapse
Transient Receptor Potential Cation Channel Subfamily M Member 7	TRPM7	Ion channel formation; homeostasis
Zinc finger and BTB domain-containing 20	ZBTB20	Transcription factor involved in immune response
CD164 molecule	CD164	Cell adhesion and cell signalling
ATRX chromatin remodeler	ATRX	Transcriptional regulation and chromatin remodeling
Zinc Finger Protein 106	ZNF106	Transcriptional regulation and cellular differentiation

PINK1 = PTEN-induced kinase 1; QKI = Quaking; CD = cluster of differentiation 164; ATRX = alpha-thalassemia/mental retardation X-linked.

**Figure 4.** A Venn diagram showing the comparison between this study and the previous studies that used yeast two-hybrid assay.

process, molecular function, and cellular component. However, pathway enrichment analysis using ShinyGO identified significant associations with oxidative stress-related pathways, including chemical carcinogenesis - reactive oxygen species, Parkinson disease, and necroptosis (Table 3).

DISCUSSION

Downregulation of host immune responses, remodelling of host membranes, and manipulation of host metabolic and signalling pathways contribute to the pathogenesis of viruses with minimal genomes such as RNA viruses through physical interactions between viral and host proteins (Durmuş & Ülgen, 2017; Kim *et al.*, 2020; Nagy & Pogany, 2012). The flavivirus NS5 protein has a methyltransferase domain for viral RNA methylation and RdRp domain for viral RNA replication at N and C termini, respectively (Fernandes *et al.*, 2021; Duan *et al.*, 2019). Modification of the viral RNA through methylation capping by methyltransferase domain of NS5 protein facilitates viral infection through evasion of host immune responses by mimicking of RNA of the host (Dong *et al.*, 2012; Li *et al.*, 2013). This dual-function characteristic of flavivirus NS5 protein underscores its importance in survival mechanisms and replication in infected hosts. The interactions of flavivirus NS5 protein with host cellular proteins result in suppression of host interferon responses through different interferon-suppression mechanisms (Biswal *et al.*, 2024; Gracias *et al.*, 2023). The conserved nature of flavivirus NS5 protein across flaviviruses and its roles in flavivirus replication and immune evasion makes it one of the most promising and attractive targets for

Table 3. List of top three pathways from Gene Ontology (GO) enrichment analysis of the identified human brain cDNAs

Pathways	Enrichment FDR	Fold Enrichment	Genes
Chemical carcinogenesis-reactive oxygen species	2.6E-02	16.2	UQCRC2, VDAC2, CBR1
Parkinson disease	2.6E-02	13.6	PINK1, UQCRC2, VDAC2
Necroptosis	5.9E-02	15.2	TRPM7, VDAC2

broad spectrum anti-flavivirus therapeutic (Fernandes *et al.*, 2021). There are several potent inhibitors of these enzymatic domains of flavivirus proteins (Lim *et al.*, 2015). However, rapid emergence of escape drug-resistant viral mutants is the major setback for this drug development. Therefore, host-oriented therapeutic targeting of host proteins has become important for flaviviral therapeutic development.

To achieve protein-protein interactome generation, an *ex situ* binding assay that depends on a systemic biology approach was used (Gillen & Nita-Lazar, 2019). Using a high-throughput yeast two-hybrid assay, we identified 20 human brain proteins physically interacting with the JEV NS5 protein. These newly identified human brain cellular partners considerably expand the number of brain proteins potentially involved at some point in the JEV life cycle and subsequent neuropathology. Of the various JEV-NS5 interacting proteins reported in different studies using various mammalian cell origins and human cDNA libraries from the liver, brain, spleen, and bronchial epithelia (Kovanich *et al.*, 2019; Le Breton *et al.*, 2011), only CBR1 was identified with the uncharacterized DKFZp434H018 gene in this study. It is therefore worth noting that most of the brain cellular targets identified in this study have not been previously reported in the literature and are required to be validated as JEV host factors interacting partners. Among the identified genes, QKI, ZNF597, ZBTB20, ATRX, and ZNF106 represent transcriptional and translational regulation, suggesting that JEV NS5 interacts with host proteins that are important for cell replication and repair mechanisms (Sehrawat *et al.*, 2021; Yang *et al.*, 2009; Ye *et al.*, 2010, 2017).

Pathway enrichment analysis of these genes identified three important pathways associated with JEV neuropathology; chemical carcinogenesis-reactive oxygen species, Parkinson's disease and necroptosis. JEV-infected rat models have been used to study neurodegenerative processes seen in Parkinson's disease due to the ability of the virus to induce Parkinson's disease pathological features and alteration of the brain catecholamine (Hamaue *et al.*, 2006). This study, however, confirms the Parkinsonian features seen in a survivor of JE (Tadokoro *et al.*, 2018). Furthermore, it also highlights the importance of Parkinson's disease biological pathway in JEV neuropathology. JEV receptors such as GRP78, Src, caveolin-1, TLR7, dopamine receptor D2 and pro-inflammatory mediators such as IL-6 and IL-1 β in JEV-induced inflammatory responses in murine neuroblastoma, human astrocytoma and astrogloma cell lines have been implicated in promoting tumor invasion and metastasis by inducing oxidative stress mechanism (Dong *et al.*, 2010; Mishra *et al.*, 2008; Yadav *et al.*, 2022). Therefore, this study requires further research to unravel the roles of genes identified for chemical carcinogenesis-reactive oxygen species biological pathway (UQCRC2, VDACC2, CBR1) in JEV induced malignant progression. Additionally, TRPM7 and VDACC2 are genes enriched for necroptosis in JEV neuropathology. These genes are different from mixed-lineage kinase domain-like protein (MLKL) mediated necroptosis in JEV neuroinflammation (Bian *et al.*, 2017), which requires further study of their roles in necroptosis in JEV neuropathology.

CONCLUSION AND RECOMMENDATIONS

This study emphasizes the importance of flavivirus NS5 protein in immune evasion and neuropathology in JEV infections. Notably, the interaction of JEV NS5 protein with brain proteins implicated in transcriptional and translational regulation, neurodegenerative processes, and tumorigenesis highlights the role of chemical carcinogenesis-oxidative stress, Parkinson's disease, and necroptosis biological pathways in JEV neuropathology. Further research should focus on these novel brain proteins and investigate their roles (UQCRC2, VDACC2, CBR1, TRPM7) in JEV-related neurodegeneration and cancer-related pathways to advance therapeutic strategies against flavivirus-induced neuropathology. Furthermore, this study

could be expanded by performing Y2H screening using multiple host libraries since JEV infection evolves in multiple hosts including its reservoir (pig, ardeid bird), vector (mosquito), and dead-end hosts (human, horse). Moreover, validation of Y2H assay results would be useful to understand the mechanism of protein. For example, Western blotting assay may detect specific JEV NS5 protein among a mixture of proteins in the yeast system which in turn will provide insight on the engagement of functional viral protein during Y2H assay. In addition, by performing a mammalian two-hybrid assay, the resulting positive host-virus protein-protein interactions from the Y2H assay could be useful since the post-translational changes of foreign proteins are more likely to display their true conformations due to the mammalian two-hybrid experiments are carried out in mammalian cells, which better stimulates the real *in vivo* interactions.

Conflict of Interest

The authors declare that they have no conflicts of interest.

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