Journal of NeuroVirology 7: 35–42 2001
 ISSN 1355–0284/01 $12.00+.00

Increased frequency of JC virus type 2 and of dual infection with JC virus type 1 and 2 in Italian progressive multifocal leukoencephalopathy patients

Pasquale Ferrante*[,1], Monica Mediati[1], Rita Caldarelli-Stefano[1], Loredana Losciale[1], Roberta Mancuso[1], Anna Elisabetta Cagni[2] and Renato Maserati[3]

[1]*Laboratory of Biology, Chair of Virology, Don C. Gnocchi Foundation, IRCCS (Research Hospital), University of Milan, Milan, Italy;* [2]*Infectious Diseases Department, St. Gerardo's Hospital, Monza, Italy and* [3]*Infectious Diseases Department, Policlinico St. Matteo, IRCCS (Research Hospital), Pavia, Italy*

To verify the possibility of different role of JC virus genotypes in the etiology of progressive multifocal leukoencephalopathy, we analysed several JC virus isolates amplified from AIDS patients with and without progressive multifocal leukoencephalopathy and healthy controls by nucleotide sequencing. Cerebrospinal fluid (CSF), peripheral blood mononuclear cells (PBMCs) and urine from 52 AIDS patients suffering from various neurological diseases including 21 cases of progressive multifocal leukoencephalopathy, and PBMCs and urine from healthy subjects were evaluated by nested polymerase chain reaction (PCR) for the presence of DNA belonging to the highly conserved large T antigen (LT) of JC virus. The different JC virus subtypes were identified by nucleotide sequence analysis of the virion protein (VP1) genomic region. JC virus DNA was detected in all the CSF samples from the progressive multifocal leukoencephalopathy patients, but not in the CSF from non-progressive multifocal leukoencephalopathy cases, while the frequency of JC virus DNA detection in the PBMCs and urine did not differ among the three groups studied. JC virus type 2 was detected only in progressive multifocal leukoencephalopathy patients, and in particular in 52.4% of their CSF samples. Moreover, in the CSF of 19.0% of the progressive multifocal leukoencephalopathy cases, dual infection with both JC virus types 1 and 2 was found. The data obtained in this study indicate that the unexpected involvement of JC virus type 2, a strain not common in Italy, and the high frequency of dual infection with both JC virus types 1 and 2 in progressive multifocal leukoencephalopathy CSF, can be indications of risk factors for progressive multifocal leukoencephalopathy development. *Journal of NeuroVirology* (2001) **7,** 35–42.

Keywords: JC virus genotypes; progressive multifocal leukoencephalopathy; nucleotide sequence; cerebrospinal fluid; JC virus neurotropism

Introduction

Progressive multifocal leukoencephalopathy is a demyelinating disease usually occurring in immunocompromised individuals and caused by JC virus (Major *et al*, 1992), an agent that infects a large part of the general population worldwide during childhood and thereafter establishes a persistent infection in the kidney (Chesters *et al*, 1983; Shah, 1996). Throughout all the life span, JC virus is frequently reactivated, with excretion of viral particles in urine and without any apparent clinical symptoms (Kitamura *et al*, 1994). However, in immunosuppressed subjects, JC virus reaches the brain and causes progressive multifocal leukoencephalopathy (Atwood *et al*, 1992; Shah, 1996), mostly as a consequence of a lytic infection of oligodendrocytes (Astrom *et al*, 1958; Berger and Concha, 1995).

The mechanism that leads a common and usually benign virus like JC virus to induce progressive multifocal leukoencephalopathy is largely unclear,

*Correspondence: P Ferrante, Laboratory of Biology, Don C. Gnocchi Foundation, IRCCS, Via Capecelatro, 66, I-20148, Milan, Italy
Received 28 June 2000; accepted 24 August 2000

but it is evident that besides host immunosuppression, other unknown factors are involved.

It is likely that the immune system of normal individuals maintains the virus in a latent state but that an immunocompromised condition could allow viral reactivation and the passage from latency to a lytic phase (Major and Ault, 1995). Molecular studies of JC virus suggest that the viral regulatory region could play a role in the selective tropism of the virus for glial cells (Kenney *et al*, 1984; Feigenbaum *et al*, 1987; Khalili *et al*, 1987), but it is not clear how these findings can explain the neuropathogenicity of the virus.

