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An Experimental Trial to Prepared γ1 34.5 Herpes Simplex Virus 1 Immunogene by cloning Technique

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ABSTRACT
The herpes simplex virus (HSV) family offers particular advantages for use as a viral oncolytic. The engineered vectors that make up oncolytic HSVs (oHSVs) have demonstrated remarkable safety in clinical trials, with some evidence of efficacy. The past decade has seen a focus on increasing the efficacy of oncolytic vectors by adding exogenous transgenes to enhance tumor destruction.
This study aimed to prepared the Recombinant γ_1 34.5 - Infected Cell Protein (ICP34.5) Herpes Simplex Virus-1 Immunogenic by cloning technique from the wild type hsv-1, through the following steps; firstly propagation the virus on Vero cell line o estimate the cytopathic effect; after then it was diagnosed by immunofluorescent microscope in cell culture supernatant; followed by evaluation the titration of hsv-1 by plaque assay. Secondly, used specific set of primers for (γ_1 34.5 gene) sequence, then cleaved this sequence by restriction enzyme; followed it cloned this sequence by appropriate vector (PSL1180), then was mixed with another vector contain green fluorescent protein to ensure was gained these sequence. Thirdly, transport this sequence to vero cell line by specific transporter for development and produce there gene expression (ICP34.5) immunogenic.
Keyword: HSV-1, cytopathic effect, γ_1ICP34.5, vero cell line, Immunofluorescence
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INTRODUCTION

All of the herpesviruses (more than 100 known viruses) share a common virion morphology and a group of conserved genes that are required for virus replication (Miranda-Saksena *et al*., 2018). Herpes simplex virus (HSV) is a relatively large enveloped virus with a 152-kb linear double-stranded genome, that codes for about 90 RNA transcripts, 84 of which appear to encode proteins. The overall sequence homology between herpes simplex virus type 1 (HSV-1) and herpes simplex virus type 2 (HSV-2) is about 50%, with homologous sequences distributed over the entire genome (Widener, *et al*., 2014).

The herpes viruses are DNA viruses able to cause lytic and latent infections in both human and animal populations. Herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) are common, lifelong infections, which often have no symptoms (Tronstein, *et al*., 2011). People with symptoms may have painful blisters or sores at the site of infection. The viruses are transmitted through contact with an infected person's lesion, mucosal surface, or genital or oral secretions (Bernstein, *et al*., 2013).

Herpes Simplex Virus encodes several viral proteins to counter the host innate response to infection. γ_1 34.5, the major HSV-1 neurovirulence gene, has been deleted in all oHSV vectors clinically evaluated in the brain (1716 and G207) (Zemp *et al*., 2010). Among these, the multifunctional viral protein $\gamma_1$34.5 is central to countering several effector pathways in the host type I interferon (IFN) response. HSV γ_1 34.5 is present in two copies in the repeated regions of the viral genome, and although initially described as a late gene, its expression is actually "leaky late," with γ_1 34.5 functioning to counter the host response after late viral DNA synthesis but also in the first hours of infection. Within γ_1 34.5 are domains that specifically target host shutoff of protein synthesis HSV γ_1 34.5 is required for full virulence in the murine brain, however, the evidence suggests that γ_1 34.5 may function differently in newborn models of HSV disease compared to the adult (Wilcox *et al*., 2015).

ICP 34.5 is encoded by the $\gamma_1$134.5 gene of HSV-1 and HSV-2. The HSV-1(F) ICP34.5 consists of 263 amino acids and can be divided into three domains: an amino-terminal domain, a linker region of ATP (Ala-Thr-Pro) repeats, and a carboxyl-terminal domain (Li *et al*., 2011).

This study is aiming to an experimental Trial to Prepared γ_1 34.5 .ICP34.5 -*Herpes Simplex Virus* 1 Immunogene by cloning technique.

MATERIALS AND METHODS

- Cells and virus

African green monkey kidney (Vero) cells (ATCC- CCL-81) were purchased from the National Cell Bank of Iran (NCBI, Pasteur Institute of Iran, Tehran, Iran). Vero cells were cultured in (RPMI 1640 medium/ Euro clone - Italy) supplemented with (10% FBS / Biowest - France) and 50µl Penicillin- streptomycin antibiotic (10,000 unit penicillin /ml + 10,000 µg streptomycin/ml +29.2 mg L-glutamine in 0.9 % saline/ Euro clone - Italy). The cell lines were incubated at 37°C, under 5% CO2 and 95% humidity. HSV-1 wild type was collected from Iraqi patients infected with hsv-1 labial lesion (Marjan medical city). The samples were filtrated before use through (0.22µ) filter paper and then virus stocks were generated from low-multiplicity infections.

- DNA Extraction of HSV-1 from infected vero cells

Vero cells were plated into 6-cm2 culture dishes and incubated at 37°C with 5% CO2. After 24-hour incubation, the cells were infected with HSV-1 at a multiplicity of infection (MOI). On the next day, infected vero cells were harvested after observation of total cytopathic effect. The cells were collected by centrifugation at 300 xg at 4°C for 5 minutes, After centrifugation at 2000 xg at 4°C for 10 minutes, then supernatant was collected and kept at -20C0.

The DNA was extracted from supernatant by QIAgen DNA/RNA extraction kit depending on kit instruction.

