Laboratory Diagnosis of Central Nervous System Infections with Herpes Simplex Virus by PCR Performed with Cerebrospinal Fluid Specimens

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Until recently, the laboratory diagnosis of central nervous system (CNS) infections with herpes simplex virus (HSV) has been limited by poor sensitivity and/or specificity. We assessed the diagnostic utility of PCR for detection of HSV in over 2,100 specimens referred to the Mayo Clinic from August 1993 to May 1996. DNA extracted from cerebrospinal fluid (CSF) samples with IsoQuick was amplified by PCR with oligonucleotide primers directed to the DNA polymerase gene of HSV, yielding a 290-bp amplicon. HSV DNA was detected in 150 (135 by gel electrophoresis, 15 by Southern blotting only) of 2,106 (7.1%) specimens. PCR-positive CNS disease occurred in patients ranging in age from 13 days to 89 years; 59% of the cases occurred in patients between the ages of 30 and 69, and 21 (14%) of the patients were infants. Genotype analysis was not routinely performed; however, amplification of a 335-bp product within the thymidine kinase gene of HSV revealed 13 positions within a span of 80 nucleotides that accurately identified the two serotypes of the virus according to 14 reference strains. We conclude that PCR detection of HSV DNA in CSF specimens should be considered an emerging "gold standard" for the laboratory diagnosis of CNS infections with this virus.

Herpes simplex virus (HSV) is the most common cause of sporadic focal central nervous system (CNS) disease; without prompt antiviral therapy with acyclovir, the mortality rate may be as high as 70% (25, 26). HSV is the most frequently detected virus in the clinical virology laboratory; however, the virus is rarely recovered from cerebrospinal fluid (CSF) except from infants with neonatal CNS disease or from other patients with meningitis (24). Although isolation of the virus from brain biopsy tissue is considered the definitive laboratory diagnostic test for CNS infection due to HSV, collection of this specimen for microbial diagnosis is very uncommon in routine medical practice. Immunologic analyses of CSF for the presence of specific antigens or antibodies to HSV and recovery of the virus from this source has not yielded either sensitive or rapid tests for the laboratory diagnosis of HSV-related CNS disease (9).

In 1990, Rowley et al. demonstrated detection of specific HSV gene sequences by PCR in CSF samples from four patients with brain tissue-proven HSV encephalitis (18). Importantly, CSF specimens from six other patients with other documented causes of infectious encephalitis were negative for HSV DNA by PCR. This seminal report led to several publications that corroborated the high sensitivity and specificity of PCR compared with brain biopsy or immunologic diagnosis of HSV encephalitis (1, 22). Most recently, in a collaborative study, HSV nucleic acid sequences were detected in CSF samples from 53 of 54 patients with biopsy-proven and clinically correlated HSV encephalitis (sensitivity, 98%) and in 3 (6%) of 47 patients for whom brain tissue was culture negative (13). In the United Kingdom, CNS disease due to HSV was detected by PCR in 26 of 143 patients (18%); most cases reported were due to enterovirus (54%) (16). Taken together, these studies suggest that PCR detection of HSV DNA in CSF should be

considered the new standard for laboratory diagnosis of CNS disease caused by this virus (13, 16).

In this report, we describe detection of HSV DNA in CSF of 150 (7.1%) of 2,106 specimens submitted to a clinical reference laboratory over a 3-year period from August 1993 to May 1996. We also describe the direct determination of the HSV genotype from the CSF by restriction fragment length polymorphism analysis and direct DNA sequencing.

MATERIALS AND METHODS

Study population. PCR for HSV DNA was conducted with 2,106 consecutive CSF specimens submitted to the clinical reference laboratory between August 1993 to May 1996. Demographic information was available for 139 (93%) of 150 patients in which HSV DNA was detected in the CSF, and clinical information was available from 59 (39%) patients. In addition, the medical records of 117 consecutive Mayo Clinic, Rochester, Minn., patients whose CSF specimens were submitted to the clinical laboratory for detection of HSV DNA were evaluated between January 1995 and May 1996. These records were reviewed for demographic, clinical, and outcome data by one of the authors after the PCR results were available.

