

Technical advance

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Comparison of nested PCR and real time PCR of Herpesvirus infections of central nervous system in HIV patients

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Abstract

Background: Molecular detection of herpesviruses DNA is considered as the reference standard assay for diagnosis of central nervous system infections. In this study nested PCR and real time PCR techniques for detection of Herpes simplex virus type 1 (HSV-1), Cytomegalovirus (CMV) and Epstein-Barr virus (EBV) in cerebrospinal fluid of HIV patients were compared.

Methods: Forty-six, 85 and 145 samples previously resulted positive for HSV-1, CMV and EBV by nested PCR and 150 randomly chosen negative samples among 1181 collected in the period 1996–2003 were retrospectively reassessed in duplicate by real time PCR and nested PCR.

Results: Samples giving positive results for CMV, HSV-1 and EBV with nested PCR were positive also with real time PCR. One of the negative samples resulted positive for HSV and one for EBV. Real time PCR showed comparable sensitivity and specificity vs nested PCR.

Conclusion: Real time PCR proved to be a suitable method for diagnosis of herpesvirus infections in CNS, showing comparable sensitivity and being less time consuming than nested PCR.

Background

Opportunistic infections as well as tumors and vascular and metabolic disorders are common in HIV-infected patients [1-3]. Generally, opportunistic viral infections are caused by a broad spectrum of different species with similar clinical patterns, especially those affecting the central nervous system (CNS), where differential diagnosis requires simultaneous screening of a wide range of different viruses [4,5]. Moreover, immunodeficiency induced by HIV infection favours reactivation of herpesviruses which could cause important diseases by themselves [6].

Although the rate of CNS complications is relatively low, if compared with the high prevalence of herpesviruses in the population, these viruses represent the most important pathogens associated with viral encephalitis and meningitis [7,8], being cytomegalovirus (CMV) the most frequently identified virus in HIV-positive patients, followed by Epstein-Barr virus (EBV) and Herpes simplex virus type 1 (HSV-1) [9]. CMV, that is often responsible of asymptomatic infections in immunocompetent host, is able to cause serious manifestations such as retinitis, pneumonia and encephalitis in presence of an alteration of immunoresponse, while the recovery of Epstein-Barr

virus (EBV) in cerebrospinal fluid (CSF) seems to be a prognostic sign for the development of cerebral tumors in patients with AIDS [10,11]. HSV-1 is the most commonly detected virus in diagnostic laboratory, being cause of a variety of clinical symptoms in different anatomical sites such as skin, lips, oral cavity and, especially in immunocompromised patients, CNS [12].

One-step or nested polymerase chain reaction (PCR) has rapidly replaced immunological assays based on virus specific Ig antibodies in CSF for laboratory diagnosis of Herpesvirus infections, even if serological methods are considered an additional tool for defining clinical diagnosis.

Although nested PCR is considered the method of choice in terms of specificity [9,13,14], some additional aspects should be considered.

In the last years, introduction of real time PCR has markedly increased the ease and the speed in the virology laboratory due to the relevant technology that permits rapid temperature cycling within a close system. Considering the importance of relationship between viral load in CSF and severity and outcome of disease, an additional advantage of Real Time PCR is the capability to perform simultaneous qualitative and quantitative analysis.

Here is reported our experience gained in the diagnosis of herpesvirus infections of the CNS in HIV patients by means of nested PCR and Real Time PCR, which has been recently applied in our laboratory.

Methods

CSF samples collection

A total of 1181 CSF samples collected in the period 1996 – 2003 from HIV patients attending at Luigi Sacco Teaching Hospital of Milan (Italy) affected by acute encephalitis

or meningitis or encephalopathy or other neurological syndromes were considered.

Particularly, they consisted of 684, 954 and 933 CSF samples previously tested for HSV-1, CMV and EBV, respectively, by means of nested PCR. Of these, all the positive samples and 150 negative samples randomly chosen were retested by means of nested PCR and real time PCR. Each sample was re-extracted and run in duplicate.

CSF positive and negative samples were stored at -80°C until analysis.

Clinical data of patients with positive samples were available only in the 20% of the total. These patients generally recorded meningoencephalitis signs such as central motor or sensory alterations, consciousness loss, seizures defects.

Nucleic acid extraction

Spin-column based QIAamp Mini Kit (Quiagen, Hilden, Germany) protocol extraction for CSF was used as indicated by the manufacturer.

This procedure allowed for the rapid purification of DNA from 200 µL of CSF and comprised four successive steps carried out using QIAamp Spin Columns in a standard microcentrifuge. Purified DNA was concentrated at a final volume of 20 µL.