- HSV-1 wild type Plaque Assay and γ_1 34.5 immunogene Plaque Assay

After seeding the vero cell on 6 wells plate before 2-3 days different 6-fold dilutions (10^1-10^6) of HSV-1wild type with RPMI medium containing samples were incubated on monolayers of Vero cells (6 well plate) at 37C0 with 5% CO2 for 1-1.5 h to allow the virus to attach and entry into cells. Then supernatants were aspirated, cells washed two times with PBS, and then we added ta each well 3ml of nitromethyl cellulose mixed with FBS 2% then incubated for 3 days at 37C0 with 5% CO2 to allow plaque formation. For plaque counting, the cells were fixed with ice-cold 100% methanol for 20 min at 25C0 then stained with 0.5% Gemsa stain and washed with tap water. HSV-1 titer was calculated through plaque counting and expressed as PFU/ml, as the following equation: PFU/ml = Avg. of plaques / DXV

D= dilution, V= volume of diluted virus added to the plate (Marconi and Manservigi, 2014; Baer and Kehn-Hall, 2014; Fabiani *et al*., 2017).

- Immunofluorescent Assay for Detection the HSV1 Wild Type on Vero cell.

The samples were Fixed in 3-4% paraformaldehyde in PBS pH 7.4 for 15 min at room temperature then the samples washed twice with ice cold PBS. After that target protein was prmeablized because it is expressed intracellularly,

Then these samples were incubated for 10 min with PBS containing 0.25% Triton X-100 (or 100 μM digitonin or 0.5% saponin). Followed that washed in PBS three times for 5 minutes.

These cells incubated with 1% BSA in PBST for 30 min to block nonspecific binding of the antibodies. we incubated in the mixture of two primary antibodies (Anti rabbit HSV-1 Biotin / abcam/USA) with 1% BSA in PBST in a humidified chamber for overnight at 4C°, then the mixture solution was decanted and cells were washed three times in PBS, 5 mins each wash.

After that the cells were incubated with the secondary antibodies (conjugated with Fluorescein isothiocyanate - FITC) in 1% BSA for 2 hours at room temperature in dark. Finally the mixture of the secondary antibody solution was decanted and washed with PBS for 5 min each and Mounted coverslip with a drop of mounting medium, then Sealed coverslip with nail polish to prevent drying and movement under microscope.

- Primers and pcr conditions for HSV-1 γ_1 34.5 gene

After the extraction of HSV-1 DNA from cell culture supernatant, conventional PCR was performed to amplify selected gene by using two specific primers set for γ_1 34.5 gene from the Pasteur institute of Iran with product size 830bp

(F -5 'AGAAGATCTGAGTAGTGCTTGCCTGTCTAACTCG-3', R-5 'AAAGGATCCCGACCTGATTAAGTTTTGCAGTAGCG-3'

Under the pcr condition involve (intial temp. = 95C^0 /10 min, Denaturation = 95C^0 /30 Sec, Annealing = 70.3 C^0 /45 Sec, Extension = 72C^0 /5 min, Final extension. = 72C^0 /5 min.

- Cloning of γ_1 34.5 gene

In the present study, the method for cloning γ_1 34.5 gene was performed using pSL1180 vector. The PCR products and plasmid vectors (pSL1180/Amersham Biosciences, USA)) were digested with restriction endonucleases (Hind III/ Thermoscientific, USA) Ligation of digested DNA fragments was performed using T4 DNA .ligase. Subsequently, GFP expression cassette-CMV promoter-EGFP-BGH poly A signal was subcloned from (pEGFP-N1/Vivantis, USA) and then inserted into pSL1180- γ_1 34.5 gene by using specific restriction enzyme (BglII / Thermoscientific, USA)

We Transfection and detection the new pSL-ICP34.5-CMV GFP-pA segment into the Vero cell line on the day before transfection, Vero cells were seeded onto 6-well plates and incubated at 37°C and 5% CO2. Afterwards, pSL-ICP34.5-CMV GFP-pA was transfected to vero cells using (lipofectamine 2000/ Thermoscientific, USA) according to the manufacturer's instructions. Transfected cells were incubated at 37°C overnight. The next day, cells were observed with an inverted fluorescent microscope to investigate fluorescent dye expression.

RESULTS

Figure (1) shows the development of vero cell line after thawing it and monitoring their growth from (1- 4 days) to reach a density around 90% at this moment the cells are ready for trypsinase , freezing and inoculation with the virus.