Different viral subtypes located in various geographic areas are known to infect humans and they could represent a tool suited to the study of the evolution of DNA viruses and human migrations (Sugimoto *et al*, 1997; Guo *et al*, 1998; Jobes *et al*, 1998). On the basis of RFLP analysis, Yogo *et al* (1991) have divided JC virus strains into groups A and B, while using sequence analysis of the VT-intergenic region, Ault and Stoner (1992) have defined types 1 and 2. A new type of JC virus (type 3) has recently been detected in the urine of Tanzanian HIV-1 positive patients (Agostini *et al*, 1995), while another new African strain has been amplified from the urine of healthy individuals residing in Ghana (Guo *et al*, 1996). This latter strain, termed type C, has also been demonstrated to be similar to type 6 identified in an African-American progressive multifocal leukoencephalopathy patient (Agostini *et al*, 1997a). In addition, another new type has been isolated (type 4), probably resulting from a recombination of two different strains on the occasion of a double infection (Agostini *et al*, 1996b). Based on its complete genomic sequence, it has been classified as a type 1 subgroup member (type 1C) (Agostini *et al*, 1998a). The reported findings of a JCV type 5 reclassified as a member of the JC virus type 3 subgroup, and the more recent isolation and characterization of a JC virus type 6 should also be mentioned here (Jobes *et al*, 1998). The epidemiological distribution of the various JC virus strains in the world still needs to be better defined, however, considered overall, reports from different authors indicate that JC virus type 1 is prevalent in Europe, type 2 in Asia and type 3 in East Africa. The epidemiology of JC virus types seems to be more complex in the USA where types 1 and 2 are present, with a higher frequency of type 1 (64%) in the general population, while JC virus type 3 is predominant in the African-American group (Guo *et al*, 1996; Agostini *et al*, 1996a,b, 1997c; Jobes *et al*, 1998).

As already suggested (Stoner *et al*, 1996; Agostini *et al*, 1997b; Ferrante *et al*, 1998), another possibility requiring further study is that the different JC virus types could show a variable pattern of neurotropism and neurovirulence and therefore play different roles in the pathogenesis of progressive multifocal leukoencephalopathy.

This study represents the first comparison by nucleotide sequence analysis of the frequency of JC virus genotypes in cerebrospinal fluid (CSF), peripheral blood mononuclear cells (PBMCs) and urine collected on the same day from AIDS patients with and without progressive multifocal leukoencephalopathy and in CSF and urine from healthy subjects.

The data obtained show a high frequency of JC virus type 2 in the CSF of progressive multifocal leukoencephalopathy patients and the occurrence of dual infection with types 1 and 2 only in progressive multifocal leukoencephalopathy patients. The results therefore suggests that JC virus type 2 probably has more pronounced neurotropic properties and that infection with two different JC virus genotypes is an additional risk factor.

Results

The distribution of JC virus large T antigen DNA among the three patient groups is reported in Table 1, subdivided by the CSF, PBMCs and urine samples. It is possible to note from this table that all of the 21 CSF samples collected from progresive multifocal leukoencephalopathy patients were positive, whereas all of the CSF samples from the 30 AIDS patients without progressive multifocal leukoencephalopathy were negative for JC virus large T antigen DNA. JC virus DNA was found in the PBMCs from 12 (57.1%) progressive multifocal leukoencephalopathy patients, from 10 (33.3%) non-progressive multifocal leukoencephalopathy AIDS patients and from eight (26.7%) of the 30 healthy controls. The frequency of JC virus DNA in the PBMCs was significantly higher in the progressive multifocal leukoencephalopathy patients than in the healthy controls ($P<0.05$; OR=3.67). No significant differences emerged from the comparison between the progressive multifocal leukoencephalopathy cases and AIDS patients without progressive multifocal leukoencephalopathy and between the healthy subjects and AIDS patients without progressive multifocal leukoencephalopathy. Viruria was a frequent finding: JC virus DNA

Table 1 Detection of JCV DNA belonging to the LT region in the CSF, PBMC and urine samples from the three study groups

Samples	*AIDS patients with PML* n=21	*AIDS patients without PML* n=30	*Healthy Controls* n=30
CSF	21 (100%)	0	not tested
PBMCs	12 (57.1%)*	10 (33.3%)	8 (26.7%)*
Urine	10 (47.6%)	14 (46.7%)	12 (40.0%)

* AIDS patients with PML *versus* healthy controls: $P<0.05$.

was detected in the urine from 10 (47.6%) PML subjects, 14 (46.7%) non-progressive multifocal leukoencephalopathy AIDS patients and 12 (40.0%) healthy controls. The PCR employed is capable of simultaneously amplifying JC virus and BK virus DNA, and we detected the simultaneous presence of both BK virus and JC virus DNA in the urine samples from one progressive multifocal leukoencephalopathy patient.

