MINIREVIEW

Molecular Approaches To Detecting Herpes Simplex Virus and Enteroviruses in the Central Nervous System

Thomas W. Smalling,¹ Susan E. Sefers,¹ Haijing Li,² and Yi-Wei Tang^{1,2*}

*Departments of Pathology¹ and Medicine,² Vanderbilt University School of Medicine,
Nashville, Tennessee 37232*

The initial presentation of symptoms and clinical manifestations of viral central nervous system (CNS) infectious diseases often makes a specific diagnosis difficult and uncertain. For example, the clinical features of CNS disease due to non-polio enteroviruses (EV) can overlap those caused by herpes simplex virus (HSV), which sometimes occur in young infants and adults in the absence of cutaneous lesions (4, 12, 39). The etiological agent and type of infection can determine potential prognoses as well as optimal treatment modality for the patient. Enterovirus infections often do not require antiviral intervention, but at least two new antivirals are undergoing trials, the more prominent of which is pleconaril (16, 22). Also, if a mean time of 24 h to diagnosis was assumed, 1.2 to 2.8 days of hospitalization per patient could be saved over current averages (1, 13, 18, 26). Mortality from HSV, however, before the use of acyclovir was 60 to 70% and has been reported to drop to 19 to 38% with therapy (27, 38). Early diagnosis and treatment of viral CNS infections, therefore, will decrease morbidity and mortality rates.

Unfortunately, laboratory tests commonly used for the diagnosis of CNS infectious diseases have the disadvantage of either being nonsensitive and nonspecific or giving results difficult to distinguish from the norm. Laboratory findings, including analyses of cerebrospinal fluids (CSF) for cell count, glucose, and protein, may be difficult to assess for newborns and infants because they have normal ranges different from those typically seen for older children and adults (4). Antigen capture or detection assays are not suitable due to the extensive antigenic heterogeneity and lack of cross-reacting antigens (30). Antibody assays have been unsatisfactory for diagnoses and differentiation. Enteroviruses lack a common antigen among various serotypes, making immunoglobulin M levels inconsistent (18, 28, 30). The HSV antibodies in plasma cannot distinguish between a primary and secondary infection or between peripheral and CNS infections (25). Local production of HSV immunoglobulin G antibodies in CSF can be used in diagnosis; however, the presence of antibody is delayed from the start of infection to day 10 or 12 and peaks at about day 20 (25). The culture sensitivity for EV from CSF is only 65 to 75% and requires a mean growing time of 3.7 to 8.2 days (8, 21).

Some serotypes of enteroviruses, especially coxsackievirus A strains, grow very poorly in tissue culture or are unculturable (4). The HSV culture sensitivity from CSF is very poor, especially in HSV type 2 (HSV-2)-related recurrent cases (1). Culture results are usually not available before the patient is discharged; therefore, culturing has a minimal impact on the management of infants hospitalized to rule out meningitis (1, 8, 26).

With the advent of molecular diagnostic clinical procedures, it is now possible to diagnose viral etiologies specifically and with high sensitivity and accuracy. Of the molecular methods, PCR has proven to be much more sensitive than viral culture in numerous clinical studies in terms of EV and HSV detection in CSF (1, 9, 11, 14, 17, 20, 21, 23, 40). Besides high sensitivity, the quick turnaround time of this procedure makes it the optimal test for detecting enteroviruses (1, 10, 18, 26, 41). The application of PCR to the detection of HSV DNA and EV RNA in CSF is becoming a standard laboratory practice. In this minireview, we describe the technical aspects of the assays required for successful implementation of PCR for routine diagnostic testing.

SPECIMEN PROCESSING

Specimen sources, volume, and storage conditions. For patients suspected of HSV- and EV-caused encephalitis and/or meningitis, a CSF specimen should be submitted for PCR detection. The use of protein and leukocyte values to screen CSF specimens before employing PCR can allow these tests to be used more effectively by avoiding indiscriminate test ordering (9, 32). CSF, which is the specimen of choice for detecting HSV and EV in the diagnostic molecular laboratory, is collected by lumbar puncture and transported at 4°C in a sterile screw-top vial. Samples for HSV detection seem to be stable when stored at 4°C prior to analysis for days or even weeks (13), but RNA viruses such as EV are more labile and should be frozen at –70°C if not received in the lab within 24 h. Samples may be stored at –70°C for long-term storage; however, multiple freeze-thaw cycles should be avoided as freezing followed by thawing significantly reduces the number of viral copies in clinical samples (33, 34).