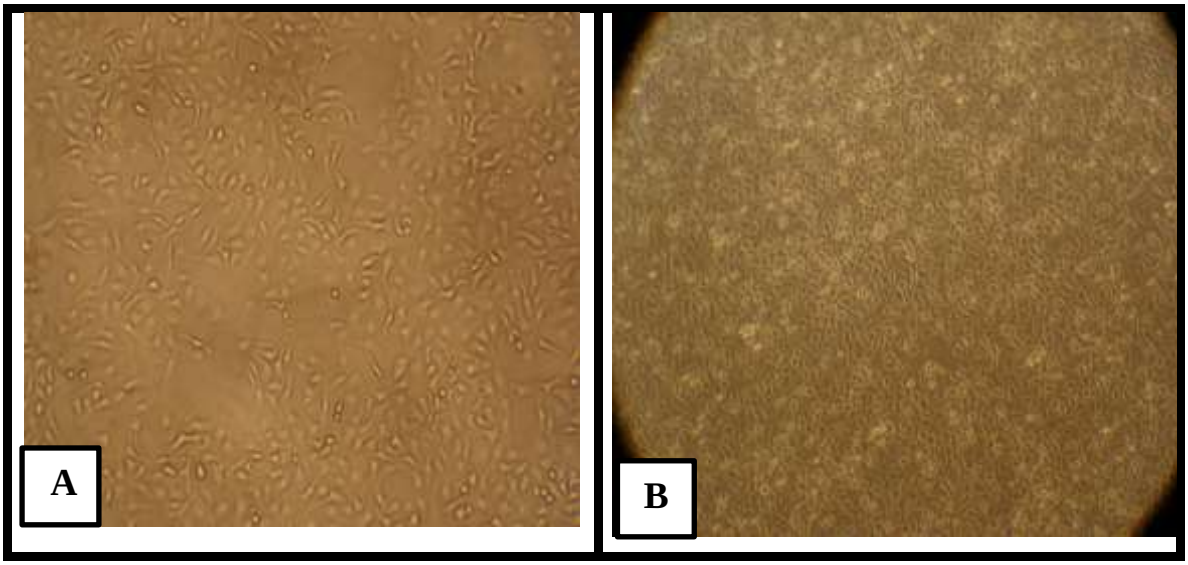


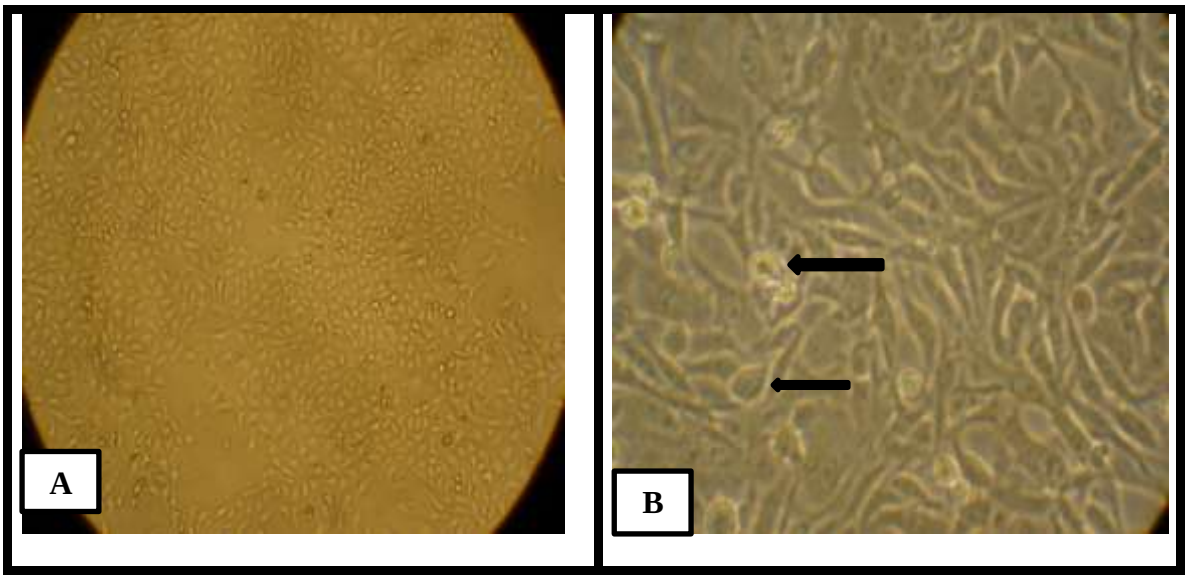
Figure 1: Propagation of Vero Cell Line on RPMI 10% FBS Medium

A-30 % confluent after 2 days of incubation -10X Magnification power.

B-90 % confluent after 4 days of incubation-10X Magnification power.

- HSV-1 wild type Plaque Assay

In order to propagation of HSV-1 on vero cell line ; were seeded 3ml of vero cell in 6cm^2 Petridis at condition (37C0;CO2 5%) for 2 days and reach the cells at 90% confluent. Filtrated 1ml of HSV1 stock via (0.22µ filter paper) and then inoculated the cells; after then waiting for 4 days to estimation the cytopathic effect (CPE) of HSV1. Three out of five HSV-1 isolates, were developed plaques as varying sizes and cytopathic effect after propagation of HSV-1 on vero cell line as shown in Figure (2).



