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*Original Article*

# Protein-protein interactions between *A. aegypti* midgut and dengue virus 2: two-hybrid screens using the midgut cDNA library

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## Abstract

Introduction: Dengue virus (DENV) is principally transmitted by the *Aedes aegypti* mosquito. To date, mosquito population control remains the key strategy for reducing the continuing spread of DENV. The focus on the development of new vector control strategies through an understanding of the mosquito-virus relationship is essential, especially targeting the midgut, which is the first mosquito organ exposed to DENV infection.

Methodology: A cDNA library derived from female adult *A. aegypti* mosquito midgut cells was established using the switching mechanism at the 5' end of the RNA transcript (SMART), in combination with a highly potent recombination machinery of *Saccharomyces cerevisiae.* Gal4-based yeast two-hybrid (Y2H) assays were performed against DENV-2 proteins (E, prM, M, and NS1). Mammalian two-hybrid (M2H) and double immunofluorescence assays (IFA) were conducted to validate the authenticity of the three selected interactions.

Results: The cDNA library was of good quality based on its transformation efficiency, cell density, titer, and the percentage of insert size. A total of 36 midgut proteins interacting with DENV-2 proteins were identified, some involved in nucleic acid transcription, oxidoreductase activity, peptidase activity, and ion binding. Positive outcomes were obtained from the three selected interactions validated using M2H and double IFA assays.

Conclusions: The identified proteins have different biological activities that may aid in the virus replication pathway. Therefore, the midgut cDNA library is a valuable tool for identifying DENV-2 interacting proteins. The positive outcomes of the three selected proteins validated supported the quality of the cDNA library and the robustness of the Y2H mechanisms.

**Key words:** dengue virus; *Aedes aegypti*; yeast two-hybrid; cDNA library; protein-protein interaction.

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## Introduction

Insect-borne diseases, particularly those transmitted by mosquitoes, are among the leading causes of mortality and morbidity in humans. Dengue hemorrhagic fever (DHF)/dengue shock syndrome (DSS) – a globally emerging insect-borne disease threatening a third of the human population [1] – is transmitted to humans by *Aedes aegypti* and *Aedes albopictus*. During a blood meal of a dengue-infected person by *Aedes* mosquito, dengue virus (DENV) is ingested into the mosquito's midgut. It is generally believed that the mosquito midgut carries important cellular membrane receptors that facilitate viral entry through receptor-mediated endocytosis [2,3], enabling replication and exocytosis, followed by DENV disseminations to salivary glands for transmission to humans [4,5].

Dengue virus, a member of the genus *Flavivirus* and family *Flaviviridae*, is an enveloped positive single-stranded RNA virus approximately 50 nm in diameter [6]. Its 11 kb RNA genome encodes a polyprotein that, after translation, is cleaved into 10 individual proteins during maturation by host proteases (*e.g.*, furin) and viral protease complex. The 10 proteins are designated as capsid (C), pre-membrane (prM), envelope (E), and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) [7,6] (Figure 1a). Despite continued studies on the existence of the fifth DENV serotype [8,9], DENV exists as four serotypes (DENV1-4) and is related to the viruses that cause yellow fever, Japanese, St. Louis, tick-borne, and West Nile encephalitis.

Most previous studies on DENV replication mechanisms have been conducted on mammals or mammalian cell lines [10-14]. These studies emphasized proteins or host cellular factors that may confer DENV susceptibility, with less research focus on DENV infection in *A. aegypti* and *A. Albopictus* mosquitoes. Although tubulin/tubulin-like protein and prohibitin were described as putative DENV receptors in mosquitoes [15,16], these studies utilized virus overlay protein binding assay (VOPBA), a technique that was not designed for direct *in vivo* biological interaction. Furthermore, the capability of the VOPBA method is very limited, as most of the identified proteins can only be reported as molecular weights [17-21].

To date, very few studies have been conducted to determine the interactome between DENV proteins and mosquito cellular proteins using sensitive and reliable protein interaction assays [22,23], with some predictions reported through a computational approach [24]. Hence, the aim of this study was to identify adult *A. aegypti* mosquito midgut proteins interacting with DENV-2 viral proteins, using an improved yeast two-hybrid (Y2H) screening system, a high-throughput screening assay that allows identification of genuine protein interacting partners *in vivo*. This study revealed a list of putative protein interacting partners in mosquitos during DENV infection. Some of these interactions were further validated by additional assays such as mammalian two-hybrid (M2H) and double immunofluorescent assay (IFA). This study provides new insights into possible virus-vector interactions.

