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**"Detecció i caracterització dels poliomavirus  
 presents en la població a partir de mostres  
 ambientals"**

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**INTRODUCCIÓ****1-41**

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**1- ELS POLIOMAVIRUS**

1.1. Característiques generals	3
1.2. Estructura del virió	4
1.3. Estructura del genoma	5
1.4. Els antígens tumorals T i t	6
1.5. Cicle replicatiu dels poliomavirus	8
1.6. Els poliomavirus humans JC i BK	
1.6.1. Història	9
1.6.2. Característiques generals dels poliomavirus humans	10
1.6.3. Infecció al ronyó. La poliomavirúria	11
1.6.4. Vies de transmissió dels poliomavirus humans	11

**2- EL POLIOMAVIRUS HUMÀ JC**

2.1. La infecció primària	13
2.2. Infecció persistent i reactivació	13
2.3. Serologia	13
2.4. Cèl.lules permissives per a la replicació de JC	14
2.5. El concepte d'arquetípic i les reorganitzacions de la regió reguladora (RR) de JC	14
2.6. La regió intergènica de JC	17
2.7. Patologies relacionades amb JC	
2.7.1. Leucoencefalopatia multifocal progressiva (PML)	20
2.7.2. Tumors	21

**3- EL POLIOMAVIRUS HUMÀ BK**

3.1. Infecció primària, infecció persistent i reactivació	23
3.2. Serologia	23
3.3. Serotips	24
3.4. La regió reguladora (RR) de BK	24
3.5. Patologies relacionades amb BK	
3.5.1. Estenosi uretèrica	25
3.5.2. Cistitis hemorràgica (CH)	26
3.5.3. Tumors	26
3.6. BK a l'embaràs	26

<b>4- SV40. UN POLIOMAVIRUS HUMÀ?</b>	
4.1. El descobriment de SV40	27
4.2. Les propietats transformants de SV40	28
4.3. Els hostes naturals de SV40	28
4.4. Resposta humana a la infecció per SV40	29
4.5. Prevalença d'anticossos anti-SV40 en humans	30
4.6. El genoma de SV40	30
4.7. SV40 en tumors humans	31
4.8. SV40 circula entre les poblacions humanes?	32
<b>5- L'EVOLUCIÓ DELS POLIOMAVIRUS</b>	35
<b>6-DETECCIÓ DE VIRUS EN MOSTRES AMBIENTALS</b>	
6.1. Presència de virus humans en l'ambient aquàtic	39
6.2. Detecció de virus en aigües residuals urbanes	40
<b>OBJECTIUS</b>	<b>43-47</b>
<hr/>	
<b>RESULTATS</b>	<b>49-128</b>
<hr/>	
<b>Capítol I. Detecció i caracterització des poliomavirus presents en aigües residuals urbanes.</b>	
• Resum	53
• "Documenting the Epidemiologic Patterns of Polyomaviruses in Human Populations by Studying their Presence in Urban Sewage"	57
<b>Capítol II. Presència i estabilitat de JC i BK en mostres ambientals. Estabilitat dels poliomavirus humans al pH àcid. Implicacions en la seva via de transmissió.</b>	
• Resum	67
• "Potential Transmission of Human Polyomaviruses through the Gastrointestinal Tract after Exposure to Virions or Viral DNA"	75
<b>Capítol III. Anàlisi de les soques de JC excretades. Infectivitat i potencial transmissió oral.</b>	
• Resum	87
• "Analysis of the Excreted JCV Strains and their Potential Oral Transmission"	99

**Capítol IV. Detecció de SV40 en mostres ambientals.**

- Resum 115
- "Detection of SV40 in the Environment of a Naturally Infected Colony of Cynomolgus Monkeys" 119

**CONCLUSIONS I DISCUSSIÓ GENERAL 129-135**

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**BIBLIOGRAFIA 137-145**

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# 1-ELS POLIOMAVIRUS

## 1.1. Característiques generals

Els poliomavirus són petits virus icosaèdrics, sense embolcall, que presenten un genoma de DNA circular, tancat, sobrecargolat i de doble cadena que mesura aproximadament 5,2 Kb. Els poliomavirus infecten una àmplia varietat de vertebrats: humans, altres primats, rosegadors, conills i ocells. Tots els poliomavirus són similars pel que fa a mida i organització. Fins fa molt poc temps, els poliomavirus constituïen un gènere dins la família *Papovaviridae* juntament amb els papilomavirus amb els quals comparteixen algunes característiques tot i que difereixen bastant en la seva organització. Recentment els poliomavirus han passat a constituir una família independent: la família *Polyomaviridae*.

**Taula 1.** Membres de la família *Polyomaviridae* i hostes als quals infecten. Els noms oficials de les espècies de poliomavirus apareixen en negreta a diferència dels noms d'espècies temptatives, noms alternatius, soques o serotips (Virus Taxonomy, 7th report ICTV).

Virus	Hoste
<b>Poliomavirus de mico verd africà (AGMPyV)</b>	Mico verd africà
Poliomavirus limfotrópic B (LPyV)	Mico verd africà
<b>Poliomavirus JC (JCPyV)</b>	Humans
<b>Poliomavirus BK (BKPyV)</b>	Humans
<b>Virus de simi 40 (SV-40)</b>	Micos <i>rhesus</i>
<b>Poliomavirus boví (BPyV)</b>	Bovins
Virus de macac "Stump-tailed"	Micos <i>rhesus</i>
Virus fetal de ronyó de rhesus	Micos <i>rhesus</i>
<b>Poliomavirus de hámster (HaPV)</b>	Hámster
<b>Poliomavirus murí (MPyV)</b>	Ratolí
<b>Poliomavirus murí neurotròpic (MPTV)</b>	Ratolí
Poliomavirus de Kilham (KPyV)	Ratolí
<b>Poliomavirus de mandril 2 (PPyV)</b>	Mandril
<b>Poliomavirus vacuolant de ronyó de conill (RKV)</b>	Conill
<b>Virus de la malaltia de <i>Budgerigar fledgling</i> (BFDV)</b>	Periquito
<b>Virus de simi 12 (SV-12)</b>	Mandril

El poliomavirus murí (MPyV), l'espècie tipus dels poliomavirus, va ser el primer poliomavirus descobert. L'any 1953, Gross el va aïllar quan estudiava la leucèmia en ratolins (Cole, 1996). Va ser anomenat poliomavirus perquè causava tumors sòlids en

llocs diversos. El poliomavirus murí i SV40 han estat els poliomavirus més estudiats. El fet de posseir un material genètic en forma de "minicromosoma" i de ser potencialment oncogènics ha convertit als poliomavirus en excel·lents models de transcripció, oncogènesi, replicació i altres mecanismes cel·lulars. Darrerament s'han descrit noves espècies de poliomavirus tot i que encara no se'n coneix la seqüència completa: el poliomavirus hemorràgic que infecta l'ànec (Guerin i col., 2000) i una espècie que infecta micos *cynomolgus* (Van Gorden i col., 1999).

Aquest treball es centra en l'estudi d'aspectes relatius a SV40 i als poliomavirus humans JCPyV i BKPyV als quals ens referirem a partir d'ara com a SV40 i JC i BK respectivament.

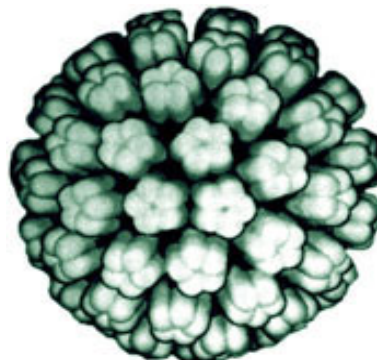
## 1.2. Estructura del virió

Els poliomavirus presenten virions no embolcallats de 42-45 nm de diàmetre. La càpside vírica està formada per 3 proteïnes: VP1, VP2 i VP3. VP1 constitueix un 70% de la massa del virió (360 còpies) i té un pes molecular de 44 kDa. La càpside presenta entre 30 i 60 molècules de VP2 i VP3.

Les càpsides estan constituïdes per 72 capsòmers pentamèrics i presenten simetria icosaèdrica trobant-se 60 d'ells coordinats hexagonalment i 12 pentagonalment.

La manca d'embolcall fa que aquests virus siguin resistents a l'èter, als àcids i a tractaments per calor, sobreviuen més d'una hora en aigua a 50°C però són inestables en les mateixes condicions en presència de  $MgCl_2$  1M (Virus Taxonomy, 7th report ICTV).

Els virions presenten una densitat de 1,34 g/ml en gradients de CsCl mentre que les càpsides buides presenten una densitat de 1,29 g/ml. El coeficient de sedimentació en gradients de sucrosa és de 240S. La partícula vírica està constituïda per un 88% de proteïna i un 12% de DNA (Cole, 1996).

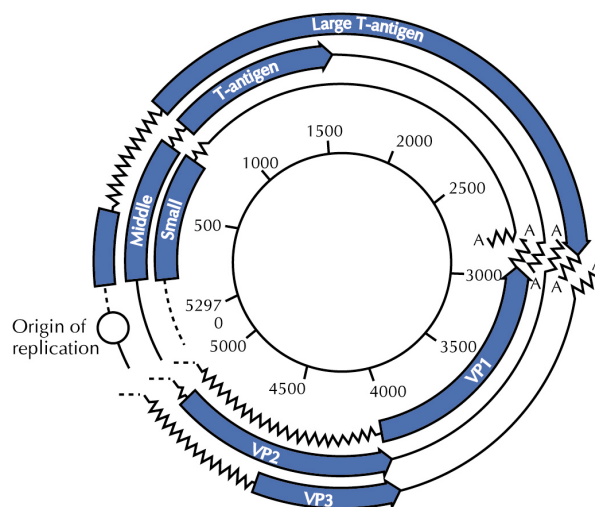


**Figura 1.** Aspecte que ofereixen els virions dels poliomavirus (Frisque, 1999).

### 1.3. Estructura del genoma

El genoma dels poliomavirus és de doble cadena, circular, covalentment tancat, sobrecargolat i la seva replicació té lloc al nucli. L'ADN es troba associat a 4 histones cel·lulars (H2A, H2B, H3 i H4) formant un minicromosoma. L'organització genòmica està dissenyada per codificar la màxima informació en tan sols unes 5,2 kb i  $3,2 \times 10^6$  Da.

Cadascuna de les dues cadenes d'ADN codifica per aproximadament un 50% de la informació genètica. El genoma dels poliomavirus es troba subdividit en una regió primerenca que codifica pels antígens tumorals T i t (AgT i Agt) i una regió tardana que codifica per les proteïnes estructurals que formen la càpside. També existeix una regió reguladora (RR) també anomenada regió del control transcripcional (TCR) d'uns 400 pb que presenta promotors de la transcripció i l'únic origen de replicació dels virus (ori). El nucleòtid 1 del genoma dels poliomavirus és el nucleòtid central del palíndrom que forma part de l'ori. La transcripció s'origina a la regió reguladora bidireccionalment, la regió primerenca es transcriu en sentit contrari a la direcció en que es transcriu la regió tardana i a partir d'una cadena diferent.



**Figura 2.** Esquema de l'organització genòmica que presenta el poliomavirus murí (MPyV) (Cann, 2001).

La regió codificant de VP1 es troba solapada a la regió C-terminal comú a VP2 i VP3. VP2 conté la seqüència sencera de VP3 en l'extrem C-terminal i presenta 115 aa addicionals a l'extrem N-terminal. Els ARN missatgers que codifiquen per aquestes proteïnes s'originen per *splicing* alternatiu d'un únic pre-ARN missatger. La traducció de VP1 té lloc mitjançant una pauta de lectura diferent a la de VP2 i VP3. Les 3 proteïnes constitueixen la regió tardana del genoma, expressant-se durant i després de la replicació.

Els antígens tumorals T i t conformen la regió primerenca i són produïts al principi del cicle lític, són proteïnes no estructurals i no s'incorporen als virions madurs.

La seva funció és vital en la replicació i transformació. Després de la infecció, els antígens T i t són fàcilment detectables en el nucli de les cèl·lules on, un cop acumulats, provoquen l'activació de la regió del genoma que codifica per les proteïnes estructurals.

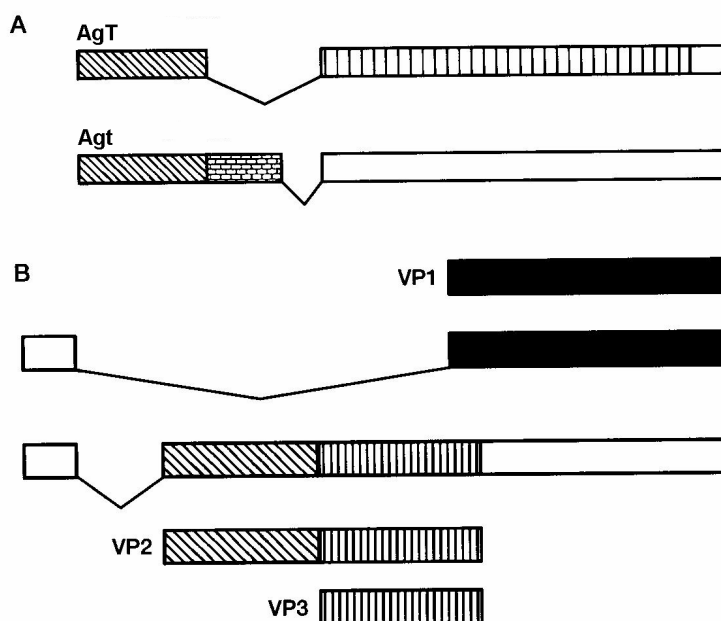
L'AgT i l'Agt comparteixen l'extrem N-terminal. Els ARN missatgers que codifiquen per aquestes proteïnes són resultants de l'*splicing* alternatiu d'un únic pre-ARN missatger. El poliomavirus murí, el que infecta conills i dues espècies que infecten rosegadors presenten, a més a més, un antigen T mitjà (AgTm).

Els poliomavirus humans i SV40 presenten una sisena proteïna: l'agnoproteïna (LP1), Aquesta proteïna de 60-70 aminoàcids s'acumula a la regió perinuclear durant la fase avançada del cicle infectiu. Sembla ser que la seva funció és la de regular negativament la transcripció i replicació de l'ADN viral, tan la basal com la induïda per l'AgT. També està involucrada en la formació de la càpside (Sabath i Major, 2002).

#### 1.4. Els antígens tumorals T i t

Els antígens tumorals reben aquest nom al ser els antígens dominants en animals portadors de tumors que han estat induïts viralment (Black i col., 1963).

L'AgT és una fosfoproteïna nuclear de 700 aa i l'Agt en té 172 en el cas de JC i BK i 174 en el cas de SV40.



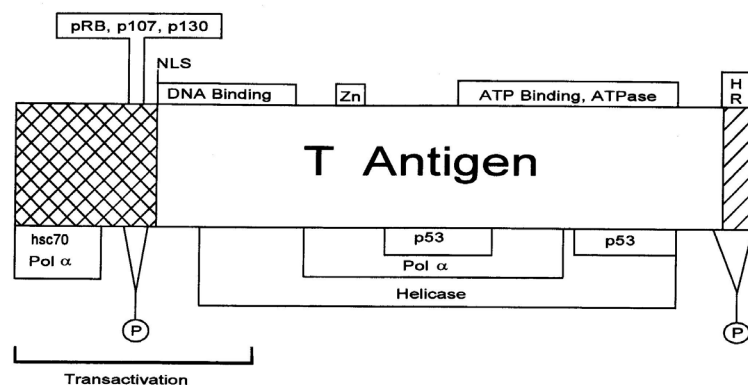
**Figura 3a.** Transcrits primaris dels antígens tumorals dels poliomavirus. La part ratllada diagonalment representa les seqüències comunes a ambdós antígens mentre que les parts quadrículada i ratllada verticalment representen la seqüència exclusiva per l'antigen t (Agt) i T (AgT) respectivament. Les línies representen els introns. **Figura 3b.** VP1, VP2 i VP3 s'expressen a partir de 2 ARN missatgers diferents. La regió codificant de VP1 es troba solapada a la regió C-terminal comú a VP2 i VP3. VP2 conté la seqüència sencera de VP3 en l'extrem C-terminal i presenta 115 aa addicionals a l'extrem N-terminal. Els ARN missatgers que codifiquen per aquestes proteïnes s'originen per *splicing* alternatiu d'un únic pre-ARN missatger (Imperiale, 2001).



