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PCR-based approach for qualitative molecular analysis of six neurotropic pathogens

R. FERESE[1], L. SCORZOLINI[1,2], R. CAMPOPIANO[1], V. ALBANO[1], A. M. GRIGUOLI[1], E. GIARDINA[3,4], S. SCALA[1], L. RYSKALIN[5], C. D'ALESSIO[1], S. ZAMPATTI[1,3], R. FANTOZZI[1], M. STORTO[1], F. FORNAI[1,5], S. GAMBARDELLA[1]

[1]IRCCS Neuromed, Località Camerelle, 86077, Pozzilli (IS), Italy; [2]Infectious Disease Unit, Department Public Health and Infectious Disease, "Sapienza" University of Rome, Italy; [3]Molecular Genetics Laboratory UILDM, Santa Lucia Foundation, Rome, Italy; [4]Department of Biomedicine and Prevention, School of Medicine, University of Rome 'Tor Vergata', Rome, Italy; [5]Department of Translational Research and New Technologies in Medicine and Surgery, University of Pisa, Pisa, Italy

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Summary. – In the last few years, polymerase chain reaction analysis is frequently required to improve the detection of pathogen infections in central nervous system as a potential cause of neurological disorders and neuropsychiatric symptoms. The goal of this paper is to set up a fast, cheap and reliable molecular approach for qualitative detection of six neurotropic pathogens. A method based on PCR has been designed and implemented to guarantee the qualitative DNA detection of herpes simplex virus types 1 and 2 (HSVI/II), Epstein-Barr virus (EBV), cytomegalovirus (CMV), varicella-zoster virus (VZV), rubella virus (RUBV) and *Toxoplasma gondii* in the cerebrospinal fluid, where otherwise they are barely detectable. Each PCR assay was tested using dilutions of positive controls, which demonstrated a sensitivity allowing to detect up to 10^2 copies/ml in PCR and 10 copies/ml in real-time PCR for each pathogen. Once been set up, the protocol was applied to evaluate the cerebrospinal fluid from 100 patients with suspected infectious diseases of the central nervous system and 50 patients without any infection. The method allowed to identify 17 positive cerebrospinal fluid with polymerase chain reaction and 22 with real-time PCR (RT-PCR), respectively. Therefore, application of RT PCR allows a fast and sensitive evaluation of neurotropic DNA pathogens in the course of diagnostic routine within neurological units.

Keywords: central nervous system; neurotropic infections; PCR; real-time PCR

Introduction

A wide range of viruses, including herpes simplex virus types 1 and 2 (HSV I/II), cytomegalovirus (CMV), varicella-zoster virus (VZV), Epstein-Barr virus (EBV), human herpes virus 6 (HHV-6), the enterovirus group (echoviruses, polioviruses, coxsackieviruses), adenoviruses, John Cunningham (JC) and BK viruses, arenaviruses, paramyxoviruses, rabies and arboviruses, are associated with neurological diseases of the central nervous system (CNS). The identification of these pathogens, especially within cerebrospinal fluid (CSF), is crucial for establishing specific therapy of patients affected by neurological and neuropsychiatric symptoms. For instance, detection of acute EBV infection in the CNS is critical due to its association with meningoencephalitis and CNS lymphomas (CNSLs). This is significant since complications from EBV infections range from 1 up to 18% of patients with infectious mononucleosis and include encephalitis, meningitis, cerebellitis, polyradiculomyelitis, transverse myelitis, cranial and peripheral neuropathies and psychiatric abnormalities (Connelly and Witt, 1994).

In the last few years, PCR is considered as a valuable tool for identifying pathogen DNA within CSF. In fact, this technique is based on the exponential amplification of a template DNA, which allows to detect a pathogen-related DNA even

E-mail: ferese.rosangela@gmail.com; phone: +390865915209.

Abbreviations: CMV = cytomegalovirus; CNS = central nervous system; CSF = cerebrospinal fluid; EBV = Epstein-Barr virus; HSV I/II = herpes simplex virus types 1 and 2; HV = herpes virus; JC = John Cunningham; RT-PCR = real-time PCR; RUBV = rubella virus; TGR = *Toxoplasma gondii*; VZV = varicella-zoster virus

when this is present in a very low amount. Further improvements of this protocol, like real-time PCR (RT-PCR), allow to further improve the detection sensitivity compared with PCR. Thus, RT-PCR represents the most sensitive method to detect pathogen infections within CNS based on CSF samples.

