Diagnosis of neurological herpesvirus infections: real time PCR in cerebral spinal fluid analysis

Aurelia Gaeta, Simona Verzaro, Latte Maria Cristina, Carlo Mancini, Cristina Nazzari

Clinical Microbiology, Department of Science and Public Health, Sapienza University of Rome

SUMMARY

Human herpesviruses (HHVs) cause many serious acute and persistent central nervous system (CNS) disorders. Because these infections manifest with various, often non-specific, symptoms and signs, and because specific therapy is often available, accurate diagnosis is essential.

Cerebrospinal fluid (CSF) from 146 patients with acute meningitis or meningoencephalitis and 9 with "other neurological disorders" were analyzed by using an automatic system for nucleic acid extraction and quantitative real-time polymerase chain reaction (PCR) for herpes simplex 1 and 2 (HSV-1, HSV-2), Epstein-Barr virus (EBV), human cytomegalovirus (HCMV), herpesvirus-6 (HHV-6), and varicella-zoster virus (VZV).

HHVs DNA was detected in 52 of 155 (33.5%) analyzed samples. In 39 CSF samples from patients with meningoencephalitis we found: VZV in 13, HSV-1 in 12, EBV in 6, HHV-6 in 4, and HSV-2 in 4. Co-infections of EBV and HSV-2, HSV-1 and HSV-2, HSV-1 and VZV were also disclosed in four cases. In addition, two patients with Guillain-Barré syndrome had HCMV and one showed HHV6 positivity, two patients with myelitis / polymyeloradiculitis had VZV and HCMV respectively, HHV-6 DNA was found in one patient with lateral amyotrophic sclerosis. Three CSF specimens from HIV-infected patients with CNS complications had HHV-6 or EBV DNA.

Moreover quantitative data were also correlated to clinical conditions to obtain more information on the virus aetiopathogenic role.

KEY WORDS: Herpesvirus, Real Time PCR, Cerebrospinal fluid

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INTRODUCTION

Human herpesviruses (HHVs) cause various acute, subacute and chronic neurological diseases which are associated with significant morbidity and even mortality in both immunocompetent and immunocompromised subjects. HHVs cause neurological dysfunction and tissue damage directly by infecting neural cells, peripheral nerves or spinal ganglia, and/or through immunologically-mediated mechanisms (Kupila *et al.,* 2004; Skoldenberg *et al.,* 2006; Tyler, 2004). Herpes simplex 1 (HSV-1) and less frequently herpes simplex 2 (HSV-2), as well as varicella-zoster virus (VZV) and human cytomegalovirus (HCMV) are recognized causes of encephalitis, while VZV and HSV-2 also cause acute meningitis.

Meningoencephalitis and myelitis/radiculitis is caused by Epstein-Barr virus (EBV). Myelitis is associated with VZV, HCMV, EBV and HSV-2, while a concurrence of ventriculitis and encephalitis is caused by VZV and HCMV (Kupila *et al.,* 2006; Skoldenberg, 1996).

Immunocompromised subjects manifest different incidences and patterns of HHVs infections (Mommeja-Marin *et al.,* 2004). For example, EBV-associated primary central nervous system (CNS) lymphomas or VZV-mediated leukoencephalitis are restricted to this class of patients (Kleinschmidt-De Masters *et al.,* 2001). In addition, HHV6 - both variants A and B - exhibit neurotropism and are implicated as an important

Corresponding author
Aurelia Gaeta
Clinical Microbiology
Department of Science and Public Health
School of Medicine
Sapienza University
P.le A. Moro, 5 - 00185 Rome, Italy
E-mail: aurelia.gaeta@uniroma1.it