Aquests antigens són proteïnes multifuncionals. Algunes de les funcions que presenten resideixen en dominis funcionals discrets i altres requereixen d'interaccions entre diferents dominis i/o l'acció independent de dos o més dominis (Brodsky i Pipas, 1998). L'AgT s'uneix a proteïnes de la família del retinoblastoma (pRb) inactivant-les i degradant-les. Per tal de fer això li és necessari posseir un motiu LXCXE i un domini J.

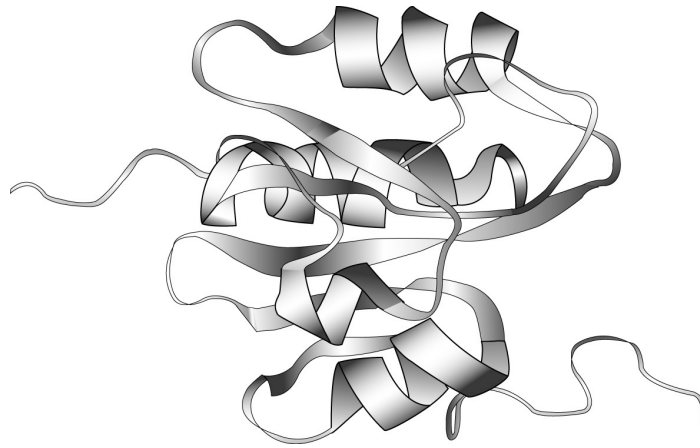
La unió a pRb provoca el desplaçament físic i alliberament del factor de transcripció E2F fet que estimula la transcripció de gens involucrats a la entrada de la cèl·lula en fase S de síntesi d'ADN. La resposta cel·lular a aquest fenomen sol ser activar p53 i provocar la mort cel·lular per apoptosi però l'AgT uneix p53 i la inactiva per augmentar la longevitat de l'hoste i assegurar un nombre elevat de partícules víriques descendents. L'AgT s'uneix a seqüències específiques de l'ori de l'ADN víric i recluta la maquinària sintètica de la cèl·lula hoste unint el complex ADN polimerasa  $\alpha$ /primasa i la proteïna de replicació A (RPA). L'AgT presenta, a més a més, activitat helicasa.

En cultiu cel·lular l'AgT és imprescindible per dur a terme infeccions productives. L'AgT, encara que no absolutament essencial, contribueix en la infecció i transcripció. Si es bloqueja la infecció productiva però continua havent-t'hi expressió de l'AgT es quan tenen lloc fenòmens de transformació cel·lular. Això és el que normalment succeeix en hostes no permissius o en hostes permissius que han patit mutacions a l'ori. L'AgT comparteix moltes funcions amb les proteïnes E1A i E1B dels adenovirus (Rao i col., 1992).



**Figura 4.** Dominis funcionals de l'antigen T dels poliomavirus. L'extrem amino terminal (quadrícula) interacciona amb proteïnes cel·lulars involucrades en la replicació i la transformació incloent la polimerasa  $\alpha$  i hsc70 (domini J), i les proteïnes de la família pRb (domini LXCXE). En aquest extrem també s'hi localitza una seqüència de localització de proteïnes cap al nucli (NLS) i un lloc de fosforilació (P) de serines i treonines. La regió central uneix la seqüència d'ADN GAGGC, el zinc (Zn), l'ATP i p53 i presenta activitats polimerasa i helicasa. La regió carboxi-terminal (ratllada en diagonal) inclou un domini de rang d'hoste (HR) i un segon lloc de fosforilació (Kim i col., 2001).

Recentment, s'ha descobert en SV40 i MPyV un nou antigen tumoral al qual se l'ha anomenat *tiny* degut a la seva mida doncs comparteix amb l'antigen T els primers 131 aminoàcids. El seu paper no es coneix massa bé (Zerrahn i col., 1993).



**Figura 5.** Aspecte que ofereix la molècula de l'antigen T dels poliomavirus (Cann, 2001).

### **1.5. Cicle replicatiu dels poliomavirus**

L'espectre d'hostes de cadascuna de les espècies de poliomavirus es força restringit i quan infecten altres hostes produeixen infeccions no productives. En hostes permissius els poliomavirus causen infeccions lítiques mentre que en hostes no permissius es produeix un bloqueig de la replicació que condueix a una infecció abortiva o a un procés oncogènic.

SV40 i MPyV infecten una àmplia varietat de cèl·lules de mamífer en cultiu tot i que en molts tipus la infecció causada és no productiva. Altres poliomavirus com JC són més exigents pel que fa a l'espectre i tipus cel·lulars a infectar (Cole, 1996).

La infecció comença amb la unió dels virus a les cèl·lules hoste. Els mecanismes mitjançant els quals els poliomavirus s'uneixen a les cèl·lules hoste no es coneixen del tot bé. Es creu que VP1 i l'àcid siàlic hi juguen un paper important i més concretament, en el cas de SV40, l'antigen MHC de classe I sembla estar-hi implicat (Norkin, 1999). Els poliomavirus entren al citoplasma per processos de pinocitosi ajudats per una interacció prèvia de VP2 i VP3 amb la superfície cel·lular. Els virus semblen ser transportats del citoplasma al nucli mitjançant el citosquelet i entren al nucli per fusió de membranes o

mitjançant els porus nuclears. Un cop al nucli, els virus són desencapsidats en un procés en el que intervenen VP2 i VP3 (Cole, 1996).

Al nucli la regió primerenca del genoma es transcriu i es produeixen els antígens T i t que condueixen a la cèl·lula a una fase S del cicle cel·lular on la maquinària de replicació de l'hoste es posa al servei del virus. L'AgT s'uneix a la RR inhibint la transcripció de la regió primerenca i activant a la vegada la transcripció de la regió tardana. El mecanisme mitjançant el qual l'AgT inhibeix la transcripció primerenca no es coneix amb detall.

Un cop la transcripció de la regió tardana comença, es produeixen dues molècules d'ARN missatger, l'una serà traduïda a VP1 i l'altra a VP2 i VP3 i, depenent de l'espècie de poliomavirus, l'agnoproteïna també serà traduïda. Les proteïnes són transportades al nucleoplasma on té lloc la formació de la càpside. L'alliberament de les partícules víriques té lloc mitjançant lisi de les cèl·lules hoste o mitjançant vesícules citoplasmàtiques (Cole, 1996). El cicle replicatiu dels poliomavirus dura unes 36-44 hores.

## **1.6. Els poliomavirus humans JC i BK**

### **1.6.1. Història**

La primera evidència de l'existència dels poliomavirus humans es remunta als anys 60 quan es van observar partícules víriques de poliomavirus per microscòpia electrònica en els nuclis engrandits d'oligodendròcits del cervell de malalts de leucoencefalopatia multifocal progressiva (PML) (Zu Rhein i Chou, 1965).

L'any 1971, JC va ser aïllat del cervell d'un pacient de PML en cultiu primari de cèl·lules gials fetals humanes (Padget i Walker, 1971). JC són les inicials del nom d'aquest pacient. El mateix any, BK va ser cultivat per primera vegada en cèl·lules Vero a partir de l'orina d'un pacient trasplantat de ronyó amb inicials B.K que patia estenosi uretèrica (Gardner i col., 1971; Padget i Walker, 1976; Gardner, 1977). Ambdós descobriments, duts a terme per grups d'investigadors diferents, van ser publicats al mateix número de *Lancet*.

JC va ser aïllat per primera vegada fa poc més de 30 anys. Des d'aleshores JC i la malaltia que aquest virus causa, la PML, han estat relegats a les darreres pàgines dels llibres de virologia. Fins fa poc la PML era una malaltia rara, a més a més resulta força

difícil treballar amb JC al laboratori, ambdós fets han contribuït a que JC fos considerat un virus poc important. Als anys 80 però tot va canviar degut principalment a l'epidèmia de la SIDA ja que un 4% de malalts de SIDA pateixen PML (Berger i col., 1987). La diagnosi de la PML en malalts de SIDA ha estat en els darrers anys facilitada per l'ús de tècniques de detecció d'ADN. Més de 20 anys d'estudi han fet que JC hagi esdevingut un virus d'interès considerable per la implicació que encara té en el desenvolupament de la PML però també pel paper que potencialment podria jugar en diversos tipus de tumors del cervell i de colon. JC és també una eina mitjançant la qual es poden dur a terme estudis de migracions de les poblacions humanes.

Si JC ha estat un virus poc estudiat fins fa poc BK ho ha estat encara menys. El seu paper en patologies del tracte urinari ha esdevingut però més notori en l'era dels trasplantaments convertint-se un virus de gran interès clínic. Darrerament i al igual que a JC se li ha atribuït un paper potencial en algun tipus de càncer de cervell (Corallini i col., 2001).

### 1.6.2. Característiques generals del poliomavirus humans

BK i JC presenten una elevada homologia entre les seves seqüències de nucleòtids, sent la regió del genoma que codifica per les proteïnes de la càpside la més conservada. La soca prototip de JC és Mad-1, aïllada a Madison, Wisconsin (USA) a partir del cervell d'un malalt de PML (Frisque i col., 1984). La soca prototip de BK s'anomena BK (Apperley i col., 1987).

**Taula 2.** Percentatges d'homologia d'aminoàcids (proteïnes) i de seqüència nucleotídica (ADN) dels poliomavirus humans i SV40 (Imperiale, 2001).

	BK-JC	BK-SV40	JC-SV40
<b>Proteïnes</b>	87	88	85
<b>VP1</b>	88	86	83
<b>VP2</b>	84	81	78
<b>VP3</b>	90	84	82
<b>Antigen T</b>	90	84	82
<b>Antigen t</b>	86	78	76
<b>ADN</b>	72	69	68

BK i JC transformen cèl·lules en cultiu i són oncogènics en animals de laboratori. JC transforma cèl·lules de cervell de hámster i cèl·lules amniòtiques humanes, BK

transforma cèl·lules de hàmmster, rata, conill, mico i ratolí. Ambdós virus són oncogènics en ratolins nous nats. JC provoca neoplasmes en algunes espècies de micos constituint l'únic model de tumor que afecta al sistema nerviós central de primats (London i col., 1978; London i col., 1983). JC i BK comparteixen també la propietat d'hemaglutinar eritròcits humans.

### **1.6.3. Infecció al ronyó. La poliomavirúria**

Els poliomavirus humans BK i JC s'excreten en l'orina i persisteixen en els ronyons dels individus infectats. Tot i que JC i BK han estat detectats a cèl·lules epitelials del tracte urinari (Beckmann i Shah, 1983) no es coneix quin és el tipus cel·lular específic on JC i BK resideixen quan es troben al ronyó.

JC ha estat detectat en 20-80% d'orines d'individus adults depenent del grup ètnic i l'edat de la població estudiada (Agostini i col., 1997; Kitamura i col., 1994).

L'excreció asimptomàtica de poliomavirus en l'orina (poliomavirúria) és comú durant la infecció primària i en períodes d'immunosupressió. L'excreció de BK i JC també es dona en individus sans sobretot en edats avançades i durant l'embaràs (Markowitz i col., 1991; Markowitz i col., 1993; Kitamura i col., 1994, Sundsfjord i col., 1994). En pacients trasplantats de ronyó s'ha descrit excreció de JC i BK en un 14-65% (Priftakis, 2001). Tot i que l'adquisició de BK té lloc abans en la infància, el nombre d'excretors en la població sana sol ser més baix (Shah i col., 1997), en canvi, la majoria de la població sana excreta JC en un moment o altre de la seva vida. La detecció de JC en orina no té doncs valor clínic a l'hora de fer diagnòstic de PML.

### **1.6.4. Vies de transmissió dels poliomavirus humans**

Tot i que la via de transmissió dels poliomavirus humans és encara desconeguda, la majoria d'investigadors coincideixen en que l'entrada podria tenir lloc pel tracte respiratori o l'alimentari (Sundsfjord i col., 1994). Se sap que JC i BK infecten persistentment el tracte urinari i els limfòcits i, en el cas de JC, també el cervell.

BK ha estat aïllat de cultius de teixit de les amígdales d'afectats de malalties respiratòries (Goudsmit i col., 1982) i de l'orina de nens amb amigdalitis aguda (Goudsmit i col., 1981). Sundsfjord i col·laboradors (1994) van aïllar ADN de BK de

2/201 mostres del tracte nasofaringi de nens que presentaven problemes respiratoris aguts. Ni JC ni BK van poder ser aïllats per aquests autors a partir de mostres de saliva d'individus adults immunosuprimits i immunocompetents cosa que els va fer proposar el tracte alimentari com a via d'entrada dels poliomavirus a l'organisme.

JC ha estat detectat en amígdals i limfòcits obtinguts a partir d'amígdals (Monaco i col., 1998a; Monaco i col., 1998b) i també en teixits del tracte gastrointestinal (Ricciardiello i col., 2000).

El fet de que es desconeix on té lloc la infecció primària fa difícil respondre la qüestió de quina és la via d'entrada dels poliomavirus a l'organisme. S'ha especulat una possible transmissió transplacentar d'ambdós virus. Això donaria sentit a la reactivació dels virus que normalment té lloc durant l'embaràs (Coleman i col., 1980). No obstant això, el nadó està en íntim contacte amb la mare després del part cosa que fa difícil discernir entre la transmissió transplacentar i altres possibles vies de transmissió. Aquesta possible transmissió transplacentar a edats primerenques no es correspon amb l'edat de seroconversió tot i que s'especula que potser la seroconversió podria ser el resultat d'una reactivació deguda a canvis hormonals en la pubertat més que a una infecció primària.

## **2- EL POLIOMAVIRUS HUMÀ JC**

### **2.1. La infecció primària**

La infecció primària per JC té lloc en la infantesa i primera adolescència (Taguchi i col., 1982, Gardner, 1973; Padget i Walker 1973). Aquesta infecció és normalment asimptomàtica. Fins al moment i com ja s'ha comentat, es desconeix quina és la via d'entrada de JC a l'organisme i quina és la seva ruta de transmissió. El fet de què en alguns casos provoquin molèsties respiratòries i la detecció del virus en amígdals ha donat força a la teoria d'una via d'entrada a través del tracte respiratori (Goudsmit i col., 1982; Monaco i col., 1998a). JC es detecta en el líquid cefaloraquídi (LCR) de la majoria de malalts de PML i gairebé mai en el LCR d'individus sans.

### **2.2. Infecció persistent i reactivació**

En individus immunocompetents JC persisteix al ronyó. També s'ha descrit en limfòcits, cervell, amígdals, pulmó, fetge, melsa, nòduls limfàtics, moll de l'os i teixits gastrointestinals (revisat a Jensen i Major, 2001) tot i que el paper que juga en aquests teixits no està massa clar ja que s'ha trobat en individus no immunosuprimits i en immunosuprimits que no han desenvolupat PML.

La reactivació dels virus té lloc durant períodes d'immunosupressió conduint a l'excreció dels virus en orina i a l'aparició de patologies com la PML. No obstant, se sap que un elevat percentatge de la població sana excreta intermitentment JC a l'orina.

