

Evaluation of the Polymerase Chain Reaction for Diagnosis of Herpes Simplex Virus Encephalitis

ELISABETH PUCHHAMMER-STÖCKL,^{1*} FRANZ X. HEINZ,¹ MICHAEL KUNDI,²
THERESE POPOW-KRAUPP,¹ GEORG GRIMM,³ MICHAEL M. MILLNER,⁴ AND CHRISTIAN KUNZ¹
Institute of Virology,¹ Institute of Environmental Hygiene,² and Department of Internal Medicine,³ University of Vienna, A-1095 Vienna, and Pediatric Neurology Unit, Department of Pediatrics, University of Graz, Graz,⁴ Austria

Received 1 May 1992/Accepted 2 October 1992

Cerebrospinal fluid samples from 257 patients with suspected herpes simplex virus encephalitis were prospectively analyzed by herpes simplex virus polymerase chain reaction. The polymerase chain reaction indicated herpes simplex virus encephalitis in 9 serologically proven cases and in 14 additional patients. Increased polymerase chain reaction signals were observed together with more severe neurological symptoms ($P < 0.01$) and within the first days of acyclovir treatment ($P < 0.05$).

Herpes simplex virus encephalitis (HSE) is the most common cause of endemic, fatal encephalitis in industrial countries (15). Treatment with acyclovir, when initiated soon after the onset of symptoms, can decrease the high mortality rate associated with this disease. Until recently, however, the early diagnosis of HSE has been restricted to the detection of herpes simplex virus (HSV) in brain biopsy material. Recently, the polymerase chain reaction (PCR), allowing the detection of HSV DNA in cerebrospinal fluid (CSF) samples, has been shown to be a promising diagnostic tool (1, 5, 10-14).

In the present study, the value of a previously established HSV PCR assay (12) applied to HSE diagnosis under routine conditions was investigated. Some 257 patients with clinically suspected HSE, from whom at least one CSF sample was obtained within the first 10 days of disease, were investigated by HSV PCR. As described previously (12), PCR was done in a 100- μ l reaction mixture containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2 mM MgCl₂, 200 μ M each deoxynucleoside triphosphate (dATP, dCTP, dGTP, and dUTP), 100 pM each of the primers HS 13 (ACGAC GACGTCCGACGGCGA) and HS 14 (GTGCTGGTGCTG GACGACAC) (7, 12), 10 μ l of CSF, and 2 U of *Taq* polymerase (Perkin-Elmer-Cetus). The mixture was overlaid with paraffin oil, and 35 PCR cycles were performed (94°C-40°C-68°C, each for 1 min). Strict precautions were taken to exclude contamination during the PCR procedure (6, 12). For the specific detection of the amplified fragments, slot blot hybridization was carried out as described previously (12). The hybridization results were analyzed semiquantitatively, using primary human foreskin fibroblasts infected with HSV type 1 strain 539 in different concentrations as positive standards. The result obtained from the dilution corresponding to 10 infectious units was used as a cutoff value (12). After hybridization, the radioactive signals of the probe bound to the individual samples were determined. The sample counts per minute were compared with the test cutoff counts per minute and are indicated as *x*-fold cutoff counts per minute. All CSF and serum samples available were screened for the presence of HSV-specific antibodies by complement fixation test (4), using 2 U of antigen, and by

immunoglobulin M- and G-specific solid-phase enzyme-linked immunosorbent assays, using HSV- type 1 strain 539-infected HeLa cells as antigen (3). Samples were tested in serial twofold dilutions (1:100 to 1:51,200). HSE was considered serologically proven when at least a fourfold increase in the CSF/serum HSV antibody ratio was observed, not accompanied by a similar increase in unrelated antigens (9).

As shown in Table 1, the PCR proved to be superior to serology, detecting 14 additional HSE cases. It also allowed a much earlier diagnosis than antibody testing in all cases. In two patients, however, only one of two CSF samples was positive by PCR. The diagnosis of HSE was in agreement with clinical and radiodiagnostic findings in all PCR-positive patients. In 105 PCR-negative cases, another cause for the clinical symptoms was proven. No information about follow-up was obtained from most of the remaining PCR-negative patients.

We then assessed whether, in the absence of HSE, latent or reactivated HSV infection may cause positive PCR results. Control CSF samples from 145 latently HSV-infected patients (presence of HSV-specific immunoglobulin G antibodies), with diseases of the central nervous system other than HSE, were all negative by PCR. In addition, CSF samples from three patients who were just undergoing HSV reactivation, proven by serology or virus isolation, without

TABLE 1. Diagnosis of clinically suspected HSE by PCR and serology in 257 patients

Increase of CSF/ serum antibody ratio	No. of patients PCR:	
	Positive	Negative
>4-fold ^a (n = 9)	9 (0) ^b	0
<4-fold (n = 38)	5 (0) ^c	33 (9)
NA (n = 210) ^d	9 (0) ^c	201 (96)

^a Serologically proven HSE.