PCR-based protocols have been included in a number of guidelines for the management of patients with CNS infectious diseases. For instance, PCR approaches represent the gold standard to detect meningitis, encephalitis caused by a variety of viruses, HSV encephalitis, HV and JC in CSF (Read *et al.*,1997; Mamoojee and Chadwick, 2011) and viruses in brain biopsies for the diagnosis of progressive multifocal leukoencephalopathy (Lindquist *et al.*, 1988; Webet *et al.*, 1994; Lakeman and Whitley 1995; Read *et al.*,1997; Tang *et al.*, 1999; Boivin, 2004; Mamoojee and Chadwick, 2011).

Although for certain tests PCR is currently more cost-effective than previous diagnostic tools, the average cost of a PCR-based test is considered quite expensive when compared with microbiological approaches (Lakeman and Whitley, 1995). This limit can be overcome considering that PCR allows a quick and sensitive diagnosis, which reduces hospitalization and the bias of wrong diagnosis with mis-treatment costs (Fredricks and Relman, 1999; Chadwick and Lever, 2002).

The goal of this paper is to set-up a qualitative, fast, cheap and reliable molecular approach to detect a range of neurotropic viruses potentially involved in neurological and neuropsychiatric disorders such as HSV I/II, EBV, CMV, VZV, RUBV as well as *Toxoplasma gondii* (TGR) in CSF samples from patients with suspected infectious CNS diseases, where the pathogen DNA or RNA may be present in a very low amount.

Materials and Methods

Clinical specimens. In the time interval 2012–2015 at Neuromed Institute of Pozzilli, Isernia, Italy, we collected 100 CSF from patients admitted to the hospital with signs and symptoms of likely, possible or unlikely CNS infection according to the criteria by Jeffery et al. (1997), which were modified ad-hoc considering the kind of patients routinely admitted at Neuromed Institute (patients with recent neurosurgery and fever, patients with known neurological disorder and worsening of mental status etc). In addition, we obtained other tissues (blood, broncho alveolar lavage, and sputum) from patients affected by neurological disorders with a recent history of infection to rule out the bias of any ongoing extracranial infection. Moreover, we tested 50 CSF from patients without any infection. A written informed consent was obtained from each patient.

We considered the presence of a CNS infection as likely when one of the two following conditions was present: i) any rise of white cell count within CSF accompanied by one or more of the following: meningism, headache of fever (≥ 37.5°C) with no other explanation; ii) onset of altered consciousness or focal neurological signs accompanied by fever (≥ 37.5°C) or headache with no other explanation. A CNS infection was considered as possible in the presence of a combination of various signs and symptoms. A CNS viral infection was considered unlikely in the following conditions: i) the concomitancy of a specific neurological diagnosis (for example, multiple sclerosis or bacterial meningitis); ii) the absence of specific neurological symptoms (for example, fever of unknown cause, fever in recent neurosurgery and worsening of mental status in already known neurological disorder in the absence of signs of CNS infections). A CSF specimen was defined as abnormal if any of the following was present: white blood cell count (WCC) > 5 cells/mm^3, protein level > 0.5 g/l; CSF glucose < 2.2 or CSF:serum glucose ratio < 0.4. Clinical data were collected from all enrolled patients. Demographics, clinical, radiological and laboratory data were collected and CSF examinations were correlated with the PCR results. We considered separately immunosuppressed patients, including the following: HIV seropositive, patients receiving chemotherapy, transplants recipient or other immunodeficiency.