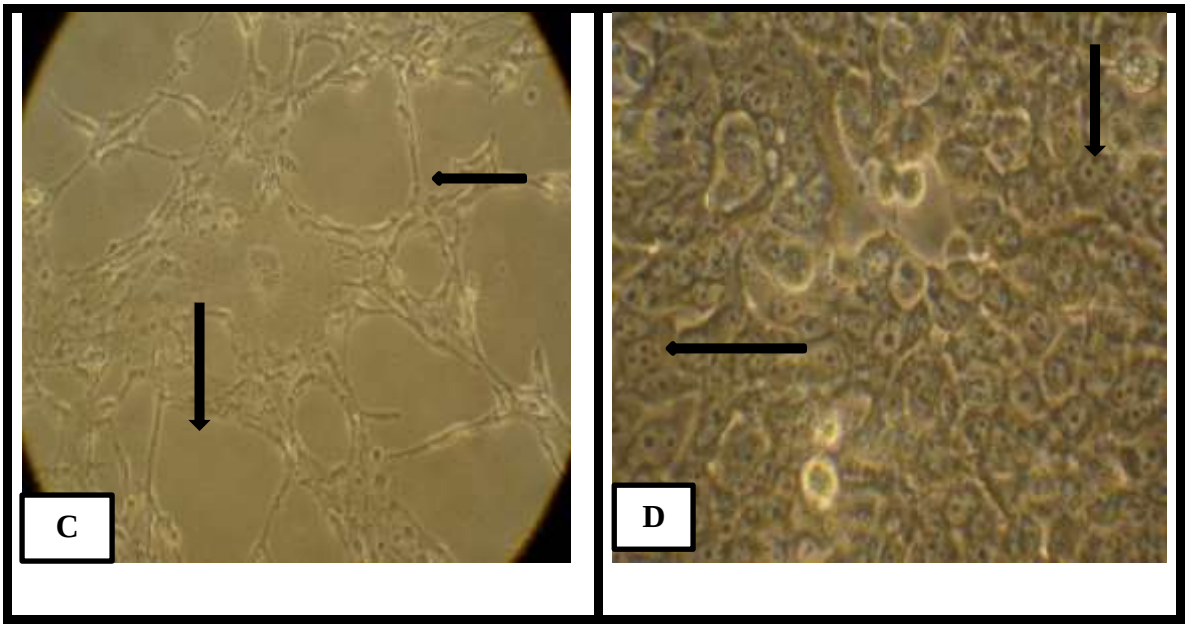


Figure 2: Cytopathic Effect of Wild Type HSV-1 on Vero Cell Line

A-Normal Vero Cell Line-10X M.P B-Ballooning CPE After 4 Days Of Infection-40X M.P C-Syncytium CPE- 10X M. D-Intra Cytoplasmic Inclusion Bodies- 40 X M.P

- Evaluation of HSV-1 Wild Type by Plaque Assay Method

Plaque assay method was used to evaluate the HSV-1 titration in biological specimens, as well as determine the quantify of HSV1 titer in supernatants from culture of HSV-1-on vero cell line. Firstly, preparation serial dilutions of HSV-1 from 10-1 to 10-8. Secondly, we took the dilution from 10-3 to 10-8 and propagation of HSV1 on 6 wells of Vero cells plated.

The titration of HSV1 were measured depending on the following equation:-

PFU/ml = No. of plaques / dilution of virus X volume of diluted virus that added

Figure (3) showing the results of HSV1-titeration as the following: plate (1) 1250 X 104 PFU/ml.; plate (2) 940 X 105 PFU/ml.; plate (3) 100 X 106 PFU/ml; plate (4) 28 X 107 PFU/ml; plate (5) 16 X 108 PFU/ml; plate (6) 10 X 109 PFU/ml .

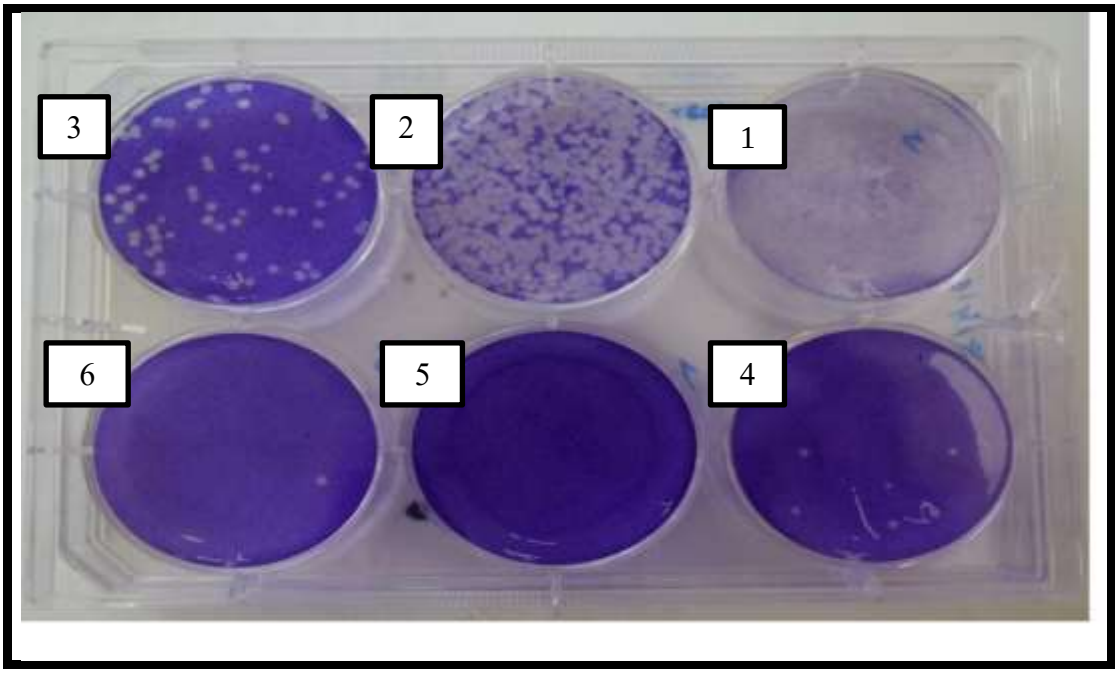


Figure 3: The Titeration of HSV-1 on Vero Cell by Plaque Assay Method (diluted 10^{3}-10^{8}).

