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Epidemiology Report

Excretion and transmission of JCV in human populations

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The potential transmission of JCV through the environment has been analyzed by studying the JC viruses present in raw sewage of urban populations from widely divergent geographical areas. High numbers of JCV were found. JCV was detected in 98% (51/52) of sewage samples from different geographical areas in Europe, Africa, and USA by applying a Nested-PCR procedure. The mean estimated concentration of JCV in sewage was of 10^2–10^3 viral particles/ml. Sequence analysis shows that JCV found in environmental samples present an archetypal structure in the regulatory region as it has been described in urine samples. Cerebrospinal fluid samples (CSF) of PML (progressive multifocal leucoencephalopathy) patients were also analyzed as control samples in this study presenting tandem repeats and rearrangements at the regulatory region (RR). Sequence analysis of the intergenic region (IGR) allowed the typification and phylogenetic analysis of the JCV sequences detected in sewage. JC viral particles were also found to be stable in sewage samples at 20°C for more than 70 days. This data suggest the idea that the intake of water or food contaminated with JCV could constitute a portal of entry for the virus or the viral DNA to the human organism. *Journal of NeuroVirology* (2001) **7,** 345–349.

Keywords: excretion; JC virus DNA; polluted food; polluted waters; polymerase chain reaction; sequence analysis

Human polyomaviruses (BK and JC) are frequently shed in urine as they persistently infect the kidneys of infected people. JCV has been detected in 20–80% of the adult urines tested, depending on the ethnic group (Agostini *et al*, 1997) and on the age (Kitamura *et al*, 1994). JCV is the causative agent of PML, it has also been related to some human glial tumors (astrocytomas, ependymomas, oligodendrogliomas, glioblastomas) although there is no direct evidence for tumorigenesis of JCV in the human brain. JC viral DNA has also been described in the intestinal epithelia and could be related to human colorectal cancer (Laghi *et al*, 1999; Riccardiello *et al*, 2000). Human infections with JCV appear to be population associated: the genotype of JCV excreted by individuals of defined ethnicity is strongly influenced by the geographical origin of the ethnic group (Agostini *et al*, 1997). If JCV is excreted in the urine, and a significant percentage of population is shedding this virus, it should be present and detectable, using the appropriate methodology, in urban sewage. If the viral particles are stable in the environment, JCV will also be detected in shellfish. Shellfish are organisms that act as biosensors of fecal contamination in seawater as they filtrate large volumes of water and concentrate the viral particles.

In previous studies, we have developed a methodology for detecting different viruses in environmental samples (Puig *et al*, 1994). We have adapted this methodology to study the presence of human polyomaviruses in sewage from different geographical areas in Europe, Africa, and the United States. The analysis of the relative viral output of a community through analysis of sewage samples could be very useful to study the regional prevalence of some viruses and future changes in the epidemiological patterns of some viral infections.

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Results and discussion

The results obtained after the analysis of the sewage samples are shown in Table 1 and reflect a very high level of excretion of JCV. Ninety-eight percent (51/52) of the samples from all the areas studied were positive for JCV at a mean concentration of 10^2–10^3 viral particles/ml. There were no significant differences between the results obtained in each of the areas, considering that the concentration of JCV in the samples collected in the United States was particularly high. JCV was also detected in 5 of 10 shellfish samples tested. Those high levels of viruses are in accordance with the fact that JCV has been detected in 20–80% of the adult urines tested (Kitamura *et al*, 1994; Agostini *et al*, 1997) from different geographical areas. Renal JCV seems not to be latent because it is also excreted by immunocompetent individuals (Shah *et al*, 1997) where JCV replicates to generate progeny that is excreted in urine. So, the persistence of JCV in the kidney may be characterized by continuous viral replication and shedding.

The procedure applied is highly sensitive. The number of polyomavirus-like particles counted in a urine sample using electronic microscopy was 8.7×10^5 per ml, and by nested PCR, the estimated sensitivity was of 5 viral particles/ml or higher.