Selection of the genomic regions. According to literature data, the most conserved and well-known DNA and cDNA regions for each pathogen were selected. These genomic regions include: i) 35-fold repeated B1 gene for TGR (Burg *et al.*, 1989; Robert-Gangneux *et al.*, 1999; Sterkers *et al.*, 2012; Teixeira *et al.,* 2013), ii) UL23 gene of the thymidine kinase that catalyses a necessary phosphorylation step of acyclovir for HSV I/II (Van der Beek *et al.*, 2013), iii) conserved regions of EBV genome encoding capsid protein gp220 (Adldinger *et al.*, 1985; Telenti *et al.*, 1990), iv) glycoprotein E for VZV (Cooray *et al.*, 2006; Grahn *et al.*, 2011; Thomsson *et al.*, 2011) v) the variable region within the E1 gene for RUBV (Cooray *et al.*, 2006). As far as the detection of CMV is concerned, a variety of amplification targets so far have been used based upon the immediate-early region (IEA1) (Jiwa *et al.*, 1989; Gerna *et al.*, 1991; Nyberg *et al.*, 1994; Espt *et al.*, 1995). This region of the CMV genome has been shown to possess sporadic sequence variation among clinical strains, and primer mismatching has been shown to reduce the amplification efficiencies of PCR assays (Chou, 1992). To avoid this, U8, another well-characterized region coding for membrane proteins of CMV was selected. The selected regions for each pathogen were considered for the design of primer pairs suitable for PCR applications (Table 1). Primers have been improved for PCR, not considering nested PCR approaches characterized by a higher risk of contamination and decreased sensitivity (Rozenberg and Lebon, 1991; Johnson *et al.*, 2000).

RNA extraction and reverse transcription PCR. For rubella analysis, total RNA was isolated from CSF using TRIzol Reagent (Invitrogen, Life Technologies) according to the manufacturer's instructions. The concentration and purity of RNA samples were

Table 1. Primers used for PCR and RT-PCR

Primer	Sequence	PCR band size	Gene	Acc. No.
TGR-FW	5'-TGA AGA GAG GAA ACA GGT GGT CG-3'	131 bp	B1	NW_002234552
TGR-RW	5'-CCG CCT CCT TCG TCC GTC GTA-3'			
HSV-I/II-FW	5'-ATC AAC TTC GAC TGG CCC TTC-3'	179 bp	UL30	NP_044632
HSV-I/II-RW	5'-CCG TAC ATG TCG ATG TTC ACC-3'			
EBV-FW	5'-GGC TGG TGT CAC CTG TGT TA-3'	239 bp	gp220	NC_007605
EBV-RW	5'-CCT TAG GAG GAA CAA GTC CC-3'			
CMV-FW	5'-GGA TCC GCA TGG CAT TCA CGT ATG T-3'	408 bp	US7	NC_006273
CMV-RW	5'-GAA TTC AGT GGA TAA CCT GCG GCG A-3'			
VZV-FW	5'-CAT AAC TCA CCT TAT ATA TGG CC-3'	677 bp	Glycoprotein E	NC_001348.1
VZV-RW	5'-AAA AGC TCC AAG TCT CGG TGT-3'			
RUBV-FW	5'-CAA CAC GCC GCA CGG ACA AC-3'	185 bp	E1	NC_001545
RUBV-RW	5'-CCA CAA GCC GCG AGC AGT CA-3'			
β-Actin-FW	5'-CAC ACT GTG CCC ATC TAT GAG G-3'	411 bp	Exon 1	NM_001101.3
β-Actin -RW	5'-GAA GAA ATG AGG GCG GAC TTA G-3'			

determined using Nanodrop 2000 (Thermo Scientific, Life Technologies). Total RNA (1 μg) was reverse transcribed (RT) with SuperScript® VILO™ (Invitrogen, Life Technologies) with Oligo dT primers.

DNA extraction. DNA extraction from CSF and other tissues (blood, broncho alveolar lavage, and sputum) has been performed through the NucleoSpin® (MACHEREY-NAGEL GmbH & Co. KG).

PCR conditions. The PCR was performed using 25 μl DNA (100 ng) or cDNA (1 μl of reverse transcription), 1x PCR Buffer, 1 mmol/l $MgCl_2$, 0.2 mmol/l dNTPs, 25 pmol/μl specific forward/reverse primer, 1.25 U/μl GoTaq Flexi DNA polymerase (Promega, Sunnyvale, CA, USA), using the following cycling conditions: initial denaturation 95°C for 3 min, 95°C for 1 min and 50 s, 62°C for 45 s, 72°C for 2 min, for 40 cycles, final extension 72°C for 10 min and 4°C for 1 min. PCR amplification of each pathogen genome was tested using serial dilution of positive controls ranging from 10^4 to 10 copies (AB Analitica S.r.l.).

PCR detection. PCR products were detected by agarose gel electrophoresis using 2% agarose gel in 1x TBE buffer.