^b Number in parentheses is the number of patients from whom another etiology of the clinical symptoms was later proven.

^c In one patient only one of two different CSF samples obtained within the first 10 days of disease was positive.

^d NA, not available; only one CSF-serum pair was available early in the course of disease. In the absence of follow-up samples, HSE could not be proven serologically.

* Corresponding author.

TABLE 2. Influence of patient's age on amount of HSV DNA in CSF

Age (yr) of patients	No. of CSF samples ^a	Mean sample cpm/cutoff cpm ^b
0-4	9	3.9
14-32	11	6.5
46-70	9	8.4

^a The different numbers of CSF samples in this table and Table 3 are due to the fact that some patient clinical data were only partially available.

^b The mean counts per minute result of three independent PCR runs from each CSF sample was used.

clinical evidence of HSE also yielded negative PCR results. Thus, positive PCR results obtained with CSF samples are highly specific for HSE.

As false-negative results were obtained with two CSF samples (Table 1), we analyzed which factors could possibly influence the amount of HSV DNA present in CSF during HSE. The PCR signal intensity obtained with 10 individual CSF samples from the PCR-positive patients shown in Table 1 could be compared with the CSF cell number available from these samples. The statistical evaluation (Kendall τ correlation) indicated no significant correlation between signal intensity and CSF cell number ($\tau = 0.09$; $P > 0.1$). The PCR signal intensity did show, however, a slight association with the patient's age (Table 2) ($\tau = 0.24$; $P < 0.1$) and a significant correlation to the clinical severity of disease (Table 3) ($\tau = 0.46$; $P < 0.01$).

Another factor possibly influencing PCR signal intensity is the time of CSF sampling in the course of disease. We have investigated whether there is a relationship between PCR signal intensities and the length of the interval between onset of disease and onset of acyclovir treatment or the duration of therapy. The PCR results from 13 patients from whom exact data concerning onset of disease and therapy were available were statistically analyzed. Two different CSF samples were included from four patients. A second-order polynomial regression was performed (Fig. 1), and this indicated a statistically significant ($P < 0.05$) relationship between initiation of acyclovir therapy and increase of PCR signals, independent of when in the course of disease the treatment was started. The highest signals were reached at day 5 to 6 of therapy. These unexpected results may be explained by the generation of a high amount of small HSV DNA fragments when HSV replicates in the presence of acyclovir (8). This would also be consistent with previous findings showing that HSV DNA is present as free DNA in the CSF during HSE (13). A significant signal decrease was observed starting on day 6 of therapy, clearly confirming the efficiency of the acyclovir treatment. CSF samples were not investigated for the existence of PCR inhibitors as no correlation of inhibitors with the independent variables was expected. To exclude that the presence of acyclovir in the CSF samples may

TABLE 3. Influence of severity of disease on amount of HSV DNA in CSF

State of consciousness	No. of CSF samples ^a	Mean sample cpm/cutoff cpm ^b
Comatose	4	21.2
Noncomatose	12	4.4

^a See footnote a, Table 2.

^b See Table 2, footnote b.

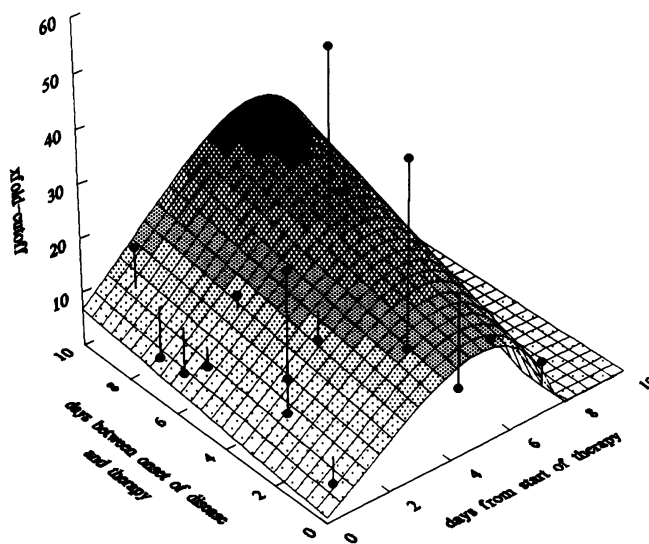


FIG. 1. Regression surface of the PCR signals obtained from 13 HSE patients with respect to the days between onset of symptoms and start of therapy and to the days after onset of acyclovir treatment. The counts per minute of the CSF samples represent the mean counts per minute obtained with these samples in three different PCR runs.

directly influence the PCR, however, infected fibroblasts containing 100 and 1,000 infectious units of HSV were subjected to PCR in the presence of acyclovir at concentrations of 25, 2.5, 0.25, and 0.025 μg and in its absence. No influence on the PCR was observed even with acyclovir concentrations that were more than 200-fold higher than those achieved during the treatment of a patient with 10 mg of acyclovir/kg of body weight/8 h (2).

