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High diversity of human polyomaviruses in environmental and clinical samples in Argentina: Detection of JC, BK, Merkel-cell, Malawi, and human 6 and 7 polyomaviruses

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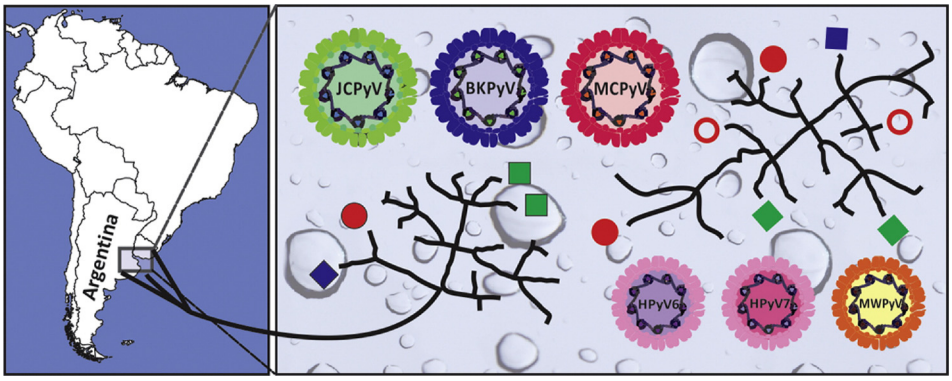
HIGHLIGHTS

- Environmental surveillance was used to study viral contamination of human origin.
- Polyomaviruses could be good, widely present and non-seasonal viral indicators.
- Different genotypes and variants for each polyomavirus were found.

GRAPHICAL ABSTRACT



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ABSTRACT

New human polyomaviruses have been recently described. The aim of this work was to detect and characterize human polyomaviruses circulating in Argentina by recovering viruses from environmental and sewage samples and evaluating their potential role as viral indicators of human waste contamination. Analysis was performed in a wider context including viruses from clinical samples from an immunocompromised population. River water and sewage samples were analyzed as a strategy to study the molecular epidemiology of viruses excreted by millions of people. Samples belonged to the Matanza-Riachuelo River (2005–2006: n = 25 and 2012: n = 20) and sewage from Buenos Aires city and suburbs (2011 and 2013: n = 24). Viral detection was performed by PCR and the amplified viral genomes were characterized by phylogenetic analysis. Polyomaviruses were detected in 95.8% of sewage samples, identifying BKPyV (87.5%), JCPyV (83.3%), MCPyV (8.3%) and HPyV6 (8.3%). Besides, one sample

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collected in 2009 resulted positive for HPyV7. In 2005–2006, polyomaviruses were detected in 84.0% of river water samples, with the highest detection for MCPyV (52.0%), followed by BKPyV (44.0%), JCPyV (20.0%) and MWPyV (4.0%). In 2012, polyomaviruses were detected in 85.0% of river samples, finding JCPyV (85.0%), BKPyV (75.0%), MCPyV (25.0%) and HPyV6 (25.0%). Also, polyomaviruses, including JCPyV, BKPyV and MCPyV, were detected in 63.2% of urine samples from patients infected with HIV (n = 19). Characterization indicated the coexistence of different genotypes and variants for each virus, particularly in sewage. MCPyV sequences (the only sequences from Argentina) formed a monophyletic group with the single sequence available for South America (French Guiana). The high level of detection and viral diversity found by environmental surveillance, which involved the characterization of viruses not previously described in South America, reinforces the usefulness of this approach to monitor viral contamination and describe the viral epidemiology in the general population.

1. Introduction

In the last years, new human polyomaviruses have been discovered, predominantly through the use of next generation sequencing techniques (DeCaprio and Garcea, 2013) and added to the already known JC and BK polyomaviruses (JCPyV, BKPyV). Following the order of reports, these viruses are: KI (KIPyV), WU (WUPyV), Merkel-cell (MCPyV), human polyomavirus 6 (HPyV6), human polyomavirus 7 (HPyV7), trichodysplasia spinulosa-associated polyomavirus (TSPyV), human polyomavirus 9 (HPyV9), polyomavirus Malawi (MWPyV/MXPyV/HPyV10), polyomavirus Saint Louis (STLPyV), human polyomavirus 12 (HPyV12) and New Jersey polyomavirus (Allander et al., 2007; Feng et al., 2008; Gaynor et al., 2007; Korup et al., 2013; Lim et al., 2013; Mishra et al., 2014; Schowalter et al., 2010; Scuda et al., 2011; Siebrasse et al., 2012; van der Meijden et al., 2010).

Although polyomavirus infection is usually asymptomatic, under some circumstances -such as the immunocompromised host resulting from the increase in frequency of tissue transplantation, use of immunomodulators and AIDS incidence- it can be associated with specific pathologies. The most commonly associated clinical diseases are as follows: i—the progressive multifocal leukoencephalopathy (PML) (caused by JCPyV), a frequently fatal disease of the central nervous system in patients with HIV/AIDS or in individuals under immunomodulatory therapies with monoclonal antibodies, such as those with multiple sclerosis; ii—the polyomavirus-associated nephropathy (PVAN) or hemorrhagic cystitis (caused by BKPyV) in patients that have received a solid organ transplant or a bone marrow transplant, respectively; iii—the trichodysplasia spinulosa (caused by TSPyV), a rare skin dysplasia seen in immunocompromised individuals, and iv—the Merkel cell carcinoma (caused by MCPyV), an aggressive skin cancer arising in the elderly and in chronically immunosuppressed individuals (Dalianis and Hirsch, 2013).

Due to their specificity for human host, prevalence in the population, routes of excretion, and chemical and physical resistance, human polyomaviruses (HPyV) have been proposed as viral indicators of human sewage contamination in environmental samples (Albinana-Gimenez et al., 2009; Bofill-Mas et al., 2006, 2000). JCPyV and BKPyV have been the viruses most frequently evaluated as viral indicators of human sewage contamination, but other HPyV have been found in sewage and environmental waters (Bofill-Mas et al., 2010; Cantalupo et al., 2011). However, different polyomaviruses might display a different epidemiology around the world and more indeed, widely distributed viruses might show a different regional distribution of subtypes, as observed for JCPyV and BKPyV, and recently proposed for MCPyV (Agostini et al., 1997; Martel-Jantin et al., 2014; Sugimoto et al., 1997; Zheng et al., 2007).

Epidemiological data related to polyomaviruses in South America and Argentina are scarce and most reports have been limited to the study of closed populations or individual cases (Bonaventura et al., 2005; Comerlato et al., 2015; Fernandez-Cobo et al., 2002; Machado et al., 2011; Reyner and Juan, 2008; Sanjuan et al., 2010; Schiavelli et al., 2014). In addition, little is known about the viral types circulating in the general population or about the recently described viruses.

Thus, the aim of this work was to describe the molecular epidemiology of the different human polyomaviruses circulating in Argentina and to evaluate their potential role as viral indicators of human waste contamination.