Specimens should be shipped, received, and processed in a manner that will not cause cross-contamination of samples (33, 34). Dedicated accessioning and aliquoting procedures should be established. Inappropriately collected specimens including

* Corresponding author. Mailing address: Molecular Infectious Disease Laboratory, 4605 TVC, Vanderbilt University Hospital, Nashville, TN 37232-5310. Phone: (615) 322-0126. Fax: (615) 343-8420. E-mail: yiwei.tang@vanderbilt.edu.

TABLE 1. DNA and RNA extraction procedures used at VUMC for CSF specimens^a

Step no.	Procedure	
	IsoQuick (HSV)	RNAzol B (EV)
1	Add 100 μ l of specimen to a 1.5-ml tube	Add 100- μ l of specimen to a 1.5-ml tube.
2	Add 100 μ l of lysing solution Mix. Incubate 5 min at room temperature.	Add 1,000 μ l of RNAzol B.
3	Add 700 μ l of extraction matrix.	Add 100 μ l chloroform. Mix. Incubate 5 min at 4°C.
4	Add 400 μ l of extraction buffer. Mix.	Spin at 12,000 $\times$ g for 15 min at 4°C.
5	Spin at 12,000 $\times$ g for 15 min at room temperature.	Transfer aqueous phase to a new tube ($\approx$ 500 μ l).
6	Transfer aqueous phase to a new tube ($\approx$ 400 μ l).	Add 2 μ l of pellet paint.
7	Add 40 μ l of sodium acetate.	Add 500 μ l of isopropanol. Mix. Incubate 15 min at 4°C.
8	Add 2 μ l of pellet paint.	Spin at 12,000 $\times$ g for 15 min at 4°C.
9	Add 440 μ l of isopropanol mix.	Aspirate supernatant.
10	Spin at 12,000 $\times$ g for 15 min at room temperature.	Add 1 ml of 70% ethanol to RNA pellet. Mix.
11	Aspirate supernatant.	Spin at 12,000 $\times$ g for 5 min at 4°C.
12	Add 1.0 ml of 70% ethanol cooled to 4°C to the DNA pellet. Mix.	Aspirate supernatant.
13	Spin at 12,000 $\times$ g for 5 min at room temperature.	Air dry RNA pellet at room temperature for 10 min.
14	Aspirate supernatant.	Add 55 μ l of sample diluent. Rehydrate pellet 30 min at 4°C.
15	Air dry DNA pellet at room temperature for 10 min.	
16	Add 25 μ l of RNase-free water. Rehydrate pellet for 30 min at room temperature.	

^a Manufacturer's instructions with minor modifications.

those that have peripheral blood contamination or overgrowth of bacteria may give false-negative results due to the presence of inhibiting factors.

Nucleic acid extraction techniques, comparison, and recommendation. Several commercial methods for the extraction of viral nucleic acid for amplification procedures have been described previously elsewhere (33). Ideally, such a method should isolate the nucleic acids, concentrate the isolate, and provide a pure product free of inhibitors. Inhibiting materials can be extracted along with the nucleic acids and can interfere with the amplification process, reducing efficiency and lowering the sensitivity of the PCR assay (19). In our laboratory, IsoQuick (ORCA Research Inc., Bothell, Wash.) and RNAzol-B (Leedo Medical Laboratories, Inc., Friendswood, Tex.) kits are used for the extraction of HSV DNA and EV RNA, respectively. IsoQuick, which utilizes the chaotropic qualities of guanidine thiocyanate, which disrupts cellular integrity and inhibits nucleases, detects as few as 1 to 10 copies of plasmid HSV DNA in 50 μ l of CSF (35). RNAzol-B kits use guanidinium thiocyanate-phenol extraction followed by ethanol precipitation (26, 31). Due to the inherent greater fragility of RNA, all specimens, all reagents except isopropanol, and RNA are kept at 4°C, and ice, bench-top coolers, and refrigerated centrifuges

are used during the extraction process. The extraction protocol, according to the manufacturer's instructions, with minor updates, is given in Table 1. No false-negative results due to "inhibitors" have been noticed on DNA or RNA extracts prepared by either the IsoQuick or RNAzol-B kit.

