

Simultaneous detection of viruses and *Toxoplasma gondii* in cerebrospinal fluid specimens by multiplex polymerase chain reaction-based reverse hybridization assay

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SUMMARY

The lack of rapidity and the low sensitivity and specificity of traditional laboratory methods limits their usefulness in the laboratory diagnosis of viral central nervous system (CNS) infections. This study describes the use of a commercially available multiplex polymerase chain reaction (mPCR)-based reverse hybridization assay (RHA) for the simultaneous detection of the genomes of 8 viruses and *Toxoplasma gondii* in cerebrospinal fluids (CSF) from 181 patients suspected of having viral meningitis. Twenty-two/181 (12.15%) CSF samples resulted positive by mPCR. Eighteen/22 were positive for 1 viral pathogen, whereas a dual infection was detected in 4/22 samples. Epstein-Barr virus (EBV) was the most commonly detected virus (6/22), followed by herpes simplex virus type-1 (HSV-1) (5/22) and -2 (HSV-2) (4/22). Cytomegalovirus (CMV), human herpesvirus-6 (HHV-6), and Epstein-Barr virus (EBV) were detected in 1 specimen each. Two CSF samples were co-infected by HSV-1/HSV-2, 1 sample by HHV-6/*T. gondii*, and 1 sample by EBV/EBV, respectively. Our data support the usefulness of mPCR as a rapid molecular method for the simultaneous detection of major viral pathogens and *T. gondii* in aseptic meningitis also to allow the earlier application of specific antiviral therapy.

KEY WORDS: CNS infection, Multiplex PCR assay, Reverse hybridization assay (RHA), Viruses, *Toxoplasma gondii*

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INTRODUCTION

Aseptic meningitis is frequently caused by a wide range of viruses and a potential cause of infection is also represented by *Toxoplasma gondii* (Rice, 2005).

The traditional laboratory methods for detection of the viral pathogens suffer from limitations, whereas testing for various pathogens using multiple primer sets in multiplex PCR (mPCR) reac-

tions offer significant benefits in terms of costs, time and accurate diagnosis (Elnifro, *et al.*, 2000, Markoulatos, *et al.*, 2002). Therefore, the rapid identification of the causative agents in cases of aseptic meningitis contributes to better patient outcome, also reducing unnecessary treatments (e.g. broad-spectrum antibiotics). This study describes the use of a commercially available mPCR-based reverse hybridization assay (RHA) allowing the simultaneous identification of up to 9 common, clinically significant pathogens, on a larger number of cerebrospinal fluid (CSF) specimens from patients suspected of having aseptic meningitis. In particular, the assay screened for herpes simplex virus type 1 (HSV-1), herpes simplex virus type 2 (HSV-2), human herpesvirus type 6 (HHV-6), cytomegalovirus (CMV), Epstein-Barr virus (EBV), varicella-zoster virus (VZV), JC papovavirus (JCV), enterovirus (EV) and *T. gondii*.

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MATERIALS AND METHODS

Specimens

CSF samples were collected consecutively from 181 patients suspected of having aseptic meningitis at different Departments of the Ospedali Riuniti, Foggia, Italy, during the period between January 2006 and March 2008. Four of the 181 patients were HIV-infected. Aseptic meningitis was suspected on the basis of either clinical signs and symptoms or pleocytosis (>5 white blood cells/mm³) in CSF as well as the negative cultures for bacteria and fungi (Rice, 2005). Patients' ages ranged from a few days to 80 years, with 71 subjects for whom age was not determined. No other clinical information was available for this analysis. Upon receipt in the diagnostic laboratory, the specimens were processed or stored at -70°C until the study.