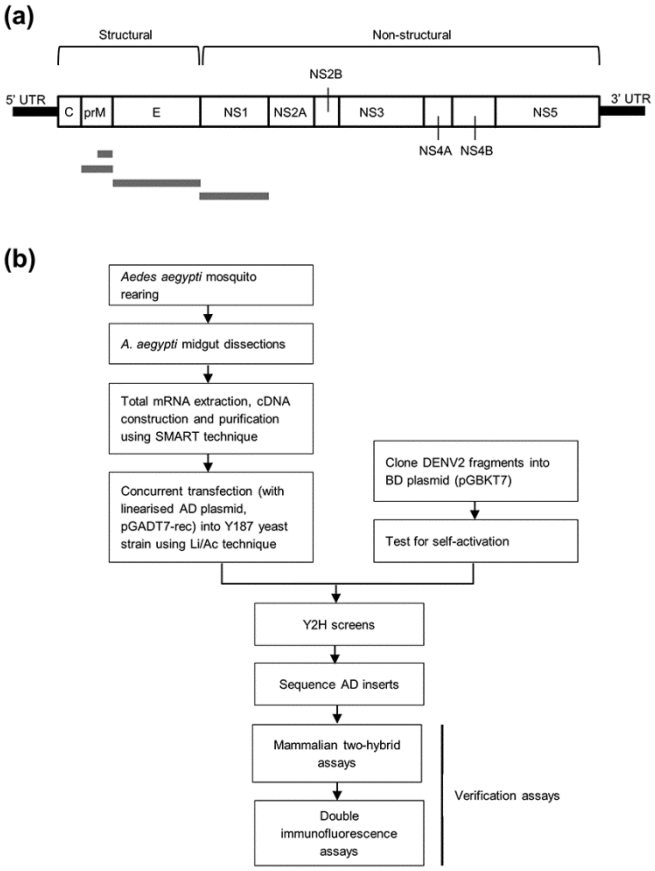
## Methodology

The workflow of this study is summarized in Figure 1b. The protocols of each experiment are stated in detail in the following sections.

*DENV-2 and the cloning of viral genes*

DENV-2 (clinical sample number: MY89-88549; GenBank accession number: AJ556804) was kindly provided by Professor Sazaly Abu Bakar of the University of Malaya. DENV-2 was propagated in C6/36 (ATCC CRL-1660) and Vero cells (ATCC CCL-81) until the development of a cytopathic effect. The virus titer was determined at $10^6$ $TCID_{50}$/mL. The nucleotide sequences of DENV-2 genes (E, prM, M, and NS1) were obtained from the National Centre for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/). These genes were amplified using *Pfu* DNA polymerase and cloned in-frame into Y2H bait vector pGBKT7 (Clontech, Mountain View, USA), namely pGBKT7-E, pGBKT7-prM, pGBKT7-M, and pGBKT7-NS1 (Figure 1a), respectively. All plasmid constructs were sequenced with neither missense nor nonsense mutation.

**Figure 1.** Overview of yeast two-hybrid (Y2H) screens to identify putative *A. aegypti* midgut cellular proteins interacting with DENV-2 proteins. (**a**) The organization of DENV-2 genome and fragments (in grey) used in Y2H screens with *A. aegypti* midgut cDNA library. (**b**) Flowchart of the Y2H approach to screen for *A. aegypti* proteins targeted by DENV-2.




*Mosquito rearing*

Mosquito (*A. aegypti*) colonies (Linnaeus) [25,26] were established and maintained at 28 ± 1°C under 70%–75% relative humidity, with a light/dark cycle of 12 hours/12 hours. Newly hatched larvae were reared in trays, each containing 750 mL of mineral water, with a water level of about 2.5 cm. Each tray was provided with five cat food pellets (Friskies Senior). Adult mosquitoes were given *ad libitum* access to 10% sucrose solution. No blood was provided throughout the lifespan of the mosquitoes.

**Table 1.** Identification of putative *Aedes aegypti* mosquito midgut proteins interacting with their respective DENV2 proteins through Y2H screens

| DENV2 proteins | *Ae. aegypti* gene ID (VectorBase) | Gene names (*Aedes aegypti*) | Size of CDS (bp) |
|---|---|---|---|
| NS1 | AAEL003582[a] | Ribosomal protein S15p/S13e | 456 |
| | AAEL008169 | 40S ribosomal protein S12 | 333 |
| | AAEL008481 | 60S ribosomal protein L18 | 570 |
| | AAEL015803 | 5.8S ribosomal RNA | 153 |
| | AAEL005732 | Acyl-coa dehydrogenase | 1,209 |
| | AAEL000393 | Suppressors of cytokine signaling | 597 |
| | AAEL012713 | Clip-domain serine protease family C | 1,092 |
| | AAEL007683 | DNA topoisomerase | 3,066 |
| | AAEL002827 | ATP synthase beta subunit | 1,515 |
| | AAEL012947 | Hypothetical protein | 2,070 |
| | AAEL001872 | Voltage-dependent anion-selective channel | 849 |
| | AAEL003393 | ATP synthase beta subunit | 1,515 |
| | AAEL007948 | Glutathione-s-transferase theta | 672 |
| | AAEL004869 | Hypothetical protein | 1,578 |
| | AAEL015740 | Small subunit ribosomal RNA, 5' domain | 592 |
| | AAEL004493 | Ribosome biogenesis protein tsr1 | 2,430 |
| prM | AAEL007696 | TOLL pathway signalling NF-kappaB Relish-like transcription factor | 1,740 |
| | AAEL005508[a] | NADH-ubiquinone oxidoreductase 24 kda subunit | 723 |
| | AAEL006447 | GATA transcription factor (GATAb) | 2,313 |
| | AAEL000987 | 60S ribosomal protein L8 | 786 |
| | AAEL005097 | cold induced protein (BnC24A) | 432 |
| | AAEL011282 | Ribosomal RNA small subunit methyltransferase b (sun) | 2,439 |
| | AAEL008599 | Zinc carboxypeptidase | 1,182 |
| | AAEL005981 | Class B scavenger receptor (CD36 domain) | 1,494 |
| | AAEL015740 | Small subunit ribosomal RNA, 5' domain | 592 |
| | AAEL004493 | Ribosome biogenesis protein tsr1 | 2,430 |
| E | AAEL010974 | Hypothetical protein | 3,021 |
| | AAEL003530 | Acidic ribosomal protein P1 | 339 |
| | AAEL009151 | 30S ribosomal protein S8 | 393 |
| | AAEL001863 | Zinc carboxypeptidase | 1,239 |
| | AAEL005374 | Sensory neuron membrane protein-1 | 1,590 |
| | AAEL007818[a] | Trypsin | 765 |
| | AAEL008599 | Zinc carboxypeptidase | 1,182 |
| M | AAEL007974 | Hypothetical protein | 2,166 |
| | AAEL009888 | Bumetanide-sensitive Na-K-Cl cotransport protein | 3,192 |
| | AAEL009588 | Expressed protein (HR3) | 1,407 |
| | AAEL012781 | Protease m1 zinc metalloprotease | 1,635 |
| | AAEL000511 | Acetylcholinesterase | 2,109 |
| | AAEL007649 | Cell cycle checkpoint protein rad17 | 3,243 |
| | AAEL005981 | Class B scavenger receptor (CD36 domain) | 1,494 |
| | AAEL007818 | Trypsin | 765 |
| | AAEL005374 | Sensory neuron membrane protein-1 | 1,590 |

[a] Protein interactions were validated by additional assays: mammalian two-hybrid and double immunofluorescent assays; CDS: coding sequence.

