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Research Article

DIRECT DETECTION OF ENTEROVIRUS GENOME IN CELL-CULTURE NEGATIVE CEREBROSPINAL FLUID FROM ASEPTIC MENINGITIS CASES IN BRAZIL

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ABSTRACT

Enteroviruses are the main etiologic agents involved in outbreaks and sporadic cases of aseptic meningitis. Viral isolation in permissive cells is a traditional method for diagnosis, but it is time consuming and may be unsuccessful due to low viral titers and because some enteroviruses do not replicate in routine cell cultures. Therefore, molecular techniques are increasingly being used for detection of these agents. In the present work, 267 cerebrospinal fluid (CSF) samples from aseptic meningitis and meningoencephalitis cases, received between 2008 and 2009, were tested by RT-PCR. These clinical samples were previously negative for virus isolation in cell culture. Enterovirus genome was detected in 59 CSF samples (22.1%) and identification was confirmed by partial nucleotide sequencing. Some demographics of the patients included in the study were analyzed. Direct detection of enterovirus genome from CSF is appropriate to increase sensitivity in aseptic meningitis and meningoencephalitis cases.

Keywords: Enterovirus, RT-PCR, Aseptic Meningitis, CSF.
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INTRODUCTION

Meningitis and meningoencephalitis are acute infections involving the central nervous system mainly of viral etiology, and enteroviruses are responsible for more than 80% of reported cases (Pallansch & Roos 2001). Different serotypes of enteroviruses, such as echovirus 4 (E4), E6, E13, E30 and coxsackievirus B5, are involved in outbreaks and sporadic cases of aseptic meningitis around the world (Choi et al. 2010; Dos Santos et al. 2006; Grenón et al. 2008; Hayashi et al. 2009; Juliá et al. 2009; Kmetzsch et al. 2006; Mirand et al. 2008; Papa et al. 2009; Tavakoli et al. 2008; Thoelen et al. 2003).

Enteroviruses belong to the Picornaviridae family, are spherical and non-enveloped viruses, with a genome consisting of a positive-sense single-stranded RNA. The 5' terminal untranslated region (5' UTR) is highly conserved and is useful for molecular identification purposes (Pallansch & Roos 2001; Racaniello 2001).

The diagnosis of aseptic meningitis can be carried out by several laboratory methods in association with clinical findings. Viral isolation in cell culture is a traditional method and it is still considered the gold standard method of diagnosis of enterovirus (WHO 2004). This method, however, may require up to 10 days for a positive result (Lee & Davies 2007; Pallansch & Roos 2001). In addition, virus isolation may be unsuccessful due to low viral titers in some clinical samples and to the fact that some viruses, such as Coxsackievirus A, do not replicate in routine cell cultures (Ishiko et al. 2002; Jaques et al. 2003; Santos et al. 2002).

Molecular techniques, such as RT-PCR, nested PCR, real-time PCR, multiplex PCR and nucleotide sequencing, have been increasingly used for detection of these agents in cerebrospinal fluid (CSF) (Archimbaud et al. 2004; Brown et al. 2003; Heim & Schumann 2002; Jacques et al. 2003; Lee et al. 2002; Leitch et al. 2009; Nix et al. 2006; Oberste et al. 1999, 2006; Verstrepen et al. 2002). These methods are more sensitive, compared to cell culture, allowing the detection of a small number of copies of the viral genome present in clinical specimens, with high specificity and fast turnaround time (Benschop et al. 2010; Jaques et al. 2003; Oberste et al. 1999; Ooi et al. 2010).

This study aimed to detect enteroviral genome in CSF specimens which yielded negative results by virus isolation in cell culture.

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MATERIAL AND METHODS

Clinical Specimens

We analyzed 267 CSF samples from patients of several locations in Brazil with clinical diagnosis of meningitis or meningoencephalitis of viral etiology, during 2008 and 2009. All samples had previously tested negative for the presence of enterovirus by isolation in RD and HEp-2 cell cultures.

All experiments were performed in compliance with the laws and institutional guidelines, and in accordance with the ethical standards of the Declaration of Helsinki. The institutional committee CEP-IPEC/FIOCRUZ approved the experiments.

Extraction of Viral RNA and cDNA Synthesis

Total RNA was extracted from CSF samples using QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, NRW, Germany), according to the protocol provided by the manufacturer. The cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA), starting with10 µl of total RNA.

