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Molecular analysis of JC virus genotypes circulating among the Italian healthy population

Elisabetta Pagani,[1] Serena Delbue,[2] Roberta Mancuso,[1] Elisa Borghi,[2] Letizia Tarantini,[2] and Pasquale Ferrante[1,2]

[1]Laboratory of Biology, Don C. Gnocchi Foundation ONLUS, IRCCS, Milan, Italy; [2]Section of Virology, Department of Biomedical Science and Technology, University of Milan, Milan, Italy

JC virus (JCV) is an unique virus, but eight different JCV genotypes and various subtypes have been individuated, with a geographic distribution that has been described in general but still needs to be detailed. To define JCV genotype epidemiology in Italy, the authors collected urine from 211 healthy individuals living in Northern, Central, and Southern Continental Italy, and in the two main Italian islands. As screening, JCV DNA was searched using a polymerase chain reaction (PCR) designed to amplify the highly conserved large T (LT) antigen–coding region. Then, to define JCV genotype and trascriptional control region (TCR) organization, fragments of major capsid protein (VP1) and TCR region were amplified and subjected to nucleotide sequencing. The mean frequency of JCV viruria was of 46%, without differences among the four geographically divided groups and between females and males. JCV types 1 and 4 were the most frequently detected, whereas JCV type 2 was rare, and type 3 was found only in one subject. The low frequency of JCV type 2 contrasts with the reported high frequency of this subtype in progressive multifocal leukoencephalopathy (PML), and supports its specific role in PML. The data indicate that JCV genotype epidemiology in Italy is quite different from that of other European countries; moreover, differences between the various Italian regions have been observed. An unexpected high frequency of a new variant of JCV type 4 with a stable point mutation (C → G) at nucleotide 1851 was found. Furthermore, all the urinary strains had a TCR showing an archetypal organization. *Journal of NeuroVirology* (2003) **9,** 559–566.

Keywords: JCV genotypes; JCV subtypes; JCV viruria; transcriptional control region

Introduction

JC virus (JCV) is a human polyomavirus that, after the primary infection occurring during the childhood, persists throughout life in the kidneys of up to 80% of the human healthy population worldwide, often reactivating with excretion of viral particles in the urine (Brooks and Walker, 1984). The primary infection caused by JCV is asymptomatic, and usually the virus also does not induce any identifiable disease when reactivated and is excreted in the urine. In immunocompromised individuals, JCV, by damaging with a lytic replication cycle the myelin-forming glial cells, causes progressive multifocal leukoencephalopathy (PML), a fatal demyelinating disease of the central nervous system (CNS) (Hou and Major, 2000). Before the acquired immunodeficiency syndrome (AIDS) epidemic, PML was a very rare disease, affecting subjects undergoing natural or drug-induced immunosuppression, such as patients with cancers or with autoimmune disorders treated with immunosuppressive drugs, but has become more frequent

Address correspondence to Pasquale Ferrante, MD, Laboratory of Biology, Don C. Gnocchi Foundation, IRCCS, Via Capecelatro, 66, I-20148, Milan, Italy. E-mail: pferrante@dongnocchi.it

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in AIDS patients, being the cause of death in up to 8% of them (Berger *et al*, 1987; Brooks and Walker, 1984).

Besides its association with PML, JCV has been also suggested as etiological cofactor with different types of tumors, including colon cancer and in particular brain tumors of children and adults (Laghi *et al*, 1999; Boldorini *et al*, 1998; Khalili *et al*, 2001; Gordon *et al*, 2000).

JCV, like BK virus, the other known human polyomavirus, is a unique virus; however, sequence analysis of the genome has led to the identification of eight genotypes and various subtypes that are distributed in the different geographic areas of the world. By convention, JCV genotypes are designed with a progressive number from 1 to 8, and JCV subtypes with the number followed by a capital letter (A, B, C). JCV genotypes and subtypes have a difference in nucleotide sequence of about 1% to 3% and 0.5% to 1%, respectively (Stoner *et al*, 2000; Agostini *et al*, 2001a).