The 215 bp fragment of the VP1 region amplified with our PCR contains polymorphic sites that offer the possibility of defining JC virus genotypes 1, 2, 3 and 4 and, among types 1 and 2, the variants 1A, 1B, 1C and 2A, 2B and 2C. The distribution of the different JC virus variants in the CSF, PBMCs and urine samples of the three groups studied is reported in Table 2. As is observable, the majority of JC virus type 2 isolates were detected in samples collected from progressive multifocal leukoencephalopathy patients, while among the other groups, JC virus type 2 was amplified only in one urine sample from a healthy control. Among the progressive multifocal leukoencephalopathy cases, the highest frequency of JC virus type 2 detection was observed in the CSF samples with 11 amplified strains (44% of the total JC virus amplified from CSF samples). The JC virus type 2 strain was detected only in two urine samples (18.2% of the total JC virus amplified from urine samples), whereas no JC virus type 2 was found in the PBMCs. Of the 11 JCV type 2 strains amplified from CSF, five were a type 2A variant, four a type 2B variant and two a type 2C variant. A JC virus type 2A strain was also detected in the urine sample from one progressive multifocal leukoencephalopathy subject. In addition, a JC virus type 2B strain was detected in one urine sample from a progressive multifocal leukoencephalopathy patient and JC virus type 2C variant was found in the urine sample from one healthy control.

JC virus type 1 was detected in the CSF (52.0% of the total amplified strains) and more frequently in the PBMC (91.7%) and urine (73.7%) samples from the AIDS patients with progressive multifocal leukoencephalopathy. It was absolutely predominant in the PBMCs and urine samples collected from the AIDS patients without progressive multifocal leukoencephalopathy and the healthy controls. As regards JC virus type 1 strains, it can be noted that only one isolate detected in the urine sample from a healthy control proved to be type 1C. The most frequent strain was JC virus type 1B, accounting for 58.7% of total JC virus type 1 isolates, while JC virus type 1A was less frequent (40.0%). As can be noted from Table 2, the number of JC virus strains amplified in the CSF and urine samples collected from the healthy controls, was greater than the number of samples analysed. This difference is due to the detection of a dual infection with both types 1 and 2 in four CSF samples and in one urine sample from the progressive multifocal leukoencephalopathy patients.

Due to the high variability of the JC virus subtype detected among the progressive multifocal leukoencephalopathy patients, the data obtained by analysing VP1 amplified products in the CSF, PBMCs and urine samples from the 21 progressive multifocal leukoencephalopathy cases are reported in detail in Table 3. A dual infection with both JC virus type 1 and type 2 was detected in four (19.0%) CSF samples, and in the urine sample from progressive leukoencephalopathy case 9. Moreover, in progressive multifocal leukoencephalopathy patient 1, JC virus type 2B was detected in the CSF and in the urine sample, while type 1A was found in the PBMCs. Considered overall, a total of six (28.6%) progressive multifocal leukoencephalopathy patients had dual infections with JC virus type 1 and 2 in the same or in different samples.

Table 4 compares the alignment of the JC virus strains amplified from CSF with the 10 typing sites included in the 215-bp fragment. One may note that the strain amplified from the PBMCs and the urine sample from progressive multifocal leukoencephalopathy case 6 is similar to type 1B, with two

Table 2 Detection of the different JCV subtypes among AIDS patients with and without PML and healthy subjects. Numbers and percentages (in brackets) refer to the total amplified JCV strains

		PML AIDS patients			*Non-PML AIDS patients*		*Healthy controls*	
		CSF	*PBMC*	*Urine*	*PBMC*	*Urine*	*PBMC*	*Urine*
JCV Genotypes		(21 samples)	(12 samples)	(10 samples)	(10 samples)	(14 samples)	(8 samples)	(12 samples)
JCV Type 1	A	4	6	3	4	7	3	3
	B	9	5	5	6	7	5	7
	C							1
	Total	13 (52.0%)	11 (91.7%)	8 (73.7%)	10 (100%)	14 (100%)	8 (100%)	11 (91.7%)
JCV Type 2	A	5		1				
	B	4		1				
	C	2						1
	Total	11 (52%)		2 (18.2%)				1 (8.3%)
Unclassified JCV		1	1 (8.3%)	1 (9.1%)				
Total JCV isolates		25	12	11	10	14	8	12

mismatches at position 1818 (G>C), which is typical of type 2, and at 1843 (T>G), which is typical of type 1A. Therefore, this strain has been defined as JC virus type 1X in the table.

