Evaluation of a Quantitative Competitive PCR Assay for Measuring Herpes Simplex Virus DNA Content in Genital Tract Secretions

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Previous studies have shown an association between the approximate titer of herpes simplex virus (HSV) DNA in clinical specimens and the ability to isolate HSV from genital secretions. To control for variance in amplification conditions, we developed a competitive quantitative PCR (QC PCR) for the detection of HSV DNA. The assay accurately measured from 10 to 10^6 copies of HSV DNA. We compared the QC PCR with our previous semiquantitative detection method and found concordance for 61 of 63 positive specimens. We also evaluated the HSV DNA content from individual swabs of genital secretions obtained from individual sites of the genital tract (cervix, vulva, and rectum) with that from one swab with secretions from all three sites. The concordance for detecting HSV DNA was 91%; for only 4 of 143 collection days was there a >1 log difference between the two collection methods. A single swab with secretions from all three genital sites and evaluated in a QC PCR format can accurately measure the frequency of subclinical and clinical shedding of HSV and the titer of HSV shed from the genital region. Such an approach should be very useful in the evaluation of antiviral chemotherapy for HSV.

Detection of viral nucleic acids in clinical specimens has been shown to be associated with replicating virus, and in vivo and in vitro inhibition of viral replication by an antiviral compound can be monitored by quantitation of the nucleic acids (5, 7, 11). We have previously shown the association between a semiquantitative measurement of herpes simplex virus (HSV) DNA levels and the ability to isolate HSV in genital lesions (4). More recently, we have shown the association between the approximate titer of HSV in clinical specimens and the isolation of HSV in tissue culture (11). To control for variance in amplification conditions on a run-to-run and sample-to-sample basis, most authorities have recommended that quantitative PCR-based assays be performed in a competitive format (1, 6). Competitive strategies involve coamplification of a range of concentrations of a modified PCR product with the target DNA and determination of the point at which the modified DNA and the target DNA give the same amount of PCR product. Ramakrishnan et al. (10) have described a quantitative competitive PCR method for HSV type 1 (HSV-1) DNA detection involving a modified PCR product with a single base change that produces a restriction enzyme site, which allows for the differentiation between products on the basis of the product size after digestion.

We describe a competitive quantitative (QC) PCR strategy for HSV-2 DNA detection in which a PCR product from which 82 bp have been deleted is used as a competitor. The assay accurately measured amounts of HSV-2 DNA from 10 to 10^6 copies. We compared the results obtained by this assay with those obtained by our previously published method (4), in which dilutions of positive specimens are run by PCR and the results are visually compared with the results from separate PCR assays with known amounts of HSV-2 DNA. During this study, we also compared the results obtained with swabs of genital secretions from individual sites of the genital tract (cervix, vulva, and rectum) with those obtained with a single swab containing secretions from all three sites.

MATERIALS AND METHODS

Collection of specimens. We selected one woman who, in previous studies, had been shown to have both frequent clinical and subclinical genital herpes (12). She gave informed consent for a protocol approved by the University of Washington Human Subjects Review Committee. We used a prospective crossover design in which the patient obtained specimens on a daily basis for 174 consecutive days. During the initial 80 days she was treated with acyclovir at 800 mg three times a day, and during the subsequent 94 days she received no antiviral therapy. This study design was selected to evaluate the specificity and utility of the QC PCR assay during a period of limited viral shedding compared to those during active viral shedding. On each day, the woman collected samples on four separate swabs upon awakening. Swabs were taken first from individual sites: the cervicovaginal, the vulvar, and the perianal areas, in that order. Then, a single swab was used to collect secretions from all three sites in the same order (a “mixed” swab). The swabs were placed in digestion buffer and were sent to the PCR laboratory, where they were stored at −20°C until they were processed. DNA was prepared as described previously with phenol and phenol-chloroform extraction and ethanol precipitation (2). Samples were obtained on 143 of the 173 study days.

PCR methods. The PCR amplified part of the HSV-2 glycoprotein B gene, as described previously (3). Briefly, the primers used were HSV2a-1 (5'-CTGGTCTGCTTCTCTTTGGTACGA) and HSV2a-2 (5'-CAGGTCGTGCAGCTGGTTGC). Amplification was for 35 cycles, and 20% glycerol was included in each reaction mixture. Products were detected by liquid hybridization, gel electrophoresis (on 2% agarose for standard noncompetitive PCR or 3% NuSieve–1% agarose for QC PCR) and autoradiography. In select experiments with high DNA input (10^6 copies or greater), liquid hybridization was not performed and gels were stained with ethidium bromide.

