

Evaluation of Peripheral Blood Lymphocytes as Cell Line for the Propagation of Human Herpes Simplex 1

تقييم خلايا اللمفوسايت المعزولة من الدم المحيطي للإنسان كخط زرع لتتبع فيروس الحلا البسيط من النوع الأولي

***Dr. Khalida K. Abbas Al-Kelaby**

***PhD in Virology/Department of Clinical and laboratory sciences/College of Pharmacy/ Kufa University.**

E-mail: khalidahk.abbas@uokufa.edu.iq

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الهدف: تهدف هذه الدراسة إلى تقييم مدى إمكانية استخدام خلايا اللمفوسايت المعزولة من الدم المحيطي للإنسان كخط زرع لتتبع فيروس الحلا من النوع (Herpes simplex 1) والكشف عن ذلك باستخدام الطرق التشخيصية الحديثة.

المنهجية: تضمنت هذه الدراسة جمع 40 عينة من حالات الإصابة بالتهابات جلدية تم فحصها باستخدام تقنية بلمرة الحامض النووي Real-time polymerase chain reaction والخاص بتشخيص فيروس الحلا البسيط من النوع الأول والثاني وباستخدام العدة التشخيصية المسماة بـ Bosphore® HSV 1-2 Genotyping Kit v1 والمنتجة من شركة اناتوليا التركية (Anatolia geneworks,Turkey).

النتائج: أظهرت هذه الدراسة أن فيروس الهيربس انتقاء خمس عينات موجبة لدراسة التي . وبإستخدام تقنية الزرع وتم فصل هذه الخلايا واستزاعها كخط زرع أظهرت هذه الخلايا استجابة متفاوتة في إظهار المرض المتعلق بالفيروس وتم تأكيد الإصابة الخلوية بتقنية الومضان المناعي غير المباشر indirect immunoflourescent assay المعتمدة على الأضداد وحيدة النسيلة monoclonal antibodies والخاصة بفيروس الحلا البسيط من النوع الأول.

الاستنتاجات: إن العدة التشخيصية Bosphore® HSV 1-2 Genotyping Kit v1 نموذجية في تشخيص الحالات المتسببة عن فيروس الهيربس من النوع وان تقنية بلمرة الحامض النووي هي الأسرع والأدق في تشخيص الفيروس قيد الدراسة كما إن الخلايا المفروية ممكنة الاستخدام للتقصي عن الامراضية المتسببة عن الفيروس بتقنية الزرع الخلوي.

التوصيات: Real-time polymerase chain reaction يعتبر جانب مكمل لتشخيص فايروس الهيربس ويفضل استخدام بادئات خاصة بعوامل الضراوة المتعلقة بالفايروس ومتابعة امراضيتها.

Abstract

Objectives: This study was planned to evaluate the use of peripheral blood lymphocytes as cell line for propagation of human herpes simplex 1, by the using of modern diagnostic techniques.

Methodology: Primarily, 40 samples were collected from dermal lesions, investigated by RT-PCR technique directed to certify human herpes simplex1 infections Bosphore® HSV 1-2 Genotyping Kit v1(Anatolia geneworks,Turkey) was used for the detection protocol.

Results: the results revealed that HSV1 was correlated with 23(57.5%) of the total cases investigated. Five of HSV1 positive samples were selected and applied to the assay of in vitro studying of specific cytopathic effects(CPE) via cell culture technique. Peripheral blood lymphocytes were isolated and propagated as a cell line. The demonstration of specific HSV1 cytopathic effect was demonstrated by indirect immunoflourescent antibody technique. This approach was revealed different degrees of sensitivity for supporting the growth of human herpes simplex1 virus; these cells were sensitive enough to support the growth of HSV1 virus.

Conclusions: We concluded that Bosphore® HSV 1 Genotyping Kit v1 allows very rapid detection of HSV DNA in dermal leisions. peripheral blood lymphocytes were efficient enough for the studying of CPE of HSV1, while PCR assay was more efficient and more précised as a diagnostic technique.

