Diagnostic study on *Herpes simplex-1*

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Abstract

The study was planned to evaluate the prevalence of HSV-1 infection, and the use of rhabdomyosarcoma and L20B as cell lines for the primary propagation of human herpes simplex1, by using modern diagnostic techniques. Study were involved a collection of 60 samples from dermal lesions, randomly selected from population of ages ranges from 15 to 45 years. These samples were collected during a period extended from February to September 2013. Primarily, these samples were investigated by RT-PCR technique directed to certify human herpes simplex-1 infections. Bosphore® HSV-1&2 Genotyping Kit v1(Anatolia geneworks, Turkey) was used for the detection protocol. From total of 47 HSV-1 positive samples in PCR step, 20 samples were cultured using two cell lines (Rhabdomyosarcoma (RD) and L20B cell lines) in an attempt for virus isolation and evaluation capability of these cells for HSV-1 propagation. Results of applied PCR revealed that HSV-1 DNA was correlated with 47(78.3%) positive of the total cases investigated. In the attempt to HSV-1 isolation, both RD and L20B cell lines were demonstrate a specific HSV-1 cytopathic effect. Herpes simplex virus type 1 had been propagated in 19 (95%) of the 20 PCR positive isolates by RD cell line, while 17:20 (85%) HSV-1 isolates were positive on L20B cells. In conclusions: The thermal protocol for Bosphore® HSV-1 Genotyping Kit v1allows very rapid detection of HSV-1 DNA in dermal lesions. It is finding to be laborsaving and show sufficient sensitivity. The RD and L20B cell lines are efficient as cell lines for the propagation of HSV-1, for that It is well recommended to achieve future therapeutic studies on HSV-1.

Key words: RD and L20B cell lines, cell culture, herpes simplex-1, real-time PCR, Fluorescent assay.

دراسة تشخيصية على فيروس الحلأ البسيط من النوع الاول

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الخلاصة

تهدف الدراسة إلى تقييم مدى انتشار الاصابة المتسببة عن فيروس الحلأ البسيط من النوع الاول وإمكانية استخدام خلايا ال rhabdomyosarcoma وال L20B كخطوط زرعيه لتنمية فيروس الحلأ من النوع الأول (Herpes simplex 1) ، والكشف عن ذلك باستخدام الطرق التشخيصية النوعية والخاصة بالكشف عن الفيروس قيد الدراسة. تضمنت الدراسة جمع 60 عينة من حالات الإصابة بالتهابات جلدية من اشخاص تتراوح اعمارهم بين 15-45 سنة ، وقد جمعت هذه العينات للفترة من شباط 2013 ولغاية ايلول من نفس العام. وقد تم فحص هذه العينات باستخدام تقنية بلمرة الحامض النووي Real-time polymerase chain reaction والخاص بتشخيص فيروس الحلأ البسيط من النوع الأول والثاني وباستخدام العدة التشخيصية المسماة ب Bosphore® HSV 1-2 Genotyping Kit v1 والمنتجة من شركة اناتوليا التركية(Anatolia geneworks, Turkey). وقد تم انتقاء عشرين عينة موجبة لدراسة التأثير المرضي Cytopathic effect الناجم عن الفيروس على خلايا ال rhabdomyosarcoma وال L20B وباستخدام تقنية الزرع الخلوي. أظهرت النتائج أن فيروس الهربس من النوع الأول كان مسبباً ل 47 إصابة وبنسبة مئوية قدرها 78.3% من العدد الكلي للعينات قيد الدراسة. وقد تم تأكيد الاصابة على خلايا ال RD في 19:20 عزلة فيروسية وبنسبة95 % من مجموع 20 عينة موجبة لفحص الPCR ، في حين كانت هذه الاصابة الموجبة تشكل 17:20 وبنسبة 85 % على خلايا ال L20B. نستنتج من ذلك ان تقنية بلمرة الحامض النووي Real-time polymerase chain reaction وباستخدام العدة التشخيصية المسماة ب

Bosphore® HSV 1-2 Genotyping Kit v1 فعالة في الكشف عن الإصابة بفيروس الحلأ البسيط من النوع الاول ، وان خلايا ال RD وخلايا ال L20B هي خطوط زرعيه ناجحة لتنمية الفيروس داخل المختبر، كما ونوصي انه من الضروري جدا اجراء الدراسات العلاجية على الفيروس مستقبلاً.