We conclude that the semiquantitative PCR assay is a reliable, specific, and sensitive tool for the early diagnosis of HSE. According to our data, however, a negative PCR result cannot completely exclude HSE and does not itself provide justification for the termination of acyclovir treatment.

We thank Sophie Agoston, Barbara Dalmatiner, and Heide Dippe for excellent technical assistance and Steve Allison for help with the preparation of the manuscript.

REFERENCES

1. Aurelius, E., B. Johansson, B. Sköldenberg, A. Staland, and M. Forsgren. 1991. Rapid diagnosis of herpes simplex encephalitis by nested polymerase chain reaction assay of cerebrospinal fluid. *Lancet* 337:189-192.
2. Blum, M. R., S. H. Liao, and P. De Miranda. 1982. Overview of acyclovir pharmacokinetic disposition in adults and children. *Am. J. Med.* 73:186-192.
3. Denoyel, G. A., A. Gaspar, and C. Nouyrigat. 1980. Enzyme immunoassay for measurement of antibodies to herpes simplex virus infection; comparison with complement fixation, immunofluorescent-antibody, and neutralization techniques. *J. Clin. Microbiol.* 11:114-119.
4. Hawkes, R. A. 1979. General principles underlying laboratory diagnosis of viral infections, p. 3-48. In E. H. Lennette and N. J. Schmidt (ed.), *Diagnostic procedures for viral, rickettsial and chlamydial infections*. American Public Health Association, Washington, D.C.
5. Klapper, P. E., G. M. Cleator, C. Dennett, and A. G. Lewis. 1990. Diagnosis of herpes simplex encephalitis via southern blotting of cerebrospinal fluid DNA amplified by polymerase

- chain reaction. *J. Med. Virol.* **32**:261-264.
6. **Kwok, S.** 1990. Procedure to minimize PCR-product carry over, p. 142-145. *In* M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White (ed.), *Pcr protocols*. Academic Press, Inc., San Diego, Calif.
 7. **McGeoch, D. J., M. A. Dalrymple, A. J. Davison, A. Dolan, M. C. Frame, D. McNab, L. J. Perry, J. E. Scott, and P. Taylor.** 1988. The complete DNA sequence of the long unique region in the genome of herpes simplex virus type 1. *J. Gen. Virol.* **69**:1531-1574.
 8. **McGuirt, P. V., J. E. Shaw, G. B. Elion, and P. A. Furman.** 1984. Identification of small DNA fragments synthesized in herpes simplex virus-infected cells in the presence of acyclovir. *Antimicrob. Agents Chemother.* **25**:507-509.
 9. **Nahmias, A. J., R. J. Whitley, A. N. Visintine, Y. Takei, and C. A. Alford, Jr.** 1982. Herpes simplex virus encephalitis: laboratory evaluations and their diagnostic significance. *J. Infect. Dis.* **145**:829-836.
 10. **Pohl-Koppe, A., C. Dahm, M. Elgas, J. E. Kühn, R. W. Braun, and V. ter Meulen.** 1992. The diagnostic significance of the polymerase chain reaction and isoelectric focusing in herpes simplex virus encephalitis. *J. Med. Virol.* **36**:147-154.
 11. **Powell, K. F., N. E. Anderson, R. W. Frith, and M. C. Crosson.** 1990. Non-invasive diagnosis of herpes simplex encephalitis. *Lancet* **335**:357-358.
 12. **Puchhammer-Stoeckl, E., T. Popow-Kraupp, F. X. Heinz, C. W. Mandl, and C. Kunz.** 1990. Establishment of PCR for the early diagnosis of herpes simplex encephalitis. *J. Med. Virol.* **32**:77-82.
 13. **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.
 14. **Rozenberg, F., and P. Lebon.** 1991. Amplification and characterization of herpesvirus DNA in cerebrospinal fluid from patients with acute encephalitis. *J. Clin. Microbiol.* **29**:2412-2417.
 15. **Whitley, R. J.** 1990. Herpes simplex viruses. *In* B. N. Fields and D. M. Knipe (ed.), *Virology*. Raven Press, New York.