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Comparing the Efficiency of Cell Culture Method to Real-Time PCR for Diagnosis of Genital Infections with Herpes Simplex Virus Type 1 and Type 2

Genital Herpes Simpleks Tip 1 ve Tip 2 Tanısında Kullanılan Gerçek Zamanlı PCR ve Hücre Kültürü Yöntemlerinin Etkinliğinin Karşılaştırılması

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SUMMARY

Introduction: *Herpes simplex viruses (HSV) are enveloped, large, double-stranded DNA viruses from the Herpesviridae family. HSV-1 causes lesions primarily in the mouth, tongue, lips, pharynx, and eyes, while HSV-2 usually causes genital lesions. Although cell culture has been accepted as the gold standard for the diagnosis of mucocutaneous HSV lesions, it has some disadvantages, such as requiring a long time and specific laboratory equipment.*

Materials and Methods: *In this study, we investigated HSV-1 and HSV-2 by using shell vial cell culture and TaqMan real-time polymerase chain reaction (PCR). We utilized direct fluorescent antibody (DFA) method to distinguish between HSV-1 and HSV-2 in genital smear samples. Genital smear samples were collected from 98 patients between the ages of 18-50 years with suspected genital HSV-1/2 infection in the Gynecology Clinic of Istanbul University Faculty of Medicine.*

Results: *HSV-1 positivity was detected at first passage in 1 (1.02%) sample. HSV-2 positivity was detected in 17 (17.3%) samples with cell culture. One (1.02%) sample was positive for HSV-1 and 21 (21.42%) samples were positive for HSV-2 by real-time PCR method.*

Conclusion: *Our data revealed that the sensitivity and specificity of real-time PCR method were 100% and 95%, respectively.*

Key Words: *Genital herpes, Cell culture*

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ÖZET

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Giriş: *Herpes simpleks virüsler (HSV), Herpesviridae ailesinden zarflı, büyük çift zincirli DNA virüsleridir. HSV-1 öncelikli olarak ağız, dil, farenks ve göz infeksiyonlarına sebep olurken, HSV-2 sıklıkla genital lezyonlara sebep olur. Mukokütanöz HSV lezyonları tanısında hücre kültürü altın standart bir yöntem olmasına rağmen, uzun zaman ve spesifik laboratuvar aletleri gerektirmesi gibi dezavantajları vardır.*

Materyal ve Metod: *Bu çalışmada, hücre kültürü ve TaqMan gerçek zamanlı polimeraz zincir reaksiyonu (PCR) yöntemleri kullanılarak HSV-1 ve HSV-2 araştırılmıştır. Genital smear örneklerinden direkt floresan antikor (DFA) yöntemi kullanılarak HSV-1 ve HSV-2 ayırımı yapılmıştır. İstanbul Üniversitesi Tıp Fakültesi Jinekoloji kliniğine başvuran 18-50 yaş arası HSV infeksiyonu şüpheli 98 hastadan genital smear örnekleri alınmıştır.*

Bulgular: *Hücre kültüründe yapılan ilk pasajda 1 (%1.02) örnekte HSV-1, 17 (%17.3) örnekte HSV-2 tespit edilmiştir. Gerçek zamanlı PCR yönteminde ise 1 (%1.02) örnekte HSV-1, 21 (%21.42) örnekte HSV-2 saptanmıştır.*

Sonuç: *Bu sonuçlara göre gerçek zamanlı PCR yönteminin duyarlılığı %100, özgüllüğü %95 olarak bulunmuştur.*

Anahtar Kelimeler: *Genital herpes, Hücre kültürü*

INTRODUCTION

Herpes simplex viruses (HSV) are enveloped, large, double-stranded DNA viruses with icosahedral capsid symmetry from the *Alphaherpesvirinae* subfamily of *Herpesviridae*[1-3]. There are two separable types of the virus, herpes simplex virus type 1 (HSV-1) and herpes simplex virus type 2 (HSV-2), which can be distinguished by their biological properties and glycoproteins[4-6].

