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Evaluation of an Integrated Cell Culture-based and PCR Assay for Diagnosis of Genital Herpes in Women

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ABSTRACT Diagnosis of genital herpes requires a combination of clinical presentation and laboratory studies. Laboratory diagnostics allow us to clearly establish the etiology (HSV-1 or HSV-2) in order to determine the course of infection and prognosis. Decisive factors in the selection of the appropriate test are: diagnostic goals, patient population, specimen type, and implementation of conditions for the specific method. In total, 187 samples collected during a routine gynecological examination from 120 women were examined for the presence of HSV-1 and HSV-2 in the genital area. Two methods were used to test swabs: cell culture isolation and PCR. HSV-1 was the dominant type of virus in both study groups. The cytopathic effect was observed in 67 (35.8%) cultures with clinical material. HSV-1 and HSV-2 DNA were detected by PCR in 73 (39.0%) cell cultures infected with clinical samples. We did not observe typical, virus related cytopathic changes in 13.7% DNA HSV positive cell cultures, but on the other hand we did not detect viral DNA in 6% of positive cell cultures. High values of the parameters, defining the usefulness of diagnostic tests (sensitivity, specificity, and predictive values) in both groups, are determined by previous viral replication in cell culture.

KEY WORDS: cell culture, cytopathic effect, diagnosis, genital herpes, HSV, PCR

INTRODUCTION

Genital herpes is one of the most common sexually transmitted diseases (STDs) worldwide. Herpes simplex viruses type 1 and 2 (HSV-1, HSV-2) responsible for the disease belong to the genus *Simplexvirus* within the *Alphaherpesvirinae* subfamily of the *Herpesviridae* family. According to updated nomenclature, there are two species of HSVs, human alphaherpesvirus 1 and human alphaherpesvirus 2. It is well-established that while HSV-1 is the causative agent of cold sores, HSV-2 is the predominant cause of genital herpes. However, it has also been demonstrated that either type can be transmitted through oral, genital, or anal sexual contacts. According to current knowledge, HSV-1 is responsible for about a half of the new cases of genital infections in some countries, and the frequency of isolation of HSV-1 from genital lesions has been increasing and requires constant monitoring (1-7).

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Clinical presentation of genital herpes depends on both the host's immune status and whether the infection is primary or recurrent. In women, typical skin or mucosal manifestations include small (1-3 mm) vesicles on an erythematous base. After 24-48 hours, vesicles rupture and form painful ulcerations. The appearance of vesicles may be accompanied by dysuria, watery or mucous discharge from the urethra, and non-physiological secretions from the cervix. Inflammation and lesions are located mainly on the cervix and/or the vulva and vagina but can be present in the urethra, on the inner surfaces of the thighs, buttocks, and anal area as well. Common signs include fever and enlargement of inguinal lymph nodes (3,4,6,8-10).

Typical clinical symptoms occur in only 20-40% of women with primary genital herpes infection, regardless of the type of virus. As viral shedding can occur not only in the presence of lesions but also when symptoms are mild or do not occur at all, diagnosis based solely on clinical features is neither sensitive nor specific (2,5,8). The characteristic stage when the vesicles are present may be short-lasting, e.g. a few hours, especially if the changes are located on mucosal membranes. Furthermore, lesions may appear in atypical or inaccessible locations. All these factors result in the fact that only approximately 20% of patients with genital herpes have been correctly diagnosed (4,8).

Thus, diagnosis should rely on a combination of clinical presentation and laboratory examinations, which includes light microscopy cytology, virus cultivation, detection of viral antigens, molecular methods, and serology. Diagnostic goals, patient population, specimen type, and conditions for the implementation of the specific method are of decisive importance in the selection of the appropriate test. Only the selection of an appropriate method provides reliable interpretation and, as a consequence, implementation of algorithms for therapeutic and prophylactic procedures (4,9,11,12). Laboratory diagnosis allows clear determination of the virus type (HSV-1 or HSV-2) in order to determine the course of infection and prognosis (10). Clinical signs of genital HSV-1 and HSV-2 outbreaks are similar, but the prognoses are quite different. Genital HSV-1 infection recurs less frequently compared with HSV-2. It is well known that in HIV-infected persons, HSV-2 coinfection leads to increased quantities of HIV RNA in genital secretions and plasma, and shedding of HSV-2 is associated with higher frequency and amount of HIV-1 RNA in genital secretions (8). Studies report that HSV-2 accounts for nearly 70% of cases of neonatal herpes, a majority of which are due to intrapartum asymptomatic viral shedding in mothers without any sing and symptom of genital herpes (13). Unfortunately, according to our knowledge, it is not known what the contribution of HSV-1 in neonatal infections is, especially in populations where this type of virus is the one dominant in genital infection.