Although the identification of new viral variants requires the examination of the entire genome, nucleotide sequence analysis of a 215-bp fragment between nucleotide positions 1710 and 1924, sequence numbering based on prototype Mad-1 (Frisque *et al*, 1984), overlapping the 5′ end of the major capsid protein (VP1)-coding region, could be enough to define the patterns of point mutations that allow to define the eight genotypes and the various subtypes described (Agostini *et al*, 1999; Stoner *et al*, 2000). The overall picture of JCV genotypes distribution indicates that genotypes 1 and 4 are predominant in Europe, types 2 and 7 in Asia, types 3 and 6 in Africa, whereas JCV type 8 has been detected in Papua New Guinea. A detailed definition of JCV subtypes distribution has been proposed in a recent review (Agostini *et al*, 2001b), which suggests that among the other subtypes, JCV type 2A is detected in America and Asia, and type 2B in Eurasia.

The relation between the various JCV genotypes and neuropathogenicity represents a controversial issue, but, because VP1 encompasses the receptor-binding domain that is crucial in virus attachment to the cell, it has been suggested that amino acid variation of VP1 observed in the different genotypes could induce difference in the tropism and pathogenic properties of JCV (Dubois *et al*, 2001). In this regard, recent reports indicated that JCV type 2 can be significantly associated with PML development in AIDS patients (Agostini *et al*, 1997; Ferrante *et al*, 2001).

A very important feature of JCV genome is represented from the presence of the noncoding transcriptional control region (TCR) that is very often found in rearranged form. It is generally thought that JCV, when latent in the kidney and excreted in the urine, has a nonrearranged archetypal TCR organization, whereas JCV detected in brain and cerebrospinal fluid (CSF) of PML patients shows rearranged TCR, characterized by unique and extensive rearrangements. The criteria of classification of the JCV regulatory region genome features have recently been reorganized in a compass scheme by Jensen and Major (2001). All the known variants of JCV TCR have been arranged into quadrants according to the integration of particular sequence sections and repetition of sequence section groups. Briefly, the type II singular (IIS) is the conserved archetype form that is generally found in the urine of healthy individuals; type I repeats (IR) and type IIR (archetype derived) are the rearranged forms, with tandem repeats and deletions that have been detected in different body compartments, but especially present in the brains and CSF of PML patients (Jensen and Major, 2001). It has been suggested that only JCV strains characterized by a rearranged TCR could reactivate and pass from the kidney to the other body districts. In contrast, JCV DNA amplified directly from urine generally carries a highly stable archetypal promoter-enhancer regulatory region, with the presence of minor changes, consisting of one or more short deletions or duplications, and only rarely shows the complex PML-type TCR rearrangements (Elsner and Doerries, 1998). The TCR region has a central role in viral replication and it has been proposed that rearranged patterns could generate viral variants with mutated tissue tropism and pathogenic capability, whereas the stable configuration is present in the infectious viral strains (Ciappi *et al*, 1999; Vaz *et al*, 2000).

In this paper, we report the results of a study performed in order to define, for the first time, the frequency of JCV viruria, the distribution of JCV genotypes and subtypes, and the organization of the TCR, in the urine of a significant number of healthy individuals born and living in Northern, Central, and Southern Continental Italy and in Sardinia and Sicily, the two largest Italian islands.

Results

JCV urinary excretion

As it is possible to see in Table 1, 97 (46%) out of the 211 healthy individuals, investigated by nested polymerase chain reaction (n-PCR), were positive for DNA belonging to the highly conserved JCV large T (LT) antigen–coding region. No significant differences were observed among the four geographical Italian areas, because JCV DNA was detected in comparable percentages in the urine samples collected from the individuals from North, Center, South of Continental Italy and the two major Italian islands. The frequency of JCV viruria did not significantly differ between females (43.1%) and males (50%); however, on the contrary, JCV viruria correlated with the age of the studied subjects because it was significantly increased in the individuals with more then 40 years of age in comparison to the 20 to 39 years age group ($P < .001$) (Figure 1).