2. Material and methods

2.1. Samples

2.1.1. River water samples

The rivers in the metropolitan area of Buenos Aires city, which are closely related to the population of Buenos Aires city and its surroundings, are part of the Río de la Plata basin (Río de La Plata, Riachuelo-Matanza and Luján Rivers). One of these tributaries, Matanza–Riachuelo River, is one of the most contaminated rivers in Argentina. Chemical contaminants, organic material and anoxic conditions are present along the path of the Matanza–Riachuelo River through Buenos Aires city as a result of the activity of several industries, wastewater treatment plants and unregulated discharges of fecal material. This river has an average flow of 7.0 m^3/s with no severe flow fluctuation. This river receives discharges of sewage effluents from wastewater treatment plants, which perform primary, secondary and tertiary treatment.

The samples from the Matanza–Riachuelo River were collected in two periods: 1) October 2005 to February 2006, every two weeks at three points located downstream from the wastewater treatment plant (n = 25), and 2) January to September 2012, every two months in these three points and in an additional one (n = 20) (Supplementary file 'Sampling points.kmz'). The sampling points were selected to represent areas where dense populations live along the banks of the river. Virus concentration was performed from 2-l water samples by the adsorption–elution method on negatively charged membranes, as described previously with minor modifications (Blanco Fernandez et al., 2012). Briefly, the pH of samples was adjusted to pH 3.5 and a final concentration of $MgCl_2$ 0.1 N was achieved by addition of $MgCl_2$ 5 N. The samples were then filtered through glass fiber prefilters (MSI, G15WP14225) and a negatively charged membrane (Millipore, HAWP14250). After filtration, prefilters were removed, and the membrane was rinsed with 25 ml of 0.14 N NaCl. The viral concentrate was obtained by elution with 7.5 ml of buffer glycine–NaOH 0.1 N (pH 11.5). Viral recovery by this method, evaluated in different river water samples by using bacteriophage PP7 as viral control, according to the procedure previously described (Poma et al., 2013), ranged between 28.1 and 55.0%.

2.1.2. Sewage samples

Raw sewage samples from the income channel of the two wastewater treatment plants and a sewage lift station were taken every two months from March to December 2011 and from April to November

Table 1
Primers for broad nested-PCRs used for detection of human polyomaviruses.

Reaction		Primers	Number of mismatches[a]									
			JCPyV	BKPyV	TSPyV	MCPyV	MWPyV	HPyV9	KIPyV	WUPyV	HPyV6	HPyV7
PCR A	1st round	Forward										
		S-AP1: CCTSAMTGGATGTTGCCT (from 1399 to 1416)[b]	0	0	0	6	5	5	–	–	–	–
		S-AP2: CCAGATTGGATKCTWCCTC (from 1399 to 1417)[b]	6	6	4	1	4	1	–	–	–	–
		S-AP3: CCTGATTGGATGCTACAAT (from 1399 to 1417)[b]	6	6	6	5	0	4	–	–	–	–
		Reverse										
		AS-AP1a: ATGTGGGAGGCTGTNACY (from 1789 to 1772)[b]	0	0	2	3	4	2	–	–	–	–
		AS-AP1b: AGASACAGCYTCCCACAT (from 1789 to 1772)[b]	2/3	1/2	0	3	5	1	–	–	–	–
		AS-AP2: TGCCTCCCACATMTRCAA (from 1783 to 1766)[b]	4	3/4	6	0	0	5	–	–	–	–
		Size of expected amplicon (bp)[c]	391	415	440	446	505	443	–	–	–	–
	2nd round	Forward										
		S-AS1a: AARAGGAGGAGTAGAAGT (from 1531 to 1548)[b]	0	0	8	2	6	2	–	–	–	–
		S-AS1b: TAAAGGAGGGGTRGAAGT (from 1531 to 1548)[b]	3	2	8	1	4	1	–	–	–	–
		S-AS2: TAAGGGTGGGATAGATGT (from 1531 to 1548)[b]	7	6	6	6	0	5	–	–	–	–
		S-AS3: GAAGGGCAATATAGAGGT (from 1532 to 1549)[b]	9	8	0	9	6	8	–	–	–	–
		Reverse										
		AS-AS1: ACAGGTHAGRTCCTCATT (from 1759 to 1742)[b]	0	0	0	1	2	2	–	–	–	–
		AS-AS2: ACAGGTCATRTCTTCATT (from 1759 to 1742)[b]	3	3	3	2	0	0	–	–	–	–
		Size of expected amplicon (bp)[c]	229	229	235	244	220	235	–	–	–	–
PCR A1	1st round	Forward										
		JC1: AATGTGCAATCTGGTGAATT (from 1333 to 1352)[b]	0	5/6	–	–	–	–	–	–	–	–
		BK1: TACATTCAGGAGAGTTTATAGA (from 1432 to 1453)[d]	5/6	0	–	–	–	–	–	–	–	–
		Reverse										
		AS-AP1a										
		Size of expected amplicon (bp)[c]	457	477	–	–	–	–	–	–	–	–
	2nd round	Forward										
		JC3:CCTTTACTTTTAGGGTTGTAC (from 1414 to 1434)[b]	0	3/4	–	–	–	–	–	–	–	–
		BK3:CCTTTRCTTCTAGGCCTG (from 1559 to 1526)[d]	3	0	–	–	–	–	–	–	–	–
		Reverse										
		AS-AS1										
		Size of expected amplicon (bp)[c]	346	370	–	–	–	–	–	–	–	–
PCR A2	1st round	Forward										
		S-AP2, S-AP3										
		Reverse										
		AS-AP1b, AS-AP2										
		Size of expected amplicon (bp)[c]	–	–	–	446	505	443	–	–	–	–
	2nd round	Forward										
		S-AS1b, S-AS2										
		Reverse										
		AS-AS1, AS-AS2										
		Size of expected amplicon (bp)[c]	–	–	–	244	220	235	–	–	–	–
PCR B	1st round	Forward										
		S-BP1: CCTGACTGGATTTTRTAT (from 1416 to 1433)[e]	–	–	–	–	–	–	0	0	7	6
		S-BP2: CCTCAATGGMTGCTTTTT (from 1235 to 1252)[f]	–	–	–	–	–	–	6	6	0	0
		Reverse										
		AS-BP1: TTCTRTACAGCTCCCATA (from 1888 to 1871)[e]	–	–	–	–	–	–	0	0	7	6
		AS-BP2: TCTAWAGGCYTCCCAAAC(from 1595 to 1578)[f]	–	–	–	–	–	–	5	5	1	1
		Size of expected amplicon (bp)[c]	–	–	–	–	–	–	473	452	361	370
	2nd round	Forward										
		S-BS1: TMARAAAAGGAGGGGTAG (from 1583 to 1600)[e]	–	–	–	–	–	–	0	0	2	2
		S-BS2: ARCTTCCCAGAGTRATAA (from 1324 to 1341)[f]	–	–	–	–	–	–	8	8	0	0
		Reverse										
		AS-BS1: TGATTWGGWATATCAGGG (from 1847 to 1830)[e]	–	–	–	–	–	–	0	0	6	5
		AS-BS2: GCMTCAGGAATTTCAGGC(from 1555 to 1538)[f]	–	–	–	–	–	–	7	5	0	0
		Size of expected amplicon (bp)[c]	–	–	–	–	–	–	265	232	232	232

[a] According to the GenBank reference sequences (for JCPyV and BKPyV, different genotypes were considered).
[b] Positions numbered according to the JCPyV GenBank reference sequence NC_001699.
[c] Estimated according to the GenBank reference sequence of each polyomavirus.
[d] Positions numbered according to the BKPyV GenBank reference sequence NC_001538.
[e] Positions numbered according to the KIPyV GenBank reference sequence NC_009238.
[f] Positions numbered according to the HPyV6 GenBank Reference sequence NC_014406.

