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Review

Molecular analysis of cerebrospinal fluid in viral diseases of the central nervous system

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Abstract

The use of nucleic acid (NA) amplification techniques has transformed the diagnosis of viral infections of the central nervous system (CNS). Because of their enhanced sensitivity, these methods enable detection of even low amounts of viral genomes in cerebrospinal fluid. Following more than 10 years of experience, the polymerase chain reaction or other NA-based amplification techniques are nowadays performed in most diagnostic laboratories and have become the test of choice for the diagnosis of several viral CNS infections, such as herpes encephalitis, enterovirus meningitis and other viral infections occurring in human immunodeficiency virus-infected persons. Furthermore, they have been useful to establish a viral etiology in neurological syndromes of dubious origin and to recognise unusual or poorly characterised CNS diseases. Quantitative methods have provided a valuable additional tool for clinical management of these diseases, whereas post-amplification techniques have enabled precise genome characterisation. Current efforts are aiming at further improvement of the diagnostic efficiency of molecular techniques, their speed and standardisation, and to reduce the costs. The most relevant NA amplification strategies and clinical applications of to date will be the object of this review.

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1. Introduction

Cerebrospinal fluid (CSF) examination is an essential part of the diagnostic work-up of patients with suspected central nervous system (CNS) viral infections. It is often the means to achieve an

etiologically diagnosis, either by the direct identification of the responsible virus, or by the demonstration of an intrathecal specific immune response. Traditional direct virological techniques, including virus isolation, antigen detection and microscopy examination, usually have low sensitivity. Only virus isolation in cell culture may be of some value for diagnosis of aseptic meningitis, with enteroviruses found in almost half of the cases. Virus isolation, however, is insensitive for

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other viruses, such as herpes simplex virus type 1 (HSV-1) and most arboviruses. Virus antigen detection techniques and light or electron microscopy are of limited value, as they require a high number of CSF-infected cells for virus identification (Rubin, 1983). On the other hand, indirect diagnosis by detection of intrathecally produced antibodies generally has poor sensitivity during the early stages of the disease.

Over the last decade, nucleic acid (NA) amplification-based techniques, primarily the polymerase chain reaction (PCR), have revolutionised the diagnosis of CNS infections, especially those caused by viruses (Fredricks and Relman, 1999). Their advantages, accounting for their success in diagnostic neurovirology, have been the extraordinary sensitivity and rapidity. These techniques were first applied to CSF in the early 90s, for the diagnosis of herpes simplex encephalitis (HSE), and enteroviral meningitis (Puchhammer-Stöckl et al., 1990; Rotbart, 1990). Since then, the number of scientific reports in this field has rapidly increased and NA amplification-based assays are now routinely performed in most diagnostic laboratories.

NA amplification-based techniques have become the test of choice for some viral CNS infections, such as HSE (Darnell, 1993; Tyler, 1994; Weber et al., 1996; Cinque et al., 1996a). Furthermore, they have been useful to establish a viral etiology in neurological syndromes of dubious origin, e.g., Mollaret's meningitis (Tedder et al., 1994), or to help recognise unusual or poorly characterised CNS diseases, such as mild forms of herpes encephalitis (Schlesinger et al., 1995a; DeVincenzo and Thorne, 1994; Domingues et al., 1997; Fodor et al., 1998) or cytomegalovirus (CMV) ventriculoencephalitis in human immunodeficiency virus (HIV)-infected patients (Arribas et al., 1996). Molecular methods have made it possible to identify viruses in CSF normally causing extracerebral infections, such as rotavirus, parvovirus B19, CMV or human herpesvirus 6 (HHV-6), thus supporting their etiological role in inducing CNS disease (Kondo et al., 1993; Suga et al., 1993; Cassinotti et al., 1993; Ushijima et al., 1994; Studahl et al., 1995; McCullers et al., 1995; Barah et al., 2001). Finally, molecular techniques

have been of unique value for identification of novel viruses responsible of CNS disease (Chua et al., 2000; Cardosa et al., 1999).

2. Nucleic acid amplification

2.1. Methods

2.1.1. CSF preparation

CSF treatment is usually required prior to NA amplification, in order to release NA from cells and to remove substances that may degrade NA or inhibit amplification. However, the relatively simple CSF composition may obviate, at least for DNA viruses, the need for NA purification. The simplest approaches include heating to high temperatures or repeated freeze-thawing of the specimens, procedures that facilitate cell membrane disruption and release of DNA (Table 1). These procedures have the advantage of being rapid, requiring small CSF volumes, and reducing the risk of sample contamination during NA purification steps. However, the lack of removal of inhibiting molecules might interfere with the

Table 1
CSF preparation prior to nucleic acid amplification

Principle	Method (examples)
CSF cell lysis	Heating to 95 °C, freezing thawing
CSF cell lysis-protein digestion	Detergents (SDS), proteases (protease K), chaotropic agents (guanidinium thiocyanate) ^a
Nucleic acid concentration	Ultracentrifugation Ethanol precipitation of nucleic acids
Nucleic acid extraction	Phenol–chloroform, spin column, silicate absorption, magnetic separation

Methods for cell lysis, and concentration and extraction of nucleic acids can variably be combined. Required time varies from 10 min (e.g., by mechanical cell lysis) to ≥ 1 h (e.g., protease K digestion and/or complex nucleic acid procedures, e.g., phenol–chloroform). Required volume varies from 2–5 μ l (e.g., mechanical cell lysis) to ≥ 1 ml (use of CSF concentration procedures).

enzymes used for amplification. NAs may also be concentrated and/or purified from CSF, by a number of in-house procedures or commercial kits (Table 1). Although some extraction methods seem to perform better than others in comparative studies (Casas et al., 1995; Fahle and Fischer, 2000), none of the known protocols has been shown to be clearly superior. Practically, the choice of one CSF preparation method is supported by a number of considerations, including the type of NA target and the amplification protocol employed, as well as the individual laboratory experience.

2.1.2. NA amplification techniques

NA amplification techniques enable the amplification of small quantities of target NA molecules to considerably larger amounts (over 10^6 copies), which can be visualised by means of common laboratory procedures. This results in a very high sensitivity, which is the main advantage of these techniques. PCR is the most popular amplification method, but a number of other techniques have been described, including the ligase chain reaction, the strand displacement assay, the transcription mediated amplification, the nucleic acid sequence based amplification (NASBA), the branched DNA technique, and the hybrid capture assay (Tang and Persing, 1999).

PCR has been widely used for detection in CSF of both DNA and RNA viruses. In the case of RNA viruses, a complementary DNA (cDNA) needs to be generated from RNA prior to amplification, by means of a reverse transcriptase (RT). A variant of the classical procedure, largely used for analysis of CSF and other biological fluids containing few viral particles, is the ‘nested’ PCR. This consists of two separate amplification reactions using two primer sets, the second of which is located between the first one, thus increasing substantially both sensitivity and specificity of detection (Tang and Persing, 1999).

NA amplification techniques other than PCR that have been applied to detect viral genomes in CSF include the NASBA and the branched DNA assays. NASBA, like PCR, is based on target NA amplification, but the synthesis of new molecules occurs through an isothermal reaction and re-

quires three different enzymes. Furthermore, the template consists of RNA (Kievits et al., 1991). Examples of NASBA studies of CSF include those performed to detect the CMV pp67 late gene transcripts in HIV-infected patients with CMV encephalitis; enterovirus, West Nile (WN) or St. Louis encephalitis virus RNA in patients with aseptic meningitis or encephalitis (Zhang et al., 2000; Bestetti et al., 2001; Lanciotti and Kerst, 2001; Heim and Schumann, 2002; Fox et al., 2002), and to assess HIV-1 RNA levels in patients with HIV infection at different disease stages (McArthur et al., 1997; Shepard et al., 2000). The branched DNA assay is based on signal amplification rather than target-amplification (Urdea, 1994). This assay has been used to measure CMV DNA and HIV-1 RNA levels in the CSF of HIV-infected patients (Flood et al., 1997; Stingle et al., 2001).