Molecular Detection of Enterovirus

A pair of primers (EVF = 5'-CTC CGG CCC CTG AAT GCG GCT A-3' and EVR= 5'-ATT GTC ACC ATA AGC AGC C-3') flanking a fragment of 153bp of the 5' UTR, conserved in genomes of all known human enteroviruses, was used for genomic amplification. This pair of primers is used routinely in the Enterovirus Laboratory for the molecular diagnosis of enterovirus (Dos Santos et al. 2006). Four microliters of cDNA were added to the PCR mix, composed of 50 µM EVR, 50 µM EVF, 12.5 µl of GoTaq Green Master Mix (Promega, Fitchburg, WI, USA) and PCR water to a final volume of 25 µl. PCR was performed with a prior denaturation step of 3 minutes at 95° C and 35 cycles of 45 seconds at 95° C, 45 seconds at 55° C and 45 seconds at 70° C, with a final extension of 7 minutes at 70° C in a thermocycler GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA). The visualization of the PCR amplified productswas done by electrophoresis on 10% acrylamide gels, using the 50bp marker (Invitrogen, Carlsbad, CA, USA), staining with 0.1 µg/ml ethidium bromide and scanning with the Universal Hood II Photo-Documentation System with UV light (BioRad, Hercules, CA, USA).

Confirmation of Enterovirus Detection by Nucleotide Sequencing

Cycle-sequencing reactions were performed using 100 ng/µl of gel-extracted PCR products using the BigDye Terminator v3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA). The nucleotide sequence analysis to confirm the virus identity was carried out by comparison with the GenBank database using BLASTn program (Altschul et al. 1990).

Analysis of Patients Records

Some aspects of the patient records included in this study, such as state of origin, age and gender, were analyzed. The following age groups were defined: <1 year, 1-6 years, 7-12 years, 13-17 years, 18-30 years, 31-45 years and > 46 years.

RESULTS

Molecular Detection of Enterovirus

RT-PCR technique carried out from CSF extracted RNA was able to detect enteroviruses in 59 samples of the 267 CSF analyzed (22.1%). All enterovirus positive samples were confirmed by nucleotide sequencing. When compared with other sequences available at GenBank, all sequences were identified as partial 5' UTR of enterovirus.

Analysis of Patient Profiles

Among the 267 patients included in this study, 121 (45.3%) were female and 146 (54.7%) were male. The distribution of cases by state of origin, by age and gender is shown in Table 01.

Table 01. Epidemiological data of patients.

Epidemiological findings		Total (267) Nº (%)	Positive (59) Nº (%)
Gender	Female	121 (45,3%)	23 (39%)
	Male	146 (54.7%)	36 (61%)
Age Group	< 1 year	26 (9.7 %)	5 (8.5 %)
	1 - 6 years	65 (24.3 %)	15 (25.4 %)
	7 - 12 years	37 (13.9 %)	9 (15.3 %)
	13 - 17 years	10 (3.7 %)	4 (6.7 %)
	18 - 30 years	36 (13.5 %)	9 (15.3 %)
	31 - 45 years	20 (7.5 %)	4 (6.7 %)
	> 46 years	21 (7.9 %)	2 (3.4 %)
	Non Informed	52 (19.4 %)	11(18.7 %)
State of Origin	Bahia	94 (35.2%)	16 (27.1%)
	Distrito Federal	23 (8.6%)	5 (8.5%)
	Minas Gerais	27 (10%)	7 (11.9%)
	Paraná	31 (11.6%)	10 (17%)
	Piauí	29 (10.8%)	9 (15.2%)
	Rio de Janeiro	56 (21%)	12 (20,3%)
	Santa Catarina	07 (2.6%)	0 (0%)

In the analysis of age groups, almost 10% of patients were under one year and 24.3% were between two and six years of age. In addition, 21 patients (7.9%) were older than 46 years. The age of patients was, on average, 36 years, ranging from four days old to 72 years.

The age of 52 patients (19.4%) was not informed.

DISCUSSION

The objective of this study was to improve the laboratorial performance of the enterovirus-related meningitis and meningoencephalitis diagnosis by detecting enterovirus RNA directly from patient CSF specimens. Current protocol for diagnosis involves virus isolation from clinical specimens such as feces, CSF and rectal swabs in cell culture. The cell lines currently used are permissive for most recognized enteroviruses (WHO 2004), but genome detection is certainly more sensitive than recovery of viable viruses.

