Herpes encephalitis is a disease of middle aged and elderly people: polymerase chain reaction for detection of herpes simplex virus in the CSF of 516 patients with encephalitis

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Abstract

Objective—To assess the diagnostic potential of the polymerase chain reaction (PCR) in herpes simplex virus (HSV) encephalitis.

Methods—Samples of CSF from 516 patients with encephalitis were studied for HSV-DNA by PCR.

Results—Samples taken one to 29 days from the onset of symptoms from 38 patients (7.4%) were positive, 32 (6.2%) for HSV-1 and six (1.2%) for HSV-2. At follow up, eight of 28 patients studied were still HSV-PCR positive. A diagnostic serum:CSF antibody ratio to HSV but not to other viruses was detected in 25 of the 38 HSV-PCR positive patients thus supporting the initial PCR findings. Patients positive by HSV-PCR were concentrated in the age group ≥ 40 years, and especially in patients aged 60–64 years, of whom nine of 24 (37.5%) were positive. The HSV-PCR was negative in all other patients with encephalitis of known or unknown aetiology. This group included 34 patients with a diagnostic serum:CSF antibody ratio to other viruses. A dual infection, HSV and another microbe, was considered possible in seven patients.

Conclusions—The HSV-PCR is a rapid and useful diagnostic method during the early phase of encephalitis. It may be useful in monitoring the efficacy of treatment and allowing the recognition of new features in the appearance of herpes encephalitis. The HSV-PCR test and antibody determinations from serum and CSF are complementary methods, which should both be applied in pursuit of clinical laboratory diagnosis of these conditions.

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The polymerase chain reaction (PCR) was introduced for the diagnosis of herpes simplex virus encephalitis (HSVE) in 1990 when Powell *et al*¹ reported the rapid detection of HSV-DNA in CSF and Rowley *et al*² reported the detection of HSV-DNA in four patients

with HSVE. Samples of CSF from six patients with other CNS infections were negative for HSV. Since then the technique has been used in many laboratories interested in the diagnosis of HSVE.^{3–9} For example, in the study reported by Lakeman *et al* CSF-HSV was detected by PCR in 53 of 54 patients with HSVE established by brain biopsy, and in all 18 CSF samples obtained before brain biopsy.³ Additionally, HSV-PCR was positive in three of 47 patients negative on brain biopsy. These studies clearly show the diagnostic potential of the test. Brain biopsy is an invasive and demanding method for diagnosis. The HSV-PCR makes the diagnosis of HSVE easier and the clinical manifestations of HSVE will, without doubt, be recognised more thoroughly. We present our experience on 516 patients with encephalitis.

Patients and methods

We studied CSF samples from 516 patients with encephalitis who were cared for at central or university hospitals throughout Finland. Encephalitis was defined as acute onset of neurological symptoms reflecting focal or generalised involvement of brain tissue presenting as pareses, convulsions, linguistic or mental dysfunction, or lowered level of consciousness, with or without meningeal signs and symptoms. Most specimens were sent for laboratory diagnosis at the time of admission of the patient to hospital. The HSV-PCR test was performed if HSVE was suspected by the clinician, or antibodies to HSV were detected in the CSF, or a high antibody titre to HSV was found in serum. The age of the patients ranged from neonate to 86 years.

A PCR was performed on CSF immediately after receipt in the laboratory (one to three days after collection of the sample). Some samples were frozen and preserved at -20°C until they were assayed. For HSV-PCR a 130 μL CSF sample was centrifuged at 4500 rpm for five minutes at 4°C ; 100 μL of the supernatant was treated with proteinase K buffer and the DNA extracted using phenol/diethyl ether and ethanol precipitation. DNA was amplified in a 100 μL reaction mixture containing 50 mM KCl, 10 mM Tris HCl (pH 8.3), 1.5 mM MgCl_2 , 0.01% (w/v) gelatin, 200 μM each of the four deoxyribonucleoside triphosphates, 2.5 U of AmpliTaq polymerase

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Figure 1 HSV-PCR positivity after the onset of the disease on admission to the hospital. Only the initial positive finding of the patient is included. The figures refer to the number of patients studied on each day. Eleven of 57 patients studied on the first day of symptoms were retested on days five to 14 and all were negative on that test except the positive patient who was still positive on day 9.