الكلمات المفتاحية: RD and L20B cell lines ، الزراعة النسيجية ، تقنية البلمرة ، فيروس الحلأ البسيط.

Introduction

Herpes simplex virus type 1 (HSV-1), a member of the *herpesviridae*, causes a variety of human viral diseases globally. Although a series of antiviral drugs are found for the limitation of dissemination and treatment of infection, HSV-1 remains highly prevalent worldwide(1,2). Herpes virus was named for the tendency of some herpes infections to produce a creeping rash, this virus family was characterized by obvious features with its tendency toward viral latency and recurrent infections (3). The aim of the study were to studying the prevalence of HSV-1 by real time polymerase assay, and evaluation of L20B and rhabdomyosarcoma cell lines for the viral propagation and confirmation of the detection by fluorescent assay.

Materials and methods

Collection of Samples and case definition

Sixty (60) samples from dermal lesions related to persons suffering from acute and chronic signs of facial and oral lesions, ages ranging from 15 to 50 years, all of these cases were from Najaf governorate. Data about each patient were obtained according to the statement of questionnaires including information about age, sex, duration of the disease, being treated or not and the date of case admission. The specimens were collected aseptically via wetting the end of sterilized cotton swab with the lesion discharge or tissue fluid from scraped pustules, were extracted in two vials with 2ml volume of transport media containing Nystatin, Penicillin, streptomycin and phosphate buffer saline solution. One of them is transported to be frozen at -70C° to be used subsequently in virus isolation. Nucleic acids were extracted from the second vial, the residual amount and the extracted material has been stored at -70 to be ready for amplification of HSV-1 DNA by PCR, subsequently**.**

Viral 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 (4).

Inoculation of cell culture

RD and L20B cell lines were prepared in 25 cm3 flask and lab-tek slides for detection of virus by indirect immune-fluorescent antibody assay. pH of growth and maintenance media were adjusted at 6.8-7.2. The cells was incubated at 37 °C and examined daily until complete monolayer was formed. Growth medium was supplemented with 10% fetal calf serum (FCS) and maintenance was supplemented with 2 % fetal calf serum,100IU/ml penicillin and 100µg/ml streptomycin for cells culture, then, was inoculated with 0.5 ml volume of treated sample to each 25 cm3 cell culture flask. After virus adsorption at 37 °C for 1 hr. (with continuous rolling every 10 min), flasks were washed three times with maintenance medium then incubated at 37 C° and checked daily for virus growth by inverted microscope for detection of any cytopathic effect (CPE)(5).

Processing of indirect IF assay

The conjugate as well as counter stain were prepared according to SANTA CRUZ company instruction as follows: 10 µl of murine monoclonal antibodies specific for the detection of human HSV-1 gD protein and goat anti mouse IgG-labeled with fluorescein isothiocyanate were added to 3990 µl of PBS, to give a dilution ratio of 1:400, pH 7.2. The cell cultures were checked for virus growth for several passages by IFAT with antisera to human HSV-1. The

methods of (5) and (6) were modified for application with HSV-1 and used in tissue culture by indirect immune fluorescent technique and by using specific monoclonal antibodies for HSV1.

Statistical analysis: Statistical analyses were performed using SPSS 18.0 for windows. Inc. Data were expressed as Mean ± SD, two-tailed T test unless otherwise stated by ANOVA test. All tests statistically significant at $P<0.05$. R2 was calculated by Pearson Correlation Coefficient.

Results

Primary detection of HSV1 in dermal lesion samples by real-time PCR assay

After completing of DNA extraction, DNA purity was determined by applying nano drop system. The results of 206/280 ratio exhibited that all extracted DNA specimens were raised within acceptable values ranging between 1.8 -2.0 and this means that it has good DNA purity and ready to the PCR protocol application. A total of 60 samples of dermal lesions were applied to PCR assay for the detection of HSV1 antigens. Out of the total samples investigated, 47 cases (78.3%) were HSV1 positive, while the rest cases, 13(21.7%) were negative (Fig. 1).