NUCLEIC ACID AMPLIFICATION

Target selection, comparison, and recommendation. For HSV, several viral genes, including thymidine kinase (TK), glycoprotein (gp) B, gpD, and DNA polymerase have been successfully selected as the targets for PCR amplification (11, 14, 17, 23, 35). Primers for HSV in our lab are selected from the TK genome (14, 35). This degenerate primer set amplified all HSV variants reported, including both HSV-1 and HSV-2 (Table 2). One advantage of choosing the TK gene is that the amplicon products may be used for detecting drug resistance-related point mutations by direct sequencing (5, 7). Enterovirus primers, which are ubiquitously aimed at the highly conserved 5' nontranslated region of the viral genome, detect 60 of the 67 enterovirus serotypes, including the serotypes most commonly associated with meningitis (1, 20, 21, 40). Detailed

TABLE 2. Primers and probes used for HSV and EV detection at VUMC

Primer or probe	Sequence for:	
	HSV	EV
Primers (5'-3')		
TK-A	GAC MAG CGC CCA GAT AAC AA ^a	
TK-B	MCA GCA TRG CCA GGT CAA GC ^a	
EV-1		ATT GTC ACC ATA AGC AGC CA
EV-2		GGC CCC TGA ATG CGG CTA AT
Probes (5'-3')		
TK-H (Type 1 specific)	ACA AAC ATC GTG TTG GGG GC ^b	
TK-G (Type 2 specific)	ACG AAC CTG GTC CTG GGT GT ^b	
EV-3		GAA ACA CGG ACA CCC AAA GTA ^b

^a M is A or C; R is A or G.

^b 5' biotinylation.

TABLE 3. PCR amplification set-up protocol used at VUMC^a

Stock reagents	HSV		EV	
	Vol (μl) added	Final concn	Vol (μl) added	Final concn
10× buffer II	5.0	1×		
MgCl ₂ (25 mM)	3.0	1.5 mM		
5× EZ buffer			5.0	1× ^a
dETP mix (1.25 mM for A, C, G; 0.625 mM for T; and 0.5625 mM for U)	8.0	A, C, G, 200 μM each; T, 100 μM; U, 90 μM		
dUTP mix (A, C, G, 10 mM, U, 9.5 mM)			1.5	A, C, G, 300 μM each; U, 285 μM
Digoxigenin 11-dUTP (1 mM)	0.5	10 μM	0.75	15 μM
Primer no. 1 (50 pmol/μl)	1.0	1 μM	0.5	0.5 μM
Primer no. 2 (50 pmol/μl)	1.0	1 μM	0.5	0.5 μM
50% glycerol	10.0	10%	7.0	18%
Ampli ^{Taq} gold DNA Polymerase (5 U/μl)	0.25	0.025 U/μl		
ABI <i>rTth</i> Polymerase			3.0	0.15 U/μl
HK UNG (1 U/μl)	0.5	0.01 U/μl	0.5	0.01 U/μl
RNase-free water	10.75		6.25	
Sample nucleic acids	10		25	
Total volume	50		50	

^a 25 μl of RNA extraction was eluted in 1× EN buffer.

information on primers and probes for HSV and EV are listed in Table 2.

Although a kit for detecting enterovirus was commercially available from Roche Molecular System, Inc. (the Amplicor Enterovirus test) (36, 41), Roche no longer produces this kit. Therefore, both HSV PCR and enteroviral reverse transcription (RT)-PCR usually follow a user-developed (“home brew”) protocol. We developed and validated a colorimetric microtiter plate PCR system for detecting both HSV (35) and EV. In the PCR amplification procedure, a special molecule, digoxigenin 11-dUTP, is incorporated into the amplicons for further identification. For EV detection, a *Tth* polymerase was used to obtain a one-step RT-PCR procedure (15, 31). A detailed setup protocol for both HSV PCR and EV RT-PCR is described in Table 3.

Thermal cycler programs. Components, reagents, and thermal cycling conditions can vary according to the instrument used. Our laboratory uses a program for each of the viral groups, which has been optimized for the Applied Biosystems GeneAmp System 9700. The approximate run times are 2.2 and 3 h for HSV PCR and EV RT-PCR, respectively (Table 4).

