

Molecular detection, quantification and characterization of human polyomavirus JC from waste water in Rio De Janeiro, Brazil

Tulio Machado Fumian, Flávia Ramos Guimarães, Beatriz Jesus Pereira Vaz, Marcus Tullius Teixeira da Silva, Flávia Fontenelle Muylaert, Silvia Bofill-Mas, Rosina Gironés, José Paulo G. Leite and Marize Pereira Miagostovich

ABSTRACT

Polyomavirus JC (JCPyV) is largely excreted by the human population through the urinary route and has been recognized as a potential viral marker for human waste contamination. This study aims to investigate the dissemination of JCPyV in waste water from a sewage treatment plant (STP) located in Rio de Janeiro, Brazil, and to describe the prevalence of JCPyV subtypes currently present in this population. Raw and treated sewage samples were collected bimonthly during one year, and examined for the presence of JCPyV using nested polymerase chain reaction (nPCR) and quantitative real time PCR (qPCR). JCPyV was detected by nPCR in 96% and 43% of raw and treated sewage samples, respectively. The concentration of JCPyV present in the samples ranged from 1.2×10^3 to 3.2×10^5 and 2.6×10^2 to 6.2×10^3 genome copies per 2 ml of concentrated raw and treated sewage sample, respectively. The strains were characterized and the obtained nucleotide sequences indicated that the detected JCPyV strains clustered with subtypes of East African, West African and European origin. To our knowledge, this is the first study describing the incidence and diversity of JCPyV strains in raw and treated sewage in Brazil.

Key words | Brazil, JCPyV, polyomavirus, quantitative PCR, waste water

Tulio Machado Fumian (corresponding author)
Laboratório de Virologia Comparada e Ambiental,
Pavilhão Hélio & Peggy Pereira,
Avenida Brasil 4.365, Manguinhos,
CEP 21040-360, Rio de Janeiro RJ,
Brazil
Tel.: +55-21-2562-1851
Fax: +55-21-2562-1851
E-mail: tuliofmf@ioc.fiocruz.br

Flávia Ramos Guimarães
José Paulo G. Leite
Marize Pereira Miagostovich
Laboratory of Comparative and
Environmental Virology,
Oswaldo Cruz Institute,
Fiocruz,
Rio de Janeiro,
Brazil

Beatriz Jesus Pereira Vaz
Marcus Tullius Teixeira da Silva
Flávia Fontenelle Muylaert
Institute of Clinical Research Evandro Chagas,
Fiocruz, Rio de Janeiro,
Brazil

Silvia Bofill-Mas
Rosina Gironés
Department of Microbiology, Faculty of Biology,
University of Barcelona,
Barcelona,
Spain

INTRODUCTION

Polyomavirus JC (JCPyV) belongs to the family *Polyomaviridae*, which is classified as a group 1 virus in the Baltimore classification scheme. It is non-enveloped with a genome that encompasses a single 5.1 kb molecule of circular double-stranded DNA (Neu *et al.* 2009). *Polyomavirus* is a single genus designation in this virus family and this genus contains 14 different species that infect at least 8 different mammalian species.

Recently, three new human polyomaviruses (WUPyV, KIPyV and MCPyV) have been described (Allander *et al.* 2007; Gaynor *et al.* 2007; Feng *et al.* 2008). JCPyV were associated initially with progressive multifocal leukoencephalopathy (PML) and the role of these viruses in the development of human cancer has been suggested (Imperiale 2000). Seroepidemiological studies have shown that JCPyV infection is widespread in the human population, with a

doi: 10.2166/wh.2010.090

prevalence rate ranging from 58 to 92% in adults (Shah *et al.* 1997; Knowles 2006; Egli *et al.* 2009).

The transmission of human polyomaviruses is still unknown. It has been reported that JCPyV can replicate in tonsillar B lymphocytes and stromal cells, supporting the notion that the respiratory tract is the primary site of viral infection (Monaco *et al.* 1998). A study performed in urban sewage from different geographical areas detected the high level of JCPyV excretion and supports the idea previously described that urine–oral transmission will probably happen soon *in vivo*, inside the family or from closely related people and less frequently later in life from other polluted sources (Bofill-Mas *et al.* 2000). Zheng *et al.* (2004) supported that the transmission of JCPyV is via a parent-to-child transmission during cohabitation.

