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Role of cell surface vimentin in Chandipura virus replication in Neuro-2a cells

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\textbf{A B S T R A C T}

The neurotropic behavior of Chandipura virus (CHPV) is partly understood in experimental animals. Under in vitro conditions, neuronal cells could be a useful tool to study the CHPV interaction with neuronal proteins. The information gathered from such studies will help to design the new therapeutics for CHPV infection. This study identified the surface vimentin protein involved in adsorption of CHPV on Neuro-2a cell line (mouse neuroblastoma). The decrease in CHPV infectivity to Neuro-2a cells was observed in the presence of recombinant vimentin or anti-vimentin antibody. Vimentin mRNA expression remains unaltered in CHPV infected Neuro-2a cells. Furthermore, \textit{in silico} analysis predicted the residues in vimentin and CHPV glycoprotein (G); probably involved in cell-virus interactions. Overall, we conclude that surface vimentin in Neuro-2a cells interact with CHPV and facilitate the binding of CHPV to the cells; it could be acting as a co-receptor for the CHPV. Further investigation is necessary to confirm the exact role of vimentin in CHPV infection in neuronal cells.

\section{Introduction}

Information on viral-host interactions allows the thorough characterization of the viral life cycle and the potential to reveal important information that could be targeted for drug therapy. For many viruses, little information is available regarding the virus-host interaction. Adsorption of the virus on to the host cells is a highly specific course of action. Some viruses need single specific receptor while others need more than one receptor or co-factors for entry into host cells (Bielefeldt-Ohmann \textit{et al.}, 2001). Enveloped viruses attach to cells by binding of their surface membrane protein to a specific cell receptor. Various components of the host cell membrane can act as virus receptors like CD4 molecules for Human immunodeficiency virus type 1 (Sattentau and Weiss, 1988), α-Dystroglycan for Lyssa Fever virus (Cao \textit{et al.}, 1998) or intercellular adhesion molecule-1(ICAM-1) for Rhinovirus (Greve \textit{et al.}, 1989). To date, no-host cell receptors have been reported for Chandipura virus (CHPV).

CHPV is an arbovirus belonging to the genus Vesiculovirus of the \textit{Rhabdoviridae} family known for causing encephalitic complications among the children in India (Rao \textit{et al.}, 2004). It has a single-stranded negative-sense RNA genome. Structurally, it comprises of nucleocapsid surrounded by an envelope made from host cell lipids and trimeric viral glycoprotein (G). The mature G protein is about 500 amino acids long. This is the lone spike protein of CHPV that enables virus adsorption, assembly, and budding. It also elicits antibody response thus acting as a major antigenic determinant (Benmansour \textit{et al.}, 1991; Lefrancois and Lyles, 1983). Most of the functional and structural information related to CHPV proteins have been derived from studies on Vesicular stomatitis virus (VSV), a prototypic vesiculovirus, as the amino acid sequences of CHPV proteins and VSV proteins are evolutionarily conserved. The amino acid sequence of CHPV G protein shares a 40% identity and 65% similarity with VSV G protein (Masters \textit{et al.}, 1989). The comparison study between different strains of CHPV associated with past outbreaks revealed that CHPV G protein is stable and its antigenic determinants are conserved (Pavitrakar \textit{et al.}, 2018).

Usually, rhabdoviruses utilize clathrin-mediated endocytic pathways for entering into host cells. It was proposed that low pH-induced conformational change in the G protein within endosome after viral entry enables membrane fusion to release core particles in two sequential steps into the host cytoplasm (Blanc \textit{et al.}, 2005). VSV was found to interact with SMAD2, CD44, SCNK and FRS2 proteins of host cells (Moerdyk-Schauwecker \textit{et al.}, 2009) while the Rabies virus (RV) utilizes Nicotinamine acetylcholine receptor (AchR) from neuronal cells as its putative receptor (Gastka \textit{et al.}, 1996). The interactome dataset of other rhabdoviruses is generally considered as a standard to validate the virus-host interactions in CHPV (Guleria \textit{et al.}, 2011). A structural similarity-based computational approach has been employed to predict the protein interactions between CHPV and human host proteins.
(Rajasekharan et al., 2013).