VISUALIZING AMPLIFICATION PRODUCTS

Amplicon identification and formats. Detection and identification of amplification products, or amplicons, has become a routine procedure in the molecular diagnostic laboratory. After amplification, the accumulated millions of target DNA copies are still invisible to the naked eye; therefore, an appropriate detection method is needed to visualize the amplified DNA molecules. Moreover, an additional identification system enhances the validity of the PCR amplification procedure, including both sensitivity and specificity. There are several methods that can be used for amplicon detection after the PCR is completed. The three main methods being used in clinical laboratories today are gel electrophoresis, Southern blotting with probe hybridization, and the enzyme-linked immunosorbent assay (ELISA) (21, 29, 35, 41). Several laboratories are

starting to convert their PCR assays to a real-time format, in which PCR amplification and amplicon identification happen simultaneously (6, 24, 37).

We have developed a PCR-ELISA (Roche Biochemicals, Indianapolis, Ind.)-based colorimetric microtiter plate (MTP) system for amplicon identification (31, 35). The procedure is simple and user-friendly, and the results can be obtained in 2 to 4 h. An additional antigen-antibody reaction was included as a signal amplification step, which significantly enhances test sensitivity.

Working conditions and concentrations. The amplicons are denatured and mixed with a hybridization solution containing a biotin-labeled DNA capture probe specific for the microorganism of interest. The mixtures are combined in wells of MTP modules, which are precoated with streptavidin and postcoated with a blocking reagent. The MTP is incubated at 37°C with gentle swirling for 1 to 3 h. The biotin-labeled probe hybridizes to the target, if present, and the biotin of the resulting complex binds to the streptavidin-coated bottom of the MTP well. Wash buffer is used to remove unreacted components, and a conjugate of antidigoxigenin and peroxidase is added to the well. After a 30-min incubation at 37°C with shaking, the plates are washed again and a substrate buffer containing ABTS (2,2'-azinobis(3-ethylbenzthiazolinesulfonic acid) is added. The MTPs are allowed to develop color during a 30-min incubation

TABLE 4. Thermal cycling profiles used at VUMC for HSV and EV amplification

Step no.	Thermal cycling conditions for:	
	EV	HSV
1	50°C for 5 min	50°C for 5 min
2	65°C for 30 min	
3	94°C for 3 min	95°C for 10 min
4	50 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 45 s	50 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 45 s
5	72°C for 7 min	72°C for 7 min
6	4°C soak	4°C soak

TABLE 5. Simplified working procedure for amplification product identification^a

Step no.	Procedure
1.....	Add hybridization solution to blank well.
2.....	Denature kit control, patient samples, and controls for 10 min at room temperature.
3.....	Prepare probe solutions and add to appropriate wells.
4.....	Add denatured amplicon to appropriate wells.
5.....	Cover with Mylar sheet and incubate with shaking for 3 h at 37°C.
6.....	Wash plate five times with wash buffer.
7.....	Add anti-digoxigenin-peroxidase.
8.....	Cover with Mylar sheet and incubate with shaking for 30 min at 37°C.
9.....	Wash plate five times with wash buffer.
10.....	Add ABTS solution.
11.....	Cover with Mylar sheet and incubate with shaking for 30 min at 37°C.
12.....	Read plate at dual wavelengths (405 and 490 nm) with blank correction.

^a Modified from manufacturer's instructions (Roche Biochemicals).

at 37°C with shaking and are read on a Microwell Plate Reader at 405 and 490 nm (31, 35). The working procedure for HSV and EV amplicon identification is given in Table 5.

Result interpretation. The presence of HSV DNA in a sample is determined by relating the absorbance of the specimen well to the intrinsic extinction of the ABTS solution well. A clinical specimen with an $A_{405-490}$ value of ≥ 0.1 should be interpreted as positive for the presence of HSV DNA or EV RNA. A clinical specimen with an $A_{405-490}$ value of < 0.1 should be interpreted as negative. Based upon which probe(s) yields a positive result, the specimen is identified as being positive for HSV-1 (probe TK-G is positive) and/or HSV-2 (probe TK-H is positive) or EV (probe EV-3 is positive).