CSF samples. A total volume of at least 0.5 ml of CSF was collected from each patient by lumbar puncture. In the laboratory, 200 μl of CSF was added to each of two microcentrifuge tubes. CSF specimens were added directly (*n* = 100) or boiled and then added (*n* = 540), or they were extracted with IsoQuick (Orca Research, Inc., Bothell, Wash.) (*n* = 1,466). Twenty micrograms of glycogen was added to each extracted sample as a carrier during the isopropanol precipitation step by the IsoQuick technique. After extraction, separation of the aqueous phase, and precipitation of the nucleic acid with isopropanol, 70% ethanol was added, and the sample was centrifuged at 12,000 × *g* for 5 min. Extracted nucleic acids were suspended in water, and 5 μl was added to 45 μl of PCR master mix as previously described (6). Isopsoralen compound 10 (HRI Associates, Concord, Calif.) was incorporated into all reaction mixtures to control the effects of amplicon contamination (7, 20). Preparation of the master mix, specimen extraction and addition, and analysis steps were performed in separate air-controlled rooms. Similarly, the technical precautions recommended by Kwok and Higuchi were strictly followed (12).

PCR. Two reaction mixtures for each specimen were placed in a thermal cycler programmed for a three-step protocol: 2 min at 94°C for one cycle and then 1 min of denaturation at 94°C, 1 min of annealing at 60°C, and 1 min of extension at 72°C for 60 cycles. After the PCR program was completed, the tubes were placed in an HRI-300 UV photochemical reaction chamber for 15 min at 4°C to inactivate amplified DNA. The 290-bp amplicon was detected by gel electro-

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TABLE 1. Characteristics of the primers used in this study

Gene target	GenBank accession no.	Primer (nucleotide position)	Sequence (5′→3′)
DNA polymerase	M16321	HSV-J (2836–2857)	TAC ATC GGC GTC ATC TGC GGG G]−Primer
		HSV-K (3104–3125)	AGT TCG GCG GT G AGG ACA AAG T
		HSV-3 (2870–2891)	TCA AGG GCG TGG ATC TGG TGC G]−Probe
		HSV-4 (3064–3085)	GGT CGG TGA TGC GCC GAT GGG C
DNA polymerase	X04771	HSV (1558–1579)	ATG GTG AAC ATC GAC ATG TAC GG
		HSV-1 469 (2004–2026)	CCT CGC GTT CGT CCT CGT CCT CC
		HSV-2	CCT CCT TGT CGA GGC CCC GAA AC
TK	X03764 (type 1)	TK-A (631–650)	GAC MAG CGC CCA GAT AAC AA
	X01712 (type 2)	TK-B (966–985)	MCA GCA TRG CCA GGT CAA GC
	Type 1	TK-E (sequencing primer)	AGG CGG TCG ATG TGT CTG TC
	Type 2	TK-F (sequencing primer)	AGG CGG TCG GCG TGT CGC GC

phoresis (3%; 2% NuSieve–1% SeaKem [FMC, Rockland, Maine]) (Table 1 and Fig. 1). PCR products were transferred to a nylon membrane (Nytran; Schleicher and Schuell) and detected by Southern blotting with an enhanced chemiluminescence (ECL; Amersham, Arlington Heights, Ill.)-labeled 216-bp probe (Table 1) (primers [nucleotide sequences 5′ to 3′], TCA AGG GCG TGG ATC TGG TGC G and GGT CGG TGA TGC GCC GAT GGG C).