Qualitative real-time PCR (RT-PCR). PCR primers were applied to RT-PCR with SYBR® Green Master Mix (Invitrogen Life Technologies, Carlsbad, CA) (Watzinger *et al.*, 2004). RT-PCR amplification was performed using 50 ng/μl DNA or cDNA in a 25 μl of reaction with 300 nmol/l of forward and reverse primer specific for each pathogen (Table 1). The reaction mixtures were amplified using the following thermal cycling conditions: an early denaturation and polymerase activation step for 10 min at 95°C followed by 40 cycles of 95°C for 45 s and 62°C for 60 s. Amplification and detection were performed on a CFX Connect™ Real Time System (Bio-Rad Life Science, CA) and Rotor-Gene™ 3000 (Corbett Research, Qiagen). For each sample β-actin was amplified to confirm the efficiency of DNA extraction.

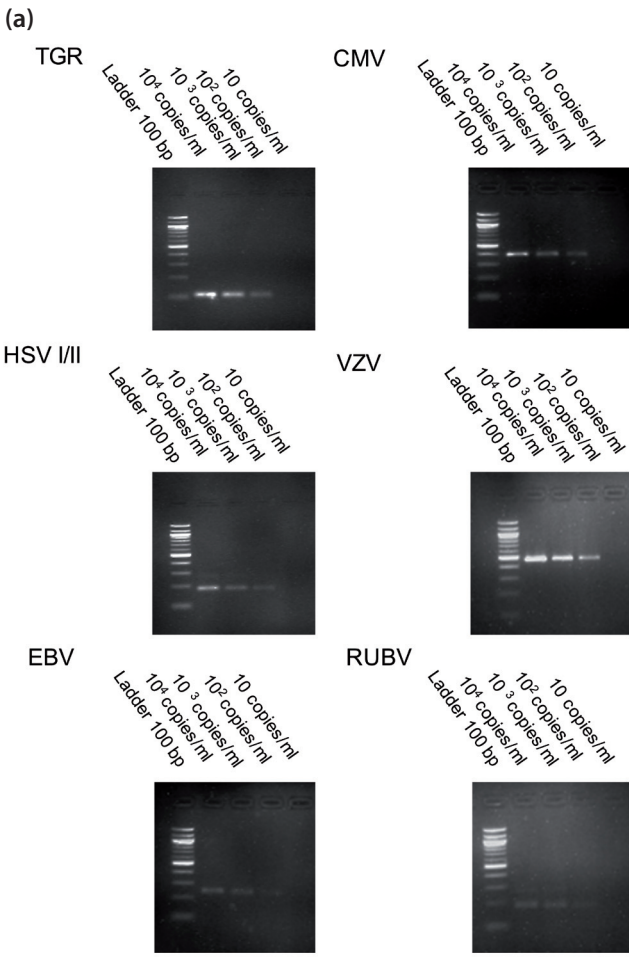
The cycle threshold (Ct) values from unknown samples were plotted on these standard curves and the ratio of the number of virus genome per cell was calculated. Curves were generated by the Sequence Detection System software, plotting Ct against the number of viral genome copies. Run acceptability was defined as a correlation coefficient (R2) > 0.98 and a slope between -3.6 and -3.1, corresponding to reaction efficiencies between 90 and 110%, according to the equation: Efficiency = 10(-1/slope) – 1 (Yuan *et al.*, 2006).