Table 1 Demographic characteristic of the Italian subjects and frequency of detection of JCV DNA in their urine samples

Italian areas	*Enrolled subjects*	*Gender*		*Group by age in years*			*JCV positive subjects (%)*
		Male	*Female*	*21–40*	*41–60*	*61–80*	
North	56	25	31	16	15	25	23 (41.1)
Center	53	21	32	11	15	27	26 (49.1)
South	51	23	28	11	19	21	27 (52.9)
Sardinia and Sicily	51	19	32	6	21	24	21 (41.2)
Total	211	88	123	44	70	97	97 (46)*

*Males 44 (50%), females 53 (43.1%).

Identification of JCV genotypes

The distribution of various JCV genotypes circulating in the different geographical Italian areas was investigated by nucleotide sequence analysis of VP1 region amplified from the above-mentioned 97 urine samples positive for the LT DNA.

JCV type 1, amplified in 57.7% of the examined samples, was the predominant Italian variant; JCV type 4, recovered in 27.8% of the subjects, was the second most frequent, whereas genotype 2 was less frequent (13.4%) and JCV type 3, known to be predominant in Africa, was detected in the urine of only one individual (Table 2). No correlation has been observed between excretion of the different JCV genotypes and the gender and the age of the subjects.

Nucleotide sequence analysis of the amplified fragment of the VP1 region allows the definition not only of the JCV genotypes but also of the JCV subtypes. In our study, with respect to JCV type 1, we have detected subtypes 1A and 1B with a similar frequency (42.9% and 57.1%, respectively). Of particular interest are the data regarding JCV type 4, because 11 (40.7%) of the 27 type 4 strains showed a nucleotide substitution (C → G) at nucleotide 1851, suggesting an unexpected high frequency of this variant already observed in Europe (Agostini *et al*, 2001a) and this strain was named 408. Three subtypes of JCV type 2 have been found: the most frequent (53.8%) was subtype 2B, followed by the subtype 2C (38.5%) and subtype 2A (7.7%). The single JCV type 3 strain we detected in the urine of a northern Italian subject was defined as a type 3A.

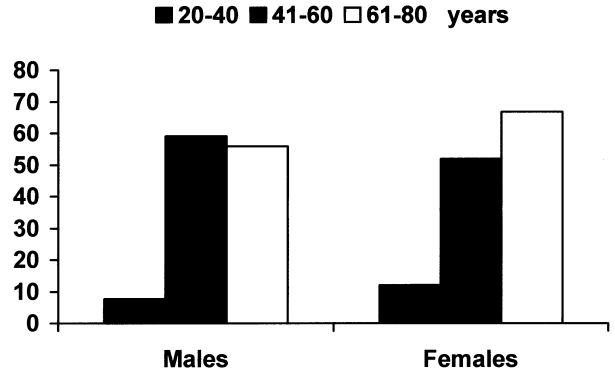

Figure 1 Frequency of JCV DNA detection in the urine according to the gender and age of the studied Italian subjects.

The distribution of JCV genotypes and subtypes in the different geographic areas of Italy showed some interesting variations (Figure 2). At first, it can be noted that in Northern Italy, more different subtypes have been observed in comparison to the rest of the country. JCV type 1, also described as the most frequently circulating genotype in European countries, was significantly more frequent in Sardinia and Sicily and in the South of Continental Italy in comparison to central ($P < .05$) and northern ($P < .05$) Italy.

JCV type 2, detected in the urine of 26.1% of northern, 15.4% of central, 3.7% of southern Italians, and of 9.5% of individuals living in Sardinia and Sicily, was significantly more frequent in the North of Italy in comparison to the South ($P < .05$) and to the two islands ($P < .05$).

The frequency of JCV type 4 did not show relevant differences among the geographical areas, but the strain 408 was observed with higher, although not significant, frequency in the North of Italy (four out of seven subjects) and in the islands (two out of three subjects) in comparison with the Center and the South of Italy (Table 2). The excretion of this particular variant was not related to the age and gender of the studied individuals (data not shown).

Transcriptional control region characterization

To verify the organization of TCR of the JCV strains circulating among the Italian healthy subjects, we performed nucleotide sequence analysis of 21 TCR amplified from the urine of individuals living in the various geographical areas and belonging at least to one of the JCV single subtype. The data obtained indicate that all the analyzed TCRs had an organization similar to the archetype (IIS) form, with few point mutations, and without the rearrangement usually observed in the TCR found in PML patients.

