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Efficient expression of Gag protein by recombinant Modified Vaccinia Ankara Virus (MVA) with HIV-1 Indian Subtype C *gagprotease* gene segments

Research Article

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Abbreviations: Human Immunodeficiency Virus (HIV); chick embryo fibroblasts, (CEF); Modified Vaccinia Ankara, (MVA); early late promoter, ($P_{E/L}$); virus-like-particles, (VLPs); polymerase chain reactions, (PCRs); Tween 20-Tris buffer Saline, (TTBS); phosphate buffered saline, (PBS); thymidine kinase, (TK); virus like particles, (VLPs);

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Summary

The AIDS epidemic in the developing world particularly in India represents a major threat where the infections are due to non-B clades of HIV-1. Most human immunodeficiency virus (HIV) vaccines currently under development are based on clade B strains of HIV-1. Since in India clade C is the predominant strain of HIV-1, it is imperative to develop an effective vaccine candidate from the locally circulating HIV strains. We describe here the development of recombinant Modified Vaccinia Ankara (MVA) expressing gag protein of HIV-1 Indian subtype C. Plasmid transfer vector, *pSC 65*, was used to transfer *gag-protease* gene segment of HIV-1 subtype C strain 49587 to MVA and gene segment was placed under the control of synthetic early late promoter ($P_{E/L}$). The recombinant MVA was selected by BrdU/X-gal selection. The recombinant MVA was found to express gag protein in BHK-21 cells and the expression was evaluated by p24 antigen capture ELISA, immunoblotting, immunoflourescence and formation of virus-like-particles (VLPs) in infected cells by electron microscopy. The construct showed stable and high expression of HIV-1 *gag* gene in eukaryotic cells.

I. Introduction

Since the time Human Immunodeficiency Virus (HIV) was first identified as the causative agent of AIDS, this epidemic has continued to spread beyond all expectations in severity as well as in scale of its impact. If the AIDS pandemic continues to spread at its current rate, there will be an additional 45 million new infections by 2010 with nearly 70 million deaths from the disease. (Stover et al, 2002; Klausner et al, 2003).

In the developing countries particularly, the AIDS epidemic has devastating consequences. Because of considerable expense and logistical difficulty in providing antiviral drugs to the infected individuals through out the world, it is clear that the best option for control and prevention of HIV/AIDS at present time is a preventive vaccine that can be applied widely at a low cost. More than 20 candidate vaccines have entered into the early phases of clinical trials**.** Most of these vaccines are based on naked DNA and/or live viral based constructs from HIV-1 subtype A and B.

The potential use of Modified vaccinia virus Ankara (MVA) as an efficient gene delivery vector is increasingly being recognized in novel vaccine development strategies. MVA, a highly attenuated strain of vaccinia virus, was derived from the vaccinia virus Ankara strain by over 570 passages in primary chick embryo fibroblasts (CEF) (Mayr et al, 1978). A number of large genetic deletions attenuated its replicative potential in a variety of mammalian cell lines including human cell lines in which it could either no longer replicate or replicate very inefficiently (Meyer et al, 1991). Nonetheless, MVA grows efficiently in primary CEF and baby hamster kidney cells (Carrol and Moss, 1997). However, the viral protein synthesis remains unimpaired even in non-permissive mammalian cells. This important property of MVA accounts for its usefulness as a safe yet efficient expression vector (Sutter and Moss, 1992). For construction of stable MVA expression vectors transfer plasmids have been developed (Sutter and Moss, 1992;

Antoine et al, 1996)). In recent studies, non-human primates immunized with a combination of DNA vaccine construct and recombinant MVA as prime-boost protocol have shown control of the pathogenic challenges by SHIV-89.6P (Amara et al, 2002).