2.1.3. Detection of amplified products

There are different procedures to detect the products of NA amplification. The simplest technique is based on the visualisation of DNA bands of the expected size by agarose gel electrophoresis after staining with ethidium-bromide. Alternative or supportive methods include hybridisation with DNA probes complementary to the target DNA, following DNA transfer to a filter, tubes, or microplates. Probes are labelled with enzymes or other molecules that lead to signal detection on appropriate stimulation. Colorimetric enzyme-linked immunosorbent assays (ELISA), in which the amplified products is captured by a probe coated to a microplate, have proved to be very practical. For this reason colorimetric ELISAs have largely been adapted to commercial kits and used for CSF analysis for a number of viruses (Rotbart et al., 1994; Ellis et al., 1997; Bestetti et al., 2001).

2.1.4. Variants of PCR: Multiplex PCR and PCR with consensus primers

Since similar neurological pictures may result from different CNS infections, it may be practical to use PCR assays that detect more than one virus or infectious agent in the same reaction. The most obvious advantage of this approach is that the

number of tests are reduced, with substantial time and cost savings. Two main strategies are used for this purpose: multiplex PCR and PCR with consensus primers.

Multiplex PCR enables the identification of more than one DNA sequence by means of two or more primer pairs, each specific for one sequence (Fig. 1). An important requirement of this approach is that the amplification conditions, i.e. reagent mixture composition and thermocycling profile, are similar for all the primer pairs, in order not to compromise amplification efficiency for each primer pair. Developments of multiplex PCR might lead to universal diagnostic protocols, based on the use of several primer pairs with fixed thermocycle programs and reagent composition (Kuno, 1998). Multiplex PCR assays are largely employed in diagnostic neurovirology, e.g. for simultaneous detection of HSV-1 and HSV-2 (Kimura et al., 1990; Cassinotti et al., 1996; Cinque et al., 1998a), or of a larger number of herpesviruses (Tenorio et al., 1993; Baron et al., 1996; Pozo and Tenorio, 1999; Quereda et al., 2000; Markoulatos et al., 2001). Protocols have also been designed for simultaneous amplification of viruses causing similar clinical pictures. These include assays for herpesvirus and enterovirus (Read and Kurtz, 1999; Casas et al., 1999), for measles, rubella and parvovirus B19 (del Mar Mosquera et al., 2002) or for different combinations of mosquito-transmitted viruses (Lee et al., 2002). A duplex PCR protocol for the amplifica-

tion of Epstein–Barr virus (EBV) and *Toxoplasma gondii* has also been proposed in AIDS patients to help distinguish CNS lymphoma from toxoplasmosis (Roberts and Storch, 1997).

PCR with consensus primers is used to amplify conserved sequences in common to different viruses, but belonging to the same family. Following amplification, the product may be identified by DNA sequencing, by hybridisation with specific probes or by restriction-enzyme analysis (Fig. 2), or, when possible, by visualisation of bands of different size on agarose gel. This strategy has widely and successfully been used with herpesviruses: to amplify simultaneously HSV-1, HSV-2, CMV and EBV by means of primers targeting conserved region of the herpesvirus DNA polymerase gene (Rozenberg and Lebon, 1991), to detect all the known human herpesviruses in two PCR assays (Johnson et al., 2000) or most of them in a single reaction through the use of ‘stair primers’ (Minjolle et al., 1999; Bouquillon et al., 2000). Further examples include protocols designed to detect most enterovirus strains using primers that recognise conserved sequences within the 5′ noncoding region of the picornavirus family (Kammerer et al., 1994), the three human polyomaviruses JC virus (JCV), BK virus and SV40 (Arthur et al., 1989; Fedele et al., 1999) or different flaviviruses (Harris et al., 1998; Scaramozzino et al., 2001; Mousavi-Jazi and Lundkvist, unpublished observation).

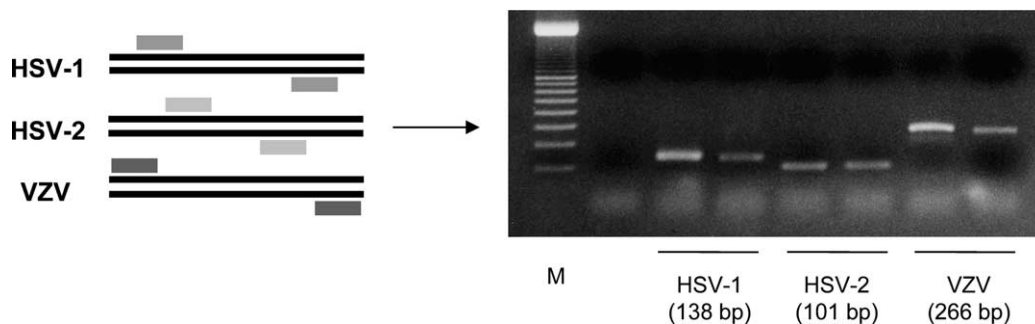


Fig. 1. Example of multiplex PCR. Three unrelated sequences of HSV-1, HSV-2 and VZV, are amplified simultaneously in the same test tube by using three different primer pairs, specific for each virus. The amplification products can be differentiated on agarose gel if the amplified fragments yield bands of different size (M: 100 bp DNA ladder marker). Alternatively, amplification products can be identified through an additional step, by hybridization with specific probes, restrictions enzyme analysis, nested PCR with specific internal primers, or DNA sequencing.

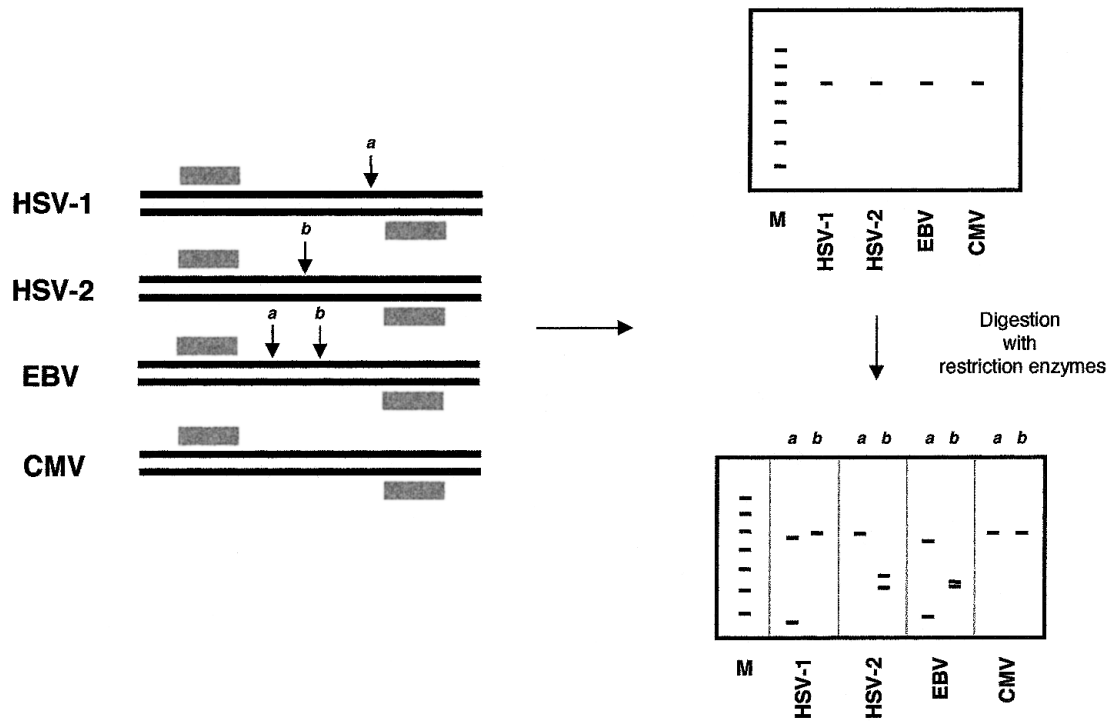


Fig. 2. Example of PCR assay with consensus primers (adapted from Rozenberg and Lebon, 1991). Conserved DNA sequence from the polymerase genes of HSV-1, HSV-2, EBV and CMV are amplified simultaneously in the same tube by a consensus primer pair targeting regions in common to these viruses. Following agarose gel electrophoresis, the amplified products have similar length (top) but each virus can be distinguished by using restriction enzymes (bottom) (a, *Sma*I and b, *Bam*HI; M: DNA marker). Alternatively, viruses can be differentiated following hybridization with virus-specific probes or DNA sequencing.

