

Análise molecular de patógenos pela reação em cadeia da polimerase no líquido cefalorraquiano de pacientes infectados com HIV

Molecular analysis of pathogens in cerebrospinal fluid by the polymerase chain reaction in HIV-infected patients

RESUMO

Introdução: A reação em cadeia da polimerase (PCR) foi teste de grande impacto no diagnóstico das meningites e encefalites linfocíticas durante a última década. Esse método foi extensivamente usado no diagnóstico das infecções do sistema nervoso central (SNC), devido a sua habilidade em detectar amostras mínimas de DNA-alvo no líquido cefalorraquiano. **Objetivo:** O objetivo deste estudo foi identificar a prevalência dos patógenos oportunistas responsáveis por causar problemas neurológicos em pacientes infectados com o vírus da imunodeficiência humana (HIV) e avaliar sua associação com os achados clínicos, laboratoriais e da tomografia computadorizada cerebral (TCC). **Pacientes e métodos:** Um estudo transversal foi realizado em 203 amostras de líquido cefalorraquiano (LCR) de pacientes do sul do Brasil infectados com HIV e com aparente encefalite e meningite linfocíticas. As amostras foram analisadas para os seguintes agentes pelo método da reação em cadeia da polimerase "nested" ou dupla (N-PCR): citomegalovírus, vírus do Epstein-Barr, vírus do herpes simplex tipos 1 e 2, vírus da *varicella zoster*, vírus do herpes humano tipo 6, vírus JC, *Toxoplasma gondii* e micobactérias. **Resultado:** Pelo menos um patógeno foi encontrado em 77 (38%) dos indivíduos. O Epstein-Barr foi o mais prevalente, com 40 casos (19,7%), seguido pelo citomegalovírus, com 12 casos (15%) e pelo vírus JC, em 9 casos (4,4%). Um N-PCR positivo mostrou associação com aumento de proteínas e de celularidade ($P=0,001$), meningismo ($P=0,017$) e tomografia computadorizada anormal ($P=0,006$). **Conclusão:** O painel de PCR empregado foi efetivo na identificação de infecções neurológicas severas em pacientes HIV positivos.

UNITERMOS: Reação em Cadeia da Polimerase, Líquido Cefalorraquiano, Infecções Oportunistas, HIV, Vírus Epstein-Barr, Encefalite Viral.

ABSTRACT

Introduction: Polymerase chain reaction (PCR) has had great impact on the diagnosis of lymphocytic meningitis and encephalitis over the last decade. It has been extensively used in the diagnosis of central nervous system (CNS) infections for its ability to detect small amounts of target DNA in the cerebrospinal fluid (CSF). **Objective:** The aim of this study was to identify the prevalence of opportunistic pathogens responsible for neurological disorders in patients infected with human immunodeficiency virus (HIV) and to evaluate its association with clinical, laboratory and cerebral computed tomography (CCT) findings. **Patients and methods:** A cross-sectional study was performed on 203 cerebrospinal fluids (CSF) from HIV-infected patients from Southern Brazil, with apparent lymphocytic meningitis and encephalitis. CSF samples were analyzed with probes for cytomegalovirus, Epstein-Barr virus, herpes simplex virus types 1 and 2, varicella zoster virus, human herpes virus type 6, JC virus, *Toxoplasma gondii* and mycobacterium in nested polymerase chain reaction (N-PCR). **Results:** At least one pathogen was found in 77 (38.0%) individuals. Epstein-Barr virus was the most prevalent with 40 cases (19.7%), followed by cytomegalovirus with 12 cases (5.9%) and JC virus with 9 cases (4.4%). Positive N-PCR showed association with high spinal fluid protein and cell count ($P=0.001$), meningism ($P=0.017$) and abnormal CCT ($P=0.006$). **Conclusion:** The PCR panel used was effective in screening several neurological infections in HIV positive patients.

KEYWORDS: Polymerase Chain Reaction, Cerebrospinal Fluid, Opportunistic Infections, HIV, Epstein-Barr Virus, Viral Encephalitis.

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I NTRODUCTION

Polymerase chain reaction (PCR) has had great impact on the diagnosis of lymphocytic meningitis and encephalitis over the last decade. It has been extensively used in the diagnosis of central nervous system (CNS) infections for its ability to detect small amounts of target DNA in the cerebrospinal fluid (CSF) and in short times (1-3).