India has second largest population of HIV infected individuals after South Africa. More than 4.5 million have been infected with this virus since its first detection in 1986 (NACO, 2003 www.naco.nic.in). More than 95% of these infections are due to subtype C of HIV-1. Subtype C also accounts for 74% of infections in South Africa and 96% in North Africa (UNAIDS. 2003). On the basis of different cell tropism and the pathogenicity of the subtype C viruses *vis-à-vis* subtype B viruses, it has been suggested that a vaccine should be designed and targeted according to the local circulating subtype of HIV-1. Therefore, development of HIV-1 subtype C based vaccinogens, capable of eliciting humoral and cell mediated immune responses, was undertaken in our laboratory under Prime Minister's *Jai Vigyan* Mission program of the Government of India. In the present study, we report development of a recombinant MVA construct expressing gag proteins with *gag-protease* gene from an Indian HIV-1 subtype C isolate. The expression of gag protein makes this construct as a promising vehicle for use as a live viral vector for immunogenicity testing in animal models.

II. Materials and methods

A. Cells, virus, plasmids

BHK-21 (Clone 13) cell line was obtained from National Center for Cell Science (Pune, India). It was grown and maintained MEM (E) medium with 10% fetal calf serum. Modified Vaccinia Ankara (MVA) and its plasmid transfer vector *pSC 65* were obtained from Dr. Bernard Moss, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland. pGEMTeasy *gag-pol* construct (7.2 kb) of HIV-1 Indian Subtype C *gagpol* genes (4.5 kb) was prepared at the Department of Microbiology, AIIMS, New Delhi and was used for the construction of the recombinant MVA with *gagprotease* gene (Chugh and Seth, 2003b).

B. Primers

The following primers were synthesized by the Commonwealth Biotechnology Incorporated (Richmond, VA, USA) and used in polymerase chain reactions (PCRs) for amplification of gag-protease gene segment.

*Gag*FP01 (139-173): 5'TTTGACTAGCGGAGGCTAGCAGGA GAGAGATGGGT3'

DP11 (1949-1923): 5'-CCATTCCTGGCTTTAATTTTACTGGT A-3'

C. Cloning of *gag-protease* gene in *pSC 65* vaccinia transfer vector

Gag-protease fragment of 1.8kb was amplified using *Gag*FP01 and DP11 from pGEMT*gagprotease49587* using Vent DNA polymerase (NEB, USA) with proofreading activity, which produced blunt end product. The vector (*pSC 65*) was digested with *SmaI* and purified. The insert was ligated to the linearised vector using blunt end ligation protocol. The resultant construct was named pSC*gagprotease49587*. The correct orientation of the insert was checked using *PstI* restriction digestions, which produced two bands of 5961bp and 3091bp size respectively, whereas the reverse orientation gave two bands of 6561bp and 2491 bp size. Further confirmation of recombinant plasmid pSC*gag-protease49587* was done by PCR for gag and protease genes and sequencing of the insert using Big Dye Terminator kit and ABI 310 sequencer.

D. Generation of recombinant MVA

A semi-confluent BHK-21 was infected with MVA (0.01-0.05 m.o.i.) and then transfected with 6ug pSC*gagprotease49587* construct using 'Lipofectamine plus' reagent (Invitrogen, USA) as per the manufacturer's instructions. The transfection mixture was replaced with fresh 5 ml medium after 4 hours incubation. Forty-eight hrs later the virus was harvested by freeze–thawing the contents thrice. The MVA recombinants were screened from non-recombinant virus preparation by standard X-gal staining technique with minor modifications (Earl et al, 1991). Briefly, cell culture medium was aspirated and BHK-21 cells infected with MVA *gagprotease49587* were fixed for 5 minutes at 4ºC with glutaraldehyde fixative in PBS. The fixative was then aspirated and X-gal staining solution (5mM each of ferroisothiocyanate and ferrus-isothiocyanate containing 1mg/ml X-gal and 2mM $MgCl_2$) was added. Cells were incubated until color developed. All positive cells were stained blue (shades of from deep, royal, to light).