QUALITY ASSURANCE AND QUALITY CONTROL

Test validation and regulation. Quality assurance programs constitute an integral part of diagnostic laboratories. Because of the requisite sensitivity of nucleic amplification procedures, quality assurance programs in molecular microbiology are more complicated and expensive than those used in other laboratories. Since current HSV and EV PCR assays are home

brewed, establishment of the performance characteristics of accuracy, precision, sensitivity, specificity, predictive values, and reference ranges must be strictly CLIA-88 compliant (i.e., in accord with the Federal Clinical Laboratory Improvement Amendments of 1988). Sufficient specimens, including positives and negatives, should be included for such test verification and validation. The Association for Molecular Pathology has suggested that home-brewed assays should be validated with 50 specimens known to contain microbes and 100 analyte-negative specimens (2).

Controls. Controls used at Vanderbilt University Medical Center (VUMC) for the detection of HSV and EV by PCR are listed in Table 6. For some assays, it may be difficult to collect positive samples, as there are rarely any positives for that assay and, when there is a positive sample, the lack of specimen volume does not allow for aliquoting for future use. In these cases, it may be helpful to use spiked samples. These spiked samples can be loaded with either virus isolated from standard viral tissue cultures or manufactured plasmids containing the target sequence. These are diluted with negative CSF samples so that the diluted matrix is the same as would be seen in a real patient specimen.

Diminishing carryover contamination. Carryover contamination can be controlled by modification of amplification protocols, careful design of the laboratory layout, and strict adherence to specific workflow patterns. It is recommended that a minimum of three separate rooms or benches be used, one dedicated to the preparation of reagents, another for the aliquoting of samples and the isolation of DNA and RNA, and a third for PCR amplification and product detection. The one-way workflow policy, from the reagent preparation area to specimen preparation, then to amplification, and finally to the detection areas, is applied to laboratory workers, directors, specimens, and work cards (34).

Special techniques can be incorporated before or after amplification steps to control carryover contamination. Addition of isopropyl alcohol (HRI Associates, Herndon, Va.) to a master mixture has been described for postamplification inactivation of PCR products. After amplification, the unopened tube is exposed to UV light at a wavelength of 300 to 400 nm, which covalently binds the isopropyl alcohol to the nucleic acid polymers,

TABLE 6. Controls used at VUMC for the detection of HSV and EV by PCR

Type of control	Preparation	Comments
Positive	Pooled previously detected positive patient CSF samples.	It is recommended that a large quantity be prepared, aliquoted, and stored at -70°C . When limited, "spiking" controls could be prepared by adding positive cell cultures into pooled negative CSF specimens.
Negative	Pooled patient CSF samples which have been tested and found negative by PCR can be used.	It is recommended that a large quantity be prepared, aliquoted, and stored at -70°C .
No template	This is the blank provided by the manufacturer in the ELISA detection kit.	Used to indicate the absence of nonspecific binding of enzyme and the subsequent lysis of substrate in the absence of target. The absorbance of the blank is subtracted from the control and patients to correct for minor variations in photometry instrument background and reagent absorbance, allowing a more precise threshold value.
Kit	The ELISA kit control is a commercially prepared amplicon and amplicon-specific probe supplied by the manufacturer of the ELISA detection kit.	Ensures hybridization reaction conditions and the enzyme conjugation and substrate catalysis steps which follow.
"Housekeeping"	A master mix containing primers for β -actin or other housekeeping gene, which should be present in all DNA/RNA extractions.	This control should be included to detect possible PCR "inhibitors" in the specimen DNA and/or RNA, when assaying nonpreferred specimens which have not been fully validated.

preventing any further amplification (3). We have used a pre-amplification inactivation technique, which combines dUTP and uracil *N*-glycosylase (UNG; Epicentre Technologies, Madison, Wis.). The dUTP is incorporated into the polymer backbone instead of dTTP, and if products containing dUTP are present as a carryover contamination in subsequent sample preparations, the UNG will selectively recognize and cleave uracil residues from the polymer backbone, preventing further amplification (31, 35; B. Furrer, U. Candrian, P. Wieland, and J. Luthy, Letter, *Nature* **346**:324, 1990).

PT. After a procedure has been validated and placed into clinical diagnostic use, for confidence in the assay results it is necessary to be able to show correlation with other laboratories. Comparing and validating results with laboratories running the same or a different procedure ensures that the assay is measuring the absolute presence of a specific organism's nucleic acids. One way to accomplish this is to participate in a national accreditation organization's proficiency testing (PT) program. The College of American Pathologists (CAP) now has a proficiency program developed for molecular assays for HSV and EV (CAP Nucleic Acid Amplification Survey Code ID), which randomly contains samples either positive or negative for the presence of a specific organism. Each laboratory is sent identical samples for assay and is scored on the results obtained in comparison with all labs performing that assay. It is also important that clinical laboratories be enrolled in a program of this nature to meet CAP accreditation requirements (34).