PCR has been evaluated for the detection of several infectious agents in HIV positive patients with neurological manifestations (4-5). Although hi-

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ghly active antiretroviral therapy has decreased the incidence of opportunistic infections in the CNS, neurological manifestations still remain as important factors in the mortality and morbidity of HIV-infected patients in developing countries (6).

PCR testing for pathogens in the CSF has clarified the etiology of certain clinical manifestations of infection in the CNS. Currently, we know that in addition to causing meningitis without genital lesions, herpes simplex virus type 2 (HSV-2) has also been found in CSF of patients presenting benign recurrent aseptic meningitis, like Mollaret's syndrome (7-8). Detection of HSV DNA in the CSF by PCR, with equal or higher sensitivity than brain biopsy, has become the choice for diagnosing herpetic encephalitis (9-10).

Cytomegalovirus (CMV) detection by PCR has been reliable in the diagnosis of CMV-related CNS lesions, with sensitivity and specificity around 75%-100% and 90.5-100% respectively (11-13). High levels of protein in CSF are also indication of CMV infection as it occurs in approximately 60% of CMV infected patients (13).

Primary CNS lymphoma (PCNSL), which has a strong association with Epstein-Barr virus (EBV), occurs in 2-13% of patients with AIDS (14-15). EBV is found in 75-100% of PCNSL biopsies in HIV infected patients (16). Data from prospective and retrospective studies show that PCR for EBV-DNA in CSF is a less invasive tumor marker than cerebral biopsy for patients with tumor signals, with sensitivity of 80-100% and specificity of 78-100% (11, 12, 16, 17).

Cinque *et al* (1997) reported that CSF PCR was used to detect varicella zoster virus (VZV) in HIV-infected patients who had neurological manifestations associated with the virus as well as sub-clinical reactivation of infection. In this study, more than two-thirds of patients with radiological and clinical findings suggestive of infections caused by other pathogens had VZV DNA in the CSF. PCR results for VZV should therefore be reevaluated

together with clinical and laboratory findings (18).

Human herpes-virus type 6 (HHV-6) and HIV-1 are viruses that infect and replicate in CD4+ and CD8+ T cells and natural killer cells (19). Studies were performed to investigate the possible role of HHV-6 in producing disease in HIV infected patients, alone or associated with CMV, as well as the hypothesis that HHV-6 accelerates the progression to AIDS (7). PCR protocols employing HHV-6 primers in a multiplex PCR have been developed enabling simultaneous analysis of several herpes-virus agents commonly involved in diseases with undefined symptoms in HIV-infected patients (1, 20, 21).

De Luca *et al* (1996) developed a nested PCR protocol to improve the sensitivity of DNA detection of JC virus (JCV) for diagnosis of progressive multifocal leukoencephalopathy (PML) in CSF samples from AIDS patients. They were able to obtain sensitivity of 90% and specificity of 99% with the method. Their results showed that JCV concentration in the CSF is very low, not exceeding 50 genomes eq/ μ L of CSF. The authors stress the need for a second spinal tap after one week, when PCR is negative and PML is highly suspect (4).

Joseph *et al* (2002) published a PCR assay in CSF to detect encephalitis caused by *Toxoplasma gondii* in AIDS patients. The authors achieved positive and negative predictive values of 100% and 97%, respectively (22). Vidal *et al* (2004) published a PCR protocol in CSF for diagnosis of *Toxoplasma gondii* in Brazilian AIDS patients. The study showed that samples from all patients with toxoplasmosis presented positive PCR results with 100% sensitivity (23).

Some studies reported the PCR performance in the detection of tuberculous meningitis, with 70-91% sensitivity and 95-100% specificity (24, 25). Therefore, considering the low sensitivity rates, negative PCR results do not exclude the diagnosis of tuberculous meningitis. *Mycobacterium tuberculo-*

sis may sometimes lead to persistent neutrophilic meningitis in patients that are HIV positive (26).

Chesky *et al* (2000) analyzed 383 CSF samples from patients suspected of having lymphocytic meningitis and encephalitis and attended in emergency rooms of 5 hospitals in the city of Porto Alegre, Southern of Brazil. After testing by PCR for 17 pathogens, they found that at least one of the pathogens was present in 46 CSF specimens (12%). These results correlated well with clinical, laboratory and neuro-imaging findings (27).

