Type Specific Identification of Anogenital Herpes Simplex Virus Infections Using a Commercially Available Nucleic Acid Amplification Test

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Abstract

Background: Herpes infections are among the most common STI but diagnostic methods for genital herpes have not kept pace with the movement toward molecular testing. Here we describe an FDA-approved molecular assay that identifies and types HSV infections for use in routine clinical settings.

Methods: Paired samples from anogenital lesions were tested using the BD ProbeTec HSV Q* (HSVQ*) system, HSV culture and a lab-developed PCR assay. Family planning, OB/GYN or STD clinics in the US served as recruitment sites. Sensitivity and specificity estimates, head-to-head comparisons, measures of agreement and latent class analyses were performed to provide robust estimates of performance.

Results: 508 participants (174 men and 334 women) with anogenital lesions were included. 260 HSV-2 and 73 HSV-1 infections were identified. No differences in test performance based on gender, clinic type, location of lesion or type of lesion were observed. Sensitivity for HSV-2 detection ranged from 98.4-100% depending on the analytical approach while specificity ranged from 80.6%, when compared to the less sensitive culture method, to 97.0% when compared to PCR. For HSV-1 the sensitivity and specificity ranges were 96.7-100% and 95.1-99.4%, respectively.

Conclusions: This assay may improve the ability to accurately diagnose anogenital lesions due to herpes infection.
Introduction

Genital herpes is a widespread sexually transmitted infection directly and indirectly linked to a spectrum of morbidities which include adverse outcomes of pregnancy and increased risk for HIV infection. In 2008, herpes simplex virus type-2 (HSV-2), the predominant cause of genital herpes, was estimated to affect approximately 17% (over 45 million) of Americans, most of whom are unaware that they are infected. (9, 17) Serological testing has helped to define the prevalence of infections and is a useful tool for evaluation of selected patient groups, however, it is not recommended for routine screening of the general population. (5) In contrast, microbiologic demonstration of virus provides information critical to clinical management of infection. Laboratory testing for herpes simplex virus (HSV) is critical for accurate diagnosis of genital herpes infections and may have a profound impact on management decisions for persons with and at risk for genital herpes.

Multiple factors currently contribute to underdiagnosis of herpes infections. Important contributors include the varied clinical presentations of genital herpes lesions, failure to test lesions, and the lack of sensitivity of culture when testing is performed. Further, even experienced clinicians have been shown to be incorrect (e.g. categorizing a lesion as herpes when it is not) about 20% of the time when a clinical diagnosis of genital herpes is made strictly on the basis of clinical presentation. (12) Testing for the presence of HSV is currently most often performed using herpes culture methods despite the fact that nucleic acid amplification tests have been repeatedly demonstrated to be 3-5 times more sensitive for virus detection than carefully performed culture. (5)
Type-specific testing of herpes viruses also plays an important role in management decisions for persons with genital herpes since an increasing proportion of genital herpes is now due to HSV-1. The natural history of genital HSV-1 markedly differs from that of genital HSV-2. Compared to genital HSV-2, HSV-1 genital outbreaks recur less frequently and are less likely to cause asymptomatic viral shedding (8). These factors may influence risk for transmission of infection to others and thus, management decisions including what schedule of herpes treatment, if any, should be offered to a patient.

Here we present the results of a multicenter study evaluating the performance of a recently FDA-approved, commercially-available, type-specific nucleic acid amplification test for HSV: the BD ProbeTec HSV Qx (HSVQx) system.

