A two-year prospective study of clinical criteria and polymerase chain reaction assay of cerebrospinal fluid for the diagnosis of viral infections of the central nervous system

Marzia Boaretti, Giovanna Scalet, Roberta Fontana

Department of Pathology, Section of Microbiology, University of Verona, Italy

───── **SUMMARY** ─────

Cerebrospinal fluid specimens from 226 patients with suspected viral infections of the central nervous system (CNS) were tested by polymerase chain reaction (PCR) to identify the most frequent viruses involved in these infections. A positive PCR result was obtained in 18 patients (7 cases positive for herpes simplex viruses, 5 for enterovirus, 3 for JC virus, 2 for varicella-zoster virus, and 1 for Epstein-Barr virus). All patients with positive PCR results had a definite diagnosis of CNS viral infection. However, a negative result did not rule out the possibility of viral infection of the CNS.

KEY WORDS: Polymerase chain reaction, Viral infections of central nervous system, Cerebrospinal fluid

INTRODUCTION

Prior to the advent of molecular techniques, the laboratory diagnosis of viral infections of the central nervous system (CNS) relied on virus isolation in cell cultures, detection of specific antibody production in cerebrospinal fluid (CSF) or, in the case of herpes simplex virus encephalitis, detection of viral antigen in brain biopsy specimens. Apart from this latter procedure, which is very sensitive but highly invasive, the other laboratory methods yield only a circumstantial or retrospective diagnosis of viral infection, which is of no use for acute patient management.

───────

Corresponding author
Marzia Boaretti
Microbiology Section
Department of Pathology
Verona University
Strada Le Grazie, 8
37134 Verona Italy
E-mail: marzia.boaretti@univr.it

Techniques based on polymerase chain reaction (PCR) on CSF specimens have revolutionised the diagnosis of CNS viral infections thanks to their speed, sensitivity and specificity, but the absence of accepted "gold standard" tests for purposes of comparison calls into question their clinical validity, except in the cases of herpes simplex virus encephalitis, enterovirus meningitis and progressive multifocal leukoencephalopathy (Davies *et al.*, 2005; DeBiasi and Tyler, 2004; Huang *et al.*, 2004; Hukkanen and Vuorinen, 2002).

To evaluate the usefulness of the PCR technique for the diagnosis of CNS viral infections in our clinical setting, we designed a two-year study to relate the PCR results to the clinical presentation and diagnosis in patients with suspected CNS viral infection.

Clinical data were collected from 226 patients with suspected CNS viral infection both at the time of CSF specimen submission and at follow-up. A broad range of viruses associated with CNS infection were sought with a battery of PCR tests.

MATERIALS AND METHODS

Two hundred and twenty-six consecutive CSF specimens from 226 patients with a presumptive diagnosis of viral meningo-encephalitis were sent to the Laboratory of Microbiology, Immunology and Virology of the Azienda Ospedaliera of Verona from June 2005 to June 2007 and were tested without selection. At the time of CNS specimen submission, clinical and laboratory data of patients were collected by asking clinicians to fill in a questionnaire with information regarding the presumptive diagnosis, clinical features and CSF physico-chemical parameters. A review of the clinical records was then performed at patient discharge.

The presence of enterovirus, herpes simplex virus types 1 and 2 (HSV1-2), Epstein Barr virus (EBV) and varicella-zoster virus (VZV) was initially investigated in all CSF samples. If none of these viruses were detected, a search for cytomegalovirus (CMV), polyoma virus JC (JCV), measles virus, mumps virus and rubella virus was subsequently carried out on the same sample.

Viral DNA was extracted from 300 µl of CSF using the Extragen kit and viral RNA from 200 µl of sample using the Extrazol kit (both from Amplimedical Diagnostic Group, Turin, Italy) according to the respective manufacturers' instructions. Before the extraction, the internal controls CPE-DNA (the sequence of human betaglobin) and CPE-RNA (phagic MS2 genomic RNA) (Amplimedical Diagnostic Group) was added to each specimen; the master mix containing virus-specific primers and opportune primers for detection of the respective inhibition controls was added to each extracted sample (Deck and Reck, respectively, Amplimedical Diagnostic Group). Viral DNA was amplified in single-step or nested PCR (for herpes simplex virus only) using the platinum *Taq* DNA polymerase kit (Invitrogen Corporation, Carlsbad, CA). For detection of RNA viruses "one step RT-PCR" was performed using Superscript One-step RT-PCR for the long template (Invitrogen Corporation). Primers and thermal profiles for PCR of each virus were those reported in the respective published protocols: HSV-1 and HSV-2 (Read *et al.*, 1999), VZV (Davison, 1993), CMV (Buffone *et al.*, 1990), EBV (Wagner *et al.*, 1992), enterovirus (Smalling *et al.*, 2002), polyoma virus JC (Tominaga *et al.*, 1992),

mumps virus (Forsey *et al.*, 1990), measles virus (Godec *et al.*, 1990) and rubella virus (Ho-Terry *et al.*, 1990). Positive and negative controls were inserted in each PCR test. The positive controls were represented by:

1. supernatants from specific positive cell-culture for enterovirus, HSV and CMV added into pooled negative CSF specimens;
2. pooled previously detected positive CSF samples for JCV and EBV;
3. the live attenuated vaccines MMRII (Aventis Pasteur MSD) for measles (EDMONSTON 749 D strain), mumps (Jeryl LYNN strain) and rubella (WISTAR RA 27/3 strain) and VARIVAX vaccine (Aventis Pasteur MSD) for VZV (Oka/Merck strain). The negative controls were represented by pooled patient CSF samples which were tested and found negative by PCR.

In our laboratory the proficiency of the assays used was evaluated on the basis of viral genome detection in simulated CSF specimens regularly supplied by the United Kingdom National External Quality Assessment Scheme (UK NEQAS) twice a year.

Amplification products were analysed on a 2% agarose gel (Sigma-Aldrich, Milan, Italy) containing ethidium bromide and visualised by the ImageMaster Gel Analysis System (Amersham Pharmacia Biotech, Milan, Italy). The PCR products were distinguished with reference to their molecular weight. All PCR-positive specimens were tested twice to confirm the results obtained. Data were analysed using Microsoft® Excel 2000. The odds ratio (OR) was used to express the strength of the association between clinical and laboratory data and PCR results. The χ^2 test with Yates' correction was used to determine the p value. A p value of 0.05 or less was used to define statistical significance.

RESULTS

Of the 226 patients enrolled in the study, clinical and laboratory data were available for all patients, either collected by questionnaire (in 91 cases) or by consultation of clinical records (in the other 135 cases): 128 (56.6%) were male; 30 (13.3%) were between 0 and 15 years old, 28 (12.4%) between 16 and 30 years old, 92 (40.7%) between 31 and 60 years old and 76 (33.6%) older than 60

years. The presumptive diagnosis in most cases was meningitis and encephalitis (104/226); in the remaining cases, other generic CNS diseases such as radiculitis, myelitis, demyelinating diseases (80/226) or generic neuropathy (42/226) were diagnosed. On the basis of clinical and laboratory data, the clinical episodes were classified into four groups in relation to the likelihood of CNS viral infection (definite, probable, possible, no CNS viral infection), according to published criteria (Davies *et al.*, 2005; Jeffery *et al.*, 1997) (Table 1). Viral DNA or RNA was detected by PCR in CSF samples from 18 patients (7.96%). Herpes simplex virus accounted for the largest fraction of the positive PCR results (7 cases), followed by enterovirus (5 cases), JCV (3 cases), VZV (2 cases) and EBV (1 case).

Table 2 shows the association between virus detection by PCR and the clinical or laboratory data of the respective cases. The presence of fever and a CSF white-cell count of 5/ µl or more were statistically significant and each was independently associated with a positive PCR result. No statistically significant association was noted between detection of virus in CSF and any of the other clinical or laboratory findings.

The PCR results within each diagnostic category are shown in Table 3. According to the criteria for classification of CNS viral infections using clinical and laboratory data, of the 226 cases considered, we classified 9 (4%) as cases of definite viral infection of the CNS, 98 (43.4%) as probable cases; 17 (7.5%) were judged as possible cases and 102 (45.1 %) as cases with no CNS viral infection.

PCR of CSF was positive and an altered white-cell count associated with fever and/or headache were recorded in all 9 cases classified as definite. Two of these patients had HSV infection confirmed by production of virus-specific intrathecal antibodies; four patients had enterovirus in-

TABLE 1 - *Classification of CNS viral infections using clinical and laboratory data*.*

Definite CNS viral infection (one or more of 1, 2, 3 plus either A or B)

1. Virus positive culture of CSF, brain biopsy, throat, stool or genital swab.
2. ≥4-fold increase in virus-specific antibody titre or detection of virus-specific IgM antibody in serum or demonstration of virus-specific intrathecal antibody synthesis.
3. Virus-specific rash (herpes genitalis or shingles).