Synthesis of competitor DNA. For the QC PCR method, a shortened version of the HSV2 PCR product was made. The modified product had the HSV2a-1 and HSV2a-2 primer sequences at each end but was 82 bp shorter (260 bp compared with 342 bp for the HSV2 PCR product). To synthesize this product, a new primer was used in place of HSV2a-1. This primer (HSVQC) contained 18 bases of the internal HSV2a PCR product sequence along with the HSV2a-1 primer sequence and 10 bases adjacent to it, attached to the 5’ end (Fig. 1). Amplification of HSV-2 DNA (10^6 copies) with this modified primer and the HSV2a-2 primer generated a 260-bp product. This product was separated on 3% NuSieve–1% agarose and visualized by ethidium bromide staining. The band was cut out, melted at 65°C, and diluted 5 x 10^8-fold in 10 mM Tris (pH 8.0). A total of 5 µl of the diluted DNA was amplified with the HSV2a-1 and HSV2a-2 primers to give the final 260-bp competitor DNA. This product was again gel purified to remove the primers.
FIG. 1. Construction of the competitor DNA. HSV-2 DNA was amplified with the HSV QC primer and the HSV2a-2 primer to give the 260-bp competitor DNA.

FIG. 2. Serial 10-fold dilutions of HSV-2 DNA from $10^6$ to 10 copies were each amplified with 7 different 3.16-fold (half-log) dilutions of competitor DNA. Products were detected by liquid hybridization, gel electrophoresis, and autoradiography (A) or by gel electrophoresis and ethidium bromide staining (B). The equivalence points (arrows), at which the 342-bp band and the 260-bp band have equal intensities, were determined by visual inspection. For panel A, the equivalence point in one dilution set was between the two competitor concentrations.
The purified competitor DNA was then quantitated by PCR. Dilutions of this DNA were amplified with the HSV2a-1 and HSV2a-2 primers, and the products were detected by liquid hybridization. An estimate of the copy number was made by comparison of the intensity of the bands obtained with those generated by amplification of known amounts of HSV-2 DNA. The precise copy number was then determined by coamplification of serial twofold dilutions of the DNA with 1,100 copies of linearized plasmid pHS108, which carries the entire glycoprotein B gene (8). Products were detected by liquid hybridization. The dilution giving equivalent intensities of the two bands (the 342-bp HSV2a product from the plasmid and the 260-bp product from the competitor) was then adjusted to contain 1,000 copies in 5 μl (the volume used in the PCR).

RESULTS

QC PCR method validation. We initially evaluated the linearity of the QC reaction by coamplifying the competitor with known copy numbers of authentic HSV-2 DNA. This HSV-2 DNA had been purified by phenol and phenol-chloroform extraction and ethanol precipitation, as described previously (2), from human diploid fibroblasts infected with a laboratory strain of HSV-2. The DNA level was quantitated by repeated comparison with known amounts of pHS108 DNA by PCR. In the validation experiment, 10-fold dilutions containing from 10 to 10^6 copies of HSV-2 DNA were coamplified with 7 half-log (3.16-fold) dilutions of the competitor DNA (Fig. 2). Products were detected either by ethidium bromide staining of gels or, for lower copy numbers, by liquid hybridization. In each case, the copy number of the competitor giving equivalent intensities of the 342- and 260-bp bands was determined. In each case it was found to equal the known copy number of the added HSV-2 DNA.

Detection of HSV DNA by noncompetitive PCR. Figure 3 illustrates the detection of HSV DNA in the genital tract by anatomic site, therapy, and day of observation. As described in Materials and Methods, specimens with copy numbers greater than 10^3 were diluted serially and rerun to get an estimate of the copy number. HSV was isolated in tissue culture on 2 of 72 days while the study subject was on acyclovir therapy, whereas it was isolated on 21 of 71 days while she was on no therapy. One clinical recurrence lasting 4 days was noted while she was on acyclovir therapy, whereas three recurrences, lasting a total of 33 days, occurred while she was on no therapy. HSV DNA was detected by standard PCR on 7 of 72 days while the subject was on acyclovir therapy, whereas it was detected on 51 of 71 days while she was on no therapy.

Comparison of QC PCR with dilution titers of HSV DNA. We then evaluated all 63 of the 137 separate samples in which HSV DNA was detected in the noncompetitive assay at ≥10^3 copies per reaction (after dilution of the specimen DNA when necessary). A comparison of the QC PCR with the dilution method is presented in Table 1. There was excellent concordance between the two methods, with identical titers being present in 36 of the 63 clinical specimens and titers within 1
4 days PCR product at Of the 13 days on which discrepant results were obtained, for specimens from individual sites were negative for HSV DNA. Three sites did not, and on 5 days the swab with specimens from all days. On 8 days, swabs with specimens from individual sites contained HSV DNA, while the swab with specimens from all three sites contained HSV DNA, whereas the swabs with specimens from individual sites were negative for HSV DNA. Of the 13 days on which discrepant results were obtained, for 4 days PCR product at <10 copies in the positive sample was involved.