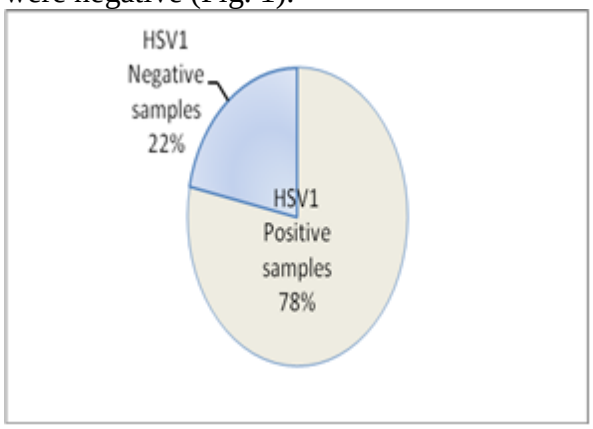

Fig.(1): Prevalence of HSV1 infection in dermal lesion samples detected by rtPCR.

Florescence data (FAM filter) collection during 60 C^{o} extension for HSV1, their curves higher than threshold line were positive results and the negative result the curves under than threshold line in quantitative PCR (qPCR) for HSV1 detection. The electrophoretic pattern of HSV1 amplified gene was also revealed in (Fig. 2).

Prevalence of HSV1 according to the duration period and patients history

Among the total positive samples, 8 cases (17.02%) were related to HSV1 chronic infections, while the majority of positive samples (82.98%) were related to cases with A cute infections (Fig. 3).

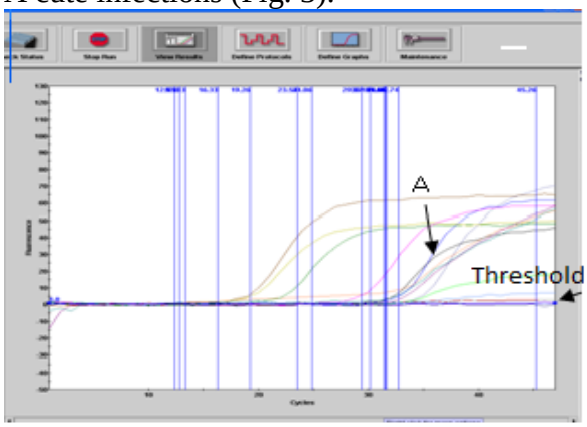

Fig. (2): PCR for HSV-1 detection. A: positive control. Other lines are extracted genomes.

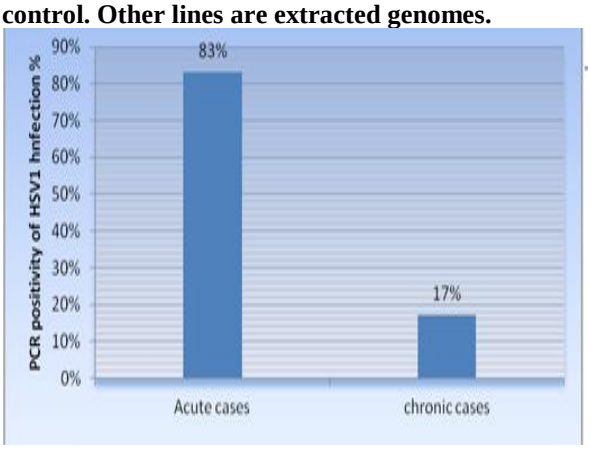

Fig. (3): Prevalence of HSV1 infection according to the duration period.

prevalence of HSV1 according to the gender and age groups

This study showed non-significant differences of the PCR positivity between males and females ($P<0.05$). However, 31 cases (65.96%) were from females community, while only 16 cases (34.04%) were males, with female to male ratio of about (2:1.15). The HSV1 infection was more dominant in 21-25 years age group, it comprised 44.7% of the total positive cases, followed by of 15-20 years age group, with a

prevalence of 21.27%, with significant differences (P<0. 05) as compared with other age groups (Fig.4).

