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Research Article

SMALL SCALE EXPRESSION, SOLUBILIZATION, AND CHARACTERIZATION OF CHIKUNGUNYA VIRUS STRUCTURAL PROTEINS

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ABSTRACT

Objective: The severity and spread of Chikungunya fever in absence of effective antiviral therapy presents a serious public health threat. The present investigation aims to generate soluble and purified viral structural proteins that can be utilized to facilitate generation of reagents for development of both diagnostic and therapeutic measures.

Methods: Bacterial expression system was used for optimization of expression and solubilization of structural proteins of CHIKV (Capsid, 6K and envelope proteins 1-3 [E1, E2 and E3]) as fusions with large (GST) and small (His and Strep) tags on a single platform. Affinity chromatography was used for small scale purification of viral proteins.

Result: The effect of different tags, inducer concentrations, temperatures and duration of induction on solubilization of proteins has been optimized and small scale purification of all the structural proteins has been attempted. Utility of these solubilized proteins has been shown by analyzing the interaction of E2 with all the structural proteins using pull down assay.

Conclusion: Small scale purification of all five structural proteins and ectodomains of envelope proteins E1 and E2 has been standardized. The data and reagents generated can be utilized for large scale purification and studying CHIKV biology.

Keywords: Chikungunya virus, Protein expression, Solubilization, Purification.

INTRODUCTION

Chikungunya virus (CHIKV), a member of the genus Alphavirus and family Togaviridae has re-emerged as a major threat to public health by being responsible for the largest epidemics among Alphaviruses, infecting around 1.4-6 million people across different regions of the world [1-6]. CHIKV, a ~70-100 nm diameter wide enveloped virus, has a single-stranded, positive-sense RNA genome approximately 11.8 kb long. The viral genome bears a resemblance to host mRNAs, as it possesses 5′ and 3′ untranslated regions, with a 5′ terminal methylguanylate cap and a 3′ terminal polyadenylate tail. The genome is comprised of two distinct open reading frames: The 5′ two-thirds of the genome, encodes the nonstructural polyprotein (nsP1-nsP2-nsP3-nsP4), and the remaining 3′ one-third, encodes the structural polyprotein (capsid-E3-E2-6K-E1) [7].

Each viral protein performs specific functions during various stages of the viral life cycle. The nonstructural proteins of CHIKV are essential for viral replication while structural proteins (capsid, E1 and E2) form the virion structure and are majorly involved in receptor identification, fusion with cell membrane and elicitation of antigenic response in host cells [8-14]. Capsid, after autoproteolytic cleavage by its protease activity, forms viral particles and packages the viral genomic RNA [15]. The E1 and E2 proteins control viral entry into host cells: E1 mediates virus fusion to cell membranes in low pH conditions [16,17], while E2 interacts with a cellular receptor [18,19]. Small proteins E3 and 6k do not form the part of CHIKV structure. E3 is responsible for the proper localization of the structural polyprotein and its cleavage from E2 is essential for spike maturation [20,21]. 6K is a classified viroporin that has been shown to be involved in membrane permeabilization which facilitates virus budding [22,23]. Re-emergence of CHIKV has been especially attributed microevolution of its genome that has facilitated in the increased vector specificity of the virus [24].

Since CHIKV has emerged from a neglected mosquito-borne tropical disease to a viral infection of epidemic scope; there is an urgent need for the development and acquisition of reagents which will facilitate understanding of disease mechanism, detection, and effective treatment of CHIKV infection. In this direction, efforts are being focused towards purification and studying proteins of CHIKV. The present work investigates the effects of different tags, inducer concentrations, temperatures, and duration of induction on solubilization of structural proteins of CHIKV followed by their small scale purification and characterization.

