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ORIGINAL ARTICLE

Diagnosis of Herpes Simplex Virus 1 and 2 in Clinical Specimens by Tissue Culture and Polymerase Chain Reaction

[1]Eman I.A. Mahmoud, [1]Walaa S. Khater*, [2]Marwa K. Assaad, [3]Laila A. Farid, [1]Aly M. Zaki

[1]Medical Microbiology & Immunology Department, Faculty of Medicine, Ain Shams University
[2]Dermatology Department, Faculty of Medicine, Ain Shams University
[3]Obstetrics and Gynecology Department, Faculty of Medicine, Ain Shams University

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Corresponding Author:
Walaa Shawky Khater: Medical Microbiology and Immunology Department, Faculty of Medicine, Ain Shams University, Cairo, Egypt
Tel.: +201222475354
walaa_khater@med.asu.edu.eg, drwalaakhater@gmail.com

ABSTRACT

Background: *Herpes simplex viruses (HSVs) are responsible for a variety of human diseases. Although lesions are usually self-limited, severe manifestations can occur, particularly in compromised hosts. Effectiveness of therapy for such infections relies upon rapid administration of appropriate antivirals which in turn creates the need to establish a prompt diagnosis and necessitates diagnostic testing that is rapid, sensitive and affordable especially for laboratories in developing countries. The specificity of tests is also crucial, since clinical manifestations of HSV are relatively nonspecific and overlap other potentially severe infections.* ***Objectives:*** *This study aimed at comparing the performance of two relatively affordable diagnostic assays; conventional PCR and tissue culture; in the detection of HSV in different clinical specimens.* ***Methodology:*** *Seventy participants were included and divided into two groups. Group I: comprised 50 patients with suspected herpetic lesions. Group II: comprised 20 subjects without any herpetic clinical manifestations. Samples from participants were tested for HSV pol gene by conventional PCR. Tissue culture was performed by inoculating the samples on Vero cell line.* ***Results:*** *Conventional PCR showed perfect agreement with the gold standard (κ= 1) with sensitivity, specificity, and accuracy of 100%. Tissue culture assay detected 15 (21.4%) of all positive cases showing substantial agreement with the gold standard (κ= 0.632) with sensitivity, specificity and accuracy of 57.7%, 100% and 84.29%, respectively.* ***Conclusion:*** *Though tissue culture has its own advantages, conventional PCR could serve as a gold standard for the diagnosis of HSV infection.*

INTRODUCTION

Herpes simplex viruses 1 (HSV-1) and 2 (HSV-2) are both members of the *Herpesviridae* family[1]. They cause a wide spectrum of clinical manifestations ranging from oral and genital mucocutaneous lesions to serious central nervous system (CNS) manifestations [2]. Although lesions are usually self-limited, severe disease can occur, particularly in compromised hosts, pregnant women, and neonates[3]. HSVs can remain latent in dorsal root ganglia following primary infection leading to lifelong carriage and may reactivate in situations when the immune status is compromised causing life threatening conditions [4].

Despite the absence of cure for latent infections, effective therapy exists that could alleviate intensity of symptoms, shorten out-breaks duration and treat life-threatening conditions [2].

Infections with HSVs are common worldwide with seroprevalence approaching 55% for HSV-1 and 20% for HSV-2 in adult populations in the United States and is believed to be more common in developing countries[2,5].

On the other hand, HSV-1 is considered the commonest cause of fatal sporadic viral encephalitis worldwide [6].

Failure to reach diagnosis and start prompt antiviral therapy result in elevated mortality rates, lifelong neurologic sequels in survivors or disseminated disease as in case of neonatal HSV-2 infection. In these situations, the availability of rapid, sensitive and affordable HSV diagnostic assays especially for laboratories in developing countries is crucial[7]. Specificity of testing is also central, since clinical manifestations of HSV are relatively nonspecific and overlap other potentially severe infections [8]. Various methods have been described for the diagnosis of HSV infections including tissue culture, direct antigen detection and molecular assays[9].