Genomic extraction

From 400 µl of CSF specimens the total nucleic acids were extracted using the EZ1 Virus Mini Kit v 2.0 (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Extractions were performed by using the BioRobot® EZ1 workstation (QIAGEN). Nucleic acids were suspended in 60 µl of DNase/RNase-free sterile distilled water (Sigma, Milan, Italy) and used in mPCR assay along with negative controls.

mPCR-based assay

mPCR assay was performed using the kit RHA CNS (Labo Bio-medical Products B.V., EV Rijswijk, The Netherlands) designed for the simultaneous amplification of a single-stranded RNA genome of EV and DNA genomes of HSV-1, HSV-2, VZV, HHV-6, CMV, EBV, JCV, and *T. gondii*. As reported by the manufacturer, species-specific primers and probes have been designed, on the basis of already published sequences. The simultaneous amplification in the mPCR assay was performed using 3 oligonucleotide PCR-mix in 3 separate reactions, "reaction 1" for the reverse transcription PCR for EV RNA, "reaction 2" for mPCR for HSV-1, HSV-2, VZV, and HHV-6, and "reaction 3" for mPCR for CMV, EBV, JCV, and *T. gondii*, respectively. mPCRs were carried out in a final volume of 50 µl of each PCR-mix along with the primers specifically designed to amplify the regions of pathogens. In each set of tested samples

50 µl of PCR-mix without DNA-target were used as negative controls, while the positive controls were represented by previous positive specimens. The cycling conditions were as follows: pre-incubation for 15 min at 94°C for activation of the AmpliTaq® Gold DNA polymerase (GeneAmp®, Applied Biosystems, Foster City, CA, USA) followed by 40 cycles, comprising 30 s at 94°C for denaturation, 30 s at 54°C for annealing and 30 s at 72°C for primers extension. The final elongation step was prolonged by 10 min at 72°C to ensure a complete elongation of the amplified DNA.

Analysis of mPCR products

mPCR products were analyzed using the RHA CNS kit (Labo Bio-medical Products B.V., EV Rijswijk, The Netherlands) based on the reverse hybridization principle (van Hamont *et al.*, 2006). In the assay, the denatured biotinylated amplicons were hybridized with specific oligonucleotide probes, and immobilized as parallel lines at known locations on a nitrocellulose strip. The results were visually interpreted as purple precipitates in correspondence of hybrids.

RESULTS

A total of 181 CSF samples from patients with suspected aseptic meningitis were tested by mPCR-based-reverse hybridization assay (mPCR-RHA). Twenty-two/181 (12.15%) CSF samples resulted positive on the basis of the hybridization signals with specific oligonucleotide probes. Eighteen/22 CSF samples were positive for 1 pathogen whereas 4/22 samples were positive for 2 pathogens (Table 1).

DISCUSSION

The typical syndrome of viral meningitis comprises both specific and non-specific symptoms and represents a difficult diagnostic problem. Prompt detection of etiological agents and diagnosis are critical because a delayed recognition may lead to life-threatening conditions. Although most cases are caused by EV (70-90%) and HSV or mumps, a wide range of viruses can be involved (Rice, 2005). Conventional laboratory methods, such as viral culture and serology, are unsatisfac-

TABLE 1 - Detection of pathogens' genome in 181 CSF samples from patients with suspected aseptic meningitis by mPCR based-RHA assay.

Pathogen	No. of positive samples	% of positive patients
CMV	1/22	4.5
HSV-1	5/22	22.7
HSV-2	4/22	18.1
EBV	6/22	27.2
HHV-6	1/22	4.5
VZV	0	-
JCV	0	-
EV	1/22	4.5
<i>T. gondii</i>	0	-
HSV-1/HSV-2	2/22	9.0
HHV-6/ <i>T. gondii</i>	1/22	4.5
EBV/EV	1/22	4.5

n.d. not determined. HSV-1, herpes simplex virus type 1; HSV-2, herpes simplex virus type 2; HHV-6, human herpesvirus type 6; CMV, cytomegalovirus; EBV, Epstein-Barr virus; VZV, varicella-zoster virus; JCV, JC papovavirus; EV, enterovirus.