- Detection of HSV1 in Direct Specimens by Immunofluorescence Assay Using a Monoclonal Antibody

Immunofluorescence assay was used in current study as a reliable and rapid diagnostic the presence of HSV-1 in vesicular lesion specimens. The HSV-1 was incubated with a mouse IgG primary antibody followed by incubation with a DAPI -labeled goat anti–mouse IgG secondary antibody. After removal of the secondary antibody, the specimen viewing on the fluorescence microscope. Figure (4) shows the positive specimens for HSV-1 by immunofluorescence assay.

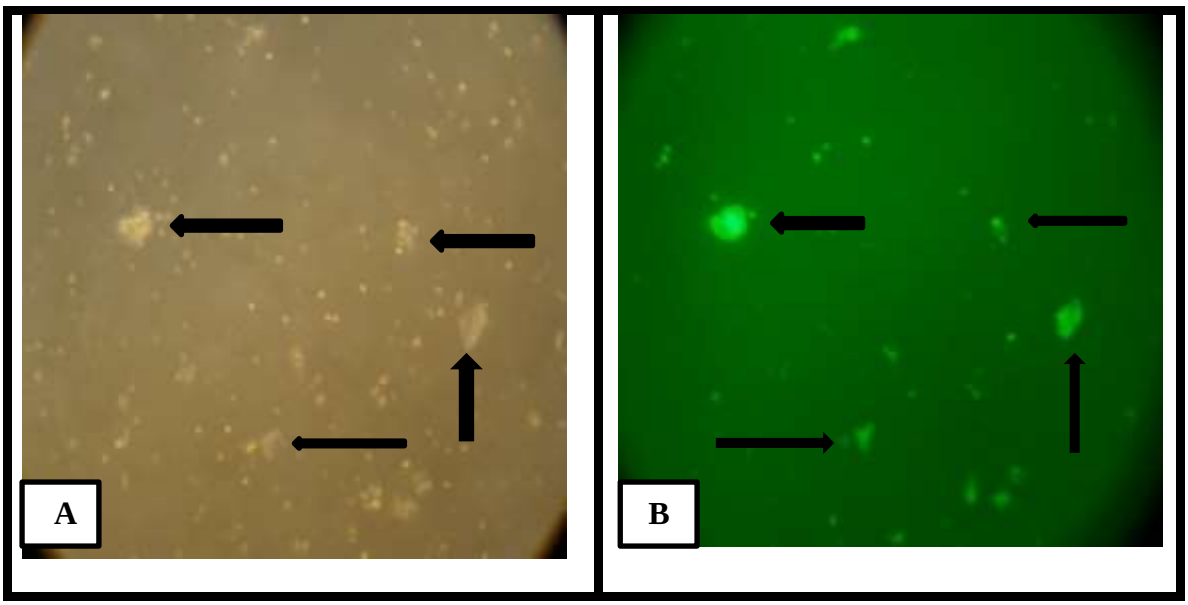


Figure 4: Photograph of Herpes simplex virus type 1 specific immunofluorescence by using monoclonal antibody 10X M.P. A-without fluorescent vision B-under fluorescent vision

- Formation of Immunogenic HSV-1 Virus (Infected Cell Protein-ICP34.5) by Using Cloning Process

Before the generation the immunogenic virus was must be extraction the HSV-1 nucleic acid from the cell culture supernatant by using (QIAgen DNA/RNA viral extraction kit) and amplification this sample via conventional PCR depending on specific primer HSV1- γ_1 34.5.

After amplification process the PCR product inserted into vector plasmid (PSL1180) by using specific restriction enzyme (*Hind III*) to perform homologous recombination PSL1180- γ_1 34.5, followed this process was used the GFP expression cassette-CMV promoter-EGFP-BGH poly A signal from pEGFP- N1 vector to recognized these new segment and sub -cloned into pSL1180- γ_1 34.5by using another specific restriction enzyme (*BglII*). Finally, the pSL-γ_1 34.5 -EGFP vector was generated with GFP reporter gene for generation of the immunogenic virus (Figure: 5, 6, 7)