Viral culture was considered the gold standard method, against which the performance of any other method is tested[10]. Nevertheless, it had been criticized by being timely, laborious, needs highly skilled

personnel, subjective, and results are affected by collection technique and transport conditions[3]. Some reports have shown that advanced molecular assays are more sensitive and rapid for the diagnosis of HSV in dermal and genital samples and could be considered the standard diagnostic assay for detecting herpes infection of the CNS as a better alternative than brain biopsy[9]. Yet, their relative high cost and inability to perform antiviral susceptibility testing are their main disadvantages[11].

This study aimed at comparing the performance of two relatively affordable diagnostic assays; conventional PCR and tissue culture; for detection of HSV in different clinical specimens

METHODOLOGY

Study Design:

This observational study was conducted during the period from January to August 2017. It included 70 participants who were divided into two groups; group I: comprised 50 patients presenting to the Outpatient Clinics of Ain Shams University Hospitals (ASUHs) with clinical manifestations suggestive of HSV-1 or 2 infections. Whereas group II comprised 20 subjects who were apparently healthy or presenting to the ASUH Clinics with Clinical manifestations other than herpes.

All subjects had participated in the study after obtaining informed consents. The work had been approved by ASU Ethics Committee and in accordance with the ethical guidelines of the Declaration of Helsinki, 1975.

Data collection:

Data were collected using a standardized data collection form. Recorded data included demographic characteristics, antiviral treatment intake and the stage of the suspected lesion (for patients in group I).

Microbiologic Study

Samples collection, transport and storage:

Orolabial or genital samples were collected by cotton swabs that were then soaked in 3 mL Viral Transport Medium (3% (w/v) tryptic soy broth, 0.5% (w/v) gelatin, 0.002% (w/v) gentamycin) (Gibco, USA). Specimens were stored at –70°C in Medical Microbiology and Immunology Department Laboratory until use.

Tissue culture on Vero cell line:

The specimens were thawed for testing and inoculated into the Vero cell line (Vacsera, Egypt) in cell culture flasks (200 µl per flask). All manipulations were done in a laminar-flow hood (BioAir, Italy). Tissue culture was performed as described by Phelan et al [12] .The cell cultures were incubated at 37°C and inspected for cytopathic effects (CPEs) (figure 1) by inverted microscope (Nikon, Japan) during a two weeks' period after which negative samples were discarded.

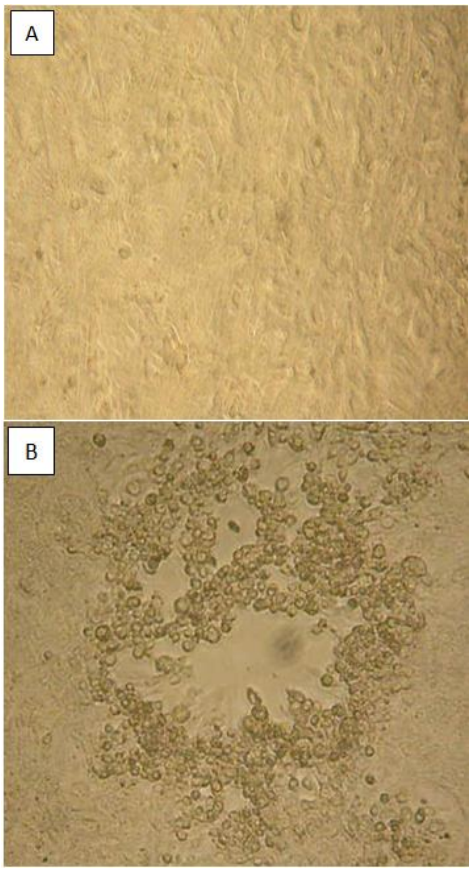

Fig. 1: A) Normal Vero cells. B) Vero cells showing CPE (ballooning and detachment) caused by HSV replication.

Detection of HSV polymerase (pol) gene by conventional PCR:

DNA extraction:

A commercial DNA isolation kit (High Pure Viral Nucleic Acid Kit, Roche, Switzerland) was used to extract DNA from samples as per the manufacturer's protocol for DNA purification. The eluted DNA was stored at -20°C until further processing.