Recommendations: Real-time polymerase chain reaction is complementary to cell culture technique in diagnosis of HSV, and it's preferred to use specific primers of viral virulence factors and monitoring their pathogenicity.

Key words: lymphocytes. cell culture, herpes simplex1, real-time PCR .fluorescent assay.

INTRODUCTION

Herpes simplex virus 1 and 2 (HSV-1 and HSV-2) are two species of the herpes virus family, Herpesviridae, which cause infections in humans (1). The structure of herpes viruses consists of a relatively large double-stranded, linear DNA genome encased within an icosahedral protein capsid, which is wrapped in a lipid bilayer called the envelope. The envelope is joined to the capsid by means of a tegument. The complete particle known as the virion(2). The genome of HSV-1 and HSV-2 contain at least 74 genes (or open-reading frames, ORFs) within their genomes(3), although speculation over gene crowding allows as many as 84 unique protein coding genes by 94 putative ORFs(4). These genes encode a variety of proteins involved in forming the capsid, tegument and envelope of the virus, as well as controlling the replication and infectivity of the virus.

In the case of a herpes virus, initial interactions occur when a viral envelope glycoprotein called glycoprotein C (gC) interact with a cell surface particle called heparan sulfate. A second glycoprotein, glycoprotein D (gD), binds specifically to a receptor called the herpesvirus entry mediator receptor (HVEM) and provides a strong, fixed attachment to the host cell. These interactions bring the membrane surfaces into mutual proximity and allow for other glycoproteins embedded in the viral envelope to interact with other cell surface molecules. Once bound to the HVEM, gD changes its conformation and interacts with viral glycoproteins H (gH) and L (gL), to form a complex. The interaction of these membrane proteins results in the hemifusion state. Afterward, gB interaction with the gH/gL complex creates an entry pore for the viral capsid (5). Glycoprotein B interacts with glycosaminoglycans on the surface of the host cell.

OBJECTIVES:

- 1- Studying the prevalence of HSV 1 as a cause of dermal lesions.
- 2- Evaluation of peripheral blood lymphocytes as cell line for viral propagation in vitro.

MATERIAL AND METHODS:

Collection of dermal lesion Samples

These specimens were collected aseptically via wetting the end of sterilized cotton swab with the lesion discharge or tissue fluid from scraped pustules. the swabs then putted into sterilized test tube containing 2 ml of PBS that should applied to antibiotic pretreatment, then stored at -80°C till be used.

DNA extraction ;

DNA extraction from the skin lesion swabs and cell culture supernatants was done by the using of Bosphore® viral DNA extraction spin kit (Bosphore®, Anatolia geneworks,Turkey). This kit is highly compatible with the kit of Bosphore® HSV 1-2 Genotyping Kit v1(Anatolia geneworks,Turkey), which is based on the Real-Time PCR method. The DNA extraction is based on the silica membrane column separation method, involve 4 main steps; lysis, binding, wash and elution, starting with a sample volume of 200µl and DNA recovery (elution) volume is 60 µl.

The concentration and the purity of the extracted total DNA were determined by measuring the absorbance ratio at wavelength 260 nm over 280 nm using scandrop spectrophotometer(analyticajena- Germany).

Real-Time PCR

The amplification reaction occurs by the repeating cycles of heating and cooling. real-time PCR were performed using PCR kit (Bosphore® HSV 1-2 Genotyping Kit v1(Anatolia gene works,Turkey)). The real-time PCR assay was performed in a total reaction mixture(25 μ l) containing 10 μ l of DNA extracts, 15 μ l of PCR master mix Amplification and real-time fluorescence detection were performed using the (smart cycler) real-time PCR system (Germany) and the following protocol: an initial denaturation and polymerase activation step for 14.5 min at 95 C, followed by 50 cycles of denaturation at 97 C for 30 sec and 60 C for 1.30min with a final step (holding step). Real-time fluorescent measurements were recorded and a Ct value for each sample was calculated by determining the point at which the fluorescence exceeded the threshold line. Positive controls for HSV1 and HSV2, Negative controls were added to each run. Real-time PCR products were electrophoresed on 1.5% agarose gel in TBE buffer (40 mM of tris and 2 mM of EDTA, with PH value of 8.0) containing ethidium bromide for 1.5 hr at 100 volt for detection of HSV 1 DNA bands.