Primary infection occurs during childhood and is mostly subclinical. Following infection, the virus persists asymptomatically in renal tissue (Arthur & Shah 1989; Kitamura *et al.* 1990). Reactivation of JCPyV can occur at any time in life, as demonstrated by the detection of viral progeny in the urine of a high percentage (20–80%) of healthy individuals over 30 years of age (Agostini *et al.* 2001; Pavese 2005).

Human JCPyV infections appear to be population-associated in that the genotype of JCPyV excreted by individuals of defined ethnicities is in high proportion determined by the geographical origin of the ethnic group rather than the JCPyV genotypes that are prevalent in their current location (Bofill-Mas *et al.* 2000).

This study determines the prevalence of JCPyV in waste water from a sewage treatment plant (STP) in order to elucidate the circulation of these viruses in the local population. Waste water is the main source of pathogenic microorganisms, and thus provides information about the different strains that are infecting a population. For this purpose a one-year study was carried out in an urban STP in the city of Rio de Janeiro. Urine samples from the local population collected during the same year were analysed to compare the genotypes of JCPyV from these human and environmental samples.

To our knowledge this is the first study demonstrating the circulation of JCPyV in sewage samples in South America.

MATERIALS AND METHODS

Sewage samples

From January to December 2005, a total of 47 sewage composite samples, 24 from raw and 23 from treated sewage, were collected twice a month from an activated sludge STP in the city of Rio de Janeiro, Brazil, as described by Guimaraes *et al.* (2008). One sample from treated sewage was not in sufficient quantity to test for JCPyV. The treatment of the STP studied includes secondary aerobic digestion by activated sludge, and extended aeration with biological nitrification and denitrification, without chlorination.

Clinical samples

JCPyV detected in 12 urine clinical samples that previously tested positive using a nested PCR to amplify the T antigen region of the DNA genome (Weber *et al.* 1994) were genetically characterized. All clinical samples were obtained from HIV-1 and/or HTLV-1 patients from an Infectious Disease Public Hospital located in the same area of the STP. All patients were Brazilian citizens with ages ranging from 29 to 63 years old and four of them presented symptoms of PML disease.

Virus particle concentration method

Viral particles present in waste water samples (effluent and effluent) were concentrated using an adsorption-elution method with negatively charged membranes, which included the insertion of an acid rinse step for removal of cations, as described previously (Katayama *et al.* 2002). Briefly, MgCl₂ was added to every sample to a final concentration of 3 mM, passed through a fibreglass membrane AP20 (Nihon Millipore[®], Tokyo, Japan) to eliminate large-particulate, then through an HA (mixed cellulose esters) negatively charged membrane filter (0.45 mm pore size and 142 mm diameter Nihon Millipore[®]). The filter was rinsed with 350 ml of H₂SO₄ (0.5 mM, pH 3.0) and the viruses were eluted with 15 ml of NaOH (1 mM, pH 10.8). The filtrate was recovered in a tube containing 50 µl of H₂SO₄ (100 mM, pH 1.0) and 50 µl of 100 × Tris–EDTA buffer (pH 8.0) for pH neutralization, followed by

centrifugation using a Centriprep YM-50[®] (Millipore) at 1,500g for 10 min to obtain a final concentrate volume of 2 ml.

Extraction of viral DNA

The viral genome DNA was extracted from pre-treated sewage samples using Vertrel[®] (Sigma-Aldrich Corporation, St. Louis, Missouri, USA) with a QIAmp Viral DNA Mini Kit[®] (Qiagen, Inc., Valencia, California, USA) following the manufacturer's protocol as previously described (Guimaraes *et al.* 2008).