tory, whereas PCR, with its speed and high molecular sensitivity, has great potential for the diagnosis of CNS infections (Read *et al.*, 1997). Unlike the traditional culture methods that may yield negative results after the patient receives even small doses of antiviral drugs, CSF PCR retains its sensitivity even after short courses of antiviral therapy. Moreover, PCR is also preferable to serological testing, often requiring 2-4 weeks after acute infection for a development of a diagnostic rise in antibody titers. In current practice, EV and the principal human herpesviruses (HSV-1 and HSV-2, CMV, VZV, EBV, and HHV-6) should be screened for in cases of suspected aseptic meningitis. The use of PCR technique on the diagnosis of viral meningitis has been reported over the years (Read *et al.*, 1997; Sauerbrei and Wutzler, 2002; Hosoya *et al.*, 1998). Since this diagnostic approach requires the use of single step PCR, the usefulness of a selected range of single PCR tests

as a routine screening is limited by the wide possible range of viruses involved in the etiology of aseptic meningitis. We used mPCR-based-RHA assay to detect and simultaneously differentiate nine pathogens which are adequate for epidemiological and clinical diagnostic applications. In particular, our study showed the useful possibility of the rapid simultaneous detection of the most frequent neurotropic viruses along with JCV and *T. gondii* which are difficult to detect by conventional means.

Out of a total of 22/181 positive samples, 5 CSF samples were positive for HSV-1, 4 CSF samples for HSV-2, and 2 CSF samples for HSV-1/HSV-2. Because of their neurotropic nature, HSV has been recognized as a relatively common CNS pathogen and the presence of two herpesvirus infections determined by PCR has been reported in CSF samples (Tang *et al.*, 1997). The release of IL-6 induced by herpesvirus (Gosselin *et al.*, 1992; Kanangat *et al.*, 1996; Lagneaux *et al.*, 1996) has been hypothesized as the possible cause of the reactivation or the establishment of other infectious agents within the host, thus increasing the general severity of the disease (Yamamoto and Nakamura, 2000). Therefore a rapid and reliable diagnosis is of interest for the prognosis of the disease since acyclovir has been proved to be effective in early systemic treatment of CNS infection caused by HSV (Sköldenberg *et al.*, 1984; Whitley *et al.*, 1986). CMV was detected in one CSF sample, EBV in 6 CSF samples, and HHV-6 in one CSF sample, respectively. These findings are noteworthy since lymphotropic herpesviruses have rarely been reported as the causative agents of CNS infections in immunocompetent patients. The presence of HHV-6 in CSF has been related to CNS complications in stem cell transplant recipients (Wainwright *et al.*, 2001), while CMV encephalitis is particularly evident in immunodepressed patients (Maschio *et al.*, 1999). To the best of our knowledge, only a few reports are present in literature on CMV meningitis in immunocompetent adults (Rafailidis *et al.*, 2007) and EBV-related myelitis, meningitis and/or encephalitis (Gruhn *et al.*, 1999).

EV are considered a major viral etiological cause of aseptic meningitis particularly in children (King *et al.*, 2007; Michos *et al.*, 2007). Accordingly, our CSF sample was from a patient in the age range 0-10 yrs.

Detection of more than one virus from any clinical specimen is uncommon and documented reports of viral dual infections in immunocompetent patients have also been uncommon. On the other hand, in immunocompromised patients the possibility to detect *T. gondii* along with viruses most frequently involved in CNS infections in one CSF sample might be of particular value. *T. gondii* is a protozoan organism which can indeed reactivate in the brain of previously infected immunocompromised individuals. In this regard, one of the two dual infected CSF samples was positive for HHV-6/*T. gondii* and was from an HIV-infected patient. Taken together our results suggest that the applications of this mPCR-based RHA method are extensive and particularly valuable for the rapid and sensitive diagnosis of aseptic meningitis in both immunocompetent and immunocompromised patients.

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