### **2.3. Serologia**

L'elevat percentatge d'homologia existent entre els poliomavirus humans i SV40 ha complicat força el desenvolupament d'assaigs serològics específics. L'AgT de BK presenta reacció creuada amb sèrum anti-AgT de JC i SV40 (Walker i col., 1973). No obstant, s'observa poca reactivitat creuada entre els antígens estructurals i a més a més a VP1 trobem un antígen de la càpside específic d'espècie (Shah i col., 1977).

Dels estudis serològics de JC duts a terme fins al moment se'n desprèn que JC i BK circulen independentment a les poblacions. Al igual que en el cas de BK es coneixen tribus que sembla ser no han estat en contacte amb JC. També es coneixen poblacions on la infecció per JC és evident mentre que la infecció per BK és rara.

JC sembla adquirir-se més tard que BK. Dades obtingudes a Anglaterra indiquen que als 9 anys només una quarta part d'infants presenten evidències d'infecció per JC, entre els 15 i 50 anys un 50-60% de la població esdevé seropositiva i aquest percentatge augmenta conforme ho fa l'edat de la població fins assolir valors superiors al 75%. Hi ha dades que indiquen que a d'altres indrets com el Japó, JC sembla adquirir-se més tard (Walker i Padget, 1983).

En resum, aproximadament un 50% d'individus de diferents poblacions a tot el món estan infectats per ambdós virus quan assoleixen l'edat adulta.

#### **2.4. Cèl·lules permissives per a la replicació de JC**

JC presenta un creixement en cultiu cel·lular molt restringit. JC va ser aïllat d'un malalt de PML mitjançant cultiu primari en cèl·lules glials humanes. Aquesta població heterogènia de cèl·lules representa encara la forma més eficient de cultivar aquest virus *in vitro*. Derivats d'aquest tipus de cèl·lules com la línia SVG de cèl·lules humanes fetals glials transformada amb l'AgT de SV40 (Major i col., 1985) constitueixen en l'actualitat el sistema més eficient per cultivar-lo. Jensen i Major (1999) han fet recentment una revisió exhaustiva de cèl·lules permissives i semipermissives de ser infectades per JC. Els receptors cel·lulars de JC no es coneixen del tot bé però se sap que el virus s'uneix a cèl·lules glials, cèl·lules estromals de les amígdals i limfòcits B (Wei i col., 2000). El rang de línies cel·lulars on BK pot créixer sembla ser força més ampli que el de JC.

#### **2.5. El concepte d'arquetípic i les reorganitzacions de la regió reguladora (RR) de JC**

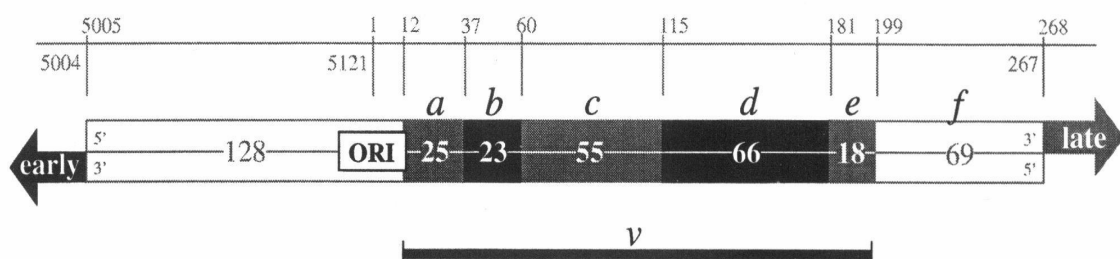
La RR de JC és hipervariable cosa que afecta marcadament a la capacitat de replicació del virus i al seu tropisme cel·lular. La RR conté diferents motius d'unió de factors de transcripció (NF1, CRE, p53...) (Ault, 1997).



La RR de JC es pot dividir en 7 seccions (Figura 6). La primera, de 128 pb, conté l'ori i és una regió força conservada. Dotze pb després del centre de l'ori es troben les seccions *a* (25 pb), *b* (23 pb), *c* (55 pb), *d* (66 pb), *e* (18 pb) i *f* (69 pb) que conformen una estructura de la RR a la que s'anomena arquetípica (Ault i Stoner, 1993).

En alguns casos, aquesta estructura arquetípica es veu modificada per duplicacions o delecions d'alguna de les seccions o parts d'aquestes. L'estructura *a*, *c*, *e*, *f* darrera l'ori resulta en una estructura que confereix al virus un elevada activitat mentre que la presència de les seccions *b* i *d* sembla inhibir l'activitat vírica. Duplicacions o duplicacions parcials de qualsevol secció *a*, *b*, *c*, *d*, *e* o *f* potencien l'activitat de JC en cultiu cel·lular.

Aquestes RR reorganitzades són les comunament aïllades del cervell o de líquid cefaloraquídi de malalts de PML, és per això que s'associa reorganització amb patogenicitat. Aquestes reorganitzacions donen lloc a un ventall molt extens de variacions que fa de les RR reorganitzades seqüències úniques que es classifiquen segons el tipus de reordenació que pateixen. Així podem distingir formes duplicades llargues (duplicació de *c* i delecio de *d*), formes triplicades curtes, formes retenedores de *d*, formes pseudoarquetípiques... La possibilitat de classificar aquestes reorganitzacions en diferents grups fa pensar que les reordenacions no tenen lloc l'atzar i que hi ha punts del genoma més susceptibles de patir talls (Ault i Stoner, 1993) i regions més conservades que altres com les necessàries per fer la funció de promotor (TATA, lloc d'unió a NF-1...). La soca tipus de JC, Mad-1, presenta delecions de *b* i *d*, quedant doncs una estructura *a*, *c*, *e* que, a més a més, està repetida en tàndem formant una estructura força típica de dos repeticions de 98 pb.

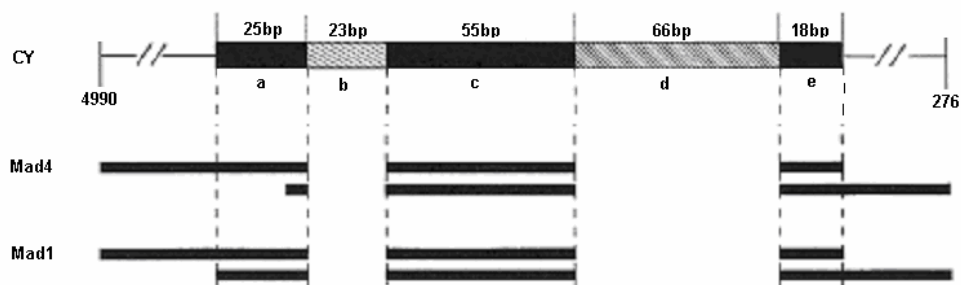


**Figura 6.** Esquema de l'estructura de la regió reguladora arquetípica del virus JC. La barra *v* indica aquelles seccions susceptibles de patir reordenacions en les soques tipus PML. La numeració està basada en la soca prototip Mad-1 (Jensen i Major, 2001)

Ambdues organitzacions, l'arquetípica i la reorganitzada, s'han trobat en orina, ronyó, cervell, pulmons, nòduls limfàtics, cèl·lules de la sang i de les amígdals (revisat a Jensen i Major, 2001) tot i que cada tipus d'organització predomina en uns llocs o en altres: dels estudis fets de les RR de JC obtinguts a partir de l'orina d'habitants de diferents parts del món se'n desprèn que, gairebé sense excepció, totes presenten estructures arquetípiques o arquetípiques amb petites variacions (*archetypal-like*). La majoria de soques aïllades de ronyó també presenten aquesta estructura i es creu que les soques arquetípiques són les que circulen majoritàriament entre les poblacions. En canvi, soques aïllades de fetge, pulmons, nòduls limfàtics, moll de l'os i cèl·lules epitelials del tracte digestiu presenten majoritàriament soques reorganitzades en la seva RR (revisat a Jensen i Major, 2001).

S'ha demostrat que les soques reorganitzades són capaces d'infectar cultius cel·lulars mentre que les arquetípiques difícilment ho fan. Aquest fet podria ser extrapolat i es podria concloure que les soques reorganitzades, també anomenades soques tipus PML, infecten més eficaçment les cèl·lules gials del cervell (Yogo i col., 1990) i per això apareixen sempre relacionades amb patogenicitat.

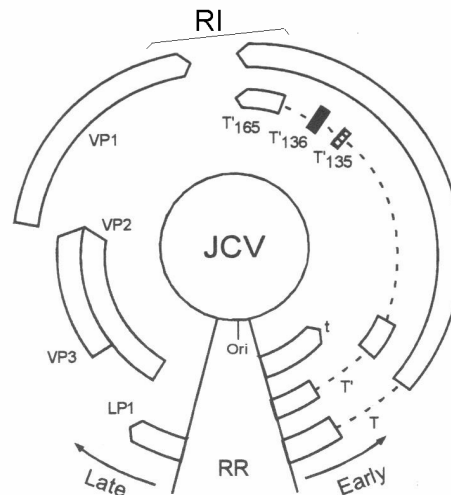
Es creu que els limfòcits actuen com a vehicles del virus des d'òrgans perifèrics cap al sistema nerviós central. De fet, els limfòcits es troben en molts òrgans i es creu que els JC detectats en aquests òrgans podrien provenir de limfòcits persistents en ells. Els limfòcits B, que presenten la maquinària necessària per produir i modificar anticossos, es creu que podrien ser els responsables de les reorganitzacions de la RR de JC. Són diversos els autors que postulen que les soques reorganitzades provenen de soques arquetípiques que experimenten diferents tipus de variacions (Yogo i col., 2001).



**Figura 7.** Estructura de la regió reguladora arquetípica de JC (soca CY) i de l'estructura de dues soques reorganitzades típiques (Mad-1 i Mad-4) que presenten duplicacions senceres o parcials de les seccions *a*, *c* i *e* i delecció de les seccions *b* i *d* (Daniel i col., 1996).

## 2.6. La regió intergènica de JC

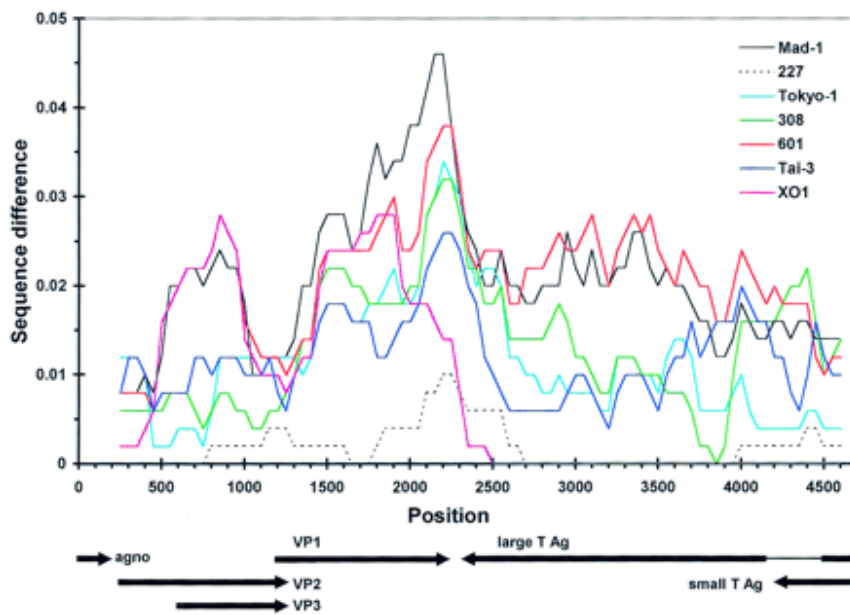
L'any 1984 Frisque i col·laboradors van seqüenciar per primera vegada un genoma complet de JC al que van anomenar Mad-1 i el qual és encara el prototip de JC. L'any 1988, quan es van seqüenciar 2 genomes més de JC (Loeber i Dörries, 1988), es va observar que els genomes presentaven diferències bàsicament en dues zones: la regió reguladora i la intergènica.



**Figura 8.** Esquema del genoma de JC on apareixen la regió reguladora (RR), la regió intergènica (RI) i les regions primerenca (*early*) i tardana (*late*), (Kim i col., 2001).

La regió intergènica que es localitza entre l'extrem C-terminal de VP1 i l'extrem C-terminal de l'AgT, és la zona del genoma de JC, exceptuant en alguns casos la RR, on hi ha una variabilitat nucleotídica més alta (Figura 9).

L'any 1987 Matsuda i col·laboradors i l'any 1990 Yogo i col·laboradors, presentaven evidències de que existien diferències entre les soques asiàtiques i europees de JC. Naixia l'idea dels genotips de JC. S'ha demostrat que les diferències existents en les regions intergèniques de diferents soques de JC responen a diferències geogràfiques, és a dir, es pot traçar quin és l'origen geogràfic d'una soca de JC si es coneix quina és la seqüència de la seva regió intergènica.



**Figura 9.** Esquema de la diversitat de la soca GS/B de JC comparada amb altres soques de JC de diferents tipus i subtipus. S’observa una gran diversitat en la regió intergènica compresa entre VP1 i l’AgT (Hatwell i Sharp, 2000).

Actualment i degut a que 2 grups van estudiar simultàniament les variacions del genoma de JC s’utilitzen dues terminologies diferents per parlar de genotips de JC: la relació entre aquestes dues terminologies s’exposa a la taula 3.

**Taula 3.** Comparació de les dues nomenclatures utilitzades per descriure els genotips de JC (Agostini i col., 2001).

Agostini, Jobes i Stoner	Yogo, Sugimoto i col.	Origen geogràfic
Tipus 1A, B	A (EU)	Europa
Tipus 4	A (EU)	Europa (paleotipus)
Tipus 2A	B (MY)	Japó i nadius americans
Tipus 2B	B1-c	Euràsia
Tipus 2D	B1-b	Índia
Tipus 2E		Oceania i Austràlia
Tipus 3A, B	B (AF2)	Àfrica (asiàtic)
Tipus 5		
Tipus 6	C (AF1)	Àfrica (paleotipus)
Tipus 7A	B (SC)	Sud de Xina
Tipus 7B	B (CY)	Nord de Xina
Tipus 7C1	B1-a	Xina
Tipus 7C2	B2	Xina
Tipus 8A, B		Papua Nova Guinea

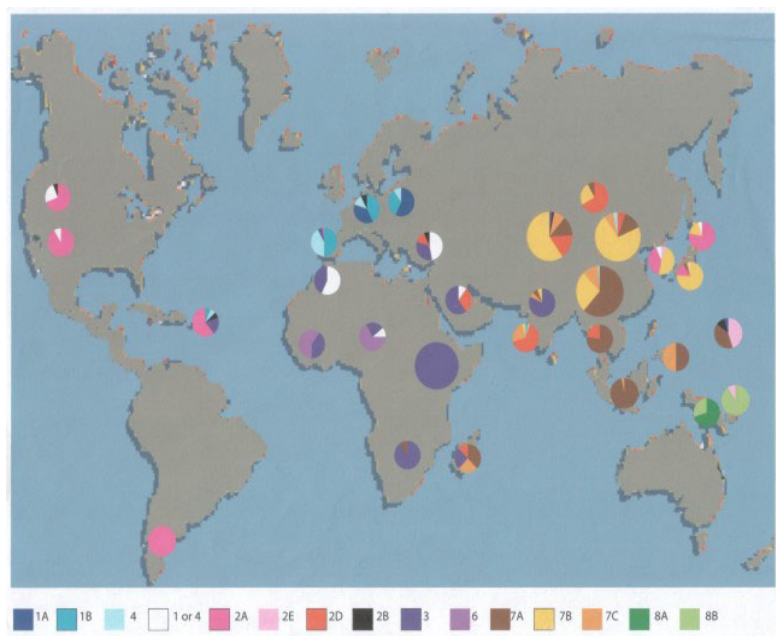
La classificació de JC en genotips es va dur a terme estudiant principalment la regió intergènica, actualment també es considera part de VP1 i fins i tot el genoma complet cosa que permet afinar més el genotipatge (Hatwell i Sharp, 2000).