Controls. (i) Positive. DNA was extracted from HSV-infected cell cultures. Serial dilutions of this virus suspension were amplified by PCR. Products were electrophoresed, and Southern blotting with probe hybridization was performed. The highest dilution of PCR product that produced a detectable band by gel electrophoresis was selected for the strong positive control, and the highest dilution of PCR product that produced a weakly detectable band on gel electrophoresis was selected for the weak positive control. An aliquot of each control dilution was added to pooled CSF (see negative control) and processed as for patient specimens; this preparation was included in every test sample run.

(ii) Negative. CSF samples collected from patients without evidence of infectious disease were pooled. An aliquot of this CSF was extracted, amplified, and analyzed as for patient specimens.

Samples for HSV genotype analysis. Three methods were used for HSV strain identification by molecular methods (Table 1 and Fig. 1). (i) Reaction of the 290-bp amplicon (DNA polymerase gene) with a restriction endonuclease, *Ksp*I, resulted in two fragments (164 and 126 bp). Type 1 strains contain the restriction site, whereas type 2 strains are not cut by the enzyme. (ii) Allele-specific amplification of HSV DNA was performed with a common upstream primer and two type-specific downstream primers designed to produce amplicons with sizes of 469 bp (HSV-1) and 391 bp (HSV-2) (11). (iii) Direct nucleotide sequencing of the thymidine kinase (TK) locus was performed, which generated a 335-bp product which was then analyzed to determine nucleotide sequence information unique for HSV-1 and HSV-2.

Sequencing for the TK locus. Primers for the TK region were selected by comparative sequence alignment of HSV genomes comprising types 1 and 2. Regions conserved in both viruses were further analyzed with the Oligo software package, and selected primers were tested empirically to achieve the highest detection sensitivity. After considerable use, these primers were modified to be in better accord with the consensus sequence for HSV-1 and -2. The primers were used to amplify a 335-bp portion of the TK locus, followed by gel electrophoresis. Positive specimens were subjected to direct DNA sequencing for determination of the HSV genotype. Briefly, 6 μl of crude PCR mixture was digested for 30 min at 37°C with 1 U of shrimp alkaline phosphatase and 10 U of exonuclease I, followed by heat inactivation for 15 min at 80°C. This treatment eliminates excess deoxynucleotides and primers prior to standard dye termination cycle sequencing. Reactions were run on an ABI Prism 377 DNA sequencer. Sequence data files were analyzed with ABI Sequence Navigator software to determine HSV genotypes.

Assay methods ii and iii (above) were evaluated with 14 known strains of HSV (8 were HSV-1, and 6 were HSV-2) which were recovered in cell cultures. Of these strains, four HSV-1 isolates were tested for which acyclovir resistance had been determined (three were resistant, and one was susceptible). All of the reference HSV strains were antigenically serotyped with reference monoclonal antibodies (Syva, Palo Alto, Calif.). In addition, 10 CSF specimens from the study population, known to contain HSV, were reamplified with the mixture of common and type-specific primers. Nucleic acids from these specimens were extracted by the IsoQuick technique.

RESULTS

Among 2,106 CSF specimens tested, 150 (7.1%) were positive for HSV DNA. Over a two and one-half year period of time, test requests increased from an average of 23 to about 140 per month, but the trend in the rate of detection of HSV-DNA from CSF did not increase in proportion to the number of specimens submitted (Fig. 2).

Detection of HSV DNA was obtained with the CSF specimens of patients who ranged in age from 13 days to 89 years (Fig. 3). Fifty-nine percent of the positive specimens and 47% of the total samples with age data (1,855) were from patients between the ages of 30 to 69; however, 21 (14%) of the patients were infants (less than 1 year of age). Similarly, the rate of detection of HSV CNS disease (no. of cases/number of specimens) occurred in patients between the ages of 30 and 69 (87 of 880 [9.9%]) and in infants (21 of 262 [8.0%]). Seventy-four of the patients were male; 65 were female. For 11 patients, this information was not provided. Review of clinical information from 59 of 150 patients with HSV DNA in CSF specimens revealed 7 individuals who presented with Mollaret's meningitis, a recurrent form of CNS disease due to HSV infection.

