

Rapid Detection and Typing of Herpes Simplex Virus DNA in Clinical Specimens by the Hybrid Capture II Signal Amplification Probe Test

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A second-generation signal amplification, nucleic acid-based test for the rapid detection and typing of herpes simplex virus (HSV) DNA was developed and evaluated with artificial and clinical specimens. The analytical sensitivity of the Hybrid Capture II (HC II) HSV DNA assay was determined by testing either cloned HSV DNA or total genomic HSV DNA titrations and resulted in detection thresholds of between 5×10^3 and 1×10^4 copies per assay. Specificity was assessed by testing a panel of bacteria and viruses commonly found in the female genital tract. Sensitivity was assessed by testing 112 ulcerative genital lesions by the HC II assay and comparing the results to those obtained by routine cell culture. Discrepant results were resolved by PCR testing. After resolution of the discrepant results, the sensitivity of the HC II assay compared to the consensus result (the results of two of three tests, the HC II assay, culture, and PCR, were in agreement) was 93.2% (41 of 44 specimens), and the specificity was 100% (60 of 60 specimens). Culture gave a sensitivity of 84.1% (37 of 44 specimens) and a specificity of 100% (60 of 60 specimens) compared to the consensus result. The results of HSV typing by the HC II assay and culture agreed in all cases. The HC II assay is a rapid and accurate assay for detecting and typing HSV types 1 and 2, with a sensitivity comparable to that of culture and greater ease of use than culture.

Herpes simplex virus (HSV) infects mucocutaneous sites, causing both symptomatic and asymptomatic infections and subsequent latent infection of nerve cells. Symptomatic HSV infections have diverse clinical presentations with symptoms which overlap the symptoms of other infections. Therefore, laboratory diagnosis of HSV is required for patients with symptomatic cases of infection and also for certain asymptomatic individuals.

The "gold standard" for detecting HSV in routine clinical laboratories is viral culture in susceptible cells. With the exception of PCR, cell culture is considered to be the most sensitive test available. Culture detects most cases of HSV infection after 2 to 3 days. However, cases of low-titer HSV infection, such as in asymptomatic individuals who are shedding virus or who have very early- or late-stage lesions, require cultures to be routinely held for 7 days (8). Confirmation of HSV culture results and discrimination between HSV type 1 (HSV-1) and HSV-2 is usually performed by immunofluorescence staining with monoclonal antibodies.

PCR testing can detect asymptomatic HSV infection, but it may be problematic as a routine screening assay because it is subject to contamination and variations in sensitivity (2, 15, 29, 30). PCR may also detect clinically insignificant levels of HSV DNA (7). Quantitation of HSV is important for some applications, such as monitoring patient therapy and for new drug discovery research, and PCR is not easily performed in a quantitative fashion with crude specimens (3, 28) from the anogenital tract. Therefore, a simple, rapid, sensitive, and quantitative diagnostic method for HSV detection and typing is needed. We adapted the Hybrid Capture System signal amplification DNA detection technology to meet this need by cloning and

characterizing HSV DNA sequences for use as probes and optimizing their use in the Hybrid Capture System. The resulting Hybrid Capture II (HC II) test is a second-generation, rapid, sensitive method for detecting and typing HSV in a single day.

The HSV HC II was formulated to detect both HSV-1 and HSV-2 in the initial test, with subsequent typing of the viruses accomplished by using separate probe cocktails derived from HSV-1 and HSV-2 genomic DNA. The probe cocktails are not completely type specific, but through the use of stringent hybridization conditions and comparative analyses of the results obtained with each probe, individual specimens can be typed accurately. In this study, we evaluated the performance characteristics of the assay HC II and applied it to the detection and typing of HSV in a symptomatic population. (Digene is willing to make HC II assay reagents available to investigators who are interested in repeating this work or in performing similar, pertinent experiments.)

MATERIALS AND METHODS

Genomic DNA. Purified HSV-1 Macintyre and HSV-2 G were purchased from Advanced Biotechnologies Inc. (Columbia, Md.). The virions were propagated in Vero cells, the cells were lysed and treated with DNase and RNase, and the virions were purified by sucrose density gradient centrifugation. Preparations were negatively stained and virions were quantitated by electron microscopy. Purified virions were then treated at Digene with sodium dodecyl sulfate and then proteinase K digestion followed by phenol-chloroform extraction and ethanol precipitation, and the resulting genomic DNA was resuspended in 0.4 ml of 10 mM Tris-1 mM EDTA (pH 8.0) (17).

