Diagnosis and quantitative detection of HSV DNA in samples from patients with suspected herpes simplex encephalitis

ABSTRACT

Diagnosis of herpes simplex encephalitis (HSE) is based on the detection of herpes simplex virus (HSV) DNA in patients' CSF samples. HSV DNA quantitation has the potential for estimating the effects of antiviral therapy. The aim of this study was to diagnose HSV DNA in HSE suspected patients and the quantitative analysis of its genome using real-time PCR to assess the value of the viral load in the course of antiviral treatment. The CSF samples were collected from 236 consecutive HSE suspected patients from November 2004 to May 2008. Upon DNA extraction, the samples were analyzed by Real-Time PCR assay. A set of primers amplified a common sequence of HSV glycoprotein B gene. The copy numbers of unknown samples were expressed via a standard curve drawn with a known amount of amplified cloned plasmid. Of the 236 samples, 137 (58%) came from males and 99 (42%) from females. The HSV genome was detected in 22 (9.3%) patients by PCR, 13 males/9 females. Serial CSF samples were available from 10 of the 22 patients. The range of the HSV DNA copy numbers in the clinical samples ranged from 2.5×10^2 to 1.7×10^6 copies/mL of CSF. Quantitative PCR results can be helpful in evaluating the efficacy of antiviral therapy in the above-mentioned patients. There is an association between the initial viral load and the duration of treatment course.

Keywords: encephalitis; herpes simplex; viral load; acyclovir; quantitative analysis.

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INTRODUCTION

Herpes simplex encephalitis (HSE) is the most common form of sporadic viral encephalitis; but it is still an uncommon disease, with an estimated incidence of 1 in 250,000 to 1 in 500,000 persons per year.[1,2] Acute HSE is associated with high morbidity and mortality. In untreated patients or those on ineffective antiviral therapy, the mortality is approximately 70% and out of surviving patients, important neurologic sequalae occurs in nearly 97%.[3] Early diagnosis and use of acyclovir as a therapeutic agent are highly effective in reducing the mortality rate due to HSE; however, permanent neurologic complications are common in survivors.[4] Herpes simplex virus (HSV) DNA detection by PCR assay of cerebrospinal fluid (CSF) samples is very efficient in establishing the diagnosis of HSE and has become the diagnostic method of choice.[5-7] It is a reliable method, highly sensitive and specific. Quantitative HSV-PCR can be used to monitor the effect of treatment,[8] but its correlation with prognosis has not been established.[9]

The aim of the present study was to evaluate HSV DNA in the CSF of the HSE suspected patients, as well as the effect of acyclovir therapy on the number of HSV DNA copies in CSF by a Real-Time TaqMan probe PCR.

MATERIALS AND METHODS

Patients

In the present study, 236 consecutive patients older than 16 years with suspected HSE were prospectively enrolled. The patient sample included 137 (58%) men and 99 (42%) women, with ages ranging between 16 and 81 years (mean ± standard deviation: 44.24 ± 15.45 years). The patients' CSF specimens were submitted to the Professor Alborzi Clinical Microbiology Research Center, Namazi Hospital, Shiraz, Iran from November 2004 to May 2008 for diagnosis of HSV infection. The inclusion criteria were behavioral abnormality, progressive alteration of sensorium, focal or generalized seizures with or without focal neurological

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deficit, preceded by a history of prodromal phase of headache, fever and other constitutional symptoms. The initial samples were drawn from 0 to 5 days after the onset of clinical symptoms, before treatment. In the PCR positive patients, CSF samples were obtained before treatment, as well as whenever possible during treatment. CSF specimens were kept at -20°C until PCR assay.

Empirical treatment with acyclovir was started for every patient with suspected HSE, while waiting for the PCR result. Acyclovir therapy continued for patients with positive PCR results and also for a few patients whose physicians decided to administer treatment based on clinical findings and other workups like focal electroencephalographic (EEG), computed tomography (CT), and/or magnetic resonance imaging (MRI) results (intravenous acyclovir, 30 – 40 mg/kg/day, for 8 – 21 days).