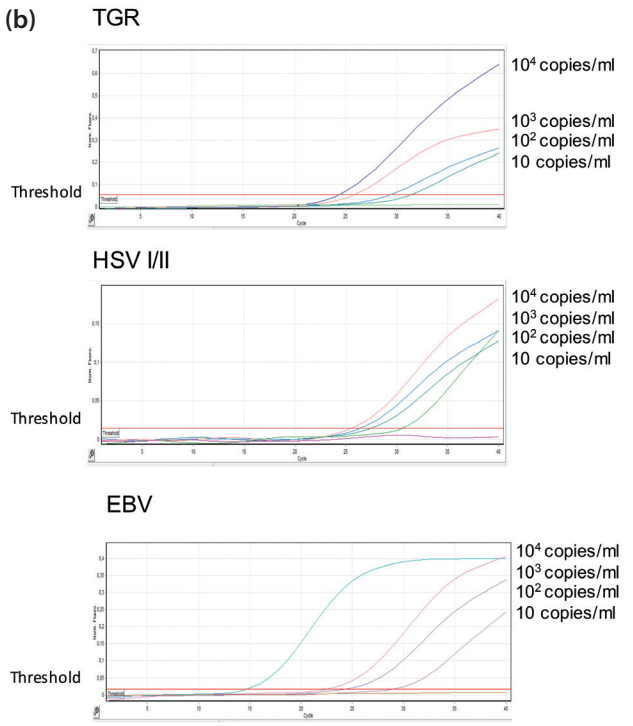
Results

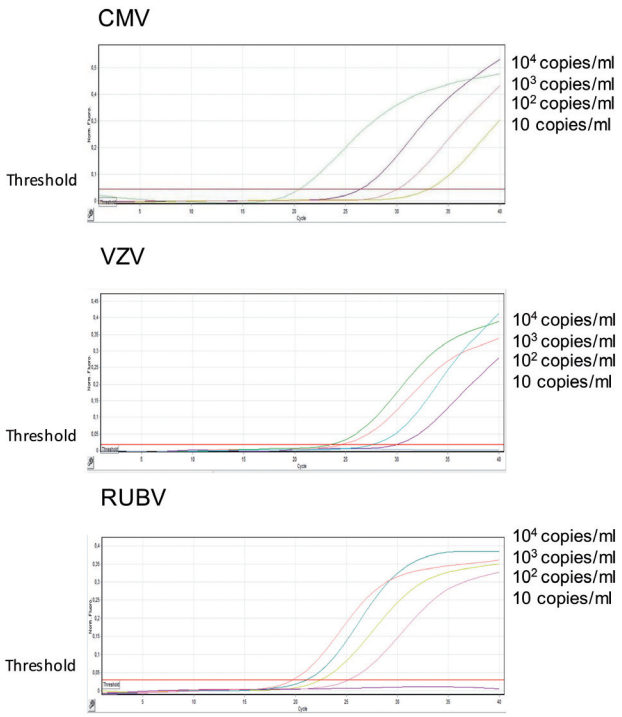
In this work we designed and implemented a PCR-based approach for qualitative detection of six neurotropic pathogens (HSV I/II, EBV, CMV, VZV, RUBV and TGR). According to the literature the most conserved and well-known DNA and cDNA regions for each pathogen were selected, and primers pairs suitable for PCR applications were designed (Table 1). Each PCR product was tested using as positive controls six commercially available genomic DNA (HSV I/II, EBV, CMV, VZV and TGR) or cDNA (RUBV), in four different dilutions ranging from 10^4 to 10 copies (Fig. 1).

As shown in Fig. 1a, PCR on 2% agarose gel was able to detect each pathogen in a concentration of 10^4, 10^3 and 10^2 copies/ml, but could not detect lower concentrations (10 copies/ml).

To improve the detection below 10^2 copies/ml, the same set of primers and positive controls were evaluated in RT-PCR application using SYBR green (Fig. 1b). RT-PCR application strongly improved the detection rate up to 10 copies/ml for each pathogen.







Then, the PCR-based protocols (PCR and RT-PCR) were evaluated in 100 CSF from patients admitted to the hospital with signs and symptoms of likely, possible or unlikely CNS infection, as reported in materials and methods.

PCR detected 17 positive samples (1 for TGR, 1 for HSVI/II, 6 for EBV, 7 for CMV, 1 for VZV and 1 for RUBV), while RT-PCR detected 22 positive samples (1 for TGR, 1 for HSVI/II, 8 for EBV, 10 for CMV, 1 for VZV and 1 for RUBV). The application of RT-PCR protocol improved the detection rate of 5 out of 100 patients (2 positive samples for EBV and 3 positive samples for CMV). In support of this, in Fig. 2 are shown molecular analyses for EBV (Fig. 2a) and CMV (Fig. 2b) respectively, in CSF from two different patients who were diagnosed as negative by PCR on agarose gel and positive by RT-PCR approach.

The same PCR-based protocols (PCR and RT-PCR) were evaluated in 50 CSF from patients without CNS infection, and no healthy positive subjects and false-positive were detected.