METHODS

Construction of plasmids expressing CHIKV structural proteins

The constructs for protein expression and pull-down were generated by amplifying genes from clones in TOPO vectors [25] encoding capsid, E3, E2, 6K, and E1 genes, using forward and reverse primers compatible for cloning in prokaryotic expression vectors pGEX-4T3 glutathione S-transferase (GST tag), pCAK (Strep tag) and pLTA (His tag). The polymerase chain reaction was performed and the purified amplicons were either digested with restriction enzymes (for cloning in pGEX-4T3 vector) or treated with T4 DNA polymerase and dTTP to generate BsaI compatible ends (for cloning in pCAK/pLTA vectors)and ligated into corresponding vectors as explained earlier by the authors [26-28].

Expression and solubilization of CHIKV fusion proteins

Escherichia coli BL21 (DE3) cells transformed with individual recombinant vectors (plasmids pGEX-4T3, pLTA, and pCAK containing CHIKV structural genes) having N-terminal fusion tags were cultured in LB medium supplemented with appropriate antibiotic (100 µg/ml ampicillin for pGEX-4T3 and pLTA vectors; 30 µg/ml kanamycin for pCAK vector) at 37°C overnight with shaking at 220 rpm. The secondary cultures were induced at 0.3 OD_{600} at different conditions of temperature, induction time, and inducer concentration (Table 1).

At desired expression levels, the cells were harvested by centrifugation at 4°C for 6 minutes at 6000 rpm and analyzed by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). This was followed by cell lysis to obtain soluble fractions of fusion proteins by lysis buffer (IBA-GmbH, Germany) according to the manufacturer's protocol. Protease inhibitor cocktail (final conc. 1X; Clontech, USA), lysozyme (1 µg/ml), and nucleases (DNase [20 ng/ml], RNase [6 ng/ml]) were supplemented in the lysis buffer. The total bacterial proteins were then fractionated into soluble and insoluble fractions by centrifugation at 4°C for 40 minutes at 12,000 rpm. Both the fractions were analyzed by 10% SDS-PAGE and Western blot to check the presence of viral protein in the soluble fraction (supernatant) or/and the insoluble fraction (pellet). The proteins which were insoluble after cell lysis (present in the pellet fraction) were solubilized using sarkosyl following the protocol previously used by the authors [26].

Batch purification of CHIKV structural proteins

The soluble fractions of capsid, full length E1 and E2 along with the ectodomains of E1 and E2 (TrE1 and TrE2, respectively) as Strep fusion; and E3 and 6K as GST fusion were subjected to batch purification using strep-tactin (IBA GmbH, Germany) and Glutathione resins (Clontech, USA), respectively. Approximately 1-2 ml of supernatant containing fusion proteins were loaded onto 250 µl of the equilibrated resin and allowed to bind at 4°C for 30 minutes. The column was washed with 10 column volume (CV) of wash buffer (containing 1 mM ethylenediaminetetraacetate [EDTA], 100 mM Tris-Cl and 150 mM NaCl for strep-tactin resin and 1X PBS for glutathione resin). The bound protein was eluted with 1 CV of elution buffer (containing 1 mM EDTA, 100 mM Tris-Cl, 150 mM NaCl and 2.5 mM desthiobiotin for Strep fusion proteins and 10 mM reduced glutathione in 50 mM Tris-Cl for GST fusion proteins). The eluted fractions were analyzed by 10% SDS-PAGE for purity level.

Table 1: The conditions used for optimizing expression of CHIKV structural proteins using three different bacterial expression vectors

Parameters	Expression vector		
	pGEX 4T3	pCAK	pLTA
Tag	GST	Strep	His
Inducer	IPTG	Arabinose	Tetracycline
Inducer concentration	1 mM	0.3% 0.5%	20 ng/mL 50 ng/mL
Induction time	4 hrs	4 hr, 2 hr	4 hr, 2 hr
Induction temp	25°C, 37°C	25°C, 37°C	25°C, 37°C

CHIKV: Chikungunya virus

Analysis of interactions of solubilized proteins by pull-down assay

The functionality of soluble CHIKV proteins was analyzed by pull-down assay. E2 (strep tagged) was allowed to bind with structural proteins (E1, E2, Capsid, E3, and 6K expressed as GST fusions) in order to find its interactors. Binding of strep-E2 with only GST was taken as an experimental control while the known interacting pair GST-nsP1 and strep-nsP3 was taken as a positive control and non interactors GST-nsP4 and strep-nsP3 was taken as a negative control [28].