*cDNA library construction*

Adult mosquitoes were harvested and stored at -80°C. Midguts of 50 female adult *A. aegypti* mosquitoes were dissected as previously described [27], and were subjected to total RNA extraction by a combination method of both Trizol reagent (Life Technologies, Carlsbad, USA) and Dynabeads mRNA purification kit (Life Technologies) [28]. Purified mRNA served as the template for the first- and second-strand cDNA synthesis, using Make Your Own "Mate & Plate" Library System (Clontech). This system uses the SMART (switching mechanism at 5' end of the RNA transcript) cDNA synthesis technology that allows the construction of cDNA libraries from any tissue source. Double-stranded cDNA was purified, and nucleic acid less than 400 bp was discarded using Chroma Spin TE-400 Columns (Clontech). These were unwanted oligonucleotide products of incomplete first- and second-strand cDNA synthesized. To allow *in vivo* recombinational cloning, purified double-stranded cDNA, in conjunction with 3 µg of linearized pGADT7-rec vector, and 200 µg of denatured Yeastmaker Carrier DNA (Clontech) were co-transformed into competent yeast Y187 cells using the lithium acetate (LiAc) method as previously described [29]. Transformed yeast cells were mixed with Yeast-Peptone-Dextrose (YPD) Plus medium (Clontech) for better transformation efficiency. Then, pelleted cells were re-suspended in 15 mL of 0.9% (w/v) NaCl solution (primary cDNA library), prior to spreading on leucine-depleting agar plates (SD/-Leu). After four-day incubation at 30°C, surviving colonies were pooled and kept in 50 mL or 1 mL aliquots for storage in -80°C until use (amplified cDNA library). The transformation efficiency, library titer of primary and amplified cDNA library, and library quantity were calculated according to the formulas described previously [30]. These were checked by spreading 100 µL of $10^{-1}$, $10^{-2}$, $10^{-3}$, and $10^{-4}$-diluted yeast culture on SD/-Leu agar plates (diluted in 0.9% NaCl solution), incubated for four days at 30°C. Then, yeast colony-forming unit/mL (cfu/mL) was determined and cell density was measured using a hemocytometer. On the other hand, the library complexity test was conducted by randomly selecting 20 independent colonies (out of 75 colonies on the SD/-Leu agar plate of $10^{-3}$ dilution). The colonies were screened for cDNA inserts using Matchmaker 5' and 3' AD LD-Insert Screening Amplimers (Clontech). The polymerase chain reaction (PCR) cycling parameters were as follows: 98°C for 2 minutes; 40 cycles of 98°C for 10 seconds, 60°C for 15 seconds, 68°C for 2.5 minutes; 68°C for 5 minutes; hold at 4°C.

*DNA-binding domain (DNA-BD) yeast preparation*

Yeast strain Y2HGold was transfected with respective bait plasmids, and maintained on synthetic dropout (SD) medium lacking tryptophan (SD/-Trp). All DNA-BD yeast clones were tested for autoactivation in the absence of prey protein. No blue colonies were observed on SD/-Trp and SD/-Trp/X-α-gal agar plates, and no surviving colony was found on SD/-Trp/X-α-gal/Aureobasidin A (Aba, 125 ng/mL) plates. This indicated the inability of the DNA-BD yeast clones to autoactivate the reporter genes in the absence of interacting prey protein.

*Yeast two-hybrid screenings*

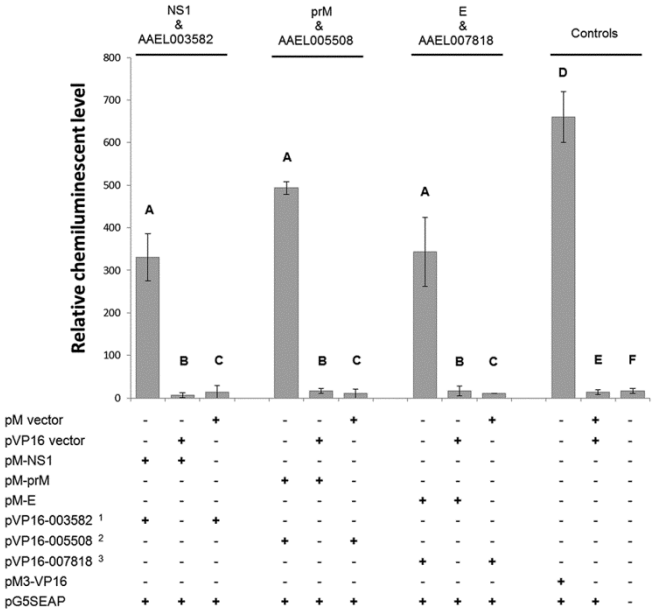
Y2H was conducted using Matchmaker Gold Yeast Two-Hybrid System (Clontech), according to the manufacturer's instructions. In brief, an overnight 5 mL Y2HGold culture and a 1 mL library aliquot were mixed, and mating was allowed for 24 hours in 50 mL of 2x YPDA broth (30°C, 45 rpm). Yeast cells were plated and incubated at 30°C for two days on low-stringency agar plates (SD/-Trp/-Leu, DDO) in the presence of Aba and X-α-gal, followed by high-stringency agar plates (SD/-Leu/-Trp/-Ade/-His, QDO) supplemented with Aba and X-α-gal. Agar plates were incubated in 30°C for four to six days. Blue colonies were marked and subjected to plasmid extractions and DNA sequencing for cDNA identification. Identified genes were classified into different categories according to their consistent descriptions of gene products across databases by Gene Ontology Consortium (http://geneontology.org/), through EnsemblMetazoa (*Aedes aegypti* Assembly and Gene Annotation, http://metazoa.ensembl.org/Aedes_aegypti/Info/Annotation).