2013 (n = 24). These facilities receive the domiciliary effluents from a population of 9,300,000 inhabitants of Buenos Aires city and surrounding areas. Additionally, one sewage sample collected in 2009 was also analyzed. Samples were concentrated by ultracentrifugation as previously described (Pina et al., 1998). Briefly, samples (45 ml) were ultracentrifuged (110,000 ×*g* for 1 h at 4 °C) and sediment was resuspended in 4 ml of 0.25 N glycine buffer, pH 9.5, on ice for 30 min. Suspended solids were removed by centrifugation (12,000 ×*g* for 15 min) and the viruses in the supernatant were pelleted by ultracentrifugation (110,000 ×*g* for 1 h at 4 °C) and resuspended in 2 ml of PBS. Viral recovery by this method, evaluated using bacteriophage PP7 as viral control, ranged between 12.2 and 61.6%.

2.1.3. Patient samples

Urine samples were collected from epidemiologically unrelated subjects (n = 19), diagnosed as infected with HIV, attending the "Hospital General de Agudos Dr. Juan A. Fernández", Buenos Aires, Argentina, from 2012 to 2013. Urine samples (20 ml) were concentrated by ultracentrifugation (100,000 *g* for 1 h at 4 °C) and the virus-containing pellet was suspended in 200 μl of PBS (Bofill-Mas et al., 2000). The study was

Table 2
Results of molecular analysis of river water, sewage and urine samples.

Sample	Period	Positive/analyzed samples (%)	Polyomaviruses detected (n samples)	Polyomaviruses sequenced in VP2/VP1 partial region (n samples)	Polyomaviruses sequenced in VP1 (n samples)	Polyomaviruses characterized: viral type (n samples)
River water	2005–2006	21/25 (84.0)	JCPyV (5) BKPyV (11) MCPyV (13) MWPyV (1)	JCPyV (5) BKPyV (11) MCPyV (12) MWPyV (1)	MCPyV (1)	MCPyV: European/North American genotype (n = 5), South American genotype (n = 6), mixed (European/North American and South American genotypes) (n = 2)
River water	2012	17/20 (85.0)	JCPyV (17) BKPyV (15) MCPyV (5) HPyV6 (5)	MCPyV (5) HPyV6 (5)	JCPyV (10) BKPyV (5)	JCPyV: subtype 2A (n = 9), 3A (n = 1). BKPyV: subgroup Ib1 (n = 4), subtype Ia (n = 1). MCPyV: European/North American genotype (n = 1), South American genotype (n = 4)
Sewage	2011 and 2013	23/24 (95.8)	JCPyV (20) BKPyV (21) MCPyV (2) HPyV6 (2)	MCPyV (1) HPyV6 (5)	JCPyV (6; 10 clones) BKPyV (5; 10 clones) MCPyV (1)	JCPyV: subtype 2A (n = 4), subtype 1B (n = 2 and 10 clones from one of these samples) BKPyV: subgroup Ia (n = 1), Ib1 (n = 2), Ib2 (n = 1), II/III (n = 1), and subtypes Ib1 (n = 2) and Ib2 (n = 8) in 10 clones from the sample characterized as Ib2. MCPyV: South American genotype (n = 2).
Urine	2005–2006	12/19 (63.2)	JCPyV (5) BKPyV (7) MCPyV (2)	MCPyV (1)	JCPyV (4) BKPyV (4) MCPyV (1)	JCPyV: subtype 2A (n = 3), 3B (n = 1). BKPyV: subgroup Ib1 (n = 1), Ib2 (n = 1), IVc2 (n = 2) MCPyV: European/North American genotype (n = 2)

carried out according to the World Medical Association Declaration of Helsinki and approved by the Ethical Committee of the "Facultad de Farmacia y Bioquímica de la Universidad de Buenos Aires", Argentina.

2.2. Extraction of nucleic acids from sample concentrates

Nucleic acids from environmental and sewage samples were extracted with the QIAamp® Viral RNA Mini Kit (Qiagen, USA) according to the manufacturer's instructions. The nucleic acids from urine concentrates were extracted by the phenol/chloroform method (Bergallo et al., 2006).

2.3. Polyomavirus detection

Nested broad-spectrum PCRs for the detection of different human polyomaviruses were designed and directed to the overlapping genetic region that encodes for VP2 and VP1 proteins (hereinafter denominated as "VP2/VP1 partial region"). Four different PCRs were performed using 2.5 μl of DNA, 0.625 U of GoTaq DNA Polymerase (Promega, USA), 1X GoTaq Green Reaction Buffer, primers (Table 1) at a final concentration between 0.2 and 0.4 μM each, 3.0 mM Mg^{+2}, 0.2 mM dNTPs and nuclease free H_2O in a final volume of 25 μl. For the first round of the PCR A, A1 and A2 described in Table 1, the cycling conditions were: 2 min at 94 °C, 10 cycles of 30 s at 94 °C, 30 s at 45 °C (with a decrease of 0.5 °C per cycle), 40 s at 72 °C, 26 cycles of 30 s at 94 °C, 30 s at 40 °C, 40 s at 72 °C, and a final extension of 5 min at 72 °C. For the second round, the conditions were as follows: 2 min at 94 °C, 8 cycles of 30 s at 94 °C, 30 s at 42 °C (with a decrease of 0.5 °C per cycle), 40 s at 72 °C, 28 cycles of 30 s at 94 °C, 30 s at 38 °C, 40 s at 72 °C, and a final extension of 5 min at 72 °C. The last conditions were used for both rounds of PCR B. The approximate limits of detection of these PCRs are 25–50 genome copies (gc) (PCR A), 125–250 gc (PCR A1), 25–50 gc (PCR A2) and 10–25 gc (PCR B). The different PCRs were implemented following an algorithm that included two steps: first, two generic nested PCRs were performed for the detection of JCPyV, BKPyV, MCPyV, TSPyV, HPyV9 and MWPyV (PCR A) and for KIPyV, WUPyV, HPyV6 and HPyV7 (PCR B). When PCR A was positive, two nested PCRs were performed for the detection of JCPyV, BKPyV and TSPyV (PCR A1), and MCPyV, HPyV9 and MWPyV (PCR A2). When PCR A was negative, it was repeated with a 1/5 dilution of the extracted DNA to reduce the effect of PCR inhibitors in the samples.