JC ha estat usat com a marcador de migracions humanes. El seu genoma de mida petita, el fet de que es transmet a l'infantesa, la seva presència a la població sense causar patologia en condicions d'immunocompetència i la facilitat per trobar-lo en orina fan de JC una eina ideal per a aquest propòsit.

Els 8 genotips descrits fins al moment difereixen un 1-2,6% en el seu genoma complet (sense considerar la RR). Aquests genotips es correlacionen amb poblacions que habiten en regions geogràfiques específiques. Aquesta distribució pot ser explicada si es considera que JC va estar lligat a la dispersió de l'home modern des de l'Àfrica fa 100.000 anys com s'explicarà amb detall més endavant.

Quan s'estudia la RI de diferents soques de JC, es poden obtenir diferents arbres filogenètics mitjançant l'ús de diferents mètodes (*neighbor joining*, màxima parsimònia...) però hi ha 3 branques molt clares que apareixen sempre: Europa (tipus 1 i 4), Àfrica (tipus 3 i 6) i Àsia (tipus 2 i 7). Àsia ofereix una elevada complexitat de genotips de JC, especialment pel que fa a soques de tipus 2. El continent americà presenta una barreja de genotips: la majoria d'americans descendents d'europaus presenten el tipus 1 i 4 i alguns presenten JC de tipus 2. Nadius americans, com els Navajo de Nou Mèxic, presenten genotips asiàtics i els afro-americans presenten genotips europeus i el genotip africà de tipus 3 (però no el 6) en menor percentatge, es creu que després de 400 anys de barreja ètnica el genotip europeu ha suposat pels afro-americans alguna avantatge selectiva. La població hispana d'Amèrica presenta genotips molt diversos fruit de la barreja genètica dels nou vinguts i la població indígena. Les Illes del Pacífic presenten els tipus 8 i 2E (revisat a Agostini i col., 2001).

**Figura 10.** Esquema dels diferents genotips de JC que prevalen en la població humana en diferents localitzacions geogràfiques (cedit per G.L. Stoner).



## **2.7. Patologies relacionades amb JC**

### **2.7.1. Leucoencefalopatia multifocal progressiva (PML)**

L'any 1930, Hallervorden descriu una patologia, mai observada abans al cervell, que es caracteritza per presentar múltiples focus de desmielinització. L'any 1958, Åström i col·laboradors descriuen la malaltia com a leucoencefalopatia multifocal progressiva (PML). L'any 1968, ZuRhein i Chou descriuen que diferents casos de pacients amb aquestes lesions al cervell presentaven partícules semblants a papovavirus, però en aquells moments no es coneixia cap poliomavirus capaç d'infectar humans. Uns anys després, quan es va descobrir JC (Padgett i Walker, 1971), la hipòtesi de ZuRhein i Chou es va confirmar. En aquells moments la PML va ser considerada una malaltia rara ja que es donava en pacients que patien malalties immunosupressives poc comuns. Des del seu descobriment l'any 1958 fins l'any 1984, quan Brooks i Walker en van fer una revisió extensiva, s'havien comptabilitzat 230 casos en tot el món.

Holman i col·laboradors (1991) van descriure quasi 136.000 individus morts de SIDA que havien patit PML entre el 1981 i el 1990 als Estats Units. Es considera que en l'actualitat aproximadament un 4% de pacients de SIDA pateixen PML (revisat a Berger i Nath, 2001).

La PML és una malaltia amb les seves pròpies característiques patològiques i clíniques i un cop comença segueix el seu curs independentment al de la malaltia immunosupressiva que l'acompanya (Åström i col., 1958). La PML és una gliopatia que afecta cèl·lules de la macroglia causant primer desmielinització i més tard degeneració d'oligodendròcits. Tomografies computeritzades de cervells de malalts de PML revelen lesions hipodenses que afecten la matèria blanca. La PML provoca desmielinització en múltiples focus suggerint una dispersió del virus per via hematològica. Les lesions poden tenir des d'un mil·límetre fins a uns quants centímetres (Åmstrom i col., 1958). Els nuclis dels oligodendròcits apareixen allargats i hipercromàtics. Un examen per microscòpia electrònica revela la presència de JC en aquestes cèl·lules.

Els símptomes de la PML inclouen entre d'altres debilitat, demència progressiva, desordres motors, deficiència visual, problemes de parla i pèrdua sensorial. La mitjana de supervivència en malalts de PML és d'uns 6 mesos (Berger i col., 1998) tot i que alguns pacients poden sobreviure anys (Kepes i col., 1975). Les teràpies antiretrovirals han aportat un increment en la supervivència dels afectats. L'arabinòsid de citosina i el cidofovir, els fàrmacs més assajats fins el moment per a la prognosi de la PML, no han

demonstrat tenir un efecte massa clar (revisat a Berger i Nath, 2001). En l'actualitat diverses teràpies estan sent assajades.

### **2.7.2. Tumors**

JC és un potent inductor de tumors de cervell en hàmmster i en primats, la seva oncogènia en models animals, sobretot en primats, i en cultiu cel·lular ha estat àmpliament descrita per diferents laboratoris arreu del món. Aquesta capacitat s'ha provat que li ve donada pel fet de codificar per l'AgT.

Són moltes les descripcions de tumors de cervell associats a PML (revisat a Del Valle i col., 2001) sobretot astrocitomes, limfomes de sistema nerviós central, ependimomes i gliomes. JC ha estat també detectat en càncer de colon (Laghi i col., 1999; Ricciardiello i col., 2000; Enam i col., 2002).





## **3- EL POLIOMAVIRUS HUMÀ BK**

### **3.1. Infecció primària, infecció persistent i reactivació**

La infecció primària per BK té lloc molt aviat durant l'infantesa (Taguchi i col., 1982, Gardner, 1973; Padget i Walker 1973). Aquesta infecció és asimptomàtica encara que en alguns casos pot ocasionar lleugers símptomes respiratoris o molèsties al tracte urinari (Goudsmit i col., 1981; Hashida i col., 1976; Padgett i col., 1983). La infecció primària provoca una virèmia i el virus assoleix diferents òrgans de l'organisme on hi roman indefinidament en estat latent. El ronyó és el principal lloc on BK estableix infeccions persistents tot i que ha estat aïllat de diversos òrgans incloent leucòcits i el cervell (revisat a Corallini i col., 2001). La reactivació de BK s'associa a un estat d'immunosupressió (Padget i Walker, 1976) conduint a l'excreció dels virus en orina i a l'aparició de patologies. La via mitjançant la qual es transmet es desconeix.

### **3.2. Serologia**

BK és un virus ubiqü present en gairebé totes les poblacions (Padget i Walker, 1976). Poblacions aïllades de Paraguai, Brasil i Malàisia presenten evidències de no d'haver estat en contacte amb el virus (Brown i col., 1975).

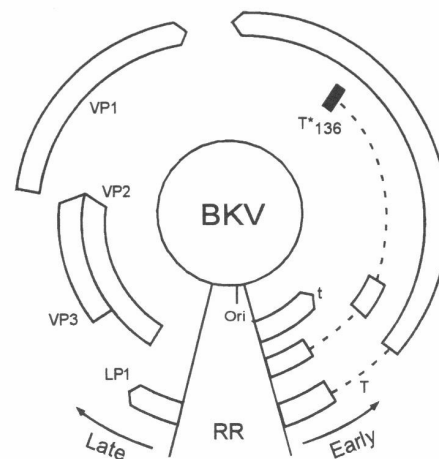
Als 3 anys un 50% de nens presenta anticossos per BK. Als 10 anys la majoria de la població està infectada pel virus (Shah i col., 1973). Les dades sobre la prevalença de BK en la població són molt dispars i van del 50% al 90% (revisat a Knowles, 2001).

Aquesta disparitat ha estat atribuïda a que BK pot ser un virus introduït molt recentment en determinades poblacions (Major i Neel, 1998). Tot i que BK sembla adquirir-se abans que JC, s'excreta menys que JC (Shah i col., 1997). Sembla ser que l'estat immunològic de l'hoste influeix directament en el grau d'excreció del virus, cosa que en el cas de JC sembla ser no tan determinant (Markowitz i col., 1993).

### 3.3. Serotips

BK presenta variants antigènics que van ser inicialment reconeguts immunològicament. La base molecular d'aquestes diferències ve donada per diferències existents a la regió amino-terminal de VP1. Els diferents serotips de BK presenten una semblança nucleotídica superior al 95% si es considera tot el genoma, si només es considera la regió amino terminal de VP1 la semblança és de només un 70%. Aquesta regió de VP1 codifica per un *loop* hidrofílic que es situa a la part externa del virió i té molta importància antigènica. Totes les soques de BK estudiades fins al moment es classifiquen en 4 serotips (I, II, III i IV). No s'han descrit diferències en la distribució dels genotips depenent de les poblacions (com passa en el cas de JC). El subtipus I és el més àmpliament distribuït i I i II són habitualment detectats en mostres d'orina de dones embarassades.

El fet de que BK presenti diferenciació en serotips, cosa que no passa amb JC, podria indicar una major història evolutiva de BK en humans o alternativament els diferents serotips podrien representar diferents salts a d'altres espècies de primats. Aquest punt serà discutit més endavant.

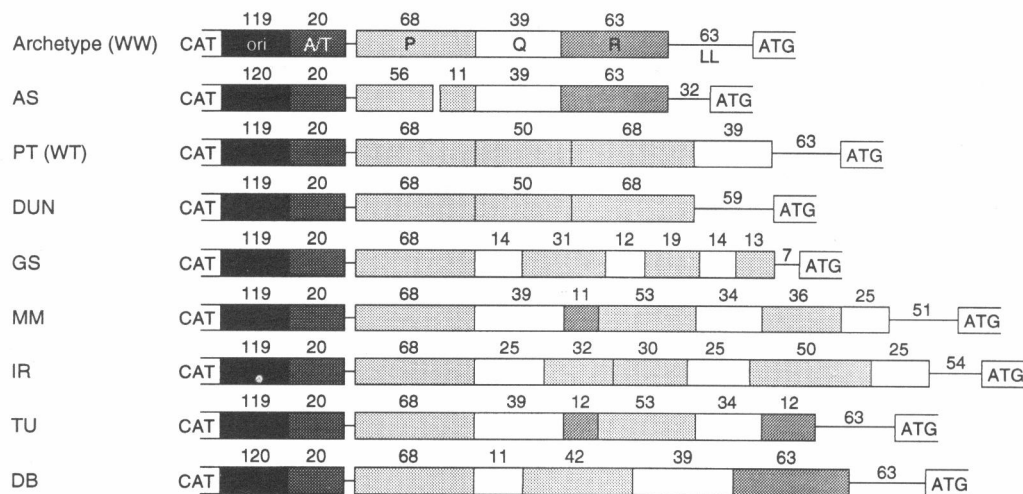


**Figura 11.** Esquema del genoma de BK on apareixen la regió reguladora (RR), la regió primerenca (*early*) i la regió tardana (*late*), (Kim i col., 2001)

### 3.4. La regió reguladora (RR) de BK

La regió reguladora de BK segueix el mateix patró que la de JC: BK-WW presenta un RR arquetípica freqüentment trobada en els virus excretats en l'orina mentre que altres soques de BK com Dun, MM, GS... presenten delecions i duplicacions en aquesta regió (Figura 12). Les diferents regions en que es pot dividir la RR de BK

són: l'ori, la regió A/T (zona rica en A i T) i les seccions P (68 bp), Q (39 bp), R (63 bp) (Markowitz i Dynan, 1988) i LL (*late leader*). P, Q i R són regions d'unió de factors de transcripció. Les reorganitzacions d'aquesta RR s'observen després de diversos pases en cultiu cel·lular i també han estat detectats en orina sobretot de pacients immunosuprimits. Es creu que les reordenacions en la RR confereixen al virus major capacitat lítica i/o transformant sent els virus amb RR arquetípiques difícilment cultivables en monocapes cel·lulars.



**Figura 12.** Comparació de la RR de diferents soques de BK. La seqüència CAT representa el codó d'inici de les proteïnes primerenques (en polaritat oposada) i ATG representa el codó d'inici de l'agnoproteïna. Les regions de l'ori, A/T, P, Q i R es troben representades en blocs diferents. WW representa la seqüència arquetípica mentre que la resta representen variacions d'aquesta estructura (Jin i Gibson, 1996).

### 3.5. Patologies relacionades amb BK

#### 3.5.1. Estenosi uretèrica

Aquesta patologia es dona en malalts trasplantats renals i en pacients terminals de SIDA i es caracteritza per una proliferació de cèl·lules epitelials que condueixen a l'obstrucció dels urèters de 1 a 3 mesos després del trasplantament requerint la majoria dels casos intervenció quirúrgica. Un diagnòstic ràpid és essencial per controlar la replicació vírica que requereix reducció del tractament immunosupressor (Binet i col., 1999).

### **3.5.2. Cistitis hemorràgica (CH)**

La cistitis hemorràgica (CH) és una complicació que apareix en pacients post-trasplantats de moll de l'os amb una incidència d'un 5-34% (Azzi i col., 1994; Priftakis, 2001). La CH es caracteritza per dolor abdominal i hematúria (Arthur i col., 1986). Un dels agents etiològics que pot provocar aquesta complicació és la infecció o reactivació del poliomavirus BK. La CH és majoritàriament una infecció autolimitant que es pot tractar amb analgèsics, i si és necessari amb l'aplicació de catèters i/o transfusió sanguínia.

Alguns treballs descriuen com a fàrmacs eficaços pel tractament de la CH la vidarabina i el cidifovir (Priftakis, 2001).

### **3.5.3. Tumors**

S'ha detectat BK en tumors de pàncreas, neuroblastoma i tracte urinari (Imperiale, 2000) però també s'ha detectat BK en teixits normals. De vegades s'ha trobat ADN integrat al cromosoma hoste i reorganitzacions a l'origen de replicació cosa que pot interferir amb el desenvolupament d'una infecció productiva. En alguns tumors on s'ha detectat BK també s'ha detectat producció d'AgT (Khalili i col., 1999).

## **3.6. BK a l'embaràs**

Es creu que els canvis immunològics i hormonals que tenen lloc durant l'embaràs són els responsables de la reactivació de BK (Coleman i col., 1983; Moens i col., 1994).

La reactivació és freqüent durant el tercer trimestre quan les concentracions d'estrògens i progesterona assoleixen els seus nivells més alts (Coleman i col., 1980). S'ha descrit una incidència d'excreció de BK en dones embarassades d'un 15-47% per BK i d'un 7% per JC (Markowitz i col., 1991; Jin i col., 1995).

## 4-SV40. UN POLIOMAVIRUS HUMÀ?

### 4.1. El descobriment de SV40

El descobriment de SV40 està lligat al desenvolupament i distribució de vacunes de la polio accidentalment contaminades i distribuïdes arreu del món exposant cents de milions de persones al virus durant els anys 1955-1963 (Shah i Nathanson, 1976). La vacuna de Salk, inactivada amb formalina, i amb menys repercussió la vacuna oral de la polio, prelegal en aquells moments, així com altres vacunes eren preparades en cultius primaris de cèl·lules de ronyó de micos *rhesus*, els hostes naturals de SV40.

El període de captivitat dels *rhesus* joves importats de la Índia, el ronyó dels quals era utilitzat per preparar els cultius primaris, va permetre que SV40 es disseminés entre els animals captius. Les monocapes de cèl·lules eren habitualment preparades a partir de ronyons de diferents animals homogeneïtzats de manera que molts lots de cèl·lules podien contaminar-se encara que un sol animal estigués infectat. En alguns casos es va dur a terme una pràctica que van fer decreïxer la probabilitat de contaminació: es van preparar cultius utilitzant els ronyons de diferents animals per separat (Strickler i Goedert, 1988).

