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POLYMERASE CHAIN REACTION FOR THE LABORATORY DIAGNOSIS OF ASEPTIC MENINGITIS AND ENCEPHALITIS

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ABSTRACT - A protocol for testing cerebrospinal fluid specimens using a range of PCR assays for the diagnosis of central nervous system infection was developed and used to test prospectively 383 specimens. PCR assays were used for the detection of adenovirus, *Borrelia burgdorferi*, enteroviruses, Epstein Barr virus, cytomegalovirus, herpes simplex virus, human herpes virus type 6, JC virus, *Leptospira interrogans, Listeria monocytogenes*, lymphocytic choriomeningitis virus, measles virus, mumps virus, *Mycobacterium sp.*, *Mycoplasma pneumoniae, Toxoplasma gondii* and varicella zoster virus. Of the 383 specimens tested in this study, 46 (12.0%) were found to be positive. The microorganisms detected were CMV, enterovirus, Epstein Barr virus, herpes simplex virus, human herpes virus type 6, JC virus, *L. monocytogenes*, Mycobacterium genus, *Toxoplasma gondii* and varicella zoster virus. The introduction of the PCR protocol described has improved the diagnosis of a range of central nervous system infections in our laboratory. We believe however that further evaluation of these assays in immunocompromised patients is necessary to better determine the predictive value of positive PCR results in these patient groups.

KEY WORDS: polymerase chain reaction (PCR), cerebrospinal fluid (CSF), central nervous system (CNS), lymphocytic meningitis, encephalitis.

Reação em cadeia da polimerase no diagnóstico laboratorial das meningites e encefalites assépticas

RESUMO - Foi desenvolvido um protocolo de PCR para analisar os principais patógenos que infectam o sistema nervoso central (SNC). Foram testadas 383 amostras de líquido-cefalorraquidiano (LCR), para detecção de: adenovirus, *Borrelia burgdorferi*, enterovirus, vírus Epstein Barr , citomegalovírus, vírus herpes simplex, herpes humano tipo 6, vírus JC, *Leptospira interrogans, Listeria monocytogenes,* vírus da coriomeningite linfocitária, vírus do sarampo, vírus da caxumba, *Mycobacterium sp, Mycoplasma pneumoniae, Toxoplasma gondii e vírus da* varicela zoster. Das 383 amostras de LCR testadas a PCR foi positiva em 46 (12,0%). Os microrganismos detectados foram: citomegalovírus, enterovirus, vírus Epstein Barr , herpes simplex, herpes humanao tipo 6, vírus JC, *Listeria monocytogenes, Mycobacterium sp, Toxoplasma gondii e* varicela zoster. A introdução da técnica de PCR através de um protocolo para análise do LCR otimizou o diagnóstico etiológico das infecções do SNC e tem se revelado um instrumento de grande potencial diagnóstico. Estudos futuros necessitam ser realizados para melhor avaliar os resultados da PCR em pacientes imunocomprometidos e assim determinar o valor preditivo positivo deste ensaio neste grupo de pacientes.

PALAVRAS-CHAVE: reação em cadeia da polimerase (PCR), líquido cefalorraquidiano (LCR), sistema nervoso central (SNC), meningite asséptica, encefalite.

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The inflammatory processes which most commonly affect the central nervous system (CNS) are aseptic meningitis and encephalitis. Generally these diseases are caused by viruses, however, *Toxoplasma gondii* and Mycobacteria have been identified as causes of lymphocytic meningitis and encephalitis in immunocompromised patients. Conventional laboratory diagnostic methods, such as growth of a microorganism in culture and detection of specific antibody, are generally poor in the diagnosis of CNS infection. They are generally too slow, have low sensitivity and consequently they are of little use in diagnosing and treating the patient during the acute phase of the disease[1,2]. In cases of aseptic meningitis and encephalitis, the rapid identification of the etiologic agent not only serves to elicit important epidemiological information, but, with the availability of antiviral drugs, helps to direct clinical procedures. More sensitive diagnostic assays may also improve our understanding of the etiology of CNS infections.

In the 1980's, the development of the polymerase chain reaction (PCR) technique revolutionized the analysis of nucleic acids, allowing the detection of DNA and RNA sequences in different types of clinical material[3]. PCR has widely used in infectious disease diagnosis, having a major impact in those areas that have been diagnostic difficulties, either because organisms cannot be cultivated, grow only slowly, or require a highly specific culture medium, such as viruses, certain bacteria, fungi, and protozoans[4-6].

