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A TWO STEP PURIFICATION STRATEGY FOR CHIKUNGUNYA VIRIONS PURIFICATION USING SUCROSE BUOYANT DENSITY GRADIENT SEPARATION

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Abstract- An improved method for purifying Chikungunya virus from cell culture supernatant of infected African Green Monkey Kidney cells is described. It employs a combination of polyethylene glycol precipitation and sucrose gradient ultracentrifugation. The infected cell culture supernatant was precipitated using polyethylene glycol and concentrated virus was banded at the interface of 20-60% (w/v) discontinuous sucrose gradient using rate zonal ultra centrifugation. Using a combination of SDS-PAGE and western blotting techniques, Chikungunya virus structural proteins were detected only in the fractions collected from the interface. The authenticity of the purified Chikungunya virions was further confirmed by reverse transcriptase polymerase chain reaction and transmission electron microscopic study. The developed method has immense application and is useful in making large quantity of purified Chikungunya virus for its characterization, virus morphological study and purification of Chikungunya virus like particles expressed from heterologous system.

Keywords- Chikungunya virus, PEG precipitation, buoyant density, ultracentrifugation

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Introduction

Chikungunya (CHIK) virus is an enveloped single stranded positive sense RNA virus that belongs to the family *Togaviridae* and genus Alphavirus [1]. CHIKV is transmitted by Aedes species mosquitoes, primarily *A.aegypti* and recent 2005-2006 CHIKV epidemic has proved that *A.albopictus* can also transmit the virus between humans [2,3]. This disease is re-emerging and has become medically important as it can incapacitate the infected individual for several days to many years [4]. The illness in humans is often characterized by sudden onset of fever, headache, fatigue, nausea, vomiting, rash, myalgia and severe joint pain. In India, Chikungunya virus infection occurred during 2006-2007 affected more than 1.39 million people [5] and Kerala outbreak during 2007 had high epidemic potential with reported mortality cases [6]. The viral RNA is approximately 12,000 nt long with 5'-methylguanylate cap and a 3'-polyadenylate tail. The genome encodes four non-structural (NS1-4), three main structural proteins (capsid, E2 and E1) and two small peptides (E3 and 6 K) in two open reading frames. The E2 and E1 exist as hetero dimers at neutral pH, in which E2 forms spikes on the virion surface that interact with cellular receptors. The E1 protein lies below E2 and mediates fusion of the viral and cellular membranes during viral entry [7]. Purified Chikungunya virus preparations are often required for characterization and immunological studies.

The viruses can be concentrated from cell culture supernatant by ultracentrifugation, or precipitation with ammonium sulphate, alum, or polyethylene glycol [8-10]. There exists many reports on the purification of several viruses using rate zonal centrifugation, equilibrium density gradient or gel filtration methods [8,9,11]. However till date, very limited information is available on purification of Chikungunya virus. Multiple steps of differential and density gradient ultracentrifugation have been previously reported to purify CHIKV [11]. However, this procedure is time consuming and may result in considerable loss of infectivity of the virus as seen in other viruses [12]. An earlier report by Fernie, et al [13] indicated that sucrose in concentrations above 15% has a stabilizing effect on the viral proteins during purification. Mbiguino and Menezes [14] found that fractionation on sucrose gradients gave better virus recovery than percoll, renografin and metrizamide gradients. Sucrose allows the virus to be purified under iso-osmotic conditions and thus better preserves the integrity and functionality of the virus particles. With this background, the present study was aimed at developing a rapid method for purifying the Chikungunya virions from the infected cells that is substantially free of cellular and serum components, confirmation of the purified virus through RT-PCR and morphological characterization of virions by transmission electron microscopic examination. A purification procedure was developed based on the method of ultracentrifugation in sucrose gradient.

Materials and Methods

Chikungunya Virus

Chikungunya virus isolated from an infected patient during 2007 outbreak in Kerala, India [East-Central-South- African (ECSA) E1: A226 V isolate] obtained from Division of Virology, DRDE, Gwalior was used in the present study. The stock virus was passaged and propagated in African Green Monkey Kidney (*vero*) cell line.

Cell Culture and Virus Infection

African green monkey kidney (*vero*) cells were cultured in four T-175 cm^2 cell culture flask (Greiner, USA) at 37°C, 5% CO_2 in Dulbecco's modified Eagle's medium (Sigma, USA) supplemented with 8% heat-inactivated fetal bovine serum (FBS; Sigma, USA) and 100 units of penicillin and 100 µg/ml streptomycin. The normal vero cells having approximately 80% confluency were infected with CHIKV at a multiplicity of infection (m.o.i.) of 1, for 1 hr. After infection, fresh medium containing 2% of FBS was added to the cells and the cells were incubated until cytopathic changes were observed. The virus was harvested after observing cytopathic changes in the whole monolayer, snap-frozen in liquid nitrogen and stored at -80°C. On thawing, the infected cell suspensions were clarified by centrifugation at 3000 x g for 20 min at 4°C using Sigma centrifuge (Sigma, USA).