Nested PCR

Nested PCR was carried out in a 50 µl mixture containing 40 µl of first amplification mix (outer primers, buffer, dNTP)-(Amplimedical SpA-Bioline Division-Italy), 2U/µl Taq DNA polymerase (Roche Diagnostics-Germany) and 5 µl of purified DNA. Primer pairs selecting for *glycoprotein D* gene of HSV-1 [15], for the late protein *gp58* of CMV [16], and for *Bam HI-W* region of EBV [17] are shown in Table 1.

Table 1: Nucleotide base sequence of primers used in nested PCR

Virus	Region		Primer (5'-3')
HSV-1	GpD	Outer	ATCACGGTAGCCCGGCGCTGTGACA CATACCGGAACGCACCACACAA
		Inner	CCATATCGACCACACCGACGA GGTAGTTGGTCGTTGCGCGCTGAA
CMV	MIEA	Outer	AAGCGGCCTCTGATAACCAAGCC AGCACCATCCTCCTCTTCTCTG
		Inner	AGTGTGGATGACCTACGGGCCATCG GGTGACACCAGAGAATCAGAGGAGC
EBV	BamH I – W	Outer	GAGACCGAAGTGAAGGCCCT GGTGCCTTCTTAGGAGCTGT
		Inner	GCCAGAGGTAAGTGGACTTTAAT GAGGGGACCTGAGACGGGT

Table 2: Nucleotide base sequence of primers used for real time PCR

Virus	Region		Primer (5'-3')
HSV-1	GpD	Forward	CATACCGGAACGCACCACACAA
		Reverse	CCATATCGACCACACCGACGA
CMV	MIEA	Forward	AAGCGGCCTCTGATAACCAAGCC
		Reverse	AGCACCATCCTCCTTCTCCTGG
EBV	EBNA-1	Forward	ATCAGGGCCAAGACATAGAGATG
		Reverse	CCTTTGCAGCCAATGCAACT

After an initial 2 min denaturation at 94°C, 35 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec were carried out, followed by a 5 min extension at 72°C using a thermal cycler (Gene Amp PCR System 2400-Applied Biosystem – Monza – Italy). The reaction mixture for the second amplification round was the same as for the first one, except for the "inner" primers used instead of the "outer" primers. In the second amplification round 44 µl of amplification mix and 1 µl of the first amplification round PCR product were used. The thermal cycling was repeated as for the first amplification round but using 30 cycles after the initial 2 min denaturation. Each amplification run contained a negative control, consisting of water and a positive plasmidial control.

Analysis for the PCR products was performed by means of 4 % agarose gel electrophoresis followed by visualization with ethidium bromide (0.4 µg/mL) staining and UV illumination to confirm the expected products.

Real Time PCR

For diagnostic real time PCR Taq polymerase RT PCR Kit (Amplimedical SpA-Bioline Division-Turin Italy) was used. Target regions for HSV and CMV were the same as in nested PCR, while EBNA-1 gene was amplified for EBV [9] as shown in Table 2..

The RT PCR was performed in 25 µl mixture containing 20 µl of amplification mix (buffer, dNTPs, Taq gold polymerase, Rox passive fluorochrome, primers and MGB Eclipse probe) and 5 µl of purified DNA.

The amplification program included an initial decontamination with uracile N¹-glycosilase at 50°C for 2 min, followed by denaturation at 95°C for 10 min and 45 two steps of 15 sec at 95°C and 1 min at 60°C.

The RT PCR products were detected by measuring fluorescence with passive reference dye in Sequence Detection System ABI Prism 7000 (Applied Biosystem). Control threshold (C_t) values were calculated by determining the point at which the fluorescence exceeded a background

Table 3: Results from nested PCR and real-time PCR

	NESTED PCR		REAL TIME PCR	
	POS	NEG	POS	NEG
CMV	85	150	85	150
HSV-1	45	150+1 ^a = 151	45+1 ^b = 46	149+1 ^a = 150
EBV	145	150	145+1 ^b = 146	149

^a: sample resulted positive in previous nested PCR

^b: samples resulted negative in nested PCR

limit of 0.04. Each analytical session comprised also a negative control (distilled water).

Quantification was carried out by analysing four positive plasmidial standards at 10², 10³, 10⁴ and 10⁵ copies/reaction. The standards were obtained by cloning the target amplification product in a plasmid, which was transformed and cultured in *Escherichia coli*. Plasmidic DNA was purified with a commercial kit (Qiagen) and its concentration determined spectrophotometrically. Then, plasmidic DNA was serially diluted in a stabilizing buffer to the final desired concentration. Amounts of copies/mL in each sample were determined by means of a quantification software (Amplimedical), by considering an extraction recovery of 80%.

Results

Nested PCR

Of 954 CSFs previously examined for CMV by means of nested PCR, 85 samples resulted positive. Among the 684 CSFs tested for HSV-1, 46 samples were found positive, while, 145 of 933 CSFs tested resulted positive for EBV.