In addition, the JC virus strain detected in the CSF of progressive multifocal leukoencephalopathy patient 21 is reported as unidentified because it has a type 1A pattern with a point mutation (G->C) in position 1818, which is typical of type 2.

Discussion

The AIDS epidemic and the consequent increasing frequency of progressive multifocal leukoencephalopathy worldwide have created the opportunity to define many aspects of this neurological disease which was once considered very rare. Although significant progress has been made in terms of diagnosis, epidemiology and the clinical definition of progressive multifocal leukoencephalopathy, the molecular basis of its pathogenic mechanism still need to be clearly defined (Major *et al*, 1992; Weber *et al*, 1994; Major and Ault, 1995).

It is evident that immunosuppression is a fundamental predisposing factor since progressive multifocal leukoencephalopathy is observed only in subjects with heavy immunological deficits. However, given that not all the immunosuppressed individuals have progressive multifocal leukoencephalopathy, it is also clear that other factors are at work in the development of this disease. Viral factors and host factors probably counteract in JC virus infected individuals to favour the clinical onset of progressive multifocal leukoencephalopathy, and as in the cases of other viral diseases, it could be possible that different JC virus strains possess variable neurotropic properties. Support for this theory is provided by reports of a higher frequency of JC virus type 2 in progressive multifocal leukoencephalopathy patients compared to HIV-infected non-progressive multifocal leukoencephalopathy patients (Agostini *et al*, 1997b, 1998b). In their papers, Agostini *et al* analyse JC virus genotypes detected in a large number of brain tissue or CSF specimens from progressive multifocal leukoencephalopathy patients and compare their distribution with JC virus strains excreted in the urine of control subjects. Thus the evidence showing a higher frequency of JC virus type 2 in progressive multifocal leukoencephalopathy is indirect to some degree. The present study represents the first comparison of the genotype pattern of JC virus strains amplified from CSF, PBMCs and urine collected on the same day from Italian AIDS patients with and without progressive multifocal leukoencephalopathy and from urine samples and PBMCs of healthy subjects. The data we obtained clearly indicate that JC virus type 2 is significantly associated with progressive multifocal leukoencephalopathy as this type was found almost solely in the CSF from progressive multifocal leukoencephalopathy patients and only in two urine samples from

Table 3 Distribution of different JCV subtypes in CSF, PBMCs and urine from the 21 PML patients with JCV-positive CSF

Patients	*CSF*	*PBMCs*	*Urine*
1	2B	1A	2B
2	1B	1B	1B
3	1A	1A	–
4	1B	1B	1B
5	1B+2A	–	–
6	1B+2A	1X	1X
7	1B	1B	1B
8	1A	1A	1A
9	2A	1A	1A+2A
10	2C	–	–
11	2C	–	–
12	1B	1B	1B
13	1A+2B	1A	1A
14	1B+2B	–	–
15	2A	–	–
16	1B	–	1B
17	2A	–	–
18	2B	–	–
19	1B	1B	–
20	1A	1A	–
21	1A*	–	–

*indicates a strain with a nucleotide sequence similar but not identical to the reported JCV variant; 1X indicates unclassified strains

Table 4 Genotype definition based on short typing fragments amplified from CSF of PML patients

		Nucleotide position									
PML patients	*JCV types*	1753	1771	1786	1804	1818	1837	1843	1850	1869	1870
3,8,13,20	1A	A	C	G	T	G	T	G	A	G	G
2,4,5,6,7,12,14,16,19	1B	A	C	G	T	G	T	T	G	G	G
5,6,9,15,17	2A	A	A	G	T	C	T	T	A	G	A
1,13,14,18	2B	A	A	T	T	C	C	T	G	G	A
10,11	2C	A	A	T	T	C	T	T	G	G	A
21		A	C	G	T	C	T	G	A	G	G
6 (PBMC; Urine)	1X	A	C	G	T	C	T	G	G	G	G

In the last row, the sequence of the unclassified strains detected in PBMCs and urine from Patient 6. Nucleotide positions are based on Mad-1 prototype strain (Frisque *et al*., 1984).

progressive multifocal leukoencephalopathy patients and in one from a healthy control.