Virus Concentration

The clarified viral supernatant was subsequently concentrated by adding 50% (w/v) PEG 6000 in NT buffer (100 mM NaCl, 50 mM Tris-HCl, pH 7.2) to a final concentration of 20% (v/v). The virus particles were then precipitated for 3 hrs. at 4°C with moderate stirring on a magnetic stirrer, followed by centrifugation at 8000 × g for 30 min at 4°C in Sorvall centrifuge. The supernatant was removed and the pellet was then re-suspended in 2ml of ice-cold NT buffer (total volume).

Chikungunya Virus Purification by Ultracentrifugation

Discontinuous sucrose gradients were prepared by sequentially layering 60 and 20% sucrose solution (w/v in buffer containing 50 mM Tris-HCl, pH 7.2, 1mM EDTA,100 mM NaCl) into 13 ml centrifuge tubes (Thermo scientific, USA), and the gradients were used immediately. PEG-concentrated virus samples were layered over a discontinuous sucrose gradient and centrifuged in a TH-641 swinging bucket rotor (Thermo Scientific, USA) for 16 h at 35,000 rpm at 4°C. The gradient fractions along with the opaque band at 20-60% interface were harvested separately. The presence of Chikungunya virus in fractionated samples was determined by SDS-PAGE, western blotting and finally by reverse transcriptase polymerase chain reaction as described below.

SDS-PAGE and Western Blot Analysis of Purified CHIKV

The fractions collected after ultra centrifugation was analyzed by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by Coomassie staining [15]. The same fractions were also subjected to western blotting using mouse polyclonal antibodies raised against recombinant Chikungunya E2 protein (obtained from Division of Virology, DRDE, Gwalior). In brief, for the western blot analysis, the purified fractions along with molecular weight marker was electrophoretically transferred onto polyvinyl difloride (PVDF) membrane (Millipore, USA) using semidry transfer unit (Biorad, USA). The membranes were blocked overnight at 4°C in 3% BSA solution prepared in phosphate-buffered saline (PBS). The blots were washed with PBS-T [(PBS containing 0.05% Tween-20(v/v)] thrice for 5 min and incubated with anti-mouse HRP conjugated antibody for 1 h at 37° C. The blots were then thoroughly washed thrice with PBS-T for 5 min each and developed with DAB-H_2O_2 chromogen-substrate mixture.

Confirmation of Purified Virus through RT-PCR for CHIKV Genome

The genetic identity of the Chikungunya virus after purification was confirmed by reverse transcriptase polymerase chain reaction (RT-PCR). Genomic RNA was extracted from the purified virus after ultracentrifugation. The region encoding the partial CHIKV poly protein was further amplified by RT-PCR using specific primers (Fwd: 5' TAGGATCCTATGGAGTTCATCCCAACCC 3' and Rev: 5' CACGTGACCTCGAGC CCTTCA 3') to confirm the genetic identity of the purified virus.

Transmission Electron Microscopy (TEM) Study

The purified Chikungunya virions were fixed in 4% formaldehyde (made in PBS), negatively stained with 1.5% phosphotungstic acid (PTA), pH 7.2 as described previously [16] with few modifications. Briefly, we placed 1.0 µl of the virus sample onto a carbon coated Formvar-filmed copper grid (TAAB, UK) and allowed the Chikungunya virions to attach to the surface for one minute. The grids were washed three times in sterile triple distilled water by floating the grid on water droplets for 30 seconds each to remove sucrose and excess sample. Finally the virions were negatively stained by floating the grid on droplet of 1.5% PTA solution. The grid was dried on what man filter paper and was examined using Transmission electron microscope (FEI Tecnai, Japan) operated at 75 kV at All India Institute of Medical Sciences, New Delhi.

Results

The Chikungunya virus (ECSA E1: A226 V isolate) used in the present study had a low infectivity titer even after four days of post infection. Hence, for purification, large volumes of cell culture fluid were necessary in order to achieve high virus titers in the purified product. The virus in the clarified culture supernatants was concentrated by PEG precipitation and resuspended in NT buffer containing 100 mM NaCl. After ultra-centrifugation of PEG-concentrated virus through discontinuous sucrose gradients, a visible opaque band was observed at the 20-60% sucrose interface. SDS-PAGE analysis of different fractions revealed that majority of chikungunya virions are localized in the 20-60% sucrose interface [Fig-1]. As seen in [Fig-1], envelope glycoproteins E1 and E2 of CHIK virus migrate very close to one another on the SDS-PAGE gel and form a single band at the region corresponding to ~55kDa. The Capsid protein was seen as a separate band at the region corresponding to the size of ~32KDa. No band corresponding to the size of E3 was visible on the SDS-PAGE gel.