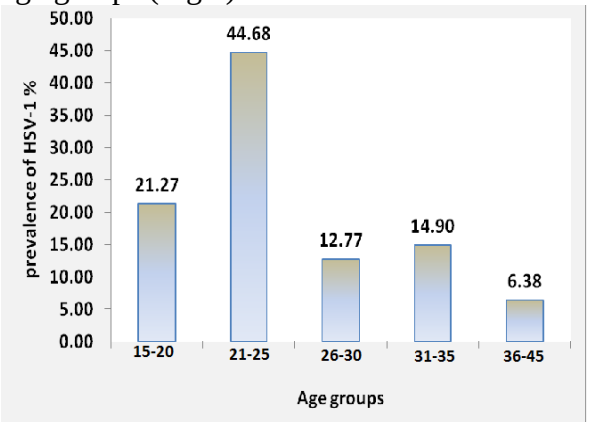

Fig. (4): Prevalence of HSV1 infections according to the age groups.

In vitro propagation and isolation of HSV1

The results of applying the technique of tissue culture by using L20B and Rhabdomyosarcoma (RD) on 20 from total 47 of HSV1 positive samples revealed that the two types of cells supported the growth of HSV1. Passage numbers (7–22) of Rhabdomyosarcoma cell line were used for primary propagation and isolation of HSV-1and the normal pattern of RD cells was revealed in (Fig.5-A). Rhabdomyosarcoma cells showed an obvious appearance of cytopathic effect (CPE), the main characteristic of this (CPE) was firstly observed during 24-96 hrs post infection (PI). The infected cells were seen swelling, rounding, and in few numbers, which were increased from the 5th day PI and accompanied by syncytial multinucleated giant cells (Fig.5 E, F). The specific (CPF) was also confirmed by indirect fluorescent assay (Fig. 5 G, H). These characteristics appeared much more rapid in the subsequent passages. On RD cell line 3 isolates (15%) revealed infection after 24hrs PI. more than 50% (11:20) that comprise 55% of selected isolates gave specific HSV1 (CPE) after 48hrs. Three isolates (15%) revealed an obvious (CPE) appearance after 72hrs and finally 2 isolates (10.5%) gave the same cytopathic effects after 96 hrs. PI. since L20B cell line showed the same obvious appearance

Table (1): HSV1 Cytopathic effect (CPE) positivity according to the incubation period

Types of cell	Post inoculation (hr.) percentage of positive specimens				
	Total	24 hr.	48 hr.	72 hr.	96 hr.
RD	19:20 (95%)	3 (15%)	11 (55%)	3 (15%)	2 (10%)
L20B	17:20 (85%)	2 (10%)	5 (25%)	8 (40%)	2 (10%)

of (CPE) but slower than RD cell line. Two isolates(10%) of total 20 cultured specimens revealed infection after 24hrs PI, 5 isolates that comprise 25% gave specific HSV1 (CPE) after 48hrs, 8 isolates (40%) revealed an obvious (CPE) appearance after 72hrs and finally 2 isolates (10%) that gave the same (CPE) after 96 hrs. PI. These results showed significant differences in time of (CPE) appearance and both cell lines were sensitive to HSV-1 isolation (Table 1). The most common (CPE) characteristics were cytopathic granulation, rounding, and sloughing of the cells, followed by formation of syncytial cells and large empty plaques two days later. After that the dead cells began be detached from flask surface and floated in media, (Fig. 5 B-D).

