JOURNAL OF CLINICAL MICROBIOLOGY, Dec. 1996, p. 2929–2932 Vol. 34, No. 12
0095-1137/96/$04.00+0

Analysis of PCR as a Tool for Detection of JC Virus DNA in Cerebrospinal Fluid for Diagnosis of Progressive Multifocal Leukoencephalopathy

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Received 6 May 1996/Returned for modification 17 July 1996/Accepted 13 September 1996

Two polyomaviruses, JC virus (JCV) and BK virus (BKV), affect humans. JCV is the causative agent of progressive multifocal leukoencephalopathy (PML), and detection of JCV in the central nervous system (CNS) is a prerequisite for confirmation of the disease. BKV is generally not associated with neurological disease, but involvement of BKV in patients with CNS disorders has been reported. In the present study polyomavirus DNA was detected by a nested PCR at a sensitivity corresponding to the detection of 10 copies of JCV DNA in 10 μl of cerebrospinal fluid (CSF). CSF samples from 212 patients with neurological symptoms and immunodeficiencies were investigated for the presence of polyomavirus DNA. Of 128 human immunodeficiency virus (HIV)-infected patients, 14 (11%) had JCV DNA in their CSF, and all 14 patients had clinical PML. BKV DNA was detected in one HIV-infected patient with neurological symptoms not compatible with PML. Among 84 HIV-negative patients, 6 (7%) had detectable JCV DNA in their CSF, confirming PML in patients with clinical conditions of T-cell lymphoma, chronic lymphatic leukemia, status postliver transplantation, congenital immunodeficiency, sarcoidosis, and immunodeficiency of unknown origin. The specificity of the PCR was confirmed by a clinical follow-up study which showed full agreement between the detection of JCV DNA in CSF and clinically manifest PML. The described PCR is a rapid, reproducible, and easily performed assay.

Progressive multifocal leukoencephalopathy (PML) was first described in 1958 in a patient with chronic lymphocytic leukemia (2). In 1971 a polyomavirus was isolated from the brain of a patient with PML and Hodgkin's lymphoma (14). The virus was named JC virus (JCV) after the initials of the patient. PML is recognized as a fatal disorder in immunocompromised individuals and earlier was considered a rare disease. However, with the human immunodeficiency virus (HIV) pandemic, an increasing number of PML cases are seen, and JCV is the cause of serious opportunistic infections in 3.8% of AIDS patients (3). Primary JCV infections are acquired at an early school age and are generally asymptomatic. After infection, JCV establishes latency, probably in the kidneys and possibly also in B lymphocytes (18). Reactivation occurs during cell-mediated immune deficiency and may result in PML. JCV causes lytic infection of the oligodendrocytes, which results in foci of demyelinization in the white matter of the brain (15). The clinical diagnosis of PML may be based on the presence of multifocal symptoms from the central nervous system (CNS) in combination with defects of the immune system. The diagnosis can be supported by computed tomography (CT) or magnetic resonance imaging (MRI), or both. Characteristic histopathological changes are seen in the white matter of the cerebral hemispheres, the brain stem, and the cerebellum. However, a variety of infectious agents cause CNS lesions in AIDS patients. The ultimate proof of PML is obtained by showing the presence of JCV in the CNS. Besides the original isolation of JCV in tissue culture (14), electron microscopy (7, 23) and in situ hybridization (1) of brain material have been used for diagnosis. After establishing a nested PCR for the detection of polyomavirus DNA in 1992, we have found the assay to be sensitive enough to detect polyomavirus DNA in cerebrospinal fluid (CSF) (4, 5). Similar studies have been performed by other groups (9–13, 17, 21), and the detection of JCV DNA in CSF samples from patients with and without clinical PML has been reported.

The aim of the present study was to evaluate the accuracy of PCR as a routine method for the detection of JCV DNA in CSF for the diagnosis of PML. The study included AIDS patients as well as patients with other immunological disorders. We also emphasize the importance of differentiating between JCV and BK virus (BKV), since CNS infection with BKV, although extremely rare, may occasionally occur (19, 20).