On the contrary, all of the strains amplified from the PBMC and urine samples from AIDS patients without progressive multifocal leukoencephalopathy and from healthy controls, with the exception of the type 2 positive urine sample mentioned previously, belong to JC virus type 1. On the whole, these data are in keeping with previous studies by other authors (Agostini *et al*, 1997b, 1998b), and with our preliminary observations (Ferrante *et al*, 1998). Moreover, they are of a particular significance because the study was performed on subjects living in Italy, a country where JC virus type 1 should be the most common as in the rest of Europe (Yogo *et al*, 1991). As yet, a clear, extensive information on the molecular epidemiology of the JC virus among the Italian healthy subjects and AIDS patients is not available. Thus our results showing that among the healthy subjects and AIDS patients, JC virus type 1 is absolutely predominant, can also be seen as a preliminary contribution to the understanding of JC virus epidemiology in Italy. The detection of JC virus type 2 in the urine of a healthy subject indicates that though very rarely, this genotype can be found in the Italian population and suggests the need for extensive epidemiological studies.

The PCR employed is capable of identifying the subtypes of JC virus type 1 and type 2 that are respectively defined as 1A, 1B, 1C and 2A, 2B and 2C, and, although phylogenetic analysis performed on longer fragments indicates that types 1A, 1B and 1C (Jobes *et al*, 1998), and types 2A and 2C (Sugimoto *et al*, 1997) constitute two single clusters, we found this classification more suitable for our study. In a recent paper (Agostini *et al*, 1998b) it has been suggested that JC virus type 2B is the most represented type in progressive multifocal leukoencephalopathy patients, but our results did not confirm this observation, given that no significant differences were observed in the distribution of JC virus type 2A and 2B among the progressive multifocal leukoencephalopathy CSF samples. On the other hand, it is interesting to note that JC virus type 2A, a strain usually found in Japan and in Native Americans (Agostini *et al*, 1997c), was detected in five CSF samples and one urine sample from our Italian progressive multifocal leukoencephalopathy patients. The origin of this strain and its route of diffusion* (o: circulation) among Italian progressive multifocal leukoencephalopathy patients is unclear. However, considered as a whole, our results suggest some possibilities that will be discussed later.

Another important aspect that can be drawn from our data lies in the unexpectedly high frequency of dual infection with both JC virus types 1 and 2 among the progressive multifocal leukoencephalopathy patients. A total of six progressive multifocal leukoencephalopathy patients had a dual infection and in four of the latter, the two JC virus strains were detected in the same CSF sample, while the other two revealed JC virus type 2 in the CSF sample and type 1 in the urine sample. Dual infection with different JC virus genotypes has already been observed in brain tissue, CSF and urine from AIDS patients (Martin and Foster, 1984; Agostini *et al*, 1996b; Stones *et al*, 1996). However, this is the first report of a very high frequency of dual infection with JC virus types 1 and 2 among progressive multifocal leukoencephalopathy patients and particularly with detection resulting from their CSF.

Various hypotheses could be postulated in order to explain the occurrence of the double infection and the higher frequency of JC virus type 2 in the progressive multifocal leukoencephalopathy patients. Our results suggest that as a consequence of the HIV-induced immunosuppression, in these subjects, a second JC virus infection could have occurred and JC virus type 2, which has been shown to circulate in the United States (Agostini *et al*, 1996b; Guo *et al*, 1996), may have infected Italian HIV-positive individuals. This route of viral diffusion has already been observed in the case of human T-lymphotropic virus type II (HTLV-II), for Italian HTLV-II-positive intravenous drug users are mainly infected by HTLV-IIb4, a subtype probably acquired by contact with infected individuals from the United States (Vallejo *et al*, 1996).

In conclusion, we may state that the results indicate that besides immunosuppression, other viral and host-related factors are needed for the development of progressive multifocal leukoencephalopathy. They suggest that the involvement of specific JC virus variants and the occurrence of a double infection with two JC virus genotypes could be important risk factors for the disease development.

Materials and methods

Subjects

The study was performed by means of analyses of CSF, PBMCs and urine samples collected from 21 AIDS patients suffering from progressive multifocal leukoencephalopathy, from 30 AIDS patients without progressive multifocal leukoencephalopathy and on PBMCs and urine samples collected from 30 healthy subjects. All the subjects enrolled in the study were Italians and residents in the area of Milan in northern Italy. Moreover, the healthy controls were matched for age with the AIDS patients. In addition to the detection of JC virus DNA in CSF, the diagnosis of progressive multifocal leukoencephalopathy was based on routine CSF examination, on brain magnetic resonance imaging and/or computed tomography, carried out for all of the AIDS patients according to the criteria described in detail in a previous study (Ferrante *et al*, 1997).