HSV infections are globally common due to their high prevalence and transmissibility rates[1,2,6]. The two pathogenic HSV agents, HSV-1 and HSV-2, typically result in lifelong recurrent disease in symptomatic patients, including mild skin or mucous membrane lesions such as neonatal lesions, skin lesions (cold sores), genital lesions, blinding eye lesions, and fatal encephalitis. Asymptomatic patients, on the other hand, have no history of recurrent disease [7-10].

Although both types of HSVs are capable of causing infections on the skin in any part of the body, in general, HSV-1 causes lesions primarly above the waist (around the mouth, tongue, lips, pharynx and eyes). HSV-1 can also cause genital herpes; however, it is mostly HSV-2 that causes infections below the waist and leads to genital lesions. Eighty to ninety percent of genital HSV infections are caused by HSV-2, but because of the different approaches to sexuality, HSV-1 is becoming a more common agent [1,8,6]. A serious result of genital herpes is that it can cause neonatal herpes via transmission from mother to infant during birth. The agent, which is HSV-2 in most cases, is transmitted through the birth canal during delivery in approximately 85% of the patients[1,8,11-13].

HSV infections are contagious and spread from person to person by close contact with herpetic lesions or even from contact with virus-shedding, asymptomatic skin, saliva, or genital secretions [14,15]. After the primary infection of the host, HSV propagates through the neurons and invades the local sensory nerves, where it remains latent (transcriptionally inactive) for life. Factors such as physical and emotional stress, hormonal changes, sun exposure, and fever can trigger

the reactivation of the virus. After reactivation, the virus can reinfect the primary infection site[10].

Culture isolation has been accepted as the traditional gold standard for HSV diagnosis. Although virus isolation is a sensitive and specific diagnostic method, it requires a long time, specific laboratory equipment and intensive labor, which may lead to delays in diagnosis and treatment of the disease[16,17]. Viral antigen detection can be considered as a suitable alternative to culture isolation. Direct fluorescent antibody (DFA) staining of smears is a sensitive way to demonstrate the presence of HSV antigen in HSV lesions. However, the sensitivity of the antigen detection tests depends largely on obtaining a high-quality specimen. Preparing the slides for fluorescence microscopy is also very important for accurate results, as having less than 50 intact cells on each slide may lead to inconclusive results[16].

Molecular diagnostic methods for HSV DNA detection are more sensitive than the cell culture. A major problem with the polymerase chain reaction (PCR) technique is the risk of false-positive results due to post-amplification sample contamination. The real-time PCR system does not require post-amplification handling, thereby minimizing the false-positive results. Although the equipment required for real-time PCR is relatively expensive, it has become more widely used. It minimizes the risk for post-handling contamination by performing the analysis in a closed system, enables working on several samples in small volumes with minimal hands on time, and gives fast results, all of which make the real-time PCR method very cost-effective[16,17].

In our study, we investigated HSV-1 and HSV-2 by using shell vial cell culture (gold standard) and TaqMan real-time PCR. We used DFA method for the separation of HSV-1 and HSV-2 in genital smear samples (perianal, vulva, vagina, cervix) collected from patients with suspected genital HSV infection from the Gynecology Clinic of Istanbul University Faculty of Medicine. Our aim was to compare the specificity and sensitivity of the TaqMan real-time PCR method with that of the shell vial cell culture.

MATERIALS and METHODS

Sample Collection

Genital smear samples of 98 patients between the ages of 18-50 years with suspected HSV-1/2 infection were collected. Genital mucus was removed from the areas before collecting the samples. Virus-infected cells were taken from the basal regions of the lesions with dacron sterile plastic ecuvion by pushing firmly and rotating over the lesion. In the presence of a vesicle, vesicle fluid was also taken with sterile syringe after opening the vesicle. The ecuvion was immediately placed in a special viral transport medium (VTB) after taking the samples and was sent to the Department of Microbiology at Istanbul University Faculty of Medicine.