La quantitat de partícules víriques de SV40 infeccioses presents en els diferents lots de vacunes podia doncs variar considerablement. Alguns lots de la vacuna inactiva probablement no contenien el virus mentre que altres podien contenir elevades concentracions de SV40.

Poc temps després del descobriment de SV40, es va saber que SV40 podia induir tumors en animals i posseïa la capacitat de transformar certa varietat de tipus cel·lulars en cultiu. Cap a l'any 1961, entre un 80 i un 90% dels infants nord-americans menors de 20 anys havien estat vacunats, almenys un cop, amb la vacuna de la polio contaminada amb SV40 (Shah i Nathanson, 1976). Els primers estudis sobre els efectes potencials de SV40 en humans no van mostrar evidències significatives de que el virus hagués causat un increment en la incidència del càncer (Shah i Nathanson, 1976).

Strickler i Goedert (1988) van fer una revisió profunda de les diferents investigacions epidemiològiques sobre la incidència del càncer després de la immunització de la població amb les vacunes contaminades. Un elevat nombre d'estudis de cohorts no mostraven una correlació entre el desenvolupament de càncer i el fet d'haver rebut o no la vacuna contaminada. La informació obtinguda a partir d'estudis de

cas-control, tot i que presentar algunes limitacions, arribaven a les mateixes conclusions.

## 4.2. Les propietats transformants de SV40

Les propietats transformants de SV40 han estat caracteritzades en diversos tipus cel·lulars de diferents espècies incloent humans, altres primats, rosegadors i bovins (Arrington i Butel, 2001). Diferents tipus cel·lulars presenten variacions en la seva permissivitat a ser infectats amb SV40. Les cèl·lules de mico es consideren permissives i presenten mort cel·lular per lisi. Les cèl·lules de ratolí es consideren no permissives i en alguns casos el genoma viral es pot trobar integrat a l'ADN hoste (Grodzicker i Hopkins, 1980). Les cèl·lules humanes es comporten com a semipermissives, la replicació viral hi té lloc tot i que es produeixen nivells baixos de progènie vírica. Molts tipus cel·lulars humans han estat immortalitzats amb gens de SV40 (Bryan i Reddel, 1994).

Els models animals han jugat un paper important a l'hora de definir a SV40 com a un virus ADN causant de tumors. L'any 1962 es va demostrar que cèl·lules de ronyó de mico induïen tumors en hámsters nounats en ser injectades (Eddy i col., 1962). La injecció de SV40 per via intracranial, intravenosa, intrapleural, intracardial o intraperitoneal produeix diferents tipus de tumors en diferents tipus d'hoste (revisat a Arrington i Butel, 2001).

SV40 és doncs un agent oncogènic important, l'associació de l'antigen T amb les proteïnes repressores de tumors són factors a tenir en consideració a l'hora d'intentar elucidar el possible paper que SV40 juga en la tumorigènesi humana.

## 4.3. Els hostes naturals de SV40

SV40 és un virus que infecta micos *rhesus* de la Índia, Japó, Taiwan i Vietnam (revisat per Shah i col., 1971). SV40 no té hostes coneguts a altres països (Shah, 2000). En captivitat, algunes espècies properes als *rhesus* com els micos *cynomolgus* i el mico verd africà poden esdevenir susceptibles a la infecció.

SV40 pot causar una infecció estesa en micos i ser detectat en diferents òrgans. La presència de SV40 al cervell de micos immunodeficients va demostrar que SV40 és un virus neurotròpic a més de presentar tropisme pels ronyons. La presència del virus

en la melsa i la sang suggereix que podria estendre's utilitzant rutes hematològiques (Butel i col., 1999).

Shah i Nathanson (1976), van descriure que després d'una inoculació intranasal, intragàstrica i subcutània de SV40 en *rhesus* el virus es multiplicava produint virèmia i resposta immunològica. La infecció sembla benigna i només s'observa patologia en micos immunodeprimits en els quals el virus pot ocasionar PML (leucoencefalopatia multifocal progressiva) (Gribble i col., 1975) i deficiències renals (Horvath i col., 1992). S'ha suggerit que SV40 podria jugar algun paper en el desenvolupament de tumors en el cervell dels micos (Hurley i col., 1997), també s'ha proposat que SV40 podria ocasionar lesions del sistema nerviós central diferents a la PML (Simon i col., 1999).

El virus s'excreta a l'orina i estableix infeccions latents al ronyó (Hull, 1968). Totes aquestes característiques suggereixen una associació ben establerta entre SV40 i els micos *rhesus*. El que es coneix fins al moment de la infecció de SV40 en humans no suggereix que l'espècie humana pogués ser un hoste primari massa eficient per SV40.

SV40 creix millor que els poliomavirus humans en cultiu cel·lular, sobretot en línies cel·lulars de ronyó de mico verd africà com CV-1 i BSC-1.

#### **4.4. Resposta humana a la infecció per SV40**

Hi ha estudis que descriuen la resposta humana a SV40 administrat per diferents vies. Voluntaris humans que van ser exposats al virus per via respiratòria van romandre lliures de signes d'infecció després d'un mes de la inoculació (Morris i col., 1961).

Després d'administrar el virus per via oral, alguns nens van excretar-lo en petites quantitats a la femta per un període superior a les 5 setmanes. La resposta immunològica va ser indetectable (Melnick i Stinebaugh, 1999). Després de la inoculació subcutània de vacunes de la polio contaminades amb SV40 el títol d'anticossos va ser alt i detectable de 8 a 13 anys després de la immunització (Gerber, 1967). SV40 va ser aïllat dels esputs de voluntaris als 7 i 11 dies post-inoculació tot i que el virus va ser indetectable en femta (Sweet i Hilleman, 1960).

#### 4.5. Prevalença d'anticossos anti-SV40 en humans

Shah i col·laboradors (1971), van descriure un 20% de prevalença d'anticossos en nens de 12 a 14 anys la majoria dels quals havien rebut la vacuna l'any 1961. El títol d'anticossos va ser moderadament elevat. Al mateix estudi, Shah revisa altres investigacions dutes a terme en diferents tipus de població: a la Índia, la distribució de la vacuna de la poliomelitis va ser molt limitada abans del 1961. El Nord de la Índia és una àrea excepcional per estudiar la presència de SV40 en humans ja que la població de la zona ha tingut contacte amb l'hoste natural. Un 27% de gent que treballava amb micos presentava anticossos neutralitzants per SV40 mentre que només un 8.7% de residents i un 5.3% de pacients amb càncer presentaven els anticossos.

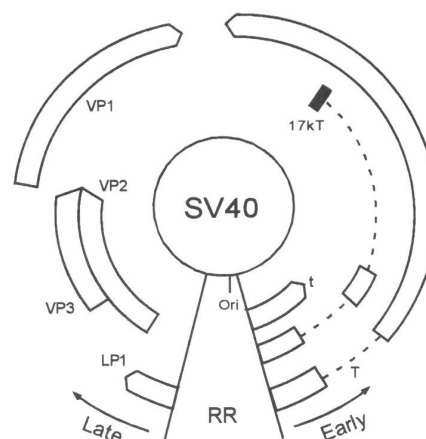
Shah descriu que un 3.8% de pacients amb càncer als Estats Units presentaven anticossos independentment d'haver rebut o no la vacuna contaminada. Un 3.2% de infants nascuts després de l'any 1961 i un 2% d'individus la sang dels quals va ser obtinguda abans de l'administració de la vacuna contaminada presentaven anticossos.

Totes les poblacions estudiades presentaven baixa freqüència de seropositius, baixos títols d'anticossos i cap evidència d'increment del títol al llarg del temps.

#### 4. 6. El genoma de SV40

El genoma de SV40, d'organització similar a la dels poliomavirus humans, (Figura 13) presenta també 2 regions d'elevada variabilitat. A la regió C-terminal de l'AgT s'observen variacions puntuals mentre que a la RR s'observen duplicacions i delecions d'una estructura a la que s'anomena arquetípica.

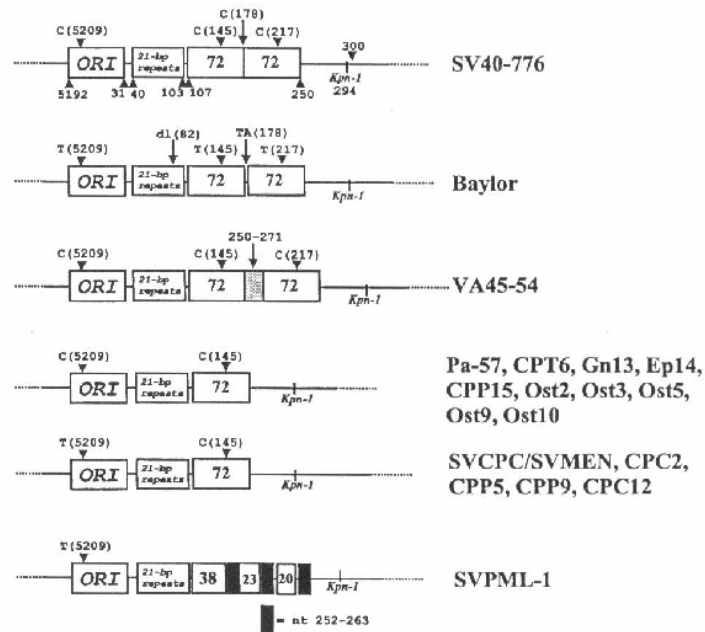
**Figura 13.** Esquema de l'organització genòmica de SV40. A l'esquema apareixen la regió reguladora (RR), la tardana (*late*) i la primerenca (*early*). La regió hipervariable del genoma de SV40 es localitza a l'extrem C-terminal del gen que codifica pel T-Ag, (Kim i col., 2001).





L'estructura arquetípica presenta una sola regió de 72 bp després de l'ori i de les 3 repeticions de 21 nt mentre que altres soques com la prototip de laboratori, 776, presenten variacions d'aquesta estructura (Figura 14).

Fins a la data, no es coneixen soques de SV40 típiques de mico i típiques d'humans (detectades en tumors) ni genotips ni serotips de SV40.



**Figura 14.** Regions reguladores de diferents aïllaments de SV40 incloent ADN aïllat de tumors en humans. SV40-776, Baylor, VA45-54, Pa-57, provenen de cultius cel·lulars de ronyó de mico no inoculats amb virus, SVPML-1 d'un cultiu cel·lular fet a partir del cervell d'un humà malalt de PML i la resta d'aïllaments de tumors en humans. A la figura es representen l'ori, les repeticions de 21 pb i la regió o regions de 72 pb. També es descriuen mutacions puntuals i altres reordenacions de la RR (Steward i col., 1998).

#### 4.7. SV40 en tumors humans

Des que l'any 1974 Soriano i col·laboradors van descriure per primera vegada una associació entre SV40 i el càncer, múltiples descripcions d'un lligam entre aquest virus i diferents tipus de tumors humans s'han anat acumulant i el paper que potencialment SV40 podria jugar en el desenvolupament de càncers en humans cal ésser considerat seriosament.

SV40 ha estat associat a mesoteliomes (tumors de la pleura), diferents tipus de tumors cerebrals com ependimomes i carcinomes del plexe coroidal (ambdós tumors pediàtrics), glioblastomes, meduloblastomes, meningiomes, oliogodendromes, astrocitomes, papilomes, gliosarcomes, i adenomes pituitaris. A més, SV40 s'ha trobat associat a d'altres tumors (osteosarcomes, limfomes tipus no Hodgkin, limfomes, limfomes relacionats amb la SIDA) i a teixits no tumorals (cèl·lules perifèriques de la sang, esperma i ronyó) (revisat a Arrington i Butel, 2001).

L'ADN de SV40 que s'ha trobat associat a tumors presenta normalment RR arquetípiques i variacions diverses en el domini C-terminal de l'AgT (Stewart i col., 1998). Fins a la data no hi ha seqüències específiques de teixit. La manca de seqüències que permetin diferenciar SV40 "humans" i SV40 de micos van portar a Stewart i col·laboradors (1998) a concloure que SV40 podria tenir un espectre d'hostes més ample del que s'havia pensat fins al moment i que existiria la possibilitat que SV40 fos una infecció natural en humans i micos *rhesus*.

La detecció de SV40 en tumors poc freqüents i en càncers pediàtrics, en adolescents i en adults contrasta amb la infecció transitòria i asimptomàtica descrita als anys seixanta després d'analitzar les possibles conseqüències de l'exposició a la vacuna contaminada.

#### **4.8. SV40 circula entre les poblacions humanes?**

Els poliomavirus són elevadament específics d'espècie i han coevolucióat amb els seus hostes. Si la informació publicada fins al moment es confirma, es podria hipotetitzar que SV40 podria ser una infecció natural d'humans i haver coevolucióat amb ells o que les vacunes podrien haver introduït el virus en les poblacions convertint a SV40 en un virus humà que es podria haver transmès entre humans. Molts individus amb tumors positius per SV40 van néixer després de que SV40 fos completament eliminat de les vacunes de la poliomeilitis. El virus podria haver estat present en les poblacions fins i tot abans de l'administració de les vacunes.

Els poliomavirus es troben latents als ronyons i s'excreten en l'orina. SV40 va ser analitzat en 166 mostres d'orina d'homes homosexuals la meitat dels quals eren seropositius pel HIV. Cap de les mostres va ser positiva per SV40 tot i que algunes ho van ser per BK i/o JC (Shah i col., 1997).

Fa molt temps que es coneix que un petit percentatge de sèrum humà presenta baixos títols d'anticossos neutralitzants de SV40. S'ha suggerit que aquesta neutralització podria ser el resultat d'una reacció creuada amb anticossos contra d'altres poliomavirus (Shah, 1972).

Cal tenir en compte que SV40 ha estat un virus àmpliament utilitzat per construir vectors d'expressió. Molts laboratoris arreu del món han estat exposats a seqüències de SV40. La possibilitat de contaminació cal ésser estrictament controlada a l'hora de dur a terme estudis de presència de SV40 en tumors humans.

Tot i això, existeixen evidències en favor de SV40 com a causant de tumors en humans: els tumors més fàcilment induïbles en hàsters mitjançant inoculació amb SV40 són curiosament mesoteliomes i tumors de cervell (Cicala i col., 1993; Brinster i col., 1984). S'ha detectat producció de AgT en tumors humans (Carbone i col., 1997; Butel i Lednicky, 1999). SV40 pot infectar persistentment cultius cel·lulars de mesotelioma humà i causar transformació (Bocchetta i col., 2000). Les soques de SV40 detectades en humans no presenten duplicacions com les que s'observen en soques de laboratori (Lednicky i Butel, 2001) i presenten elevada variabilitat en la regió C-terminal de l'AgT cosa que ajuda a eliminar la possibilitat de contaminació.

Tot i l'existència de molts estudis que descriuen la presència de SV40 en tumors humans, no existeixen estudis que descriguin les característiques de les interaccions entre SV40 i les cèl·lules tumorals. La presència d'un virus en un tumor no necessàriament implica que els virus en sigui la causa. Cal que el paper potencial que SV40 podria jugar en el desenvolupament de càncers humans sigui estudiat en detall i que estudis centrats en estudiar l'epidemiologia, patogènia i mecanismes moleculars convergeixin en conclusions coherents i suficientment contrastades.