The objectives of the present study were to identify and measure the prevalence of pathogens in HIV patients with aseptic meningitis and encephalitis, using a PCR panel for detecting pathogens in CSF (Chesky *et al*, 2000), as well as to verify the correlation between PCR results with laboratory, radiology and clinical findings (27).

PATIENTS AND METHODS

A cross-sectional study was performed in 203 CSF samples from adult HIV-infected patients with clinical suspicion of lymphocytic meningitis and encephalitis. All subjects were patients admitted in the emergency room of the Hospital de Clínicas de Porto Alegre, Brazil. A sample of CSF, as requested by the assisting physician, was sent to the molecular biology laboratory with a form containing the patient data and symptoms. Clinical examination, spinal tap, blood collection and the cerebral computed tomography (CCT) were performed within 24 hours of patients' admission.

The hospital's Ethics Committee approved the present study. Informed consent was not required because the spinal tap is a routine procedure for all patients with clinical evidence of lymphocytic meningitis and encephalitis.

The protocol included pathogens that are difficult to diagnose by conventional methods and which more frequently cause CNS disorders in immunocompromised patients: CMV, EBV,

HSV, VZV, HHV-6, JCV, *Toxoplasma gondii* and mycobacterium (10, 22, 28).

DNA extraction was performed with the Qiagen QIAamp kit for viral RNA (Qiagen, Valência, USA) that copurifies RNA and DNA. The nested PCR described by Chesky *et al* (2000) was used to test the CSF samples. In summary, primary PCR amplifications were performed in a final volume of 50µL containing 10µL of extracted nucleic acids. Secondary amplification with nested primers was performed with 2µL of the first post-PCR reaction in a total volume of 25µL. All amplifications were performed using a Perkin Elmer Applied Biosystems 9600 machine. For some pathogens, nested PCR in a multiplex protocol were used. EBV, CMV and HHV-6 were assayed in the same reaction tube as triplex PCR and HSV and VZV were tested in a duplex PCR (29, 30). The remaining pathogens were tested individually. For detecting mycobacterium DNA, oligonucleotide primers based on the sequence coding for the 65kDa antigen of mycobacterium were used and oligonucleotide primers based on the IS6110 repetitive region were used to identify *M. tuberculosis* complex (28, 31). All PCRs were repeated twice and if the results were not in agreement, the test was repeated from the DNA extraction step. Amplification products were detected by agarose gel electrophoresis and identified by their sizes. DNAs of the pathogens in the PCR panel were used as positive controls. We also used a panel of 20 coded positive and negative specimens sent by the Public Health Laboratory, Oxford, UK as an external quality control. For some of the PCR assays, a measurement of sensitivity of detection was made by determining the endpoint dilutions of well-characterized virus preparations for which positive PCR results were previously obtained (27).

Statistical analysis was performed with descriptive techniques using a 95% confidence interval. Continuous variables with normal and asymmetrical distribution were compared to PCR

results through the Student's t test and Mann-Whitney U test, respectively. For testing categorical variables with PCR results, the Fisher exact test was utilized.

RESULTS

DNA of at least one pathogen was detected by PCR in 77 (37.9%) of 203 CSF specimens. HHV-6 DNA was not detected in any sample. Concomitant infections with two pathogens were found in 12 CSF samples (5.9%) and in one sample (0.5%) 3 viruses were detected. The total number of pathogens detection in the 77 CSF positive samples was 91. The distribution of the detected pathogens was as follows: EBV 40 cases (19.7%), CMV 12 (5.9%), JCV 9 (4.4%), *T. gondii* 8 (3.9%), mycobacterium 8 (3.9%), HZV 8 (3.9%) and HSV 6 (3.0%) (Figure 1). Associations of dual infection were: EBV and CMV (2), EBV and VZV (2), EBV and mycobacterium (1), EBV and *T. gondii* (1), CMV and mycobacterium (2), CMV and HSV (2), CMV and *T. gondii* (1) and HSV and VZV (1). The patient with three viral infections (CMV, EBV and HSV) had skin lesions, fever, confusion, levels of CD4

with 8 cells/µL and hypo dense lesions on CCT.