2.1.5. False positive and false negative results

The possibility of generating false positive results by sample contamination is a major risk of NA amplification techniques. Clinical specimens or products of previous amplifications (carry-over) are the most common source of contamination. In order to minimize this risk, it is recommended to maintain CSF sterility in all the pre-laboratory and laboratory steps and to carry out the different laboratory steps in separate areas (Persing, 1991). In addition, some laboratories use the enzyme uracil *N*-glycosylase (UNG), which degrades products from previous amplifications but not native NA templates, to prevent carry-over of amplification products. This is accomplished by substituting dUTP for dTTP in the amplification

mixture, and pretreating all subsequent mixtures with UNG prior to amplification (Longo et al., 1990). More in general, the use of negative controls, usually water or known negative samples tested in parallel with the CSF specimens throughout the whole procedure, and analysis of samples in duplicate, are useful to recognise false positive results.

False negative results can be caused by the presence of inhibitors, i.e. molecules affecting the correct functioning of enzymes. Inhibition of amplification has been reported in 1–5% of CSF specimens (Tang et al., 1999). In order to reveal the presence of inhibition, it may be useful to amplify 'internal standard' molecules together with the target. In addition, amplification of

both strong and weak positive controls in parallel with CSF samples can further help monitoring amplification efficiency of the whole reaction.

2.1.6. CSF storage conditions

Since viable virus is not necessary for NA amplification-based procedures, NAs can be found in CSF samples kept stored for long periods. DNA of HSV can be recovered in CSF samples maintained for up to 30 days at -20°C , $2-8^{\circ}\text{C}$, and even at room temperature (Wiedbrauk and Cunningham, 1996). Actually, it has been recovered following storage at -20°C for several years (personal observation). From a practical point of view, it is considered safe to send CSF specimens for the search of DNA viruses to the laboratory at room temperature. However, it is preferable that CSF samples are kept at 4°C even for short-term storage, or frozen if they cannot be delivered or examined within 1 day (Cinque et al., 1996a).

On the other hand, RNA is regarded to be less stable than DNA, and its detection in plasma seems to be affected by factors such as, the type of anticoagulant used for specimen collection and storage temperature (Moudgil and Daar, 1993; Holodniy et al., 1995). However, recovery of enterovirus RNA in CSF seems not to be reduced by storage at 4°C or room temperature for 96 h after sampling (Rotbart et al., 1985). Similarly, no significant decay of HIV-1 RNA load was observed after up to 96 h of storage at 4°C (Ahmad et al., 1999). Theoretically, storage at 4°C might actually be advantageous in the case of enveloped RNA viruses, because freezing may destroy the envelope, and thus make the RNA vulnerable to nucleases.

2.2. Clinical applications

Tables 2 and 3 show an overview of NA amplification use in diagnostics, in immunocompetent and immunocompromised patients, respectively. Some of the most relevant examples will be briefly emphasised.

2.2.1. Herpes simplex encephalitis

The detection of HSV-1 DNA in CSF is one of the most convincing examples of the diagnostic use

of molecular analysis. This approach has now largely replaced the identification of HSV in brain tissue biopsies as the method of choice for the etiological diagnosis of HSE (Linde et al., 1997; Tang et al., 1999). A number of retrospective and prospective studies have demonstrated the reliability of PCR methods, reporting a sensitivity higher than 90% and a virtually 100% specificity (Aurelius et al., 1991; Lakeman and Whitley, 1995; Linde et al., 1997). Furthermore, NA amplification techniques enable the diagnosis of uncommon forms of HSV CNS infection that might otherwise go unrecognised (Schlesinger et al., 1995b; DeVincento and Thorne, 1994; Domingues et al., 1997; Fodor et al., 1998). Most importantly, CSF analysis by these techniques is rapid, making the laboratory diagnosis useful for management decisions. However, it is important that the PCR results are interpreted cautiously in relation to the clinical presentation and the duration of antiviral therapy. Initially negative PCR results have been observed very early after onset of HSE symptoms, likely to reflect a still limited virus replication (Rozenberg and Lebon, 1991; Puchhammer-Stöckl et al., 2001). On the other hand, the likelihood of finding a positive CSF PCR result is reduced following a few days of acyclovir treatment, as well as in untreated patients from whom CSF is obtained late after onset of neurological symptoms (Rozenberg and Lebon, 1991; Aurelius et al., 1991; Lakeman and Whitley, 1995).

2.2.2. Enterovirus meningitis

Although enteroviruses are frequently isolated from CSF of patients with enteroviral meningitis by cell culture, molecular techniques have significantly improved the detection rate of these infections (Jeffery et al., 1997). Protocols have been designed with primers that recognise almost all of the enterovirus serotypes, including those that can not be isolated in cell systems. Exceptions are Echo viruses 22 and 23 that diverge extremely from the other serotypes (Oberste et al., 1998). Because of the enhanced sensitivity of NA amplification methods, the virus can also be detected in CSF samples obtained a few days after onset of symptoms, where isolation of virus is usually infrequent (Yerly et al., 1996). Furthermore, the

Table 2

Diagnostic use of nucleic acid amplification technique in CSF in CNS infections of immunocompetent patients