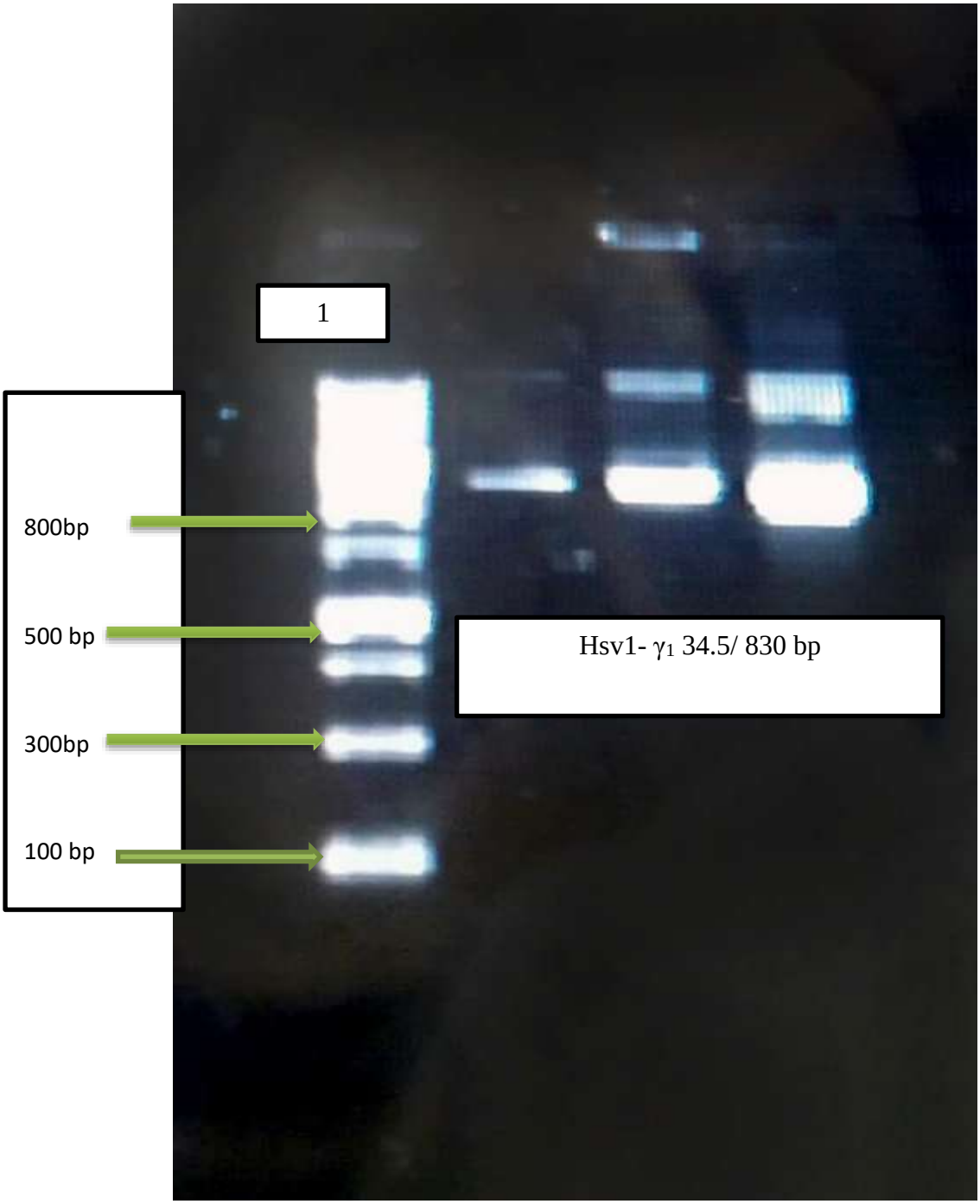


Figure 5: PCR analysis of herpes simplex virus (γ_1 34.5). PCR was performed HSV1 by using specific primers listed in table (3-5). Lane1= 1 kb DNA ladder, Lane 2 , 3 , 4 = PCR product of γ_1 34.5 hsv-1 virus showing three bands at 830 bp 1 kb DNA ladder

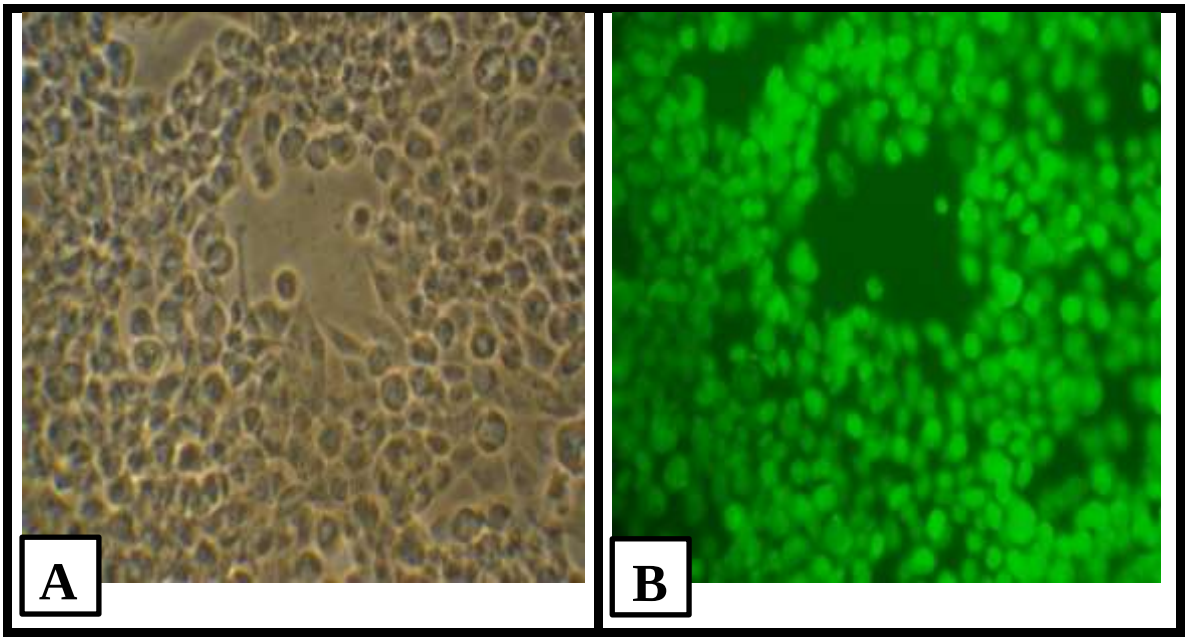


Figure 6: Positive Infected Cells by Analysis of Green Fluorescent Protein (GFP).
A-Immunogenic virus without fluorescent – 10X M.P.B-Immunogenic virus with fluorescent – 10X M.P.