HSV-specific DNA sequences were detected in the CSF of 4 (3.4%) of the 117 patients evaluated at the Mayo Clinic between January 1995 and May 1996 and whose CSF was submitted to the clinical laboratory for the detection of HSV DNA (Table 2). Three patients had a clinical syndrome compatible with HSV encephalitis and clinically improved with acyclovir therapy. One patient was diagnosed with aseptic meningitis and improved without specific antiviral therapy. Review of the medical records of the remaining 114 patients without detectable HSV sequences in the CSF revealed 10 patients with microbiologic evidence of CNS infection due to organisms
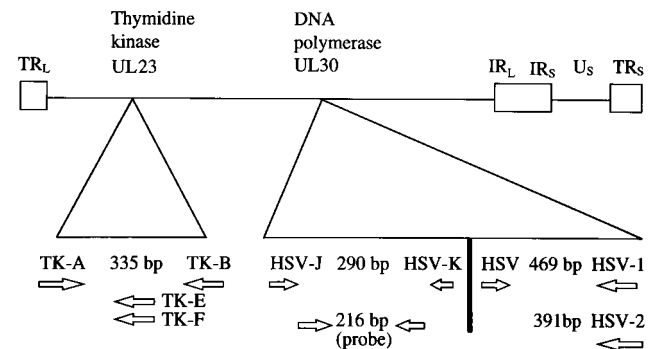

FIG. 1. Gene location and nucleotide base position of primers and probes used to detect HSV DNA. TR$_L$, terminal long repeat; IR$_L$ inverted long repeat; IR$_S$, inverted short repeat; TR$_S$, terminal short repeat; U$_S$, unique short region.
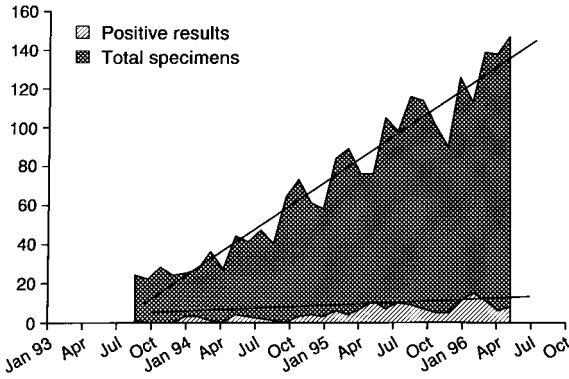
FIG. 2. CSF specimens and positive results for HSV DNA from August 1993 to May 1996.

other than HSV (Table 2). In addition, there were 10 patients with acute encephalitis in which no etiologic agent was identified despite extensive testing. One of these 10 patients had a clinical diagnosis compatible with HSV encephalitis and improved with acyclovir therapy. The three patients seen at our institution with a clinical diagnosis of Mollaret's meningitis did not have HSV-specific DNA sequences detected in the CSF. The remaining 84 patients had a wide variety of CNS disorders.

Three methods were used to process specimens for PCR. The first 100 specimens were analyzed without prior treatment. However, spike-back studies showed an unacceptably high rate of inhibition (10 to 15%). Boiling of the specimens was performed for the next 540 specimens, which reduced inhibition, but higher detection was ultimately shown to be achieved after chaotropic lysis and extraction. In a direct comparison of 93 CSF specimens processed by boiling and by IsoQuick extraction, 6 were positive for HSV DNA, of which 3 were detected exclusively by the IsoQuick technique. The remaining 1,466 analyses were performed by this method. All CSF specimens now submitted for HSV PCR analysis are extracted by the IsoQuick technique and amplified in duplicate.