RESULTS AND DISCUSSION

Expression and solubilization of CHIKV proteins

Expression profile of all the proteins expressed as N-terminal fusion was analyzed by SDS-PAGE. It was observed that the optimal expression levels were obtained at 25°C for 4 hrs with 0.5% arabinose for pCAK, 50 ng/ml anhydrotetracycline for pLTA and 1 mM IPTG for pGEX-4T3 (data not shown). Since the expression of proteins with all three tags yielded cytoplasm associated recombinant proteins; cell lysis was performed to obtain the soluble fraction. Following Western blot analysis, it was observed that E1, E2 and capsid proteins, as all fusions (GST, His and Strep), were present in insoluble fraction (data not shown), while E3 and 6K (as GST fusions; His and Strep fusions could not be detected)were soluble (data not shown). Interestingly, when the induced cell pellets were treated with 5 mM ß-mercaptoethanol and 0.2% each of triton X-100, tween 20, and CHAPS during lysis, capsid protein appeared in the soluble fraction. However, E1 and E2 proteins still remained insoluble by this treatment and were subsequently solubilized using sarkosyl (Fig. 1a and b).

Since it is known that the hydrophobic transmembrane region of viral spike proteins is responsible for their insoluble nature, E1 and E2 proteins were expressed as ectodomains (without transmembrane regions) with N-terminal strep tag to see the effect of transmembrane region on their solubility. The solubilization profile showed that the ectodomains (TrE1 and TrE2) were soluble without any treatment with detergent suggesting that the transmembrane region was responsible for the insolubility of these proteins (data not shown).

Batch purification of *E. coli* generated recombinant CHIKV proteins

The purification of structural proteins was performed using strep fusion proteins because, (i) strep tag is very small in size so does not affect the