Cell Culture

Shell vial culture method was applied by using Hep-2 (human larynx epidermoid carcinoma) cells for the isolation of HSV. All stages of cell culture studies were carried out in a Class 2A cabin that was previously sterilized using 70% alcohol and ultraviolet rays. After being trypsinized, Hep-2 cells were passaged into 10 mL culture tubes (25 cm^2) as 50.000 to 400.000 cells. Passaged cells were incubated at 35-37°C in a 5% CO_2 incubator until they formed a complete layer. Clinical samples were cultured into 200 µL cell culture tubes with pastor pippets. The medium in the tubes was completely evacuated after incubation in the CO_2 oven for 1 hour at 37°C. 2 mL continuing plates were placed on them and were incubated at 37°C in the CO_2 oven again. Cells were examined for cytopathic effects every other day for seven days with inverted microscope. HSV antigen was investigated with DFA method in all tubes in which HSV-specific cytopathic effect occurred after seven days' follow-up.

Direct Fluorescent Antibody (DFA) Test

We used DFA test using the HSV-1 and HSV-2 kit Light Diagnostics™ Chemicon International, USA, to verify the growth and determine the type of HSV in the shell vial cell culture after incubation at 35-37°C for 48 hours[16]. Samples were fixed with 2 mL acetone. Slides were prepared by dropping 25 µL of HSV-1 and HSV-2 monoclonal antibodies labelled with fluorescein onto the samples and were examined by fluorescent microscope at a magnification of 400x. Samples with typical green color were considered as positive for HSV-1 or HSV-2.

Real-Time PCR

DNA was extracted from 98 samples using RTP DNA/RNA Virus Mini Kit (INVITEK). Real-time PCR

was performed according to the TaqMan method in a 20 µL volume using 0.5 mM forward and reverse primers, 0.5 mM TaqMan probe (FAM/TAMRA) and 18.5 mM 1X RT-PCR Master Mix. An ABI PRISM 7000 Sequence Detection System was used with the following amplification conditions: (1) 95°C 10 min, (2) 95°C 30 s and (3) 60°C 1.5 min, for 40 cycles.

For HSV-1 detection by real-time PCR, HSV-1 forward (5'TCGAACAGCTCCTGGCCGATTTT-3'), HSV-1 reverse (5'CGGTTGATAAACGCGCAGT-3') polA gene including primers and HSV-1 TaqMan probe (5'-FAM-CGTCTGGACCAACCGCCACA-CAGGT-TAMRA), and for HSV-2 detection, HSV-2 forward (5'-GTGCGGTTGATAAACGCGCAGT-3'), HSV-2 reverse (5'-ATCATCTACGGGGACACG-GACT-3') polA gene including primers and HSV-2 TaqMan probe (5'-FAM-CTCGCTTAGATGGCC-GATCCCAATC-TAMRA) were used. The lower detection limits for HSV-1 and HSV-2 were 0.9 copies/mL and 0.47 copies/mL, respectively[12,18].

RESULTS

Ninety-eight genital smear samples were examined by both shell vial cell culture and real-time PCR methods. HSV-1/2 could not be detected by either method in 76 samples. HSV-1 positivity was detected at first passage in 1 (1.02%) sample. HSV-2 positivity was detected in 17 (17.3%) samples at the first and second passages by the shell vial cell culture (Table 1). Examining the same 76 samples by real-time PCR revealed that 1 (1.02%) sample was positive for HSV-1 and 21 (21.42%) samples were positive for HSV-2. 1 HSV-1 and 17 HSV-2 samples identified to be positive by the shell vial cell culture method were also positive by real-time PCR. HSV-2 positivity in 4 samples confirmed by real-time PCR could not be detected by the shell vial cell culture method (Table 2). The 4 samples were planted to shell vial culture once more and no viruses were detected, suggesting that these HSV-2 positive samples by real-time PCR were actually false-positives.