Reassessment of these positives and of 150 negative samples confirmed results previously obtained with the same method, with the exception of one HSV-1 positive sample, which resulted negative when retested, as shown in Table 3.

Table 4: Sensitivity and specificity of nested PCR and real-time PCR

	Real-time PCR	
	Sensitivity (%)	Specificity (%)
CMV	85/85 = 100%	150/150 = 100%
HSV-I	45/45 = 100%	150/151 = 99%
EBV	145/145 = 100%	149/150 = 99%

Real Time PCR

Results from real time PCR are reported in Table 3. All the samples for which nested PCR gave positive results were confirmed by Real Time-PCR for CMV, HSV-1 and EBV. Of the 150 samples resulted negative by nested PCR, 148 were negative, while 1 sample resulted positive for HSV and 1 for EBV. The positive sample giving negative result when reassessed by nested PCR was negative also by real time PCR.

Sensitivity and specificity

By considering nested PCR as gold standard [9,13], sensitivity and specificity of real time PCR are described in Table 4. Comparable sensitivity (100%) and specificity (99–100 %) were found for real time PCR in respect to nested PCR.

Discussion

Introduction of PCR into routine diagnostic has rapidly gained a pivotal role for diagnosis of a wide range of diseases, supplanting, in many cases, other methods, such as the classical serodiagnosis. This is particularly true for diagnosis of herpes virus infections in immunocompromised patients, where diminished or suppressed virus-specific antibody responses do not reflect possible reactivated herpes mediated aetiologies [18].

Real time PCR has represented a further step forward, since it allows for quantitative detection of target DNA in a single sample over a large range, remaining possible qualitative detection. Even if contradictory results have been found [19-21], quantification of DNA could represent an important issue to evaluate the severity and outcome of herpesvirus encephalitis, and it may be also used to monitor the success of antiviral therapy. This strategy has already been used for monitoring of patients at risk for CMV infections, when viral load kinetic patterns are used to identify patients who are more likely to have recurrence of CMV disease after the initiation of therapy, as well as to identify patients needing treatment [22-24].

Moreover, in these cases, use of a highly sensitive assay could be of crucial value.

In the last years several real-time PCR methods have been developed for detection of herpesviruses in different biological specimens [25-27]. Real-time PCR has been well recognized to offer several advantages over nested PCR other than allowing quantification of viral load: it reduces the risk of amplicon contamination, being a close-system, is a safer laboratory protocol by not using ethidium bromide, and it allows a notable reduction of time required for response.

In the present work, we compared a real time PCR panel for detection of herpesvirus DNA in CSF with methods employing nested PCR. Our results indicate an overall agreement between the two methods, as reported by other authors [9,27,28]. Differences between the two assays were observed for HSV-1 and EBV analysis, where one negative sample was found positive by real time PCR.

Since extraction panels and primers for HSV and CMV were uniform for the both types of assays, inhibitors present in the DNA preparations could not explain the different results obtained for these samples. Moreover, being nested PCR generally considered as the gold standard for diagnosis of herpes virus in CSF, we tried to use similar primers, chemistry and amplification conditions in order to limit differences for a better comparison of the performance of the two methods. Thus, discrepancies could be likely attributed to the different detection of amplification products, although real time PCR has been reported to be as sensitive as nested PCR [29]. This suggestion is also supported by the fact that the two patients with CSF negative for HSV-1 and EBV with nested PCR showed clinical syndromes compatible with viral encephalitis and clinically improved with antiviral treatment (data not shown). These data seem to suggest that real time PCR could be more sensitive than nested PCR. Since the limit of detection is generally calculated by using plasmidial DNA, it may be possible that, although molecular sensitivity is reported to be similar for nested PCR and real-time PCR [27,28], some differences may occur for biological specimens. The sample classified positive for HSV-1, which resulted negative when retested with both methods, was one of the oldest in our collection, dating 1996, and it might have degraded over the 7 years storage. From this point of view, development of standardized quality controls might be very helpful [30].

Conclusions

Data obtained in this study confirms the validity of real-time PCR method for detection of herpesvirus DNA in CSF specimens of HIV patients, being sensitive, rapid and quantitative. Since specific and rapid diagnosis is the

main target in the case of CNS infections, real time PCR could be considered the method of choice, due to its high specificity, sensitivity and rapidity, once proper quality controls will be available.

Competing interest

The company Amplimedical SpA-Bioline Division-Italy will pay the article processing fees for the manuscript.

Authors' contributions

LD conceived of the study and participated in its design and coordination, AL carried out real time PCR, EDV participated in data analysis and drafted the manuscript, GG performed statistical analysis, RB carried out nested PCR, MRG participated in design and coordination of the study. All authors read and approved the final manuscript.

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