Cells and culture assay.

Peripheral blood mononuclear cells (5×10^7) were isolated from healthy donors by sedimentation in Lymphoprep™ (Nyegaraa, Oslo, Norway) and cultured in RPMI 1640 (Gibco, USA) with L-Glutamin and 10 mM HEPES (pH 7.5) supplemented with 5% fetal calf serum, glutamine (300 μ g/ml), and penicillin-streptomycin (20 μ g/ml each), (Alembic Limited, India). Peripheral blood lymphocytes were seeded onto 25cm² volume cell culture flasks. At that time, the cells were counted and assessed for viability by 0.5% trypan blue exclusion. The percent of viable cells were determined at indicated time intervals in both growth and maintenance media, and 2×10^6 cells were sub cultured in tissue culture flask containing 9 ml stimulation medium or growth medium, every 72 hours. The lymphocytes culture was maintained continuously by this method. Strict aseptic technique was used when subculture the lymphocytes to prevent bacterial or fungal contamination.

Monitoring of HSV-1 cytopathic effect and fluorescent assay

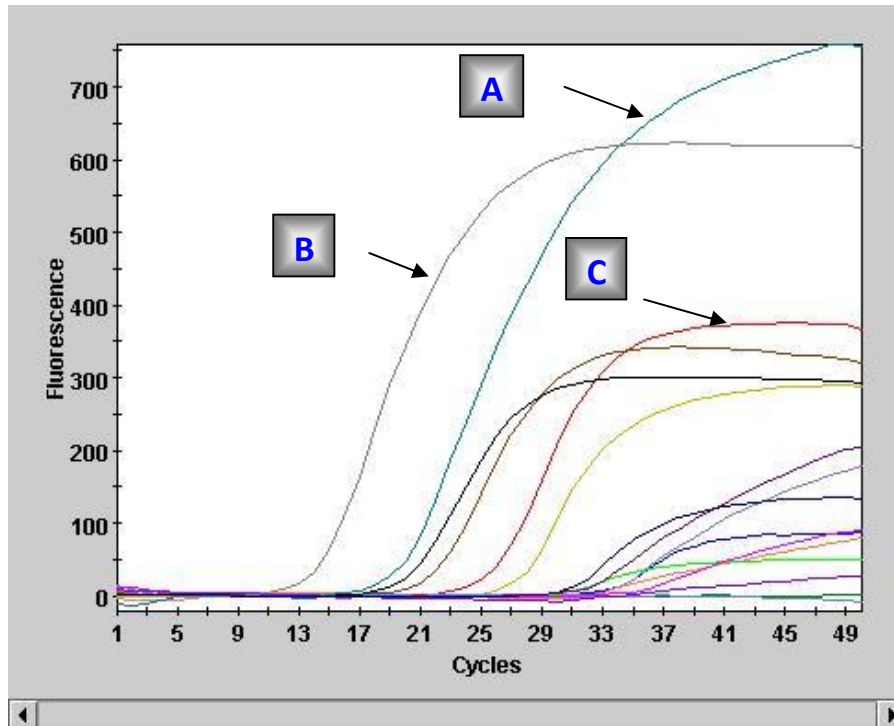
Peripheral blood lymphocytes were infected with HSV-1 for 24 h. The cells were subsequently washed twice with phosphate-buffered saline (PBS) and lysed by three freeze-thaw steps. Cell debris was removed by centrifugation, and the viral titer produced by the infected lymphocytes was determined in the supernatants. Cell supernatants were diluted six times (10^{-1} to 10^{-6}), and 100 μ l of each dilution was added for 1 hr at 37°C to confluent lymphocytes (5×10^6 cells/well) in 24-well plates.(14)