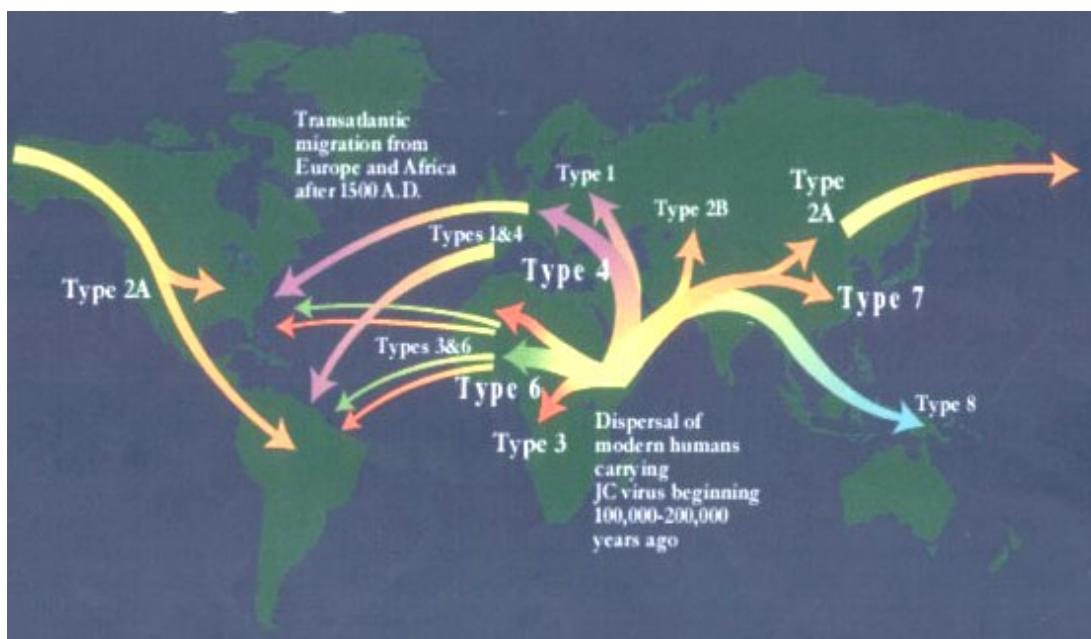


## 5- L'EVOLUCIÓ DELS POLIOMAVIRUS

La capacitat codificant dels poliomavirus es troba limitada a 5000 pb. Això fa a aquests virus molt dependents de la maquinària de l'hoste, persistents en ell i poc canviants.

Cal assumir que espècies amb elevada similaritat genòmica han evolucionat a partir d'una única espècie ancestral. La distribució del prototip víric i les seves variants és resultat d'una evolució molecular i de l'evolució i migració de l'espècie hoste.

JC és el poliomavirus que més clarament ha evolucionat amb el seu hoste com demostra el fet de que els diferents genotips de JC es correlacionen clarament amb la distribució geogràfica dels humans. Es creu que una població fundadora de 10.000 individus va deixar Àfrica fa 100.000 anys a la migració coneguda com *Out of Africa II* i que JC es va anar diferenciant fins assolir la diversitat existent a l'actualitat (Figura 15).



**Figura 15.** Proposta de la dispersió dels genotips de JC amb les migracions que van tenir lloc a l'Àfrica Fa 100.000 anys (Agostini i col. 2001).

No està clar si ja existien uns genotips fixats en el grup fundador que va deixar Àfrica. La no existència de serotips de JC indica estabilitat d'ADN i de l'estructura vírica cosa que es relaciona amb infeccions persistents de l'hoste. S'ha calculat que JC té una taxa de mutació molt baixa ( $4 \times 10^{-7}$ /posició i any). Això el fa molt vàlid com a eina per estudiar la història evolutiva ja que les mutacions reflecteixen la distància evolutiva real i la possibilitat de què es donin varies mutacions en un sol lloc, enfosquant la història

evolutiva real, és molt baixa. A l'hora de construir arbres filogenètics aplicant un mètode que mesura la similaritat nucleotídica total (*neighbor joining*) o un que mesura les mutacions a cada posició (màxima parsimònia) s'obtenen resultats similars.

L'existència dels serotips en BK és consistent amb una història evolutiva més llarga, una evolució més ràpida o a salts entre espècies. Està clar que JC i BK han seguit històries evolutives diferents i que BK sembla haver-se hagut d'adaptar a un grup més gran d'hostes com reflexa el seu més ampli espectre d'hostes *in vitro*. L'elevada similaritat que BK té amb el poliomavirus de simi SAV-12 fa pensar amb la possibilitat de que BK fos un virus de simi que es va adaptar a una nova espècie, fet que explicaria aquesta evolució més ràpida.

La teoria més àmpliament acceptada és la d'un ancestre comú de JC i BK que va deixar l'Àfrica amb la migració de l'*Homo erectus* o l'*Homo ergaster* cap a Europa i Àsia quan va tenir lloc la dispersió coneguda com a *Out of Africa I* fa 1,7 milions d'anys (Gabunia i col., 2000). JC va evolucionar en els ancestres africans d'*Homo sapiens* i es va adaptar als humans moderns mentre que BK va evolucionar independentment en les poblacions d'*Homo erectus* a Àsia i Europa i en els seus descendents com l'home de Neandertal, fet que explicaria la seva diversitat actual. Després de la segona dispersió des de l'Àfrica fa 100.000 anys BK va estendre's als humans moderns, que acabaven d'arribar de l'Àfrica, però havent evolucionat tant que ja no es donava protecció creuada entre BK i JC. Els homes moderns podrien ser infectats per dues espècies diferents de poliomavirus.

L'evolució de SV40 sembla encara més complexa. Tot indica que si els poliomavirus humans i SV40 compartien un ancestre comú (virus JBS\*) s'haurien d'haver separat fa 30 milions d'anys quan els ancestres primats es van separar. Però SV40 és més semblant als poliomavirus humans que a d'altres poliomavirus de primats. Potser altres poliomavirus es van separar abans i van coevolucionar amb el seu hoste durant més temps o potser l'ancestre comú de JC i BK va arribar via *Homo erectus* als micos asiàtics fa uns 1.5 milions d'anys i va evolucionar com a SV40.

L'homologia entre les seqüències de JC i BK i SV40 (72-74%) és massa elevada per ser consistent amb una separació fa 30 milions d'anys. Això podria implicar també salts de SV40 entre espècies un o més cops (revisat a Agostini i col., 2001).

Tot i que les teories exposades són força compartides per diversos autors encara no es coneix quants poliomavirus de primats hi ha i quins són els seus hostes, quines d'aquestes espècies han coevolucinat amb els seus hostes i quines han saltat d'espècie.

El futur descobriment de noves espècies de poliomavirus i la seqüència nucleotídica d'aquestes haurien de fer més clar el panorama de l'evolució dels poliomavirus i haurien d'aportar dades que ajudessin a discernir quin o quins són els veritables hostes naturals de SV40.





## 6- DETECCIÓ DE VIRUS EN MOSTRES AMBIENTALS

### 6.1. Presència de virus humans en l'ambient aquàtic

Els ecosistemes aquàtics presenten concentracions variables de virus que constitueixen la comunitat planctònica microbiana. La concentració de virus en aigües naturals no contaminades (aigües marines o llacs d'alta muntanya) pot arribar a ser de  $10^5$ - $10^8$  partícules víriques per ml (Berg i col., 1989; Børshheim, 1993; Maranger i Bird, 1995; Pina i col., 1998a). En zones properes a nuclis urbans el medi aquàtic rep aportacions amb elevades càrregues víriques excretades per l'home i altres animals en la femta i l'orina d'individus que pateixen infeccions víriques, de vegades asimptomàtiques.

Depenent del tipus de virus, la seva supervivència en aquests ambients pot ser més o menys llarga segons sigui la seva resistència als processos d'inactivació natural i de tractaments artificials de les aigües. Hi ha virus que poden sobreviure a aquests tractament i constituir un risc potencial de noves infeccions per l'home. Les vies de transmissió dels virus presents en el medi hídic són molt diverses i es troben esquematitzades a la Figura 16.

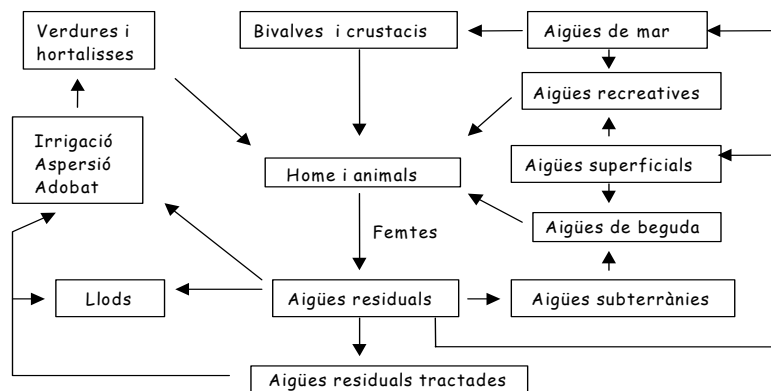


Figura 16. Cicle de contaminació del medi hídic (Schwartzbrod, 1991).

Els principals virus d'origen humà excretats a l'ambient, que causen patologies en humans i que han estat estudiats fins al moment pel seu interès sanitari són: els enterovirus, els adenovirus, els virus de l'hepatitis A i E, els virus del grup de Norwalk, els calicivirus, els rotavirus, els astrovirus i els reovirus.

## 6.2. Detecció de virus en aigües residuals urbanes

L'aigua residual ofereix la possibilitat d'estudiar els virus que són excretats pel conjunt d'una població que habita en una àrea determinada en un moment donat.

La baixa concentració de virus que es poden trobar en les aigües residuals limita les tècniques de detecció potencialment aplicables (veure Taula 4). Els virus habitualment presents en aigües residuals són petits (enterovirus de 20-30 nm fins a adenovirus de 70-80 nm) i requereixen de tècniques de microscòpia electrònica per a la seva visualització. El nombre de virus presents en aigües residuals que disposen de línies cel·lulars capaces de suportar el seu creixement en cultiu és limitat. Es requereix d'una elevada sensibilitat metodològica per detectar la baixa concentració d'aquests virus en mostres ambientals. L'aplicació de mètodes de concentració per tal de recuperar partícules víriques en elevades concentracions a partir de mostres d'aigua ha estat una metodologia àmpliament utilitzada i esdevé un pas necessari a l'hora d'estudiar virus presents en mostres ambientals. Els mètodes immunològics i basats en la detecció d'àcids nucleics, com la reacció en cadena de la polimerasa (PCR), són actualment els mètodes més ràpids, sensibles i específics per la detecció de virus en l'ambient (Metcalf i col., 1995) malgrat que no permeten saber si una partícula vírica és infecciosa i presenten problemes d'inhibició enzimàtica.

**Taula 4.** Mètodes de detecció i identificació de virus (Pina, 2001).

Mètode	Avantatges	Inconvenients
<b>Inoculació en animals</b>	Estudis patogènia	Lent i costós Aplicable a alguns virus
<b>Cultiu cel·lular</b>	Estudis variabilitat Estudis partícules infeccioses	Sensibilitat variable Aplicable a alguns virus Precisa de personal preparat Lent i car Precisa combinar tècniques immunològiques i moleculars
<b>Microscòpia electrònica</b>	Ràpid Informació morfològica	Possibilitat de confusió Baixa sensibilitat
<b>Tècniques immunològiques</b>	Detecció de l'antigen	Baixa sensibilitat
<b>Tècniques moleculars (hibridació, PCR)</b>	Molta rapidesa Elevada sensibilitat Elevada especificitat No requereix infectivitat Permet fer estudis filogenètics Permet fer estudis epidemiològics	Cal conèixer la seqüència No informa sobre viabilitat Precisa personal preparat Precisa infraestructura Sensible a inhibidors enzimàtics

El nostre grup de recerca ha desenvolupat protocols per a la detecció de virus presents en l'ambient: virus de l'hepatitis A i E (VHA i VHE), adenovirus humans (AdH), adenovirus bovins i porcins, virus del grup de Norwalk (Pina i col., 1998b; Formiga-Cruz i col., 2002; Clemente-Casares i col., 2003; Maluquer de Motes i col., sotmès per publicació).

L'objectiu d'aquesta tesi doctoral ha estat el d'adaptar aquesta metodologia a la detecció i estudi en mostres ambientals, especialment aigües residuals urbanes, dels poliomavirus humans JC i BK i de SV40 amb l'objectiu d'estudiar l'excreció d'aquests virus per part de poblacions de diverses àrees geogràfiques i definir els possibles mecanismes de transmissió d'aquests virus.

El potencial efecte cancerígen que SV40 ha pogut causar a la població que va rebre la vacuna de la poliomelitis contaminada ha esdevingut un tema de gran controvèrsia científica i social sobretot per les implicacions que això podria suposar a nivell legal.

Els poliomavirus humans JC i BK s'excreten en l'orina. Sembla plausible pensar que si SV40, que és comunament excretat pels primats infectats, infecta a la població humana hauria de comportar-se de manera similar a JC i BK i probablement ser excretat en orina i/o femta. L'objectiu d'aquesta tesi ha estat el d'adaptar una metodologia ja assajada pel nostre grup de recerca en altres virus presents a l'ambient per tal de detectar poliomavirus en aigües residuals urbanes.

Si SV40 està present en la població i és excretat per aquesta hauria d'estar present, en menor o major concentració, en l'aigua residual urbana. Això permetria saber en quines poblacions circula SV40 i quines són les característiques de les soques excretades.

De la mateixa manera ens vam plantejar analitzar la presència dels poliomavirus humans JC i BK en aquestes mostres ja que no es disposa d'informació de la presència d'aquests virus en l'ambient i recentment també s'han relacionat amb el desenvolupament de tumors en humans. La detecció i caracterització genètica de les soques excretades per la població i detectades en aigües residuals urbanes de diferents àrees geogràfiques així com dades sobre l'estabilitat i capacitat infectiva de les partícules víriques excretades podrien aportar dades interessants sobre l'epidemiologia d'aquests virus i sobre la seva via de transmissió, que roman encara desconeguda.

En resum, els principals objectius d'aquesta tesi doctoral van ser:

- 1) Dissenyar un protocol i uns iniciadors específics per detectar i caracteritzar genèticament JC, BK i SV40 en mostres d'aigua residual urbana mitjançant PCR (reacció en cadena de la polimerasa) després de concentrar les partícules víriques presents en les mostres.
- 2) Analitzar la presència de poliomavirus humans i SV40 en aigua residual urbana de diferents zones geogràfiques.
- 3) Analitzar genètica i filogenèticament les soques víriques detectades.

- 4) Estudiar la potencial transmissió oral dels poliomavirus humans així com l'estabilitat de les partícules víriques en l'ambient i la seva estabilitat a tractaments amb pH àcid.
- 5) Avaluar la infectivitat en cultiu cel·lular de les soques aïllades.

El resultat obtingut durant la realització d'aquesta tesi s'exposen en 4 capítols diferenciats. Els 3 primers capítols consten d'un article publicat o en procés de publicació. En el darrer capítol es presenten les dades que apareixeran en un article en procés de preparació. Els capítols que s'exposen a continuació són:

### **Capítol I. Detecció i caracterització dels poliomavirus presents en aigües residuals urbanes.**

Documenting the Epidemiologic Patterns of Polyomaviruses in Human Populations by Studying their Presence in Urban Sewage.

Sílvia Bofill-Mas, Sonia Pina, Rosina Girones.

Applied and Environmental Microbiology, 2000. 66(1):238-245.

### **Capítol II. Presència i estabilitat de JC i BK en mostres ambientals. Estabilitat dels poliomavirus humans al pH àcid. Implicacions en la seva via de transmissió.**

Potential Transmission of Human Polyomaviruses through the Gastrointestinal Tract after Exposure to Virions or Viral DNA.

Sílvia Bofill-Mas, Meritxell Formiga-Cruz, Pilar Clemente-Casares, Francesc Calafell, Rosina Girones.