Virus overlay protein binding assay (VOPBA) is one method that has been successfully applied to identify the cellular receptors for many viruses including New Castle Disease virus (Holguera et al., 2014), Human Respiratory syncytial virus (Holguera et al., 2014; Tayyari et al., 2011), Lymphohcytic Choriomeningitis virus (Borrow and Oldstone, 1992) and Dengue virus (Jindadamrongwech and Smith, 2004; Salas-Benito and Angel, 1997). In this study, we attempted the VOPBA method to identify the proteins involved in CHPV adsorption on Neuro-2a cells. This study can guide future experiments to understand the molecular mechanisms of virus-cell interaction.

2. Materials and Methods

2.1. Cell lines and virus

Neuro-2a (mouse Neuroblastoma cells) cell line (ECACC Cat. No. 89121404, Sigma, USA) and Vero African green monkey kidney cell line (Vero) (ECACC Cat. No. 84113001, Sigma, USA) were grown and maintained in Dulbecco’s modified eagle medium (DMEM; HyClone, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, USA), 100 U/ml penicillin (100 μg/ml) and streptomycin (100 μg/ml). The CHPV strain (NIV id: 034267) was originally isolated from the CHPV outbreak in Andhra Pradesh in 2003 (Rao et al., 2004). The viral titer was determined by the plaque assay (Jadi et al., 2010).

2.2. Neuro-2a cell membrane protein extraction

The cell membrane proteins from Neuro-2a cells were extracted by a method described earlier (Salas-Benito and Angel, 1997; Valle et al., 2003). Briefly, confluent grown Neuro-2a cells in 75 cm² tissue culture flask were detached by treating the cells with 5 ml of phosphate buffered saline (PBS, pH 7.4) supplemented with 5 mM EDTA for 10 min at room temperature (RT). After centrifugation, the cell pellet was resuspended in ice-cold buffer M (100 mM NaCl, 20 mM Tris [pH 8.0], 2 mM MgCl₂, 1 mM EDTA, and 1 mM beta-mercaptoethanol) and sonicated (Sonics, Vibra cell, USA) thrice at maximum capacity at 10 sec interval on ice. Nuclei and cell debris were removed by centrifugation at 1500 xg (Sorvall Biofuge Primo B, Thermo scientific) for 5 min at 4 °C. The membrane proteins were pelleted at 20,000 rpm for 30 min at 4 °C using the Sorval SS-34 rotor (SORVALL RC-6 Plus, Germany) and resuspended in buffer M without beta-mercaptoethanol. The concentration of protein was quantified by the Bradford method.

2.3. Virus Overlay Protein Binding Assay (VOPBA)

Neuro-2a cell membrane proteins were separated by 15% Sodium Dodecyl Sulphate-Polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membrane (BioRad, USA) using Large Semaphore Transphor Unit (Amershams Biosciences, USA) at 150 mA for 1 hr. The membrane was blocked for 2 hr using 5% non-fat dried skimmed milk-PBS at RT. The VOPBA assay was performed by incubating the PVDF membrane with polyethylene glycol (PEG) precipitated CHPV in 5% milk solution (10 μg/ml) for overnight at 4 °C with continuous shaking. The membrane was further incubated with anti-CHPV rabbit immune sera (in house antibody) for overnight at 4 °C. Subsequently the membrane was incubated with horseradish peroxidase (HRP) enzyme-conjugated goat anti-rabbit IgG (Sigma, USA) for 45 min at RT. The virus reactive bands were visualized after developing the signals using Western blot Quant HRP substrate (TaKaRa, Japan) and captured on photographic films.

2.4. Protein Identification by Q-TOF LC/MS

The area corresponding to the reactive band in VOPBA film was excised from the colloidal Coomassie Brilliant Blue G-250 (CBB G-250) stained SDS-PAGE gel and subjected to mass spectrometry. Mass spectrometry was performed at Amrita Agilent Analytical Research Centre, Kollam, Kerala, India.