MATERIALS AND METHODS

CSF samples. A total of 236 CSF samples from 212 patients with CNS disorders were submitted to the Swedish Institute for Infectious Disease Control for analysis for the presence of JCV. The age of the patients ranged from 6 months to 79 years. The specimens were collected from 1 week to 2 months after the onset of neurological symptoms. The majority of the patients (60%) were HIV seropositive, and most of them had developed AIDS. The non-HIV-infected patients had, in most cases, an underlying disease consistent with immunological impairment and focal neurological symptoms without a known etiology. As controls, CSF samples from 14 patients with neurological conditions not associated with PML were analyzed.

Retrospective study of patients with PML and suspected PML. The results of the PCR for polyomavirus in CSF were confirmed by a limited clinical follow-up study including all patients with JCV DNA in their CSF as well as 25 of the JCV- and BKV-negative patients with symptoms similar to those of PML. Information on the patients' clinical diagnosis, results of MRI, CD4 lymphocyte counts at the time of onset of CNS symptoms, survival times, and autopsy results was collected retrospectively.

Nested PCR for detection of polyomavirus DNA. All samples were frozen (−20 or −70°C) before analysis. Blood-contaminated CSF samples were centrifuged before freezing, and samples from HIV-seropositive patients were heated to 95°C for 10 min before testing.

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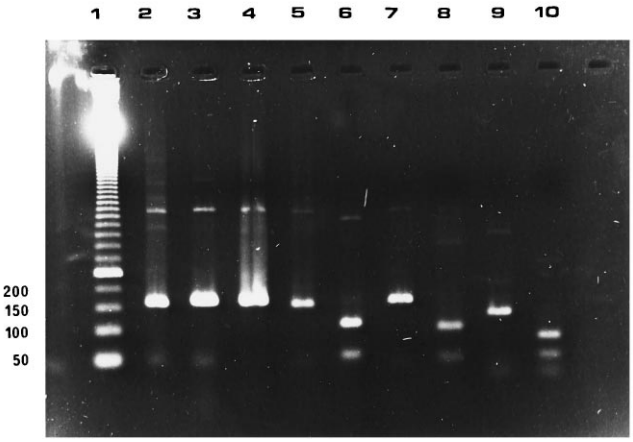

FIG. 1. Differentiation of SV40, JCV, and BKV DNAs by restriction enzyme analysis of amplified PCR products. Lane 1, DNA marker (50-bp ladder [the numbers on the left are in base pairs]); lanes 2, 3, and 4, SV40, JCV, and BKV DNA amplimers (undigested), respectively; lanes 5, 6, and 7, SV40, JCV, and BKV DNA amplimers, respectively, digested with *Bam*HI; lanes 8, 9, and 10, SV40, JCV, and BKV DNA amplimers, respectively, digested with *Hin*fI. *Bam*HI cleaves the JCV amplimer in two fragments of 120 and 53 bp respectively, while the amplimers of SV40 and BKV remain uncleaved. *Hin*fI cleaves the SV40 amplimer in three fragments (110, 54, and 4 bp, respectively), the JCV amplimer in two fragments (141 and 32 bp, respectively), and the BKV amplimer in three fragments (90, 54, and 32 bp, respectively).

A previously described nested PCR that detects human polyomavirus DNA was performed with CSF samples (4). Ten microliters of the CSF was used for amplification. The chosen primers also detected DNA from simian virus 40 (SV40), which is closely related to JCV and BKV, but which does not normally infect humans. The two sets of primers were complementary to the early conserved region of the virus genomes, encoding for large and small T antigens. The sequences of the outer primer set were 5′-GTA TAC ACA GCA AAG GAA GC-3′ and 5′-GCT CAT CAG CCT GAT TTT GG-3′, respectively. The first round (20 amplification cycles) with the outer primer set amplified a fragment of 631 bp for JCV and one of 372 bp for BKV. The second round (40 amplification cycles), performed with the inner primer set (sequences 5′-AGT CTT TAG GGT CTT CTA CC-3′ and 5′-GGT GCC AAC CTA TGG AAC AG-3′, respectively), resulted in a fragment of 173 bp for JCV and one of 176 bp for BKV. The PCR products were visualized with UV light and ethidium bromide staining after agarose gel electrophoresis.