The titration of immunogenic HSV1 were measured depending on the following equation:
PFU/ml = No. of plaques / dilution of virus X volume of diluted virus that added)

Figure (7) showing the results of immunogenic HSV1-titeration as the following: plate (1) 75 X 104 PFU/ml.; plate (2) 30 X 105 PFU/ml.; plate (3) 23 X 106 PFU/ml; plate (4) 20 X 107 PFU/ml; plate (5) 7 X 108 PFU/ml; plate (6) 1 X 109 PFU/ml .

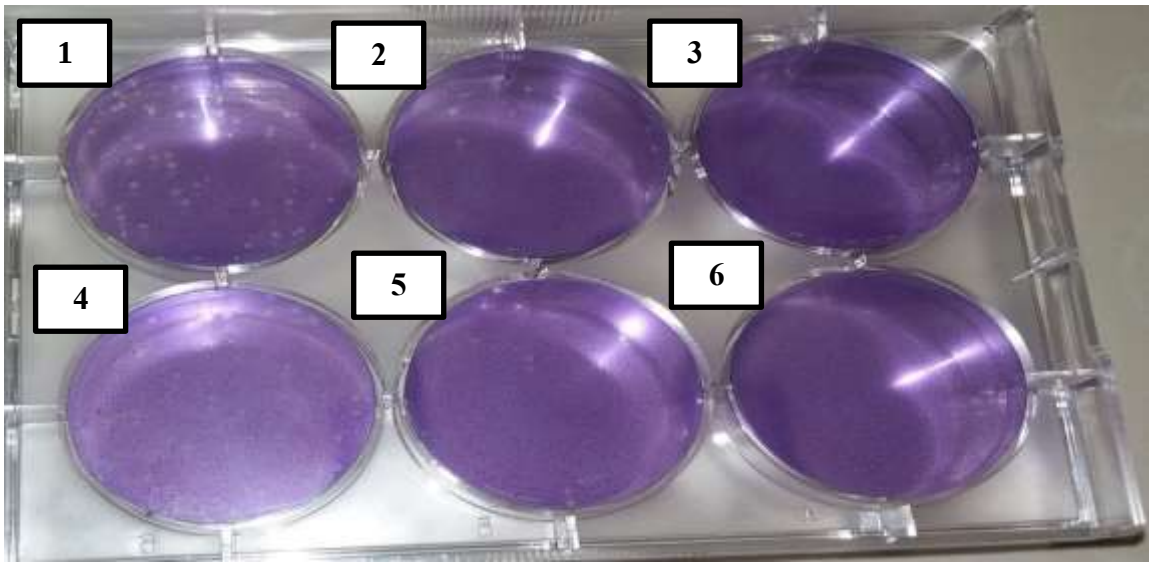


Figure 7: Isolation and Purification of Immunogenic Virus by Plaque Formation Test (diluted 10^3-10^8).

DISCUSSION

Vero cell line is amongst the commonly used continuous cell lines in the tissue culture laboratory and used in combination for the isolation of commonly encountered clinical pathogens like HSV1, HSV2, measles, enteroviruses, adenoviruses. The preservation of cell lines is important particularly to maintain the lowest number of passage for a longer time, in order to preserve the genetic stability and support the growth of virus to express proper viral antigens. Cell lines as well as infected cell cultures are processed in liquid N 2 (-196ºC) as a customary. But the procedure demands good expertise and timely handling of the cell lines before putting inside the liquid N 2 (Mishra *et al*., 2010).
The study of the wild-type HSV-1 strain based on the cytopathogenic features and are important in the detection and discrimination the virus. Vero cell lines showed the highest efficacy for the propagation of HSV. The cytopathic effect was detected 24 h post-infection (p.i.) and the presence of syncytia was remarkable 48 h p.i. The present study, is consistent with the study of Stanfield *et al*., (2014)

who used the vero cell to propagation and replication the immunogenic hsv-1 virus (VC2 strain).

The current results, are consistent with the Nozawa *et al*.,(2014) ; Nabavinia *et al* .,(2015) and Dilnessa and Zeleke, (2017), who found the CPE was observed 24-48h after the inoculation of the clinical specimens. Infected cells presented increased cytoplasm refringence, cell rounding and clumping, initially in a focal pattern.

Characteristically, giant multinucleated cells were observed after HE staining of infected cells in epithelial keratinocytes. The isolation of HSV in cell culture as a diagnostic method for HSV infection has been recognized for its efficiency, rapidity and low cost, as has been demonstrated elsewhere. However, it has been shown that less than 60% of HSV infections are detected Nozawa *et al*.,(2014). The results of Shintani *et al* ., (2011) and Fabiani *et al*.,(2017) were found the HSV-1 make plaque are correspond with our result.