The evaluation of the stability of JC viral particles in sewage showed that JC viral particles were present in sewage samples kept at 20°C during 92 days. The results of these experiments are shown in Figure 1. We have computed a linear regression model and we estimated JCV values of T_{90} and T_{99} of 26.7 days and 61.5 days, respectively. When we used DNase before the nucleic acid extraction, we found no effect showing that genomes were protected by well-structured capsids. Also, preliminary data show stability of JC viral particles in sewage samples after being exposed to pH 5 (data not shown).

Table 1 Results obtained after analyzing 52 sewage samples for the presence of JCV

Area (number of samples)	*No. positive samples/No. total samples Mean estimated concentration*
Barcelona (25)	23/24 10^1–10^2 PV/ml
Nancy (4)	4/4 10^2–10^3 PV/ml
Umeå (4)	4/4 10^2–10^3 PV/ml
Pretoria (4)	4/4 10^1–10^2 PV/ml
El Cairo (4)	4/4 10^2–10^3 PV/ml
Patras (5)	5/5 10^2–10^3 PV/ml
Washington (6)	6/6 10^3–10^4 PV/ml
Total samples = 52	51/52 (98%) 10^2–10^3 PV/ml

The JC viral genomes detected were also analyzed by sequencing the regulatory (RR) and the intergenic regions (IGR). All sequences obtained from the RR of JCV detected in sewage samples and from the urine sample used as a control presented identical sequence in the 187 nucleotides analyzed, being identical with the archetypal consensus sequence. In contrast, clinical samples analyzed (CSF) presented diverse genetic rearrangements as has been described for PML-related JC viral strains (Yogo *et al*, 1990).

It has been also reported that archetypal JCV do not seem to be highly infective in cell cultures, although some data exist on the infection of cell cultures with archetypal strains (Hara *et al*, 1998). Rearranged strains seem to be those that cause PML in the brain, although archetypal strains are believed to be those that spread throughout the population and cause persistent infections. The analysis of the sequences at the regulatory region gives us information about the frequency and distribution of the JC viral types in different populations.

We analyzed 461 bp of the IGR of 19 samples positive for JCV and 3 CSF samples from PML patients. Sequences analyzed confirmed the specificity of the nested PCR amplification. We observed differences in the IGR of the JCV sequenced. These results showed the absence of cross-contamination in our experiments. All sequenced samples from different countries in Europe, samples from the north of Africa (Egypt), and most United States samples were strongly related to those strains described in data banks as European types (Type 1). Only one of the isolates from the United States presented similarity with Asian strains (Type 2). We sequenced 2 samples from the south of Africa (Pretoria, South Africa): one presented similarity to strains previously isolated from north Africa and belonging to Type 1, whereas the other was similar to sub-Saharan types (Type 3).

The portal of entry of JCV into humans has not been elucidated. Tonsil tissue has been suggested as a possible site of the initial infection (Monaco *et al*, 1998) and also the digestive tract (Riccardiello *et al*, 2000). Our results show that JCV is persistently present in sewage and in contaminated food and the environment. Consequently, the human population is being exposed with high frequency to JCV when ingesting water and food as shellfish or vegetables exposed to fecal pollution.

JCV DNA has recently been found in different sites through the digestive tract (Riccardiello *et al*, 2000). It has been proven in animals using the bacteriophage M13 (Schubbert *et al*, 1997) that ingested noninfectious viral DNA can reach peripheral leukocytes and some organs of the human body. Not only could JC viral particles represent a source of infection, but the intake of JCV DNA through the intestine may also affect human cells.

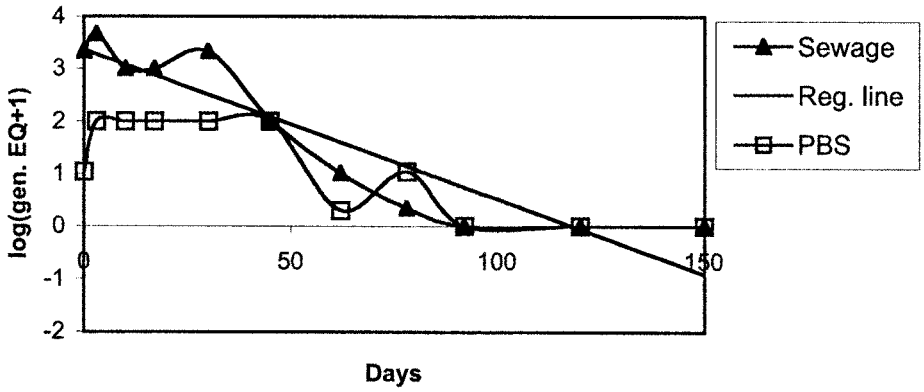

Figure 1 Stability of JCV in sewage. The regression line, the transformed values of the averages of genome equivalents detected by nested-PCR in the 3 samples (sewage), and the genome equivalents of spiked PBS control (PBS) are represented.