The expression of gene coding for gpD of HSV-1 was monitored by the IFAT assay using peripheral blood lymphocytes, cultured for 24 h. Subsequently, cells were inoculated with infectious HSV-1 at an MOI of 1 for a further 7 h,(14). Then indirect immunofluorescence studies were performed. Cells were fixed with 2% paraformaldehyde (Merck, Darmstadt, Germany) and subsequently permeabilized using 0.1% Triton X-100 (Sigma) for 4 min and

blocked with 1% bovine serum albumin (Sigma) for 30 min. The cells were then stained for 30 min with a monoclonal antibody specific to HSV-1 (SANTA CRUZ,USA), Following extensive washing steps in PBS, cells were incubated for 30 min with appropriate secondary antibodies conjugated to FITC (SANTA CRUZ,USA).

Results and Discussion

1- Results of polymerase chain reaction



(Figure-1) Polymerase chain reaction for HSV-1 gD replication: fluorescence data (FAM) collection during 60C extension for HSV1 virus, their curves higher than Threshold line were positive results and the negative result the curves under than threshold line in qPCR for HSV1 detection.(A: positive control; B: product of cell culture propagated HSV1; C: samples extracted genomes applied to pcr).

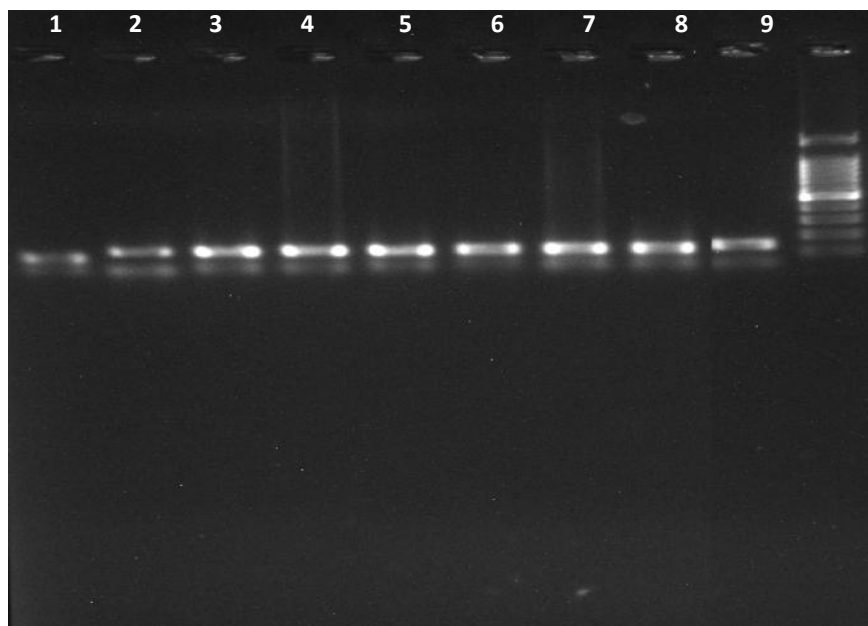


Figure-2. The product of PCR electrophoresis picture: the gel constitute of 1.5 % agarose with 2µg/ml ethidium bromide and e electrophoresis was done under 100V for 1.5 hr. The picture was taken by gel documentation system(Biometra, UK). (Lane 1: positive control of HSV2; Lane 2: positive control of HSV1; 3,4,5,6,7,8 and 9 were positive HSV1 samples ; lane 10: Ladder of 1kbp.)