Plaque assay method for HSV-1 titration which allows to easily and quickly analyze viral replication and infectivity in swab specimens. These features, it is particularly suitable in experimental virology and especially for the screening of antiviral compounds. Moreover, this method can be exploited for different viruses causing cytopathic effect or not (CPE) (Kumar *et al*., 2012). This method is based on the counting of discrete plaque caused by virus-induced CPE, considered a marker of infectious viral units unfortunately, it is time consuming (e.g., 48–72 for HSV-1), especially because virus-induced CPE may take long time to be visualized as plaques. Furthermore, it general susceptibility or to cell overlay systems (Borisevich *et al*. 2007; Baer and Kehn-Hall, 2014) and potential human via introduced by manual plaque counting.

Our method allows a quicker detection of HSV-1 infectious foci (e.g., 24 h for HSV-1), does not need the use of cell overlay systems, requires a smaller volume of samples (3 ml in 6 well seeded cells =18 ml of cells with medium) – thus also taking advantage of multichannel pipettor use for handling large amount of samples.

The standard plaque assay (SPA), in this method, a confluent monolayer of permissive cells is incubated with the biological sample potentially containing HSV-1, and then covered with an immobilizing semisolid overlay medium to prevent indiscriminate spreading of neo-formed virions. Thus, viral infection and replication are constrained to the surrounding cells and individual plaques, or zones of cell death, become detectable and countable. The HSV-1 titer results from the number of formed plaques per milliliter of sample, and it is expressed as plaque forming unit per ml (PFU/ml) (Fabiani *et al*., 2017).

Viral antigen can be easily detected by direct immunofluorescence (IF) assay using fluorescein-labelled typespecific monoclonal antibodies on smears, or by enzyme immunoassay (EIA) on swabs (LeGoff *et al*., 2014).

Diagnosis of HSV infection with tissue culture has low sensitivity because HSV is isolated from lesions in about 80% of primary infections but in only 25–50% of recurrent lesions, and in even fewer people whose lesions have begun to heal. Thus, fluid collected from intact blisters (vesicular or pustular lesions) will grow out in culture more than 90% of the time. By the time the lesions have crusted over, only about 25% of cultures will be positive. Failure to detect HSV by culture does not indicate an absence of HSV infection (Wald *et al*., 2003). Typing of HSV using cell culture can be performed directly on infected cell cultures using fluorescein-labelled type specific monoclonal antibodies by direct immunofluorescence which constitutes the most practicable procedure, or, eventually, by testing the cell supernatant by molecular assays (Ustaçelebi, 2001).

However, up to our best knowledge, this is the new report of replacing γ_1 34.5 with distinct fluorescent dye (green dye). In addition, generated a single-deleted γ_1 34.5 virus, HSV-GFP, which has been reported so far and has shown that it gives higher titers than common dually deleted γ34.5 strains, as well as the original wild-type strain. Deletion of γ34.5 has been shown to attenuate HSV-1-based viruses these result is consistent with Kanai *et al*., (2012).

γ_1 34.5 is an HSV-1 gene product that promotes dephosphorylation of eIF-2 and is necessary for robust HSV-1 replication (Mullen *et al*., 2002). γ 34.5 gene has been demonstrated to decrease replication competency of recombinant virus in comparison with parental virus (Duebgen *et al*.,2014).

In the current study, was used the immunogenic HSV-1 ICP34.5 as live attenuated vaccine and this is corresponds with Leib *et al*.,(2009) and Zhu *et al*., (2014) who used ICP34.5 as live attenuated vaccine of HSV-1and HSV2 ,respectively.

ICP34.5 of herpes simplex viruses is a virulent factor with multiple cellular functions. ICP34.5 contains a consensus binding site for PP1. The PP1 binding motif is required for the formation of a high-molecular-weight complex that contains the dephosphorylation activity toward eIF2. On the other hand, ICP34.5 truncation mutations or replacement of the conserved amino acids completely abolishes the dephosphorylation of eIF2 (Li *et al*., 2011).

The herpes simplex virus 1 (HSV-1) protein ICP34.5, encode the γ_1 34.5 gene, interferes with several host defense mechanisms by binding cellular proteins that would otherwise stimulate the cell's autophagic, translational-arrest, and type I interferon responses to virus infection. ICP34.5 also plays a crucial role in determining the severity of nervous system infections with HSV-1 and HSV-2 (Korom *et al*., 2014).

In the present study, a fluorescent expressing oHSV, named HSV-G, was constructed through the deletion of γ_1 34.5 copy and insertion of GFP and green-Cherry coding sequence into deletion sites. These vectors could be isolated easily by fluorescent microscopy or fluorescence activated cell sorting for rapid isolation or enrichment of recombinant viruses , these result is agree with the result from published paper by Popov *et al*.,(2009).This property is more evident with HSV-GR. New recombinant viruses could be easily detected and separated from other viruses by the selection of one specific color (e.g. green). These vectors were constructed by conventional homologous recombination approach (Yuan *et al*., 2016).

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CONCLUSION

Based on results was concluded, the γ34.5 Gene and Infected Cell Protein- (ICP34.5) Expression as initially immunogenic ability of these gene to induce high serum neutralizing antibody titers activity without deleterious changes in the cell.

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