*Mammalian two-hybrid analyses*

Three protein interactors (AAEL003582, AAEL005508, and AAEL007818) were selected for M2H analyses. All three genes were cloned in-frame into plasmid vector pVP16 (DNA-AD) using primers specific to ribosomal protein S15p/S13e (forward: GGGAATTCGGTCGTATGCACGCTCCCGGTAAG; reverse: ACGGATCCGGCAACCAGGGCCGAGGCGGTG), NADH-ubiquinone oxidoreductase (forward: GGGAATTCCTGACAAACTCCTTTAAAATAATTC; reverse:

ACGGATCCAAAGCCTGTTTGCATTCCGAATC), and trypsin (forward: GGGAATTCAACCAATTTCTCTTTGTCAG; reverse: ACGGATCCAACCTCGGAAACCTCTCGGATC). Restriction sites are underlined. As shown in Table 1, their respective viral protein interacting partners were cloned in-frame into pM (GAL4 DNA-BD) cloning vector (Matchmaker Mammalian Assay Kit 2, Clontech). Using calcium phosphate transfection technique [31], along with pG5SEAP, all plasmids were co-transfected into Vero cells (ATCC CCL-81), according to the scheme in Figure 2. This assay was conducted in biological triplicate. Transfected cells were incubated at 37°C, 5% $CO_2$ for 48 hours before culture media were harvested for SEAP activity measurements using GreatEscAPe SEAP Chemiluminescence Detection Kit (Clontech). The intensity of chemiluminescence signals were measured by VICTOR X5 Multilabel Plate Reader (PerkinElmer, Massachusetts, USA).

**Figure 2.** Mammalian two-hybrid (M2H) assays to validate the the interaction between *A. aegypti* midgut proteins and DENV-2 proteins in Vero cells. Columns show mean values, error bars standard deviation of three samples (n = 3). Each column represents a treatment and is labelled "+" for the plasmid transfected and "-" for the plasmid that is absent. Each independent experiment was constituted of 3 assays, labelled A, B, and C. Cells were transfected with: (**A**) pM & pVP16 vectors with respective cDNA inserts; (**B**) pM with cDNA inserts & empty pVP16 vectors; (**C**) empty pM vectors & pVP16 with cDNA inserts. For controls, cells were transfected with: (**D**) positive control plasmid pM3-VP16; (**E**) empty pM & pVP16 plasmids. (**F**) Non-treated controls.




[1] *Aedes aegypti* Ribosomal protein S15p/S13e (AAEL003582)
[2] *Aedes aegypti* NADH-ubiquinone oxidoreductase (AAEL005508)
[3] *Aedes aegypti* Trypsin (AAEL007818)

*Double immunofluorescence assays*

The cDNA of *A. aegypti* proteins (AAEL003582, AAEL005508, and AAEL007818) were amplified and cloned in-frame into pIB/V5-His vector (Invitrogen, Carlsbad, USA). These plasmid constructs were transfected into C6/36 (ATCC CRL-1660) of 80% confluency using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. After 24-hour incubation at 28°C, cells were infected with DENV-2 (Multiplicity of infection (MOI): 1.0), followed by an additional 48-hour incubation under the same conditions. Then, cells were fixed with 4% paraformaldehyde (15 minutes, room temperature). Background blocking was performed with 1% bovine serum albumin (BSA) in 1x saline sodium citrate (SSC) buffer (20 minutes, room temperature). Proteins of interest were captured by anti-V5 antibody (Invitrogen) and each respective anti-DENV-2 antibody (GeneTex Inc.: GTX103346, GTX128093, GTX103345), followed by fluorescent staining using AlexaFluor 488 and 594 (Invitrogen). Cells were observed using an Olympus IX81 fluorescent microscope. Image processing was performed using cellSens software (Olympus, Tokyo, Japan).

## Results

*Detection of the transformation efficiency, library titer, and library quantity of the cDNA library*

The transformation efficiency of the primary cDNA library was calculated at $1.125 \times 10^6$ transformants (> $1 \times 10^6$ transformants), and the library titer was $7.5 \times 10^4$ cfu/mL (> $6.7 \times 10^4$ cfu/mL was suggested to be optimal by the manufacturer). After library amplification, cell density was adjusted to $8.23 \times 10^8$ cells/mL (> $2 \times 10^7$ cells/mL) by reducing the volume of the suspension by centrifugation. The amplified cDNA library titer was determined to be $2.13 \times 10^7$ cfu/mL, with the library quantity of $1.491 \times 10^9$ cfu (Table 2).

*Identification of the length of the inserts*

In Figure 3, most of the fragments were more than 250 bp, and samples 11, 14, 15, and 20 carried fragments of more than 3 kb. Since the cDNA inserts with various lengths were detected in 20/75 colonies at $10^{-3}$ dilution, this reflects the high complexity of cDNA inserts of various genes present in the undiluted library.