A. CSF white-cell count ≥5/ml and presence of meningism, headache, or fever (with no other explanation).
B. Clouding of consciousness or focal neurological signs and fever or headache (with no other explanation).

Likely CNS viral infection (any one of the following)

1. CSF white-cell count ≥5/µl and one or more of meningism, headache or fever with no available corroborative evidence of a viral infection (with no other explanation).
2. Clouding of consciousness or focal neurological signs and fever or headache, with no corroborative evidence of viral infection (with no other explanation).
3. Acute ascending polyneuropathy or myelopathy (with no other explanation).
4. In transplant recipients or HIV-positive patients, imaging evidence of intracranial lesion when toxoplasmosis is not clinically suspected.

Possible
Clinician's final diagnosis of a CNS viral infection and any other combination of symptoms and signs.

NO CNS viral infection (any one of the following)

I. Another definite clinical diagnosis (e.g. bacterial meningitis).
II. Non-specific diagnosis (e.g. febrile convulsions) and clinical or laboratory features not consistent with the above categories.
III. No definite diagnosis (e.g. fever of unknown cause and clinical or laboratory features not consistent with the above categories).

*Classification adapted from Jeffery et al.

fection: two cases were confirmed by demonstration of virus-specific intrathecal antibody production and two by serological testing in paired serum samples. Two patients had VZV infection confirmed by extensive thoracic shingles in one and by synthesis of virus-specific intrathecal antibodies in the other. One case with a positive PCR result for EBV was confirmed by serological testing of paired serum samples obtained during acute infection and convalescence.

Of the 98 cases classified as probable, 8 had a positive PCR result; 4 patients had untyped HSV, 3 had JCV and 1 had enterovirus. Three patients who were positive in the search for the HSV genoma in CSF had abnormal CNS neuroimaging with specific involvement of the temporal lobes, often considered one of the clinical criteria for diagnosis of herpes simplex encephalitis (HSE). All three patients with positive PCR for the JC virus were immunocompromised: one was HIV-seropositive and the other two were renal transplant patients.

TABLE 2 - *Logistic regression of association between the PCR result of virus detection in CSF and clinical or laboratory data of the respective cases.*

Clinical and laboratory data	Positive PCR result (n=18*)	Negative PCR result (n=208*)	Odds ratio (95% CI)	p value
Fever (≥37.5°C)	13/18 (72.2%)	52/158 (32,9%)	5.3 (1.75-16.01)	0.0026
Meningism	09/18 (50%)	62/158 (39.2%)	1.55 (0.57-4.2)	0.529
Headache	07/18 (38.8%)	48/154 (31.2%)	1.41 (0.5-3.93)	0.691
Focal neurology	11/18 (61.1%)	94/168 (55.9%)	1.24 (0.45-3.42)	0.866
Confusion	08/18 (44.4%)	68/146 (46.6%)	0.92 (0.34-2.51)	0.936
Immunosuppression	06/18 (33.3%)	20/146(13,7%)	3.15 (1.04-9.56)	0.07
Abnormal CSF WCC	13/18 (72.2%)	60/204 (29.4%)	6.24 (2.08-18.68)	0.0006
Abnormal CSF protein	13/18 (72.2%)	106/204(51.9%)	2.40 (0.81-7.14)	0.16
Abnormal EEG	03/06 (50%)	30/152 (19.7%)	4.07 (0.76-21.89)	0.201
Abnormal CNS imaging	05/10 (50%)	86/186 (46.2%)	1.16 (0.32-4.26)	0.925

*Not all clinical and laboratory features were recorded for each patient. CSF = cerebrospinal fluid; WCC = white-cell count; EEG = electroencephalogram; CNS = central nervous system.

TABLE 3 - *Correlation between clinical classification of CNS viral infection and specific viruses detected in CSF by PCR.*

Category of CNS viral infection	PCR positive result for					PCR positive result	PCR negative result
	HSV	JCV	EV	VZV	EBV		
Definite	2		4	2	1	9	0
Probable	4	3	1			8	90
Possible	1					1	16
No infection						0	102
Total	7	3	5	2	1	18	208

Of the 17 cases judged as possible, only one had a positive PCR result for HSV.