Detection of HSV1 in cell culture by IFAT

The demonstration of specific HSV1 antigen in infected cells at each passage was accomplished by indirect fluorescent antibody technique (IFAT) on Lab-Tek slides, which had been carried out after 24 hrs post appearance of (CPE). The character increased with subsequent passages represented by the appearance of fluorescence cells with bright intranuclear fluorescence inclusions which occupied most of the nucleus in severe extensive infection of passages of each type of cells used (Fig. 5 E,G, 6 D). On the other hand, results were highly significant according to the incubation period differences and appearance of specific (CPE) of HSV-1. The main characteristics of

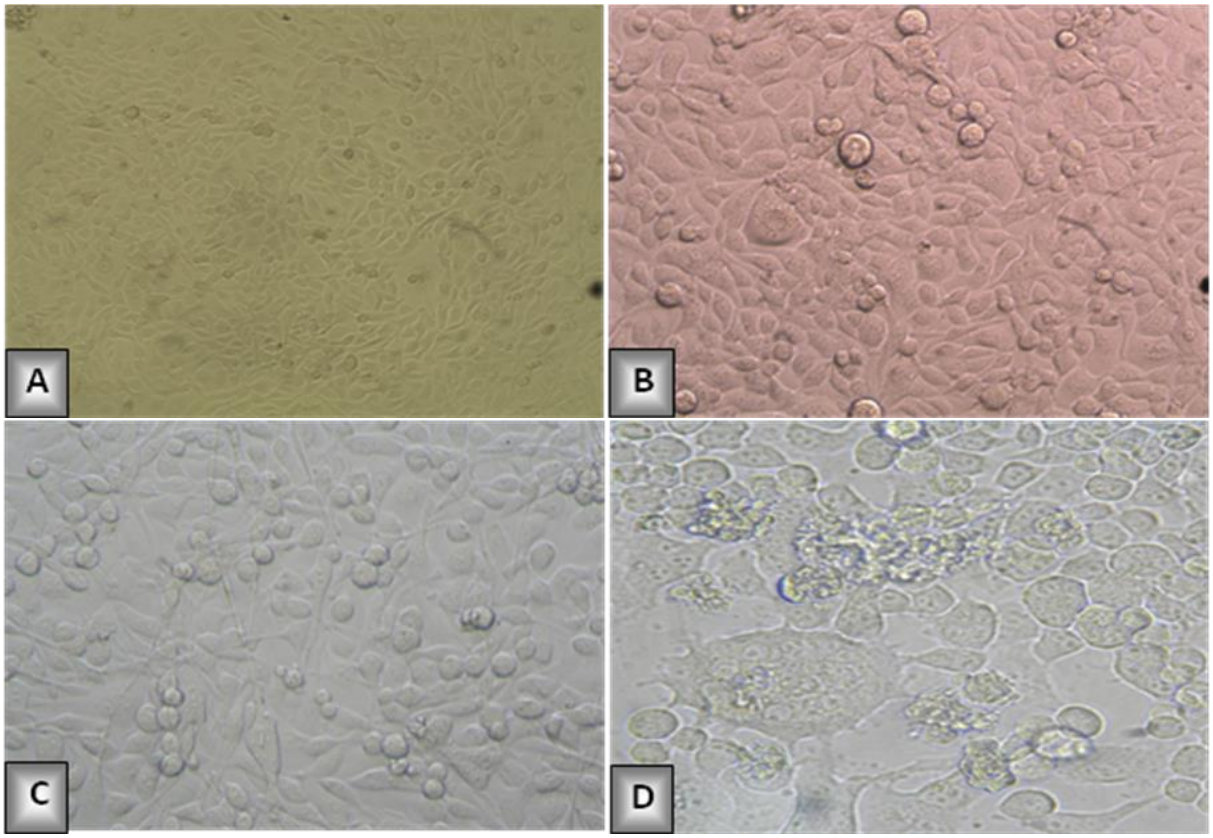

Fig. 5 (A-D): Cytopathic effect of HSV 1 on RD cells according to different periods of infection intervals. A-Normal RD cells B- Infected RD cells after 48 hrs. C-Infected RD cells after72 hrs. D- RD cells after 96 hrs.