The polyomaviruses detected were identified as follows: i. by amplicon size for PCR A1, which allows identifying JCPyV and BKPyV, and ii. by sequencing of the PCR A2 or PCR B amplicons or, from the PCR A amplicons if the second step PCRs were negative. Viruses amplified by PCR A or PCR A1 from the period 2005–2006 were also sequenced to confirm the identification by amplicon size.

2.4. Polyomavirus characterization

VP1 gene sequences were obtained from the viruses classified as JCPyV (n = 19), BKPyV (n = 14) and MCPyV (n = 3) that we were able to amplify by using virus-specific nested PCRs (Table A.1). To confirm the viral diversity expected in complex samples such as sewage, genomic fragments of JCPyV and BKPyV were cloned from PCR products of two sewage samples. The purified PCR products were cloned into a pGEM-T Easy Vector System II (Promega, USA). Ten clones from each sample were sequenced.

Phylogenetic analyses were performed on sequences from Argentina and reference sequences obtained from GenBank, belonging to different genotypes and subgroups/subtypes. Sequences were aligned with ClustalX v2.1 (Larkin et al., 2007) and edited with BioEdit v7.0 (Hall, 1999). Phylogenetic trees were built using Bayesian inference with MrBayes v3.2 (Ronquist et al., 2012). For each dataset, analyses were performed using an appropriate substitution model according to the Akaike Information Criterion, estimated with the jModelTest v2.1 software (Darriba et al., 2012). Analyses were run for 1 million generations and sampled every 1000 generations, in the CIPRES Science Gateway server (Miller et al., 2010). Convergence was assessed by effective sample size values higher than 200 using the Tracer v1.6 software (Rambaut et al., 2014), and 10% of the sampling was discarded as burn-in.

In addition, sequences of the VP2/VP1 partial region obtained for the identification of MWPyV, HPyV6 and HPyV7 were also analyzed along with all available sequences from GenBank, performed as described above.

2.5. GenBank accession numbers

Nucleotide sequences larger than 200 bp reported in this work were deposited in GenBank under the accession numbers KT354779-KT354848. Sequences shorter than 200 bp were included in Appendix B.

3. Results

3.1. Polyomavirus detection

A nested broad-spectrum PCR was used for human polyomavirus detection in environmental, sewage and urine samples. This strategy included an algorithm of sequential PCRs and a simple differentiation of JCPyV and BKPyV according to the amplicon size or further characterization by sequencing.

Polyomavirus detection ranged from 84.0 to 91.7% for river water and sewage samples without any variation in the percentage of positive samples along the year (Table 2). In more detail, in river samples from the period 2005–2006 polyomavirus detection was 84.0% (21/25), with JCPyV (5/25; 20.0%), BKPyV (11/25; 44.0%) and MCPyV (13/25; 52.0%) as the main viruses, and MWPyV (1/25; 4.0%) as a minor component. The simultaneous detection of different polyomaviruses was found in 6 out of 25 samples (24.0%).

In river samples from 2012, viral detection reached 85.0% (17/20). These viruses were classified as JCPyV (17/20; 85.0%), BKPyV (15/20; 75.0%), MCPyV (5/20; 25.0%) and HPyV6 (5/20; 25.0%). More than one polyomavirus was found in 75.0% (15/20) of samples.

On the other hand, the broad-spectrum nested PCR was positive in 95.8% (23/24) of the sewage samples collected in 2011 and 2013. Viruses were classified as JCPyV (20/24; 83.3%), BKPyV (21/24; 87.5%), MCPyV (2/24; 8.3%) and HPyV6 (2/24; 8.3%). Besides, the additional sample collected in 2009 resulted positive for HPyV7.

Finally, polyomaviruses were detected in 63.2% (12/19) of urine samples from patients infected with HIV. These were classified as JCPyV (5/19), BKPyV (7/19) and MCPyV (2/19). Among the positive samples, the following coinfections were also found: JCPyV/BKPyV (1/12), JCPyV/MCPyV (1/12) and BKPyV/MCPyV (3/12).

3.2. Polyomavirus characterization

Viruses identified as JCPyV, BKPyV and MCPyV that were amplified on the VP1 gene region or, alternatively, on the VP2/VP1 partial region (for MCPyV), were sequenced and characterized (Table 2). Samples from sewage showed higher genetic diversity for JCPyV and BKPyV than samples from river water. This higher diversity was evidenced by the identification of several genotypes but also by the observation of mixed nucleotides in direct sequencing (arising from the concomitant presence of different genotypes, subgenotypes or even distinct genetic variants within a subgenotype).

Phylogenetic analysis of VP1 JCPyV sequences from sewage indicated that four out of six samples analyzed belonged to subtype 2A and that the other two samples resulted as subtype 1B (Fig. 1). Molecular cloning of one of these subtype 1B samples (which presented mixed nucleotides in direct sequencing) showed that all 10 clones grouped with subtype 1B and that eight of these clones formed a monophyletic cluster (Fig. 1). In river waters, most of the samples were characterized as subtype 2A (9/10), whereas the remaining sample showed the presence of subtype 3A (1/10). In addition, samples from patients showed JCPyV subtype 2A (3/4) and 3B (1/4) (Fig. 1 and Table 2).

Phylogenetic analysis of VP1 BKPyV sequences from sewage showed the presence of subgroups Ia (1/5), Ib (Ib1 (2/5), Ib2 (1/5)) and II (1/5) (Fig. 2). Molecular cloning of the sample classified as Ib2 (according to direct sequencing) showed the presence of subtype Ib2 (8/10 clones) but also of subtype Ib1 (2/10 clones). Besides, three clones identified as Ib2 formed a highly supported monophyletic cluster with a sample from a patient. In addition, the viral genomes from river water samples were characterized as Ia (1/5) and Ib1 (4/5), while samples from patients showed BKPyV subtype Ib1 (1/4), Ib2 (1/4) and IVc2 (2/4) (Fig. 2 and Table 2).

Regarding MCPyV, the analysis of the VP2/VP1 partial region and the VP1 sequences from Argentina indicated the presence of at least two viral types in Argentinean samples (Table 2). While sequences of one of these viral types were similar to European/North American sequences (8 out of 22 sequences), other sequences (12 out of 22 sequences) showed three nucleotides only present in the South American VP1 sequences (which were considered as molecular markers of the group). Besides, direct sequencing of two samples showed mixed nucleotides that would correspond to the European/North American and South American groups, suggesting the concomitant presence of both viral types.

Notably, two VP1 sequences from Argentina were phylogenetically close and grouped with the single VP1 sequence from South America available so far, isolated from French Guiana (Fig. 3), whereas one VP1 sequence from a patient grouped with European sequences.

On the other hand, sequences obtained from the VP2/VP1 partial region for identification of MWPyV, HPyV6 and HPyV7 were also analyzed. Particularly, the sequence of MWPyV from Argentina grouped with a sequence from China and would be the most ancient MWPyV virus sequenced so far (given that is was collected in 2005) and the only sequence from South America reported to date (Fig. 4 (a)). Meanwhile, HPyV6 sequences from Argentina were similar to the only HPyV6 sequences available so far, from the USA, Australia and China, and no clustering was observed (Fig. 4 (b)). In addition, despite the small size of the region analyzed, the HPyV7 sequence from Argentina clustered with viral genomes from the USA (Fig. 4 (b)).