Family (nucleic acid) ^a	Virus	Main Common Clinical Syndromes	Significance of NA detection in CSF ^b	Comments	References
Herpesviridae (dsDNA)	HSV-1	Herpes encephalitis (HSE), neonatal infection	Diagnosis of HSE (test of choice), etiological characterization and diagnosis of atypical HSE forms, diagnostic potential in neonatal infections	> 90% sensitivity vs. brain biopsy	Aurelius et al. (1991), Kimura et al. (1991), Lakeman and Whitley (1995), Linde et al. (1997), Kimberlin et al. (1996)
	HSV-2	Aseptic meningitis, recurrent meningitis, neonatal infection	Diagnosis of aseptic meningitis, etiological characterization and diagnosis of recurrent meningitis, diagnostic potential in neonatal infections		Kimura et al. (1991), Aurelius et al. (1993), Tedder et al. (1994), Schlesinger et al. (1995b), Kimberlin et al. (1996)
	VZV	Varicella and herpes zoster (HZ) complications	Diagnosis of aseptic meningitis and others VZV-associated CNS diseases	NA detection also in cases of uncomplicated HZ	Puchhammer-Stöckl et al. (1991), Echevarria et al. (1994), Haanpaa et al. (1998)
	CMV	Aseptic meningitis, encephalitis, neonatal infection	Etiological characterization and diagnosis of various neurological syndromes, diagnostic potential in neonatal infections		Darin et al. (1994), Troendle Atkins et al. (1994), Studahl et al. (1995)
	EBV HHV-6	Aseptic meningitis, encephalitis Febrile seizures, encephalitis	Diagnostic potential Association with febrile child seizures and encephalitis		Imai et al. (1993), Landgren et al. (1994) Kondo et al. (1993), Suga et al. (1993), Caserta et al. (1994), McCullers et al. (1995), Hall et al. (1998)
	HHV-7	Febrile seizures	Association with febrile child seizures and other neurological conditions		Torigoe et al. (1996), van den Berg et al. (1999), Yoshikawa et al. (2000), Komatsu et al. (2000), Pohl-Koppe et al. (2001)
Polyomoviridae (ssDNA)	BKV	Encephalitis?	Occasional association with encephalitis		Voltz et al. (1996)
Reoviridae (ssDNA)	Rotavirus	Aseptic meningitis, encephalitis	Etiological characterization and diagnosis of rotavirus CNS diseases		Keidan et al. (1992), Nishimura et al. (1993), Ushijima et al. (1994), Abe et al. (2000)
Parvoviridae (ssDNA)	Parvovirus B19	Aseptic meningitis	Etiological characterization and diagnosis of parvovirus B19 meningitis		Cassinotti et al. (1993), Okumura and Ichikawa (1993), Druschky et al. (2000), Barah et al. (2001)
Picornaviridae (ss + RNA)	Enterovirus	Aseptic meningitis	Diagnosis of aseptic meningitis (test of choice)	> 90% sensitivity vs. virus isolation	Rotbart (1990), Glimaker et al. (1993), Jeffery et al. (1997)
Togaviridae (ss + RNA)	Rubella	Aseptic meningitis, subacute panencephalitis, neonatal infection	Occasional association with encephalitis		Date et al. (1995)
Faviviridae (ss + RNA)	Dengue viruses	Encephalitis	Diagnostic potential		Lum et al. (1996), Cam et al. (2001)
	Japanese encephalitis	Encephalitis	Diagnostic potential		Igarachi et al. (1994)
	West Nile	Encephalitis	Diagnostic potential		Briese et al. (2000), Lanciotti et al. (2000), Lanciotti and Kerst (2001)
	Tick borne encephalitis	Encephalitis	Diagnostic potential		Günther (1997), Puchhammer-Stöckl et al. (1995)

Table 2 (Continued)

Family (nucleic acid) ^a	Virus	Main Common Clinical Syndromes	Significance of NA detection in CSF ^b	Comments	References
	Saint Louis encephalitis	Encephalitis	Diagnostic potential		Huang et al. (1999b), Lanciotti and Kerst (2001)
Bunyaviridae (ss–RNA)	Jamestown Canyon	Encephalitis	Diagnostic potential		Huang et al. (1999a,b)
	La Crosse	Encephalitis	Diagnostic potential		Huang et al. (1999b)
	Toscana	Aseptic meningitis	Diagnosis of aseptic meningitis		Valassina et al. (1996, 2000)
Ortomyxoviridae (ss–RNA)	Influenza	Encephalitis	Etiological characterization and diagnostic potential in influenza associated CNS disease		Fujimoto et al. (1998), McCullers et al. (1999), Ito et al. (1999), Togashi et al. (2000)
Paramyxoviridae (ss–RNA)	Mumps	Aseptic meningitis	Diagnosis of aseptic meningitis	> 90% sensitivity vs. virus isolation	Poggio et al. (2000)
	Measles	Acute encephalitis, subacute encephalitis, subacute sclerotizing panencephalitis (SSPE)	Diagnostic potential in acute encephalitis and SSPE		Matsuzono et al. (1994), Nakayama et al. (1995), Tomoda et al. (2001)
	Nipah	Encephalitis	Diagnostic potential		Paton et al. (1999)
	Hendra	Meningitis, encephalitis	Diagnostic potential		O'Sullivan et al. (1997)
Arenaviridae (ss–RNA)	Lassa	Encephalitis	Occasional association with encephalitis. Etiological characterization of Lassa virus associated encephalopathy?		Gunther et al. (2001)
Rhabdoviridae (ss–RNA)	Rabies	Rabies	Diagnostic potential		Crepin et al. (1998), Wacharapluesadee and Hemachudha (2001)
Retroviridae (RNA, NA)	HTLV-1	HTLV-associated myelopathy (HAM)	Diagnostic potential	Detection of cell-associated DNA	Kompoliti et al. (1996), Furuya et al. (1998), Cavrois et al. (2000)

NAs of other viruses have also been found in the CSF, but without clear association with CNS disease, e.g., hepatitis C virus (HCV), TTV, coronavirus, SV40 (Maggi et al., 1999; Dessau et al., 1999; Cristallo et al., 1997; Maggi et al., 2001; Tognon et al., 2001). HSV-1, herpes simplex virus type 1; HSV-2, herpes simplex virus type 2; VZV, varicella-zoster virus; CMV, cytomegalovirus; EBV, Epstein–Barr virus; HHV-6, human herpesvirus 6; HHV-7, human herpesvirus 7; BKV, BK virus.

^a dsDNA, double-stranded DNA virus; ssDNA, single-stranded DNA virus; dsRNA, double-stranded RNA virus; ss+RNA positive stranded RNA virus; ss–RNA, negative stranded RNA virus (van Regenmortel et al., 2000).

^b PCR has been the most commonly employed NA amplification technique.

Table 3
Diagnostic use of nucleic acid amplification techniques in CSF in viral CNS infections of immunocompromised patients

Family (nucleic acid) ^a	Virus	Main clinical syndromes	Significance of NA detection in CSF	Comments	References
Herpesviridae (dsDNA)	HSV-1	Subacute encephalitis	Diagnosis of HSV-associated clinical syndromes in HIV-infected patients	100% sensitivity, 99% specificity (HIV-infected patients)	Tan et al. (1993), Cinque et al. (1998a)
	HSV-2	Subacute encephalitis	Diagnosis of HSV-associated clinical syndromes in HIV-infected patients	100% sensitivity, 99% specificity (HIV-infected patients)	Miller et al. (1995), Cinque et al. (1998a)
	VZV	Varicella and herpes zoster (HZ)	Diagnosis of VZV-associated clinical syndromes in HIV-infected patients		Burke et al. (1997), Cinque et al. (1997b), Iten et al. (1999)
	CMV	Subacute encephalitis, polyradiculopathy	Diagnosis of CMV-associated clinical syndromes in HIV-infected patients	82–100% sensitivity, 89–100% specificity (HIV-infected patients)	Cinque et al. (1992), Gozlan et al. (1992), Wolf and Spector (1992), Clifford et al. (1993), Fox et al. (1995), Cinque et al. (1998b)
	EBV	Lymphoproliferative disorders (transplanted patients), PCNSL (HIV-infected patients)	Tumor marker in HIV-associated PCNSL	88–100% sensitivity, 89–100% specificity (PCNSL in HIV-infected patients)	Cinque et al. (1993), Arribas et al. (1995a), de Luca et al. (1995), Cinque et al. (1996b)
	HHV-6	Encephalitis (transplanted patients)	Diagnostic potential in transplanted patients. Lack of clear association with CNS disease in HIV-infected patients		Knox et al. (1995), Wang et al. (1999), Bossolasco et al. (1999), Singh and Paterson (2000)
Polyomaviridae (dsDNA)	JCV	Progressive multifocal leukoencephalopathy (PML)	Diagnosis (non-invasive test of choice)	72–100% sensitivity, 92–100% specificity (HIV-infected patients)	Weber et al. (1994), Fong et al. (1995), McGuire et al. (1995), Cinque et al. (1996b), de Luca et al. (1996)
	BKV	Encephalitis	Occasional association with meningoencephalitis		Bratt et al. (1999)

HCV RNA has also been found in the CSF of HIV-infected patients, but without clear association with CNS disease (Maggi et al., 1999; Morsica et al., 1997; Gazzola et al., 2001). PCNSL, primary CNS lymphoma.