In order to examine the authenticity of the purified virus, different fractions collected from the buoyant density gradient were analyzed *via* western blotting using Chikungunya envelope protein (E2) specific antibodies. As seen in [Fig-2], the E2 protein was recognized by the specific antibodies only from the 20-60% interface band and the fractions that were very close to the interface. The genomic RNA obtained from the 20-60% interface band amplified the partial region of CHIKV polyprotein through RT-PCR [Fig-3A] and thus confirmed the authenticity of the purified virions. No such amplification was seen from the other fractions tested (data not shown). The

negative staining of the fraction collected from 20-60% interface band revealed the presence of both cores and complete virions under transmission electron microscope [Fig-3B]. The average diameter of Chikungunya cores was approximately 40 nm and complete virions had varied sized particles ranging from 50-65 nm.

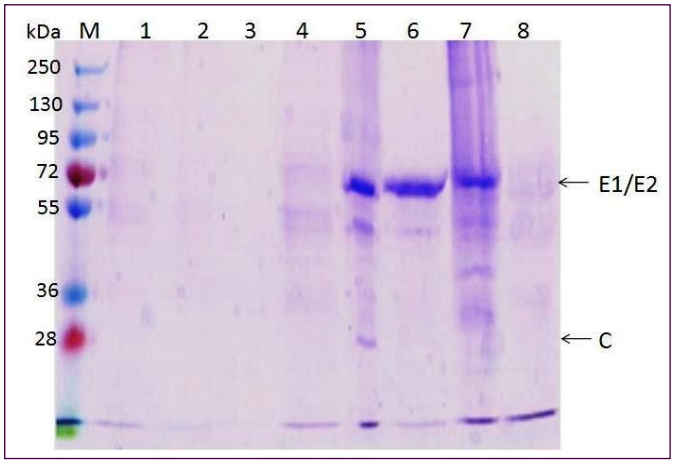

Fig. 1- SDS-PAGE analysis of purified Chikungunya virus fractions from discontinuous sucrose gradient

The PEG concentrated Chikungunya virus was separated on discontinuous sucrose gradient (20-60% w/v) and the fractions collected were analyzed on SDS-PAGE.

Lane M: prestained molecular weight marker (Fermentas, USA); *Lanes* 1-4: fractions collected from 20% sucrose layer; *Lane* 5: fraction slightly above 20-60% interface; *Lane* 6: band collected from 20-60% interface; *Lane* 7: fraction collected below 20-60% interface, *Lane* 8: fraction from 60% sucrose layer.

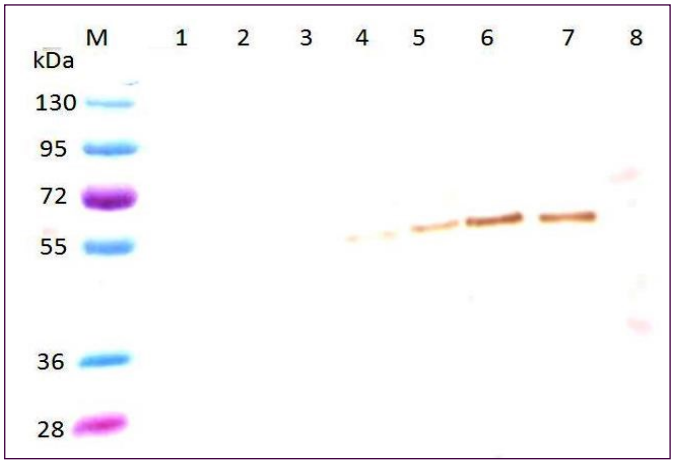

Fig. 2- Western blot analysis of purified Chikungunya virus fractions from discontinuous sucrose gradient

The Chikungunya virus was purified on discontinuous sucrose gradient (20-60% w/v) and the presence of virions in different fractions was confirmed by western blotting using mouse anti-E2 polyclonal antibodies raised against recombinant E2 protein of Chikungunya virus.

Lane M: prestained molecular weight marker (Fermentas, USA); *Lanes* 1-4: fractions collected from 20% sucrose layer; *Lane* 5: fraction slightly above 20-60% interface; *Lane* 6: band collected from 20-60% interface; *Lane* 7: fraction collected below 20-60% interface, *Lane* 8: fraction from 60% sucrose layer.