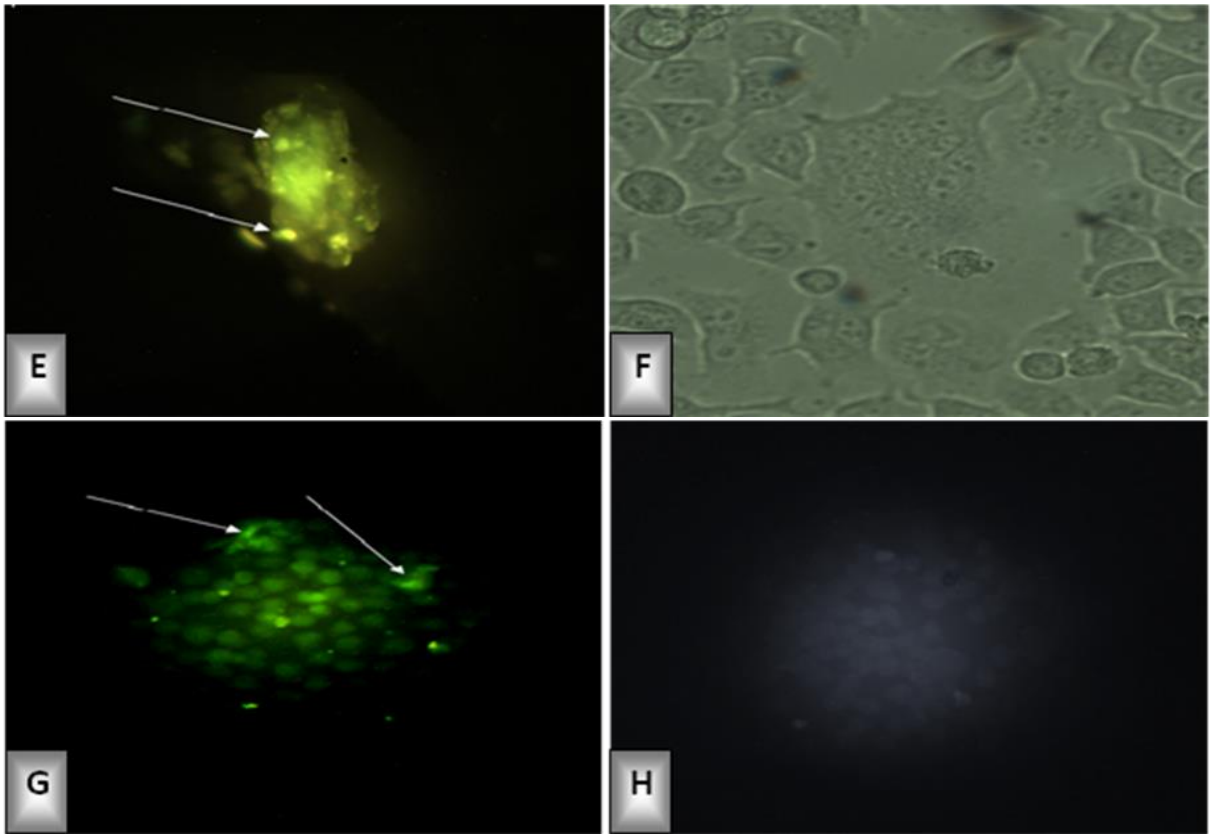

Fig. 5 (E-H): Cytopathic effect of HSV-1 infection detected by IFAT on RD cells. E and F: mulinucleated giant cells. G: HSV1, H: Non infected RD cells. Arrows refer to viral inclusions.