Table 3 evaluates the titers of HSV in samples from 143 days in which both mixed and individual swab specimens were collected. The concordance between the two collection methods was again excellent. On 112 of 143 collection days the titers for the swabs containing specimens from all three sites were identical to the highest titer for a swab specimen from an individual site, and on only 5 of 143 collection days was there >1 log difference between the titers obtained by the two collection methods.

**DISCUSSION**

This study extends current methods of accurately quantitating HSV DNA levels in swab specimens. We have developed a QC PCR method which can be used to quantitate HSV levels over a large linear range and which more completely controls dilution of each other (1 log) being present in 61 of 63 specimens. For both of the other two specimens, QC PCR showed the copy number to be 2 logs higher than that by the dilution method.

**Comparison of single-site versus multiple-site swab specimens.** We then compared the accuracy of detecting HSV DNA from a swab containing secretions from all three anatomic sites with that of detecting HSV DNA from the swabs containing secretions from the individual sites. The results for the detection of HSV DNA were concordant between the two collection methods on 91% of the days (Table 2). Both collection methods were negative for HSV DNA on 80 days and positive on 50 days. On 8 days, swabs with specimens from individual sites contained HSV DNA, while the swab with specimens from all three sites did not, and on 5 days the swab with specimens from all three sites contained HSV DNA, whereas the swabs with specimens from individual sites were negative for HSV DNA. Of the 13 days on which discrepant results were obtained, for 4 days PCR product at <10 copies in the positive sample was involved.

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**TABLE 1. Comparison of QC PCR with dilution titers for HSV-2 DNA in the genital tract**

<table>
<thead>
<tr>
<th>Titer by QC PCR</th>
<th>No. of samples with the following titers by dilution:</th>
<th>% Discrepant results</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>$10^3$</td>
<td>$10^4$</td>
</tr>
<tr>
<td>$10^3$</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>$10^4$</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>$10^5$</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>$10^6$</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>$10^7$</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>1</td>
<td>13</td>
</tr>
</tbody>
</table>

*a* Boldface numbers indicate equivalence.

**TABLE 2. Comparison of samples containing secretions from all three sites and samples containing secretions from individual sites for detection of HSV-2 DNA in the genital tract**

<table>
<thead>
<tr>
<th>Result for specimen</th>
<th>No. of days with the following result for individual specimens:</th>
<th>% Discrepant results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>All negative</td>
<td>Positive at one or more site</td>
</tr>
<tr>
<td>Negative</td>
<td>80</td>
<td>8<em>a</em></td>
</tr>
<tr>
<td>Positive</td>
<td>5<em>a</em></td>
<td>50</td>
</tr>
</tbody>
</table>

*a* On 4 of these 13 days, trace amounts of PCR product which do not amplify reproducibly were involved.

for the difference in amplification variability seen with diverse clinical specimens. Quantitation of HSV DNA levels in clinical specimens is particularly helpful in the evaluation of the response to antiviral chemotherapy (5). When subjects have clinical lesions, HSV DNA is detected by PCR in high copy numbers. In this setting, the QC PCR offers advantages because of the large linear range and could be a useful tool for evaluating the rapidity with which antiviral agents influence HSV clearance from mucosal sites.

Another point of interest in our study was the high concordance of the results for swabs with secretions collected from several anatomic areas compared to those for swabs with secretions taken from individual sites. Our subject collected samples very systematically. Our subject obtained all samples upon awakening in an established order and after a detailed instruction period. Sample collection is an important aspect in evaluating any diagnostic assay for HSV infection (9). Both the frequency of detection and the titer of virus obtained were similar between swabs containing specimens from all three sites and those containing specimens from a single site. Differences of >1 log in the amounts of virus were seen in only 5 of 143 specimens. Thus, it appears that sampling of sites collectively may be as accurate a means of detecting the presence of HSV DNA in the genital tract on a single day compared to sampling of individual sites. This is an important advance for studies of systemic antiviral agents.

In summary, we have described a QC PCR assay which accurately quantitates HSV DNA levels. While this assay is labor-intensive, it may be useful in research settings. Moreover, it appears that a single swab specimen with secretions taken from all three genital sites and evaluated in a QC PCR format can qualitatively and quantitatively establish the frequency and titer of subclinical and clinical shedding of HSV in the genital tract.

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**REFERENCES**