**Table 2.** The transformation efficiency, insert sizes, and quality of the *Aedes aegypti* mosquito midgut cDNA library

| | cDNA library | Expected |
|---|---|---|
| Transformation efficiency | $1.125 \times 10^6$ transformants/3 µg pGADT7-Rec | $\geq 1 \times 10^6$ transformants/3 µg pGADT7-Rec |
| Total number of independent yeast colonies | $1.125 \times 10^6$ | $\geq 1 \times 10^6$ |
| Insert size (kb) | | |
| Minimum screened | 0.15 kb | |
| Maximum screened | 3.24 kb | |
| Average | 1.40 kb | |
| Cell density of frozen library (cells/mL) | $8.23 \times 10^8$ cells/mL | $> 2 \times 10^7$ cells/mL |
| cDNA library titer (cfu/mL) | | |
| Primary cDNA library | $7.5 \times 10^4$ cfu/mL | $6.7 \times 10^4$ cfu/mL |
| Amplified cDNA library | $2.13 \times 10^7$ cfu/mL | $> 1 \times 10^7$ cfu/mL |
| cDNA library quantity | | |
| Primary cDNA library | $1.125 \times 10^6$ cfu | |
| Amplified cDNA library | $1.491 \times 10^9$ cfu | |

CFU: colony-forming unit

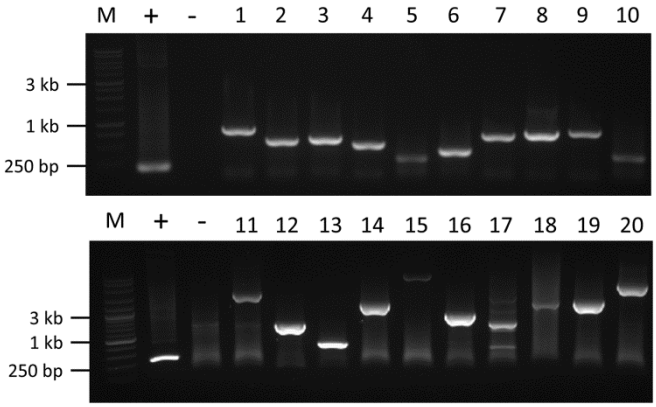
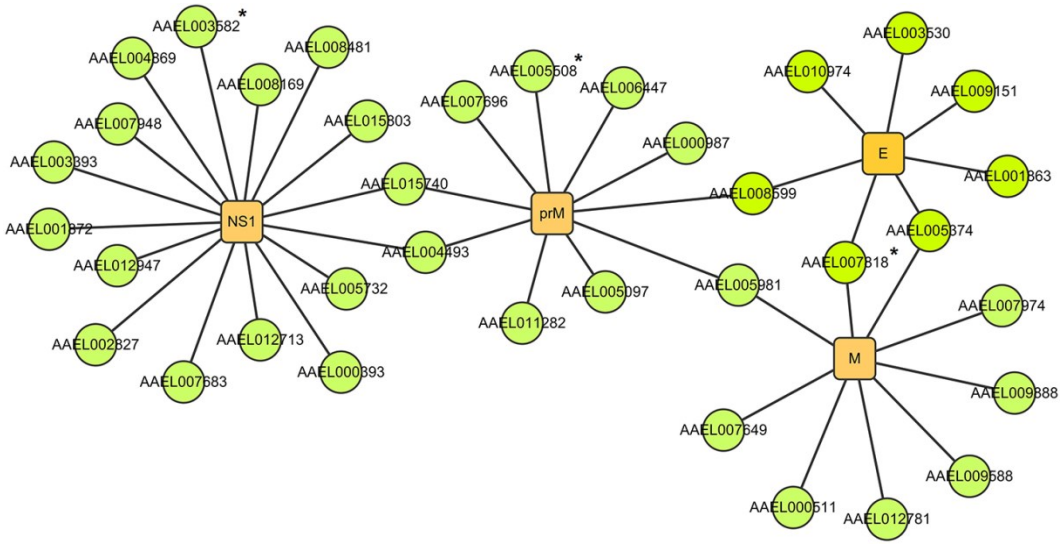



**Figure 3.** Library complexity check via colony PCR amplification from 20 randomly-selected yeast colonies. (+) Positive controls, amplification of the empty vector pGADT7 using Matchmaker 5' and 3' AD LD-Insert Screening Amplimers. (-) Negative controls, template was substituted by nuclease-free water. (**M**) DNA marker. (**1-20**) 20 randomly selected yeast colonies from $10^{-3}$-dilution SD/-Leu agar plate yielded individual cDNA fragments ranged between 300 bp to 4,500 bp. This indirectly reflects the high cDNA complexity present in the undiluted library.

**Figure 4.** The putative *A. aegypti* midgut cellular protein candidates which were suggested to interact with their respective DENV-2 proteins. Square nodes represent DENV-2 proteins while round nodes represent proteins identified from female adult *A. aegypti* midguts. Nodes are connected to indicate the protein-protein interactions found in our yeast two-hybrid screenings in this study. Proteins marked asterisks (*) were subjected to mammalian two-hybrid and double immunofluorescent assays. Protein IDs are in accord with VectorBase Bioinformatics Resource Centre.