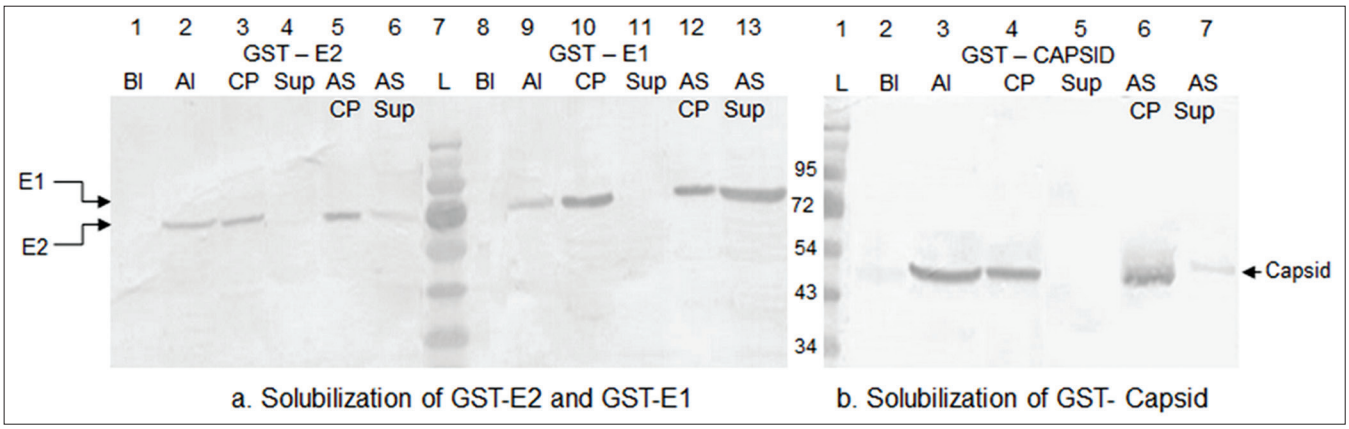

Fig. 1: Representative western blot for solubilization of E2, E1, and capsid proteins of Chikungunya virus (CHIKV) as glutathione S-transferase (GST) fusion using sarkosyl: CHIKV proteins expressed with N-terminal GST tag in *Escherichia coli* BL-21 (DE3) cells were checked for solubilization after cell lysis using IBA lysis buffer followed by treatment of insoluble fraction with 10% sarkosyl. Soluble and insoluble fraction were then analyzed by western blotting using anti-GST monoclonal antibodies. Panel a lanes 3, 4 and 10, 11 show the localization of E2 and E1 in the cell pellet (CP) after cell lysis. Similarly, capsid is visible in CP (b4 and b5). Lanes a (6), a (13) and b (7) show the solubilized E2, E1 and capsid, respectively, after treatment with sarkosyl. BI-before induction; AI-after induction; CP; sup-supernatant, AS CP-after sarkosyl CP and AS sup-after sarkosyl supernatant. L is the prestained protein ladder (molecular sizes are indicated in kDa, Fermentas). All the proteins are indicated by arrow heads

folding of protein and is biochemically inert; (ii) it has been reported to purify protein in active form [29]; (iii) more specific binding with strep-tactin resin as compared to the non-specific binding associated with purifying his fusions and; (iv) under gentle, physiological conditions it is especially suited for generation of functional proteins [29,30]. Purification of E3 and 6k was carried out as GST fusions since expression was not observed for strep construct for either protein. The one-step affinity purification of GST-E3 and GST-6K using glutathione resin yielded purified fusion protein. Capsid, E1, E2, TrE1 and TrE2 proteins were purified using strep-tactin resin. After affinity chromatography purification, the eluted fractions were analyzed by SDS-PAGE. A typical protein elution profile of the CHIKV proteins from strep or GST column is shown in Fig. 2a-g. SDS-PAGE analysis of purified proteins revealed that more than 90% purification has been achieved for each of the CHIKV proteins (Fig. 2a-g).

Interaction analysis of CHIKV structural proteins by GST pull-down

The functionality of the expressed proteins was assessed by studying the intra-viral protein interactions. This was achieved by pull-down assay where GST-tagged viral structural proteins was used as bait while strep tagged viral protein E2 was the prey. The eluted fractions were probed with both anti-strep and anti-GST monoclonal antibodies, and the presence of the band in anti-Strep blot was indicative of protein interaction. Among the five test pairs studied, E2 was found to interact with E1, capsid and 6k proteins (Fig. 2h and i, lanes 5, 6, and 8). The association of capsid protein with the cytoplasmic tail of E2 has been reported previously for other Alphaviruses and is responsible for lateral association of viral spikes during assembly [30-33]. The interaction among spike proteins E1 and E2 has been known to be critical for viral budding and envelope formation [33]. However, the association among E2 and 6k was shown for the first time for Alphaviruses. Though the functional relevance of this interaction is yet to be studied, it can be speculated that 6k could be interacting with E2 during the ER transport of remaining structural polypeptide after cleavage of capsid in cytosol. It is also possible that at late stage of infection when 6K increases membrane permeability that facilitates virus budding, it may be interacting with E2 protein.

CONCLUSION

In this study, bacterial expression system was used for the expression and purification of the structural proteins of CHIKV as three different fusions. Previously, E1, E2, and capsid proteins were purified using baculovirus expression system and the purified proteins were used for serodiagnosis [34-38]. However, small-scale purification of all