E. Screening and plaque purification for recombinant MVA

Recombinant MVA virus was plaque purified on agarose containing selective medium containing BrdU+X-gal by standard technique (Earl et al, 1991). Briefly, BHK-21 cell monolayer was infected with 10-fold serial dilution of the recombinant virus preparation and were overlaid with selective plaque medium without phenol red but containing BrdU (0.05mg/ml) and 1% low melting point agarose and allowed to solidify at room temperature. The plates were incubated for 48 hours at 37°C in a CO_2 incubator. Thereafter, a second agarose medium overlay containing 0.4mg/ml X-gal was overlaid and the cultures were returned to 37°C incubator for 8-10hrs for color development. Blue plaques were picked and resuspended individually in 100 ul of MEM (E) and subjected to three cycles of freeze thawing. The virus suspension was diluted appropriately and subjected to plaque purification as mentioned above. The process was repeated six times to purify the recombinant MVA containing the insert. Finally, the recombinant MVA was purified on 36% sucrose cushion (10mMTris.Cl,pH 9.0) at 13,800 rpm for 80 min at 4°C using SW 45 rotor (Sorvall). Viral Pellet was finally resuspended in PBS.

F. p24 antigen capture ELISA

A time course experiment was performed to examine the expression of HIV-1 *gag* in BHK 21 cells infected with the recombinant virus. Cells and supernates were harvested at various time intervals post infection .The expression of p24 antigen was detected by HIV-1 p24 antigen capture ELISA kit (Innogenetics, Belgium). ELISA was performed as per the manufacturer's instructions. Standard curve was plotted for the absorbance recorded for standard provided in the kit and concentration of p24 antigen in samples was determined using standard curve. The negative controls included uninfected BHK-21 cells and cells infected with vector alone (MVA).

G. Immunoblotting

In-vitro expression of MVA *gagprotease49587* was also tested in infected BHK-21 cell lysates by immunoblotting. At 72 hrs post infection, cells were harvested and freeze-thawed thrice to prepare cell lysate. The proteins in the cell lysate were resolved by SDS-PAGE and were transferred onto a nitrocellulose membrane by Electrophoretic Transfer Cell (Bio Rad Laboratories) For further analysis by Western Blot, HIV-1 positive human polyclonal serum was used as a source of antibody. Cells infected with MVA parental vector served as control. The membrane was treated with non-fat powdered milk in TTBS (Tween 20- Tris buffer Saline) for 1 hr at room temp. HIV-1 positive human polyclonal serum (at a dilution of 1:200) in TBS was added to the membrane for 1hr at room temperature. After washing thrice with TTBS, the membrane was incubated at room temperature for 1 hr. with goat anti-human IgG conjugated with alkaline phosphatase (1:10,000). Membrane was then washed thrice with TTBS and incubated with the substrate (Sigma fast BCIP/NBT tablet dissolved in 10ml of deionized water, Sigma Chemicals Co., St. Louis) for 30 minutes in dark, at room temp with constant shaking. The reaction was stopped by rinsing the blot with deionized water.

H. Transmission electron microscopy

MVA*gagprotease49587* infected BHK-21 cells were scraped off from the plates with cell scraper, washed in phosphate buffered saline (PBS pH7.4) and fixed using Karnowsky fixative (2.5% glutaraldehyde and 2% formaldehyde solution) for two hours on ice. Cells were then washed with PBS thrice and post fixed with 1-% osmium tetroxide in PBS for two hours. After washing with PBS and distilled water, fixed cells were stained with 1-% uranyl acetate in 20% acetone for half an hour. The sample was dehydrated by treatment with acetone and cleared with toluene. Thereafter, infiltration was done with toluene araldiet mixture at room temperature and then infiltration was repeated at 50ºC temperature. The sample was embedded in epoxy resin, sectioned and viewed under TEM at various magnifications.

J. Immunofluorescence for the detection of HIV-1 gag expression

BHK-21 cell monolayers were grown on glass coverslips and infected with MVA- *gagprotease49587* at an m.o.i. of 5. After 24 hours, cells were washed twice with PBS and fixed in acetone at -20°C for 30 minutes. The cells were incubated with HIV positive human serum (1:50 dilution) for 30 min. After washing with PBS, the cells were reincubated with FITC conjugated goat anti-human IgG (Sigma) for 30 min. After washing with PBS, cover slips were counterstained with Evans Blue and were mounted in glycerol buffer for visualizing under fluorescent microscope.