None of the 102 cases classified as "no CNS viral infection" had a positive PCR result. The majority of these patients were hospitalised in neurology divisions and the definitive diagnosis was reported as degenerative disease of the CNS, such as myelitis or demyelinating disease.

Comparison of probable, possible and no viral infection of the CNS with definite cases indicated a positive predictive value (PPV) of 50%, a sensitivity of 100% and a specificity of 96%. A diagnosis of definite viral infection of the CNS was 24 times as likely to occur in a patient with a positive PCR result as in patient with a negative PCR result (likelihood ratio positive 24.11, CI 95%: 12-41). Evaluation of the possibility of a diagnosis of CNS viral infection in a patient with a negative PCR result was based on comparison of definite, probable and possible viral infection with no viral infection: the negative predictive value (NPV) was 49% and the likelihood ratio negative 0.85 (CI 95%: 0.79-0.92).

DISCUSSION

Previous studies reported a positivity of PCR of CSF for the diagnosis of CNS viral infections ranging from 6.6 to 14.3% (Davies *et al.*, 2005; Jeffery *et al.*, 1997; Huang *et al.*, 2004; Hukkanen and Vuorinen., 2002). The incidence reported in our study (7.9%) was comparable to those reported by Jeffery *et al.* (6.6%) and Hukkanen *et al.* (7.8%) but was lower than those reported by Davies *et al.* (12%) and Huang *et al.* (14.3%). However, if we consider that our classification of patients into diagnostic groups revealed that a substantial number of cases (102/226, corresponding to 45%) were finally classified as "no viral infection", this might explain the lower positivity. In fact, various infectious or inflammatory neurological diseases may mimic viral infections of the CNS, and therefore a large number of cases are included in the diagnostic assessment of CNS viral infections.

In our study HSV was the most common etiological agent detected in CSF samples; the lower prevalence of CNS disease caused by enterovirus than that reported by some authors (Jeffery *et al.*, 1997; Huang *et al.*, 2004) could be explained by

the age of our cohort of patients (only 13.5% of patients younger than 16 years). It is known that CNS infections caused by enterovirus are predominant in children and in adolescents (DeBiasi and Tyler, 2004; Hukkanen and Vuorinen, 2002). An examination of clinical records of patients showed that a positive HSV PCR result was commonly associated with severe neurological disorders like encephalitis or meningoencephalitis and was most prevalent in adults older than 55 year, whereas enterovirus infections were associated with benign course of meningitis with most incidence in young patients. The value of employing PCR for the diagnosis of CNS viral infection was undeniable in our clinical setting since all the positive PCR results were consistent with either definite or probable or possible viral infections.

Moreover, since PCR can actually be considered the gold standard test for diagnosis of CNS infections due to HSV, enterovirus and JC virus (Davies *et al.*, 2005; DeBiasi and Tyler, 2004; Huang *et al.*, 2004; Hukkanen and Vuorinen, 2002) are no longer need to relate the PCR results to the various clinical and laboratory data, as previously suggested by Jeffery *et al.* On the basis of this consideration our results could be interpreted without close correlation with numerous clinical and laboratory variables and all PCR-positive cases, including 8 classified as likely CNS viral infection as well as 1 classified possible, could be considered definite CNS viral infections.

Indeed the PCR, in particular for HSV and enterovirus CNS infections diagnosis, represents a rapid, sensitive and specific test and could be very useful as regards the clinical approach to early anti-viral treatment and for improvement of patient management (DeBiasi and Tyler, 2004; Steiner *et al.*, 2005), as we also observed from clinical records review in our institution.

On the other hand, virus was not detected in about 46% (106/226) of episodes where a CNS viral infection was considered probable or possible. Consequently, as some authors have emphasised, a negative PCR result cannot be relied upon to rule out a CNS viral infection (Davies *et al.*, 2005; Jeffery *et al.*, 1997). A number of factors could account for these findings, such as the interval between the time of onset of the neurological symptoms and the time of CSF sampling (Colimon, 2002; Huang *et al.*, 2004), or unusual (undetected) or unknown infectious agents in-

volved in the neuropathy (Davies *et al.*, 2005). Furthermore, although admitting the benefits obtained by PCR technique for the diagnosis of CNS viral infections, many authors indicated that the interpretation of PCR results should be made in the context of some clinical, laboratory and neurodiagnostic evaluations (Kimberlin, 2005; Sauerbrei and Wutzler, 2002; Steiner *et al.*, 2005). In conclusion, molecular assay for viral nucleic acid research in CSF is a fundamental diagnostic tool in suspected CNS viral infection to provide a prompt and accurate diagnosis and to aid clinicians for their subsequent diagnostic and therapeutic decisions.