Detection of JCV-specific DNA. For differentiation of the almost equally sized amplimers obtained from the two human polyomaviruses, JCV and BKV, digestion with the restriction enzyme *Bam*HI was performed. The enzyme cleaved the amplified JCV DNA amplimer into two fragments of 120 and 53 bp, respectively; these fragments were subsequently visualized by agarose gel electrophoresis (4). In order to distinguish between all three polyomaviruses (JCV, BKV, and SV40) the restriction enzyme *Hin*fI was used to cleave the DNA from each virus into distinct segments (Fig. 1). Amplification of nonrelevant viral DNA by PCR was excluded in experiments with tissue culture-grown herpes simplex virus types 1 and 2, human herpesvirus 6, cytomegalovirus, Epstein-Barr virus, varicella-zoster virus, and HIV.

Controls, DNA detection level, and external quality assessment. Two positive controls, tissue culture-grown BKV and a urine sample containing JCV, were used for all PCRs. A negative control, consisting of the PCR mixture containing water instead of template, was inserted between all clinical specimens throughout the test. To establish the sensitivity of the PCR, titration of the JCV (strain Mad-1) genome cloned into the *Eco*RI site of the pGem vector (provided by Virus Reference Laboratory, Central Public Health Laboratory, London, England) was performed. Furthermore, a quality control panel provided by The European Union Concerted Action group on Virus Meningitis and Encephalitis was tested for confirmation of the sensitivity and specificity of the JCV PCR. The panel consisted of 10 CSF samples with different concentrations of JCV DNA, which were unknown to the investigator.

RESULTS

Detection of polyomavirus DNA in CSF. JCV DNA was detected in CSF samples from 20 (9%) of the 212 investigated patients with neurological disorders. The majority of the positive patients (14 of 20; 70%) had AIDS (Table 1). The remaining positive patients (6 of 20; 30%) had underlying conditions correlated with other immunological impairments (Table 2). CSF collected from two of the AIDS patients at an early phase of the illness showed a negative result, but a second CSF sample taken later was found to be JCV positive. BKV DNA was found in one HIV-infected patient.

TABLE 1. Patients investigated for the presence of polyomaviruses DNA in CSF

Patient group	No. of patients[a]		
	JCV negative, BKV negative	JCV positive	BKV positive
HIV negative	78	6	
HIV positive	113	14	1
Total	191	20	1

[a] A total of 212 patients were investigated.

Retrospective study of patients with PML and suspected PML. (i) JCV DNA-positive patients. At the time of onset of PML symptoms, 13 of the 14 patients with AIDS-associated PML had a $CD4^+$-lymphocyte count of 240 cells per mm^3 (240×10^6/liter) or less; 1 patient had a $CD4^+$-lymphocyte count of 380 cells per mm^3. The mean survival time for the patients with AIDS-related PML was 3.5 months (range, 2 to 5 months) from the time of onset of neurological symptoms. Two AIDS patients could not be followed with respect to survival time. The group of non-AIDS PML patients had a mean survival time of 8 months (range, 6 to 11 months). In one patient with AIDS-related PML, stabilization of the CNS symptoms was observed after treatment with cytosine arabinoside, and the patient was alive 1 year after the onset of neurological symptoms. However, in three patients treated with cytosine arabinoside and two patients treated with high doses of intravenous immunoglobulin G, no lasting effect of the therapy was observed. All 20 JCV-positive PML patients had clinical features of the disease, such as focal symptoms, change of personality, and memory loss. Their clinical PML diagnoses were supported by MRI or CT, which showed the characteristic neuroimaging pattern with multiple lesions in the white matter of the brain. In 4 of 14 AIDS patients and 5 of 6 non-AIDS patients, the PML diagnosis was confirmed by histopathological investigations at autopsy. In the remaining patients no brain tissue was available for examination, and one AIDS patient was still alive at the end of the study period.