*Identification of the recombination rates of the cDNA library*

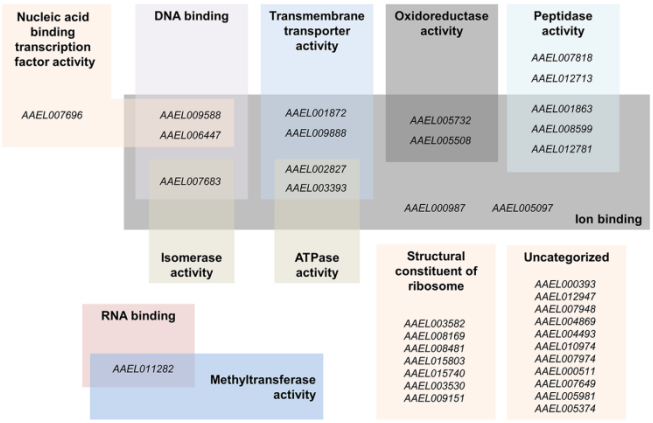
The insert fragment length of the screened cDNA library ranged from 153 bp to 3,243 bp, with an average length of 1,403 bp (Table 2). Six samples were between 0.3 and 0.5 kb (14.29%), ten samples were between 0.5 and 1.0 kb (23.81%), eight samples were between 1.0 and 1.5 kb (19.05%), seven samples were between 1.5 and 2.0 kb (17.07%), seven samples were between 2.0 and 2.5 kb (17.07%), and four samples were more than 2.5 kb (9.52%) (Table 3). These data were obtained from the identified putative protein-protein interactions listed in Table 1.

*Mapping DENV-2 interactomes in Aedes aegypti*

A total of 36 putative protein-protein interactions between the midgut cells of *A. aegypti* and DENV-2 proteins (E, prM, M, NS1) were identified. A biological network showing information of protein-protein interaction was constructed and visualized using Cytoscape software (Figure 4). There were 40 nodes (proteins) and 42 edges (interactions) in the network. Protein IDs were in accord with VectorBase Bioinformatics Resource Centre (https://www.vectorbase.org/). Table 1 tabulates the details of each interactions including gene IDs, coding sequence (CDS) lengths, deduced protein sizes (kDa), and protein identities.

The cDNA identification analysis showed a number of putative *A. aegypti* midgut protein interaction partners of their respective DENV-2 proteins. The results suggested that 16 proteins interacted with DENV-2 NS1 protein, 10 proteins with DENV-2 prM protein, 7 proteins with DENV-2 E protein, and 9 proteins with DENV-2 M protein (Table 1). Although the genome of *A. aegypti* has been published [32], several hypothetical proteins of minimal match with any gene of other species were listed (AAEL004869, AAEL010974, AAEL007974). In addition, identified genes were systemically classified into different categories according to the Gene Ontology molecular function, including structural constituent of ribosome, oxidoreductase activity, ion binding, peptidase activity, DNA binding, isomerase activity, ATPase activity, transmembrane transporter activity, nucleic acid binding transcription factor activity, RNA binding, and methyltransferase activity (Figure 5). However, categorization of a few genes was not possible since there was no information available about the molecular function of the gene products annotated as of the date the annotation was made (Figure 5).

**Figure 5.** Identified genes derived from *A. aegypti* midguts were classified into different categories according to their consistent descriptions of gene products (molecular function) across databases by Gene Ontology Consortium (http://geneontology.org/), through EnsemblMetazoa (*Aedes aegypti* Assembly and Gene Annotation, http://metazoa.ensembl.org/Aedes_aegypti/Info/Annotation). Some genes with multiple descriptions were located in the overlapped regions of various boxes labelled with different titles of molecular functions.




*Identified interactomes validated in mammalian systems*

Mammalian-two hybrid (M2H) was also used to complement the Y2H study, since mammalian cells used in the M2H system undergo more comprehensive post-translational modifications of proteins, hence may better mimic *in vivo* interactions [33]. M2H assays using Vero cells were conducted to validate the three selected *A. aegypti* putative protein candidates found interacting with their respective DENV-2 proteins. These proteins were ribosomal protein S15p/S13e

**Table 3.** Insert fragments length by sequencing

| Insert size (kb) | Number of fragments | Rate (%) |
|---|---|---|
| 0.3–0.5 | 6 | 14.29 |
| 0.5–1.0 | 10 | 23.81 |
| 1.0–1.5 | 8 | 19.05 |
| 1.5–2.0 | 7 | 17.07 |
| 2.0–2.5 | 7 | 17.07 |
| > 2.5 | 4 | 9.52 |

(VectorBase: AAEL00358), NADH-ubiquinone oxidoreductase (VectorBase: AAEL005508), and trypsin (VectorBase: AAEL007818). The reporter plasmid, pG5SEAP, when activated by the physical interaction of pM- and pVP16-conjugated proteins, encodes alkaline phosphatase, which is secreted to the extracellular environment. Hence, the activity of alkaline phosphatase is a quantitative reflection of the interaction between pM- and pVP16-conjugated proteins. In Figure 2, the alkaline phosphatase activities measured from each conjugated protein partner (Figure 2A) were significantly higher than the background controls (Figure 2B, C, and E) and negative controls (Figure 2F), but lower than positive controls (Figure 2D). Cells in positive controls were transfected with pM3-VP16, which expressed fusion proteins consisting of Gal4 DNA-BD and VP16 AD.