Participation in a national PT program is vital, but samples are received for testing only a few times each year. To maintain confidence in day-to-day assay results, it is necessary to utilize an in-house PT program. Some form of internal testing program is also required under the regulations of the CLIA-88. Internal PT can be started by using leftover microbiology survey materials from CAP or specimens exchanged with other laboratories (2, 34). The quality control technologist or supervisor should integrate these samples blindly into the routine laboratory workload.

CONCLUSION

The growing ability of molecular approaches, especially PCR assays, to provide information to direct the treatment of CNS disease in a timely manner and to identify the subset of patients that do not need further medical intervention is invaluable to the clinician. PCR amplification of nucleic acids and their subsequent detection by the colorimetric MTP format combines accuracy, precision, sensitivity, and specificity. If used promptly in the diagnostic workup, these tests may significantly reduce unnecessary diagnostic tests, shorten hospitalization duration, and improve patient management.

ACKNOWLEDGMENTS

We thank David Head, Charles Stratton, and Peter Wright for critically reviewing the manuscript.

REFERENCES

1. Ahmed, A., F. Brito, C. Goto, S. M. Hickey, K. D. Olsen, M. Trujillo, and G. H. McCracken, Jr. 1997. Clinical utility of the polymerase chain reaction for diagnosis of enteroviral meningitis in infancy. *J. Pediatr.* **131**:393–397.