(ii) JCV DNA-negative patients. A follow-up study similar to that described above was performed with 25 patients (5 HIV-negative and 20 HIV-positive patients) lacking detectable JCV DNA in their CSF but with clinically suspected PML (Table 3). The final diagnoses for the five HIV-negative patients were not compatible with PML (Table 3). Of the 20 HIV-positive pa-

TABLE 2. Underlying conditions in HIV-negative PML patients

Patient no.	Age (yr)	Sex[a]	Underlying condition
1	52	F	Status postliver transplantation
2	21	F	Congenital immunodeficiency
3	46	M	T-cell lymphoma
4	54	F	Immunodeficiency of unclear etiology
5	75	M	Chronic lymphatic leukemia
6	51	M	Sarcoidosis

[a] F, female; M, male.

TABLE 3. Patients with clinically suspected PML but without detectable JCV DNA in CSF specimens

Patient group and diagnosis	Deceased	Results of postmortem, CT, or MRI
HIV-negative patients (n = 5)		
T-cell leukemia	Yes	No PML
Hodgkin's lymphoma	Yes	No PML
Meningioma	Yes	Brain tumor
Astrocytoma	Yes	Brain tumor
Multiple sclerosis (?)	No	Healthy for 1 yr
HIV-positive patients (n = 20)		
Brain stem encephalitis	Yes	No PML
HIV encephalitis	Yes	HIV encephalitis, died 1 year after clinical onset of CNS symptoms
AIDS	Yes	No PML, regression of CNS symptoms; CT normal 2 months before death
AIDS	Yes	No PML, no CNS symptoms at time of death
CNS symptoms	Yes	No PML, atypical mycobacteria in CSF
Polyneuropathy	Yes	No PML, CT not compatible with PML
Encephalitis	Yes	No PML, CT not compatible with PML
Cerebral infarction	Yes	No PML, CT and MRI compatible with cerebral infarction
Cerebral lymphoma	Yes	No PML, CT compatible with lymphoma or cerebral infarction
Polyneuropathy	No	No PML, CT not compatible with PML
Peripheral cranial nerve palsy	No	No PML, CT not compatible with PML
Peripheral cranial monoparesis	Yes	No PML, regression of CNS symptoms, CT not compatible with PML
HIV dementia complex, suspected neuropathy	Yes	No PML, CT not compatible with PML
HIV dementia complex (two patients)	Yes	No PML, no focal neurological symptoms at time of death, CT not compatible with PML
HIV dementia complex (three patients)	Yes	No PML, CT not compatible with PML
AIDS, PML(?)	Yes	PML(?), MRI not compatible with PML, no autopsy performed
AIDS, PML(?)	Yes	PML(?), no autopsy performed

tients, 18 received a final diagnosis not consistent with PML. For the two remaining HIV-positive patients, unspecified neurological disorders combined with advanced HIV infection were recorded as the cause of death (Table 3).

BKV DNA-positive patient. The neurological symptoms of the BKV DNA-positive patient were not compatible with what is seen in PML (unpublished data).

Control group of samples from patients without PML symptoms. In the control group of samples consisting of CSF samples from 14 patients ages 33 to 76 years (mean age, 53 years) with neurological symptoms not correlated with PML, no JCV DNA was found.

PCR sensitivity and external quality control assessment. The sensitivity of the PCR was established by titration of the JCV genome in the pGem vector, and the detection level was found to be 10 genome copies per 10 μl. The results of the quality control panel investigation were sent to The European Union Concerted Action group, which confirmed our PCR detection level at 10 genome copies per 10 μl.