Cloning and transcription. The initial phase of the project was the cloning of specific portions of the HSV-1 and HSV-2 genomes for use as templates to generate the RNA probes to be used in the HC II assay. The HSV genome is 145 kb; therefore, a full-length genomic HSV probe cannot be used in the HC II assay. The goal was to produce multiple, unique clones of 8 to 10 kb each such that probe cocktails consisting of greater than 20 kb could be made for each HSV type. The vector and viral DNAs were prepared for ligation by digestion with restriction enzymes (Life Technologies, Gaithersburg, Md.) according to the manufacturer's instructions. DNAs were digested with either *EcoRI*, *HindIII*, or *EcoRI-HindIII*. Vector DNAs were then treated with calf intestinal alkaline phosphatase (Promega, Madison, Wis.) according to the manufacturer's instruc-

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tions. Digested viral DNAs were ligated into their respective prepared vectors by using T4 DNA ligase (Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's protocol. Ligations were transformed into MAX Efficiency DH5 Alpha Competent Cells (Life Technologies). Transformants were grown on agar plates containing 100 µg of ampicillin per ml, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, and isopropyl-β-D-thiogalactopyranoside (Digene Corporation, Beltsville, Md.). White colonies were inoculated into Luria broth containing 100 µg of ampicillin per ml, and the mixture was shaken overnight in a 37°C incubator. The recombinant plasmid DNA was isolated by standard isolation techniques (25) and further characterized by restriction digestion to confirm the presence of the desired insert (25). Clones with inserts ranging from 3 to 15 kb were selected for probe production. Probes were prepared by *in vitro* transcription by using T7 RNA polymerase (Pharmacia Biotech).

Cross-reactivity testing. Thirty-four microorganisms (1×10^7 bacterial organisms/ml) and 19 DNAs and viruses (2×10^7 genomes/ml), many of which are commonly found in the female anogenital tract, have been tested for cross-reactivity to the combined probe for HSV-1 and HSV-2 in the HC II assay. The 34 microorganisms included the following: two species of *Acinetobacter*, two species of *Bacteroides*, three species of *Chlamydia*, *Candida albicans*, two isolates of *Escherichia coli*, *Enterobacter cloacae*, *Fusobacterium nucleatum*, *Gardnerella vaginalis*, *Haemophilus ducreyi*, *Klebsiella pneumoniae*, *Lactobacillus acidophilus*, two species of *Mobiluncus*, 2 species of *Mycoplasma*, 4 species of *Neisseria*, *Peptostreptococcus anaerobius*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Serratia marcescens*, *Staphylococcus aureus* Cowan, two species of *Streptococcus*, *Treponema pallidum*, *Trichomonas vaginalis*, and *Ureaplasma urealyticum*. The viral DNAs tested included those from adenovirus, cytomegalovirus, Epstein-Barr virus, human herpes virus 6, varicella-zoster virus, simian virus 40, hepatitis B virus, human immunodeficiency virus, and several different types of human papillomavirus.

Testing detection limits of the HC II assay. The detection limits of the HC II assays were tested by using target DNAs from two sources: purified genomic DNA and clonal DNA from the individual plasmid clones of HSV (both methods of DNA preparation as described above). DNA was quantitated by spectral analysis and was used to prepare a dilution series of genomic or clonal DNA targets in Hybrid Capture System Negative Control (100 µg of sonicated herring sperm DNA per ml in Digene Sample Transport Medium). The clonal DNAs were recombinant in equimolar ratios and dilutions were made to be equivalent to the number of copies of the HSV genome. Both of these target titrations were used to test the sensitivities of the assays.

Clinical specimens. The HC II assay was evaluated with a panel of clinical specimens from patients for routine HSV culture. All patients gave written informed consent to donate a second lesional swab for the study. Specimens were collected from ulcerative lesions of men and women as follows: open sores, fissures, or lesions were rubbed vigorously with a swab which was processed by the laboratory's routine method for HSV cell culture, and then the same lesion was rubbed vigorously with a second Dacron swab, which was then immediately placed in Digene Sample Transport Medium. Vesicular lesions were opened to reveal the parabasal cells, which were then sampled as described above. HSV cell culture was performed with all specimens in MRC-5 cells for 2 to 3 days (9), and then HSV was detected by an enzyme-linked immunosorbent assay with centrifuged cultures. For those specimens that were culture positive, fluorescein isothiocyanate-conjugated antibodies specific for HSV-1 and HSV-2 were used to determine HSV type. Specimens for analysis by the HC II assay were stored at 2 to 8°C for up to 2 weeks and were subsequently frozen. Specimens were shipped at ambient temperature to Digene for analysis by the HC II assay. All HC II assays were performed without knowledge of the culture results.