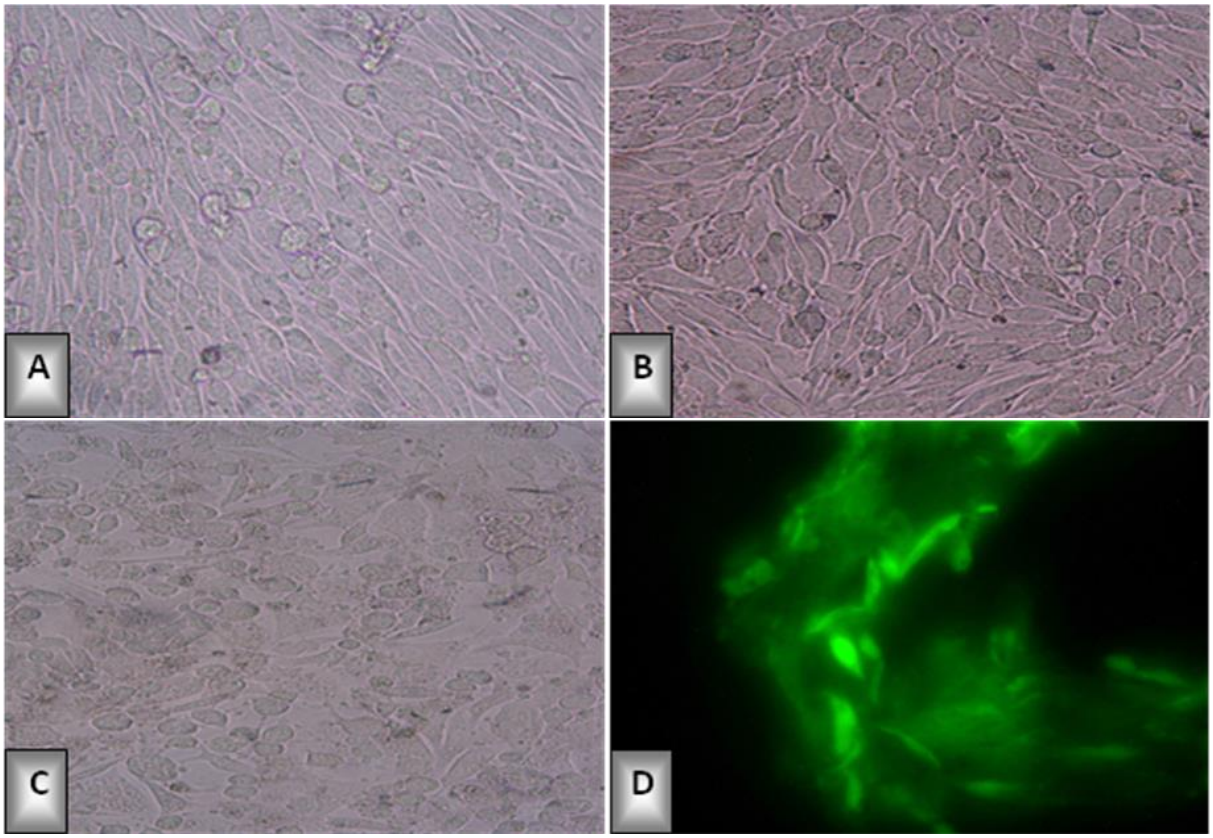

Fig. 6 (A-D): Cytopathic effect of HSV 1 on L20B cells according to different periods of infection intervals. A: Normal L20B cells B- Infected L20B cells after 24 hrs. C-Infected L20B cells after 96 hrs. PI. D- CPE as detected by IFA.

this (CPE) firstly observed within 24 hrs. post infection (PI) were swelling and rounding of few numbers of cells (Fig. 6 C) which increased by the 4th day (PI) and accompanied by syncytia cells frequently observed at 96 hrs. post infection. After 80-100% of CPE appearance (Fig. 6 D), infected cell cultures were frozen at -20˚C for further passages, while the control flasks remained unchanged and showed no CPE, (Fig. 6 A). Passages (9-25) of L20B cell line were used throughout this study.

Discussion

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. Results of RT-PCR revealed that from a total of 60 samples of dermal lesions, HSV1 was detected in 47(78.3%) of the total samples investigated, that revealed the requested and quick assay and also precise typing protocol for the diagnosis of HSV-1. PCR sensitivity was 100%; specificity was 92.9% and diagnostic accuracy of 98.4%, according to the equations of (7). Our results were came in agreement with (4) who mentioned that Bosphore® HSV 1 Genotyping Kit v1 allows very rapid detection of HSV DNA in dermal lesions, and (8) 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 study involved both viral culture and (PCR) testing, where the PCR seen more sensitive than viral culture in detecting HSV-1 infection. The use of molecular methods in clinical microbiology has increased exponentially over the past two decades. The main reason for this influx of molecular-based methods has been the continued development and improvement of PCR and the introduction of other nucleic acid amplification formats. The exquisite

sensitivity of such methods has surpassed even the traditional "gold standards" for diagnostic testing. They provided a sensitivity and specificity not achievable by classic microbiological methods. Assay turnaround time can now be as short as 1 to 2 h because of the simultaneous product amplification and detection step. Additionally, the closed system has essentially reduced the risk of contamination to negligible levels (9).