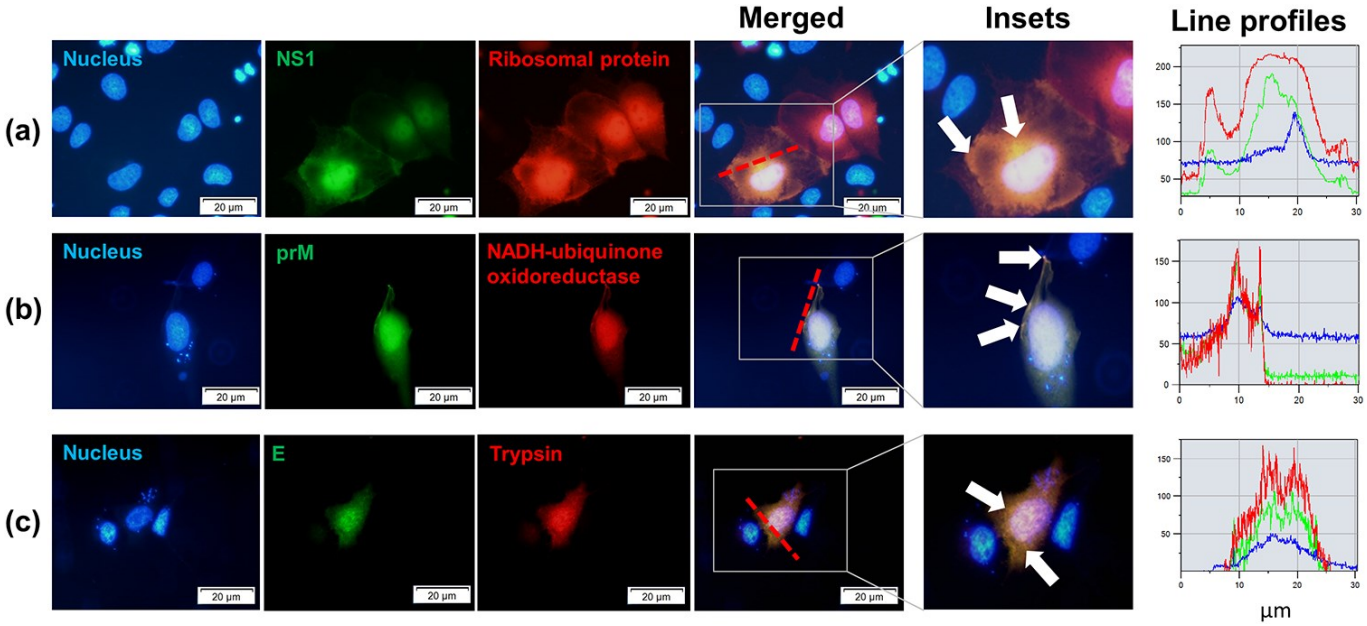
*Double immunofluorescence cellular co-localizations of mosquito and DENV-2 proteins*

Double IFA enables the visualization of intracellular proteins. Figure 6 illustrates the cellular distribution and co-localization of DENV-2 proteins (NS1, prM, and E) and *A. aegypti* midgut proteins (ribosomal protein, NADH-ubiquinone oxidoreductase, and trypsin) after transfection of V5-tagged plasmid constructs into C6/36 cells, followed by infection with DENV-2. White arrows in the merged images showed the regions where the protein-protein co-localizations may occur. Line profiles demonstrate the intensities of blue, red, and green fluorescence across the red lines shown in the merged images (Figure 6).

## Discussion

In this study, a cDNA library of the midgut of female adult *A. aegypti* was constructed and applied to Y2H screens against DENV-2 proteins (E, prM, M, and NS1). Previous Y2H technology is generally known to generate a high false-positive rate; however, the Matchmaker Gold Yeast Two-Hybrid System (Clontech) used in this study has been optimized and improved by the manufacturer for a remarkable reduction of false positivity [34]. A number of studies have revealed novel protein-protein interaction using the Matchmaker Gold Yeast Two-Hybrid System [35-37]. However, in order to validate the quality of the cDNA library constructed, and to ensure the high performance of the Y2H mechanisms employed, we performed additional assays including M2H and

**Figure 6.** Cellular co-localization of *A. aegypti* midgut proteins and DENV-2 proteins in DENV-2-infected C6/36 cells. The plasmid constructs (pIB/V5-His) carrying the cDNA of midgut proteins were transfected into C6/36 cells, which were subsequently infected with DENV-2. Midgut proteins (ribosomal protein, NADH-ubiquinone oxidoreductase, trypsin) were captured using anti-V5 antibody, while DENV-2 proteins were captured using antibody against NS1, prM, and E, respectively. Nuclei were stained with DAPI prior to viewing under a fluorescent microscope. Co-localization of: (**a**) ribosomal protein S15p/S13e (VectorBase: AAEL003582) and DENV-2 NS1 protein; (**b**) NADH-ubiquinone oxidoreductase (VectorBase: AAEL005508) and DENV-2 prM protein; (**c**) trypsin (VectorBase: AAEL007818) and DENV-2 E protein. Co-localizations are indicated by white arrows. Line profiles were shown to demonstrate the intensity of red, green, and blue fluorescence across the cross-sectional regions indicated by the red dotted lines in the merged images.



double IFA on three selected mosquito midgut proteins, *i.e.*, ribosomal protein S15p/S13e (VectorBase: AAEL003582), NADH-ubiquinone oxidoreductase (VectorBase: AAEL005508), and trypsin (VectorBase: AAEL007818), which interact with NS1, prM and E proteins, respectively.

Previously, the first draft of the mosquito protein interaction network using a computational approach was presented by Guo *et al*. The research group reported 714 *A. aegypti* proteins with closely related functions in the replication/transcription/translation, immunity, transport, and metabolic pathways [24]. In another study, Mairiang *et al*. identified several mosquito protein interacting partners of DENV-2 C, prM, NS3, NS4A, NS4B, and NS5 proteins [23]. Interestingly, the study identified similar proteins that were also identified in our study. These include human ribosomal protein and mosquito carboxypeptidase, which were suggested to interact with the DENV C protein [23]. However, although most of the earlier studies have identified putative interacting partners of DENV proteins, very limited information is available on mosquito protein interactors of DENV E and NS1 proteins. In our study, beside prM and M, we also identified mosquito midgut proteins interacting with DENV-2 E and NS1 proteins using Y2H screenings, which have not been performed elsewhere.