Nucleic acid extraction

DNA was extracted from 200 µL of CSF using spin-column based QIAamp Mini Kit (Quiagen, Hilden, Germany). Extraction protocol for CSF was used as indicated by the manufacturer.

Real-Time PCR

The Real-Time quantitative PCR was performed with oligonucleotide primer pairs and probe specific for the type-common region of HSV-1 and HSV-2 glycoprotein B (gB), as reported previously.[10] The primers used were HSV-FP (5′- TCC CGG TAC GAA GAC CAG-3′) and HSV-RP (5′-AGC AGG CCG CTG TCC TTG-3′), and the probe was HSV-TCP (5′-FAM-TGG TCC TCC AGC ATG GTG ATG TTG/C AGG TCG-TAMRA-3′). Amplification was carried out in an Applied Biosystem Sequence Detector 7500 machine, programmed for a four-step protocol: 2 min of incubation at 50°C for AmpErase activation, 10 min at 95°C for polymerase activation and for 45 cycles: 15 s at 94°C for denaturation, 60 s at 58°C for annealing, extension and data collection. Each 50 µL-PCR mixture contained 10 µL of purified DNA, 840 nM concentrations of each primer, and 100 nM probe in 1x TaqMan universal PCR master mix (Applied Biosystems, Branchburg, New Jersey USA). Negative controls were included in the extraction process between every 20 clinical samples. All negative samples were tested twice.

Target amplicons were produced based on a standard PCR protocol by the above-mentioned primer sets and separated on agarose gel, then extracted and purified from gel and cloned into the pTZ57R vector (Fermentas UAB, Vilnius, Lithuania).[11] The plasmid containing the target sequences was purified using a commercial kit (Fermentas UAB, Vilnius, Lithuania) and its concentration was determined spectrophotometrically. Quantification was carried out by drawing standard curves using the serially diluted pTZ57R plasmid containing the target amplification product. Six positive control standards at 10^1, 10^2, 10^3, 10^4, 10^5 and 10^6 copies/reaction were used. The drawn standard curve was linear in 10 to 10^6 copies/reaction of the virus (R2 = 0.98). The copy numbers of the clinical samples were calculated automatically by applied biosystem sequence detection software v 1.3.1.

Statistical analysis

Descriptive statistic, Pearson Chi-square test, independent two tailed t-test, paired t-test and Wilcoxon signed ranks test were used for analysis of the data; p-value of less than 0.05 was considered significant. SPSS version 15 (Chicago, IL, USA) was used for statistical analysis.

RESULTS

Among the 236 patients enrolled in this study, 22 (9.3%) had positive PCR results. Thirteen PCR positive patients were male and 9 were female. Sequential CSF samples were available for 10 patients. HSV DNA was detected in a total of 34/42 (81%) CSF samples from these 22 patients. The demographic data and laboratory findings are shown in Table 1. No correlation was found between age, gender and positive PCR results ($p > 0.05$).

The copy number of HSV DNA, measured by the real-time PCR assay, ranged from 2.5×10^2 to 1.7×10^6 copies/mL in the initial CSF samples; median 4.8×10^4 copies/mL. Viral load in the initial CSF specimens of patients 29 and 77, with more than one sample for each, appeared lower than that in the second obtained sample. In both patients, the virus became undetectable, following a 21-day antiviral treatment. Nevertheless, the comparison of viral loads between the first and second samples, obtained from all patients, showed a decline in the latter ($p = 0.047$).

In patients with serial samples the initial viral load in CSF was positively correlated with the duration of the acyclovir treatment course ($p = 0.014$). Although no statistically significant correlation was found between the initial viral load in CSF and the number of positive PCR results, the data shown in Table 1 are somehow indicative of such a correlation, i.e., the greater the load, the longer the PCR remains positive during the course of treatment.