REFERENCES

BUFFONE G.J., HINE E., DEMMLER G.J. (1990). Detection of mRNA from the immediate early gene of human cytomegalovirus in infected cells by in vitro amplification. *Mol. Cell Probes*. **4** (2), 143-151.

COLIMON R. (2002). Introduction to the virological diagnosis of the most frequent infections of the central nervous system. *J. Clin. Virol*. **25**, S1-S3.

DAVISON A.J. (1993). DNA sequence of the US component of the varicella-zoster virus genome. *The EMBO Journal*. **2** (12), 2203-2209.

DAVIES N.W., BROWN L.J., GONDE J., IRISH D., ROBINSON R.O., SWAN A.V., BANATVALA J., HOWARD R.S., SHARIEF M.K., MUIR P. (2005). Factors influencing PCR detection of viruses in cerebrospinal fluid of patients with suspected CNS infections. *J. Neurol. Neurosurg. Psychiatry*. **76**, 82-87.

DEBIASI R.L., TYLER K.L. (2004). Molecular methods for diagnosis of viral encephalitis. *Clin. Microbiol. Rev*. **17**, 903-925

FORSEY T., MAWN J.A., YATES P.J., BENTLEY M.L., MINOR P.D. (1990). Differentiation of vaccine and wild mumps viruses using the polymerase chain reaction and dideoxynucleotide sequencing. *J. Gen. Virol*. **71**, 987-990.

GODEC M.S., ASHER D.M., SWOVELAND P.T., ELDADAH Z.A., FEINSTONE S.M., GOLDFARB L.G., GIBBS C.J., GAJDUSEK D.C. (1990). Detection of measles virus genomic sequences in SSPE brain tissue by polymerase chain reaction. *J. Med. Virol*. **30** (4), 237-244.

HO-TERRY L., TERRY G.M., LONDESBOROUGH P. (1990). Diagnosis of foetal rubella virus infection by polymerase chain reaction. *J. Gen. Virol*. **71**, 1607-1611.

HUANG C., MORSE D., SLATER B., ANAND M., TOBIN E., SMITH P., DUPUIS M., HULL R., FERRERA R., ROSEN B., GRADY L. (2004). Multiple-year experience in the diagnosis of viral central nervous system infections with a panel of polymerase chain reaction assay for detection of 11 viruses. *Clin. Infect. Dis*. **39**, 630-635.

HUKKANEN V., VUORINEN T. (2002). Herpesvirus and enterovirus in infections of the central nervous system: a study using time-resolved fluorometry PCR. *J. Clin. Virol*. **25**, S87-S94.

JEFFERY K.J., READ S.J., PETO T.E., MAYON-WHITE R.T., BANGHAM C.R. (1997). Diagnosis of viral infections of the central nervous system: clinical interpretation of PCR results. *Lancet*. **349**, 313-317.

KIMBERLIN D.W. (2005). Diagnosis of herpes simplex virus infections f the CNS. *Expert. Rev. Mol. Diagn*. **5** (4), 537-547.

SAUERBREI A., WUTZLER P. (2002). Laboratory diagnosis of central nervous system infections caused by herpesvirus. *J. Clin. Virol*. **25**, S45-S51.

SMALLING T.V., SEFERS S.E., LI H., TANG Y. (2002). Molecular approaches to detecting herpes simplex virus and enterovirus in the central nervous system. *J. Clin. Microbiol*. **40** (7), 2317-2322.

STEINER I., BUDKA H., CHAUDHURI A., KOSKINIEMI M., SAINIO K., SALONEN O., KENNEDY P.G.E. (2005). Viral encephalitis: a review of diagnostic methods and guidelines for management. *Eur. J. Neurol*. **12**, 331-343.

TOMINAGA P.T., YOGO J., KITAMURA T., ASO Y. (1992). Persistence of archetypal JC virus DNA in normal renal tissue derived from tumor-bearing patients. *Virology* **186** (2), 736-741.

WAGNER H.J., G. BEIN, A. BITSCH, H. KIRCHNER. (1992). Detection and quantification of latently infected B lymphocytes in Epstein-Barr virus-seropositive, healthy individuals by polymerase chain reaction. *J. Clin. Microbiol*. **30** (11), 2826-2829.