A number of ribosome-related genes were identified in this study. Despite its crucial role in protein translation, ribosomal protein's involvement in RNA virus replication and dissemination has been well studied [38,39]. A few recently published reports demonstrated the versatility of ribosomal proteins in either facilitating or inhibiting viral growth during infection [40-42]. In addition, although ribosome has traditionally been thought to function as the catalytic machinery for translational elongation, Lee *et al*. also showed that ribosomal subunit protein rpL40 acts as a requisite for vesicular stomatitis virus (VSV) cap-dependent translational regulation [43].

As for proteins involved in transcription, our results suggested that *A. aegypti* Toll pathway signalling NF-kappaB Relish-like transcription factor (VectorBase: AAEL007696), GATA transcription factor (GATAb) (VectorBase: AAEL006447), and HR3 protein (VectorBase: AAEL009588) interact with DENV-2 prM or M proteins. This finding may be linked to the ability of RNA viruses (including DENV) to regulate its host cell gene expression profiles [44].

Another DENV-2 prM protein interacting partner, NADH-ubiquinone oxidoreductase, is a 24 kDa subunit (VectorBase: AAEL005508) protein with provisional function similar to NADH-ubiquinone oxidoreductase found in mammals. NADH-ubiquinone oxidoreductase catalyses NADH to $NAD^+$, reduces ubiquinone, and transports protons across the inner membrane of mitochondria. Meanwhile, this enzyme also reduces $O_2$ to superoxide, leading to cellular oxidative stress [45]. Previous studies reported the generation of superoxide in mosquito cells during DENV infection [46], and the overexpression of quinone oxidoreductase has been recognized as the major contributor to reactive oxygen species formation [47]. Since the accumulation of this enzyme in the midgut of DENV-infected mosquito was observed [48], the biological interaction between DENV-2 prM protein and *A. aegypti* NADH-ubiquinone oxidoreductase may be the major contributor in the regulation of the oxido-reduction mechanism to manipulate DENV infection in mosquitoes.

Class B scavenger receptor CD36 (SRB), a cell surface glycoprotein, is present on a variety of cell types, including *A. aegypti* hemocytes [49]. The possible interaction between *A. aegypti* SRB [VectorBase: AAEL005981] and DENV-2 prM/M proteins paved an avenue towards a more comprehensive understanding of mosquito antiviral mechanisms during DENV infection. Previously, SRB in ixodid ticks (*Haemaphysalis longicornis*) was found to play a key role in granulocyte-mediated phagocytosis to invading *Escherichia coli* and contributed to the first-line host defence against various pathogens [50]. Also, other studies on SRB in insects revealed the critical roles of this protein in cellular lipid regulation [51] and uptake of dietary carotenoids [52].

We also identified a few digesting enzymes that were found to interact with prM, E, or M, namely zinc carboxypeptidase (VectorBase: AAEL008599, AAEL001863, AAEL012781) and trypsin (VectorBase: AAEL007818). Carboxypeptidase, a well-known hydrolytic enzyme involved in C-terminal peptide cleavage, was also found to be highly regulated after blood meal [53]. In mosquito, carboxypeptidase has not only been found to be involved in sexual development of malarial protozoan parasites [54], but also in the interaction between carboxypeptidase and DENV capsid protein in the salivary gland [23] and the midgut cells [55]. In addition, carboxypeptidase D has been continuously proven to be a receptor for duck hepatitis B virus [56]. These studies show the diverse roles of carboxypeptidases in pathogen invasions, including viruses.

Our results also support the protein-protein interaction between DENV-2 E protein and *A. aegypti* trypsin. This interaction is in accord with the presence of trypsin activity in the mosquito midgut, which peaked at three hours after blood feeding; that tryptic digestion of viral surface proteins enhances the infectivity of DENV-2 in mosquito midgut cells, but was unable to support viral replication [57]. This finding has made our Y2H screening workflow, including the construction of *A. aegypti* midgut cDNA library, more reliable and accurate.

Several hypothetical proteins were identified in this study (VectorBase: AAEL012947, AAEL004869, AAEL010974, AAEL007974). These proteins were documented in VectorBase without predicted functions and identifications.

## Conclusions

In this study, we reported the construction of a new cDNA library of female adult *A. aegypti* midguts, with tests to justify its complexity, robustness, and quality for use in Y2H screening studies against DENV-2 proteins (E, prM, M, NS1). A number of putative mosquito midgut proteins that interact with DENV proteins were identified. Additional validation assays (M2H and double IFA) were conducted for a few selected mosquito midgut proteins and the results supported the quality of the cDNA library and the robustness of the Y2H mechanisms. Although investigations into the biological relevance of the reported interactions are necessary, this preliminary study has paved a pathway and direction towards the investigations of the candidate proteins on their importance for the virus replication cycle inside insect cells. The employment of several decisive methodologies, such as RNA interference (RNAi), could aid in functional validations. Besides the study of DENVs, the cDNA library can also be applied for the discovery of novel interacting proteins of other mosquito-borne viruses such as yellow fever and Chikungunya virus. This study will guide future research into dissecting and targeting these proteins in vector control or dengue prevention.

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### Authors' contributions

S.S.H., H.A. and H.T. conceived and designed the experiments. H.T., V.R.M.T.B. and M.C. performed the experiments. H.T., V.R.M.T.B. and M.C. analysed the data. H.A., S.S.H. contributed reagents/materials/analysis tools. S.S.H. and H.T. wrote the paper.

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