These results showed significant differences of the prevalence of HSV1 according to the duration of herpes virus infection. When a person has active symptoms of herpes, the condition is usually considered acute. Each outbreak is called an acute phase of herpes. Chronic herpes generally refers to infected people who have six or more outbreaks of herpes in a year time. Like those infected with eczema herpeticum, an infection with herpesvirus in patients with chronic atopic dermatitis may result in spread of herpes simplex throughout the eczematous areas (10). These frequent outbreaks are a medical concern because herpes simplex virus does create a risk for other illnesses, such as the development of viral meningitis, and a high frequency of outbreaks can make it difficult for people to track sexual relations. Most people have fewer infections per year, a fact that is interpreted as the majority of collected samples were regarded as acute infections. These results may disagree with results mentioned by (11) who mentioned that women are more likely to be seropositive than men, and likely to acquire the virus at an earlier age for each European country. However our results are statistically non-significant according to the gender differences. Pebody *et al*. (11) mentioned that HSV seropositivity becomes more common from adolescence onwards and increases in the population with age with a decline in the older age groups in some countries. Our results revealed that HSV1 infection was more dominant in 21-25 years age group, as it comprised 44.7% of the total positive cases, followed by the age group of 15-20 years, with a prevalence of 21.27%, with significant differences as compared with other age groups.

Using RD and L20B cells as cultivation lines for isolates HSV-1 from many biological specimens that exhibited cytopathogenic effects related to HSV1is agreed with (12). The RD cells seem to support viral propagation in 95% of selected samples (19:20) These results came in agreement with those results of (13, 14) that RD cells were successful to propagate HSV-1 isolates with 100 % of cases. The little variation may be related to the primary detection protocol we are applied. PCR technique that is well known for sensitivity and selectivity as higher as in cell culture technique (13). Results are also come in agreement with (15) declare that the degenerative changes in monolayer cells provide evidence of viral presence. The spectrum of change is broad, ranging from swelling, shrinking, and rounding of cells to clustering, syncytium formation, and in some cases complete destruction of the monolayer. In this study the efficacy of PCR technique is more detectable for HSV1 infections than cell culture and gave similar results to (16) affirm that nucleic acid amplification increased the detection rate of HSV DNA in particular compared to virus isolation, and concluded that Real-time PCR has the advantage of rapid amplification, a reduced risk for contamination and it is a suitable method for diagnosis of HSV in specimens from skin lesions (16). The RD cells were easy to grow and the (CPE) is very distinguishable as compared with L20B cells. Infected cells also showed fusion and syncytia formation. The RD cell line supports virus propagation in 95% of tested samples (19:20); therefore, RD cell line is applied for the propagation and isolation of HSV-1 for TCID50, and for the assessment of toxicity and antiviral activity of lycopene versus acyclovir effect. Generally RD and L20B cells are easy to grow, and the (CPE) is very distinguishable. Infected cells also showed fusion and syncytia formation. Syncytia formation resulting from virus-induced cell-

to-cell fusion can be viewed as an aberrant manifestation of the interactions of altered membranes in herpesvirus infected cells with the unaltered membranes of neighboring cells. While wild-type herpes viruses usually cause infected cells to round up and clump together, some viral mutants in both HSV-1 and HSV-2 cause cells to fuse into large 62 polykaryocytes containing many nuclei (17). Virus-induced cell-to-cell fusion has been extensively studied as a tool for analysis of the functions of viral membrane proteins, and as a model of the initial interaction between HSV and susceptible cells that results in fusion of the viral envelope with the cellular plasma membrane (18,19). In conclusion the applying technique of tissue culture and propagation, both cell lines; RD and L20B revealed obvious support of the growth as well as propagation and adaptation of HSV1.

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