Nucleic acid amplification:

The extracted DNA was tested for the presence of *pol* gene. The PCR was carried out in a 50 µl reaction mixture containing 25µl of Taq PCR master mix solution (Qiagen, UK), 13µl of double-distilled DNase-free water; a 1 µM concentration of each primer (table 1); and 10µl of the extracted sample. Cycling conditions were initiated by cell denaturation of 15 minutes at 95ºC, followed by 40 cycles of 45 seconds at 95ºC, 45 seconds at 55ºC and 1 minute at 72ºC for denaturing, annealing and extension, respectively and final extension at 72ºC for 5 minutes in the thermal cycler (Biosystems, USA).

Amplified DNA fragments were separated on 2.0% agarose by gel electrophoresis, visualized by ethidium bromide staining and photographed under UV light (figure 2).

Table 1: Oligonucleotides used in the PCR assay [15]

Oligonucleotide	Sequence (5′_3′)	Product size (bp)
pol-1F	GTG GTG GAC TTT GCC AGC CTG TA CCC	532 bp
pol-1R	TAA ACA TGG AGT CCG TGT CGC CGT AGA TGA	

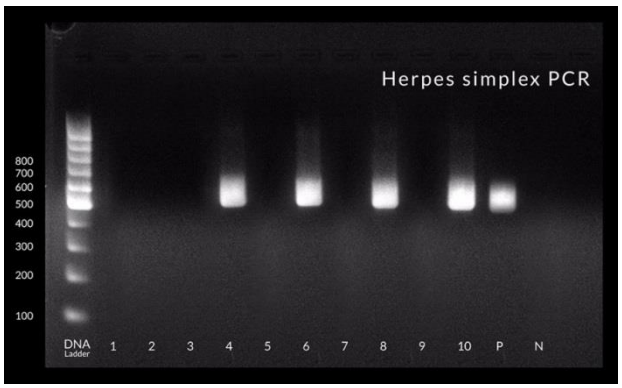

Fig. 2: Agarose gel electrophoresis of PCR products (representative gel). Lane P: positive control; Lane N: negative control; Lane 4, 6, 8,10: positive for *pol* gene; Lane 1, 2, 3, 5, 7, 9: negative for *pol* gene.

Statistical analysis:

Data were entered in an Excel file (Microsoft, Redwoods, WA, USA) and then transferred to SPSS version 20 (Statistical Package for the Social Sciences Inc, Chicago, IL, USA) for further analysis. Qualitative data were presented as number and percentages while quantitative data were presented as mean, standard deviations and ranges. Comparison between two groups with qualitative data was done using Chi-square test whereas comparison between more than two independent groups regarding quantitative data with non-parametric distribution was done using Kruskall-Wallis test. *p*-value was considered as the following: $p > 0.05$: non-significant, $p < 0.05$: significant and $p < 0.01$: highly significant. 2-by-2 contingency tables were used for the calculation of diagnostic parameters: sensitivity, specificity, positive predictive value (PPV) negative predictive value (NPV) and accuracy [13]. Kappa (κ) statistic was used to test the agreement between the two laboratory tests. κ values were interpreted as follows: κ< 0: less than a chance agreement, κ = 0.01– 0.20: slight agreement, κ = 0.21– 0.40: fair agreement, κ= 0.41–0.60: moderate agreement, κ= 0.61–0.80: substantial agreement and κ=0.81–0.99: almost perfect agreement[14].

RESULTS

The age of all participants included in this study ranged from 2 to 58 years (35.79 ± 14.61 years). They were 63 females (90%) and 7 males (10%). 17 orolabial and 33 genital swabs were collected from patients in group I which included 46 females (92%) and 4 males (8%) patients. Their age ranged from 2 to 58 years old (35.79 ± 14.61). Whereas 4 oral and 16 genital swabs were gathered from subjects in group II who were 17 females (85%) and 3 males (15%). Their age ranged from 4 to 53 years (38.65±9.51 years). There was no statistically significant difference between both groups as regards age or sex. Relevant demographic data are shown in table 2.