Discussion

The analysis of the worldwide geographical distribution of the different JCV genotypes is of relevant interest for both epidemiological and pathogenic considerations. It has been shown that JCV genotypes could

Table 2 Frequency of detection of the different JCV genotypes (in bold) and subtypes among the studied subjects according to their geographic area

		Viral genotypes and subtypes										
Italian areas	*Positive subjects*	***1***	*1A*	*1B*	***2***	*2A*	*2B*	*2C*	***3A***	***4***	*4*	*408*
North	23	**9 (39.1%)**	4	5	**6 (26%)**	—	3	3	**1 (4.3%)**	**7 (30.4%)**	3	4
Center	26	**11 (42.3%)**	5	6	**4 (15.4%)**	—	4	—	**—**	**11 (42.3%)**	7	4
South	27	**20 (74%)**	10	10	**1 (3.7%)**	1	—	—	**—**	**6 (22.2%)**	5	1
Sardinia and Sicily	21	**16 (76.2%)**	5	11	**2 (9.5%)**	—	—	2	**—**	**3 (14.3%)**	1	2
Total	97	**56 (57.8%)**	24	32	**13 (13.4%)**	1	7	5	**1 (1%)**	**27 (27.8%)**	16	11

be a useful marker for the study of the migration of human family major branches, allowing the scientists to trace new migration routes and to verify those already hypothesized (Agostini *et al*, 1999; Stoner *et al*, 2000; Sugimoto *et al*, 1997). In fact, because JCV is an ubiquitous, genetically stable virus acquired early in the life through an horizontal transmission involving the family or the close community, it has been proposed that the virus may have a coevolution with the human species throughout their history (Agostini *et al*, 2001a).

The knowledge of JCV genotypes distribution in the different population could be also of interest in order to define the relevance of the previously