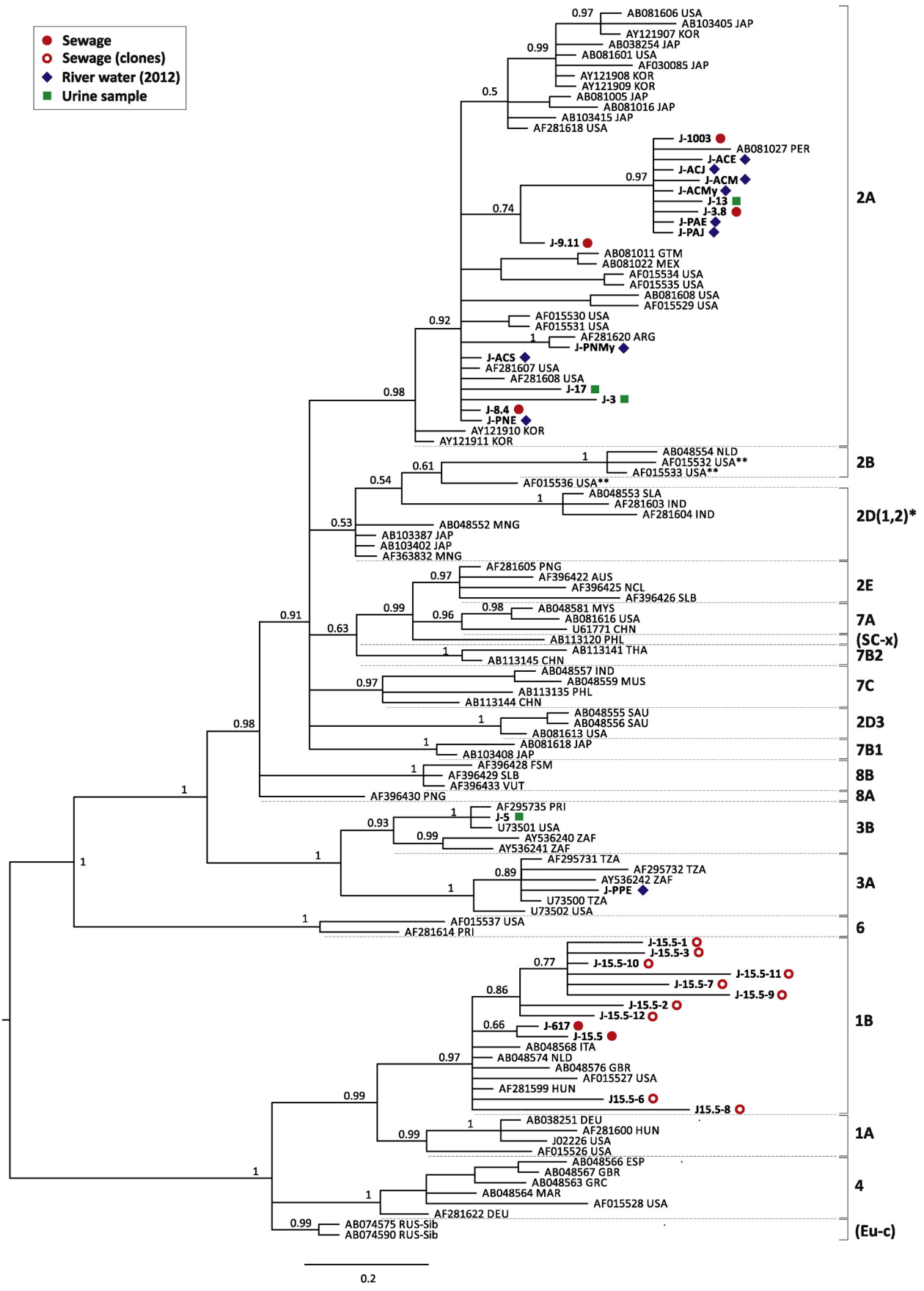
4. Discussion

In this study we presented the design of a nested broad-spectrum PCR that allowed the simultaneous detection of different polyomaviruses from environmental, sewage and clinical samples. This strategy allowed us to perform an environmental surveillance of these viruses and to evaluate the usefulness of polyomaviruses as viral indicators of human waste contamination. Further characterization of the samples allowed describing the molecular epidemiology of the human polyomaviruses circulating in Buenos Aires city, Argentina.

As compared to polyomavirus detection in other locations, polyomavirus detection in river waters from Argentina (~85%) agreed with the highest values within the range of levels found in other geographic areas (Germany, Japan, Spain and the USA), which varies from 11 to 100% of positive samples, mainly focused on the detection of JCPyV and BKPyV (Albinana-Gimenez et al., 2009; Hamza et al., 2009; Haramoto et al., 2010; Jurzik et al., 2010; Staley et al., 2012), and less frequently on that of MCPyV (Bofill-Mas et al., 2010).

On the other hand, the detection in sewage samples was high (~96%) and similar to most of the reports from different areas of Europe, Africa, the USA and Brazil, where detection ranged from 88 to 100% of samples for JCPyV, BKPyV and MCPyV (Bofill-Mas and Girones, 2001; Bofill-Mas et al., 2010, 2006, 2000; Fumian et al., 2010; McQuaig et al., 2009; Staley et al., 2012). However, a lower detection in sewage (50–75%) from some regions of Greece, Spain, Italy and Sweden has also been reported (Bofill-Mas et al., 2000; Di Bonito et al., 2014; Kokkinos et al., 2011). These differences could be attributed to the dissimilar sensitivity of methods, the viral targets or differences in their prevalence in populations.