Real-time PCR standard curve and viral titration

A pBR322 plasmid containing the whole genome of JCPyV (Mad-1 strain) was used to construct the standard curve (SC) in the quantitative assay (Albinana-Gimenez *et al.* 2006). To prepare the human JCPyV DNA SC, *E. coli* Top 10 cells were transformed with the plasmid pBR322 containing the full JCPyV genome. The plasmids were purified from the bacteria using the commercial Kit Maxiprep Purification System[®] (Promega Corporation, Madison, Wisconsin, USA) following the manufacturer's instructions and the DNA obtained was quantified with a NanoDrop[®] (Thermo Scientific) by absorbance at 260 nm. The copy number was estimated based on the plasmid and insert size (Yin *et al.* 2001). A JCPyV-specific SC was generated by a 10-fold serial dilution (5×10^6 to 5×10^0 copies per reaction) of purified JCPyV (strain Mad-1) genome DNA. Final values for absolute levels of viral genome are given as genome copies (GC)/reaction.

Real-time PCR to detect human JCPyV DNA was performed as described previously (Pal *et al.* 2006). The protocol was adapted to the ABI 7500 (Applied Biosystems[®], Foster City, California, USA) and performed using 5 μ l of the DNA sample or 5 μ l of the quantified plasmid DNA, 12.5 μ l of TaqMan Universal PCR Master Mix (Applied Biosystems[®]), and 500 and 150 nM concentrations of each primer and probe, respectively, in a final mixture reaction volume of 25 μ l.

For all molecular procedures four separate rooms were used to avoid cross contamination of samples. The samples were analysed in duplicate, and the specific prototype

Mad-1 DNA plasmid previously tested and milli-Q water were used as positive and negative controls, respectively, in all procedures.

Nested polymerase chain reaction (nPCR)

Qualitative nPCR was used for molecular detection and characterization of the JCPyV strain. The nPCR amplification was performed using a set of primers that target an intergenic region fragment spanning nucleotides (nt) 2099 to 2766 of the JCPyV genome and thermo cycling conditions, as previously described by Bofill-Mas *et al.* (2000). Every DNA extraction was diluted 10-fold, and then 5 μ l of this dilution was analysed for the presence of inhibitors in sewage samples.

JCPyV sequence reaction and phylogenetic analysis

The amplicons (668 bp) obtained in the nPCR were purified using the QIAquick[®] PCR Purification Kit (Qiagen, Inc.) following the manufacturer's recommendations and quantified with a NanoDrop[®] (Thermo Scientific) by absorbance at 260 nm. PCR products were sequenced using an ABI Prism[®] 3100 Genetic Analyzer and Big Dye[®] Terminator Cycle Sequencing Kit v. 3.1 (Applied Biosystems) in both directions using individually the same primers as in the amplification reactions. Centri-Sep Columns (Princeton Separations, CA, USA) were used for purification of the sequencing reaction products, according to the manufacturer's recommendations.

The nucleotide (nt) sequences determined in this study were aligned and edited using the BioEdit Sequence Alignment Editor, deposited in the GenBank database and compared with available sequences including reference sequences of each genotype. A phylogenetic tree was constructed using the MEGA program version 3.1 by the neighbour-joining method, with the genetic distance calculated by the Kimura 2-parameter model using bootstraps analysis with 2000 pseudo-replicas (Kumar *et al.* 2004).

Nucleotide sequences obtained in this study from sewage (30/32) and clinical samples (8/12) were submitted to National Center for Biotechnology Information (GenBank, <http://www.ncbi.nlm.nih.gov/>) under the accession numbers FJ524336 to FJ524365 and FJ524366 to FJ524373, respectively.

Table 1 | Detection and quantification of polyomavirus JC (JCPyV) in sewage samples using nested polymerase chain reaction (nPCR) and quantitative PCR (qPCR)

Sewage samples	JCPyV detection		JCPyV quantification	
	nPCR positive/tested (%)	qPCR positive/tested (%)	Viral load	Media
Raw	23/24 (96)	23/24 (96)	$1.2 \times 10^3 - 3.2 \times 10^5$	4.6×10^4
Treated	10/23 (43)	9/23 (39)	$2.6 \times 10^2 - 6.2 \times 10^3$	2.4×10^3
Total	33/47 (70)	32/47 (68)	–	–

RESULTS

Detection and quantification of JCPyV

Table 1 shows the nPCR and qPCR results used to detect and quantify JCPyV from the 47 samples collected in the STP, 23 from affluent and 24 from effluent waste water. Using nPCR, viruses were detected in 96% (23/24) and 43% (10/23) from affluent and effluent waste water samples, respectively. The medium viral load in raw waste water samples was one logarithm higher than in the finished treated water, as expected in an STP. Table 2 shows the reduction of the concentration of JCPyV in the STP demonstrated for each month during the study. The values presented in log (10) represent the difference between the medium concentration of genome copies in raw and treated sewage per month. As observed in Table 2, no JCPyV could be detected during summer months in the treated samples.