III. Results

A. Construction of recombinant MVA with Indian HIV-1 subtype C *gag-protease*-gene construct

1. Subcloning in pSC 65 Vaccinia transfer vector

To construct recombinant MVA with *gag protease* genes of HIV-1 subtype C, a 1.8kb fragment of *gag protease* was amplified from the pGEM*gag-pol* clone using *Gag*FP01 and DP11 primers with Vent DNA *polymerase* (NEB, USA). The purified insert was blunt end ligated to the *SmaI* linearised *pSC65* vector (**Figure 1a)**. The correct orientation of the clone was confirmed using restriction digestion with *PstI* (**Figure 1b**), which produced two bands of 5961bp and 3091bp size respectively, whereas the reverse orientation two bands of 6561bp and 2491bp size. The recombinant clone obtained was further confirmed by PCR of *gag* and *protease*. The correct clone was selected and named as pSC*gagprotease49587*. The clone was sequenced using primer-walking strategy with nested sets of primers and various contigues were sorted and assembled into gag and protease genes. A total of 1736 base pairs were read (**Figure 2**). The sequence was aligned with the parent sequence and was found to be the same (Genbank Accession # AF533140).

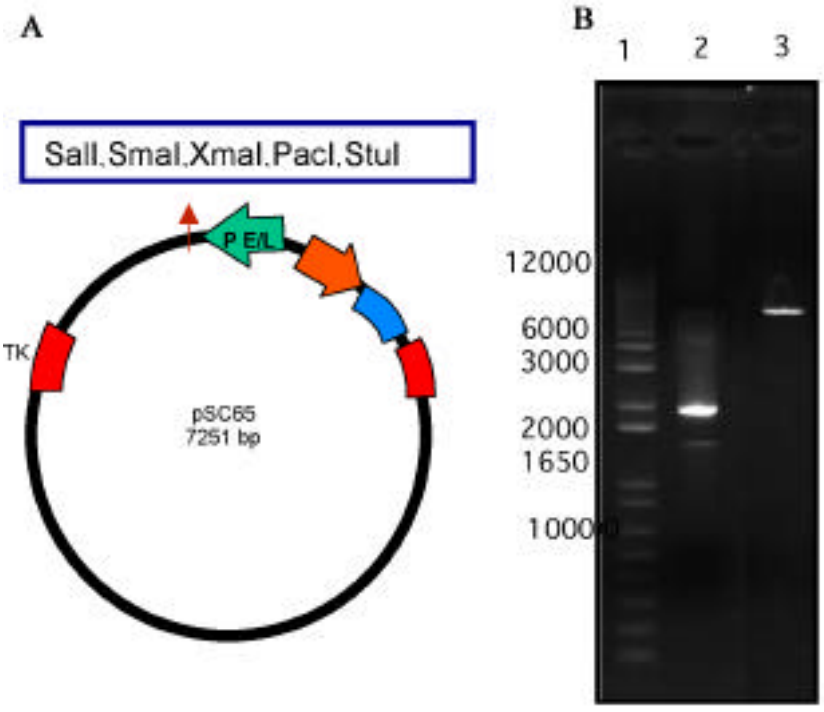


Figure 1. a) *pSC-65* vaccinia virus transfer vector and gel showing linearised vector (lane2) and amplified gagprotease gene segment (Lane3) for cloning. Lane 1 shows Ikb plus DNA Ladder (Invitrogen,USA) **b)** Recombinant plasmid pSC*gagprotease49587*. The correct orientation of the clone was confirmed using restriction digestion with *PstI*, which produced two bands of 5961bp and 3091bp (Lane2&3) size respectively. Lane 1&5 shows λ HindIII marker.