Several studies have employed PCR for diagnosing CNS infections, especially for herpes simplex virus (HSV) encephalitis and for enterovirus meningitis: in patients with herpes encephalitis, studies comparing PCR of CSF with a 'gold standard' test (viral culture or viral antigen detection in brain biopsy) have demonstrated that PCR is highly sensitive and specific, in addition to being fast and less invasive[7,8]. In cases of enterovirus meningitis, the use of PCR has allowed rapid laboratory diagnosis, thus reducing the duration of hospital stay and avoiding the need for aggressive therapeutic measures[9].

A report published in 1997 described the use of PCR assays arranged in an algorithm for the laboratory diagnosis of aseptic meningitis and encephalitis in the UK[10,11]. A similar PCR protocol was developed at Hospital de Clínicas de Porto Alegre, Brazil, with the aim of establishing a routine molecular diagnostic service for CNS infection and determining which are the most common CNS pathogens in the Porto Alegre area.

METHOD

PCR assays were used to prospectively test CSF specimens from 383 patients with presumed aseptic meningitis and encephalitis for the presence of 17 infectious agents. The following protocol was used: stage 1 comprised of assays for HSV, varicella zoster virus (VZV), and enteroviruses; stage 2 had assays for adenovirus, cytomegalovirus (CMV), Epstein Barr virus (EBV), human herpes virus 6 (HHV6), lymphocytic choriomeningitis virus (LCMV), measles virus, and mumps virus (patients with a known immunodeficiency were also tested for JC virus, Mycobacterium genus and *T. gondii*); stage 3 tested for *Borrelia burgdorferi*, *Leptospira interrogans*, *Listeria monocytogenes*, and *Mycoplasma pneumoniae*.

Microorganisms with a DNA genome were initially amplified without DNA extraction and purification: 10 µl of CSF was added directly to PCRs with release of DNA achieved by heat denaturation of organisms during PCR incubations. However, after PCR amplification inhibition by components of some CSF specimens was detected, DNA extraction using the QIAamp viral RNA kit (QIAGEN Ltd, Crawley, UK) was added to the protocol. For viruses with an RNA genome, nucleic acid was initially extracted and purified from CSF using an in-house method in which virus particles were lysed with guanidine isothiocyanate and nucleic acid was bound to a suspension of silica particles[12]. This method was replaced by the QIAamp procedure so that DNA and RNA targets could be processed with a common protocol. The QIAamp procedure was performed according to the manufacturers instructions. All CSF specimens were submitted to routine biochemical, differential cytological and bacterioscopical examination.

PCR using nested primers was used for each of the targets. For some reactions, multiplex PCR using a combination of primers was used. The combined primers had similar annealing temperatures, and it was possible to distinctly identify each PCR product according to its electrophoretic mobility. Primers for the following micro-

Table 1. Lymphocytic meningitis and meningoencephalitis: PCR and laboratorial findings in CSF.

N	Age	Leukocyte	Lymphocyte (%)	Protein mg/dl	Glucose mg/dl	PCR	Observations
1	3	87	80	172	13	EBV	Guillan-Barre syndrome; leukemia
2	*	4	*	32	73	EBV	HIV disease; clinically encephalitis
3	34	30	84	248	56	EBV	HIV disease; clinically encephalitis; HSV IgG reactive; CMV reactive
4	32	1	*	59	95	EBV	HIV disease; cerebral lesions on CT scan
5	25	1	*	53	48	EBV	HIV disease; cerebral lymphoma
6	24	32	99	72	53	EBV	HIV disease; clinically viral encephalitis
7	34	1	*	38	49	EBV	HIV disease; cerebral lesions on CT scan
8	7	1	*	30	58	EBV	Hepatic transplant; use of corticoid and cyclosporin; Burkitt's lymphoma
9	32	297	32	85	26	EBV	HIV disease; clinically encephalitis; M. tuberculosis culture positive
10	40	6	*	108	111	EBV	HIV disease; clinically encephalitis
11	70	476	98	118	44	EBV	Clinically viral encephalitis
12	45	120	10	486	27	EBV and CMV	HIV disease; clinically encephalitis; M. tuberculosis culture positive
13	Adult	16	82	52	48	CMV	HIV disease; clinically encephalitis
14	Adult	3	*	61	38	CMV	HIV disease; clinically encephalitis
15	28	32	97	75	34	CMV	HIV disease; clinically encephalitis
16	32	1100	10	204	26	CMV	HIV disease; clinically encephalitis; CMV retinitis
17	4/12	2	*	45	62	CMV	HIV disease; CMV pneumonitis; clinically encephalitis
18	1/12	3	*	287	38	CMV	Neonatal sepsis; blood and urine CMV PCR positive
19	41	81	99	92	44	CMV	HIV disease; clinically encephalitis; cerebral lesions on CT scan
20	64	1	*	55	100	CMV	HIV disease; clinically encephalitis
21	Adult	363	100	75	50	HSV	Clinically viral encephalitis
22	72	45	89	163	65	HSV	Clinically viral encephalitis
23	11/12	96	78	158	56	HSV	Clinically viral encephalitis; petechial rash