Table 2: Demographic data of the study participants (n=70)

Characteristics	Group I (n=50)	Group II (n=20)	Test value	*p*-value
Age (years)	34.64 ±16.15	38.65±9.51	1.038•	0.303 (NS)
Sex				
Female	46 (92.0%)	17 (85.0%)	0.778*	0.378 (NS)
Male	4 (8.0%)	3 (15.0%)		

*: Chi-square test; •: Independent t-test; NS: Non-Significant

In order to set a gold standard against which the two employed techniques would be compared in this study, cases were defined as "true positives" if they showed positive results by two or more of PCR, tissue culture and/or clinical diagnosis. While "true negatives" were those negative to both PCR and tissue culture.

Based on the above definition, out of the 70 processed samples, a total of 26 (37.1%) were true positives; 21 (42%) were from patients in group I and 5 (25%) were from participants in group II and a total of 44 (62.9%) samples were true negatives; 29 (58%) were from patients in group I and 15 (75%) were from participants in group II. Relevant data are shown in table 3.

Table 3: Characteristics of study participants with true positive and true negative results

Variables		Total (n=70)	HSV positive (n=26)	HSV negative (n=44)	*p* value
Age		35.79 ± 14.61	38.42 ± 15.76	34.23 ± 13.84	0.249 (NS)
Sex	Females	63 (90.0%)	25 (96.2%)	38 (86.4%)	0.187 (NS)
	Males	7 (10.0%)	1 (3.8%)	6 (13.6%)	
Type of sample	Cervical	34 (48.6%)	15 (57.7%)	19 (43.2%)	0.272 (NS)
	Vulvar	15 (21.4%)	3 (11.5%)	12 (27.3%)	
	Orolabial	21 (30.0%)	8 (30.8%)	13 (29.5%)	
Stage of lesion	Vesicle	14 (28.0%)	7 (33.3%)	7 (24.1%)	0.083 (NS)
	Ulcer	30 (60.0%)	14 (66.7%)	16 (55.2%)	
	Crust	6 (12.0%)	0 (0.0%)	6 (20.7%)	
Antiviral treatment	Negative	24 (48.0%)	14 (66.7%)	10 (34.5%)	0.025 (S)
	Positive	26 (52.0%)	7 (33.3%)	19 (65.5%)	

Data are presented as mean ± S.D. for continuous variables and as number (percentage) for categorical variables.
HSV: Herpes Simplex Virus; NS: Non-Significant, S: Significant.

Tissue culture assay detected 15 (21.4%) of all positive cases showing substantial agreement with the gold standard (κ= 0.632) with sensitivity, specificity, PPV, NPV and accuracy of 57.7%, 100%, 100%, 80% and 84.29%, respectively.

Whereas, PCR detected all 26 (100%) and showed perfect agreement with the gold standard (κ= 1) with sensitivity, specificity, PPV, NPV and accuracy of 100% in all parameters.

On the other hand, clinical diagnosis showed slight agreement with the gold standard methods (κ= 0.125) with sensitivity, specificity, PPV, NPV and accuracy of 80.8%, 31.4%, 42%, 75% and 51.43%, respectively.

Recovery rates of HSV by PCR and tissue culture assays and the performance characteristics of the employed methods of diagnosis are shown in tables 4 and 5, respectively.

Table 4: Recovery rates of HSV by PCR and tissue culture among studied samples (n=70).

Variables		Total (N=70)	Group I (N=50)	Group II (N=20)
PCR	Negative	44 (62.9%)	29 (58.0%)	15 (75.0%)
	Positive	26 (37.1%)	21 (42.0%)	5 (25.0%)
Tissue culture	Negative	55 (78.6%)	40 (80.0%)	15 (75.0%)
	Positive	15 (21.4%)	10 (20.0%)	5 (25.0%)

Table 5: Results of PCR, tissue culture and clinical diagnosis as compared to the gold standard.

Variables		Gold Standard				*K*
		Negative		Positive		
		No.	%	No.	%	
PCR	Negative	44	100%	0	0%	1
	Positive	0	0%	26	100%	
Tissue Culture	Negative	44	100%	11	42.3%	0.632
	Positive	0	0%	15	57.7%	
Clinical Diagnosis	Negative	15	34.1%	5	19.2%	0.125
	Positive	29	65.9%	21	80.8%	

κ: Kappa coefficient

High statistical significant difference was observed between positive and negative results of tissue culture as regards the stage of lesion (p=0.004). While no statistical significance was observed between results of PCR (p=0.083) in the same context (Figure 3).