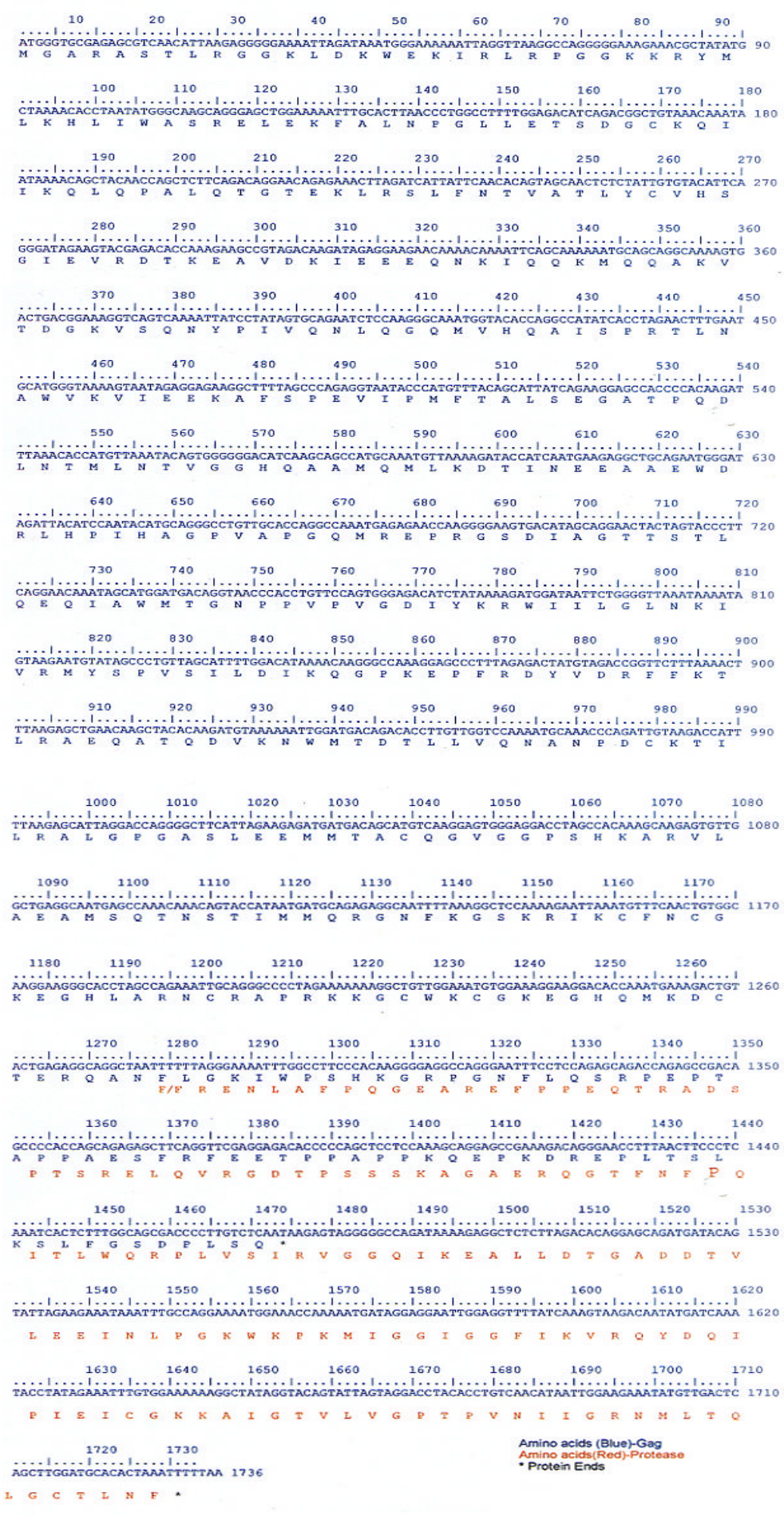


Figure 2. Complete sequence of *gag-protease* insert cloned into the vector *pSC 65*. The open reading frame of *gag* was found to be without any mutatio

2. Confirmation of double homologous recombination

Confirmation of recombination between pSC*gagprotease49587* and MVA was carried out using X-gal staining of the BHK-21 cells. The cells infected with recombinant MVA showed blue colored foci on the monolayer upon staining with X-gal stain. Thereby confirming that the expression cassette got inserted successfully into the MVA genome by means of double homologous recombination, at the *thymidine kinase(TK*) locus.