The possibility to detect up to 10 copies/ml allows to improve the detection rate of this protocol, suitable for the identification of HV in CSF from neurological patients.

Fig. 1

PCR products obtained from serial dilutions of positive control
In this figure are shown PCR products obtained from serial dilutions (10^4, 10^3, 10^2,10) of positive controls for TGR, HSVI/II, EBV, CMV, VZV and RUBV detected in PCR separated on 2% agarose gel **(a)** and detected by RT-PCR **(b).**

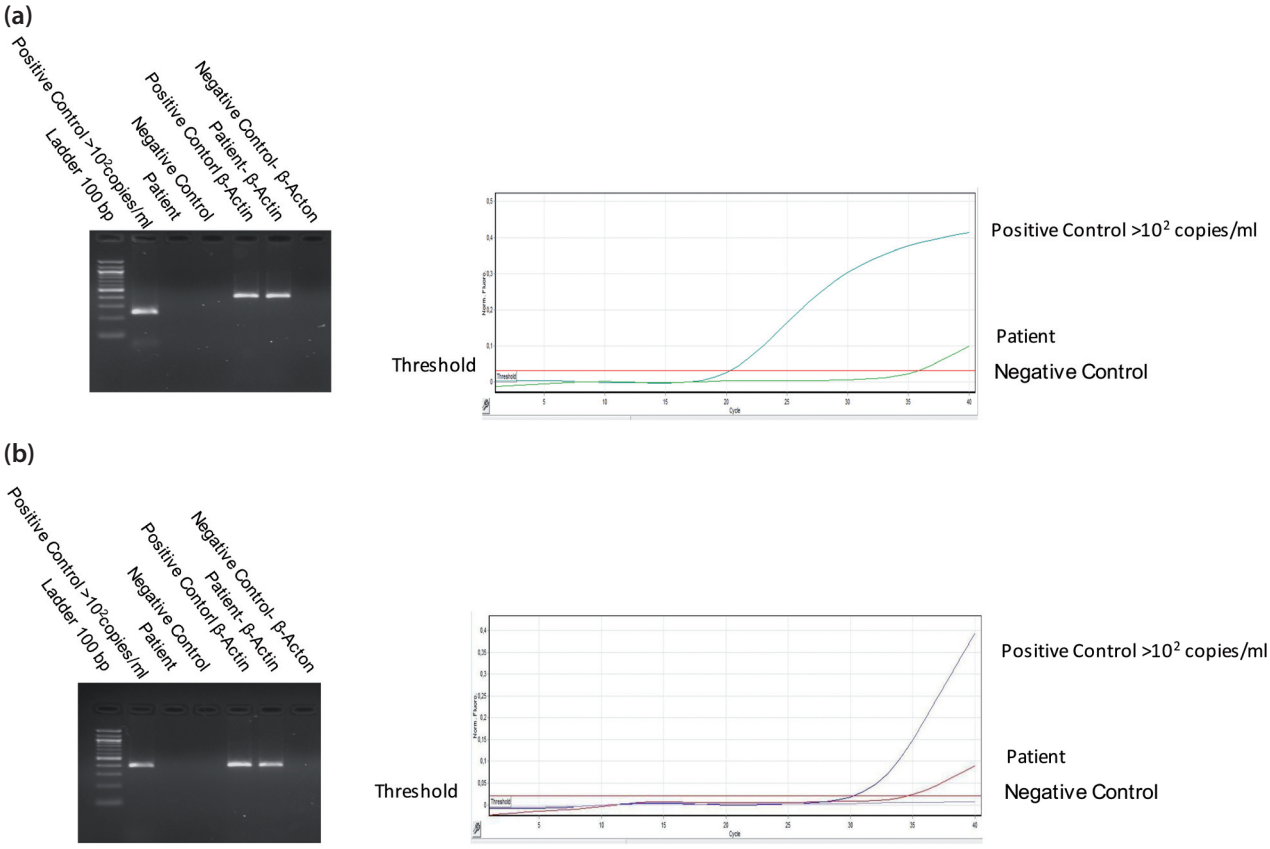


Fig. 2

PCR (left) and RT-PCR (right) analysis for EBV (a) and CMV (b) respectively, in CSF from two different patients

On the left side are shown PCR product separated on 2% agarose gel for EBV (A) and CMV (B). Lane 1: molecular weight standard (100 bp DNA Ladder); Lane 2: positive control with >10^2 copies/ml viremia; Lane 3: patient analysed; Lane 4: PCR negative control; Lane 5: positive control for β-actin; Lane 6: patient for β-actin; Lane 7: PCR negative control for β-actin. On the right side are shown RT-PCR plots for EBV (A) and CMV (B). Amplification curves: i) positive control with >10^2 copies/ml viremia; ii) patient analysed; iii) negative control.