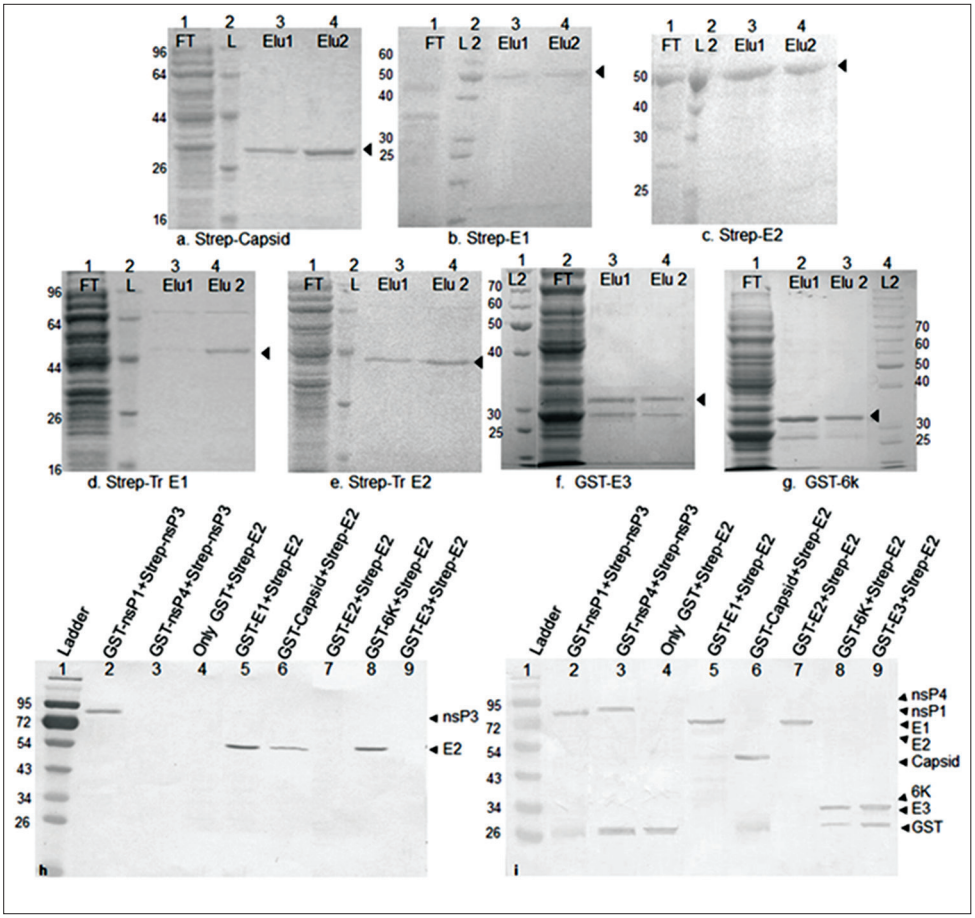

Fig. 2: The purification and interaction analysis of Chikungunya virus (CHIKV) structural proteins by affinity chromatography and glutathione S-transferase (GST) pull-down: *Escherichia coli* BL-21 (DE3) cells transformed with strep-CHIKV were induced with 0.5% arabinose for protein expression. Cells were lysed, and the soluble fraction was loaded onto the strep-tactin beads. After elution of the bound protein, samples were then analyzed by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by Coomassie brilliant blue staining. Panel (a-g) represents the purified structural proteins capsid, E1, E2, Tr-E1 and Tr-E2 as Strep fusion and E3 and 6K as GST fusion protein respectively. FT-Flow through, Elu1 and Elu 2-Elutes 1 and 2, L is the unstained protein ladder (Merck, India) and L2 is unstained protein ladder by Fermentas (molecular sizes are indicated in kDa). Interaction analysis of CHIKV structural proteins (prey) detected with anti-strep antibody, (h) and CHIKV structural proteins (bait) detected with anti-GST antibody, (i) the presence of band in the strep blot, (h) confirms interaction. Arrowheads indicate the position of each protein in respective blots. Lane 1-prestained ladder (molecular sizes are indicated in kDa, Fermentas)

five structural proteins and ectodomains of envelope proteins E1 and E2 can be utilized for studying CHIKV biology in a broader fashion. Furthermore, standardizations can be readily used for large scale protein purification. Overall, availability of purified viral proteins opens and widens the possibility of utilization of these proteins as reagents for structural analysis, understanding the structure and function of viral proteins, their role in viral life cycle; identification of host factors associated with viral proteins during viral life cycle and their potential as diagnostic and therapeutic targets.