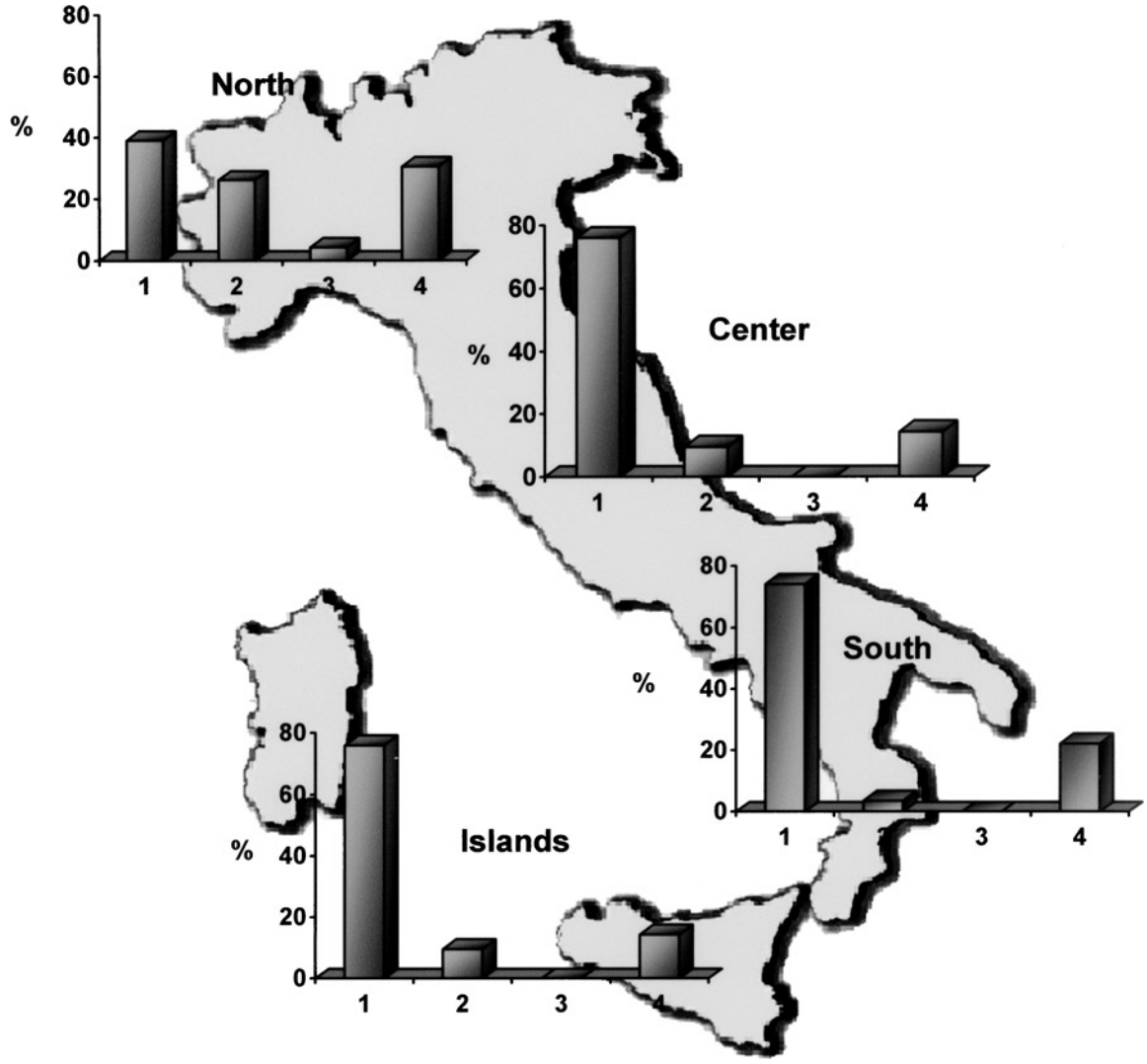

Figure 2 Distribution of the four JCV genotypes in the three different geographical areas of continental Italy and in the two main Italian islands.

reported finding of a significant association between specific JCV genotypes and PML development in AIDS patients (Agostini *et al*, 1997; Ferrante *et al*, 2001).

The data obtained in the present study allow for the first time the definition of frequency of JCV viruria and the distribution of JCV genotypes, in a consistent number of healthy Italian individuals. In agreement with the data obtained in other European areas (Agostini *et al*, 2001a), JCV DNA was found in the urine of almost half of the enrolled subjects without differences among the various geographical areas and between females and males, whereas the frequency of excretion significantly increased with age, as already reported (Kitamura *et al*, 1989; Agostini *et al*, 1996). Assuming that JCV presence in the urine is due to the reactivation of the virus, the increased detection in older individuals could be explained with a more frequent, even if transitory, decline of the specific host immunity in those subjects in comparison to the younger individuals.

Four different JCV genotypes have been detected, and JCV type 1 has been confirmed as the most frequent in Italy, as already observed in European countries (Agostini *et al*, 2001a; Dubois *et al*, 2001). Likewise, JCV genotype 4 was shown to be the second most frequent genotype, followed by type 2, whereas JCV type 3 has been found only in one subject. Both JCV subtypes 1A and 1B have been detected with a comparable frequency in the urine of Italian subjects, and this epidemiological picture is intermediate between that of Northern and Central Europe, where JCV type 1A is predominant, and that of South West Europe in which almost only JCV type 1B has been found (Agostini *et al*, 2001a; Dubois *et al*, 2001). It would be of great interest to compare JCV genotypes distribution in PML patients living in Italy with those living in Asian countries, where JCV type 2 is reported to be predominant in the general population. So far, there are few data concerning PML in Asia; however, in a recent article, Shankar and colleagues (2003) tried to understand the low prevalence of PML in South East Asia and Africa, where the infection with human immunodeficiency virus (HIV) is seriously widespread. They hypothesized, reviewing three clinically undiagnosed PML cases, that many PML cases could have similarly been missed or underreported because of the unavailability of confirmatory tests in those countries. Likewise, they suggest that other opportunistic infections, occurring in AIDS patients with higher CD4 cell count, could lead the patients to die before they develop PML. Little is know also about the JCV genotypes distribution in Asian PML patients, but a recent article reported the finding of JCV type 1 in two HIV-positive PML patients from Korea, a country where type 2 is the prevalent in the general population (Jeong *et al*, 2002). On the whole, we can speculate that the possibility that JCV infrequent genotypes in the general population (i.e., type 2 in Italy, type 1 in Asia) could represent a risk factor for PML development in HIV-positive subjects.