DISCUSSION

In this report, an in-house Real-Time quantitative PCR was used to detect the HSV DNA in CSF samples of 236 HSE suspected patients, and the amount of HSV DNA in the CSF samples from 22 HSE patients was measured. Age, gender and duration of acyclovir treatment with PCR positivity and/or levels of viral DNA loads were compared. Also, the effect of acyclovir therapy on the HSV DNA load is shown for 10 patients.

As reported in previous studies, if CSF specimens are collected within a couple of days after the onset of clinical manifestations and when CSF protein and white blood cell count

Table 1. Demographic data and laboratory findings of the 22 patients with herpes simplex virus encephalitis

Patient no.	Sex	Age (yr) after the on set (day)	CSF collection cell count (/mL)	Initial CSF leukocyte (mg/dL)	Initial CSF total protein therapy (day)	Duration of acyclovir	No. of samples (copies/mL)	Viral loads
6	F	55	3	134	103	14	1	3.7×10^4
10	F	43	2	300	98	14	1	4.1×10^4
25	M	26	1	460	135	8	1	2.5×10^2
29	M	47	1	57	35	21	4	1.6×10^4 2.1×10^4 1.5×10^3 0
40	M	32	0	65	58	14	1	8.5×10^3
44	M	57	2	525	316	21	3	1.7×10^6 4.5×10^5 3.9×10^4
55	F	44	2	137	120	14	1	7.2×10^4
65	F	26	1			9	1	3.4×10^3
77	F	24	0	140	90	21	4	6.7×10^4 8.1×10^4 2.8×10^2 0
95	M	30	1	78	53	16	1	1.8×10^5
105	M	62	3	113	88	21	1	1.3×10^6
115	M	80	2	22	97	14	3	6.9×10^4 5.4×10^3 0
132	M	40	0	17	65	8	2	2.7×10^3 0
135	F	41	2	75	93	10	2	4.1×10^3 0
139	M	68	1	235	172	18	3	2.8×10^4 1.3×10^4 4.2×10^2
156	F	35	2	502	43	21	4	3.9×10^5 2.7×10^3 7.8×10^2 0
169	M	26	2	421	76	18	1	8.3×10^5
186	F	22	3	160	81	9	2	5.6×10^3 0
193	M	50	2	193	88	14	3	6.6×10^4 8.4×103 0
206	M	44	1	6	35	16	1	5.5×10^4
218	F	58	2	25	93	14	1	4.3×10^5
232	M	61	3	12	84	8	1	5.7×10^3

F, female; M, male; CSF, cerebrospinal fluid.

are not highly elevated, the PCR test in HSE patients may turn out negative.[12,13] In the present study, there were some cases with a negative PCR test for which the antiviral treatment was pursued, based on the clinical grounds, MRI or CT scan findings. Although in most HSE cases the PCR result is positive, PCR negative infections have also been described.[14,15] In view of the fact that clinical and imaging findings cannot always serve as the basis for a definitive diagnosis of HSE,[16,17] it could be suggested that a second sample be collected and examined two days thereafter if the initial HSV PCR test was negative. In doing so, the chance of false negative results in the HSE diagnosis is reduced. Acyclovir therapy for HSE patients is usually successful and improves patient outcome.[18] Moreover, resistance to acyclovir in an immunocompetent individual from our region has not been reported.[19] Of the 10 patients with sequential CSF samples, treatment with acyclovir led to successful virus elimination in eight patients and in the remaining two it severely reduced viral load. Thus, all of them responded clinically to acyclovir therapy. The results also showed that duration of antiviral therapy of less than 8 days does not turn the positive PCR results negative.

There are a few reports indicating a correlation between the initial HSV DNA load and the patents' outcomes.[16] However, the majority of the literature on HSE patients reveals no association between the initial load of HSV DNA in CSF and the respective outcomes.[8,9,20,21] Although the present study avoided the analysis of the patients' outcomes, the results could be indicative of some association between the initial viral load and the number of subsequent positive PCR results and, more importantly, the duration of treatment. Also, it could be suggested that antiviral therapy should not be discontinued unless the PCR result becomes negative during treatment.

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