cause of neurological diseases in patients undergoing solid organ or bone marrow transplantation (Fotheringham *et al.,* 2007).
Recent advances in technological tools applied to molecular diagnosis have increased the spectrum of CNS diseases related to HHV: herpes simplex viruses have been implicated in schizophrenia (Yolken, 2004), Alzheimer's disease and radiculomyelitis (Kammerman *et al.,* 2006; Nakajma *et al.,* 1998), while HHV6 has been implicated in multiple sclerosis (Alvarez-Lafuente *et al.,* 2006), HCMV and EBV in Guillain-Barrè syndrome (Griffiths, 2004; Takahashi *et al.,* 2005).
Considering that HHVs manifest various and often not specific symptoms, accurate diagnosis is necessary and at present the polymerase chain reaction (PCR) for cerebrospinal fluid (CSF) analysis is considered the method of choice (Boivin, 2004; Gregoire *et al.,* 2006; Schovoerer *et al.,* 2006; Kleinschmidt-De Masters *et al.,* 2001). The International Herpes Management Forum (IHMF) has identified guidelines to improve diagnosis of CNS HHVs infections and PCR analysis of CSF is recommended for diagnosing HSV, CMV and VZV infections (Boivin, 2004).
PCR may also facilitate differential diagnosis between recurrent virus infection and post-infectious immune-mediated disease for which a different therapeutic management is required (Dong *et al.,* 2005; Weber, 1996). Moreover, genome quantization may be useful in assessing the severity of disease and for monitoring response to antiviral therapy, although this is not established for all HHVs infections (De Biasi *et al.,* 2002; Domingues *et al.,* 1998).
One limitation of PCR is its high sensitivity, which permits detection of minute viral genome quantities also in the absence of active viral infection, thus making it difficult to establish that a given HHV is the cause of a specific neurological illness (Kimberlin, 2005). Nevertheless, real time PCR technology is the best available approach for CSF molecular analysis as its high sensitivity and specificity provide rapid quantitative results reducing risk of contamination (De Labarthe *et al.,* 2005; Petitijean *et al.,* 2006; Rand *et al.,* 2005).
This study reports qualitative and quantitative data obtained using real time PCR in the analysis of cerebrospinal fluid. We evaluated the PCR results and the relationship with the diagnosis of meningoencephalitis and other CNS diseases related to herpesvirus infections.

PATIENTS AND SAMPLES

We analyzed 155 CSF samples collected from the same number of hospitalized adult patients between September 2006 and December 2007 (Table 1). The patients (99 men and 56 women - mean age 50.2 years ±11.74 S.D.) were from the Intensive Care Unit, Emergency, Neurology and Infectious Disease Departments of "Policlinico Umberto I" Hospital in Rome.
One-hundred and forty-six CSF out of 155 analyzed samples were obtained from patients with a presumptive diagnosis of acute meningitis or meningoencephalitis and lumbar puncture was

TABLE 1 - *Clinical evidence and demographic caracteristics of patients included in the study; age is reported as mean year (yr) ± S.D.*

Clinical evidence of neurological syndromes	*CSF**	*Male (yr ± S.D.)*	*Female (yr ± S.D.)*
Acute meningoencephalitis without focal defects	143	92 (52±18.2)	51 (37±10.1)
Acute meningoencephalitis with focal defects	3	2 (41±4)	1 (38)
Guillain-Barrè syndrome	3	1 (45)	2 (50±1)
Myelitis/radiculitis	2	1 (46)	1 (80)
Lateral amyotrophic sclerosis	1	1 (69)	-
Diffuse cerebral/cerebellar lesions in HIV pts	3	2 (38±3.5)	1 (38)

*No. of Cerebro Spinal Fluids (CSF) tested.

performed during the acute phase of neurological symptoms. Clinical conditions were characterized by headache, fever ≥37.5°C with no other explanations, meningism, confusion and CSF white-cell count ≥5/µl. Three of these patients also showed clinical symptoms of encephalitis characterized by clouding of consciousness and focal signs supported by central nervous system imaging suggestive of temporal lesions. Nine CSF specimens were obtained from subjects with "other neurological disorders": three patients had acute myelopathy consistent with Guillain-Barré syndrome, two showed myelitis and polymyeloradiculitis respectively, and one patient had supportive evidence of lateral amyotrophic sclerosis (LAS). Moreover, three patients showed signs of diffuse cerebral or cerebellar lesions thereby complicating progressive HIV infection showing as meningoencephalitis, cerebellitis and primary CNS lymphoma respectively. All CSF specimens were negative for microscopic and culture evidence of bacterial infections. Each sample was immediately processed for nucleic acid extraction and then analyzed to detect HSV-1/-2, HCMV, EBV, HHV-6, and VZV DNA presence.