In respect to the worldwide spread of JCV genotypes proposed by Stoner *et al* (2000), these data suggest that the presence of type 1 as predominant variant in Italy might be due to the late migration of Middle East populations, infected with JCV type 1, who have imposed themselves on the previous Italian population, probably carrying JCV type 4.

A higher circulation of different JCV subtypes has been observed in the North and, to a lesser degree, in the Center of Continental Italy in comparison with Southern Italy and the two main Italian islands. The higher variability in Northern Italy could be explained by the fact that in this part of Italy, already from many centuries, there has been immigration of people coming from Central and Eastern Europe, whereas Southern Italy, in the last centuries, has not been involved by immigration routes, being, on the contrary, a land of migrants.

One of the most interesting observation is the detection of a variant of genotype 4, termed strain 408 (the first digit represent the genotype number) and characterized by a C $\rightarrow$ G substitution at position 1851. This variant has been already observed in a very limited number of individuals in Europe, and in particular in one Basque, in two German Sinti, and one Polish subjects (Agostini *et al*, 2001a), but the high frequency observed in our subjects suggest that this strain is more relevant than expected among Italian population.

The data of the present study, based on a consistent number of subjects, confirm that JCV type 2 is less frequent in the urine of the healthy Italian population in comparison to genotypes 1 and 4. This observation is, in our opinion, of particular relevance because it is a further support to the hypothesis of a significant association of JCV type 2 with PML in AIDS patients (Agostini *et al*, 1997; Ferrante *et al*, 2001). Moreover in the already quoted previous work (Ferrante *et al*, 2001), we found that JCV type 2A was the most frequent strain in the CSF collected from Italian AIDS patients suffering of PML, whereas in the present study, we have detected this JCV subtype only in one individual, suggesting a more pronounced pathogenic role of JCV subtype 2A in PML development. The finding of one type 2A in Southern Italy is so far unexplained, such as the observation of a type 3A in the Northern Italy. Those two individuals did not have a previous journey to a foreign country but we could not exclude a close contact with people immigranted from areas where JCV 2A and 3A are circulating.

On the basis of the observation that also in healthy individuals a limited number of urinary JCV strains could have rearranged TCRs (Elsner and Doerries, 1998), we have performed nucleotide sequence analysis of TCR amplified from urine samples of 21 healthy Italian subjects. All the amplified TCR regions had an archetypal organization (type IIS) with

few scattered point mutations, as already observed in healthy individuals from other areas of the world (Yogo *et al*, 1990; Agostini *et al*, 1998).

In conclusion, the results of the present study add further elements to better define the worldwide geographical distribution of the different genotypes and subtypes of JCV and to trace ancient and modern human migrations. The epidemiology of JCV genotypes and subtypes is quite different in Italy from that observed in the rest of Europe; moreover, relevant differences have been also observed between the various Italian geographic areas, suggesting the need for more extensive studies to fully define JCV molecular epidemiology. From the pathogenic point of view, the data obtained are a further support to the possibility that some JCV genotypes, and in particular JCV type 2, could have a more pronounced neuropathogenic role in PML development.

Materials and methods

Subjects
The study has been performed on urine collected from 211 healthy Italian individuals who underwent to routine testing at the Clinical Chemistry Laboratory of the Don Gnocchi Foundation in Milan, Italy. According to their place of birth and of residence in the first 20 years of life, the subjects were divided in four groups corresponding to four great geographical areas: north, center, and south of Continental Italy, and Sicily and Sardinia (the two main Italian islands). The principal epidemiological characteristics of the studied population are detailed in Table 1.

Urine preparation
The urine samples were treated according to a previously described protocol (Agostini *et al*, 1995; Stoner *et al*, 1996). Briefly, 10 ml of freshly collected urine, stored at 4°C up to 2 weeks, were centrifuged at 4000 × *g* for 10 min. The pellet was washed once in sterile phosphate-buffered saline (PBS) (PBI International, Milan, Italy), and subjected to an overnight digestion at 56°C with a lysis solution (50 mM Tris, 1 mM EDTA, 0.45% NP40, 0.45% Tween 20, pH 8.0) containing 0.1 mg of proteinase K (Promega Italia, Milan, Italy).