^a See Table 2, footnotes.

use of these techniques has reduced the time for diagnosis from 4–10 days to 1 day. The diagnostic reliability of both in-house and commercial NA amplification assays has been extensively evaluated, showing a >90% sensitivity and 48–89% specificity when compared to viral isolation. The low specificity probably reflects enterovirus detection in culture-negative CSF samples from patients with true enterovirus meningitis (Glimaker et al., 1993; Lina et al., 1996; Yerly et al., 1996; Muir and van Loon, 1997; Romero, 1999).

2.2.3. Encephalitis and meningitis caused by zoonotic viruses

Although highly sensitive methods have been developed for detection of viral genomes in patient samples for most of the important arbo- and rodent-borne viruses, these methods are in many cases of limited value in the routine diagnostics. The major reason is that the time period for the viremia is often very short, i.e. the virus is usually no longer detectable at the onset of systemic or CNS disease. A typical example is tick-borne encephalitis (TBE), where attempts to use RT-PCR to tract TBE virus RNA in acute phase CSF or serum have to large extent been unsuccessful, with only a few samples having been found positive in the sero-negative, or in the IgM-positive but IgG-negative, phases (Günther, 1997; Puchhammer-Stöckl et al., 1995; Lundkvist et al., unpublished observation). Other examples where detection of viral genomes is of limited use, and the diagnostics have to be based, or at least supplemented, by serology, are Japanese encephalitis (Igarachi et al., 1994), encephalitis caused by the Western, Eastern and Venezuelan equine encephalitis, and the Dengue viruses. On the other hand, the use of RT-PCR in CSF has rendered more positive results for other flaviviruses, such as West Nile virus, or Bunyaviruses, e.g. La Crosse, Jamestown Canyon or Toscana viruses. Real-time PCR analysis of CSF samples collected from patients with serologically confirmed West Nile encephalitis during the 1999 epidemics in the New York area, revealed a 57% sensitivity and 100% specificity, respectively (Lanciotti et al., 2000). Although a correlation was observed between positivity rate and survival, this observation re-

mains to be confirmed (Briese et al., 2000). Toscana virus, a phlebotomus-transmitted virus causing an endemic infection in the Tuscany area of Italy is the responsible of benign forms of meningitis. By RT-PCR, Toscana virus sequences have been identified in CSF from as many as in 30% of patients with acute meningitis who were hospitalised in this geographic area. These findings not only indicate NA amplification analysis as a valid diagnostic support, but also as a means for estimating the relevance of this disease in the population (Valassina et al., 2000).

2.2.4. HIV-related opportunistic diseases of the CNS

Neurological complications is one of the major problems in patients with HIV infection. Although their frequency has significantly declined in the developed world following the advent of highly active anti-retroviral therapies (HAART), they still represent a major diagnostic and therapeutic challenge. CNS diseases caused by viruses include encephalitis, meningitis, myelitis or mixed pictures caused by herpesviruses, and progressive multifocal leukoencephalopathy (PML), associated with JCV infection of the CNS. In this field, the impact of diagnostic molecular techniques has been remarkable (Cinque et al., 1997a). Detection of CMV DNA in CSF has shown to be highly sensitive and specific for the diagnosis of CMV encephalitis, a disease reported in as many as one third of AIDS patients (Cinque et al., 1992; Gozlan et al., 1992; Wolf and Spector, 1992; Clifford et al., 1993; Fox et al., 1995; Cinque et al., 1998b). The identification of HSV-1, HSV-2 and varicella-zoster virus DNA in CSF has largely contributed to identify and clinically characterise the CNS complications induced by these viruses, and also provided a useful tool for their diagnosis and clinical management (Tan et al., 1993; Miller et al., 1995; Burke et al., 1997; Cinque et al., 1997a, 1998a; Iten et al., 1999). CSF PCR for JCV has partly replaced the practice of brain biopsy as diagnostic method of choice for PML, though JCV sequences are demonstrated by PCR in only two thirds of the patients, with higher rates of detection in the more advanced stages of disease (Weber et al., 1994; Fong et al., 1995; McGuire et al., 1995;

Cinque et al., 1996a; de Luca et al., 1996). Furthermore, clearance of JCV DNA from CSF has frequently been observed in patients receiving HAART, in association with PML stabilisation (Miralles et al., 1998; Giudici et al., 2000), suggesting that the rate of JCV DNA detection among PML patients might further decrease as a consequence of anti-HIV therapy. Another virus-related CNS disease in HIV-infected patients is primary CNS lymphoma (PCNSL), which is almost always associated with the presence of EBV in the tumour cells (MacMahon et al., 1991). Studies in patients with histologically proven PCNSL or CNS localization of systemic non-Hodgkin lymphomas have reported a striking association between the presence of these complications and EBV DNA detection in CSF. In some patients, EBV-DNA could even be detected days or months before the lymphoma manifested itself clinically (Cinque et al., 1993; Arribas et al., 1995a; de Luca et al., 1995; Cinque et al., 1996b; Cingolani et al., 2000).

3. Quantitative NA amplification

3.1. Methods

Measuring the amount of NAs in clinical specimens is a successful development of basic diagnostic molecular techniques. Both PCR and other NA amplification techniques have been proven to be reliable for this purpose, and a variety of semi-quantitative and quantitative PCR methods are described (Clementi et al., 1996; Hodinka, 1998; Preiser et al., 2000). Semi-quantitative techniques include methods based on limiting dilution of samples before amplification, or comparison of the extent of amplification between samples and 'external' standards at known NA concentration. The main disadvantage of these procedures is that they do not take into account the possible differences in amplification efficiency between the different samples and/or standards. Quantitative techniques allow a more accurate estimate of the NA levels, through co-amplification in the same tube of target NA and an 'internal' standard at a

known concentration, which enables control of the amplification efficiency.

New automated procedures based on real-time detection of NAs are becoming popular in diagnostic neurovirology. The real-time PCR is based on detection and quantification of a fluorescent reporter. Fluorescence emission is recorded at each cycle making it possible to monitor the PCR reaction during its exponential phase, where the amount of PCR product correlates to the initial quantity of template. Compared to classical methods where the amounts of DNA are measured at the end of amplification, when the amplification efficiency is reduced, real-time PCR is more accurate and expands the dynamic range of quantification. It also eliminates post-PCR processing of amplification products, resulting in reduced risk of contamination and increased speed (Bustin, 2000). There are two general methods for NA quantitation by real-time PCR: those based on the use of DNA-binding dyes, e.g. syber green, or of fluorescence-labelled probes. The latter are employed in the TaqMan (Fig. 3) (Holland et al., 1991; Higuchi et al., 1993; Heid et al., 1996) and LightCycler (Wittwer et al., 1997a,b) technology, that have both been described for quantitation of viral DNA in CSF (Kessler et al., 2000; Verstrepen et al., 2001; Gunther et al., 2001; Nagai et al., 2001; Read et al., 2001; Gautheret-Dejean et al., 2002; Aberle and Puchhammer-Stöckl, 2002) (Table 4). Real-time PCR also allows simultaneous quantification, in the same tube, of different genomes (Read et al., 2001), as well as virus genotyping and mutational analysis.

3.2. Clinical applications

Quantification of viral genomes in the CSF can be important at the time of diagnosis of viral encephalitis or meningitis, in order to obtain diagnostic or prognostic information, as well as for subsequent patient management, e.g. during antiviral therapy. Table 4 summarises the most significant clinical applications of quantitative molecular techniques in viral CNS infections.