The qPCR assay was also applied to the quantification of JCPyV in urine samples to calculate the viral load excretion from patients, and the data are expressed as GC per reaction. Concentration of JCPyV ranged from 1.26×10^1 to 5.19×10^6 GC/reaction. All 12 samples were confirmed as JCPyV positive using both nPCR and the quantitative assay.

Characterization of JCPyV

The amplicons of 668 bp obtained by nPCR (nt 2099 to 2766) were sequenced and confirmed as JCPyV by BLAST comparison with sequences available from GenBank using the BLAST program. JCPyV strains detected from sewage and clinical samples had a nucleotide identity with each other that ranged from 95.3 to 100%.

Phylogenetic analyses were carried out for molecular characterization of JCPyV genotypes. The results of the

Table 2 | Concentration of polyomavirus JC in the waste water treatment plant distributed along a one-year data collection

Month	Raw sewage		Treated sewage		Reduction*
	GC per 2 ml	Std dev.	GC per 2 ml	Std dev.	
January	5.66×10^3	2.42×10^3	ND [†]	–	3.75
February	4.71×10^3	3.63×10^2	ND [†]	–	3.67
March	2.97×10^5	8.79×10^2	ND [†]	–	3.47
April	7.21×10^3	7.66×10^3	ND [†]	–	3.85
May	3.98×10^3	–	5.14×10^2	–	0.89
June	1.71×10^4	1.93×10^4	2.57×10^2	–	1.82
July	1.19×10^5	–	6.00×10^2	–	2.30
August	2.95×10^4	2.48×10^4	4.37×10^3	2.54×10^3	0.83
September	3.83×10^4	3.91×10^4	4.19×10^3	1.22×10^2	0.96
October	1.69×10^4	2.22×10^4	1.45×10^3	1.20×10^2	1.06
November	2.75×10^5	6.99×10^4	ND [†]	–	5.44
December	4.18×10^4	3.24×10^4	ND [†]	–	4.62

*Accumulative elimination efficiency expressed in log (10).

[†]ND: Not detected.

maximum likelihood method applied to the phylogenetic analyses are shown in Figure 1. The strains detected in this study clustered mainly in three different groups. Sequence analyses of sewage and clinical samples from

the intergenic region of the JCPyV genome indicated that JCPyV clustered with subtypes of East Africa, West Africa and European origins.

DISCUSSION

The results produced in this study show a wide distribution of JCPyV infections in the area studied. JCPyV DNA was found in almost 100% of sewage samples, corroborating with previous studies that have reported high concentrations of viruses, mainly DNA viruses (adenovirus and polyomavirus), in residual waters worldwide (Bofill-Mas *et al.* 2000; Albinana-Gimenez *et al.* 2006; Bofill-Mas *et al.* 2006; Katayama *et al.* 2008; Rafique & Jiang 2008).

A correction factor was used to account for viral loss during the water concentration step. Based on this, virus levels found in sewage samples in this study presented a mean concentration of 10^6 and 10^4 GC/l for affluent and effluent samples, respectively. These virus concentrations were similar to concentrations found in samples of urban sewage in other geographical areas and corroborate with levels in the effluent of a waste water treatment plant (Bofill-Mas *et al.* 2006). The results clearly support the interest and applicability of using the specific human JCPyV as a microbial source tracking tool and as a marker of human faecal contamination as it has been suggested in previous studies, and furthermore for the evaluation of the virus removal efficiency in waste water treatment plants (Bofill-Mas *et al.* 2000).