Material and methods

Viruses We used CSF (cerebrospinal fluid) samples donated by José Luis Pérez from the Microbiology Department of Hospital de Bellvitge, Barcelona, as positive controls. Other positive controls of JCV used in this study were obtained after concentrating in 500 μL of phosphate-buffered saline (PBS) 48 ml of urine from a healthy 38-week pregnant woman.

Sewage samples Fifty-two raw-sewage samples from different geographical areas were analyzed. Sixteen samples were collected from the sewers of Barcelona (Spain). Each sample was collected in a sterile 500-ml polyethylene container, kept at 4°C for less than 8 hours until the viral particles were concentrated in phosphate-buffered saline (PBS), and stored at −80°C.

Five samples were collected from Patras (Greece), 4 samples were collected from El Cairo (Egypt), 6 samples were collected from Washington DC (USA), 4 were collected from Umeå (Sweden), 4 from Nancy (France), and 4 from Pretoria (South Africa). These samples were collected and shipped, frozen, to Spain, where they were concentrated in PBS and stored at −80°C.

Shellfish samples Four mussel samples (*Mytilus galloprovincialis*) and 6 oyster samples (*Crassostrea gigas*) were obtained from shellfish growing areas with different levels of fecal pollution located in the Delta of Ebro river, Tarragona, Spain. Shellfish sample analyses were carried out following a protocol slightly modified from Pina *et al* (1998) and Muniain-Mujika *et al* (2000).

Concentration of viral particles and nucleic acid extraction Following the procedure described in previous studies, we recovered viral particles from sewage samples and extracted nucleic acids (Girones *et al*, 1995; Pina *et al*, 1998). Briefly, 40 ml of sewage sample were ultracentrifuged (229,600×*g* for 1 h at 4°C) to pellet all the viral particles together with any suspended material. Then, the sediment was eluted by mixing it with 4 ml of 0.25 *N* glycine buffer pH 9.5 on ice for 30 min, and the suspended solids were separated by centrifugation at 12,000×*g* for 15 min after the addition of 5 ml of 2×PBS. The viruses were finally pelleted by ultracentrifugation (229,600×*g* for 1 h at 4°C), resuspended in 0.1 ml of 1×PBS, and stored at −80°C.

The nucleic acid extraction was based on a procedure that uses guanidinium thiocyanate (GuSCN) and adsorption of the nucleic acids to silica particles (Boom *et al*, 1990).

Enzymatic amplification Ten-μl aliquots of the extracted nucleic acids were used in each test, corresponding to 4 ml of sewage sample, 1 g of shellfish digestive system, 1 ml of urine, or 10 μl of CSF. Amplifications were carried out in a 50-μL reaction mixture containing 10 mM Tris-HCl (pH 8.3 at 25°C), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM of each dNTP, 2 units of Ampli Taq® DNA polymerase (Perkin-Elmer Cetus), and the corresponding primers (25 μM of external and internal primers). Thermal cycling of the amplification mixture was performed in a programmable heat block (Gene Amp PCR System 2400, Perkin-Elmer). The first cycle of denaturation was carried out for 4 min at 94°C. The conditions for the 29-cycle amplification were: denaturing at 92°C for 60 s, annealing for 60 s, and extension at 72°C for 75 s. Amplifications were completed with a 4-min, 72°C extension period.