In a study conducted between 1998 and 2003 in Brazil, the isolation rate of enteroviruses from cases of aseptic meningitis was 15.8% (Dos Santos et al. 2006). Even using molecular diagnostic methods, the percentage of cases with unknown etiology is still considerable. This high rate of cases of unexplained etiology may be due to factors such as problems in storage, transport of CSF and low sensitivity of cell cultures used, which may not be suitable for the isolation of some viral agents (King et al. 2007).

In the present study, we analyzed 267 CSF samples received between 2008 and 2009, which were negative by cell culture for enterovirus. After RNA extraction from CSF and cDNA synthesis, enterovirus group-specific PCR was performed and the positivity rate of 22.1% shows that the direct RNA detection from CSF should be used to increasesensitivity of enterovirus detection in this type of clinical specimen.

Some demographics of the patients with aseptic meningitis, regardless of enterovirus etiology, were analyzed. Their ages ranged from four days to 72 years. Approximately 48% of patients were under 12 years old and there was no predominance of gender. These results confirm the epidemiological characteristics of aseptic meningitis patients reported in another study in Brazil (Dos Santos et al. 2006).

Considering that all samples used in this study were negative for virus isolation, it was possible to compare the performance of the direct extraction of CSF and virus isolation in cell culture for recovery of viral nucleic acid and its subsequent molecular detection.
The use of cell culture systems for isolation is a useful and reliable tool for enterovirus isolation and for increase of viral titers. Besides, the isolated virus can be preserved for future studies. However, in the current context which demands higher sensitivity and speed of diagnostic tests, the RT-PCR can be extremely useful to detect enterovirus in CSF without need for cell culture.

Many studies report that the detection of enterovirus by PCR is more sensitive than cell culture-based tests, besides being faster and able to detect viruses which do not replicate in cultures (Benschop et al. 2010; Iturriza-Gómara et al. 2006; Nigrovic & Chiang 2000; Shoja et al. 2007; Van Doornum et al. 2007; Verstrepen et al. 2001). Buxbaum and colleagues (2001) also found greater positivity rates of enterovirus in CSF using the RNA extraction followed by RT-PCR compared to virus isolation in cell culture. These authors suggest the use of both methods for the detection of enteroviruses.

Some studies compared nested PCR and real-time PCR with viral isolation for enterovirus detection in CSF specimens, and had also found higher sensitivity in molecular tests (Archimbaud et al. 2004; Nix et al. 2006; Van Doornum et al. 2007; Verstrepen et al. 2002).

In a study conducted by Santos and colleagues (2002), 15% of stool samples previously negative for virus isolation in cell culture were positive for enterovirus by RT-PCR, while Shoja and colleagues (2007), found a rate of 10%. Perhaps this lower proportion of positivity compared to the one found in the present study (22.1%) is due to the easiness of virus isolation from stool samples in relation to the CSF. Furthermore, inhibitor factors may be in stool specimens. Due to the nature of the clinical specimens, the use of viral RNA extraction directly from CSF may be more efficient than in fecal samples. The low viral load present in CSF act as a limiting factor to use of non-molecular tests in these samples.

Direct detection of enterovirus RNA in CSF samples can improve the laboratorial diagnosis of enterovirus and support aseptic meningitis and meningoencephalitis surveillance in Brazil. This method, applicable specifically to CSF samples in which low viral titers are expected, could be used in outbreaks, where rapid results are required so that public health actions can be taken in order to prevent virus spread and disease transmission. As an alternative to traditional workflow, CSF samples would be subjected to direct extraction of RNA followed by RT-PCR alongside with inoculation in cell culture to attempt virus isolation. This should shorten the time required to obtain a positive result. Negative samples in the first passage and RT-PCR would follow the current algorithm used by the Enterovirus Laboratory for viral isolation, which includes a second passage, in order to minimize the possibility of false-negative results.

In addition to the above epidemiological implications, rapid diagnosis is crucial for patient management because it reduces the unnecessary use of antibiotics and hospitalization, and also for the health system, reducing healthcare costs (Brasil 2009; Buxbaum et al. 2001; Nigrovic & Chiang 2000; Oberste et al. 1999; Shoja et al. 2007; Verstrepen et al. 2001).

The knowledge generated from this study supports the need for increased investment in diagnostic methods for aseptic meningitis in Brazil.

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