PCR products were resolved by gel electrophoresis. After the amplification products were transferred to a nylon membrane by Southern blotting, the products were detected by a chemiluminescent probe. The copy level of HSV DNA was sufficiently high in 135 of 150 (90%) positive specimens to allow recognition of a distinct product of the expected molecular weight in both electrophoresis lanes. Ten (6.6%) and five (3.3%) CSF specimens were positive only by DNA probe anal-

TABLE 2. Results of PCR for the detection of HSV from CSF of 117 patients

Clinical diagnosis (n)	HSV PCR result
Encephalitis	
HSV (3)	+
Cytomegalovirus (2)	−
Mycoplasma spp. (2)	−
AIDS/human immunodeficiency virus related (2)	−
Epstein-Barr virus (1)	−
California encephalitis virus (1)	−
Unidentified encephalitis (10)[a]	−
Meningitis	
HSV (1)	+
Streptococcus agalactiae (1)	−
Tuberculous meningitis (1)	−
Mollaret's meningitis (3)	−
Aseptic meningitis (7)	−
Other neurologic disorders	
Cerebrovascular accident (11)	−
Seizure disorder (10)	−
Metabolic or toxic encephalopathy (11)	−
Associated with malignancy (9)	−
Multiple sclerosis (6)	−
Dementia (6)	−
Psychiatric disorder (4)	−
Headache disorder (3)	−
Idiopathic Bell's palsy (3)	−
Other (20)[b]	−

[a] One of the 10 patients had evidence of HSV encephalitis based on clinical presentation, compatible magnetic resonance imaging study, and response to parental acyclovir therapy.

[b] Acute confusional state (n = 2), CNS hemorrhage (n = 2), central nervous system vasculitis (n = 2), hydrocephalus (n = 1), relapsing inflammatory encephalopathy (n = 1), trigeminal trophic syndrome (n = 1), tuberosis sclerosis (n = 1), subacute demyelinating and necrotizing encephalitis (n = 1), Leigh's disease (n = 1), subdural empyema (n = 1), erythema multiforme (n = 1), thoracic radiculopathy (n = 1), transverse myelitis (n = 1), Creutzfeldt-Jakob disease (n = 1), cauda equina syndrome (n = 1), Cockayne syndrome (n = 1), undiagnosed neurologic illness (n = 1).

ysis in both lanes or one lane of duplicate determinations, respectively.

Only a single restriction enzyme (*Ksp*I) that could be used to differentiate the 290-bp amplicon generated by both HSV genotypes was identified. Recognition of type 2 strains could only be inferred by the lack of a restriction site; only 7 of 14 known serotype HSV strains were correctly identified by this method. In contrast, all were correctly identified by allele-specific amplifications with a commonsense primer with strain-specific, antisense primers yielding 469-bp (HSV-1) or 391-bp (HSV-2) amplicons (Fig. 1). Similarly, amplification of a 335-bp amplicon within the TK gene of HSV revealed 13 positions within a selected 80-nucleotide region which consistently differentiated HSV-1 from HSV-2 genotypes (Fig. 1). It should be noted that both the strain-specific primer (DNA polymerase; 469 and 391 bp) and sequence analysis (TK; 335 bp) required a separate reaction in addition to the initial specimen amplification.

DISCUSSION

Over the past several years, the laboratory diagnosis of CNS disease due to HSV has been facilitated greatly by PCR. The assay is rapid, sensitive, and specific enough to be used as a frontline test for the detection of HSV DNA, thus avoiding invasive and expensive brain biopsy specimen procedures which have been previously used as a benchmark for the laboratory diagnosis of HSV encephalitis. Early concerns about
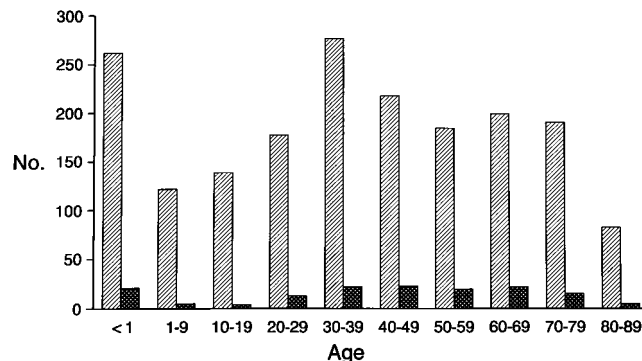