Table 1. The results of identification of herpes viruses (HSV-1, HSV-2, or both) in clinical samples using PCR

HSV-1	HSV-2	HSV-1/HSV-2
Group of women suspected of having genital herpes		
94%	3%	3%
Women without clinical signs of genital herpes		
91%	6%	3%

It is well known that screening in the general population is not indicated, but laboratory diagnosis is recommended for the confirmation of clinically suspected genital herpes, especially when symptoms or localization are atypical or for differential diagnosis with other ulcerative lesions: infectious (caused by inter alia *Treponema pallidum* and *Haemophillus ducrei*) or non-infectious (e.g. Crohn's disease, Behcet syndrome, or fixed drug eruption) (1,4,11). Correct diagnosis is important for epidemiological reasons, as well (12).

Virus isolation in cell culture is the gold standard for the diagnosis of HSV mucocutaneous infections; a high recovery rate is expected when lesions are fresh, which is why aspirated vesicle fluid samples or swabs, collected from the base of a lesion, should be tested. Proper handling and transport of the collected materials are critical for the upkeep of virus infectivity. Initial identification of the cytopathic effect requires confirmation with specific methods (4,11,13). Detection by the NAATs (nucleic acid amplification testing), such as polymerase chain reaction (PCR) or its modifications, is considered more rapid than virus isolation and more sensitive as well, even if sampling was performed when the lesions have begun to crust over. The sensitivity of PCR highly depends on the amount of viral DNA collected from the site of infection. The main disadvantage is the risk of receiving false positive results due to contamination of the sample. The next restriction of PCR is small sample volume. Additionally, procedures based on NAAT may present an increased number of positive results, with subsequent clinical dilemmas which can be related to the presence of viral DNA, but not the infectious virus (7,10,11,14).

Since the selection of a diagnostic method is highly dependent on the stage of infection, we decided to analyze the usefulness of a combination of these

diagnostic methods in the study of clinical samples taken from women without the classical signs of infection.

PATIENTS AND METHODS

Overall, 187 samples collected during a routine gynecological examination from 120 women were examined for the presence of HSV-1 and HSV-2 in the genital area.

All patients signed informed consent forms. We analyzed 83 swabs taken from women with suspected genital herpes, but without typical lesions in the form of vesicles (history of recurrent nature of symptoms or discomfort, mild symptoms in the genital area) and 104 swabs taken from asymptomatic women without prior clinical history of genital herpes. Swabs were taken from the vaginal vestibule, the cervix uteri, the vaginal part of cervix, vulva, the vaginal fornix, and the anorectal area. Depending on the patient's status, the gynecologist made the decision about the place of sampling. When the time of sample storage was shorter than 24 h, samples were stored at 4 °C; in case of longer storage time, samples were kept frozen at -20 °C. Two methods were used to test swabs: virus isolation in cell culture and polymerase chain reaction (PCR).

Specimens were obtained with soft-tipped dacron swabs and placed into 2 mL of viral transport medium; VTM (Copan, Italy). For virus isolation, confluent 24h *Vero* cell culture (ATCC CCL-81) was used. Infected cells were monitored daily for morphological changes under an inverted microscope (Olympus CK2, Germany). Detection of light refracting cells and ballooning degeneration with enlarged nuclei suggested infection caused by *Alphaherpesvirinae*. Infected cell culture supernatant was harvested at 4-5 days post-infection or earlier if >50% cytopathic effect (CPE) was observed. If CPE was not observed, 200 µl of supernatant was used for next passage. Up to five blind passages were performed for negative cultures.

Total DNA was isolated from 200 µl of the culture supernatant using DNA Mini Kit (Qiagen, Germany) according to the manufacturer's instructions (15). PCR was performed regardless of the result of the cell culture test.

The statistical analyses were performed using chi-square test with Yates correction for small groups at a confidence level of $P<0.05$. Sensitivity (SE), specificity (SP), positive predictive value (PPV), and negative predictive value (NPV) were calculated.