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REFERENCES

1. Ng LC, Hapuarachchi HC. Tracing the path of Chikungunya virus – evolution and adaptation. Infect Genet Evol 2010;10:876-85.
2. Chretien JP, Anyamba A, Bedno SA, Breiman RF, Sang R, Sergon K, et al. Drought-associated chikungunya emergence along coastal East Africa. Am J Trop Med Hyg 2007;76(3):405-7.
3. Powers AM, Logue CH. Changing patterns of chikungunya virus: re-emergence of a zoonotic arbovirus. J Gen Virol 2007;88:2363-77.
4. Sang RC, Ahmed O, Faye O, Kelly CL, Yahaya AA, Mmadi I, et al. Entomologic investigations of a chikungunya virus epidemic in the Union of the Comoros, 2005. Am J Trop Med Hyg 2008;78(1):77-82.
5. Arankalle VA, Shrivastava S, Cherian S, Gunjikar RS, Walimbe AM, Jadhav SM, et al. Genetic divergence of Chikungunya viruses in India (1963-2006) with special reference to the 2005-2006 explosive epidemic. J Gen Virol 2007;88:1967-76.
6. Chandra P, Das B, Sowmya MK, Samanta S, Thimmaraju KV. Epidemologal study on Chikungunya outbreak in Manglore District, Karnataka. Int J Pharm Biosci 2011;2(4):342-7.
7. Khan AH, Morita K, Parquet Md Mdel C, Hasebe F, Mathenge EG, Igarashi A. Complete nucleotide sequence of chikungunya virus and evidence for an internal polyadenylation site. J Gen Virol 2002;83:3075-84.
8. Byrnes AP, Griffin DE. Binding of Sindbis virus to cell surface heparan sulfate. J Virol 1998;72(9):7349-56.
9. Cheng RH, Kuhn RJ, Olson NH, Rossmann MG, Choi HK, Smith TJ, et al. Nucleocapsid and glycoprotein organization in an enveloped virus. Cell 1995;80(4):621-30.
10. Omar A, Koblet H. Semliki forest virus particles containing only the E1 envelope glycoprotein are infectious and can induce cell-cell fusion. Virology 1988;166(1):17-23.
11. Smith TJ, Cheng RH, Olson NH, Peterson P, Chase E, Kuhn RJ, et al. Putative receptor binding sites on Alphaviruses as visualized by cryoelectron microscopy. Proc Natl Acad Sci U S A 1995;92(23):10648-52.
12. Wahlberg JM, Garoff H. Membrane fusion process of Semliki Forest virus. I: Low pH-induced rearrangement in spike protein quaternary structure precedes virus penetration into cells. J Cell Biol 1992;116(2):339-48.
13. Salminen A, Wahlberg JM, Lobigs M, Liljeström P, Garoff H. Membrane fusion process of Semliki Forest virus. II: Cleavage-dependent reorganization of the spike protein complex controls virus entry. J Cell Biol 1992;116(2):349-57.
14. Lopez S, Yao JS, Kuhn RJ, Strauss EG, Strauss JH. Nucleocapsid-glycoprotein interactions required for assembly of Alphaviruses. J Virol 1994;68(3):1316-23.
15. Thomas S, Rai J, John L, Günther S, Drosten C, Pützer BM, et al. Functional dissection of the Alphavirus capsid protease: sequence requirements for activity. Virol J 2010;7:327.
16. Kielian M, Helenius A. pH-induced alterations in the fusogenic spike protein of Semliki Forest virus. J Cell Biol 1985;101(6):2284-91.
17. White J, Helenius A. pH-dependent fusion between the Semliki Forest virus membrane and liposomes. Proc Natl Acad Sci U S A 1980;77(6):3273-7.
18. Dubuisson J, Rice CM. Sindbis virus attachment: isolation and characterization of mutants with impaired binding to vertebrate cells. J Virol 1993;67(6):3363-74.