FIG. 3. Age distribution (years) of patients from whom CSF specimens were submitted for detection of HSV DNA sequences. Hatched (light) bars, negative patients; cross-hatched (dark) bars, positive patients.

the possibility that PCR would be too sensitive have proven to be largely unfounded; CSF specimens from patients without CNS infection do not commonly have detectable virus nucleic acids present in this fluid (1, 17). In addition, HSV DNA can be detected as early as 1 day after onset of clinical disease. The persistence of target DNA for an average of 14 days (range of 1 to 30 days) in CSF enhances the diagnostic sensitivity of this assay compared to that of HSV antigen or cell culture recovery of the virus, which have a narrower diagnostic window (8–10). HSV antibody testing of serum specimens may be useful in the acute-phase setting, but there is an age-dependent increase in baseline seroprevalence. This greatly diminishes the diagnostic value of seroconversion as an indicator of acute infection.

PCR is technically demanding, requiring the appropriate physical facilities for reagent preparation, specimen processing, and PCR product analysis to ensure that carryover contamination does not occur. Conditions for isopsoralen incorporation into the HSV PCR products were optimized in our laboratory, which we feel helped to minimize false-positive test results (5, 15). Appropriate positive and negative controls are integral for the accurate detection of HSV DNA in CSF specimens. An HSV reference strain, cultivated in cell culture, was adjusted to the highest limiting concentration that would still produce a detectable band by gel electrophoresis. Predilution of the positive control prior to use in the PCR laboratory may have also helped to avoid false-positive PCR results. Over a 3-year period during which this test was run, the positive control failed to produce a band by gel electrophoresis only six times. We determined that this was likely due to degradation of the positive controls after several freeze-thaw cycles and was not due to inhibition of the PCR.

Inhibition of the PCR by substances present in CSF samples is a technical concern. However, of 64 CSF samples extracted under our current protocol that were spiked with a limited number of copies of HSV DNA, only one reaction failed to amplify. These data have important implications if a laboratory intends to monitor specimen inhibition of PCR as a standard procedure. Similarly, 15 specimens from 150 patients gave negative results by gel electrophoresis and required Southern blot analysis for detection of HSV DNA. Extension of these data to our current experience of testing CSF from 260 HSV-positive specimens has revealed only 5 specimens that were detected in a single lane only by Southern blot analysis. Careful evaluation of these two procedural steps—PCR inhibition control and duplicate (two lanes) testing of each specimen by gel electrophoresis and Southern blotting—have important implications regarding control of laboratory costs for routine PCR assays. For example, spiking of each clinical CSF specimen with a limited number of copies of HSV DNA to monitor inhibitors of the PCR would double the technical workload, but increase the yield of HSV-positive results by only slightly more than 1% (1 of 64). Furthermore, since the rate of positivity for detection of HSV in CSF is approximately 7%, with the PCR assay there is a 93% likelihood that a true-negative result would occur. Thus, the introduction of DNA as an internal control at the time of specimen processing may introduce cross-contamination between samples unless the reference target produces an amplicon of different molecular weight than native DNA in the CSF. By the same rationale, assay of the DNA extract in only one lane of a gel with subsequent Southern blot analysis would reduce the technical workload by 50% and test performance sensitivity by less than 2% (5 of 260). Based on these data, we suggest single, rather than duplicate, gel and Southern blot analyses for routine testing purposes. Duplicate evaluation could be reserved for patients for whom treatment has been initiated or for whom prior results were negative.

It must be recognized that the CSF specimens in our study (n = 2,006 [95%]) were either boiled or extracted with Iso-Quick prior to amplification by PCR. These steps likely reduce or eliminate any inhibitors in CSF specimens. Conversely, untreated CSF specimens may inhibit PCR and therefore require the simultaneous processing of an inhibitor control with each reaction.