Median values of protein and leukocytes in CSF were significantly higher among the group of patients with positive PCR ($P<0.001$). However, 23 cases with CSF positive PCR had less than 5 cells/µL. The spinal fluid glucose was similar in both groups ($P=0.329$) and abnormal CCT was associated with one positive PCR in CSF ($P=0.006$) (Table 1).

Meningism was the only clinical manifestation showing correlation with pathogen positive PCR ($P=0.017$). Fever, confusion, headache, seizure, skin lesions and focal neurological signs were manifestations that appeared equally in both groups of patients (Table 2).

All patients with CMV positive PCR showed abnormal CCT. Twenty eight out of 40 patients with EBV positive PCR had abnormal neuro-imaging findings. Only 2 out of 8 patients with PCR positive for *T. gondii* had normal CCT. Eight out of 9 patients with PCR positive for JCV had normal CSF leukocytes values while 4 had neuro-imaging findings suggesting PML. Among 7 patients with HSV positive PCR, 3 had skin lesions. Two out of 8 patients with VZV positive PCR

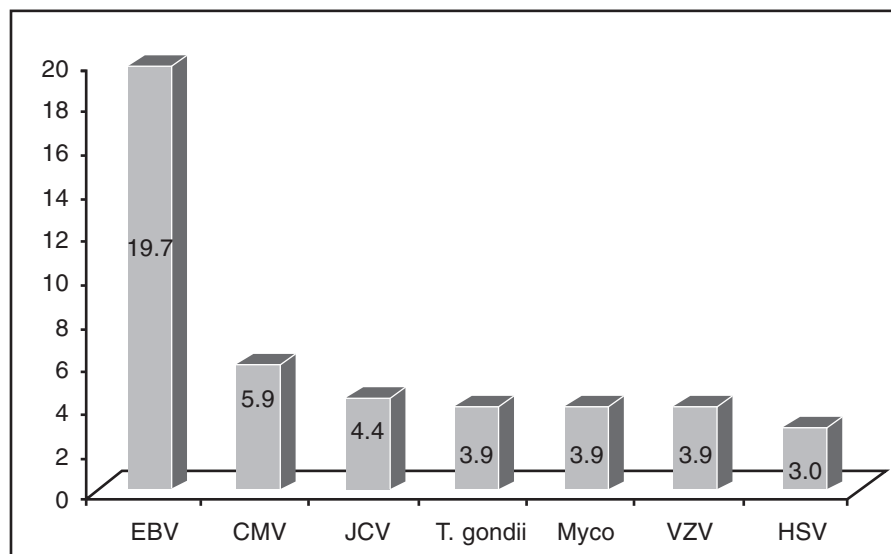


Figura 1 – Prevalence of pathogens amplified in CSF by PCR (n=91).

EBV (Epstein-Barr virus); CMV (cytomegalovirus); JCV (JC virus); *T. gondii* (*Toxoplasma gondii*); Myco (mycobacterium); VZV (varicella zoster virus); HSV (herpes simplex virus).

TABLE 1 – PCR results in CSF and correlation with laboratory findings

	Positive PCR		Negative PCR		P
	n	Mean (*)	N	Mean (*)	
Leukorrachia (µL)	75	21 (3-70)	125	2 (1-10)	0.001
Proteinorrachia (mg/dL)	74	124 (78-201)	122	58 (45-100)	0.001

* Median (percentiles 25-75).

TABLE 2 – PCR findings in CSF and correlation with clinical symptoms and CCT

	Positive PCR		Negative PCR		P
	%	(n)	%	(n)	
Meningism	40.3%	(31)	20.6%	(31)	0.017
Fever	55.8%	(43)	41.3%	(43)	0.059
FNS	22.1%	(17)	28.6%	(17)	0.328
Skin lesions	19.5%	(15)	21.4%	(15)	0.328
Confusion	46.8%	(36)	38.1%	(36)	0.242
Convulsions	14.3%	(11)	19.8%	(11)	0.349
Headache	32.5%	(25)	40.5%	(25)	0.296
CCT	78.3%	(54)	57.0%	(54)	0.006

CSF (cerebrospinal fluid); CCT (cerebral computed tomography); FNS (focal neurologic signs).

presented zoster skin activity and 3 had CCT showing brain lesions.