CMV and HIV infections of the CNS are good examples where quantitative methods are most useful. In CMV encephalitis, the measurement of

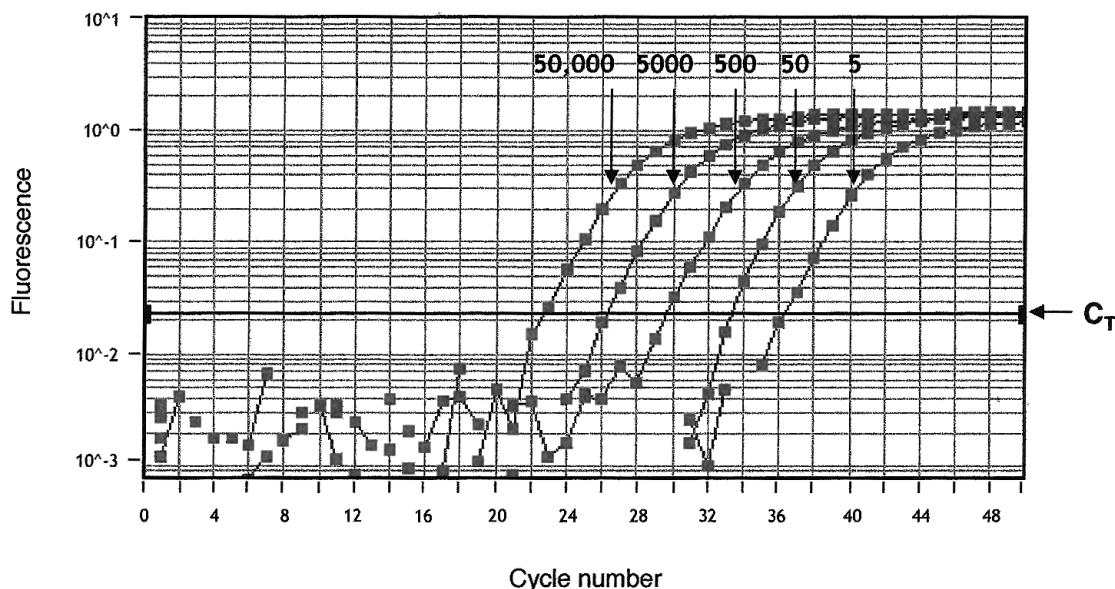


Fig. 3. Example of a standard curve consisting of 5–50 000 EBV DNA genomes/reaction generated by automated real-time PCR using the TaqMan technology. The fluorescence, proportional to the amount of amplified products, is acquired at each PCR cycle by an automated fluorometer. A threshold cycle (C_T) defines the cycle number at which the fluorescence passes a fixed threshold. Quantification of the amount of target in unknown samples is accomplished by measuring the C_T and comparing this value to the C_T values of the standard curve.

CSF CMV DNA levels at the time of diagnosis is useful in order to distinguish extensive from mild infections (Arribas et al., 1995b; Bestetti et al., 2001). In patients with CMV encephalitis or polyradiculomyelitis, the CMV DNA levels tend to decrease following antiviral therapy with ganciclovir or foscarnet, although the persistence of significant levels is frequent and it is associated with lack of clinical improvement (Cinque et al., 1995; Flood et al., 1997).

Commercial assays, including PCR, NASBA and bDNA assays, have been used for HIV-1 RNA quantification in CSF (Cinque et al., 2000). As a consequence of early virus invasion of the CNS, HIV-1 RNA can be detected in CSF at any stage of HIV infection and irrespective of the presence of neurological symptoms. However, CSF viral load is usually higher in patients with productive HIV infection of brain cells, i.e. HIV-associated dementia or encephalitis (McArthur et al., 1997; Brew et al., 1997; Ellis et al., 1997; Cinque et al., 1998c). Current anti-HIV treatments induce substantial decreases of CSF RNA levels

and quantitative molecular techniques can thus be useful to monitor the local response to treatment (Gisslen et al., 1997; Foudraire et al., 1998; Staprans et al., 1999; Gisolf et al., 2000; Price et al., 2001).

4. Post-amplification analyses

4.1. Methods

Besides their use for direct diagnosis, NA amplification techniques constitute the base for genomic analysis. These techniques can yield high amounts of genomic material, which can be analysed for different purposes. These include virus characterisation for epidemiologic and phylogenetic studies, detection of viral mutations, e.g. those associated with antiviral drug resistance, neurotropism or neurovirulence. In addition, genotyping of viruses in CSF may be used to recognise unusual viral strains or to characterise new viral pathogens involved in CNS disease. There are a

Table 4
Example of NA quantification in the CSF

Family	Virus	Quantitative techniques employed	Significance of NA quantitation in CSF	References
Herpesviridae	HSV-1	Competitive PCR, real-time PCR	Wide range of level variation (up to 10^7 copies/ml). Association of high DNA levels with bad HSE outcome? Decline of DNA levels following aciclovir therapy in HSE	Ando et al. (1993), Revello et al. (1997), Domingues et al. (1998), Kessler et al. (2000), Aberle and Puchhammer-Stöckl (2002)
	HSV-2	Real-time PCR	Narrower range of level variation in patients with HSV-2 meningitis than in patients with HSV-1 encephalitis. Highest levels found in children with congenital infection (up to 10^6 copies/ml)	Aberle and Puchhammer-Stöckl (2002)
	VZV	Semiquantitative PCR, real-time PCR	Higher levels in patients with herpes zoster complications than in those with varicella	Puchhammer-Stöckl et al. (1991), Aberle and Puchhammer-Stöckl (2002)
	CMV	Semiquantitative PCR, competitive PCR, branched DNA	Association of high DNA levels with HIV associated VE or PRP, and with lesion extension in VE Decrease of DNA following antiviral therapy in HIV-infected patients	Arribas et al. (1995b), Cinque et al. (1995), Shinkai and Spector (1995)
	EBV	Real-time PCR	Association of high levels with PCNSL or CNS localization of systemic NHL	Bossolasco et al. (2002)
	HHV-6	Real-time PCR	Low levels (below 10^3 copies/ml) in children with neurological symptoms. Decrease of DNA levels with antiviral therapy	Aberle and Puchhammer-Stöckl (2002), Gautheret-Dejean et al. (2002)
Polyomaviridae	JCV	Semiquantitative PCR, competitive PCR	Association of high levels with bad prognosis? Clearance or decrease of DNA levels with HAART	Taoufik et al. (1998), Koralnik et al. (1999), Yiannoutsos et al. (1999), Garcia de Viedma et al. (1999), Eggers et al. (1999)
Picornaviridae	Enterovirus	Competitive PCR	Not described	Martino et al. (1993), Arola et al. (1996)
Retroviridae	HIV-I	Competitive PCR, NAS-BA, branched DNA	Association of high RNA levels with presence and severity of ADC or HIV-E Decrease of RNA levels following antiretroviral therapy	Brew et al. (1997), Ellis et al. (1997), Cinque et al. (1998a), Gisslen et al. (1997), Foudraire et al. (1998), Staprans et al. (1999), Ellis et al. (2000), Gisolf et al. (2000), Price et al. (2001)
	HTLV-1	Real-time PCR	CSF proviral DNA load higher than in blood cells in patients with tropical spastic paraparesis	Nagai et al. (2001)

HSE, herpes simplex encephalitis; VE, ventriculoencephalitis; PRP, polyradiculopathy; PCNSL, primary CNS lymphoma; NHL, non-Hodgkin lymphoma; ADC, AIDS dementia complex; HIV-E, HIV encephalitis; PML, progressive multifocal leukoencephalopathy; HAART, highly active antiretroviral therapy.

number of post-amplification methods described, recently reviewed elsewhere (Arens, 1999). Among these, DNA sequencing, restriction fragment length polymorphism (RFLP) and hybridisation-based techniques have all been used in neurovirology.