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24	33	900	98	99	63	HSV	Clinically viral encephalitis
25	47	235	100	334	56	HSV	Clinically viral encephalitis
26	36	26	100	172	71	HSV	HIV disease; clinically encephalitis; herpes lesions on the skin
27	25	95	100	186	39	HSV	HIV disease; clinically viral encephalitis
28	28	132	*	100	44	HSV and Mycobacterium sp	HIV disease; clinically encephalitis; M. tuberculosis culture positive; herpes lesions on the skin
29	4	208	79	429	40	Mycobacterium sp	Mycobacterium meningitis; Increased ADA levels
30	Adult	299	*	129	16	Mycobacterium sp	HIV disease; clinically encephalitis; Ziehl-Neelsen stain positive
31	28	485	*	135	39	Mycobacterium sp	Clinically encephalitis; Ziehl-Neelsen stain and M. tuberculosis culture positive
32	34	200	35	117	34	Mycobacterium sp	HIV disease; clinically encephalitis; M. tuberculosis culture positive
33	23	200	87	308	15	Mycobacterium sp	Clinically encephalitis; M. tuberculosis culture positive
34	27	2	*	3730	24	Mycobacterium sp	HIV disease; pulmonary tuberculosis
35	5	500	*	528	2	Mycobacterium sp and enterovirus	Cerebral palsy; Mycobacterium meningitis
36	40	39	97	154	83	VZV	Clinically viral encephalitis; herpes lesions on the skin; HSV IgG reactive in CSF
37	32	5	98	226	67	VZV	HIV disease; clinically encephalitis; herpes lesions on the skin; HSV IgG reactive in CSF
38	49	1	*	74	77	VZV	Clinically encephalitis; leukemia; herpes lesions on the skin
39	33	2	*	72	66	VZV	HIV disease; clinically encephalitis; herpes lesions on the skin
40	30	3	*	82	62	Toxoplasma gondii	HIV disease; clinically encephalitis; cerebral lesions on CT scan
41	20	10	90	122	40	Toxoplasma gondii	Lymphoma; clinically encephalitis; T.gondii IgG titre=1:128
42	54	*	*	*	*	enterovirus	HIV disease; clinically viral encephalitis
43	29	8	*	*	*	JCV	HIV disease; clinically viral encephalitis
44	Adult	15	*	*	*	JCV	Brain demyelination
45	5	20	78	14	70	HHV6	Seizures; Rasmussen sindrome
46	59	11	55	15	52	Listeria monocytogenes	Clinically encephalitis; L. monocytogenes culture positive

* Data not given.

organisms were combined: enterovirus, HSV and VZV; CMV, EBV and HHV6; LCMV, measles virus and mumps virus.The other microorganisms were amplified with individual primer PCR assays.