2.5. Immunofluorescence assay (IFA) and Western blot analysis

The co-localization of CHPV with vimentin on the surface of Neuro-2a cells was determined by indirect IFA. The Neuro-2a cells cultured on glass coverslips in 24-well plate (Nucleon Delta Surface, Thermo scientific) were incubated with PEG precipitated CHPV (multiplicity of infection, MOI = 10) at 4 °C for 1 hr with intermittent shaking. The cells were fixed with freshly prepared 4% paraformaldehyde (PFA) and blocked with 3% bovine serum albumin (BSA)-PBS for 2 hr at RT. The cells were incubated with anti-CHPV mouse immune sera (in house antibody) and vimentin (D21H3) rabbit mAb (CST, USA) for 1 hr at 37 °C. Subsequently, the cells were double-stained with Alexa Fluor 488-conjugated goat anti-mouse IgG antibody (Invitrogen, USA) and Alexa Fluor 546-conjugated goat anti-rabbit IgG antibody (Invitrogen, USA). The cover-slips were mounted on microscope slides with Moiwal (Calbiochem, USA) mounting medium and viewed under laser confocal microscope (Leica TCS-NT, Germany). The pictures were captured and merged. To confirm the presence of vimentin in Neuro-2a membrane protein extract as well as cytoplasm, Western blot analysis was performed. Briefly, the cell membrane and cytoplasm extract were resolved on 15% SDS-PAGE gel and probed with vimentin (D21H3) rabbit mAb as described earlier.

2.6. Plaque assay

A cell-based assay utilizing virus mediated plaque formation was set up using Neuro-2a cells to find out the role of vimentin in virus replication. In the first experiment, the Neuro-2a cells were treated with 50 fold and 100 fold dilutions of vimentin (D21H3) rabbit mAb for 1 hr at 4 °C with intermittent shaking. The control well was treated with rabbit immunoglobulin. After incubation, cells were infected with CHPV (MOI = 1) in the presence of antibody for 1 hr at 37 °C. Cells were washed with PBS and further incubated for 12 hr in 1X DMEM supplemented with 2% FBS. Culture supernatant was collected for plaque assay to calculate the viral progeny yield. The plaque assay was performed as described by Jadi et al (2010).

In another experiment, the vimentin receptor in the virus was blocked by treating the virus with the recombinant vimentin protein. The His-tagged vimentin protein was cloned, expressed and purified in our laboratory. The PEG precipitated CHPV (50 μg) was pre-incubated with 15 μg/ml and 30 μg/ml purified vimentin protein at 4 °C for 1 hr. The virus treated with BSA was used as a negative control. Neuro-2a cells were then infected with the virus-protein complex for 1 hr at 37 °C. After virus infection, cells were washed with PBS to remove unbound virus and further incubated for another 12 hr in 1X DMEM supplemented with 2% FBS. The culture supernatant was collected for plaque assay. The percent reduction in plaque was calculated by the following formula,

\[
\% \text{plaque reduction} = \frac{\text{Number of plaques in untreated} - \text{Number of plaques in treated}}{\text{number of plaques in untreated}} \times 100
\]

2.7. Quantitative real-time polymerase chain reaction (qRT-PCR)

The total cellular RNA was extracted using RNAiso plus reagent (TaKaRa, Japan) from the CHPV infected (MOI = 0.1) and uninfected Neuro-2a cells up to 12 hr post infection (PI) at the interval of 2 hr. The cDNAs were prepared using the Avian Myeloblastosis Virus (AMV) reverse transcription system (Promega Corporation, Madison, USA) and
the mRNA levels of vimentin was quantified using 2X SYBR Green Premix Ex-Taq (TaKaRa, Japan). The qPCR reaction was carried out using the BioRad CFX96 Touch Real-time PCR machine. Results were analyzed with the inbuilt CFX Maestro software. Absolute vimentin mRNA quantification was performed by the standard curve method using vimentin clone. The qRT-PCR primers used in the current study are listed in Table 1.

2.8. In silico protein-protein interaction study

The molecular docking is a useful technique for understanding the binding mode of ligand with its receptor (Morris et al., 2009). In the present study, we have docked the structures of mouse vimentin protein with CHPV G protein (UniProtKB: 4d6w.pdb). To perform these computational studies, the three-dimensional structure of the mouse vimentin protein was generated using SWISS-MODEL (Schwede et al., 2003). The molecular docking between mouse vimentin protein and CHPV G protein was done by using AutoDock software. After completion of docking, all the images were built using structural visualization platform CHIMERA.

2.9. Statistical analysis

All the statistical analyses were performed by using GraphPad Prism 8 software (GraphPad Software, USA). The data from three independent experiments were analyzed by one way ANOVA test. The \( p \)-value < 0.05 considered as statistically significant.