Fig. 1. Bayesian phylogenetic tree of VP1 sequences of JCPyV (1080 nt) (mid-point rooted). Posterior probability values higher than 0.5 are shown at nodes for relevant groups. Subtypes are indicated in squared brackets. Sequences from Argentina are shown in bold. Country of origin is indicated when available, abbreviated in uppercase. AUS: Australia, CHN: China, DEU: Germany, ESP: Spain, FSM: Micronesia: Chuuk, GBR: Great Britain, GRC: Greece, GTM: Guatemala, IND: India, ITA: Italy, JAP: Japan, KOR: South Korea, MAR: Morocco, MEX: Mexico, MNG: Mongolia, MUS: Mauritius, MYS: Malaysia, NCL: New Caledonia, NLD: The Netherlands, PER: Peru, PHL: the Philippines, PNG: Papua New Guinea, PRI: Puerto Rico, RUS-Sib: Russia (Siberia), SAU: Saudi Arabia, SLA: Sri Lanka, SLB: Salomon Islands, THA: Thailand, TZA: Tanzania, USA: the United States, VUT: Vanuatu, ZAF: South Africa.*non-monophyletic groups. **sequences originally classified as subtype 2C.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Sewage (clones)
River water (2012)
Urine sample
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AY121909 KOR
AB081005 JAP
AB081016 JAP
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J-1003
AB081027 PER
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J-ACJ
J-ACM
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J-13
J-3.8
J-PAE
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J-17
J-3
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AF396433 VUT
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AF295735 PRI
J-5
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AY536241 ZAF
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AY536242 ZAF
J-PPE
U73500 TZA
U73502 USA
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AF281614 PRI
J-15.5-1
J-15.5-3
J-15.5-10
J-15.5-11
J-15.5-7
J-15.5-9
J-15.5-2
J-15.5-12
J-617
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J15.5-6
J15.5-8
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AF281600 HUN
J02226 USA
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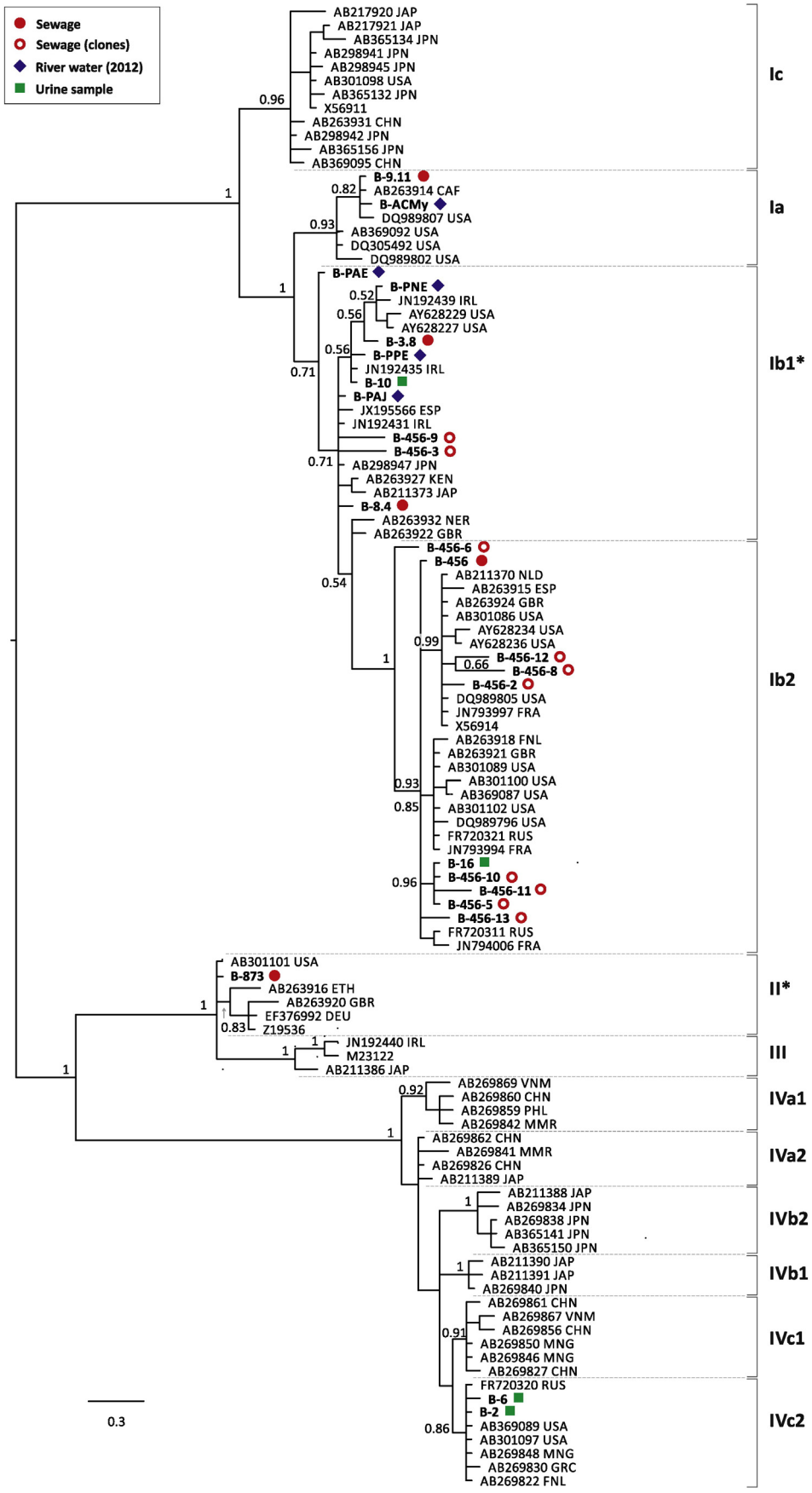
AB048566 ESP
AB048567 GBR
AB048563 GRC
AB048564 MAR
AF015528 USA
AF281622 DEU
AB074575 RUS-Sib
AB074590 RUS-Sib
2A
2B
2D(1,2)*
2E
7A
(SC-x)
7B2
7C
2D3
7B1
8B
8A
3B
3A
6
1B
1A
4
(Eu-c)
0.2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Sewage (clones)
River water (2012)
Urine sample
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Ic
B-9.11
AB263914 CAF
B-ACMy
DQ989807 USA
AB369092 USA
DQ305492 USA
DQ989802 USA
Ia
B-PAE
B-PNE
JN192439 IRL
AY628229 USA
AY628227 USA
B-3.8
B-PPE
JN192435 IRL
B-10
B-PAJ
JX195566 ESP
JN192431 IRL
B-456-9
B-456-3
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B-8.4
AB263932 NER
AB263922 GBR
Ib1*
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AB301086 USA
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B-456-2
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AB301102 USA
DQ989796 USA
FR720321 RUS
JN793994 FRA
B-16
B-456-10
B-456-11
B-456-5
B-456-13
FR720311 RUS
JN794006 FRA
Ib2
AB301101 USA
B-873
AB263916 ETH
AB263920 GBR
EF376992 DEU
Z19536
II*
JN192440 IRL
M23122
AB211386 JAP
III
AB269869 VNM
AB269860 CHN
AB269859 PHL
AB269842 MMR
IVa1
AB269862 CHN
AB269841 MMR
AB269826 CHN
AB211389 JAP
IVa2
AB211388 JAP
AB269834 JPN
AB269838 JPN
AB365141 JPN
AB365150 JPN
IVb2
AB211390 JAP
AB211391 JAP
AB269840 JPN
IVb1
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AB269850 MNG
AB269846 MNG
AB269827 CHN
IVc1
FR720320 RUS
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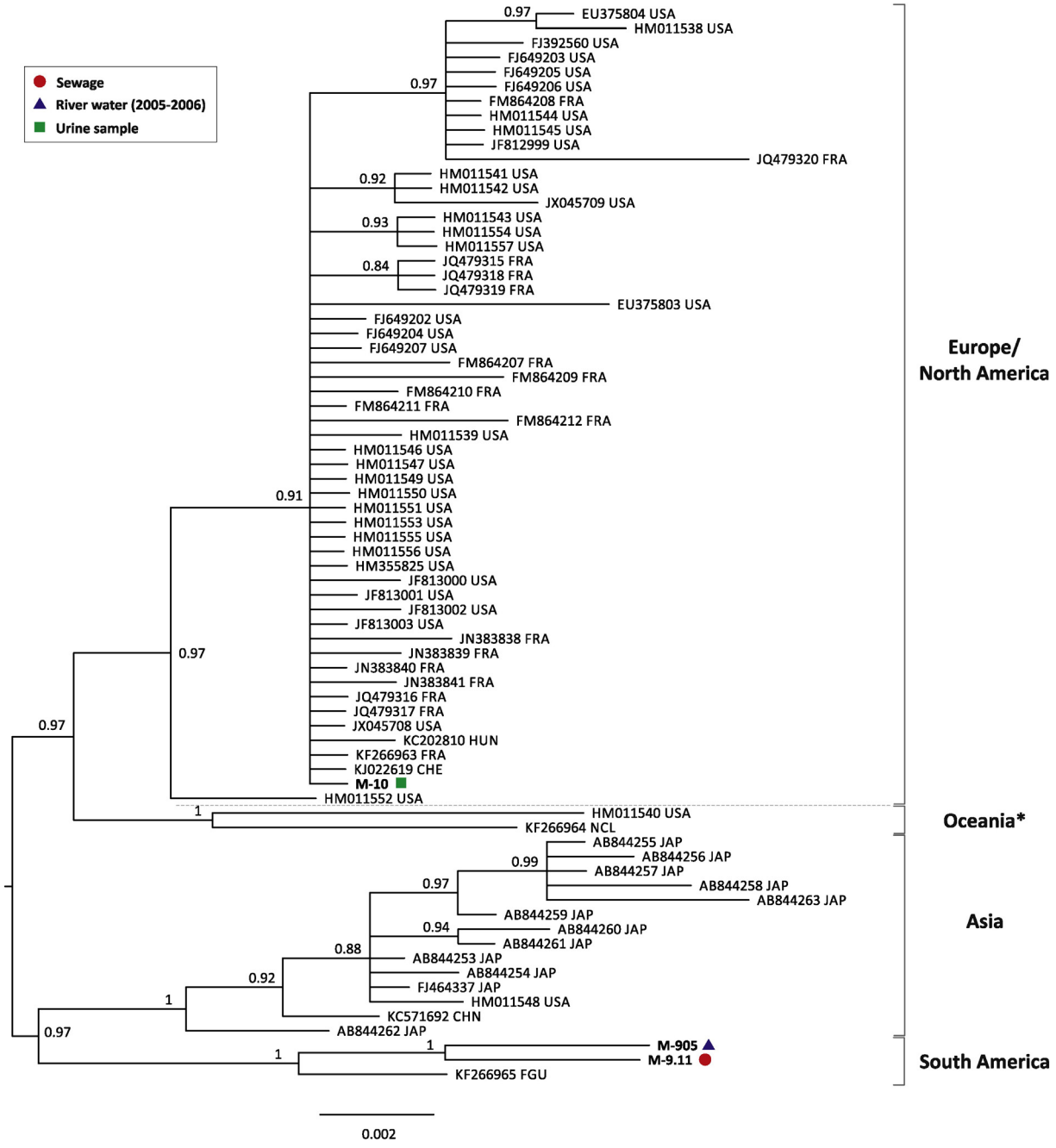