Table 1. The number of HSV-1 and HSV-2 isolations from first and second passages

	Number of HSV-1 isolations	Number of HSV-2 isolations
First passage	1	13
Second passage	0	4
Total	1	17

In this study, real-time PCR method was compared with the gold standard shell vial cell culture method. Statistical analysis of the results using the Statistical Package for the Social Sciences (SPSS) 17.0 software revealed that the sensitivity and specificity of real-time PCR method were 100% and 95%, respectively. Positive predictive value was 81% and negative predictive value was 100%. The results were found to be statistically significant ($p< 0.001$).

DISCUSSION

HSV causes several different clinical pictures, including asymptomatic infection and mild skin diseases or mucous membrane lesions to fatal clinical forms such as encephalitis and organ or systemic involvement with progressive prognosis. Undiagnosed genital HSV infection is an important obstacle to effective treatment and contamination control, since immediate diagnosis and treatment of these treatable infections are critical[8,18,19].

Although virus isolation in cell culture is a sensitive and specific diagnostic method, it has several disadvantages, such as requiring a long time, specific laboratory equipment and intensive labor. A two-week period is required for the observation of cytopathic changes. The level of virus excretion, such as the quality and transport conditions, affects the sensitivity of the method. The virus isolation rate varies among different lesions, as 52-93% for vesicular lesions, 41-72% for ulcerative lesions, and 19-27% for crusted lesions. Additionally, non-compliance for taking the samples for virus excretion during active lesions and transport and storage of clinical samples under inadequate conditions significantly reduce the sensitivity of virus isolation in the cell culture[9,20]. False-negative results in HSV diagnosis in mucocutaneous lesions by cell culture due to bacterial and fungal contamination have to be considered as well in re-evaluating the sensitivity of the method.

For all these reasons, improved molecular diagnostic methods have been taking the place of cell culture in the diagnosis of mucocutaneous HSV lesions in recent years[21-23]. Different studies have sugges-

Table 2. Comparison of the results obtained with shell vial cell culture and real-time PCR

Test	TaqMan Real-Time PCR HSV-1 (+)	TaqMan Real-Time PCR HSV-2 (+)	TaqMan Real-Time PCR HSV-1/2 (-)	Total
Shell vial cell culture HSV-1 (+)	1	0	0	1
Shell vial cell culture HSV-1 (-)	0	0	0	0
Shell vial cell culture HSV-2 (+)	0	17	0	17
Shell vial cell culture HSV-2 (-)	0	4	0	4
Shell vial cell culture HSV-1/2 (-)	0	0	76	76
Total	1	21	76	98

PCR: Polymerase chain reaction, HSV: Herpes simplex virus.

ted that PCR is powerful in investigating the samples that were negative by cell culture, especially for detecting viruses from cerbrospinal fluid (CSF) in HSV encephalitis. Moreover, recent studies have demonstrated that when compared with cell culture, PCR has an increased rate of HSV identification in mucocutaneous lesions by 11-41%[22,23]. One of the major problems with the previous PCR methods was the vulnerability to contamination. Newly developed real-time PCR techniques have decreased the contamination risk by utilizing a fully automated and closed system[9,21,23,24]. The closed environment lowers the risk of contamination by eliminating sample transfer, reagent addition and gel separation[25,26]. The PCR method also reduces the time required for HSV isolation by cell culture, which is 1-2 weeks, to 1-3 days.

In this study, we compared the sensitivity and specificity of the real-time PCR method to that of shell vial cell culture method. Our data revealed that the sensitivity and specificity of the real-time PCR technique were 100% and 95%, respectively. Positive predictive value was 81% and negative predictive value was 100%. Our results are consistent with previous studies that proposed that real-time PCR method has a higher sensitivity and specificity than cell culture by utilizing different real-time PCR methods [27-31]. We conclude that real-time PCR method is a very good alternative to cell culture method for HSV-1/2 diagnosis, as it is fast, reliable, sensitive, and easy to use, with the advantage of being able to study more than one sample at a time.

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