Methods

Population and Specimens

Participant enrollment took place at nine clinical centers comprised of 5 STD and 4 family planning clinics. Two laboratory sites provided viral culture results, three performed HSVQx testing, and one provided PCR results only. The study protocol was approved by Institutional Review Boards for Human Research (IRBs) at each participating site. All participants provided written informed consent for specimen collection. Inclusion criteria included the presence of a lesion (either vesicles or ulcers) in the anogenital ("boxer-short") region, i.e. the perineum, perianal, groin, buttocks, thigh, vulvar, penis and scrotal areas. Exclusion criteria included lesions in non-genital areas, crusted lesions, or use of anti-herpes medications (e.g. Acyclovir or
Lesions were sampled using a polyester swab, which was placed directly into universal viral transport medium (UVT) [Becton Dickinson, Sparks, MD] and then using a second, foam-tipped swab which was placed directly into the Becton Dickinson (BD) Q⁺ liquid wet swab transport medium (Q⁺ WS). UVT samples were collected first in order to optimize the integrity of culture specimens and were stored at 2-8°C for no more than 36 hours prior to aliquotting for use in multiple assays [Figure 1]. Specimens collected in UVT were divided into three aliquots: one was tested using the ELVIS®HSV ID/Typing Test System [Diagnostic Hybrids Inc., San Diego, CA], the second aliquot (500 μL) was placed into the BD Q⁺ transport medium for testing using the new assay (designated Q⁺ UVT) on the Viper instrument, and the third 500 μL aliquot was stored at -70°C for subsequent testing by PCR at the University of Washington. The second swab, the Q⁺ WS specimen was immediately placed in the BD Q⁺ transport medium and stored at 2-30°C for up to 7 days prior to testing on the Viper system. This dual swab specimen collection and aliquotting design allowed head-to-head testing of a single sample using culture, a highly regarded research PCR³,⁶,⁴, and the new assay, as well as comparison with a second swab collected directly into the Q⁺ WS transport medium. All participants had samples tested by all three methods: viral culture, PCR and HSVQ⁺. Specimens from all participants were sent to one of three laboratories (located at: Indiana University, Louisiana State University, and University of Alabama Birmingham) for testing with the BD Viper system, one of two laboratories (located at: LabCorp and Louisiana State University) performed all viral cultures and all PCR testing was
performed at the University of Washington. All testing sites participated in pre-trial training and performance validation prior to testing any participant samples.

**HSV-1 & 2 assays**

*HSVQ* using *Viper Instrumentation*  
The procedural and processing details of the BD HSVQ* assays are identical to those of the BD *Chlamydia trachomatis* Q* assay on the Viper instrument which has been described elsewhere.(14) Briefly, the steps of the assay are as follows. Samples in transport medium are warmed at 114°C to dissolve mucous and homogenize the specimen matrix. After cooling at room temperature for 15 minutes, the specimens were loaded onto the Viper instrument which then performed all steps necessary for extraction and amplification of target DNA without further user intervention. The HSVQ* amplified DNA assays are based on the simultaneous amplification and detection of target DNA using amplification primers and a fluorescently labeled detector probe. The primers for each assay target a type-specific DNA sequence (HSV1 126bp, HSV2 124bp) within the Herpes simplex virus *glycoprotein G* gene. The presence or absence of HSV DNA is determined by calculating the peak fluorescence, which is called the maximum relative fluorescent units, over the course of the amplification process and by comparing this measurement to a predetermined threshold value.

**ELVIS culture**  
The ELVIS culture system uses a genetically engineered cell line for culture that expresses β-galactosidase in the presence of HSV-1 or -2 replication.(7) Accumulation of this reporter product can be detected within 17-24 hours following inoculation with samples. Cultures demonstrating β-galactosidase reactivity are then sequentially stained
with fluorescent-tagged monoclonal antibodies for viral typing. Type-specific fluorescent staining is first performed for HSV-2. If negative for HSV-2, the cultures are re-stained with an HSV-1 type specific reagent. As a result, for those cultures giving a positive result for HSV-2, no further staining is possible since the fluorescent tag is fluorescein isothiocyanate (FITC) in both cases. Thus, dual infections with both HSV-1 and HSV-2 cannot be identified with this method.