Fig. 3. Bayesian phylogenetic tree of VP1 sequences of MCPyV (1087 nt) (mid-point rooted). Posterior probability values higher than 0.5 are shown at nodes for relevant groups. Genotypes proposed by Martel-Jantin et al. (2014) are indicated in squared brackets. Sequences from Argentina are shown in bold. Country of origin is indicated when available, abbreviated in uppercase. CHN: China, FGU: French Guiana, FRA: France, HUN: Hungary, NCL: New Caledonia, JPN: Japan, CHE: Switzerland, USA: the United States. *this group presents a sequence from the USA.

In this sense, it would be expected that strategies that use the concomitant detection of different viral targets might increase the chance of finding contaminant viruses in samples that collect viral excretion from millions of people. In fact, this strategy allowed us to detect the most frequent viral targets (JCPyV, BKPyV and less often, MCPyV) but also viruses such as MWPyV and HPyV7, which had not been described in sewage or river water samples so far, and HPyV6, which has been previously detected only once through pyrosequencing in sewage from Spain (Cantalupo et al., 2011).

Shedding of some polyomaviruses such as JCPyV, BKPyV, KIPyV, WUPyV and MCPyV by the urinary or fecal route, as well as their presence in sewage, have been described (Babakir-Mina et al., 2010; Bofill-Mas et al., 2010; Di Bonito et al., 2014; Husseiny et al., 2011). However, although viruses such as MWPyV, HPyV6 and HPyV7 have

Fig. 2. Bayesian phylogenetic tree of VP1 sequences of BKPyV (1155 nt) (mid-point rooted). Posterior probability values higher than 0.5 are shown at nodes for relevant groups. Subgroups are indicated in squared brackets. Sequences from Argentina are shown in bold. Country of origin is indicated when available, abbreviated in uppercase. CAF: Central African Republic, CHN: China, DEU: Germany, ESP: Spain, ETH: Ethiopia, FNL: Finland, FRA: France, GBR: Great Britain, GRC: Greece, IRL: Ireland, JPN: Japan, KEN: Kenya, MMR: Myanmar, MNG: Mongolia, NER: Niger, NLD: The Netherlands, PHL: the Philippines, RUS: Russia, USA: the United States, VNM: Vietnam.*non-monophyletic groups.