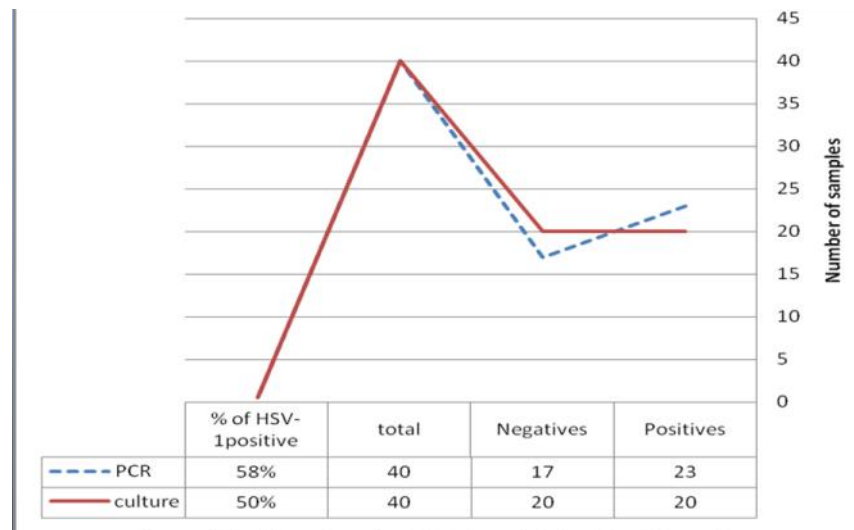


Figure (3) : Showing the HSV-1 positivity via PCR and peripheral blood lymphocytes culture technique.

Real-time PCR, also called quantitative PCR or qPCR, can provide a simple and elegant method for detection of a target gene that is present in a sample. Results of RT-PCR and electrophoresis PCR product revealed that from a total of 40 samples of dermal lesions, HSV1 was detected in 23(57.5%) of the total samples investigated. Real-time PCR was done and nucleotide segment of the gD region was amplified by the use of thermal protocol for Bosphore® HSV 1 Genotyping Kit v1 that revealed the requested and quick assay and also precise typing protocol for the diagnosis of HSV-1, since the sensitivity and specificity were 100% and 98% respectively(figure-1 and 2). Our results were came in agreement with the results of [Stránská, et al., 2004 \(12\)](#), who mentioned that The increase of HSV detection rates by PCR varied with the origin of specimen and was particularly significant for skin specimens (7/14 versus 3/14 detected by culture). The HSV-1 positivity and percentage were seen in figure-3.

This modern diagnostic tool was also achieved by different studies from the world that monitoring HSV-1(7, 8). A copy number standard curve is provided for quantification and an the internal extraction template (DNA or RNA), controls for the quality of the nucleic acid extraction and eliminates false negative results. The thermal protocol for Bosphore® HSV 1 Genotyping Kit v1 was applied, The kit is designed with the possible detection profile to ensure that all clinically HSV-1 strains are detected. our study involved both viral culture and polymerase chain reaction (PCR) testing, but PCR has been shown to be 1-2 times more sensitive than viral culture in detecting HSV-1 infection. the results revealed that 100 HSV-1 DNA copies/mL was enough to ensure the highly positive results. this came in agreement with [Xu et al.,2006 \(6\)](#).

2- Results of cell culture assay

After 24 hours , cell culture was examined under inverted microscope. Pure , rich lymphocytes were seen as shown in figure-4.