On the other hand, there were no statistical significance between positive and negative results of both assays as regards the type of processed sample (orolabial, cervical or vulvar) (Data not shown).

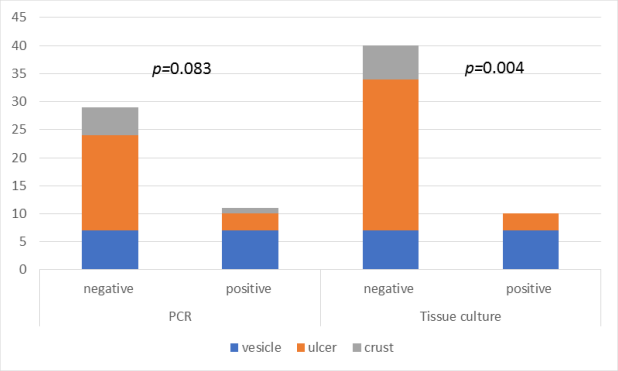

Fig. 3: Comparison between PCR and tissue culture results as regards the stage of the lesion.

The estimated cost per sample for PCR and tissue culture assays were 200 and 250 EGP, respectively. The turnaround time for detection of HSV by PCR is about 7 hours, while that by tissue culture was from 5 to 10 days.

DISCUSSION

In this study, conventional PCR and cell culture assays were done for the diagnosis of HSV infections. Compared to other recent and commercially available direct methods of viral detection, both techniques are considered relatively inexpensive.

The current study demonstrated that HSV detection rate by conventional PCR was 100% and had shown complete agreement with the gold standard method of diagnosis suggested in this study. Similar high sensitivity of conventional PCR in HSV diagnosis was reported by other investigators who worked on various sample [10,16–19]. A specificity of 100 % in conventional PCR had also been reported in a number of studies [19,20].

On the other hand, some investigators documented lower sensitivities [20–22] and specificities [18,21] of PCR for detection of HSV.

The discrepancies in PCR performance among studies is explained by the variability of specimens studied and the lack of standardized PCR protocols especially in earlier studies, such as variations in amplification conditions or the fact that some investigators utilized type-common primers (as the case in the current study), while others detected HSV-l DNA or amplified only HSV-2 DNA.

After the introduction of real-time PCR technology, its performance has been evaluated in the diagnosis of HSV infections and showed sensitivities ranging from 98% to 100% in a number of studies [3,23–25]. Kessler et al[26] compared real-time PCR to conventional PCR in diagnosing CNS herpes infections. They found comparable performance characteristics and deduced that real-time PCR was easier and more rapid. Besides, the less manipulations required for carrying out the technique, would probably lead to lower probability of false-positive results that are due to contamination. However, the main disadvantage of real-time PCR is its high cost that could hardly be afforded by many laboratories.

Prior to the advances introduced to molecular diagnostics, viral culture was the mainstay for diagnosis of HSV infection. The technique is intrinsically dependent on the quality of specimen and on its proper transport and handling so that the infectivity of the virus is maintained [2]. The sensitivity of tissue culture in this study was 57.7%, which came in accordance with the results of other studies undergone on different types of specimens in UK [27] and South Africa [28]. Other investigators reported lower levels of sensitivity in studies undergone in USA [29,30] and UK [31].

Higher levels of tissue culture sensitivity were documented by Slomka et al.[10], Filen et al.[32] and Gitman et al.[3] (80.9% , 75.4% and 86.2%, respectively). The better-than-expected culture results recorded in the later study owes to the fact that all samples were collected locally and inoculated on the day of collection without transport or freezing and thawing prior to inoculation.

The specificity of tissue culture in the current study was 100%. This was similar to many earlier studies[10,29,33,34].

The obvious higher sensitivity of PCR over culture highlights the fact that detection of HSV DNA by PCR, and isolation of HSV by tissue culture, do not reflect the same biological events. A positive result for isolation represents the existence of infectious virus particles, while the presence of DNA may represent both infectious and non-infectious particles. Infectious virions can lose viability in a clinical sample during transit which in turn offers an advantage to the PCR that can detect viral DNA in specimens that are negative by standard isolation methods. Moreover, PCR is much less affected by specimen storage, freezing, thawing or bacterial contamination that reduce virus viability [34].