Nucleotide sequencing is the most accurate method to collect information on genome composition. Automated procedures have been developed during recent years, making sequencing relatively easy to perform (Fig. 4). By RFLP, restriction enzymes are used to digest NAs into fragments of different size, which can be visualised by gel electrophoresis. This technique can be used after CSF PCR with consensus primers to distinguish individual viruses or viral strains (Fig. 2) (Rozenberg and Lebon, 1991; Arthur et al., 1989). Hybridisation-based techniques include classical procedures, such as the Southern Blot, as well as modern high stringency hybridisation. The latter identifies minimal variations in the genome composition, such as single mutations. An example is the reverse hybridisation technique, incorporated into the commercial Line Probe Assay (LIPA), that has been used to detect HIV resistance

mutations in CSF and plasma pairs (Cunningham et al., 2000). DNA microarrays, or ‘DNA chip’ technology might become an additional tool for the identification and genomic analysis of virus sequences amplified in the CSF (Pease et al., 1994; Lockhart and Winzeler, 2000; McGlennen, 2001). Despite high costs and current limited availability of technology and instrumentation, the DNA chip technology is in rapid development in virology, especially in the field of research, e.g. for measuring viral gene expression (Chambers et al., 1999; Jenner et al., 2001). Sequences from plasma or other clinical samples have initially been tested for epidemiological or diagnostic purposes, such as screening for multiple HIV-1 drug resistance mutations (Wilson et al., 2000).

4.2. Clinical applications

Table 5 summarises some of the most significant applications of genotypic analysis in neurovirology. The identification of enterovirus strains is an example of virus genotyping for both epidemiological and clinical purposes. Molecular typing of enteroviruses may be useful in epidemiological

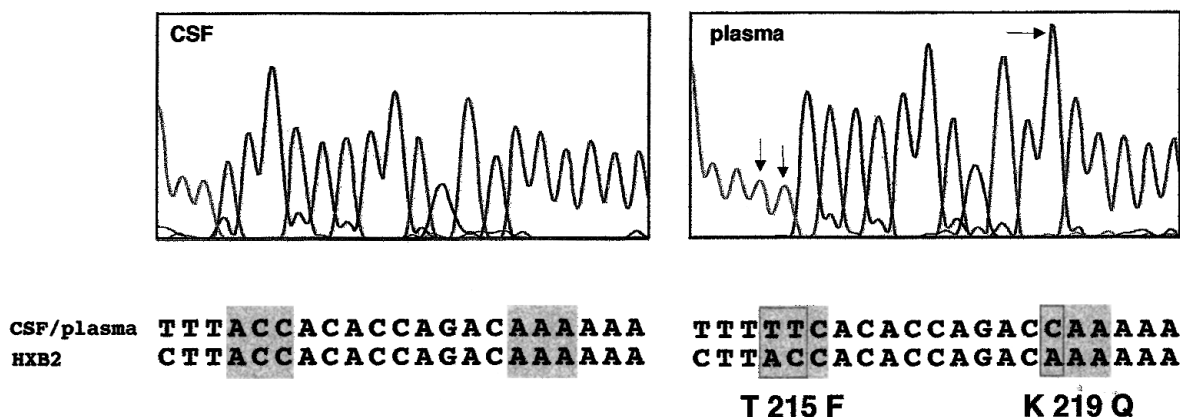


Fig. 4. DNA sequencing from paired CSF and plasma specimens. An example of nucleotide sequencing from paired CSF and plasma samples using cycle-sequencing with dye-labeled oligonucleotides. Amplified products are obtained from paired CSF and plasma specimens following nucleic acid extraction, RNA retrotranscription and PCR amplification of a fragment from the HIV-1 reverse transcriptase (RT) gene. The amplified DNA is purified from unincorporated primers and nucleotides and added to the sequencing reaction mixture. The products of the sequencing reaction are subjected to automated electrophoresis and recognized by a laser scanner. A four-color electropherogram is produced, which is translated into a linear nucleotide sequence by a computer software. The final sequence is compared to reference sequences, e.g., HXB2 for HIV-1. Three nucleotide mutations, resulting in two aminoacid substitutions at codons 215 (treonin → phenylalanin) and 219 (lysine → glutamin) are found in plasma but not in CSF (arrows). Such mutations are associated with resistance to the RT inhibitor drug zidovudine.

Table 5
Post amplification analysis of CSF

Virus family	Virus	Genomic region	Methods	Main findings and significance or post-amplification analysis of CSF	References
Herpesviridae	HSV-1, HSV-2	gD	DNA sequencing	Possible determinants for neurovirulence not found	Rozenberg and Lebon (1996)
		Tymidine kinase	DNA sequencing	Possible determinants for neurovirulence not found	Lee et al. (1999)
	CMV	UL-97	RFLP, DNA sequencing	Identification of resistance mutations in patients with CMV-induced CNS disease on long-term treatment with ganciclovir	Wolf et al. (1995)
Adenoviridae	Adenovirus	Complete sequence	DNA sequencing	Identification of a novel neurotropic virus	Cardosa et al. (1999)
Polyomaviridae	JCV	VP-1, large T, intergenic region	RFLP, DNA sequencing, DGGE	JCV genotyping (genotypes 1–4); association of genotypes 1 and 2 with PML; tracing of human migrations	Agostini and Stoner (1995), Sugimoto et al. (1997), Agostini et al. (1997), De Santis and Azzi (2000), Ferrante et al. (2001)
		Hypervariable noncoding transcriptional control (regulatory) region	RFLP, DNA sequencing	Distinction of archetypal vs. rearranged virus: association of rearranged virus with PML; association of rearranged patterns with bad prognosis of PML?	Agostini and Stoner (1995), Ciappi et al. (1999), Vaz et al. (2000), Pfister et al. (2001), Jensen and Major (2001)
	BKV	Regulatory region	DNA sequencing	Distinction of archetypal vs. rearranged virus: association of rearranged virus with BKV-induced meningoencephalitis?	Stoner et al. (2002)
Picornaviridae	Enterovirus	5' non coding region, other regions	RFLP, DNA sequencing	Monitoring EV outbreaks and transmission	Byington et al. (1999), Takami et al. (2000)
		5' non coding region	RFLP, DNA sequencing	Distinction of poliovirus (poliomyelitis) vs. vaccine virus (post-vaccination flaccid paralysis) vs. non polio EV	Furione et al. (1993), Kammerer et al. (1994), Leparc-Goffart et al. (1996)
		5' non coding region, VP-1, other regions	DNA sequencing	Potential replacement of traditional subtyping	Oberste et al. (1999), Brown et al. (2000)
Paramyxoviridae	Mumps	Haemoagglutinin-neuroaminidase	DNA sequencing	Identification of a vaccine-strain (Urabe) in association with CNS disease	Brown et al. (1991), Forsey et al. (1990)
	Measles	Nucleocapsid, haemoagglutinin	DNA sequencing	Documentation of evolutionary changes with time	Nakayama et al. (1995), Katayama et al. (1997), Kreis and Schoub (1998)
	Nipah virus	Complete genome	DNA sequencing	Identification of a novel neurotropic virus	Chua et al. (2000)
Flaviviridae	Yellow fever virus	Complete genome	DNA sequencing	Identification of a vaccine-strain (17D) in association with CNS disease	Martin et al. (2001)
Retroviridae	HIV	pol (RT, protease)	DNA sequencing, LIPA, DNA microarrays	Identification of different resistance mutations between CSF and blood strains in patients on long-term antiretroviral therapy	Cunningham et al. (2000), Venturi et al. (2000), Cinque et al. (2001), Stingelet et al. (2001)

Table 5 (Continued)

Virus family	Virus	Genomic region	Methods	Main findings and significance or post-amplification analysis of CSF	References
	env		DNA sequencing	Documentation of evolutionary differences between CSF and plasma strains	Steuler et al. (1992), Keys et al. (1993), Kuiken et al. (1995)
	env		DNA sequencing	Identification of polymorphisms possibly associated with ADC	Power et al. (1994), Di Stefano et al. (1996)

RFLP, restriction fragment length polymorphism; DGGE: denaturing gradient gel electrophoresis; LIPA, line probe assay; ADC, AIDS dementia complex; PML, progressive multifocal leukoencephalopathy; YF, yellow fever.

surveillance programmes, but also has diagnostic and prognostic significance: to correctly identify polioviruses, new enterovirus types or variants, enteroviruses responsible of severe infections, or those causing infections in neonates or immunodeficient (Muir et al., 1998). Molecular typing systems for enteroviruses might become a rapid alternative to traditional serotyping, which is time-consuming, labour-intensive and requires virus isolation in cell culture (Muir et al., 1998; Oberste et al., 1999).