3. Results

3.1. Identification of CHPV interacting protein

To identify the proteins, present in Neuro-2a cell membrane that interact with CHPV, VOPBA assay followed by LC-MS analysis was performed. The Neuro-2a cell membrane proteins were hybridized with PEG-precipitated CHPV and proteins interacting with the virus were identified using anti-CHPV antibody. VOPBA revealed the protein band of 55 kDa from the Neuro-2a cell membrane protein extract reacting with CHPV (Fig. 1a) however, no corresponding band was observed in the control membrane (b). Protein sizes were indicated on left.

![Image](Fig. 1. Virus overlay protein binding assay (VOPBA). The membrane proteins from Neuro-2a cells were resolved on SDS-PAGE and transferred to PVDF membrane. The PEG precipitated CHPV was overlaid onto the membrane and interacting proteins was detected using anti-CHPV rabbit immune sera. A protein band of 55 kDa was seen in the CHPV overlaid membrane (a) while the corresponding band was absent in the control membrane (b). Protein sizes were indicated on left.)

(Fig. 2c). The presence of vimentin in membrane extract and cytoplasm was confirmed by Western blot analysis (Fig. 3).

3.3. Blocking surface vimentin reduces the CHPV yield in infected cells

To further demonstrate the role of cell surface vimentin in CHPV binding to Neuro-2a cells, plaque reduction assay was performed. Reduction in viral yield was observed in a dose-dependent manner when the Neuro-2a cells were treated with vimentin (D21H3) rabbit mAb before the CHPV infection. Plaque assay analysis showed approximately 46 % and 68 % reduction in plaques in 100-fold and 50-fold dilution of anti-vimentin antibody-treated cells respectively as compared with plaques in the rabbit immunoglobulin treated cells. (Fig. 4)

![Image](Fig. 4. Reduction in viral yield was observed in a dose-dependent manner when the Neuro-2a cells were treated with vimentin (D21H3) rabbit mAb before the CHPV infection. Plaque assay analysis showed approximately 46 % and 68 % reduction in plaques in 100-fold and 50-fold dilution of anti-vimentin antibody-treated cells respectively as compared with plaques in the rabbit immunoglobulin treated cells.)

Similarly, a reduction in virus yield was noticed when the CHPV was pre-incubated with increasing concentration of purified recombinant vimentin protein before infecting the cells. The plaque assay analysis revealed approximately 25% and 48% reduction in plaques in the blocking experiment with 15 μg/ml and 30 μg/ml of recombinant vimentin protein respectively as compared to the BSA –virus complex infected cells (Fig. 5). These results indicate that the interaction between surface vimentin and CHPV play important role in the early phase of CHPV pathogenesis.

3.4. Vimentin mRNA expression remains unaffected by CHPV infection in Neuro-2a cells

To study the effect of CHPV infection on expression of vimentin mRNA, Neuro-2a cells were infected with CHPV. Total cellular RNA was extracted, quantified and used for gene expression analysis. The vimentin expression level was analyzed at transcription level throughout the study period. The vimentin mRNA levels were determined by qRT-PCR using SYBR Green based assay. The standard vimentin clone was used for constructing the standard curve for vimentin mRNA quantification. The results shown that, there was no significant change observed in vimentin mRNA expression in infected cells compared to the...
3.5. In silico analysis of CHPV-vimentin interaction

Specific identification of the residues involved in interaction between vimentin and CHPV G is a complex process. The docking model was established for the vimentin-CHPV G complex. The structure of mouse vimentin and CHPV G protein was modelled and docking was performed using AutoDock software. The results suggested that the amino acid argenine (ARG158) and threonine (THR165) of vimentin has strong hydrogen-bonding interactions with amino acids glutamine (GLN155), aspargine (ASP159) and valine (VAL149) of CHPV G protein with a distance of 2.49 Å, 2.43 Å and 2.42 Å respectively (Fig. 7). These residues can be predicted as hot sites involved in interaction of two proteins. These results strongly indicate the probability for vimentin to interact with VHPV G protein during initial phase of CHPV pathogenesis in Neuro-2a cells.