Real-time PCR

While there are many laboratory developed PCR assays described in the literature, a lack of thorough evaluation and standardization prevents cross-study comparison of the performance of these tests. The University of Washington PCR assay for detection and typing of HSV has been well described and is recognized as the gold-standard assay for use in FDA drug treatment and vaccine clinical trials. Thus, this assay was chose as the comparator assay for the evaluation of the HSVQ assay. Briefly, UVT samples were used for DNA extraction and the resulting DNA was amplified in two stages. In the first amplification, genus-specific primers are used in real-time PCR to amplify either HSV-1 or 2. This assay has a linear output that also provides quantification of HSV DNA present in the specimen. If positive, a second, multiplex PCR assay containing primers and probes specific for both HSV-1 and 2 is performed. Type specificity is determined based on melting curves. Although this assay is not commercially available, it is considered to be a highly sensitive detection method and has been utilized to describe the pathogenesis of HSV, the association of HSV with HIV-1 infection, and to measure clinical outcomes for an HSV vaccine trial. The assay can detect mixed infections with up to a 3 log difference in DNA copy number and is estimated to be 3-5 times more sensitive than culture performed in the same laboratory.
The results of the HSVQ\textsuperscript{x} assay, using either the Q\textsuperscript{x} WS or Q\textsuperscript{x} UVT sample, were compared head-to-head with ELVIS and with PCR results. The performance of the Q\textsuperscript{x} WS and Q\textsuperscript{x} UVT were also compared to one another, and to a patient infected standard (PIS). The patient infected standard was defined as a positive result by either ELVIS or the PCR assay. The intent of this analysis was to identify all potential infections and allow the best estimation of the sensitivity of the HSVQ\textsuperscript{x} assay. For all head-to-head comparisons, percent agreement and $\kappa$-scores were estimated. $\kappa$-scores are used for assessing the agreement between paired binary outcomes with a rule of thumb interpretation of 0.60-0.80 as good agreement and >0.80 as very good agreement. Sensitivity and specificity were calculated compared to the PIS. The analysis for HSV-1 compared to culture is based only on those samples which were negative for HSV-2 since ELVIS staining for HSV-1 could not be performed for HSV-2 positive cultures. Differences in performance based on gender, age, and location of lesion were assessed. Finally latent class analysis was used in an attempt provide an unbiased estimate of sensitivity and specificity based on the latent class of infection status\cite{2}. Using results from all of the assays, latent class analysis determines the number of classes into which results can be categorized; for diagnostic testing this is ideally two classes (infected and uninfected). After classes are identified based on the total of all testing results, an estimation of the accuracy of each assay is made based on those categorizations. Alpha = 0.05 was used for all analyses.
Results

Samples were obtained from 564 participants, 56 of whom were subsequently excluded based on pre-determined study inclusion/exclusion criteria. For the final analyses, 498 Qx WS results and 501 Qx UVT results were compared to culture and PCR for HSV-2 [Table 1]. The 189 (37.2%) HSV-2 positive culture specimens were not able to be tested by subsequent staining with the HSV-1 type-specific antibody and one HSV-1 Qx wet swab result was unable to be determined due to an extraction control failure. Thus, while all compliant Qx results were compared to PCR for HSV-1, there were 308 (62%) Qx WS and 312 (62%) Qx UVT results that could be analyzed using HSV-1 culture results. Exclusions were based on improper sample collection, improper specimen handling, or improper storage of specimens: 2 samples were not available for PCR testing, 7 were missing ELVIS results and 3 were lacking Qx WS samples.

Study participants consisted of 334 women and 174 men ranging in age from 17-71 years with an overall median age of 25 years [Table 1]. Approximately half (49.6%) were enrolled from STD clinics. Lesions tested were sampled from the following anogenital sites: glans or penis (77.0% of men), vulva (74.5% of women), perineum (15.9% of women), perianal (3.8% of all participants) and unspecified skin locations (10.2% of all participants). Based on estimates derived from the PCR assay, the positivity rate of HSV-2 in these symptomatic patients was 55.0 and 41.2% in STD and Family Planning clinics, respectively. The positivity rate of HSV-1 was lower, but remained substantial with 8.0 and 20.4% identified from patients attending STD and Family Planning clinics, respectively.
**HSV-2**

The HSVQ\^x assay detected 186/189 (98.4%) of the samples positive in the ELVIS culture system [Table 2] and yielded an additional 51 positive results from the same UVT specimens (Q\^x UVT) from specimens with negative ELVIS culture results. The Q\^x WS specimen also detected 186 of the culture positive samples while detecting an additional 60 positive results from specimens with negative cultures. The κ-scores for the Q\^x UVT samples were .782, .956 and .945 when calculated for culture, PCR or the patient infected standard, respectively. For the Q\^x WS samples the κ-scores were .746, .944 and .932. The estimated sensitivities for the HSVQ\^x assay, based on the patient infected standard, were 96.4% and 97.6% for Q\^x UVT and Q\^x WS, respectively. There was no difference in assay performance estimates by age, gender or lesion location (all p-values>0.3). The latent class analysis model was saturated with two latent classes suggesting that the results could be divided into infected and uninfected participants. For HSV-2 infections the sensitivities and specificities estimated using latent class analysis were: 79.7 ± 1.9% and 98.9 ± 0.5% for culture; 100% and 98.3 ± 0.6% for PCR; and 100% and 97.0 ± 0.8% for HSVQ\^x. The ability to detect infections based on PCR-determined organism load is shown in Figure 2 for the HSVQ\^x assay and ELVIS cultures. The data for HSVQ\^x was generated with pooled data from both sample types. Thus, the commercially available HSVQ\^x assay performs substantially better than culture and at least as well as the laboratory developed PCR assay for detection of HSV-2.