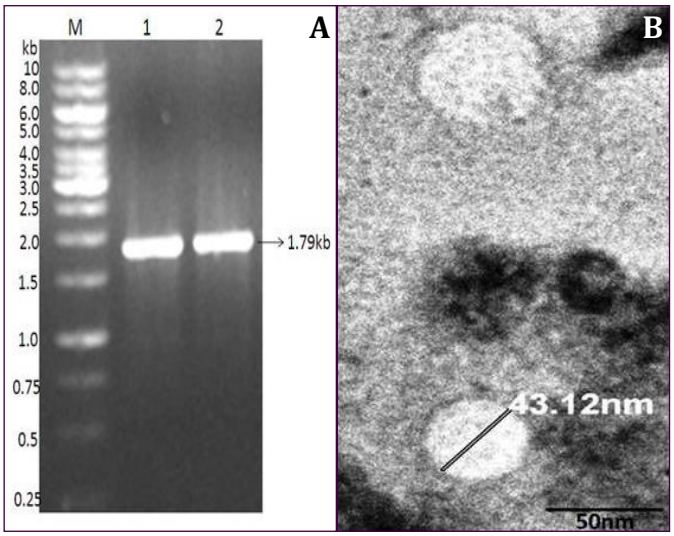

Fig. 3- (A) Confirmation of purified virus through reverse transcriptase polymerase chain reaction (RT-PCR) for CHIKV genome: The genetic identity of the purified Chikungunya virus was confirmed by RT-PCR using specific primers that amplify partial 5' region (1795bp) of the polyprotein encoding gene. *Lane* M: GeneRuler 1 kb DNA Ladder (Fermentas, USA); *Lane* 1: Amplification from pTZ57 CHIKV C-E3-E2-6K-E1 recombinant plasmid DNA (positive control), *Lane* 2: amplification from purified Chikungunya virus collected from the 20-60% interface. **(B)** Negatively stained Chikungunya virions as seen under transmission electron microscope (scale bar, 50 nm): The sucrose interface showed both cores and complete virions with external diameter 40-45 nm and 50-65 nm respectively.

Discussion

Virus purification is a routine method for several downstream applications and hence it is necessary to have an easy and simplified method that gives consistent results. In the present study we report a two step method for purifying Chikungunya virus from the infected cell culture supernatant. The procedure involves a combination of virus precipitation using polyethylene glycol and virus concentration using sucrose density gradient ultracentrifugation. We could band Chikungunya viral particles at the interface of 20-60% (w/v) discontinuous sucrose gradient using rate zonal ultra centrifugation. Upon electrophoresis of the purified virus, on denaturing PAGE, Chikungunya structural proteins E1/ E2 bands corresponding to the size of ~55kDa and capsid protein of size ~32KDa were noticed. As reported earlier, the association between E1 and E2 of CHIK virus is very strong than any other related alpha viruses and it was very difficult to separate E1 from E2 [11]. Thus the observed single band of E1/E2 is justified and is also in accordance with the earlier reports [17,18]. There was slightly increase in the size of E1/E2 proteins due to the presence of sucrose in the virus preparation which makes the protein to have slightly retarded movement. The obtained size of Capsid protein is in agreement with that of the earlier reports [19,20]. In the present study, no other proteins were noticed smaller than the capsid protein and the band intensity of capsid protein was weak in comparison to the E1/E2 protein as also reported by Simizu, et al [11]. Our study also suggests that the E3 of CHIK virus is not associated with the virions and is released into infected culture fluids which is in agreement with the previous report [11] and hence is not visible on the SDS-PAGE gel. The authenticity

of the virus protein in the purified fraction was reconfirmed Via western blotting using envelope protein (E2) specific antibodies. From our results, it is evident that 20-60% sucrose gradient is efficient as Chikungunya virus cannot not enter the 60% sucrose. The top 20% sucrose layer was sufficient enough to selectively permit the Chikungunya virions while arresting other media or host cell derived proteins. The present study is of significant importance, as we have successfully used PEG to concentrate and reduce the volume of low titer virus for ultracentrifugation. In addition, earlier studies by Eckels, et al [8] have used multiple steps that involve initial high speed ultra centrifugation and tween- ether extraction for virus concentration followed by sucrose gradients (5-30% in PBS) for purifying CHIKV virus. As the use of multiple steps and detergents may damage the virions, we have developed an improved method with only two steps for better purification of CHIKV with minimum impurities. Both cores with an approximate diameter of 40 nm and complete virions of diameter 50-65 nm were seen on transmission electron microscope. This observation is in agreement with that of recent study by Wataru Akahata, et al [19] where Chikungunya Virus like particles (VLPs) have been analyzed by Cryo electron microscopy and three-dimensional image reconstruction wherein the VLPs had an external diameter of 65 nm and a core diameter of 40 nm.

Conclusion

We have successfully purified the Chikungunya virions by an improved method using two steps with minimum impurities. The concentration and buoyant density gradient method followed in the present study particularly suits the purification of Chikungunya virus which produce low virus titers. It may also find applications in purifying Chikungunya VLPs and authentic virus for its morphological characterization.

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Conflict of Interest : None Declared.

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