DNA EXTRACTION

DNA was extracted by an automatic nucleic acid extractor (Nuclisens EasyMag - Biomerieux). Aliquots of 200 µl of each CSF sample were added to 800 µl of lysis buffer and the mix was loaded in the instrument; after lysis step (10 minutes at room temperature) 65 µl of magnetic silica, 10 µl of internal control (CPE, Nanogen Advanced Diagnostics S.r.l.), represented by purified beta globin sequence, and 55 µl of wash reagent were added to each specimen. DNA was automatically extracted, eluted in 55 µl of specific buffer, immediately used in real time PCR and an aliquot was stored at -80°C.

Real Time PCR

Extracted DNA was analyzed for the presence of HHVs by TaqMan real time PCR using a commercially available separate kit for each virus (Nanogen Advanced Diagnostics S.r.l.). The assays were performed by a multiplex format targeting both specific viral sequences and beta globin control gene by using 5' reporter dye 6-carboxyfluorescein (FAM)-labelled probes for viral genes and 5' fluorescein (VIC)-labelled probe for human beta globin (Table 2). PCR reactions were carried out with ABI PRISM 7000 (Applied Biosystem, USA) in 96-well plates, adding 5 µl of extracted DNA to 20 µl of amplification mixture. In each run we added virus-specific standards (10^2, 10^3, 10^4, 10^5 copies/5 µl) constructed with dilutions of plasmids carrying the specific viral genes. These standards were used to produce the reference curve to quantify viral DNA in samples. The real time PCR thermal profile consisted of a first cycle at 50°C for 2 minutes, a second cycle at 95°C for 10 minutes, and a third step which included 45 cycles at 95°C for 15 seconds. The analysis was performed in three hours in a single run, since it is possible to employ the same am-

TABLE 2 - *Characteristics of Real Time-PCR utilized in the study.*

	HSV-1	*HSV-2*	*CMV*	*VZV*	*EBV*	*HHV-6*
Target region	Glycoprotein D (gp D)	Glycoprotein G (gp G)	Exon 4 MIEA gene	Major DNA binding protein (ORF 29)	EBNA-1	ORF 13R region
Sensibility	10 copies/ reaction 250 copies/ml	10 copies/ reaction 250 copies/ml	10 copies/ reaction 250 copies/ml	10 copies/ reaction 250 copies/ml	10 copies/ reaction 250 copies/ml	10 copies/ reaction 250 copies/ml
Dynamic range	10-10^6 copies/reaction	10-10^6 copies/reaction	10-10^6 copies/reaction	10-10^6 copies/reaction	10-10^6 copies/reaction	10-10^6 copies/reaction
Diagnostic specificity	89.8%	95.4%	90-99 %	90-99%	>95%	>94.1%

plification thermal profile for all analyzed viral DNA sequences.
Computer software quantified the viral DNA load, reported as gen.copies/ml, taking into consideration the sample extracted volume, the final elution volume and the DNA volume utilized in amplification reaction. The analytical sensitivity, diagnostic specificity, and dynamic range of the assays are reported in Table 2. The low limit for the linear measuring range was 250 genomic copies/ml. Samples with specific fluorescence corresponding to genomic copies lower than the above values were reported as <250 gen. copies/ml.