19. Strauss EG, Stec DS, Schmaljohn AL, Strauss JH. Identification of antigenically important domains in the glycoproteins of Sindbis virus by analysis of antibody escape variants. J Virol 1991;65(9):4654-64.
20. Liljeström P, Garoff H. Internally located cleavable signal sequences direct the formation of Semliki Forest virus membrane proteins from a polyprotein precursor. J Virol 1991;65(1):147-54.
21. Bonatti S, Blobel G. Absence of a cleavable signal sequence in Sindbis virus glycoprotein PE2. J Biol Chem 1979;254(24):12261-4.
22. Melton JV, Ewart GD, Weir RC, Board PG, Lee E, Gage PW. Alphavirus 6K proteins form ion channels. J Biol Chem 2002;277(49):46923-31.
23. Madan V, Sanz MA, Carrasco L. Requirement of the vesicular system for membrane permeabilization by Sindbis virus. Virology 2005;332(1):307-15.
24. Schuffenecker I, Iteman I, Michault A, Murri S, Frangeul L, Vaney MC, et al. Genome microevolution of Chikungunya viruses causing the Indian Ocean outbreak. PLoS Med 2006;3(7):e263.
25. Dudha N, Appaiahgari MB, Bharati K, Gupta D, Gupta Y, Kumar K, et al. Molecular cloning and characterization of Chikungunya virus genes from Indian isolate of 2006 outbreak. J Pharm Res 2012;5(7):3860-3.
26. Kumar K, Rana J, Guleria A, Gupta A, Gupta A, Chaudhary VK, et al. Expression and characterization of chandipura virus proteins. Res Biotechnol 2011;2(6):27-36.
27. Kumar K, Rana J, Sreejith R, Gabrani R, Sharma SK, Gupta A, et al. Intraviral protein interactions of Chandipura virus. Arch Virol 2012;157(10):1949-57.
28. Sreejith R, Rana J, Dudha N, Kumar K, Gabrani R, Sharma SK, et al. Mapping interactions of Chikungunya virus nonstructural proteins. Virus Res 2012;169(1):231-6.
29. Skerra A, Schmidt TG. Use of the strep-tag and streptavidin for detection and purification of recombinant proteins. Methods Enzymol 2000;326:271-304.
30. Schmidt TG, Skerra A. The Strep-tag system for one-step purification and high-affinity detection or capturing of proteins. Nat Protoc 2007;2(6):1528-35.
31. Voss JE, Vaney MC, Duquerroy S, Vonrhein C, Girard-Blanc C, Crublet E, et al. Glycoprotein organization of Chikungunya virus particles revealed by X-ray crystallography. Nature 2010;468(7324):709-12.
32. Skoging U, Vihinen M, Nilsson L, Liljeström P. Aromatic interactions define the binding of the Alphavirus spike to its nucleocapsid. Structure 1996;4(5):519-29.
33. Garoff H, Sjöberg M, Cheng RH. Budding of Alphaviruses. Virus Res 2004;106:103-16.
34. Garoff H, Cheng RH. The missing link between envelope formation and fusion in Alphaviruses. Trends Microbiol 2001;9(9):408-10.
35. Cho B, Kim J, Cho JE, Jeon BY, Park S. Expression of the capsid protein of Chikungunya virus in a baculovirus for serodiagnosis of Chikungunya disease. J Virol Methods 2008;154(1-2):154-9.
36. Metz SW, Geertsema C, Martina BE, Andrade P, Heldens JG, van Oers MM, et al. Functional processing and secretion of Chikungunya virus E1 and E2 glycoproteins in insect cells. Virol J 2011;8:353.
37. Cho B, Jeon BY, Kim J, Noh J, Kim J, Park M, et al. Expression and evaluation of Chikungunya virus E1 and E2 envelope proteins for serodiagnosis of Chikungunya virus infection. Yonsei Med J 2008;49(5):828-35.
38. Tripathi NK, Priya R, Shrivastava A. Production of recombinant Chikungunya virus envelope 2 protein in Escherichia coli. Appl Microbiol Biotechnol 2014;98(6):2461-71.