The results also show that the methodologies (nPCR and qPCR) applied are feasible for the analysis of JCPyV as well as robust, allowing not only the quantification (96%) but also the genetic characterization of the identified samples. nPCR showed a slightly higher level of sensitivity than qPCR, and in treated sewage samples where concentrations of viruses are expected to be lower, more positive samples were detected by nPCR (70%) than by qPCR (68%). Previous data published elsewhere using the same samples showed a detection of 92% of hepatitis A virus (HAV) using qPCR, and 32% and 16% for treated and untreated samples, respectively (Villar *et al.* 2007). In another study, the percentage of norovirus detected ranged from 15% to 58%, depending on the methodology of detection used (Victoria *et al.* in press). Although we could not

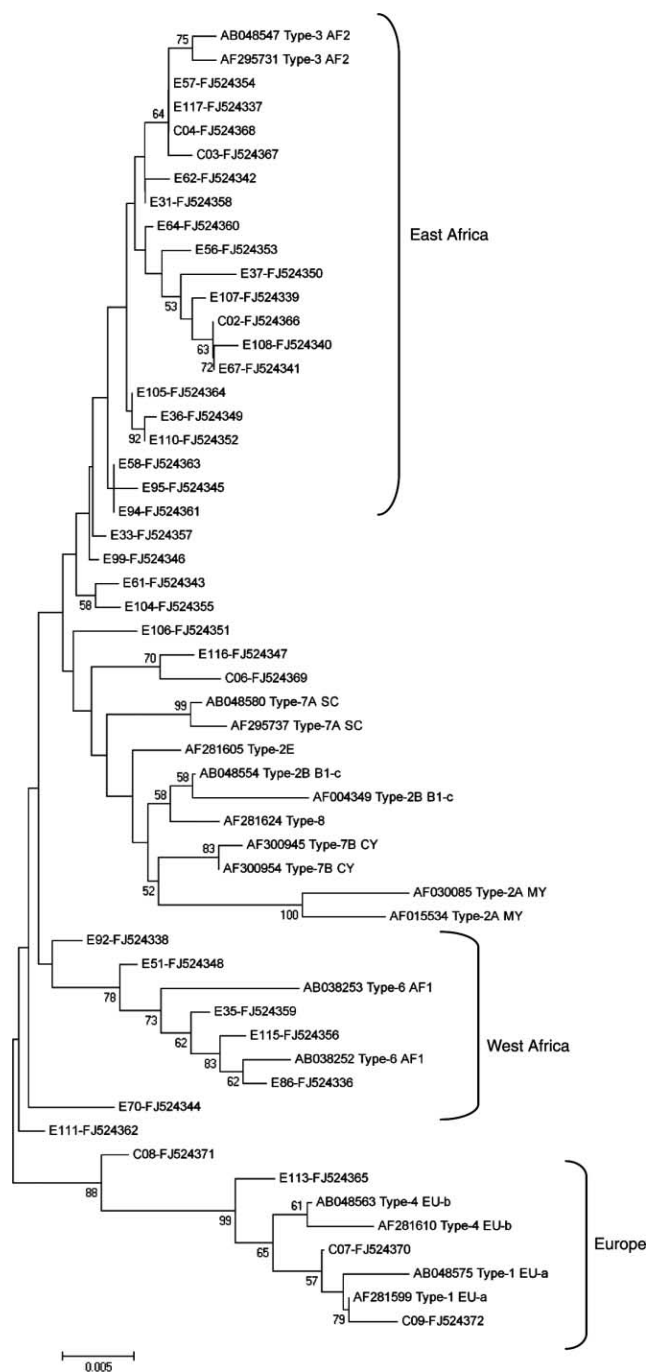


Figure 1 | Phylogenetic analyses of the intergenic regions of 38 sequenced JCPyV strains compared with previously described JCPyV prototypes. E or C before accession number means environmental and clinical samples, respectively.

demonstrate the recovery efficiency of the adsorption elution method for JCPyV, previous studies in this STP showed a recovery efficiency of 4.2% and 4.3% for raw and treated sewage samples for human astrovirus, and 7.8% and 4.6% for norovirus, respectively (Guimaraes *et al.* 2008; Victoria *et al.* in press). This same method, when used to concentrate waste water samples, presented satisfactory results for detection of enteric viruses in STPs in other studies (Haramoto *et al.* 2008; Katayama *et al.* 2008).