Hybrid capture method. For testing by the HC II assay, 50 µl of specimen was denatured by base denaturation at 65°C for 45 min and was then hybridized in solution at 65°C for 2 h with RNA probes specific for regions of the target DNA. The resulting 100-µl hybridization mixtures were transferred to a 96-well microplate and RNA-DNA hybrids were captured with specific antibodies (14, 27) for 1 h with shaking. Alkaline phosphatase-conjugated antibodies specific for the hybrids (32) were added, and the mixture was incubated at room temperature for 30 min. Specifically bound conjugate was detected by the addition of a chemiluminescent substrate (1, 4) and reading on a plate luminometer.

PCR. Primers for PCR (24) were from the HSV DNA polymerase gene and could not distinguish between HSV-1 and HSV-2 (13, 21). One primer was biotinylated, and a probe sequence internal to the primers was chosen for subsequent detection. Amplification of HSV DNA with these primers produced an amplicon of 476 bp. PCR was performed in a 100-µl volume containing 10 µl of clinical sample, 50 pmol of each primer, 2.5 mM MgCl₂, and 2.5 U of *Taq* DNA polymerase (AmpliTaq; Perkin-Elmer Cetus, Norwalk, Conn.). Amplification was performed with the Perkin-Elmer 9600 thermocycler by using 40 cycles, as follows: denaturation for 1 min at 94°C, primer annealing for 30 s at 65°C, and extension for 1 min at 72°C. The PCR products were then analyzed by the SHARP Signal System (Digene Corporation) according to the manufacturer's instructions (16). For quality control of the PCR and subsequent detection, 100 genome equivalents of HSV-1 genomic DNA was used as a control target in a PCR. The PCR was valid if this amount of target was detectable by the SHARP Signal System by an overnight incubation with the colorimetric substrate.

RESULTS

More than 60 recombinant colonies were isolated by the cloning procedure. Of these, 28 were selected for probe production on the basis of an appropriate insert size (3 to 15 kb). Nine probes were then qualified for use in the HC II assay on the basis of performance characteristics such as efficient growth of transformed bacteria, adequate plasmid yield, full-length transcription from DNA, and low probe background level when used in the HC II assay. Four probes that totaled 19.8 kb and that met these criteria were isolated for HSV-1 and 5 probes that totaled 20.9 kb and that met these criteria were isolated for HSV-2. These probes were then combined into three different probe cocktails: one that consisted of HSV-1 clones only, one that consisted of HSV-2 clones only, and one that contained all probes for HSV-1 and HSV-2.

The detection limits of the HSV-1 and HSV-2 HC II assays were between 1×10^5 and 2×10^5 virions/ml or 5×10^3 to 1×10^4 virions/assay. The results demonstrate a linear dose-response over a 3-log dynamic range (data not shown).

None of the battery of 34 microorganisms and 19 DNAs tested for cross-reactivity were positive when tested with the HSV-1 and HSV-2 combined detection system. This demonstrates the excellent specificity of the probes selected, since none of the other herpesvirus DNAs tested were recognized by the probes.

One-hundred twelve clinical specimens were tested at Digene by all three HC II HSV assays. Eight specimens were excluded from analysis because culture results were not available. The HC II assay cutoff used to analyze the data was chosen on the basis of previous experience with other Hybrid Capture assays (26, 31). The cutoff was calculated by multiplying the mean relative light unit (RLU) value of the negative control by 2. The RLUs for each specimen were then divided by the cutoff to give a ratio of RLU/cutoff. Ratios of ≥ 1.0 are considered positive in the assay, and those of < 1.0 are considered negative. A frequency distribution analysis of these data demonstrates a clear separation of culture-negative and -positive specimens by the HC II assay and indicates that the assay cutoff is midway between the values for the two populations (data not shown).