RESULTS

Real time PCR assay on CSFs analysis disclosed HHV DNA in 52 (33.5%) of the 155 investigated samples. Forty-three out of 52 HHV DNA-positive specimens were obtained from immunocompetent adult patients with typical clinical signs suggestive of acute meningitis or meningoencephalitis.
Thirty-nine (90.6%) out of 43 CSFs proved positive to a single herpesvirus genome: VZV was detected in 13 samples (30.2%), HSV-1 in 12 (27.9%), EBV in 6 (13.9%), HHV-6 in 4 (9.3%) and HSV-2 in 4 (9.3%). Among these three CSF samples positive for HSV-1 DNA were obtained from patients with meningoencephalitis complicated by focal neurological symptoms (Table 3).
Among the other four (9.3%) positive CSF samples taken from patients with acute meningoencephalitis, co-infection was observed respectively between HSV-1/VZV (2 samples), between HSV1/HSV-2 (1 sample) and finally between EBV/HSV-2 (1 sample).
Nine out of the 52 HHV-DNA positive CSF samples were from subjects with clinical symptoms reported as "other neurological disorders" (Table 4). In three patients with suspected Guillain-Barré syndrome, real time PCR proved positive in one case for HHV-6 and in two cases for the HCMV genome. VZV was found in one patient with myelitis and HCMV DNA was detected in CSF from a patient with polymyeloradiculitis. HHV-6 genome was detected in the CSF from patient with LAS.
The analysis of the three CSFs obtained from HIV immunocompromised subjects disclosed HHV-6 DNA in one case with diffuse cerebral dysfunctions. EBV DNA was detected in CSF samples from the other two HIV patients with cerebellar lesions.

TABLE 3 - *Prevalence (%) of herpesvirus DNA detected in cerebro spinal fluid (CSF) from patients with acute meningoencephalitis. For each virus quantitative results are expressed as genomic copies/ml of CSF.*

Virus detected	*RT-PCR positive CSF samples*	*Male (yr ± S.D.)*	*Female (yr ± S.D.)*	*No. of genomic copies/ml*
VZV	13 (30.2%)	8 (46±9.5)	5 (44±12.6)	<250
HSV1	12 (27.9%)	4 (4±11.3) 1* (45) 1* (37) -	5 (41±19.1) - - 1* (38)	<250 <250 3840 3570
EBV	6 (13.9%)	3 (38±20.6)	3 (37±11.5)	<250
HHV6	4 (9.3%)	4 (52±17)	-	<250
HSV2	4 (9.3%)	3 (29±7)	1 (25)	<250
HSV1/VZV	2 (4.7%)	1 (46) -	- 1 (37)	<250/1080 448620/<250
HSV1/HSV2	1 (2.3%)	1 (32)	-	<250/<250
EBV/HSV2	1 (2.3%)	-	1 (65)	<250/<250

TABLE 4 - *Prevalence of herpesvirus DNA detected in cerebrospinal fluids (CSF) from patients affected by various neurological disorders; each line referts to data obtained from a single sample. For each virus, quantitative result is expressed as genomic copies/ml of CSF.*

Neurological disorder	*SEX (age)*	*Virus detected*	*No. of gen. copies/ml*
Guillain Barrè	M (45)	HHV-6	<250
	F (51)	HCMV	<250
	F (49)	HCMV	690
Amyotrophic lateral sclerosis	M (69)	HHV-6	<250
Myelitis	M (46)	VZV	<250
Polymyeloradiculitis	F (80)	HCMV	2630
Meningoencephalitis in HIV infection	M (42)	HHV-6	720
Primary CNS lymphoma in HIV infection	M (35)	EBV	12190
Cerebellitis in HIV infection	F (38)	EBV	542

The analysis of quantitative data showed that CSFs from patients with acute meningoencephalitis generally had a low viral load: we found DNA copies lower than 250 gen. copies/ml in 37 samples positive for a single virus and in 2 CSFs with HSV-1/HSV-2 and EBV/HSV-2 coinfections. On the other hand, high HSV-1 DNA levels were detected, 3570 and 3840 gen.copies/ml respectively, in 2 out of 3 CFSs taken from patients with meningoencephalitis with focal signs,. Moreover, in two cases of HSV-1/VZV co-infection we found <250 1080 and 448620/<250 gen.copies/ml respectively.

Concerning the "other neurological disorders", quantitative data showed HCMV DNA at 2630 gen.copies/ml concentration in CSF from polymyeloradiculitis and 690 gen.copies/ml in a case of Guillain-Barré syndrome. High EBV DNA levels (12190 genomic copies/ml) were detected in cerebrospinal fluid taken from an HIV-infected patient with cerebellar lesions in which a primary CNS lymphoma was diagnosed.