**HSV-1**

As described in the Methods, the ELVIS culture system does not allow testing of specimens for HSV-1 if the culture is positive for HSV-2. As a result, 189 specimens culture positive for HSV-
2 were not available to provide HSV-1 culture results. The HSVQx assay using the same UVT (Qx UVT) samples that were used in the culture system detected 60 (96.7%) of the 62 HSV-1 culture positives. This sample type also resulted in detection of 6 additional positives that were negative by culture [Table 3]. The HSVQx assay identified 59 (96.7%) HSV DNA in 61 culture positive specimens and had an additional 12 positive results when using the Qx WS sample type. The κ-scores for the Qx UVT samples the κ-scores were .921, .992 and .973 when compared to culture, PCR or the patient infected standard, respectively. For the Qx WS samples, κ-scores were .865, .960 and .938. The estimated sensitivities for the HSVQx assay, based on the patient infected standard, were 95.9% and 97.3% for Qx UVT and Qx WS, respectively. There was no difference in assay performance estimates by age, gender or lesion location (all p-values>0.3).

For HSV-1 infections latent class analysis estimated sensitivities and specificities were: 90.2 ± 2.6% and 99.2% ± 0.4% for culture; 100% and 99.9% ± 0.1% for PCR; and 100% and 99.4% ± 0.3% for HSVQx. The detection of infections based on organism load is shown in Figure 3 for the HSVQx assay and ELVIS cultures. These data demonstrate that the HSVQx assay performs as well as the other assays on a fully automated system that provides results within a few hours rather than many days.

Discussion

The data from this large, multi-site clinical trial demonstrate the excellent performance of the HSVQx for detection of both HSV-1 and HSV-2 from anogenital lesions. This assay provides a potential mechanism for increased availability of highly sensitive detection of HSV in persons with lesions. Like well characterized, laboratory-developed research PCR assays(16) this system detected HSV from far more patients than the FDA approved, commercially available culture-
based assay used for comparison in this study. Further, when results were compared to those of PCR testing performed in a respected research laboratory; the results of the two assays were comparable. Indeed the DNA based assays had the capacity to detect dual HSV-1 and HSV-2 infections which cannot be accomplished using the culture-based system. Although the impact of not having an HSV-1 culture result was negligible in this study (only 2/504 (<0.4%) participants had dual infections [data not shown]), it is useful to have any assay that provides both results accurately and quickly for those populations in which dual infections might be more common. In addition to increased sensitivity, the HSVQ^ assay provides a convenient method for collecting specimens in transport medium which can be stocked at room temperature and easily transported to laboratories for testing. As a commercially available assay the BD Q^ assay for HSV detection also has the potential advantage of being added to a laboratory platform already in wide use for detection of gonococcal and chlamydial infections.

Our analyses were rigorous, employing multiple methods. Confirmation of the sensitivity and specificity estimated derived by comparison with the patient infected standard was obtained using latent class analysis. In fact, the latent class analysis estimated sensitivity was even higher than the estimate obtained in comparison with the patient infected standard. This is likely due to the fact that the latent class analysis does not assume that culture negative results represent uninfected individuals. The improved limit of detection of the HSVQ^ assay (Figures 2 and 3), and thus, the ability to correctly identify infections with fewer organisms in the specimen, results in improved clinical sensitivity over culture.
This study is not without limitations. This study was limited to evaluation of lesions occurring in the anogenital region, thus further study will be needed to evaluate the utility of the test for detection of HSV from oral lesions and from other locations. The study design did not allow for assessment of asymptomatic genital shedding, an important cause of HSV transmission which can be detected by PCR from about 10% of specimens in persons with genital HSV-2 infections.