HC II correctly scored 34 of the 37 (91.9%) culture-positive specimens and 60 of the 67 (89.6%) culture-negative samples. Statistical analysis of these data by McNemar's paired test gave a two-sided *P* value of 0.3428, indicating that there was not a statistically significant difference in sensitivity between the two assays. All 16 HSV-1 culture-positive specimens were correctly typed by the HC II assay. Of the 21 HSV-2 culture-positive specimens, 18 were correctly typed and 3 were not detected. The 10 specimens with discrepant results between the HC II assay and culture and an equal number of negative specimens were amplified by PCR. The seven specimens that were negative by culture but positive by the HC II assay were confirmed to be positive by PCR. The three specimens that were positive by culture and negative by the HC II assay were also confirmed to be positive by PCR (Table 1). Therefore, the sensitivity of the HC II assay compared to the adjudicated consensus result (culture, HCII, and PCR) was 93.2% (41 of 44 specimens), and the specificity was 100% (60 of 60 specimens) (Table 1). The culture result compared to the consensus result gave a sensitivity of 84.1% (37 of 44 specimens) and a specificity of 100% (60 of 60 specimens) (Table 1). The kappa values for the HC II assay indicate a stronger correlation between the HC II test result and the consensus result than those for culture. The Fisher exact test was then performed with the data set, and the calculated two-sided *P* values were < 0.0001 . These *P* values

TABLE 1. Comparative analysis of HC II HSV DNA assay and culture results to consensus result^a

Assay	Sensitivity		Specificity		Agreement		Kappa statistic
	Percent (no. of specimens positive/no. tested)	95% confidence interval	Percent (no. of specimens positive/no. tested)	95% confidence interval	Percent (no. of specimens positive/no. tested)	95% confidence interval	
HC II	93.2 (41/44)	81.3–98.6	100 (60/60)	94.0–100	97.1 (101/104)	91.8–99.4	0.940
Culture	84.1 (37/44)	69.9–93.4	100 (60/60)	94.0–100	93.3 (97/104)	86.6–97.3	0.859

^a The consensus result was the result of two of three tests (HC II assay, culture, and PCR) in agreement. The *P* value was calculated by the Fisher exact test to be <0.0001 for both the HC II assay and culture compared to the consensus result.

demonstrate an extremely significant association for both HC II assay and culture results to the consensus result.

The seven specimens shown to be culture negative but HC II assay and PCR positive were further analyzed on the basis of clinical findings. Of the seven specimens, clinical information was readily available for six. Of these, four were HSV-1 positive and two were HSV-2 positive according to typing by the HC II assay. The clinical findings for the four HSV-1-positive patients were as follows: one had a recurrent cold sore on the lip, one had an atypical, but probable, cold sore on the lip, one had genital ulcers not typical of herpes, and one had no lesions but generalized symptoms suggestive of herpes. The HC II assay quantitation associated with these four specimens showed that the first and third specimens had low levels of viral DNA, whereas the second specimen had an intermediate load and the fourth specimen had a high load. Interestingly, both of the HSV-2-positive specimens were from individuals with definitive recurrent genital herpes outbreaks. For both individuals, two separate specimens had been taken for the study. In one case, one specimen was positive by both culture and the HC II assay, whereas the other was culture negative and a high-level positive by the HC II assay. In the second case, both specimens were culture negative, while one was HC II assay negative and the other was a very low level positive by the HC II assay.

A comparison of the HSV-1 and -2 typing assay results to the combined HSV-1 and -2 assay results showed the reproducible quantitative nature of the assay. Regression analysis indicates that the two assays perform very similarly, with a slope of 1.05, an *r*² value of 0.98, and a *y* intercept of 0.124.

Also of interest is the comparative analysis of the RLU/cutoff ratio values for each sample. The HSV-1 typing data for each sample was plotted versus the HSV-2 typing data. The HSV type of each culture positive specimen was assigned on the basis of monoclonal antibody typing performed with the culture. There were no cases of double infection according to the antibody typing result. To determine the HSV type by the HC II assay, each specimen was tested with both typing probes and the results were compared. The correct type was indicated by the probe producing the highest RLU/cutoff ratio value. This analysis indicates that cross-reactivity between HSV types was significant; however, the types were distinguishable, with approximately 10-fold resolution between HSV-1 and HSV-2 RLU/cutoff ratio values. In this small data set, there were no ambiguous typing results.

DISCUSSION

The data presented here describe the development and evaluation of prototype assays for the detection and typing of HSV-1 and HSV-2 from clinical specimens. The HC II test was demonstrated to be comparable in sensitivity and specificity to culture for HSV. Of note, the time to results for the HC II

assay was approximately 6 h, whereas it takes 3 to 7 days to obtain routine culture results.