3. Plaque purification of MVA gagprotease49587

Plaque purification of the recombinant MVA was carried out using the selective medium containing BrdU and X-gal to ensure that only recombinant MVA with inactivated *TK* gene would grow in the BHK-21 monolayer. Six rounds of plaque purification were carried out under BrdU selection and blue screening in order to get rid of the parental MVA and to get pure stock of recombinant MVA expressing *gagprotease* genes. After completion of plaque purifications, a single plaque was amplified to prepare large stocks and pelleted on 36% sucrose cushion. The titer of recombinant MVA was determined by counting the number of blue plaques formed on the X-gal agarose-containing medium. Finally stocks of 10^8 p.f.u./ml were prepared in PBS and stored at -70°C for further use.

B. Expression study of MVA *gagprotease*49587

1. p24 antigen capture ELISA

The amount of protein secreted in the medium as well as in the infected BHK-21 cells was assessed by p24 antigen capture ELISA in a time course evaluation study. Supernates and cells were harvested at 12 hourly intervals for 108 hours. HIV-1 p24 antigen was detectable both in medium and cell pellet at 24-hr post-transfection. At 48 hrs, the concentration of p24 antigen in the medium as secreted protein was 445pg/ml. Whereas in the cell pellet, 309pg/ml of p24 antigen was detected at 72 hrs-post infection. Thereafter it declined, indicating thereby that the protein was being synthesized and secreted by the recombinant MVA*gagprotease49587* infected cells **(Figure 3a)**. Uninfected cells and cells infected with MVA alone served as negative control (without any insert). No p24 antigen was detected in any of the control samples.

2. Immunoblotting

The 55-kilodalton band representing gag precursor protein was detected along with another band corresponding to the 24 *KDa* position, suggesting that the gag polyprotein was successfully cloven by the HIV-1 protease **(Figure 3b**).

3. Transmission electron microscopy of transfected cells

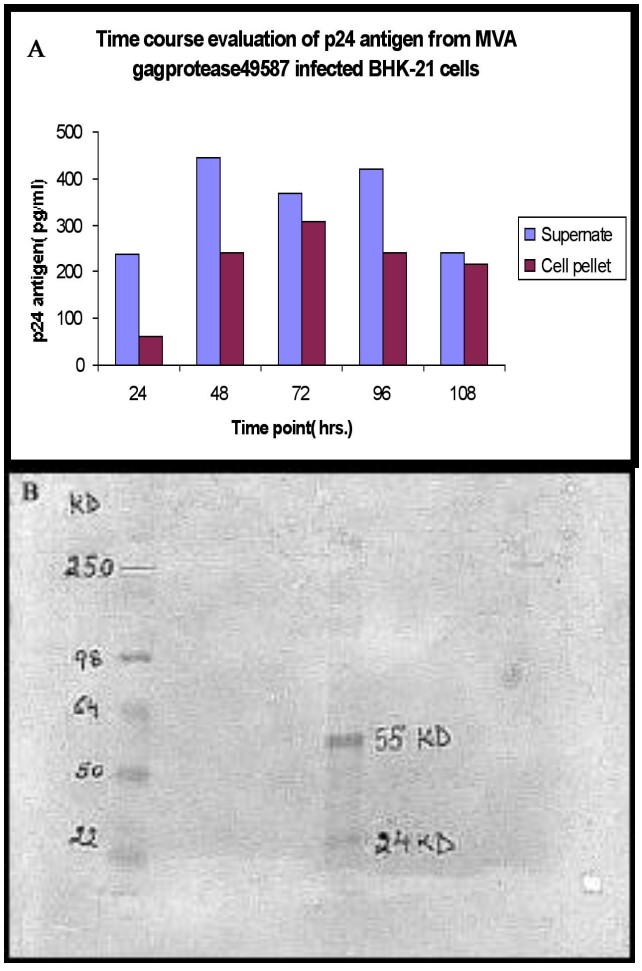
Numerous virus like particles (VLPs) were seen budding out of the cell membrane and lying outside the membrane in the intercellular spaces. The morphology of these particles corresponded to that of a pr55 VLP. These VLPs were observed in MVA *gagprotease49587* infected BHK-21 cells at 24 -48 hrs post infection. The average size of the particle was 120 -160 nm **(Figure 3c),** which were quite distinguishable from the MVA particles having size of nearly 250-300nm.