The samples were collected from the individual subjects on the same day, treated and stored as previously described (Ferrante *et al*, 1997) for examination by means of various polymerase chain reaction (PCR). Strict precautionary measures were taken to avoid contamination and all PCR reactions were performed using one negative and one positive control.

PCR for JC virus large T antigen region
JC virus DNA was searched in the collected samples with a nested PCR using oligonucleotide primers selected from within the JCV genome encoding the large T antigen region and sharing significant genomic homology with BK virus. The experimental conditions, as well as the specificity and sensitivity of this PCR, have been defined and described in a previous study (Ferrante *et al*, 1995).

PCR for VP1 region
Molecular characterization of a short fragment of major virion capsid protein (VP1) gene was performed on CSF, PBMCs and urine samples from AIDS patients with PML, on PBMCs and urine samples from AIDS patients without PML and from healthy subjects who proved to be positive for large T antigen JC virus DNA.

In order to characterize JC virus isolates, we adopted a PCR designed to amplify a 215-bp fragment of the VP1 gene that allows for the distinction between JC virus types 1 – 4 and related subtypes. The VP1 region was amplified with JLP-15 (5′-ACAGTGTGGCCAGAATTCCACTACC-3′) at position 1710 – 1734, and JLP-16 (5′-TAAAGCCTCCCCCCCAACAGAAA-3′) at position 1924 – 1902, 10 pmol each, in a 40-cycle protocol which combined annealing and elongation at 61°C (Agostini *et al*, 1997b). The reaction was carried out in a total volume of 100 μl, 50 μl of which were used for sequencing.

The amplified DNA was visualised by UV light exposure on 2% agarose gel, after staining with ethidium bromide, yielding the expected bands of 215 bp for the VP1 region.

Nucleotide sequencing of the amplified products
PCR was performed using one standard and one biotinylated primer. PCR amplified products were used as a template for direct sequencing. The sequencing reaction was done using the AutoLoad Solid Phase Sequencing kit (Pharmacia Biotech, Uppsala, Sweden) and the automated dideoxy-sequence analysis was run on an ALF*express* DNA sequencer (Pharmacia Biotech, Uppsala, Sweden). Biotinylated amplified products were immobilised on streptavidin-coated Autoload combs and sequenced according to the protocol (Hultman *et al*, 1989). Fluorescein-labelled primer and T7 polymerases were used for the sequencing reaction. The terminated sequencing products were loaded directly onto an ALF DNA sequencer by inserting the combs into a 6% Ready Mix™ gel (Lagerkvist *et al*, 1994) which contains polyacrylamide, 100 mM Tris-borate (pH 8,3), 1 mM Na_2EDTA, 7 M urea.

Genotype determination
Genotyping of JC virus strains was based on the partial VP1 sequence which includes 10 type-determining sites (Agostini *et al*, 1996a) which permit the results that have been obtained to be compared with those obtained by the sequencing of the whole JC virus genome (Agostini *et al*, 1998a). Sequence relationships and alignments were performed by DNAsis® for Windows® program of the Hitachi Software Engineering Co. (San Bruno, CA, USA). The DNA consensus sequences reported were based on the GenBank/EMBL Data Library.

Dual infection definition
As the difference between the various JC virus genotypes is based on single-base mutations in the amplified VP1 region, the length of the amplified products do not vary. Therefore, in order to identify the different genotypes, the nucleotide sequence analysis must be performed. When a sample contains two JC virus genotypes, the employed primers are capable of simultaneously amplifying both strains that differ by a few point mutations. The nucleotide sequence automatic analyser reads the sequence without any difficulty, however, at the position where the two strains have different bases, a double peak is produced. This ambiguous result is then manually resolved by employing the operative manual of the Alfexpress apparatus which provides an interpretative code for the definition of the ambiguous automatic reading. To confirm these unusual results, the amplified products showing these atypical patterns were subjected to nucleotide sequence analysis three times and the same reading was always obtained. Using this approach, dual infection of the same sample with two different JC virus strains can be detected and defined.

Statistical analysis
The frequencies of JC virus DNA detection in the CSF, PBMC and urine samples from the three study groups were compared by calculating the odds ratio (OR) and the confidence interval limits using the chi-square test with Yate's correction (EpiInfo 6.0).

Acknowledgements

This study was partially supported by grants to the Don C. Gnocchi Foundation, IRCCS, from the Italian Ministry of Health: Ricerca Finalizzata 1996, Ricerca Corrente 1999 and 1998 AIDS project (grant no. 50B19).