The detection limit of the current HC II assay determined in a model system is approximately 1×10^5 genomes/ml, or 5×10^3 genomes per well with a specimen volume of 50 μ l. There are several possible explanations for the three false-negative results by the HC II assay. It is possible that unequal sampling played a role in these cases, because the specimens used for culture were always taken first, possibly removing all or most of the HSV in the first swab. It has been well documented that sample adequacy influences diagnostic test results (10). Future studies should address this concern by randomizing the collection of each type of specimen. It is also interesting that these three specimens were all HSV-2 positive. The limit of detection data generated for HSV-2 showed that this probe cocktail is less sensitive than the probe for HSV-1. In order to improve the sensitivity of the assays, additional probes, particularly for HSV-2, could be incorporated into the probe cocktails. On the basis of experience with other hybrid capture systems, the addition of 10 to 15 kb of sequence to each probe cocktail would improve the sensitivity approximately two- to threefold.

PCR analysis and/or review of the patient's history confirmed the HC II assay results for the seven HC II assay-positive, culture-negative specimens, suggesting that the HC II assay may be slightly more sensitive than culture. Sensitivity appeared to be the main factor for three of the seven specimens on the basis of the low RLUs in the HC II test. However, for the other four specimens, the RLUs by the HC II test were relatively high, suggesting either differences in sampling or inhibition of culture in these instances.

The ability of the HC II assay to accurately distinguish between HSV-1 and HSV-2 infections is important. Discrimination between types was approximately 10-fold (the signal generated for the correct HSV type was approximately 10-fold greater than the signal generated for the incorrect HSV type). Further optimization of the probe cocktails used in the HC II assay is possible and may result in additional discrimination between types. Although typing is usually not required in the clinical setting, knowledge of the HSV type may be useful in patient management since recurrences at individual sites are reported to differ by HSV type (18, 22).

The HC II assay offers an ability to quantitate the virus, providing useful information that other rapid, nonculture assays cannot provide. The reproducible quantitation by the different HC II assays was demonstrated in this study. The fact that the slope was close to 1 and the *y* intercept was only slightly shifted from the origin demonstrates that quantitation by the HSV-1 and HSV-2 combined probe cocktail and HSV-1 and HSV-2 typing are very similar, with the detection by the probe cocktail being slightly less sensitive due to the more complex nature of the probe mixture. Quantitation of HSV may be useful for some patient management applications such as monitoring of drug therapy for immunocompromised indi-

viduals (7). High virus titers require more aggressive treatment with acyclovir or other drugs. While acyclovir is a very effective treatment for HSV infections, there have been numerous reports of acyclovir-resistant HSV infections in patients with human immunodeficiency virus infection resulting from prolonged use of the drug (6, 11). Also, detection of continuing HSV infection in the presence of acyclovir treatment is necessary prior to initiation of alternative drug therapy, such as with foscarnet or vidarabine (23). The quantitative aspect could also be used in drug discovery research, in which rapid quantitation of HSV in the presence and absence of new drugs could aid in the search for better treatments for HSV disease (19).

Due to the severe manifestations of HSV infections in neonates, early diagnosis and treatment are vital for these patients. Since many HSV-1 and most primary HSV-2 infections are asymptomatic, women in labor and their physicians may not realize the threat of viral transmission to the unborn child. Most infants who develop neonatal HSV disease are born to women who are asymptomatic at the time of delivery and have no past history of infection (5, 20). The estimated rate of occurrence of perinatal HSV infection of neonates is 1 in 2,000 to 1 in 5,000 live births. This results in about 1,000 to 1,500 cases per year (33). Approximately 30% of the infections are caused by HSV-1 and are acquired postnatally. Pathological manifestations such as a lesion may occur by days 2 to 10 after delivery in 60% of neonates (12). Due to the short duration of viral shedding, cultures of samples obtained predelivery are typically not predictive of HSV shedding during delivery. A simple and rapid screening assay performed shortly before or after birth would be required for the timely initiation of treatment for neonates. The test of choice should be sensitive enough to detect virus in asymptomatic virus-shedding women and should have a high predictive value for HSV disease in infants, with results available in less than 5 h. Future research will focus on further optimization of the HC II assay for detecting and typing HSV to meet the need for detection of HSV in asymptomatic populations.

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