4. Indirect immunoflourescense assay

The infected cells expressing gag protein showed typical apple green fluorescence. (**Figure 3d)**. It showed that the infected cells produced gag protein of HIV-1, which was absent in the MVA alone-infected BHK-21 cells.

IV. Discussion

Most vaccines currently being developed are based on clade B strains of HIV-1, which are predominately found in North America and Europe. Since in India, subtype C is the predominant strain of HIV-1, it is necessary that a vaccine based on local circulating subtype be designed. Preliminary studies from our laboratory had shown that HIV-1 Indian subtype C based DNA constructs in *pJW4304* vector backbone were highly immunogenic.



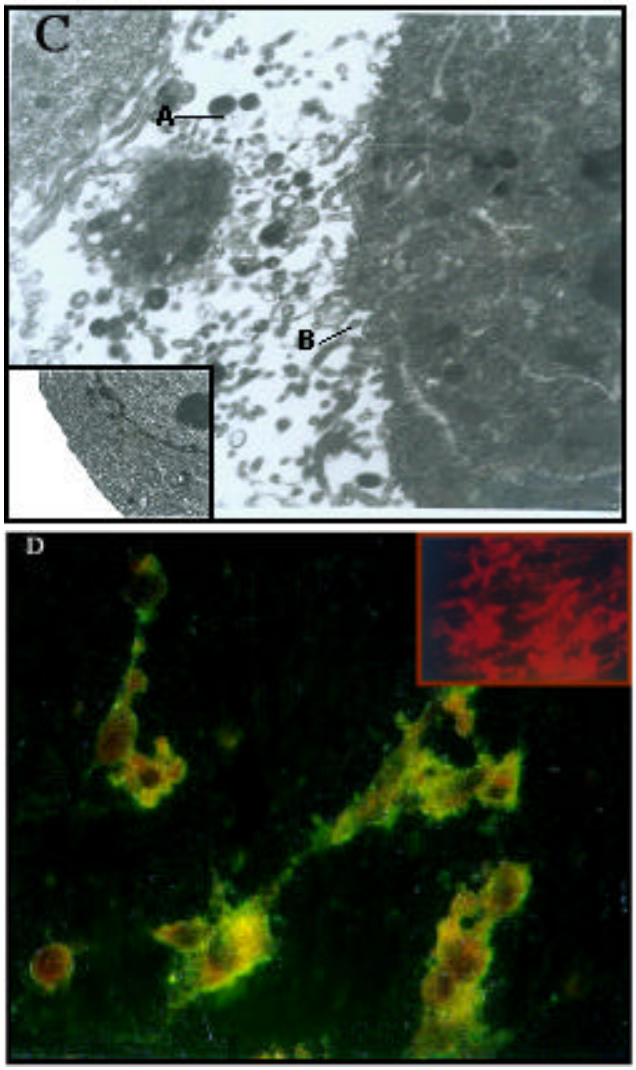


Figure 3. A) Time course evaluation of p24 antigen from MVA*gagprotease49587* infected BHK-21 cells at various time intervals in cell lysate and supernates. The relative amounts of the cell bound and secreted p24 detected by ELISA in BHK-21 infected with MVA*gagprotease* was quantified using HIV-1 p24 antigen capture ELISA .**B)** Western blot analysis of the MVA *gagprotease49587* infected BHK-21 cell lysate showed bands of 55kDa and smaller MW band corresponding to 24Kda(Lane5). No such bands were observed in MVA infected BHK-21 cell lysate (Lane8). The extract was subjected to SDS-PAGE and immunoblotting. HIV-1 positive human polyclonal serum served as the source of primary antibody. **C)** Transmission Electron Microscopy (4600X) of MVA*gagprotease49587* infected BHK-21 cells showing numerous VLPs (marked **B)** budding out from the cells. MVA particle (marked **A**) are also seen in the picture (TEM of control cells in small insert). **D)** The infected cells expressing gag protein showing typical apple green fluorescence. The control cell infected with MVA were negative (small insert in the picture). The red fluorescence of was due to Evan's Blue counter stain. HIV-1 positive human polyclonal serum served as the source of primary antibody.