(Perkin Elmer-Cetus, Norwalk, CT), and 100 pmol of the primers selected from the DNA polymerase gene for HSV-1 and HSV-2—that is, 229 and 241 base pairs for HSV-1 and HSV-2.¹⁰ The amplified fragments reaction mixture was initially heated at 95°C for two minutes followed by amplification consisting of 40 cycles at 95°C for 25 seconds (denaturation), at 55°C for 30 seconds (annealing), and at 72°C for 30 seconds (extension, allowed to continue for 10 minutes after the last cycle) (Perkin Elmer-Cetus, GeneAmp PCR 9600 thermal cycler, Norwalk, CT). The amplified frequent PCR products were electrophoresed in 3.5% agarose containing 0.2 µg/ml ethidium bromide. PhiX174 RF DNA-*Hae* III digest (Biolabs, Beverly, MA) was used as a size marker. For definitive identification of the amplified HSV-DNA, samples with putative positive results were subjected to Southern blotting and hybridised with end labelled radioactive or digoxigenin oligonucleotide probes synthesised by an Applied Biosystems 381 A DNA synthesiser. Measures to avoid contamination were applied throughout the process.¹¹

Antibodies against HSV-1, HSV-2, and varicella zoster virus were assayed using commercial enzyme immunoassay antigens (Virion®, Würzburg, Germany), and recorded in enzyme immune units (the optical density of the specimen at a 1:200 dilution divided by the optical density of the positive control and then multiplied by 100¹²). The serum:CSF antibody ratio was calculated and considered diagnostic if ≤ 20 with respect to the reciprocals of end point titres.¹³ HSV-specific IgM was determined with a commercial indirect IgM immunofluorescence kit (Gull Laboratories, Salt Lake City, UT). If positive, the test was repeated after IgG inactivation (Gullcorp absorption).

Antibody assays against adeno, influenza A and B, rota, Coxsackie B5, ECHO 22 (antigens grown and purified as described¹⁴), cytomegalo, parainfluenza 1, and respiratory syncytial viruses (Virion®) and *Mycoplasma pneumoniae* (gift from M Kleemola, National

Public Health Institute, Helsinki) were measured in serum and CSF using enzyme immunoassay tests. For chlamydial diagnostic tests, a microimmunofluorescence method measuring IgG and IgM antibodies specific to *Chlamydia trachomatis*, *Chlamydia pneumoniae*, and *Chlamydia psittaci* was used.¹⁵ Antibodies to tick borne encephalitis and Puumala hantavirus (nephropathia epidemica) were measured if indicated, using an in house indirect immunofluorescence antibody assay.^{16 17}

Virus culture was attempted on 122 CSF samples using four different cell lines; African GMK, Vero, human amniotic epithelial cells, and human embryonic skin fibroblasts.

Results

Of 516 patients, 38 (7.4%) were positive by the HSV-PCR test; 32 (6.2%) for HSV-1 and six (1.2%) for HSV-2. The test was positive in the first CSF specimen obtained, usually on admission to hospital; 82% of the samples were obtained within two weeks from the onset of symptoms. The HSV-PCR assay seemed to be of most value for confirming an aetiological diagnosis of HSVE when used during the first week after the onset of symptoms (fig 1). In one patient, HSV-PCR appeared positive (day 3) after an initial negative finding (day 2). Eight of the 28 patients, on whom follow up tests were performed, remained PCR positive six to 57 days after the first sample. In a 60 year old man with a chronic progressive disease the CSF sample was initially positive to HSV-1, but six months later to HSV-2, and in subsequent tests remained so despite prolonged treatment with acyclovir.

Patients positive for HSV-PCR were concentrated in the age group ≥ 40 years, and in the 60 to 64 year age group HSV-DNA was found in the CSF in nine of 24 patients (fig 2). In the 1 to 4 year age group no positive findings appeared in any of the 50 children studied. Similarly, in the age group 10 to 24 years samples were negative in all 58 patients with encephalitis. In patients aged 40 to 59 years

Figure 2 Distribution of HSV-PCR positive findings in different age groups. The figures refer to the number of patients studied in each age group.

Figure 3 Distribution of HSV-PCR findings in those patients who afterwards developed a diagnostic serum:CSF antibody to HSV. The day refers to the day when HSV-PCR was performed.

PCR positivity ranged from 13% to 18% of patients with encephalitis studied. Findings of HSV-2 were distributed across the age range. The rate of PCR positivity did not show seasonal variation.

The HSV-PCR was negative in all other patients with encephalitis of known or unknown aetiology including 34 patients with a diagnostic serum:CSF (S/CSF) antibody ratio to some other microbe and 10 patients with a diagnostic S/CSF ratio to HSV (fig 3). In the HSV-PCR positive group a diagnostic S/CSF antibody ratio to HSV was found in 25 of 38 (66%), thus supporting the initial PCR result. A possible dual infection appeared in seven HSV-PCR positive patients (table).

HSV-PCR positive patients with a concomitant diagnostic finding of some other microbe

Age (sex)	Date* of HSV-PCR	Microbe	Finding
64 (F)	9	HSV-1, PHV	PHV IgM positive†
62 (M)	19	HSV-1, VZV	VZV S:CSF ratio
47 (M)	4	HSV-1, CHL	CHL IgG and IgM positive†
64 (M)	6	HSV-1, TBE	TBE IgM positive†
29 (F)	10	HSV-2, VZV	VZV S:CSF ratio
61 (M)	8	HSV-1, CHL	CHL IgG and IgM positive†
NB (F)	7	HSV-1, HIV	HIV antibodies†

*Days from the onset of the diseases.