RESULTS

A cytopathic effect was observed in 67 (35.8%) cultures infected with clinical material. In 60 cultures we observed characteristically enlarged, rounded cells. In the remaining 7 cultures we observed granulation, plaque formation, presence of filopodia-like cellular membrane protrusions, and small multinucleated syncytium formation. In 20 (out of 60; 33.3%) cultures, typical CPE appeared after 48 hours of cultivation and on the fifth day in 37 cultures (61.7%). The remaining samples (3; 5.0%) were found to be CPE positive after subsequent passages, so the analysis was extended to 14 days. In the 120 (64.2%) cell cultures infected with clinical material, there were no changes in the morphological structure of *Vero* cells during the 22 days of observation.

Virus recovery rate in cell culture by mean of CPE production was similar in the group of women with non-characteristic mucosal or skin changes (30, 44.8%) when compared with the group of asymptomatic patients (37, 55.2%), and this difference was not statistically significant ($P>0.05$) (Figure 1). Similarly, the frequency of viral DNA detection in cell cultures inoculated with the clinical material obtained from women with suspected infection (31, 42.5%) was not significantly higher when compared with the group of asymptomatic women (42, 57.5%, $P>0.05$).

HSV-1 and HSV-2 DNA was detected with PCR in 73 (39%) cell cultures infected with clinical material. Viral DNA was determined both in samples considered positive by the cell culture method (63; 33.7%) and in the samples prepared from the culture without any morphological changes in the *Vero* cells (10; 5.3%), but the frequency of HSV DNA detec-

Table 2. Sensitivity, specificity, and predictive values of cell culture/PCR assay in the study of women suspected of having genital herpes and without evidence of genital herpes

TP	TN	FP	FN	SE	SP	PPV	NPV
Group of women suspected of having *genital herpes*							
28	44	10	3	90.0%	81.0%	73.0%	86.0%
Women without clinical signs of *genital herpes*							
35	64	0	7	83.3%	100.0%	100.0%	90.0%

SE: sensitivity; SP: specificity; TP: true positive results; TN: true negative results; FP: false positive results; FN: false negative results; PPV: positive predictive value; NPV: negative predictive value

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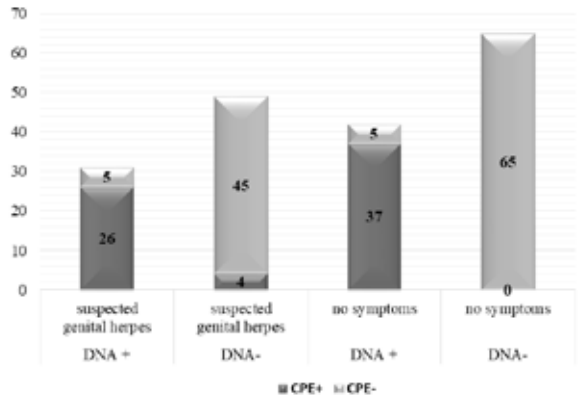

Figure 1. The detection of HSV DNA in CPE+ and CPE- samples taken from women suspected of having genital herpes and from women without local signs of infection.

tion was significantly higher (37 vs. 26; $P<0.01$) in samples where CPE was observed (Figure 1). HSV-1 was the dominant type of virus in both study groups (Table 1).

We decided to evaluate the diagnostic value of cell culture PCR assay in a study of two groups of women and compare it with the virus isolation method. Sensitivity and specificity and predictive values (positive and negative) were calculated. The results are presented in Table 2.

The sensitivity of integrated cell culture-based and PCR assay for diagnosis of genital herpes among women suspected of having infection and without clinical signs of infection was 90.0% and 83.3%, respectively. The incidence of true negative results – reflected in the specificity of the test – was higher (100% vs. 81%) in asymptomatic women. In this group of patients, we were also observed a higher negative predictive value that expresses the probability that a negative result indicated no infection (90% vs. 86%). Among women suspected of having genital herpes and in the group of women without clinical signs of genital herpes, positive test results indicated that the probability of disease was estimated at 73% and 100%, respectively. High values of the parameters in both groups were determined by previous viral replication in cell culture.

DISCUSSION

The laboratory diagnosis of genital herpes is recommended for confirmation of clinically suspected infection, especially if symptoms are atypical. We have to remember that classical herpetic blisters are observed only in a minority of infected women. Recent studies have shown that HSV reactivates much more frequently than previously thought and often sheds from skin or mucosal membranes in the absence of clinical signs. It is important to note that most genital herpes infections are transmitted in the absence of symptoms (8,12). As was shown by Wald and colleagues, the mean number of HSV DNA copies in samples taken from symptomatic and asymptomatic infected woman was $10^{4.9}$ and $10^{4.4}$, respectively (12). Tronstein and coworkers also found that the median $\log_{10}$ HSV-2 DNA copy number among the participants who reported lesions and among persons who remained asymptomatic was similar (4.8 vs. 4.1) (16). Likewise, many neonatal infections result from asymptomatic cervical shedding of HSV after an episode of genital herpes at the time of delivery (8). Therefore, there is a tremendous need to monitor certain groups of people for the presence of the virus in the the anogenital area and selection of the appropriate diagnostic method is critical (3,16).