Journal of Virology, 2001. 75(21):10290-10299.

### **Capítol III. Anàlisi de les soques de JC excretades. Infectivitat i potencial transmissió oral.**

Analysis of the Excreted JCV Strains and their Potential Oral Transmission.

Sílvia Bofill-Mas, Pilar Clemente-Casares, Eugene O. Major, Blanche Curfman, Rosina Girones.

Acceptat al Journal of Neurovirology, en procés de publicació.

**Capítol IV. Detecció de SV40 en mostres ambientals.**

Detection of SV40 in the Environment of a Naturally Infected Colony of *Cynomolgus* Monkeys.

Sílvia Bofill-Mas, Néstor Albiñana-Giménez, Pamela A. Pipkin, Phil D. Minor, Rosina Girones.

En procés de preparació.

**Documenting the Epidemiologic Patterns of Polyomaviruses in Human Populations by Studing their Presence in Urban Sewage.**

Sílvia Bofill-Mas, Sonia Pina, Rosina Girones.

Applied and Environmental Microbiology, 2000. Vol. 66(1):238-245.

**RESUM**

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**1. Objectius**

Els poliomavirus humans JC i BK fan infeccions persistents al ronyó i són excretats en l'orina. En casos d'immunosupressió poden ocasionar patologies i se'ls ha relacionat amb el desenvolupament de tumors. Fins al moment es desconeix quina és la via mitjançant la qual es transmeten. En el moment d'iniciar aquest estudi no es tenien dades sobre la presència d'aquests virus a l'ambient. El principal objectiu d'aquesta tesi va ser el d'adaptar una metodologia, ja desenvolupada per a la detecció d'altres virus en l'ambient, a la detecció de poliomavirus. Assumint que SV40 és molt similar als poliomavirus humans, si aquest circulés entre la població humana hauria de ser excretat en l'orina i/o femta i ser detectable en mostres d'aigua residual. La detecció de SV40 en aigües residuals tindria importants implicacions a l'hora d'elucidar si aquest virus va ser introduït a la població humana accidentalment mitjançant la vacuna de la polio i si juga algun paper en el desenvolupament de tumors en humans.

Els objectius d'aquest treball van ser:

- Dissenyar el protocol i els iniciadors específics per detectar JC, BK i SV40 en mostres d'aigua residual per PCR (reacció en cadena de la polimerasa) mitjançant un mètode prèviament desenvolupat per detectar altres virus.
- Valorar la sensibilitat del mètode d'anàlisi.
- Analitzar mostres d'aigua residual urbana de diferents àrees geogràfiques d'Europa i Àfrica: Barcelona, Nancy (França), Umeå (Suècia) i Pretòria (Sud-àfrica).

- Analitzar la presència d'adenovirus humans (AdH) com a control positiu de la contaminació fecal humana de les mostres analitzades.
- Caracteritzar genèticament les soques de poliomavirus detectades.

## 2. Material i Mètodes

Es van dissenyar iniciadors específics i en algun cas es van utilitzar iniciadors ja descrits a la bibliografia per analitzar per PCR diferents regions del genoma de JC, BK i SV40 (Taules 1 i 2 de l'article). Es van concentrar les partícules víriques (PV) presents en les mostres per ultracentrifugació, elució de la matèria orgànica en tampó glicina pH 9,5 i una segona ultracentrifugació. Es va dur a terme una extracció d'àcids nucleics mitjançant el mètode de Boom i col. (1990). L'amplificació dels àcids nucleics es va fer per PCR niada i semi-quantificació de la concentració vírica per assaigs de dilució límit.

Els amplicons obtinguts van ser seqüenciats.

Els mètodes de concentració, extracció d'àcids nucleics, PCR niada, seqüenciació i els controls utilitzats en aquests assaigs es descriuen detalladament a la secció de Material i Mètodes de l'article.

## 3. Resultats

- 1) El mètode assajat va resultar ser força sensible per a la detecció de poliomavirus en mostres d'aigua residual. Els resultats de les proves de sensibilitat indiquen que es detecten 5 PV de JC i de 1 a 10 PV de SV40. Els iniciadors dissenyats van resultar ser altament específics i no presentar detecció creuada entre les 3 espècies de poliomavirus que es volien estudiar.
- 2) Un 93% de mostres de Barcelona van ser positives per AdH i JC. Un 62.5% van ser positives per BK i cap per SV40 (Taula 4 de l'article). Les mostres recol·lectades a Pretoria, Nancy i Umeå van resultar ser positives per tots els virus analitzats excepte per SV40 (Taula 5 de l'article). La concentració de JC estimada en les mostres va ser de  $10^2$ - $10^4$  PV/4 ml d'aigua residual. La de BK



va ser de  $10^1$ - $10^3$  PV/4 ml d'aigua residual i la d'AdH de  $10^1$ - $10^4$  PV/4 ml d'aigua residual.

- 3) Es va seqüenciar la regió intergènica (RI) dels JC detectats i es va construir un arbre filogenètic usant seqüències prototip dels diferents tipus i subtipus descrits fins aquell moment (Taula 3 de l'article). L'arbre resultant indica una correspondència clara entre el genotip al que pertanyen els JC presents en les mostres analitzades i el seu origen geogràfic (com també s'aprecia a la Taula 6 de l'article). Cal destacar que els JC presents en 2 de les mostres de Pretoria eren genotípicament diferents: un similar a soques nord-africanes i per tant més similar a soques europees (Figura 3 de l'article) i un més similar a soques sub-saharianes.
- 4) Es va seqüenciar la regió reguladora (RR) dels JC presents en 7 mostres d'aigua residual de diferents àrees i en mostres clíniques de líquid cefaloraquidi (LCR) de 2 malalts de SIDA. Les soques presents en aigua residual presentaven una organització arquetípica d'aquesta regió tal i com s'ha descrit per soques aïllades d'orina mentre que les presents en LCR presentaven reorganitzacions de la RR (Figura 4 de l'article) tal com s'ha descrit per soques tipus PML.
- 5) Es va seqüenciar la regió VP1 de 5 mostres positives per BK. Es van observar diferències d'un 0.8-2% en el total dels 248 nucleòtids analitzats (Figura 5 de l'article).
- 6) No es va detectar SV40 en cap de les mostres analitzades. Això indicaria absència del virus en les mostres analitzades o presència en concentracions molt baixes, indetectables mitjançant el mètode aplicat.

Aquest resultat es troben més àmpliament descrits i discutits a les seccions de Resultats i Discussió de l'article.

#### **4. Conclusions**

- 1) Els poliomavirus humans són excretats amb elevada prevalència en les aigües residuals urbanes per poblacions de diferents zones geogràfiques d'Europa i Àfrica. Les concentracions d'aquests virus en aquestes mostres són elevades representant el grup de virus més abundant, dels estudiats fins al moment, en aigües residuals.
- 2) Les soques de JC detectades en mostres d'aigua residual presenten RR arquetípiques i les seves regions intergèniques es correlacionen amb el seu origen geogràfic.
- 3) SV40 no està present en les aigües residuals urbanes o bé hi està present en concentracions inferiors a 10 PV/ml.
- 4) El poliomavirus humà JC podria ésser utilitzat juntament amb els AdH com a indicadors de contaminació fecal d'origen humà.

# Documenting the Epidemiologic Patterns of Polyomaviruses in Human Populations by Studying Their Presence in Urban Sewage

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**This is the first description, to our knowledge, of the distribution of human polyomavirus and simian virus 40 (SV40) in urban sewage. Using a nested-PCR procedure, we report the detection of human polyomaviruses JC virus (JCV) and BK virus (BKV) but not SV40 in a high percentage of urban sewage samples obtained from widely divergent geographical areas in Europe and Africa. For a total of 28 samples analyzed, JCV was detected in 26, BKV was detected in 22, and none was positive for SV40. All geographical areas showed a high prevalence of these viruses with mean estimated values of JC viral particles per ml on the order of  $10^3$  in Barcelona (Spain) and Nancy (France) and  $10^2$  in Pretoria (South Africa) and Umeå (Sweden) and mean values of BK viral particles on the order of  $10^2$  in Pretoria and Barcelona and  $10^1$  in Nancy and Umeå. This compares with estimated mean values of  $10^2$  to  $10^3$  for human adenovirus that was evaluated as a control. Nucleotide sequence analysis of the amplified DNA from some of the samples is also presented and represents the sequence of the most abundant JC and BK viral strains in these samples. The nucleotide sequence of the JCV detected was also analyzed in a phylogenetic study and for genomic characterization in the regulatory region. This study has shown that human polyomaviruses are spread in high concentrations in the sewage of different geographical areas and are present in contaminated environments. The frequency and concentration of JCV detected in the environment and the absence of described animal hosts suggest that JCV may be useful as a marker for fecal pollution of anthropogenic origin. The results also support the idea previously described that the strains of JCV are closely related to the ethnic origin of the population studied. The procedure applied should also be useful in future studies of population patterns of viral excretion and as a tool in epidemiological studies for the detection of changes in the prevalence of specific viral pathogens.**

JC virus (JCV) and BK virus (BKV) are human viruses classified in the genus *Polyomavirus* of the family *Papovaviridae*. JCV is etiologically associated with a fatal demyelinating disease known as progressive multifocal leukoencephalopathy (PML) which has emerged as a frequent complication of AIDS in human immunodeficiency virus-infected individuals (9). Infection with BKV has been associated with diseases of the urinary tract including hemorrhagic cystitis and ureteral stenosis (8). Most of the primary infections with JCV and BKV occur early in childhood and are asymptomatic. In this regard, recent data suggest that some BKV infections may be transplacental (30). Human infections with JCV appear to be population associated in that the genotype of JCV excreted by individuals of defined ethnicities is in high proportion determined by the geographical origin of the ethnic group rather than the JCV genotypes that are prevalent in their current location (5). The latent infections established by these viruses persist indefinitely in infected individuals (37).

Simian virus 40 (SV40) is a simian virus that is closely related to JCV and BKV. This polyomavirus establishes latent infections in nonhuman primates and has been associated with a PML-like condition in simian immunodeficiency virus (SIV)-infected monkeys (19). SV40 has been transmitted to humans experimentally, and evidence that SV40 may be circulating in the human population is accumulating (13). Large numbers of humans were exposed to SV40 when inoculated with the polio

vaccine prepared in rhesus monkey cells between 1955 and 1961. Several recent studies have described the detection of SV40-like DNA in tumors from children and young adults who were born after SV40 was removed from the polio vaccine (11, 14, 15, 25, 26, 38). These tumors include choroid plexus tumors, ependymomas, and osteosarcomas. SV40-like DNA sequences have also been detected in mesotheliomas in adults who could have been exposed to SV40-contaminated polio vaccines (14).

JCV, BKV, and SV40 are nonenveloped virions containing double-stranded, closed-circular DNA genomes of approximately 5 kb. The genomes of these viruses have a common organizational structure and are homologous over about 75% of their respective nucleotides (37). Each of these viruses has been found to be oncogenic when injected into rodents, and each has the capacity to transform rodent and human cells in tissue culture (37). Their oncogenic capacity, their persistently latent state in infected tissues, and their association with some types of tumors make these viruses potential human tumor viruses (37).

To provide an independent means of assessing the population association of JCV and the regional prevalence of JCV, BKV, and SV40 or any of their genotypes, an analysis of the relative viral output of an entire community or locality could be useful. Since JCV and BKV are excreted in the urine and SV40 has been found in the feces of infected primates and humans, it seemed reasonable to assume that if a significant percentage of the human population were shedding these viruses in their excreta, then all three should be present and possibly detectable in urban sewage. In addition to the potential use of polyomavirus contamination as a marker of human waste in water

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TABLE 1. Oligonucleotide primers used for PCR amplification and sequencing of human Ad and BKV<sup>a</sup>

Virus type (region)	Position (nt)	Amplification reaction	Primer	Product size (bp)	Annealing temp (°C)	Sequence
Ad2 (hexon)						
Ad40 (hexon)	18858–18883	First-left	HL	301	55	5'-GCCGCAGTGGTCTTACATGCACATC-3'
Ad41 (hexon)	19136–19158	First-right	HR			5'-CAGCACGCCCGCGATGTCCAAAGT-3'
Ad2 (hexon)	18937–18960	Nested-left	NHL	143	55	5'-GCCACCGAGACGTACTIONCAGCCTG-3'
Ad2 (hexon)	19051–19079	Nested-right	NHR			5'-TGACAGTACGCGGTATCCTCGCGGTC-3'
BKV, JCV (VP2/VP3)	1352–1367	First-left	BJ1	765	46	5'-TATTGCMCCAGGAGGT-3'
BKV, JCV (VP1)	2132–2148	First-right	BJ2			5'-AACATTTTCYCCCTCTG-3'
BKV (VP1)	1762–178	Nested-left	BK4	290	50	5'-AGTAGATTTCCACAGGTTAG-3'
BKV (VP1)	1486–1506	Nested-right	BK6			5'-CCAGGGGAGCTCCAAAAG-3'
BKV (VP1)	2132–2148	Seminested-left	BK2	388	46	5'-AACATTTTCCTCTG-3'
BKV (VP1)	1758–1776	Seminested-right	BK5			5'-GGACCTAACCTGTGGAAT-3'

<sup>a</sup> M = A + C; Y = C + T. For Ad, the sequence positions are referred to the Ad2 hexon region; for BKV and JCV, the sequence positions are referred to the BKV-Dunlop sequence.

sources and other environmental locations, being able to detect and study these viruses in sewage would provide a unique opportunity to evaluate and monitor over time those strains that are prevalent in specific geographical areas. Furthermore, the detection of SV40 in sewage would be reasonable evidence that SV40 is circulating in the human population.

In a previous study (32–34), we have developed nested-PCR procedures for the amplification of viral nucleic acids to detect human adenovirus (human Ad), enteroviruses, and hepatitis A and E viruses in sewage, environmental samples, and shellfish. The aim of this study was to apply the experience gained from our previous work to initiate an assessment of the presence of JCV, BKV, and SV40 in sewage of different geographical areas. For this study, we have adapted these methodologies to look for JCV, BKV, and SV40 in urban sewage. Using our

previous data (33) on the prevalence of human Ad in sewage as a point of reference, we have detected JCV and BKV but not SV40 in urban sewage samples from widely divergent geographical locations and have developed sequencing data for the most prevalent strains of JCV and BKV that are present in these samples.