Primary PCR amplifications were performed in a solution of total volume 50 µl containing 20 µl of extracted nucleic acid solution, 16mM $(NH_4)_2SO_4$, 67mM Tris-HCl (pH 8.8 at 25°C), 0.01% (wt/vol) Tween-20 (PCR buffer, Advanced Biotechnologies Ltd, Epsom, UK), 1.5mM $MgCl_2$, 0.25mM of each deoxynucleotide triphosphate (Advanced Biotechnologies Ltd), 0.1µM of each oligonucleotide primer (R&D Systems Ltd, Abingdon, UK), and 0.625 unit of Taq polymerase (manufacturer's units; Advanced Biotechnologies Ltd). For the amplification of viruses with an RNA genome, 0.1 unit of Moloney murine leukaemia virus reverse transcriptase (manufacturer's units; Advanced Biotechnologies Ltd, Epsom, UK) was included for the specific antisense oligonucleotide primed reverse transcription of the genomic RNA. For the detection of DNA genomes, 10 µl of the extracted nucleic acid was added to the PCR, whereas for RNA genome detection 20 µl of nucleic acid was used.

Secondary amplifications with nested primers were performed on 2 µl of the primary reaction solution. Identical concentrations of reagents to those in the primary PCR were used but in a total volume of 25 µl.

PCR thermal cycling incubations used were as follows: reverse transcription (where appropriate) and initial amplification was performed in a single reaction by incubation at 37°C for 15 minutes (for RNA genome detection) and 94°C for 40 seconds preceding 33 cycles of 20 second incubations at 94°C, the appropriate annealing temperature and 72°C; further amplification with nested primers was by 33 cycles of 20 second incubations at 94°C, the appropriate annealing temperature and 72°C. All thermal cycling was performed using PE Applied Biosystems 9600 machines. Amplification products were identified by their molecular weight following electrophoresis of 10 µl of the secondary reaction mixture through an ethidium bromide-stained 2% agarose gel and UV light transillumination. The average duration of tests was 6 hours.

Carry over contamination by amplified product was avoided with strict physical separation of pre- and post- amplification processes and would have been detected using the multiple nucleic acid extraction and amplification controls incorporated into each assay batch.

External quality assessment was performed by testing a panel of 20 coded specimens (CSF, water, and diluted viral cultures) sent by the Public Health Laboratory, Oxford, UK. For some of the PCR assays an accurate measurement of the molecular sensitivity of detection was made by determining the end-point dilutions of well characterized virus preparations[11] at which a positive PCR result was obtained.

RESULTS

A total of 383 CSF specimens from patients with clinical and laboratory indication of aseptic meningitis or encephalitis were tested using the PCR protocol. A positive PCR result was obtained in 46 specimens (12.0%). The most prevalent microorganisms were EBV (11 cases); CMV (8); HSV (7); Mycobacterium genus (6); VZV (4);*T. gondii* (2); JC virus (2); HHV6 (1); enteroviruses (1) and *L. monocytogenes* (1). In three specimens, two microorganisms were detected: HSV and Mycobacterium genus, enterovirus and Mycobacterium genus, CMV and EBV. Table 1 shows the positive PCR results and their correlation with other laboratory findings.

For the first 280 specimens from which nucleic acid was either amplified without extraction (DNA genomes), or by using an in-house method (RNA genomes), 6.1% of the cases were positive by PCR. For the remaining 103 specimens from which nucleic acid was extracted using the QIAGEN QIAamp kit, the rate of positive PCR results was 28.2%.

PCR sensitivity was calculated for CMV, HSV type 1 (HSV-1), measles virus, mumps virus and poliovirus type 2 (Table 2). In the external quality assessment exercise complete concordance in the results achieved was found between the two laboratories (data not shown).

DISCUSSION

The difficulty in making a laboratory diagnosis using traditional techniques in cases of aseptic meningitis and encephalitis has stimulated the development of studies using PCR assays. So far, results for a number of organisms have shown that PCR increases diagnostic sensitivity, specificity, and speed[13-17].

Table 2. Calculation of PCR sensitivity.

	log infectivity ($TCID_{50}$/ml)[1]	log nvp/ml[2]	log ($PCRD_{50}$/ml)[3]	log nvp/$TCID_{50}$ (antilog)	log nvp/$PCRD_{50}$ (antilog)	log $TCID_{50}$/ $PCRD_{50}$ (antilog)[4]
CMV	4.5	8.58	8.0	4.08 (12023)	2.58 (380)	-3.5 (0.0003)
HSV-1	6.5	8.2	9.0	1.7 (50)	2.2 (158)	-2.5 (0.003)
measles virus	3.3	-	4.0	-	-	-0.7 (0.2)
mumps virus	4.0	-	3.0	-	-	1 (10)
poliovirus type 2	7.0	8.03	6.5	1.03 (11)	1.53 (34)	3 (1000)

[1]Infectious dosage of tissue culture in which 50% of inoculated cells were infected.
[2]Number of viral particles counted by electronic microscope.
[3]Refers to the highest dilution detectable by PCR, adjusted for concentration/ml. For example, if in 10µl the test is positive for up to 10^{-4}, the suspension contains 10^6 $PCRD_{50}$.
[4]Number of infectious units in 1ml specimen required to have a positive PCR.