DISCUSSION

The early diagnosis of PML is valuable for social reasons because of the rapidly progressive course of the disease, as well as for the exclusion of treatable infections from the brain. The majority of the CSF samples in our study were obtained 2 to 3 weeks after the onset of neurological symptoms. The precise time of onset of PML is difficult to state since HIV-infected patients in particular may be affected by several infectious agents causing symptoms similar to the initial focal symptoms of PML, such as impaired speech and vision or changes in mental state. However, an analysis for JCV DNA early in the course of disease may sometimes show a negative result because of the absence of or the presence of only low amounts of viral DNA in the CSF. In our study, CSF samples taken from two patients approximately 7 and 22 days, respectively, after the onset of neurological symptoms gave negative results by PCR. Since the suspicion of PML remained, follow-up samples were taken, and these were found to be JCV positive. Inhibitory activity (6) was not found in the initial samples. Repeat samples from 22 additional patients were tested, and they all remained negative. Thus, for samples collected early in the course of PML, a negative result does not exclude the possibility of the presence of JCV in the brain, and a follow-up CSF sample should be examined. Similar experiences have been reported previously (10).

To confirm the validity of the PCR results achieved in the study, a clinical follow-up study was performed. The study included the 20 JCV DNA-positive PML patients and 25 patients (5 non-AIDS and 20 AIDS patients) with clinical symptoms of PML but a negative result by JCV PCR of their CSF. All data regarding the 20 JCV DNA-positive patients supported the PML diagnosis. Among the JCV DNA-negative patients, all five non-AIDS patients had a final diagnosis not compatible with PML (Table 3). Likewise, the PML diagnosis was excluded for 18 of 20 AIDS patients with initially suspected PML; in the 2 additional patients, PML could not be excluded. These two patients had advanced AIDS, and the cause of death was declared as AIDS or PML. Unfortunately, no autopsies were performed on these patients (Table 3).

From the present study, we conclude that false-positive JCV PCR results were not observed because JCV DNA was not detected in CSF from patients without PML. This is in accordance with the results obtained by others (10, 21, 22) and does not suggest a subclinical JCV infection in the brain. PCR has recently been introduced for the detection of JCV DNA in brain tissue and CSF (4, 5, 9–13, 16, 17, 21). Although it has been reported that as a test material CSF has a lower sensitivity compared with that for brain tissue, the use of CSF is preferable because of the less invasive sampling procedures that are required. Furthermore, a more rapid PCR test performance is obtained with CSF compared with that with brain tissue. The correlation between the detection of JCV DNA in

CSF and corresponding brain tissue has been shown to be good in the limited number of patients whom we have examined (6). Similar results have also been reported by Moret et al. (13), who used DNA purification of CSF samples. In the present study the sensitivity of the PCR corresponded to the detection of 10 DNA copies per 10 μl. The CSF samples were investigated without DNA extraction, since all handling of the specimens increases the risk for contamination and the loss of DNA. Extraction of DNA in CSF has been suggested as a way of avoiding the failure in detecting viral DNA because of the presence of PCR-inhibiting factors (8) and increasing the sensitivity of the assay (13, 22). We have not, however, noticed any inhibitors in CSF (unpublished data), which is in accordance with reports by Gibson et al. (10). A concentration step obtained by DNA purification may improve the sensitivity, and McGuire et al. (12) have reported a sensitivity corresponding to one copy of DNA in 50 μl of CSF after purification.

In conclusion, the rapid and easy handling of the specimens combined with high specificity and sensitivity makes the nested PCR an excellent routine method. We have 3 years of experience with the described polyomavirus PCR, and so far, JCV DNA has been demonstrated only in patients with PML. However, since CNS infection with BKV may also occur, a positive polyomavirus PCR result must be followed by a test for the differentiation between JCV and BKV.

ACKNOWLEDGMENTS

We are indebted to the following people for their invaluable contributions of clinical data: E. Olding-Stenkvist (University Hospital, Uppsala, Sweden), M. Karlsson (Danderyd Hospital, Stockholm, Sweden), P.-O. Pehrson and V. Kostulas (Huddinge University Hospital, Huddinge, Sweden), P. Hedman and G. Bratt (Söder Hospital, Stockholm, Sweden), L. Hagberg (Östra Hospital, University of Göteborg, Sweden), C. Vedalen (Haukeland Hospital, Bergen, Norway), C. Beroni (Lund University Hospital, Lund, Sweden), and K. Forslund (Karolinska University Hospital, Stockholm, Sweden).

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