DISCUSSION

Herpesviruses are related to various acute, subacute, and chronic CNS diseases both in immunocompetent and in immunocompromised patients (Kleindschmidt-DeMasters *et al.,* 2001; Gilden, 2001). In this field, real time PCR has optimized several aspects of molecular analysis, so providing easier standardization and more rapid results (Rand *et al.*, 2005). In this study we used a nucleic acid automatic extraction and a Real Time PCR for the diagnosis of herpesviruses in acute meningoencephalitis and in other pathological features related to HHV infections.

Our resulting data showed the Herpesvirus genome in 33.5% of investigated CSF samples. In acute meningoencephalitis, VZV DNA was revealed in the highest percentage of cases followed by HSV-1 and EBV while HSV-2 and HHV-6 were detected only a low percentage of cases. About 10% of positive CSF samples from patients with clinical features of acute viral meningoencephalitis showed DNA representative of co-infection.

This matches the findings of other authors (Cinque *et al.,* 1996; Landergreen *et al.,* 1994; Studhal *et al.*, 1994) who reported that herpesvirus co-infections may occur in CNS disease. Tang hypothesized that, by disturbing immune homeostasis, herpesvirus could have an immunosuppressive effect which allows CNS co-infection to occur (Tang *et al.,* 1997).

An aspect still being discussed concerns the role of the herpesvirus in various CNS disorders, given that these viruses are latent pathogens that may be reactivated during other diseases where such viruses might not constitute the aetiopathogenic agents.

Although the involvement of herpesvirus has been confirmed in some CNS clinical conditions, other neurological diseases need to be further investigated. For example, EBV, HCMV and HHV-6 genome have been detected in CSF samples from patients with Guillain-Barré syndrome (Bitan *et al.,* 2004; Dewhurst, 2004; Steininger *et al.,* 2004). An aetiological role of HHV-6 in sporadic LAS has also been suggested (Cermelli *et al.,* 2003).
Another interesting aspect concerns the viral load evaluation in the analysis of CSF samples that is being increasingly applied.
It is recommended for herpes simplex encephalitis and for HCMV CNS infections to assess disease severity and to monitor antiviral therapy. However, further data are required for the interpretation of quantitative PCR results for EBV, HHV6 and VZV, experimental data on all of these being limited. Additional studies on quantitative methods may help to establish the role of HHVs infection for cases in where qualitative results may be considered indicative. Nonetheless such results do not provide definitive evidence of a viral aetiopathogenic role (Boivin, 2004; Wildemann *et al.,* 1997).
Concerning the quantitative results, our data showed the presence of VZV, EBV, HSV-2 and HHV-6 DNA in low copies in CSFs from patients with acute meningoencephalitis while in cases complicated by focal symptoms, high levels of HSV-1 DNA were detected.
In CSF samples from patients with other neurological features, we found HCMV in two cases of Guillain Barré syndrome and in a CSF from a patient with polymyeloradiculitis.
In the latter case a significant level of DNA virus was revealed (2630 gen/ml), matching other reports where the HCMV DNA load in patients with polyradiculopathy was higher than in patients with encephalitis (Shinkai *et al.,* 1995; Arribas *et al.,* 1995). HHV6 at low concentrations (<250 gen/ml) was found in CSF from a patient with Guillain Barré syndrome and in one patient with LAS.
HHV-6 DNA was revealed at 720 gen.copies/ml in a HIV positive subject with meningoencephalitis with diffuse cerebral lesions. At present HHV6 is recognized as an important agent in transplant recipients and HIV patients and, interestingly, a recent study confirmed that active HHV-6 brain infection may not be reflected in viral DNA detected in CSF (Fotheringam *et al.,* 2007).
In the present study the association of real time PCR with a new automatic nucleic acid extraction method proved to be a reliable and rapid standardized (turnaround time of three hours) procedure for the detection of the herpesvirus genome in CSF.
Taken together, the illness history, associated clinical findings and appropriate laboratory tests all provide essential elements for prompt diagnosis of CNS viral infections and specific therapeutic management.

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