2. Anonymous. 1999. Association for Molecular Pathology statement. Recommendations for in-house development and operation of molecular diagnostic tests. *Am. J. Clin. Pathol.* **111**:449–463.
3. Cimino, G. D., K. C. Metchette, J. W. Tesson, J. E. Hearst, and S. T. Isaacs. 1991. Post-PCR sterilization: a method to control carryover contamination for the polymerase chain reaction. *Nucleic Acids Res.* **19**:99–107.
4. Dagan, R. 1996. Nonpolio enteroviruses and the febrile young infant: epidemiologic, clinical and diagnostic aspects. *Pediatr. Infect. Dis. J.* **15**:67–71.
5. Englund, J. A., M. E. Zimmerman, E. M. Swierkosz, J. L. Goodman, D. R. Scholl, and H. H. Balfour, Jr. 1990. Herpes simplex virus resistant to acyclovir. A study in a tertiary care center. *Ann. Intern. Med.* **112**:416–422.
6. Espy, M. J., J. R. Uhl, P. S. Mitchell, J. N. Thorvilson, K. A. Svien, A. D. Wold, and T. F. Smith. 2000. Diagnosis of herpes simplex virus infections in the clinical laboratory by LightCycler PCR. *J. Clin. Microbiol.* **38**:795–799.
7. Gaudreau, A., E. Hill, H. H. Balfour, Jr., A. Eric, and G. Boivin. 1998. Phenotypic and genotypic characterization of acyclovir-resistant herpes simplex viruses from immunocompromised patients. *J. Infect. Dis.* **178**:297–303.
8. Hamilton, M. S., M. A. Jackson, and D. Abel. 1999. Clinical utility of polymerase chain reaction testing for enteroviral meningitis. *Pediatr. Infect. Dis. J.* **18**:533–537.
9. Jeffery, K. J., S. J. Read, T. E. Peto, R. T. Mayon-White, and C. R. Bangham. 1997. Diagnosis of viral infections of the central nervous system: clinical interpretation of PCR results. *Lancet* **349**:313–317.
10. Kessler, H. H., B. Santner, H. Rabenau, A. Berger, A. Vince, C. Lewinski, B. Weber, K. Pierer, D. Stuenkel, E. Marth, and H. W. Doerr. 1997. Rapid diagnosis of enterovirus infection by a new one-step reverse transcription-PCR assay. *J. Clin. Microbiol.* **35**:976–977.
11. Kimura, H., M. Futamura, H. Kito, T. Ando, M. Goto, K. Kuzushima, M. Shibata, and T. Morishima. 1991. Detection of viral DNA in neonatal herpes simplex virus infections: frequent and prolonged presence in serum and cerebrospinal fluid. *J. Infect. Dis.* **164**:289–293.
12. Lahat, E., J. Barr, G. Barkai, G. Paret, N. Brand, and A. Barzilai. 1999. Long term neurological outcome of herpes encephalitis. *Arch. Dis. Child.* **80**:69–71.
13. Marshall, G. S., M. A. Hauck, G. Buck, and G. P. Rabalais. 1997. Potential cost savings through rapid diagnosis of enteroviral meningitis. *Pediatr. Infect. Dis. J.* **16**:1086–1087.
14. Mitchell, P. S., M. J. Espy, T. F. Smith, D. R. Toal, P. N. Rys, E. F. Berbari, D. R. Osmon, and D. H. Persing. 1997. Laboratory diagnosis of central nervous system infections with herpes simplex virus by PCR performed with cerebrospinal fluid specimens. *J. Clin. Microbiol.* **35**:2873–2877.
15. Myers, T. W., and D. H. Gelfand. 1991. Reverse transcription and DNA amplification by a *Thermus thermophilus* DNA polymerase. *Biochemistry* **30**:7661–7666.
16. Nowak-Węgrzyn, A., W. Phipatanakul, J. A. Winkelstein, M. S. Forman, and H. M. Lederman. 2001. Successful treatment of enterovirus infection with the use of pleconaril in 2 infants with severe combined immunodeficiency. *Clin. Infect. Dis.* **32**:E13–E14.
17. Powell, K. F., N. E. Anderson, R. W. Frith, and M. C. Croxson. 1990. Non-invasive diagnosis of herpes simplex encephalitis. *Lancet* **335**:357–358.
18. Ramers, C., G. Billman, M. Hartin, S. Ho, and M. H. Sawyer. 2000. Impact of a diagnostic cerebrospinal fluid enterovirus polymerase chain reaction test on patient management. *JAMA* **283**:2680–2685.
19. Read, S. J., and J. B. Kurtz. 1999. Laboratory diagnosis of common viral infections of the central nervous system by using a single multiplex PCR screening assay. *J. Clin. Microbiol.* **37**:1352–1355.
20. Rotbart, H. A. 1990. Enzymatic RNA amplification of the enteroviruses. *J. Clin. Microbiol.* **28**:438–442.
21. Rotbart, H. A., M. H. Sawyer, S. Fast, C. Lewinski, N. Murphy, E. F. Keyser, J. Spadaro, S. Y. Kao, and M. Loeffelholz. 1994. Diagnosis of enteroviral meningitis by using PCR with a colorimetric microwell detection assay. *J. Clin. Microbiol.* **32**:2590–2592.
22. Rotbart, H. A., and A. D. Webster. 2001. Treatment of potentially life-threatening enterovirus infections with pleconaril. *Clin. Infect. Dis.* **32**:228–235.
23. Rowley, A. H., R. J. Whitley, F. D. Lakeman, and S. M. Wolinsky. 1990. Rapid detection of herpes-simplex-virus DNA in cerebrospinal fluid of patients with herpes simplex encephalitis. *Lancet* **335**:440–441.
24. Ryncarz, A. J., J. Goddard, A. Wald, M. L. Huang, B. Roizman, and L. Corey. 1999. Development of a high-throughput quantitative assay for detecting herpes simplex virus DNA in clinical samples. *J. Clin. Microbiol.* **37**:1941–1947.
25. Sauerbrei, A., U. Eichhorn, G. Hottenrott, and P. Wutzler. 2000. Virological diagnosis of herpes simplex encephalitis. *J. Clin. Virol.* **17**:31–36.
26. Schlesinger, Y., M. H. Sawyer, and G. A. Storch. 1994. Enteroviral meningitis in infancy: potential role for polymerase chain reaction in patient management. *Pediatrics* **94**:157–162.
27. Skoldenberg, B., M. Forsgren, K. Alestig, T. Bergstrom, L. Burman, E. Dahlqvist, A. Forkman, A. Fryden, K. Lovgren, and K. Norlin. 1984. Acyclovir versus vidarabine in herpes simplex encephalitis. Randomised multicentre study in consecutive Swedish patients. *Lancet* **2**:707–711.
28. Spicher, V. M., P. Y. Berclaz, J. J. Cheseaux, P. A. Morandi, S. Suter, W.