4. Discussion

The members of the Family Rhabdoviridae are known for causing infections in a wide range of organisms (plants, insects, animals, and humans). This family includes viruses like VSV known for causing infections in livestock, and human pathogens like rabies (RV) and CHPV. Despite the importance of CHPV in Public health, there is no experimental evidence available for explaining the mechanism of entry of CHPV in susceptible cells.

Rhabdovirus uses many receptors to bind the cells. The viral G protein is involved in virus attachment to host cell receptors and plays a critical role in the initial steps of virus infection. In the case of VSV, the involvement of phosphatidylycerine (Schlegel et al., 1983) and LDL receptors (Finkelstein et al., 2013) were reported in an attachment to permissive cells. But, the involvement of phosphatidylycerine in VSV attachment is a topic of debate (Coll and Miller, 2004). In the case of RV, several molecules like Low-affinity nerve growth factor receptor (P75NTR) (Tuffereau et al. 1998), Neuronal cell adhesion molecule (Thoulouze et al., 1998), Nicotinic acetylcholine receptor (Gastka et al., 1996) were proposed as virus receptors. These studies indicate that several molecules may be taking part in the process of virus binding and entry into the susceptible host cell.

In the current study, we confirmed the interaction of CHPV with vimentin on the Neuro-2a cell surface during virus adsorption. Vimentin is known to be involved in the various cellular processes like cell adhesion, organelle movements within the cells, cell signaling, and maintenance of cytoskeletal interactions (Nieminen et al., 2006). It is mainly a cytoplasmic protein intensely found around the cell membrane. Vimentin along with other cytoskeletal proteins are involved in the trafficking of many viruses within the cells. Several viruses require vimentin assistance in completing their life cycle. Human cytomegalovirus needs vimentin for completion of replication in fibroblasts (Miller and Hertel, 2009) and African swine fever virus needs vimentin re-arrangements for viral assembly (Stefanovic et al., 2005). Cell surface-expressed vimentin has been reported at different developmental stages of astrocytes, fibroblast and fibroblast-like cells in mouse brain tissue.

Fig. 3. Western blot analysis to demonstrate the presence of vimentin in cytoplasmic and membrane extract. The Neuro-2a cell membrane and cytoplasmic proteins were resolved on SDS-PAGE, transferred to PVDF membrane and probed with anti-vimentin antibody. Note the presence of vimentin in cytoplasmic and membrane fractions.

Table 2

| Sample          | Proteins Identified                        | Accession     | MASCOT Score | Molecular Mass (Da) | Sequence coverage (%) |
|-----------------|--------------------------------------------|---------------|--------------|--------------------|-----------------------|
| VOPBA2          | Tubulin beta-5 chain OS                    | TBB5_MOUSE    | 6227         | 50095              | 84                    |
| Tubulin alpha-1A chain OS | TBA1A_MOUSE    | 4898         | 50788        | 76                 |
| ATP synthase subunit alpha, mitochondrial OS | ATPA_MOUSE     | 2838         | 59830        | 64                 |
| ATP synthase subunit beta, mitochondrial OS | ATPB_MOUSE     | 1968         | 56265        | 67                 |
| 60S ribosomal protein 4 OS | RL4_MOUSE      | 1633         | 47409        | 52                 |
| Vimentin OS = Mus musculus GN | VIME_MOUSE     | 721          | 53712        | 69                 |
| Heterogeneous nuclear ribonucleoprotein H OS | HNRH1_MOUSE    | 697          | 49454        | 40                 |
| T complex protein 1 subunit OS | TCPB_MOUSE     | 445          | 57783        | 56                 |

uninfected Neuro-2a cells (Fig. 6).
and primary cell culture (Schnitzer et al., 1981).

The mass spectrometry analysis of the 55 kDa CHPV reactive band visualized in VOPBA identified multiple proteins in this particular study. The presence of multiple proteins in MASCOT search after LC/MS analysis indicated several possibilities including ATP synthase α and β subunit, α actin, β actin and vimentin. However, we have selected vimentin among the other proteins identified because the involvement of cell surface vimentin as receptor in virus binding has been reported in Japanese encephalitis virus (Jian-Jong et al., 2011), Dengue virus (Yang et al., 2016), Severe acute respiratory syndrome Coronavirus – SARS-CoV (Ting-Chun et al., 2016) and Enterovirus 71 (Ning et al., 2014).