No patients had typical symptoms in our study. We therefore decided to propagate viruses from clinical samples in culture and subsequently detect the viral genetic material. Regarded in that light, our results indicate that this integrated cell culture PCR assay is highly sensitive for diagnosis of genital herpes and can be applied even to the detection of asymptomatic infection in order to detect asymptomatic genital viral shedding (e.g. for epidemiological purposes, to detect the virus on the surface of the mucous membranes of the pregnant woman's birth canal, in HIV-infected individuals, or sexual partners of herpes infected men).

Virus isolation in cell culture is considered the gold standard for genital herpes diagnosis. The success of this method depends on the choice, collection, and transport of clinical specimens and type of cell culture used (1,4,12,13,17). Fluid aspirated from the vesicles or smear from the base of an intact vesicle is the best clinical sample (18). The sensitivity depends on the viral titer and varies from 90-95% for vesicles to 17-35% for crusted lesions (1,4,11). Standard viral isolation takes 1-7 days to the appearance of degenerative changes in cells inoculated with the virus. CPE in cells infected with HSV-1 and HSV-2 are quite distinct but not unique. Additionally, morphologic changes of the cell line induced by other viruses, toxins, or even *Trichomonas vaginalis* can be confused with HSV (19). This method classifies the virus as a part of the *Alphaherpesvirinae* subfamily. Both HSV and Varicella zoster virus (VZV) are members of the viral *Alphaherpesvirinae*, so confirmation with another method, e.g. PCR, should be subsequently conducted (3). It should be noted that the absence of visible cytopathic changes does not exclude presence of the pathogen (1,4,5,13). We would like to emphasize that

the interpretation of CPE requires substantial experience. Observed cytopathic changes may differ from those commonly described. This can be caused by mutations that affect the HSV membrane proteins, which play an important role in viral entry and fusion of infected cells with neighboring cells. The consequences of those processes are cytopathic changes in HSV infected cells, such as polykaryocytes, plaques, and syncytia (*syn* phenotype). It is worth noting that the virulence of the syncytium-forming strain has not been clarified, but researchers have suggested that these strains may present some characteristics of the attenuated virus. This hypothesis is supported by the finding that most of tested syncytial mutants were less virulent than nonsyncytial strains after corneal infection of rabbits or footpad and vaginal infection of mice (20-22).

CONSLUSIONS

In our study, we did not detect viral DNA in 6.0% of positive cell cultures, but on the other hand we detected DNA HSV in 13.7% negative cell cultures (with no CPE indicative of herpes infection). The false negatives in cell culture assay can be due to the slow growth of wild strains *in vitro* or low titer of the virus in the specimens. It is worth noting that negative results never mean the absence of herpes disease (14). Laboratory diagnosis combining two methods allowed us to achieve high test sensitivity, especially among women suspected of having infection (90%). We evaluated the feasibility of such a procedure analyzing the positive and negative predictive values. Obtained rates were high enough to recommend an integrated cell culture-based and PCR assay for the detection of asymptomatic genital viral shedding and diagnosis of genital herpes in asymptomatic infected woman or those with no characteristic sign of infection. According to the literature, laboratory diagnosis of genital herpes in men is less complicated (e.g. due to the anatomical structure of the genital organs) so we cannot confirm whether this integrated cell culture/ PCR assay is more useful than single-step diagnostics i.e. detection of viral DNA in signs located on the genital area.

We also found that analysis the material taken from different anatomic locations in the genital tract can be the good practice in women. Such an approach can increase the probability of recognition of infection and thus reduce the risk of false negative results (5).

Furthermore, virus culture allows us to perform a phenotypic drug susceptibility assay if necessary. And lastly, it is also important to provide access to laboratory diagnosis of genital herpes, especially in developing countries or selected, clearly defined populations, and then choose the most optimal method enabling the diagnosis of genital infection in specific patients. The use of improper methods or failure to comply with the procedures not only leads to false results, but also falsifies epidemiological data (12).

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