Highly sensitive and specific tools for HSV diagnosis and typing are needed. Serological testing has proven helpful in some settings but is not currently recommended for routine screening. Antibody responses may take months to develop following infection, delaying diagnosis and potentially resulting in either spread of infection to others by persons unaware that they have HSV or troublesome anxiety in persons awaiting test results. Finally, in persons with HSV infections, a positive serological test does not identify the location of infection, a fact which may hamper adoption of measures to reduce transmission to others.

HSV is the most common cause of genital lesions, irrespective of appearance worldwide. Globally it is the most common cause of genital ulceration and many lesions are not “classical”. In contrast, the most common cause of a “typical” chancre in multiple studies is still HSV. Conversely, about 20-25% of lesions identified as HSV by experts are not actually attributable to HSV and are due to other causes. As a result, with the availability of more sensitive, widely available tests, there is now an opportunity to operationalize the recommendation that all genital lesions which are not known to be a recurring problem with a known diagnosis (i.e. HSV or other dermatological process) should be tested for HSV.
The changing epidemiology of genital herpes also warrants increased testing with emphasis on determining not only the presence or absence of infection but, when herpes is diagnosed, the type of virus present. The proportion of genital HSV caused by HSV-1 is increasing and in some studies the proportion of genital herpes caused by HSV-1 equals or exceeds the proportion caused by HSV-2. While the presentation of initial genital herpes due to HSV-1 and HSV-2 is clinically indistinguishable, however, when compared to genital HSV-1, genital HSV-2 recurs more often and is associated with higher rates of asymptomatic genital shedding, differences that may impact management decisions. While there are data to encourage provision of chronic suppressive anti-viral therapy to persons with HSV-2, there are not similar recommendations for HSV-1. Addition of the HSVQ to the laboratory diagnostic menu will positively impact our ability to identify, and therefore manage, this highly prevalent infection.
References


Figure 1. Sample collection and testing scheme

Figure Legend. UVT samples were aliquoted for use in all three HSV assays (ELVIS culture, PCR and HSVQ³). HSV-1 testing in the ELVIS system could only be performed on HSV-2 negative samples. The sample collected using the assay collection kit (Q³ WS) and the UVT aliquoted into the collection kit were both tested on the HSVQ³ system.

Figure 2. Assay sensitivity by organism load for HSV-2

Figure Legend: PCR positive samples generated a viral load value. The percent positivity of the other two assays (ELVIS culture ● and HSVQ³ ■) for HSV-2 is shown based on the PCR-determined organism load.

Figure 3. Assay sensitivity by organism load for HSV-1

Figure Legend: PCR positive samples generated a viral load value. The percent positivity of the other two assays (ELVIS culture ● and HSVQ³ ■) for HSV-1 is shown based on the PCR-determined organism load.
Acknowledgements The authors would like to thank Hanne Harbison, Paula Dixon and Connie Lenderman at UAB for their assistance in this trial.

Previous Presentation
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Disclosures
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Table 1. Description of Participants and Samples Collected

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*Not applicable
Table 2. HSV-2 Performance Compared to Culture and PCR

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<th>PIS&lt;sup&gt;c&lt;/sup&gt;</th>
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<th>PIS&lt;sup&gt;c&lt;/sup&gt;</th>
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<td>240 (98.8%)</td>
<td>241 (97.6%)</td>
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<td>(-)</td>
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<td>3 (95.8%)</td>
<td>6 (95.7%)</td>
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<sup>a</sup>ELVIS Culture system results  
<sup>b</sup>PCR performed by the University of Washington  
<sup>c</sup>Patient Infected Standard  
<sup>d</sup>HSVQ<sup>x</sup> assay performed using samples collected in universal transport medium  
<sup>e</sup>HSVQ<sup>x</sup> assay performed using the wet swab transport system provided with the assay
Table 3. HSV-1

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<td>247 (95.1%)</td>
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<sup>a</sup>ELVIS Culture system. Results could only be performed on HSV-2 culture negative samples while PCR was performed on all specimens. Thus Elvis and PIS comparisons have lower total sample size.

<sup>b</sup>PCR performed by the University of Washington

<sup>c</sup>Patient Infected Standard

<sup>d</sup>HSVQ<sup>x</sup> assay performed using samples collected in universal transport medium

<sup>e</sup>HSVQ<sup>x</sup> assay performed using the wet swab transport system provided with the assay