Analysis of the intergenic region (IGR) of JCV We amplified the IGR of JCV from sewage samples. External primers used for the detection of IGR were EP1A (5′-TGAATGTTGGGTTCCTGATCCCACC-3′) and EP2A (5′-ACCCATTCTTGACTTTCCTAGAGAG-3′). Internal primers were P1A (5′-CAAGATATTTTGGGACACTAACAGG-3′) and P2A (5′-CCATGTCCAGAGTCTTCTGCTTCAG-3′). P1A and P2A were modified from the work of Kunitake *et al* (1995). The annealing temperature for first strand PCR and nested PCR were 59°C. The results were analyzed by 3% agarose gel electrophoresis using ethidium bromide as stain. Some of the positive samples were further analyzed

by sequencing using primers P1A, P2A, JCSR (5′TGATTACAGCATTTTTGTCTGCAAC-3′), and JCSL (5′-GGAAGTCCTTCTGTTAATTAAATCAG-3′).

Analysis of the regulatory region (RR) of JCV We analyzed sewage samples for the detection of the RR of JCV to elucidate if the JCV found in environmental samples presented an archetypal regulatory region as it has been described for urinary strains or, on the contrary, it presents rearrangements of this sequence as has been described for those JCV that cause PML. Primers used were described by Monaco *et al* (1998). JR1 (5′-CCCTATTCAGCACTTTGTCC-3′) and JR2 (5′-CAAACCACTGTGTCTCTGTC-3′) were used as external primers and JR3 (5′-GGGAATTTCCCTGGCCTCCT-3) and JR4 (5′-ACTTTCACAGAAGCCTTACG-3′) as internal ones. The annealing temperature for both, first strand and nested PCR, was 54°C. Some sewage samples positive for JCV, a urine sample used as a control and some clinical samples (CSF from PML patients) were sequenced using primers JR3 and JR4.

Sequencing of the nested-PCR products Products obtained after the nested-PCR were purified with the QUIAquick PCR purification kit (QIAGEN, Inc.). Thermal cycling of the amplification mixture was performed in a programmable heat block (Gene Amp PCR System 2400, Perkin-Elmer). Both strands of the purified DNA amplicons were sequenced with the ABI PRISM‘ Dye Terminator Cycle Sequencing Ready Reaction kit with Ampli Taq® DNA polymerase FS (Perkin-Elmer, Applied Biosystems) and appropriate primers following the manufacturer's instructions. The conditions for the 25-cycle amplification were: denaturing at 96°C for 10 s, annealing at 50°C for 5 s, and extension at 60°C for 4 min. The results were checked using the ABI PRISM 377 automated sequencer (Perkin-Elmer, Applied Biosystems). The sequences were compared with the GenBank and the EMBL (European Molecular Biology Library) using the basic BLAST program of the NCBI (The National Center for Biotechnology Information).

Nucleotide sequence accession numbers The sequences reported in this paper have been deposited in the GenBank database under accession number AF120240 to AF120242 for JCV RR sequences AF119345 to AF119356, AF304389, AF303943 to AF303948, and AF304386 to AF304388 for JCV IGR sequences.

Stability of JC viral particles in sewage samples Sewage samples and a PBS control spiked with JC viral particles present in a urine sample were kept in sterile containers at 20°C. Aliquots of these samples were analyzed within a 4-month period for the presence of JC viral particles. We carried out 2 different nucleic acid extractions for each sample. In 1 of them, we added DNase prior to the extraction to destroy free DNA [25 μl of DNase buffer containing Tris-HCl pH 7.5 100 mM, $MgCl_2$ 20 mM and 100 μg/ml of BSA, 25 μl of viral particles, and 1 μl of DNase I (Amersham Pharmacia Biotech, Inc) 10,000 units/ml]. We did the other DNA extraction in the same way but added 1 μl of sterile water instead of DNase.

Quality control of the amplification method Direct and 10-fold dilutions of the nucleic-acid extracts were analyzed routinely on highly polluted samples in order to avoid false negatives due to inhibition of the reactions. Standard precautions were applied in all the manipulations to reduce the probability of sample contamination by amplified DNA molecules. Separate areas were used for treatment of samples, reagents, and manipulation of amplified samples. All the samples were analyzed twice in independent experiments, and a negative control was added every two samples. Sequencing of the amplified DNA in a high percentage of the samples was the final proof that we were not detecting false positives, and the differences observed in the sequence of nucleotides ruled out the possibility of cross-contamination.

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