References

Agostini HT, Brubaker GR, Shao J, Ryschkewitsch CF, Blattner WA, Stoner GL (1995). BK virus and a new type of JC virus excreted by HIV-1 positive patients in rural Tanzania. *Arch Virol* **140:** 1919–1934.

Agostini HT, Ryschkewitsch CF, Stoner GL (1996a). Genotype profile of human polyomavirus JC excreted in urine of immunocompetent individuals. *J Clin Microbiol* **34:** 159–164.

Agostini HT, Ryschkewitsch CF, Singer EJ, Stoner GL (1996b). Co-infection with two JC virus genotypes in brain, cerebrospinal fluid or urinary tract detected by direct cycle sequencing of PCR products. *J NeurVirol* **2:** 259–267.

Agostini HT, Ryschkewitsch CF, Brubaker GR, Shao J, Stoner GL (1997a). Five complete genomes of JC virus Type 3 from Africans and African Americans. *Arch Virol* **142:** 637–655.

Agostini HT, Ryschkewitsch CF, Mory R, Singer EJ, Stoner GL (1997b). JC virus (JCV) genotypes in brain tissue from patients with progressive multifocal leukoencephalopathy (PML) and in urine from controls without PML: increased frequency of JCV type 2 in PML. *J Infec Dis* **176:** 1–8.

Agostini HT, Yanagihara R, Davis V, Ryschkewitsch CF, Stoner GL (1997c). Asian genotypes of JC virus in native Americans and in a Pacific Island population: markers of viral evolution and human migration. *Proc Natl Acad Sci USA* **94:** 14542–14546.

Agostini HT, Ryschkewitsch CF, Stoner GL (1998a). JCV virus type 1 has multiple subtypes: three new complete genomes. *J Gen Virol* **79:** 801–805.

Agostini HT, Ryschkewitsch CF, Singer EJ, Baumhefner RW, Stoner GL (1998b). JCV virus type 2B is found more frequently in brain tissue of progressive multifocal leucoencephalopathy patients than in urine from controls. *J Hum Virol* **1:** 200–206.

Astrom KE, Mancall EL, Richardson Jr EP (1958). Progressive multifocal leukoencephalopathy, a hitherto unrecognized complication of chronic lymphocytic leukaemia and Hodgkin's disease. *Brain* **81:** 93–111.

Atwood WJ, Amenaiya K, Traub R, Harms J, Major EO (1992). Interaction of the human polyomavirus, JCV, with human B-lymphocytes. *Virology* **190:** 716–732.

Ault GS, Stoner GL (1992). Two major types of JC virus defined in progressive multifocal leukoencephalopathy brain by early and late coding region DNA sequences. *J Gen Virol* **73:** 2669–2678.

Berger JR, Concha M (1995). Progressive multifocal leukoencephalopathy: the evolution of a disease once considered rare. *J NeuroVirol* **1:** 5–18.

Chesters PM, Heritage J, McCance DJ (1983). Persistence of DNA sequences of BK virus and JC virus in normal human tissues and in diseased tissues. *J Infec Dis* **147:** 676–684.

Feigenbaum L, Khalili K, Major EO, Khoury G (1987). Regulation of the host range of human papovavirus JCV. *Proc Natl Acad Sci USA* **84:** 3695–3698.

Ferrante P, Caldarelli-Stefano R, Omodeo-Zorini E, Boldorini R, Costanzi G (1995). PCR detection of JC virus DNA in brain tissue from patients with and without progressive multifocal leukoencephalopathy. *J Med Virol* **47:** 219–225.

Ferrante P, Caldarelli-Stefano R, Omodeo-Zorini E, Cagni AE, Cocchi L, Suter F, Maserati R (1997). Comprehensive investigation of the presence of JC virus in AIDS patients with and without progressive multifocal leukoencephalopathy: PCR, neuroradiological and other laboratory findings. *J Med Virol* **52:** 235–242.

Ferrante P, Mediati M, Caldarelli-Stefano R, Omodeo-Zorini E, Cagni AE, Maserati R (1998). Increased frequency of JC virus type 2 and of dual infection with JC virus type 1 and 2 in the CSF of Italian AIDS patients with progressive multifocal leukoencephalopathy. *J NeuroVirol* **4:** 349.

Frisque RJ, Bream GL, Cannella MT (1984). Human polyomavirus JC virus genome. *J Virol* **51:** 458–469.