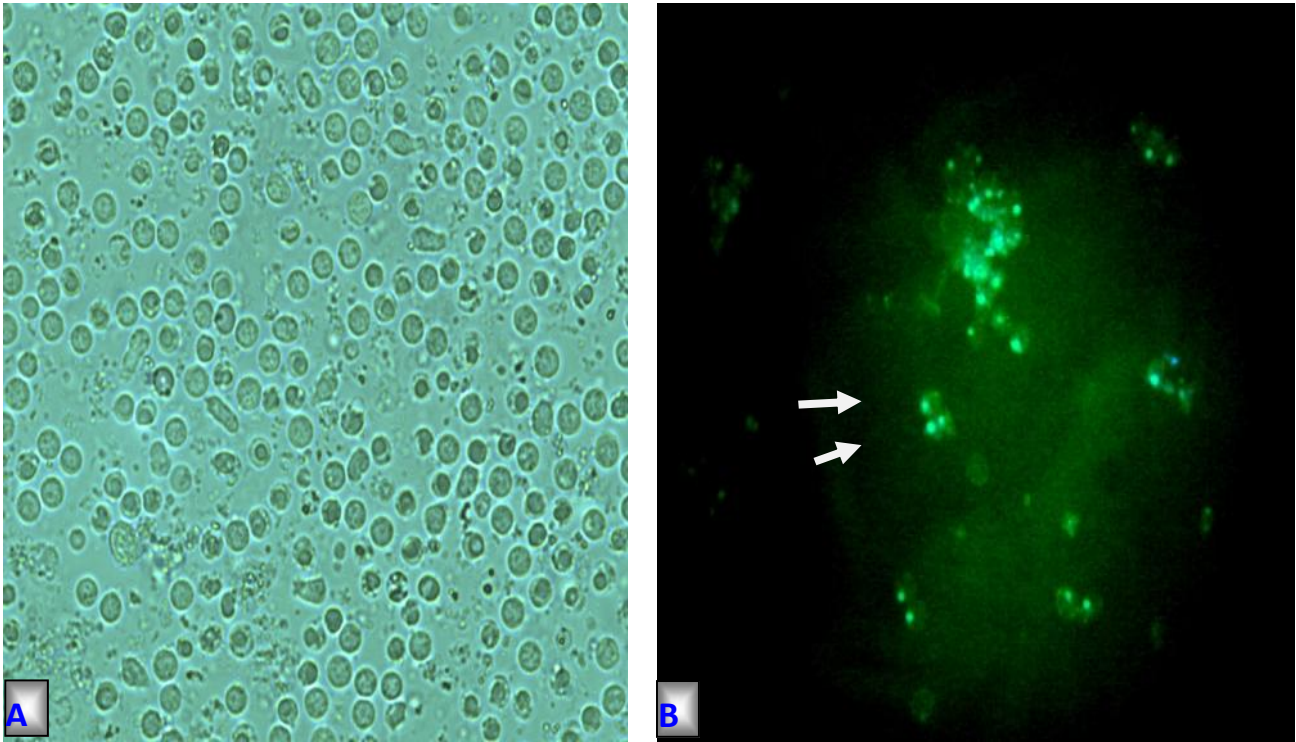


Figure (4): Cytopathic effect of HSV1 infection on peripheral blood lymphocytes: A-Normal lymphocyte culture; B- Infected lymphocytes (intranuclear inclusions) after 48 hrs PI. as detected by IFAT . Since 1975 , study of the susceptibility of human peripheral blood mononuclear cells to measles virus infection and replication was reported (10). We have therefore sought evidence for a viral aetiology in the dermal Lymphoreticular cells are infected during the pathogenesis of many viral infections and are a plausible site of viral persistence. This virus invariably grows in cultured lymphocytes from peripheral blood. The test of indirect fluorescent reaction revealed 50% of HSV-1 positivity (20:40). The specificity of the PCR assay was confirmed by testing DNA extracted from cytomegalovirus (clinical isolates) , no amplified DNA was detected from these samples. Our results showed that sensitivity, specificity and positive predictive value of PCR protocol were 100% for each, versus 86.9%, 90.9% and 88.9% respectively for cell culture assay, these results were also came in agreement with the results of Burrows, *et al.*, 2002, who mentioned that LC-PCR provided a highly sensitive test for simultaneous detection and subtyping of HSV in a single reaction tube. In addition to increased sensitivity, the LightCycler PCR provided reduced turn-around-times (2 hours) when compared to enzyme immunoassay (4 hours) or culture (4 days),(13). However, this study revealed that Peripheral blood lymphocytes can be used as a cell line for the propagation of HSV1 and revealing of the expression of region D of HSV1, and still the method that has traditionally been accepted as the diagnostic 'gold standard'.

CONCLUSIONS

1-The thermal protocol for Bosphore® HSV 1 Genotyping Kit v1 allows very rapid detection of HSV DNA in dermal lesions. It was found to be labor saving and showed sufficient sensitivity.

2- Peripheral blood lymphocytes give an alternative choice to propagate HSV-1, but still time and efforts consuming, and seemed less sensitive and specific than real-time PCR methods.

RECOMMENDATIONS:

Advanced steps via molecular assays by using of specific primers also required to assess vaccination or therapeutic approach against HSV-1.

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