In our laboratory-based experience, almost three of four patients diagnosed with CNS disease due to HSV were either infants (14%) or adults between 30 and 69 years of age. In contrast to previous studies, we detected more cases of HSV disease in infants, but fewer cases in young children and teenagers. It is possible that the submission of CSF for the PCR assay, rather than brain biopsy specimens, increased the representation of HSV CNS disease detected in infants compared with those in other patient groups. In two studies of 166 and 53 patients, 31 and 17% of the patients with carefully diagnosed cases of infection, respectively, were less than 20 years of age. Only 6 to 10% were between 6 months and 10 years of age (23, 27).

With the increasing recognition that the laboratory diagnosis of HSV CNS disease can now be made with a CSF specimen rather than a brain biopsy, this test has grown in popularity. Our laboratory has observed a greater number of specimens (slope of trendline, 3.83) submitted over time relative to the number of positive specimens (slope, 0.35) (Fig. 2). These data suggest that physicians may consider a clinical diagnosis of HSV infection in patients that perhaps do not manifest clear-cut symptomatology of this disease (14). These patients can now be evaluated by a rapid laboratory assay requiring CSF instead of brain biopsy. Due to the availability of an accurate laboratory test utilizing CSF specimens, we anticipated that the clinical spectrum of HSV CNS disease might be expanded. Previously, severe disease was recognized diagnostically only by the processing of a brain biopsy specimen for virus culture. Indeed, at least seven cases of HSV-associated meningitis disease (Mollaret's meningitis) were recognized among 59 of 150 total patients from whom clinical information was obtained. The clinical manifestations of this disease are characterized by recurrent, self-limited episodes of aseptic meningitis. In these cases, the CNS episodes last only 2 to 5 days, and the transient neurologic abnormalities that may be present disappear after the acute illness. Of the seven CSF specimens from these patients, two samples (29%) required DNA probe detection to obtain a laboratory diagnosis. These observations suggest that the copy number of HSV DNA in the CSF of patients with mild, atypical, or recurrent infections may be lower than the level of viral DNA present in more severe clinical cases (4, 15, 17, 28). A low copy number of HSV DNA target in the CSF may explain the negative results for the three samples from Mollaret's meningitis patients seen at our institution. Alternatively, other pathogens may have been present.

Monoclonal antibodies to HSV-1 and -2 allow differentiation of these two strains of virus in cell cultures. Similarly, molecular methods, such as the use of restriction endonuclease enzymes or sequence analysis, can be used to identify HSV-1 or HSV-2 genotypes (3, 10, 21, 28). Application of this method to the 290-bp product in the DNA polymerase gene did not yield consistent HSV genotype results. Type-specific PCR can yield accurate genotypic information, but this method requires reamplification of the original specimen with unique primer sets or duplicate testing of the original specimens with two sets of PCR primers. We amplified a portion of the TK gene to confirm HSV PCR results and to obtain information regarding the virus genotype directly from the CSF specimens. In previous work, mutations in the TK gene have been associated with

resistance to the antiviral drug acyclovir (2, 5). In addition, mutations in this locus have been analyzed for possible recognition of neurotropic strains (19). Initial evaluation of the TK-specific primer set has shown at least comparable performance relative to the DNA polymerase primer set used in the clinical test (data not shown). A single test that includes amplification of this target followed by determination of type-specific sequence polymorphisms and other mutations would theoretically be able to predict both HSV genotype and drug resistance without the need for a second amplification. Rapid progress in automated DNA sequencing techniques will facilitate this approach.

Based on our data and routine diagnostic experience, we recommend, in agreement with Lakeman et al. and Read et al., that PCR detection of HSV DNA in CSF be considered the test of choice for diagnosis of this CNS infection (9, 16).

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