Six out of 8 mycobacteria detected by PCR were from the *Mycobacterium tuberculosis* complex. Of the 8 CSF with mycobacterium positive PCR, five gave negative cultures, two were positive and in one sample culture was not performed. Neuro-imaging findings showed abnormalities in five of these patients. Two patients who had positive culture for mycobacterium were negative PCR and were thus classified as non-tuberculosis *Mycobacterium* (NTM).

D ISCUSSION

All patients included in this study presented some clinical, laboratory or radiological findings suggestive of lymphocytic meningitis or encephalitis. Pathogens were detected by PCR in at least 37.9% of CSF samples.

PCR for HSV in CSF is already the test of choice for the diagnosis of herpetic encephalitis, with sensitivity and specificity of 100% and 99.5% respectively (32). However, in spite of high specificity, PCR does not show the desirable sensitivity of 100% for the majority of pathogens studied.

The frequency of pathogens observed in this study was similar to that of Cinque *et al* (1996) for HIV-infected patients with neurological disorders. However, it is worth noting that in their sampling CMV are 3 times and JCV twice more prevalent than in the present study.

In another study, Quereda *et al* (2000) using PCR to study herpes-virus group in CSF from HIV-infected patients with neurological symptoms found herpes-virus DNA in 34% of patients. In the same study CMV was found with a frequency of 25%, EBV in 7%, HSV in 3%, VZV in 4% and HHV-6 in 1%. Their HSV and VZV findings were similar to those found in this study, but differently for CMV and EBV. Interestingly, dual infections had the same value as well in addition to some identical viral associations (33). Rojanawiwat *et al.* (2005) reported a prevalence of EBV and CMV of 22.1% and 11.4%, respectively. They also identified 4.3% of dual infection by EBV and CMV (34).

PCR in a multiplex format using consensus primers for searching six main human herpesviruses was tested in 23 CSF samples from HIV positive patients with clinical signs of CNS disorder (35). The authors found the fo-

llowing results: CMV, 13%; EBV, 10.6%; VZV, 5.3%; HSV-1/2, 1.3% and HHV-6, 0%.

Tang *et al* (1997) analyzing the clinical significance of CNS co-infection by different viruses of the herpes-virus group in 30 patients who apparently were HIV-negative found 3 cases (10%) of mixed infections, 1 case of CMV/EBV, 1 case of HSV/EBV and another of HSV/HHV6 (36). The first 2 patients with double infection associated with EBV died and the last one experienced slow regression of the disease but with lasting neurological damage. These outcomes suggest that co-infection with viruses of the herpes-virus group in CNS infection can have a poor prognosis.

In the present study we found EBV in 19.7% of the HIV-infected patients sampled. A more recent research (16) has found EBV in 16.32% of HIV patients. On the other hand, Corcoran *et al* (2008) detected EBV DNA in CSF of 36.4% of HIV-infected patients with neurological diseases.

In our study no case of HHV-6 in the 203 CSF specimens analyzed were observed. Quereda *et al* (2000) detected HHV-6 in 1% of patients, without apparent clinical significance. In the study of Minjolle *et al* (2002) no HHV-

6 was detected as well. Confirming our results, this virus is rarely detected in the CSF of HIV-infected patients. However, Calvario *et al* (2002) found 2 seropositive HIV patients with the associations HHV-6/CMV and HHV-6/EBV (37).

We detected two cases of NTM by culturing in which PCR testing was negative. These results can be explained by the lack of sensitivity or specificity of the 65 kD primers for amplification of some atypical mycobacterium. All CSF samples were initially tested for 65 kD primers and if any mycobacterium sequence were amplified by PCR, IS6110 primers were used to screen for *M. tuberculosis* complex.

The present study showed the prevalence of opportunistic pathogens in a group of HIV patients from Southern Brazil and the general applicability of the PCR panel used. We found differences in pathogens frequencies as compared to other published surveys. These differences are expected taking into account many factors such as the immunological status of each patient and the epidemiological profile of pathogens in a particular region. This PCR panel has been proven to be a powerful and reliable diagnostic tool for using routinely to screen opportunistic CNS infections in HIV-infected patients. It is advisable, however, to evaluate the PCR findings in the context of clinical, laboratory and radiological results for each patient.

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