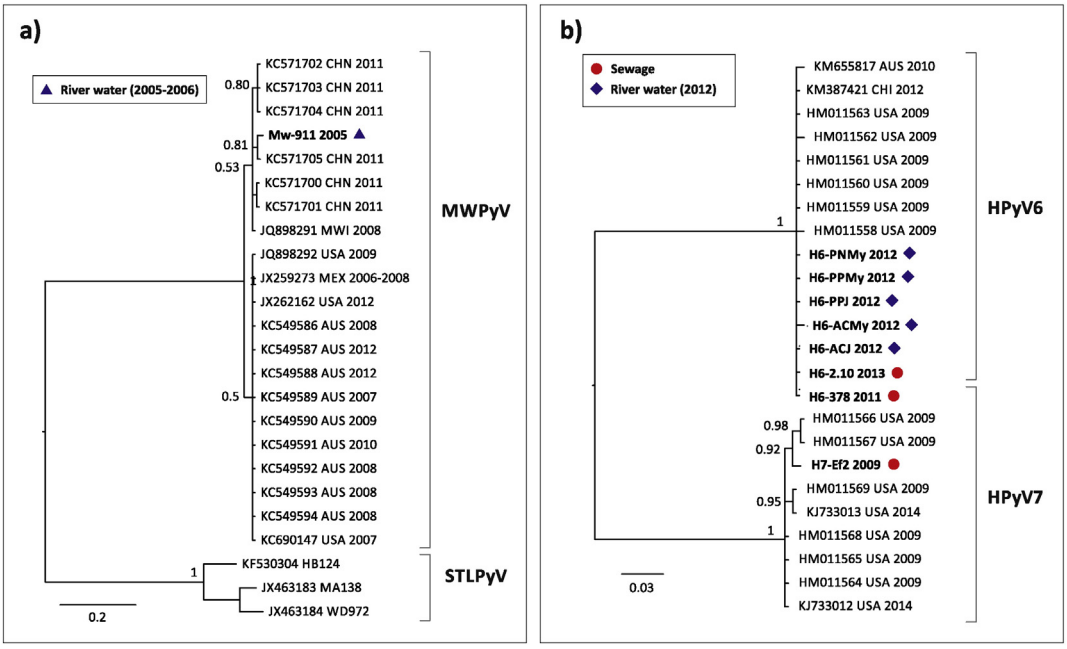

Fig. 4. a) Bayesian phylogenetic tree of VP2/VP1 partial region sequences of MWPyV (152 nt) (STLPyV was used as outgroup), and b) Bayesian phylogenetic tree of VP2/VP1 partial region sequences of HPyV6 and HPyV7 (165 nt) (mid-point rooted). Posterior probability values higher than 0.5 are shown at nodes for relevant groups. Sequences from Argentina are shown in bold. Year of isolation and country of origin is indicated when available, abbreviated in uppercase. AUS: Australia, CHN: China, MWI: Malawi, USA: the United States.

been detected in fecal samples (Rockett et al., 2013), their route of excretion has not been definitely established yet. The evidence here provided, indicating the presence of these viruses in river water and sewage, suggests that they would be excreted by the fecal or urinary routes and that they would be widely present in the Argentine population, since they should be an important component of these samples to be detected by a direct PCR. Alternatively, the presence of HPyV6 and HPyV7 in sewage could also be the result of skin peeling since the viral genome has been detected mainly in skin swabs (Schowalter et al., 2010).

Current legislation related to the control of microbial contamination of water only considers the use of bacterial indicators. However, it has been shown that these indicators do not satisfactorily reveal viral contamination: then, monitoring specific human viruses in water is particularly important (He et al., 2009; Vivier et al., 2004). The analyses of our environmental and sewage samples using a broad detection strategy indicate that polyomaviruses could be good, highly frequent, non-seasonal viral indicators.

Further characterization of the samples allowed us to describe the molecular epidemiology of the human polyomaviruses circulating in Buenos Aires with the coexistence of different genotypes and variants for each virus, particularly in sewage.

JCPyV has been classified into genotypes (1–8) and several subtypes associated with different human population (Stoner et al., 2000; Sugimoto et al., 1997; Yogo et al., 2004). In this work, subtype 2A was the main JCPyV type in all kind of samples (sewage, river water and urine), although subtypes 1B, 3A and 3B were also detected. Subtype 2A has been widely associated with the Asian and Native American population and it would have entered the American continent with the first settlers through the Bering Strait (Agostini et al., 1997; Fernandez-Cobo et al., 2002), whereas subtype 1B has been associated with Europe. Thus, considering the origin of the current population, which includes Native American (descendants from the Asian population) and European people, the finding of both types in our region is not surprising. More surprising was the detection of genotype 3, mainly associated with Africa or central/western Asia (Jobes et al., 1998; Stoner et al., 2000; Sugimoto et al., 1997). Although it is true that it could be an occasional detection, in a previous work we found an uncommon genotype of hepatitis A virus in sewage (Blanco Fernandez et al., 2012), not detected previously in clinical samples, which after a few months, was clinically detected (Altabert et al., 2015). Based on these results, this finding could indicate that the circulation of the JCPyV genotype 3 in the population could be wider than expected.

Whereas subtype 3B has been found in African and Afro-American populations, subtype 3A has spread through African, Afro-American, western/southern/central-western Asian populations and scarcely in southern Europe (Agostini et al., 2001; Dubois et al., 2001; Pagani et al., 2003; Takasaka et al., 2006).

On the other hand, BKPyV has been classified into four subtypes (I to IV) with subgroups within some of them (Ia, Ib1, Ib2, Ic and IVa1, IVa2, IVb1, IVb2, IVc1, IVc2) that show a relationship with particular geographic regions and human populations. Briefly, subtype I would be the most prevalent virus throughout the world; in particular subgroup Ib1 has been associated with Southeast Asians and subgroup Ib2 with Europeans and West Asians (Zheng et al., 2007). Subtypes II and III have been rarely detected worldwide and subtype IV is commonly distributed in East Asia and Europe (Yogo et al., 2009). BKPyV viruses found in this work corresponded mainly to subtype I (subgroups Ib1 and Ib2), but subtype II and IV (subgroup IVc2) were also found. Finding subgroups Ib1, Ib2 and IVc2 in our region is congruent with the origin of the population, as mentioned above. However, the detection of subtype II in a sewage sample – a rare genotype according to the provenance of the Argentine population – is noteworthy and encourages the analysis of the real prevalence of this type in the country. Previous reports have showed a very low prevalence worldwide (Yogo et al., 2009; Zheng et al., 2007) but a recent study has shown a seroprevalence of 69% for this viral type in a commercial serum panel (Pastrana et al., 2013).

In addition, this work reports the first sequences of MCPyV from Argentina. Previously, geographically related MCPyV genotypes were proposed: Europe/North America, Africa (sub-Saharan), Oceania, South America and Asia/Japan (Martel-Jantin et al., 2014). Two main lineages were found among MCPyV isolates from Argentina: one viral genome (from a patient) grouped with European sequences (Europe/North America clade), whereas the other two MCPyV genomes reported in this work grouped with the single VP1 sequence from South America

available so far, isolated from French Guiana, belonging to the South American genotype. Notably, these two Argentine sequences also formed a supported cluster within the South American group, which could represent a viral lineage associated with a southern location within South America.

Further analyses based on a larger number of samples from different locations in South America and larger genetic fragments will probably clarify and deepen the study of the geographic structure of these viruses.

Finally, the high level of detection and viral diversity found in sewage and environmental samples (which received the discharge of sewage effluents coming from millions of inhabitants) and the characterization of viruses scarcely reported worldwide and no previously described in South America reinforce the usefulness of this approach to study the viral contamination of human origin and to analyze the molecular epidemiological patterns of viral infections in the general population. This is particularly important given that epidemiological data from the general population are often unavailable for these viruses. Most of the viral types detected in a small number of clinical samples were also recovered from sewage and environmental samples, which indicates that environmental surveillance is a good approach to describe the epidemiology of viruses excreted on sewage.

Several limitations, such as low viral recovery, differential viral recovery depending on concentration methods or lack of information regarding viral infectivity, affect the results of environmental Virology. However, this and other previously published works show that environmental viral analysis is a powerful tool. Further analysis and methodology development are still needed to exploit this field of research.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi: http://dx.doi.org/10.1016/j.scitotenv.2015.10.047. These data include the Google maps of the most important areas described in this article.

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