DNA sequencing in the CSF has enabled the diagnosis of CNS diseases caused by attenuate vaccine strains rather than wild-type viruses. Examples are the meningitis cases caused by the Urabe vaccine against mumps in the end of the 80s (Forsey et al., 1990; Brown et al., 1991), or the more recently reported vaccine-induced yellow fever cases (Martin et al., 2001). Novel CNS pathogens have been identified following their isolation and/or amplification in the CSF. Recent examples are the two paramyxoviruses Hendra virus, transmitted by horses and causing meningitis and encephalitis in humans (O'Sullivan et al., 1997), and Nipah virus, identified in patients with encephalitis during the 1998 and 1999 outbreaks in Malaysia and Singapore (Chua et al., 2000). Similarly, a newly described B adenovirus was unexpectedly identified by molecular techniques during the 1997 epidemic of enterovirus 71-associated encephalitis in Malaysia (Cardosa et al., 1999).

Another field of application of post-amplification analyses is pharmacogenomics, a term that indicates the study of genome for treatment management. One of the most significant examples in neurovirology is the study of the HIV genome for mutations selected by anti-HIV drugs (Schinazi et al., 1997; Hirsch et al., 2000). Drug resistant viral mutants can be identified in CSF and these not infrequently show mutation profiles differing from those found in plasma or other body sites (Fig. 4) (Cunningham et al., 2000; Venturi et al., 2000; Cinque et al., 2001; Stingele et al., 2001). These findings might provide information concerning viral dynamics in different body compartments, and might also be useful for clinical management of CNS HIV-induced complications.

5. Practical considerations

It is clear that the use of molecular techniques has tremendously improved the diagnosis and clinical management of viral CNS infections. On the other hand, potential problems related to interpretation of NA amplification results, costs and standardisation of these techniques deserve particular consideration.

5.1. NA amplification in unusual viral CNS diseases

Although the diagnostic value of NA amplification techniques is well established in a number of viral CNS infections such as HSE, enterovirus meningitis or opportunistic diseases in HIV-infected patients, it is still unclear in less frequently encountered infections. Examples are encephalitis caused by certain zoonotic viruses, or complicating viral exanthemas like measles or rubella (Table 2). Viral genomes are occasionally found in the CSF of patients with these infections by PCR or other amplification techniques. However, because of the paucity of cases studied, the rate of detection in diseased patients or controls and their clinical significance are not well known, and the potential of NA amplification methods remains in many cases uncertain.

5.2. Interpretation of NA amplification results

It is not infrequent that viral NAs are found in the CSF in patients with infectious or non-infectious CNS diseases, but without a clear causative association with the disease itself. This is more frequently observed with viruses that may be latent in circulating blood cells, brain or other body sites. An example is the detection of EBV in CSF of patients diagnosed with other CNS infections, such as HSE or HIV-related opportunistic infections (Cinque et al., 1996b; Tang et al., 1997; Studahl et al., 1998; Portolani et al., 1998). Theoretically, this finding might result from sliding of virus or latently infected lymphocytes through an impaired blood-CSF barrier, but also from reactivation of EBV infection in the CNS (Bossolasco et al., 2002). Viral genomes have also been found in the CSF of patients with non-

infectious CNS diseases. For instance, both JCV and HHV-6 genomes have been demonstrated in patients with multiple sclerosis, though their etiologic role in this disease has never been confirmed (Ferrante et al., 1998; Liedtke et al., 1995).

In other instances, viral NAs can be detected in CSF prior to the onset of clinically relevant neurological symptoms, which can be advantageous in order to allow for an early diagnosis. This has been observed in AIDS patients, in whom viral agents, e.g. CMV or EBV—not yet causing clinical disease—may occasionally be identified in CSF in patients with another neurological complication (Cinque et al., 1996b).

These examples are the consequence of the extreme sensitivity of NA amplification techniques, and underline the importance of careful interpretation of NA amplification findings in CSF within the individual clinical contexts. It is likely that quantitative molecular techniques might be of help to discriminate a clinically significant infection, characterised by viral replication and high viral loads, from incidental CSF findings.

5.3. Costs and savings of NA amplification techniques

Elevated cost is a potential disadvantage of CSF examination by NA amplification techniques. Taking into account only expenses for technical equipment, reagents, and disposables, the cost per sample of a basic PCR usually varies between approximately 20 and 200 US\$ or €. In-house developed assays are the cheapest to perform and costs can be further reduced by avoiding, when possible, expensive procedures for CSF preparation and NA detection, or by using assays for simultaneous examination of multiple viruses. Commercial assays have some advantages, including standardisation and, sometimes, automation (Jungkind, 2001), but are much more expensive. In general, the savings of establishing a rapid diagnosis often overcome the costs of NA amplification techniques (Ross, 1999). In the diagnosis of HSE, the CSF PCR approach is evidently much cheaper than brain biopsy, but it seems cost-effective also when compared to empirical initia-

tion of antiviral therapy. Using a decision analysis model, the use of CSF PCR was associated with better outcome, and, on the other hand, with significant savings of acyclovir, resulting from higher rate of correct drug discontinuation in PCR-negative patients (Tebas et al., 1998). In aseptic meningitis, cost savings seem to be increased by adopting a PCR testing procedure, as compared to standard practice, especially during the year season characterised by higher enterovirus infection prevalence. Early demonstration of an enterovirus as causative agent is associated with reduced requests for other diagnostic examinations, duration of empirical antibiotic treatments and periods of hospitalisation (Swingler et al., 1994; Rice et al., 1995; Marshall et al., 1997; Ramers et al., 2000).

5.4. Quality control assessment

A major drawback of NA amplification techniques is their limited standardisation. Different protocols are in use for each virus in the different laboratories and reference standards for the evaluation of assay sensitivity are often lacking, making it difficult to compare results among laboratories (Saldanha, 2001). Furthermore, testing of CSF might be subjected to laboratory errors, due to inaccurate test validation, quality of reagents and equipment or staff training (Garrett, 2001). To help obviate these problems, quality control (QC) programmes are being carried out for viruses and other infectious agents. QC assessments for viruses responsible of CNS infections, including enteroviruses, HSV-1, HSV-2 and JCV, have been performed as a part of European Union sponsored QC in virology programmes (van Vliet et al., 1998; Muir et al., 1999; van Loon et al., 1999; Weber et al., 1997; Schloss et al., 2001). These are based on the use of panels consisting of coded samples containing known amounts of NA molecules and control samples, which are distributed to participant laboratories and therein tested blindly. Analysis of reported results has commonly revealed substantial different reports between laboratories, especially with samples containing low amounts of NA. Furthermore, a significant rate of false positive results has

been observed (Muir et al., 1999; Schloss et al., 2001). On the other hand, little or no relationship is generally found between performance and the use of in-house rather than commercial techniques.

6. Final remarks

An array of NA amplification techniques is nowadays applicable to the CSF in order to establish an etiological diagnosis of viral infections of the CNS. Over the last 10 years, the spectrum of clinical conditions that can be recognised has largely expanded and diagnostic reliability significantly improved. Quantitative methods have provided a valuable additional tool for clinical management of these diseases, whereas post-amplification techniques have enabled precise characterisation of viral genomes following their recovery in the CSF. Current efforts are aiming at improvement of the diagnostic efficiency of molecular techniques, in both frequent and less common infections. They also will increase the diagnostic speed and standardisation.

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