†In serum.

PHV = Puumala hantavirus; VZV = varicella zoster virus; TBE = tick borne encephalitis; CHL = *Chlamydia pneumoniae*; NB = newborn.

Discussion

The PCR provides a highly sensitive method for the identification of viral invasion of the CNS at an early stage of infection.¹⁸ In the present study HSV-PCR seemed to be specific as indicated by the negative results obtained from many patients with encephalitis of various aetiologies and by the positive results which were confirmed by a concomitant or subsequent antibody response in 66% of cases. The number of non-responders, with no intrathecal antibody production, was similar to the numbers reported by Nahmias *et al.*¹⁹ and Anderson *et al.*²⁰ The use of HSV-PCR represents an improvement over viral culture and antigen detection from the CSF.^{21, 22} Diagnostic brain biopsy in CNS infections is considered appropriate in prolonged and complicated cases only. Nevertheless, detection of HSV from brain tissue biopsy or necropsy remains the reference method for confirmation of HSVE.

Our findings indicate failure of treatment to eradicate HSV-DNA in the CSF, an observation similar to that found in other studies.^{8, 9, 23} In eight of 28 patients HSV-PCR was still positive six to 57 days after the first positive finding, despite treatment with acyclovir. It has been reported that the highest PCR signals are reached at days five to six of acyclovir treat-

ment.²⁴ In one of our patients, the initial HSV-1 PCR positivity disappeared, and six months later PCR showed HSV-2 positivity which in subsequent CSF samples remained positive for months despite repeated courses of acyclovir, suggestive of therapeutic failure.

In this study it is apparent that HSVE seems to affect predominantly middle aged and elderly people. Positivity on PCR was recorded for 37.5% of patients aged 60 to 64 years with encephalitis. This may reflect the prevalence of the virus genome in the brains of older people.²⁵ Among people aged ≥ 65 years, however, HSV-PCR positivity remained $< 10\%$, although herpes encephalitis was often suspected. With increasing age, other aetiological agents, such as influenza viruses appear (M Färkkilä *et al*, unpublished observations).

Only a few PCR results on children after the newborn period have been published.^{8 23 26} In our series no patients with HSVE were found in the adolescent groups. Among children, a few cases were identified. A negative HSV-PCR result may reflect a low amount of virus in the CSF, the disease process may be deep within the brain, or the primers used may not have been suitable to detect the virus type or strain causing the infection.^{27 28} The overall HSV-DNA detection rate in our series was a little lower than in the study by Puchhammer *et al*,²⁴ 7.4% *v* 8.9%. However, in our study serological confirmation rate was higher (66% *v* 39%).

In 10 patients with a diagnostic serum:CSF antibody ratio to HSV, PCR remained negative even though some samples were obtained during the first two weeks. The HSV antibody response was considered diagnostic because there was no evidence of intrathecal production of antibodies to other microbes in the CSF. It is apparent that detection of HSV specific DNA from the CSF may sometimes be problematic.²² A negative PCR result should always be critically assessed, repeated sampling may be worthwhile, and other laboratory diagnostic procedures should be performed. A nested PCR protocol may be more sensitive than a single PCR. In a clinical laboratory, however, the inherent risk of contamination of amplified products makes the use of nested PCR problematic. Enzyme linked immunosorbent assay based detection systems, using streptavidin-coated microplates, biotin-labelled probes, and luminometric reading with appropriate substrate, may enhance the sensitivity.²⁹

Dual infections were considered a possibility in seven of 38 patients. These patients had a diagnostic finding of another microbe in addition to a positive HSV-PCR. Significantly increased levels of antibodies to varicella zoster virus in CSF were found in two patients. Similar findings of dual infection have been reported previously, based on PCR results.³⁰ It may therefore be important to do multiple PCR tests on the same specimen. With regard to HSV-1 and HSV-2 dual infections, the separation of closely related bands may be difficult using gel electrophoresis. With microplate detection,²⁹ the separation is

clearcut and may be confirmed by Southern blotting.

To confirm the use of the HSV-PCR test for clinical diagnostic purposes we have studied a large series of patients with encephalitis. In older age groups, HSV seems the most prevalent cause of encephalitis and with "ideal" timing, the diagnostic hit rate may be 20% to 35%. In young adults, HSV is a rare cause of encephalitis. The specificity of the HSV-PCR test seemed good in the present study although a "gold standard" reference test is not available in encephalitis cases in general. The PCR test will improve knowledge on viral encephalitis and help to monitor the efficacy of treatment. In the early phase of a CNS disease, PCR is a rapid and sensitive method. At follow up, antibody tests may support the result and possibly establish more diagnoses. The two methods, PCR and antibody determinations, are complementary and should be performed in parallel in clinical laboratory diagnosis.

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