- Wunderli, and C. A. Siegrist. 2000. Detection of enteroviruses in the cerebrospinal fluid by polymerase chain reaction: prospective study of impact on the management of hospitalized children. *Clin. Pediatr.* **39**:203–208.
29. Stellrecht, K. A., I. Harding, F. M. Hussain, N. G. Mishrik, R. T. Czap, M. L. Lepow, and R. A. Venezia. 2000. A one-step RT-PCR assay using an enzyme-linked detection system for the diagnosis of enterovirus meningitis. *J. Clin. Virol.* **17**:143–149.
30. Storch, G. A. 2000. Diagnostic virology. *Clin. Infect. Dis.* **31**:739–751.
31. Tang, Y. W., P. J. Heimgartner, S. J. Tollefson, T. J. Berg, P. N. Rys, H. Li, T. F. Smith, D. H. Persing, and P. F. Wright. 1999. A colorimetric microtiter plate PCR system detects respiratory syncytial virus in nasal aspirates and discriminates subtypes A and B. *Diagn. Microbiol. Infect. Dis.* **34**:333–337.
32. Tang, Y. W., J. R. Hibbs, K. R. Tau, Q. Qian, H. A. Skarhus, T. F. Smith, and D. H. Persing. 1999. Effective use of polymerase chain reaction for diagnosis of central nervous system infections. *Clin. Infect. Dis.* **29**:803–806.
33. Tang, Y. W., P. S. Mitchell, M. J. Espy, T. F. Smith, and D. H. Persing. 1999. Molecular diagnosis of herpes simplex virus infections in the central nervous system. *J. Clin. Microbiol.* **37**:2127–2136.
34. Tang, Y. W., and D. H. Persing. 1999. Molecular detection and identification of microorganisms, p. 215–244. *In* P. R. Murray, E. Jo Baron, M. A. Pfaller, F. C. Tenover, and R. H. Tenover (ed.), *Manual of clinical microbiology*, 7th ed. American Society for Microbiology, Washington, D.C.
35. Tang, Y. W., P. N. Rys, B. J. Rutledge, P. S. Mitchell, T. F. Smith, and D. H. Persing. 1998. Comparative evaluation of colorimetric microtiter plate systems for detection of herpes simplex virus in cerebrospinal fluid. *J. Clin. Microbiol.* **36**:2714–2717.
36. van Vliet, K. E., M. Glimaker, P. Lebon, P. E. Klapper, C. E. Taylor, M. Ciardi, H. G. van der Avoort, R. J. Diepersloot, J. Kurtz, M. F. Peeters, G. M. Cleator, and A. M. van Loon for the European Union Concerted Action on Viral Meningitis and Encephalitis. 1998. Multicenter evaluation of the Amplicor Enterovirus PCR test with cerebrospinal fluid from patients with aseptic meningitis. *J. Clin. Microbiol.* **36**:2652–2657.
37. Verstrepen, W. A., S. Kuhn, M. M. Kockx, M. E. Van De Vyvere, and A. H. Mertens. 2001. Rapid detection of enterovirus RNA in cerebrospinal fluid specimens with a novel single-tube real-time reverse transcription-PCR assay. *J. Clin. Microbiol.* **39**:4093–4096.
38. Whitley, R. J., C. A. Alford, M. S. Hirsch, R. T. Schooley, J. P. Luby, F. Y. Aoki, D. Hanley, A. J. Nahmias, and S. J. Soong. 1986. Vidarabine versus acyclovir therapy in herpes simplex encephalitis. *N. Engl. J. Med.* **314**:144–149.
39. Whitley, R. J., and B. Roizman. 2001. Herpes simplex virus infections. *Lancet* **357**:1513–1518.
40. Yerly, S., A. Gervaix, V. Simonet, M. Cafilisch, L. Perrin, and W. Wunderli. 1996. Rapid and sensitive detection of enteroviruses in specimens from patients with aseptic meningitis. *J. Clin. Microbiol.* **34**:199–201.
41. Young, P. P., R. S. Buller, and G. A. Storch. 2000. Evaluation of a commercial DNA enzyme immunoassay for detection of enterovirus reverse transcription-PCR products amplified from cerebrospinal fluid specimens. *J. Clin. Microbiol.* **38**:4260–4261.