Amplification protocols
Each reaction was performed using a negative and a positive control. The negative control contained all the PCR components but the template; as positive control, simian virus 40 (SV40) DNA, obtained by SVG (SV40 transformed human glial cells) cell line culture (Major *et al*, 1985), was used, because it shares a significant nucleotide sequence homology with the human polyomavirus JCV. Five microliters of the urine samples were added to the PCR mixture. Amplifications were performed on an aluminum 96-well Gene Amp PCR 9700 apparatus (Applied Biosystems, Foster City, USA) and PCR products were analyzed by electrophoresis on 2% agarose gel stained with ethidium bromide.

JCV DNA detection
To detect JCV DNA, all the urine samples were first examined using a nested PCR designed to amplify the highly conserved LT-coding region and whose primers and conditions were described in details in a previous study (Ferrante *et al*, 1995).

VP1 amplification
The molecular characterization of the detected JCV was performed by amplification and nucleotide sequencing of a 215-bp fragment of the VP1 gene, using the primers JLP-15 (5′-ACAGTGTGGCCAGAATTCCACTAC-3′, 1710 to 1734) and JLP-16 (5′-TAAAGCCTCCCCCCCAACAGAAA-3′, 1924 to 1902). This region, encoding for the viral structural late protein VP, includes the genotyping sites, which distinguish the eight JCV genotypes and the different subtypes. The amplification was carried out in a total volume of 100 μl, containing 400 μM dNTPs, 2.5 mM $MgCl_2$, 2 U AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, USA), with reaction buffer 10× (PCR Buffer II), and 25 pmol of each primer. After initial denaturation at 95°C for 9 min, the amplification protocol provides an annealing temperature at 63°C and an extension temperature at 72°C, repeated for 40 cycles. The use of a DNA polymerase enzymatically inactive until the first denaturation step on high temperature reduces the formation of unspecific amplification products and represents a particular advantage for sequencing reactions.

TCR amplification
A nested PCR was used to amplify a 353-bp fragment belonging to JCV TCR. The first round of PCR was carried out in a total volume of 50 μl with 2 U of *Taq* polymerase (Roche Diagnostics) and 20 pmol of the primers JRE1 (5′-CCTCCCTATTCAGCACTTTGT-3′, 4989 to 5009) and LP2 (5′-TACGTGACAGCTGGCGAAGAA-3′, 518 to 537) in a 30-cycle protocol with annealing temperature at 59°C. Five microliters of the outer-PCR product were added to the inner PCR reaction mixture contained 20 pmol of each inner primers, RFOR (5′-GCCTCCACGCCCTTACTACT-3′, 5085 to 5104) and RREV (5′-CAGAAGCCTTACGTGACAGC-3′, 310 to 329) (nucleotide numbering is based on prototype Mad-1 from Frisque *et al*, 1984). The parameters of the 25 cycles inner PCR were 95°C (denaturation), 63°C (annealing), and 72°C (elongation).

DNA direct sequencing
Modified 5′ biotin-labeled forward/reverse primers and 5′ Cy-fluorescein-labeled reverse/forward primers (Pharmacia Biotech, Uppsala, Sweden) were used for the amplification and sequencing of both

negative and positive strands of VP1 and TCR sequences.

After immobilization of the biotinylated amplified products, the sequencing reactions were performed, using the AutoLoad Solid Phase Sequencing kit (Pharmacia Biotech), following the manufacturer's protocol. The terminated sequencing products ran on 6% polyacrylamide gel containing 7 M urea (Ready Mix gel; Pharmacia Biotech) onto an ALFexpress DNA sequencer (Pharmacia Biotech).

Sequence homology searches for genotyping and TCR pattern rearrangements analysis were performed using BLAST at NCBI (USA) according to Agostini *et al* (1999) and Vaz *et al* (2000).

Statistical analysis

The comparison of JCV DNA detection in the urine and JCV genotypes distribution among the different groups of subjects was performed using the chi-square test with Yate's correction (EpiInfo 6.0).

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