Treatments commonly applied in the STPs have significantly reduced the incidence of bacterial agents in finished water, but viral pathogens can persist following the treatment process, resulting in several human viruses being introduced back into the environment through the water system (Thompson *et al.* 2003; Bofill-Mas *et al.* 2006). Conventional waste water treatment plants that use filtration treatments and activated sludge have shown removal efficiencies of about two logarithms for JCPyV (Bofill-Mas *et al.* 2006). In this study the efficiency of the removal of viruses observed, in log (10), is highly variable ranging from 0.83 to 5.44. This variability could be explained by changes occurring in the amount of biological material observed in the STP during the summer time. In this biological treatment system, the rate of production of activated sludge is influenced by temperature: higher temperatures mean higher rates of growth. Thus, to maintain the same concentration of volatile suspended solids (measured by the amount of bacteria that degrade the sewage) there is a need for more disposal of sludge in the same period. Considering that the activated sludge adsorbs and enhances settling of material in suspension, we can explain the lower viral concentrations in the final effluent and the higher removal efficiency observed in those samples obtained from the warm season. Bacterial removal data obtained from these samples was previously published and showed to be 2.5 and 3.2 for total and faecal coliforms after sedimentation and biological secondary (activated sludge) treatment, respectively (Guimaraes *et al.* 2008).

The high prevalence of JCPyV in waste water, coupled with its high stability, qualifies this virus as a good marker for faecal pollution of human origin. The presence of JCPyV in the treated sewage shows that the virus can persist after the water treatment and that the treatment was unable to provide virus-free waste water effluent. The high stability of

JCPyV in the environment, as described by Bofill-Mas *et al.* (2006) who demonstrated a t_{90} of 63.9 days and a t_{99} of 127.3 days for this virus in sewage samples, shows the importance in reducing or eliminating viral agents from treated effluent that will be discharged into other water sources. Information on the concentration of JCPyV in raw and treated waste water has been described as useful, not only in understanding their fate, but also in assessing the risk of infection through water (Maunula *et al.* 2005).

Monitoring the occurrence of JCPyV in sewage from a waste water treatment plant could be an appropriate advance to understanding the circulation of these viruses in the population located in the area enclosed by the STP, particularly because the influent contains viruses shed from patients of asymptomatic cases. It is considered that JCPyV has been evolving with the human population since the emergence of modern humans, 100,000–200,000 years ago. The major population groups in the Americas have shown, in previous studies that analysed urine samples, genotypes characteristic of their known Old World origins: European types 1 and 4, African types 3 and 6, and Asian type 2A (Stoner *et al.* 2000). However, a much wider search in South America was required to identify the distribution of genotypes in this geographical area. The analysis of the amplified sequences in the intergenic region of the JCPyV strains showed the presence of subtypes previously associated with populations of African and European origins, corroborating with the authentic structure of the Brazilian population and supporting the idea that the strains of JCPyV are related to the ethnic origins (Parra *et al.* 2003).

To our knowledge, this is the first study describing the incidence and diversity of JCPyV strains in raw and treated sewage in South America, and more specifically in Brazil. The information provided adds evidence to the applicability of JCPyV as a marker of human faecal pollution in widely divergent geographical areas.

CONCLUSIONS

Both molecular assays used in this study (nPCR and qPCR) support the applicability of either of these techniques to investigate the presence of JCPyV in the sorts of samples analysed in this study; quantification of JCPyV in untreated

sewage samples presented suitable results and corroborate previous findings that point to JCPyV as a possible viral marker to trace human faecal pollution in environmental scenarios; the high rates of viral reduction during the warm months suggest that operations in the STP, such as increasing the frequency of sludge removal, can help in the elimination of virus.

ACKNOWLEDGEMENTS

This work was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq - Papes V - 403530/2008-3) and Fundo Nacional de Saúde/CGVAM. We would like to thank the sewage treatment plant staff for supplying the sewage samples, PDTIS DNA Sequence Platform staff at FIOCRUZ for technical support in sequencing reactions and Dr Márcia Terezinha Baroni de Moraes e Souza for laboratory support. T. M. Fumian is a PhD. student in the Cellular and Molecular Biology Post-Graduation Program - Instituto Oswaldo Cruz (IOC) and has a scholarship from IOC-FIOCRUZ.

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First received 8 June 2009; accepted in revised form 19 November 2009. Available online 9 March 2010