In earlier study, it was proved that vimentin mediated signaling was required for lba+ E. coli K1 cells to enter into human brain microvascular endothelial cells (Chi et al., 2010). These findings support the involvement of vimentin in neuronal invasion by other pathogens. Therefore, the interaction of CHPV G protein with superficial vimentin may be the possible hypothesis to explain the neuroinvasiveness and neurotropic behavior of CHPV. In the present study, immunofluorescence staining showed the co-localization of CHPV with vimentin on the Neuro-2a cell surface. Thus, the involvement of surface vimentin in CHPV pathogenesis must not be underestimated.

This study also confirmed that CHPV infection did not influence the vimentin mRNA expression in the infected cells. According to Bin et al. (2004), surface vimentin is not originated from the cell-specific mRNA but is the outcome of post-translational modification of existing vimentin mRNA (Bin et al., 2004). So, the presence of an alternative form of vimentin on the cell surface is an interesting phenomenon and needs to be thoroughly investigated.

We also observed the CHPV multiplication in Neuro-2a cells despite the treatment of cells with increasing concentration of anti-vimentin antibody and treatment of CHPV with recombinant vimentin protein before infection. These observations suggest that the surface vimentin helps in the initial stage of virus binding and additional protein molecules are involved in the process of CHPV entry into neuronal cells.

The current in silico study proposed the involvement of hydrogen bonding interactions between the residues of mouse vimentin and CHPV G protein. Structurally, the vimentin monomer consists of the head domain (amino acids 1-10), central rod domain (amino acids 102-410) and tail domain (amino acids 411-466). Two such monomer forms a coiled-coil structure which forms the basic subunit of vimentin assembly (Herrmann and Aebi, 2004). In earlier studies, it was reported

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**Fig. 4.** Plaque assay using an anti-vimentin monoclonal antibody. Neuro-2a cells were pre-treated with increasing concentration of vimentin antibody and rabbit immunoglobulin before CHPV infection. The cell supernatant was collected 12 hr PI and titrated by plaque assay using Vero cells. The result of plaque reduction obtained is represented in graphical form as mean ± SD (n = 3).

**Fig. 5.** Plaque assay using recombinant vimentin protein. CHPV was incubated with different concentrations of purified vimentin protein and BSA. Protein-virus complex was used to infect Neuro-2a cells. The supernatant was collected 12 hr PI and titrated by plaque assay using Vero cells. The result of plaque reduction obtained represented in graphical form as mean ± SD (n = 3).

**Fig. 6.** Vimentin mRNA expression in CHPV infected Neuro-2a cells. Neuro-2a cells were infected and mock infected with CHPV. The cells were collected at 2,4,6,8,10 and 12 hr PI. The absolute quantification of expression level of vimentin mRNA was performed by SYBR Green based qPCR. All the experiments were run in triplicates and presented as mean ± SD (n = 3). The data was statistically insignificant.
that the rod domain of vimentin is involved in interaction with Dengue virus on vascular endothelial cells (Yang et al., 2016). In the current study, the amino acid residues of vimentin involved in the in-silico interaction belongs to the rod domain of vimentin. This finding indicates that the interaction was specific, and hence suggests the possibility for surface vimentin to interact with CHPV G protein on cell surface. The exact function of predicted amino acids involved in CHPV-vimentin interaction needs to be further investigated.

Overall the study results conclude the involvement of surface vimentin on Neuro-2a cells in CHPV infection. These results shed some light on the possible receptor for CHPV. In the future, it will be exploited to design peptides or small molecules to inhibit the virus infection into the cells. However, further study is necessary to find out the precise role of vimentin in CHPV infection to neuronal cells.

CRedit statement

Vishal K Kavathekar: PhD fellow, has designed the experiment, performed the experiments and written the article
Anukumar Balakrishnan: Guide of Mr. Vishal K Kavathekar, conceptualized the study, analysed the data and has written the article
Maruti J Dhanavade & Kailash D Sonawane: Performed all the bioinformatics work and in-silico experiments
All the authors agreed on the submission of the final manuscript.

Author Contribution

VVK has designed and performed the experiments. AB analyzed the data. MJD and KDS performed the in-silico experiments. VVK and AB wrote the article. All the authors agreed on the submission of the final manuscript.

Declaration of Competing Interest

The authors declare no competing financial interest.

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