As evident in figure 3, the stage of the lesion had high statistical significant effect on HSV recovery rate in tissue culture, where HSV was isolated in 50% of vesicular lesions, 10% of ulcerative lesions and 0% of crusting lesions. This significance was not observed in

PCR, where HSV DNA was detected in 50% of vesicular lesions, 43.3% of ulcerative lesions and 16.6% of crusting lesions. Similarly, in a study conducted by Caviness et al.[35], on 659 pediatric patients with symptoms suggestive of mucocutaneous herpes, the sensitivity of culture technique was 48.4% for vesicular lesions and 12.9% for ulcerative lesions. The superiority of PCR over tissue culture in detecting HSV in different stages of the lesion was also documented in a study conducted by Scoular et al. [36] in UK on 236 patients with symptoms suggestive of genital herpes.

The very low sensitivity of tissue culture in isolating HSV from ulcerative lesions in our study can be explained by the fact that 50% of the patients with ulcerative lesions gave a history of antiviral intake which had probably affected the live virions but not their DNA.

In our study, 26 patients gave positive history of antiviral intake. PCR detected HSV in 7 (26.9%) of them while tissue culture was only positive in 2 (7.7%) cases. Very close findings were reported by other investigators [35,37].

The duration of lesions prior to sampling is also important when considering the reliability of a detection method. As the length of time the lesion has been present increases, the level of infectious particles decreases, while levels of HSV DNA does not necessarily follow this inverse relationship [34].

In USA, Cone et al. [30] conducted a study that proved that the average duration of HSV DNA detection was twofold greater than that of virus isolation.

However, three reasons are often cited to support the continued use of tissue culture, namely, its low cost, well-established methodology and the applicability of performing phenotypic antiviral susceptibility testing. On the other hand, PCR is more expensive and requires dedicated laboratory areas adapted to its use [6,31,38]. Although some commercial PCR assays are FDA-cleared for detection of HSV only from cutaneous and mucocutaneous lesions, currently, there are no FDA-approved assays for CSF testing, blood testing, or prenatal screening [2].

On the other hand, PCR can detect the virus DNA in subclinical episodes of viral shedding, defined as the presence of HSV in the absence of clinical lesions. Sites of shedding include the mucosal surfaces of the eyes, mouth, and genitalia. In these cases, the low numbers of infectious virions involved may result in negative isolation results [39].

The current study included 20 samples from participants with no symptoms suggestive of HSV infection. Interestingly, 5 (25%) of the samples were positive by both PCR and tissue culture. One of which was an oral and four were cervical specimens. These results feature the subclinical shedding of HSV. Wald et al. [17], in their study that was conducted on 27 HSV-2 asymptomatic but seropositive women, detected HSV DNA in genital secretions in 19 (95%) women, and HSV was isolated by culture in 15 (75%) women. The higher rate of subclinical shedding in such study compared to the current one could be attributed to the fact that they selected seropositive subjects as an inclusion criterion. Similarly, Miller et al. [39] concluded that at least 70% of the population in USA shed HSV-1 asymptomatically at least once a month, and many individuals appear to shed HSV-1 more than 6 times per month.

From an infection prevention standpoint, there is concern that such asymptomatic individuals might probably act as a reservoir of infection spreading the virus to their contacts. In fact, it has been proved that asymptomatic genital shedding of HSV accounts for the majority of HSV infections transmitted to sexual partners and neonates. Therefore, identification of such cases, is central to an effective infection prevention and control strategy.[40]

In the current study, all negative samples by PCR were found negative by tissue culture. Clinical diagnosis of HSV alone had very low specificity (31.4%) suggesting an over estimation of herpetic infection based on clinical diagnosis solely.

It is worth noting that this study is not without its limitations which include the relatively small sample size, the lack of incorporating other diagnostic modalities and HSV typing.

CONCLUSION

Though tissue culture has its own advantages, conventional PCR could serve as a gold standard for the diagnosis of HSV infection.

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