Guo J, Kitamura T, Ebihara H, Sugimoto C, Kunitake T, Takehisa J, Qun Na Y, Al-Ahdal M, Hallin A, Kawabe K, Taguchi F, Yogo Y (1996). Geographical distribution of the human polyomavirus JC virus types A and B and isolation of a new type from Ghana. *J Gen Virol* **77:** 919–927.

Guo J, Sugimoto C, Kitamura T, Ebihara H, Kato A, Guo Z, Liu J, Zheng SP, Wang YL, Na YQ, Suzuki M, Taguchi F, Yogo Y (1998). Four geographically distinct genotypes of JC virus are prevalent in China and Mongolia: implications for the racial composition of modern China. *J Gen Virol* **79:** 2499–2505.

Hultman T, Stahl S, Hornes E, Uhlen M (1989). Direct solid phase sequencing of genomic and plasmid DNA using magnetic beads as solid support. *Nucl Ac Res* **17:** 4937.

Jobes DV, Chima SC, Ryschkewitsch CF, Stoner GL (1998). Phylogenetic analysis of 22 complete genomes of the human polyomavirus JC virus. *J Gen Virol* **79:** 2491–2498.

Kenney S, Natarajan V, Strika V, Khoury G, Salzman NP (1984). JC virus enhancer-promoter active in human brain cells. *Science* **226:** 1337–1339.

Khalili K, Feigenbaum L, Khoury G (1987). Evidence for a shift in 5′-termini of early viral RNA during the lytic cycle of JC virus. *Virology* **158:** 469–472.

Kitamura T, Kunitake T, Guo J, Tominaga T, Kawabe K, Yogo Y (1994). Transmission of human polyomavirus JC virus occurs both within the family and outside the family. *J Clin Microbiol* **32:** 2359–2363.

Lagerkvist A, Stewart J, Lagerstrom-Fermer M, Landegren U (1994). Manifold sequencing: efficient processing of large sets of sequencing reactions. *Proc Natl Acad Sci USA* **91:** 2245–2249.

Major EO, Amemya K, Tornatore GS, Houff SA, Berger JR (1992). Pathogenesis and molecular biology of progressive multifocal leukoencephalopathy, the JC virus-induced demyelinating disease of the human brain. *Clin Microbiol Rev* **5:** 49–73.

Major EO, Ault GS (1995). Progressive multifocal leukoencephalopathy: clinical and laboratory observations on a viral induced demyelinating disease in the immunodeficient patient. *Cur Opin Neurol* **8:** 184–190.

Martin JD, Foster GC (1984). Multiple JC virus genomes from one patient. *J Gen Virol* **65:** 1405–1411.

Shah KV (1996). Polyomaviruses. In Fields BN, Knipe DM, Howley PM (eds): ''*Fields Virology*''. Philadelphia: Lippincott-Raven Publishers, Pp. 2027 – 2040.

Stoner GL, Agostini HT, Ryschkewitsch C (1996). A comparison of JC virus genotype profiles in progressive multifocal leukoencephalopathy brains and control urine. *Neurology* **46:** A316.

Sugimoto C, Kitamura T, Guo J, AL-Hadal MN, Shchelkunov SN, Otova B, Ondrejka P, Chollet JY, El-Safi S, Ettayebi M, Gresenguet G, Kocagoz T, Chaiyarasamee S, Zin Thant K, Thein S, Moe K, Kobayashi N, Taguchi F, Yogo Y (1997). Typing of urinary JC virus DNA offers a novel means of tracing human migrations. *Proc Natl Acad Sci* **94:** 9191 – 9196.

Vallejo A, Ferrante P, Soriano V, Calabro ML, Mancuso R, Heredia A, Mannella E, Favero A, Garcia-Saiz A, Chieco-Bianchi L, Gonzalez-Lahoz J, Hewlett IK (1996). Nucleotide sequence and restriction fragment length polymorphism analysis of human T-cell lymphotropic virus type II (HTLV-II) in southern Europe: evidence for the HTLV-IIa and HTLV-IIb subtypes. *J AIDS Hum Retrovirol* **13:** 384 – 391.

Weber T, Turner RW, Frye S, Ruf B, Haas J, Schielke E, Pohle HD, Luke W, Luer W, Felgenhauer K (1994). Specific diagnosis of progressive multifocal leukoencephalopathy by polymerase chain reaction. *J Infect Dis* **169:** 1138 – 1141.

Yogo Y, Iida T, Taguchi F, Kitamura T, Aso Y (1991). Typing of human polyomavirus JC virus on the basis of restriction fragment length polymorphism. *J Clin Microbiol* **29:** 2130 – 2138.