Discussion

Detection of HV in CSF is quite complex since these pathogens are often present in very few copies. The PCR is a powerful molecular approach that is increasingly applied to the diagnosis of infectious diseases. This technique is based on the amplification of specific nucleic acid sequences of DNA or cDNA, and this allows to detect a pathogen genome in CSF also when it is present in low copy numbers.

Thus, PCR represents a unique approach for the molecular diagnosis of CSF in CNS infection. All PCR-based assays detect pathogen nucleic acid in clinical samples and do not require growth of the organisms, and this offers the possibility of a quick, sensitive, and specific diagnostic approach. In the clinical routine of the Neurological Institute, this approach is fundamental to prioritize the optimal therapeutic treatment and to reach a fast and reliable diagnosis. For example, a large amount of patients are admitted for fever after a recent history of neurosurgery or fever, which occurs during worsening of mental status in concomitancy with a neurological disorder. In these cases, PCR detection of pathogen DNA is an important tool to confirm/exclude an empirical antimicrobial treatment and to adopt reliable examinations to assess the specificity of otherwise general neurological symptoms.

Unfortunately, molecular identification of pathogen DNA in CSF during neurological disorder is not well defined yet. This is the case of CMV DNA that is frequently detected in the CSF of patients during acute neurological disorders. Different studies show a strong association between CMV virus in CSF and Guillain-Barrè Syndrome (GBS) (Steininger *et al.*, 2004), where the re-activation of a persistent CMV infection during the course of GBS in the CNS is likely to represent the causal mechanism. Moreover, CMV was found

by PCR in 8% of post-mortem brain samples from patients without neurological disorders, to underline the specific ability of CMV to cross the blood-brain barrier (BBB) and remaining latent in the CNS after a primary infection (Sanders *et al.*, 1996).

We developed PCR-based protocols that were evaluated in 100 CSF from patients admitted to the hospital with signs and symptoms of likely, possible or unlikely CNS infection.

PCR detected 17 positive samples (1 for TGR, 1 for HSVI/II, 6 for EBV, 7 for CMV, 1 for VZV and 1 for RUBV), while RT-PCR detected 22 positive samples (1 for TGR, 1 for HSV I/II, 8 for EBV, 10 for CMV, 1 for VZV and 1 for RUBV). The application of RT-PCR protocol improved the detection rate of 5 out of 100 patients (2 positive samples for EBV and 3 positive samples for CMV), as shown in the examples.

According to the study of Davies and colleagues (Davies *et al.*, 2005), in our study we observed mostly EBV-positive PCR in patients with unlikely infection rather than HSV and CMV, but the number of specimens associated with the clinical symptoms is still too small to allow any conclusion. Previous data reported high predictive value of EBV PCR detection in HIV- associated lymphoma, but other more recent studies reported lower sensitivity (Cinque *et al.*, 1993; Ivers *et al.*, 2004). EBV-positive PCR results should therefore be interpreted with caution, also considering that in most cases the detection of EBV in CSF occurs in the absence of any CNS involvement.

Thus, EBV detection in CSF might be the result of unusual presentation of a CNS viral infection, as well as an epiphenomenon. EBV might be present in CSF either due to the reactivation of the virus after concurrent stress to the host or it may be due to the latent infection of leukocytes trafficking into the intrathecal space (Davies *et al.*, 2005). For instance, the leukocytes carriage of EBV and CMV has already been described in the synovial fluid and tissue of diseased joints (Stahl *et al.*, 2000).

In summary, although the use of commercial positive control for each genome points out the possibility to detect up to 10 genome copies/ml with RT-PCR, a large scale validation considering several positive and negative CSF is strongly required to establish the actual sensitivity and specificity of this approach. Then, this protocol could be integrated with clinical routine exams for a quick diagnosis and a better patient management.

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