HIV-1 *gag* based construct, pJW*gagprotease49587* (Chugh, 2003) has been shown to induce a broad based cellular as well as humoral immune response in Balb/C mice. The present study focuses on viral based vector system for generation of immunogenic recombinant MVA construct expressing HIV-1 Indian subtype C, *gag protease* genes. This recombinant MVA construct was given a nomenclature of MVA*gagprotease49587*.

MVA expressing different genes of HIV-1subtype B has been found to boost the immune response in the experimental animal models primed with DNA constructs (Amara et al, 2002; Ourmanov et al, 2000). In the present study, we demonstrate the construction and expression of HIV-1 Indian subtype C based recombinant MVA expressing *gagprotease* gene segments.

Gag gene is being targeted in a number of vaccine strategies involving plasmid DNA and live viral vectors as a backbone. *Gag* is the most immunogenic in HIV infected individuals and contains important helper as well as CTL epitopes (Addo et al, 2003; Chugh and Seth, 2003b) Since the first report describing *gag* based DNA vaccine (Boyer et al, 1998), a number of other *gag* based HIV DNA vaccine constructs have been prepared in the recent years (Baggarazi et al, 1999; Qiu et al, 2000; Zur Megede et al, 2000; Barouch 2001; Chugh and Seth, 2003a). Highly efficient recombinant viral based vectors that have been prepared and tested are MVA (Amara et al, 2002), canarypox (Santra et al, 2002), replication deficient adenovirus (Shiver et al, 2002) and replication competent Rhabdovirus (McGettingan et al, 2001). For construction of MVA*gagprotease 49587*, cloning of *protease* gene along with *gag* gene was necessary to provide the viral protease for processing of 55 *KDa*-gag precursor protein. Since the reading frame of *protease* gene was not altered, both the *gag* and protease proteins were synthesized as in their native infection process.

The immunoblotting study of MVA*gagprotease49587* showed 55*KDa* band along with a smaller protein of size of 24 *KDa* position, indicating thereby that the 55 kilodalton-Gag precursor was successfully cloven into respective products. The immunofluorescence study also showed expression of gag by the MVA*gagprotease49587* infected BHK-21 cells, thereby confirming the expression of the recombinant MVA construct.

The Transmission Electron Microscopy showed that numerous viral like particles (VLPs) were budding from the surface of BHK-21 cells infected with MVA*gagprotease49587* at 48 hours post infection. These VLPs were in the size range of 120-160 nm. The formation of VLPs upon infection clearly indicated the processing of precursor protein in cells infected with MVA*gagprotease49587*. This formation of non-infectious, morphological immature HIV-1 virus like particles (VLPs) depends upon the expression of myristoylated HIV-1 gag polyproteins (Goettlinger et al, 1989). Two domains located in p17 MA (a.a. 47-59) and in the p24 CA (a.a. 339-349) portions of pr55 *gag* are essential for the formation of immature HIV virion (Niedrig et al, 1994). VLPs have been seen after infection of different host cells with recombinant vaccinia virus (Karacostas et al, 1989; Haffar et al, 1990), MVA based SIV-*gagpol* construct (Sharpe et al, 2001).

To summarize, in the present study MVA construct (MVA *gagprotease49587)* was found to be express gag protein of HIV-1 in infected BHK-21 cells. Nonetheless, this study on generation of MVA*gagprotease49587* as a

potential HIV-1 Indian subtype C based vaccine strategies is limited to demonstration of efficient expression.

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