The primary objective of developing a PCR protocol for the detection of the more common organisms that cause aseptic meningitis and encephalitis was to establish a simple and robust routine diagnostic protocol. The protocol developed has assays for 17 of the pathogens that are known to commonly cause CNS symptoms. To determine testing order in this protocol these pathogens were arranged into three groups using the criteria of frequency of occurrence, immune status of the patient, and clinical suspicion. The use of three multiplex PCR assays for the more commonly performed tests (enteroviruses, HSV, VZV; CMV, EBV, HHV6; LCMV, measles virus, mumps virus) made the protocol less time consuming and less expensive. The sensitivity of detection of the viruses by these multiplex assays was not reduced when compared to the respective PCR assay performed with individual primer sets: the end point dilutions of the control cultures positive in the assays were within 1 log of each other (data not shown).

Three-hundred and eighty-three patients with suspected aseptic meningitis and encephalitis were tested using this protocol. It is interesting to compare the results of this series with those from a similar series performed in the UK and reported previously[10]. Overall, a PCR assay was positive in 46 cases (12.0%), higher than the 6.6% rate obtained in the UK. It should be noted that in that series direct detection of DNA genomes was used without nucleic acid extraction, and that when the similarly processed subset of patients from the Brazilian series is compared to the UK series a similar rate of organism detection is observed.

With the replacement of the in-house nucleic acid extraction methods by the QIAamp extraction kit in this series, the PCR positive detection rate increased from 6.1% to 28.2%. This is a much greater increase than that observed when the same change in methodologies was made in the UK: there the increase was from 6.6 to 8.2%. The changes in nucleic acid extraction were made when it was suspected that PCR inhibitors were present in some CSF specimens. The organisms most commonly detected were those with a DNA genome and this may explain the discrepancy in the positivity rate between the two series: in the UK series enteroviruses were the most commonly detected infection and this assay was unaffected by the inhibitory effects of CSF as an in-house RNA extraction system was used. In the two UK series that have been reported an increase was observed after the introduction of the QIAamp procedure in the detection rate of two herpes viruses but not of enteroviruses[18].

Of the 46 patients with a positive PCR result, 32 were immunocompromised (27 had HIV infection, 4 had lymphoreticular neoplasia, and one patient was immunosuppressed following hepatic transplant). Overall in this series 125 patients (32.6%) were known to have HIV infection, much

higher than in the two UK series (3.3% and 4.0%). It is interesting to note that among the immunocompromised patients, 14 had normal CSF cytology, and three had dual CNS infection. The large number of CSF samples positive for EBV DNA raises the question of whether this virus is directly involved in CNS disease pathogenesis, whether innocent virus reactivation is detected, or whether latent EBV genome was detected due to the high sensitivity of the technique[19,20].

In two patients with HIV infection and a clinical diagnosis of tuberculosis meningitis (Table 1, samples 9 and 12), the PCR assay failed to detect Mycobacterium DNA but *Mycobacterium tuberculosis* was grown from both samples. In one of these samples, PCR for EBV DNA was positive, and in the other PCR detected both EBV and CMV DNA. These false-negative results, and the high incidence of tuberculosis infection in the population of Porto Alegre, has lead us to introduce a PCR assay using primers specific for *M. tuberculosis*[21] and with apparent higher molecular sensitivity: when the culture positive specimens were tested with the new PCR assay *M. tuberculosis* DNA was amplified in each case.

In our laboratory, the introduction of molecular techniques and the adoption of a protocol for determining testing of CSF by PCR has greatly improved the laboratory diagnosis of aseptic meningitis and encephalitis. PCR has shown great potential for the diagnosis of CNS infection, however difficulties such as the interpretation of finding EBV and CMV in the CSF of immunocompromised patients remain.

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