MATERIALS AND METHODS

**Viruses.** SV40 DNA strain 776 (Gibco BRL) and viral particles of SV40 strain WT 800, kindly donated by Ferran Azorin from the Institute Juan de la Cierva, Consejo Superior de Investigaciones Científicas, Barcelona, Spain, were used in this study as positive controls. A urine sample from a healthy 38-week-pregnant woman was tested for the presence of JCV and BKV and found to be positive for both viruses. The viruses present in 12 ml of urine were concentrated by ultracentrifugation, suspended in 100 µl of phosphate-buffered saline (PBS), and used as a positive control. Clinical samples of cerebrospinal fluid (CSF) from PML

TABLE 2. Oligonucleotide primers used for PCR amplification and sequencing of JCV and SV40

Virus type (region) <sup>a</sup>	Position (nt) <sup>b</sup>	Amplification reaction	Primer	Product size (bp)	Annealing temp (°C)	Sequence
JCV (VP1 region)	1710–1734	Nested-left	JLP15	215	63	5'-ACAGTGTGGCCAGAATCCACTACC-3' <sup>c</sup>
JCV (VP1 region)	1902–1924	Nested-right	JLP16			5'-TAAAGCCTCCCCCAACAGAAA-3' <sup>c</sup>
JCV (IG region)	2062–2087	First-left	EP1A	737	59	5'-TGAATGTTGGGTTCTGATCCACC-3'
JCV (IG region)	2774–2798	First-right	EP2A			5'-ACCCATTCTTGACTTTCCTAGAGAG-3'
JCV (IG region)	2099–2124	Nested-left	P1A	668	59	5'-CAAGATATTTTGGGACACTAACAGG-3' <sup>d</sup>
JCV (IG region)	2742–2766	Nested-right	P2A			5'-CCATGTCCAGAGTCTTCTGCTTCAG-3' <sup>d</sup>
JCV (IG region)	2511–2536	Nested-right	JCSR	478	55	5'-TGATTACAGCATTTTGTCTGCAAC-3'
JCV (IG region)	2364–2388	Nested-left	JCSL			5'-GGAAGTCCCTTCTGTTAATTAATCAG-3'
JCV (R region)	4992–5011	First-left	JR1	586	55	5'-CCCTATTTCAGCACTTTGTCC-3' <sup>e</sup>
JCV (R region)	428–447	First-right	JR2			5'-CAAACCACTGTGTCTCTGTCC-3' <sup>e</sup>
JCV (R region)	5060–5079	Nested-left	JR3	382	55	5'-GGGAATTTCCCTGGCCTCCT-3' <sup>e</sup>
JCV (R region)	297–317	Nested-right	JR4			5'-ACTTTTCACAGAAGCCTTACG-3' <sup>e</sup>
SV40 (TAg)	2614–2633	First-left	SV1	336	46	5'-AATTTGTGATGCTATTGCTT-3'
SV40 (TAg)	2932–2950	First-right	SV2			5'-TTGGAGTTTGTAGAAAGGCT-3'
SV40 (TAg)	2746–2766	Nested-left	SV3	157	50	5'-CTACAATGTGGTATGGCTGA-3'
SV40 (TAg)	2883–2903	Nested-right	SV4			5'-AGCCAGGAAAATGCTGATAA-3'
SV40 (TAg)	2609–2631	First-left	SV1A	336	55	5'-TGTGAAATTTGTGATGCTATTGC-3'
SV40 (TAg)	2922–2945	First-right	SV2A			5'-GTTTTAGATTGGCTAAGAAACAGT-3'
SV40 (TAg)	2746–2766	Nested-left	SV5	119	50	5'-CTACARATGTGRTATGGCTGA-3' <sup>f,g</sup>
SV40 (TAg)	2844–2864	Nested-right	SV6			5'-AAGAACATGGAAGACTCAGGG-3' <sup>f</sup>

<sup>a</sup> VP1, virion protein 1; TAg, T antigen.

<sup>b</sup> For JCV, the sequence positions are relative to the JCV-Mad-1 sequence; for SV40, the sequence positions are relative to a standard SV40 sequence.

<sup>c</sup> From the work of Agostini et al. (5).

<sup>d</sup> Modified from the work of Kunitake et al. (23).

<sup>e</sup> From the work of Monaco et al. (27).

<sup>f</sup> From the work of Lednicky et al. (24).

<sup>g</sup> R = A + G.

TABLE 3. List of the JCV types used in the phylogenetic analysis<sup>a</sup>

Genotype	Strain no.	GenBank accession no.	Reference
Type 1 (Europe)			
1A	Mad-1	JO227	17
1A	124	AFO15526	3
1B	123	AFO15227	4
Type 2 (Asia)			
2A	Tokyo-1	AFO30085	4
2A	224	AFO15529	4
2A	225	AFO15530	4
2A	226	AFO15531	4
2B	223	AFO15532	4
2B	227	AFO15533	4
2C	228	AFO15534	4
2C	229	AFO15535	4
2D	230	AFO15536	4
Type 3 (Africa)			
3A	308	U73500	1
3A	312	U73502	1
3B	311	U73501	1
Type 4 (European and African American)			
	402	AFO15528	2
Type 5 (minor European)			
	501	AFO15684	4
Type 6 (West Africa)			
	601	AFO15537	Unpublished data
Type 7 (Southeast Asia)			
	Tai-3	U61771	28

<sup>a</sup> From the work of Jobes et al. (20).

patients were kindly donated by José Luis Pérez from the Microbiology Department of the Hospital de Bellvitge, Barcelona, were tested for the presence of JCV by nested PCR, and were used as a positive JCV control and for comparative sequence analysis between viral strains of clinical and environmental origin. Ad type 2 (Ad2) (prototype) was grown on A549 cells, and then viruses were partially purified and stored at  $-80^{\circ}\text{C}$ .

**Sewage samples.** A total of 28 raw sewage samples from different geographical areas were analyzed. Sixteen samples were collected in the sewers of Barcelona

TABLE 4. Nested-PCR results in urban sewage samples collected in Barcelona<sup>a</sup> (site 1)

Sample and date of collection (day-month-yr)	HAd	BKV	JCV	SV40
BCN1 (29-09-97)	+ (-1)	+ (-1)	+ (-2)	-
BCN2 (07-10-97)	+ (-1)	+ (-1)	+ (-2)	-
BCN3 (21-10-97)	+ (-1)	+ (-2)	+ (-3)	-
BCN4 (28-10-97)	+ (0)	+ (-1)	NT	-
BCN5 (07-11-97)	+ (-1)	+ (0)	+ (-1)	-
BCN6 (11-11-97)	+ (-1)	+ (-1)	+ (-1)	-
BCN7 (19-11-97)	+ (-2)	+ (-1)	+ (-2)	-
BCN8 (05-12-97)	+ (-2)	+ (-1)	+ (-2)	-
BCN9 (17-12-97)	-	-	-	-
BCN10 (30-12-97)	+ (-2)	+ (-1)	+ (-3)	-
BCN11 (09-01-98)	+ (0)	-	+ (-1)	-
BCN12 (12-01-98)	+ (-2)	+ (0)	+ (-3)	-
BCN13 (16-01-98)	+ (-1)	+ (0)	+ (-3)	-
BCN14 (23-01-98)	+ (-2)	-	+ (-2)	-
BCN15 (02-02-98)	+ (-2)	-	+ (-1)	-
BCN16 (11-02-98)	+ (-2)	-	+ (-3)	-

<sup>a</sup> Results obtained after nested-PCR analysis of 4 ml of sewage. HAd, human Ad; +, positive; -, negative; NT, not tested. Numbers in parentheses after results are the highest decimal dilutions positive by nested PCR.

TABLE 5. Nested-PCR results in urban sewage samples from different countries<sup>a</sup>

Sample	HAd	BKV	JCV	SV40
Site 2 (Umeå)				
UMEA1	+ (-2)	+ (0)	+ (-2)	-
UMEA2	+ (0)	-	+ (-1)	-
UMEA3	+ (0)	+ (0)	+ (-2)	-
UMEA4	+ (-1)	+ (0)	+ (-1)	-
Site 3 (Nancy)				
NANCY1	+ (-2)	+ (-1)	+ (-3)	-
NANCY2	+ (-2)	+ (-1)	+ (-3)	-
NANCY3	+ (-1)	+ (-1)	+ (-3)	-
NANCY4	+ (-2)	+ (0)	+ (-2)	-
Site 4 (Pretoria)				
PRETORIA1	+ (-1)	+ (-2)	+ (-2)	-
PRETORIA2	+ (-3)	+ (-1)	+ (-1)	-
PRETORIA3	+ (-3)	+ (-1)	+ (-1)	-
PRETORIA4	+ (-2)	+ (-1)	+ (-1)	-

<sup>a</sup> Results obtained after nested-PCR analysis of 4 ml of sewage for sites 2 and 3 and 1.2 ml of sewage for site 4. HAd, human Ad; +, positive; -, negative. The numbers in parentheses are the highest decimal dilutions positive by nested PCR.

(Spain) and were found to contain a mean of  $1.7 \times 10^6$  fecal coliform bacteria/100 ml with a variance of  $5.6 \times 10^5$ . These samples were collected from September 1997 to February 1998. Each sample was collected in sterile 500-ml polyethylene containers, kept at  $4^{\circ}\text{C}$  for less than 8 h until the viral particles were concentrated in PBS, and stored at  $-80^{\circ}\text{C}$ .

Four samples were collected in Umeå, Sweden, during September and October 1997. Four samples were collected in Nancy (France) during March 1998, and four samples were collected in Pretoria (South Africa) in October 1997. The sewage samples collected in Umeå and Nancy were stored at  $-80^{\circ}\text{C}$  and shipped to Spain, where they were analyzed. From each sewage sample collected in Pretoria, 30-ml aliquots were centrifuged ( $229,600 \times g$  for 1 h at  $4^{\circ}\text{C}$ ) and the 2-ml sediments were shipped to Spain at room temperature immediately (within 24 h) after being processed.

**Concentration of viral particles and nucleic acid extraction.** The method applied for the recovery of viral particles and nucleic acid extraction was based on previous studies (18, 33). Briefly, 40 ml of sewage sample was ultracentrifuged ( $229,600 \times g$  for 1 h at  $4^{\circ}\text{C}$ ) to pellet all the viral particles together with any suspended material. From concentrated Pretoria samples, 2 ml was resuspended in 6 ml of  $2 \times \text{PBS}$  and ultracentrifuged ( $229,600 \times g$  for 1 h at  $4^{\circ}\text{C}$ ). The next step in the treatment of all the sewage samples was the elution of the sediment 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  $12,000 \times g$  for 15 min after the addition of 5 ml of  $2 \times \text{PBS}$ . The viruses were finally pelleted by ultracentrifugation ( $229,600 \times g$  for 1 h at  $4^{\circ}\text{C}$ ), resuspended in 0.1 ml of  $1 \times \text{PBS}$ , and stored at  $-80^{\circ}\text{C}$ . Since the DNA extracted from 10  $\mu\text{l}$  of the viral concentrate corresponded to a sample volume of 4 ml and was used as inoculum for the PCR test, the results will be reported in reference to this volume. According to

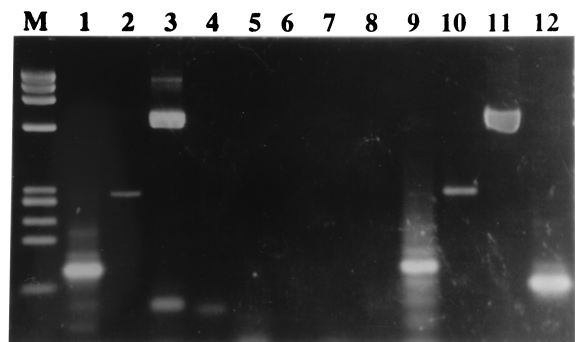


FIG. 1. Agarose gel electrophoresis showing amplified DNA after nested PCR of one sewage sample that was positive for human Ad, BKV, and JCV and negative for SV40 (lanes 1, 2, 3, and 4, respectively). Lanes 5, 6, 7, and 8 are the corresponding negative controls. Lanes 9, 10, 11, and 12 are positive controls. Lane M, molecular weight standard,  $\phi\text{X174 HaeIII}$  digest.

previous studies, the yield after adding poliovirus type 1 LSc 2ab in sewage samples with similar conditions was 70% PFU.

Viral nucleic acids were extracted by a procedure that provides clean nucleic acids for molecular studies. This procedure uses guanidinium thiocyanate and adsorption of the nucleic acids to silica particles (12).

**Sensitivity of the method.** Raw urban sewage samples were spiked with WT 800 SV40 viral particles (10<sup>5</sup>/ml) in order to evaluate the sensitivity of virus extraction and the presence of inhibitors for the PCR. The polyomavirus-like particles of a urine sample positive by PCR for JCV and BKV were quantified by transmission electron microscopy (10, 31). Briefly, 10 ml of urine was harvested directly onto electron microscope grids (400-mesh Ni grids) supported with carbon-coated Formvar film by ultracentrifugation (197,500 × g for 90 min) with a swing-out rotor. Afterwards, the supernatant was removed, the grids were air dried, and the sample was stained with 3% uranyl acetate for 20 s. Polyomavirus-like particles were counted in 200 randomly selected fields of view, by using a Hitachi-600AB transmission electron microscope at 75 kV and 80,000× magnification. Serial dilutions were used to estimate the sensitivity of the detection method.

**Enzymatic amplification.** For the detection of the specific viral genomes in raw sewage, urine, or CSF, 10-μl aliquots of the extracted nucleic acids were used in each test, corresponding to 4 ml of sewage sample, 10 μl of urine, or 10 μl of CSF.

Amplification was 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<sub>2</sub>, 200 μM (each) deoxynucleoside triphosphate, 2 U of Ampli Taq DNA polymerase (Perkin-Elmer Cetus), and the corresponding primers at their corresponding concentrations (0.08 μM external primers and 0.07 μM internal primers for Ad amplification and 0.5 μM external and internal primers for all polyomavirus amplifications). Thermal cycling of the amplification mixture was performed in a programmable heat block (Gene Amp PCR System 2400; Perkin-Elmer). In all PCR assays for human Ad detection, the first cycle of denaturation was carried out for 4 min at 94°C. The conditions for the 29-cycle amplification were as follows: denaturing at 92°C for 60 s, annealing at 55°C for 60 s, and extension at 72°C for 75 s. All amplifications were completed with a 4-min, 72°C extension period. The PCR amplifications of Ad genomes were carried out with external primers HR and HL and internal primers NHR and NHL, described and tested in previous studies (6, 7).

In all PCR assays for polyomavirus detection, the first cycle of denaturation was carried out for 4 min at 94°C. The conditions for the 29-cycle amplification were as follows: 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. Primers and annealing temperatures used in this study are described in Tables 1 and 2.

The PCR amplifications of SV40 genomes were carried out with external primers SV1 and SV2 and internal primers SV3 and SV4. External primers SV1A and SV2A and internal primers SV5 and SV6 (Table 2), which detect the SV40 variants that were described for primates by Lednický et al. (24), were used for analyzing all the samples from Pretoria, Nancy, and Umeå and four samples from Barcelona (BCN11, -12, -14, and -15). JCV genomes were amplified with BJ1 and BJ2 as external primers and JLP15 and JLP16 as internal primers (5). All the samples were also analyzed for JCV with EP1A and EP2A as external primers and P1A and P2A as internal primers (23). This second set showed higher sensitivity for the amplification of JCV genomes.

BKV genomes were amplified with BJ1 and BJ2 external primers and BK4 and BK6 internal primers. All the primers used in this study are represented in Tables 1 and 2.

The results were analyzed by agarose gel electrophoresis with ethidium bromide as a stain.

**Quality control of the amplification method.** To reduce the probability of sample contamination by amplified DNA molecules, standard precautions were applied in all the manipulations. Separate areas of the laboratory were used for reagents, treatment of samples, and manipulation of amplified samples. All the samples were analyzed twice in independent experiments, and a negative control was added every two samples (a negative control is an amplification reaction mixture with the same reagents as in the test tubes of the samples but without the inoculum of viral nucleic acids). Treatment with uracil-DNA-glycosylase for the degradation of amplified material that could contaminate the samples was performed in previous studies (34), but they were not considered necessary for the routine analysis. Direct extract and a 10-fold dilution of the nucleic acid extracts were analyzed routinely on highly polluted samples in order to avoid false negatives because of inhibition of the reactions. This could occur only in a minority of the samples, according to the positive results observed previously with sewage samples supplemented with viruses.

**Analysis of the viral genomes.** The amplicons of 12 JCV-positive samples were sequenced with the primers for amplification of the intergenic (IG) region, P1A, P2A, JCSR, and JCSL (23). Nine of these 12 JCV-positive samples were also sequenced with primers for amplifying the regulatory (R) region, JR1, JR2, JR3, and JR4 (27). The amplicons of five BKV-positive samples were also sequenced with BK2 and BK5 primers.

Nested-PCR products were purified with the QUIAquick PCR purification kit (Qiagen, Inc.). Both strands of the purified DNA amplicons were sequenced with the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit with

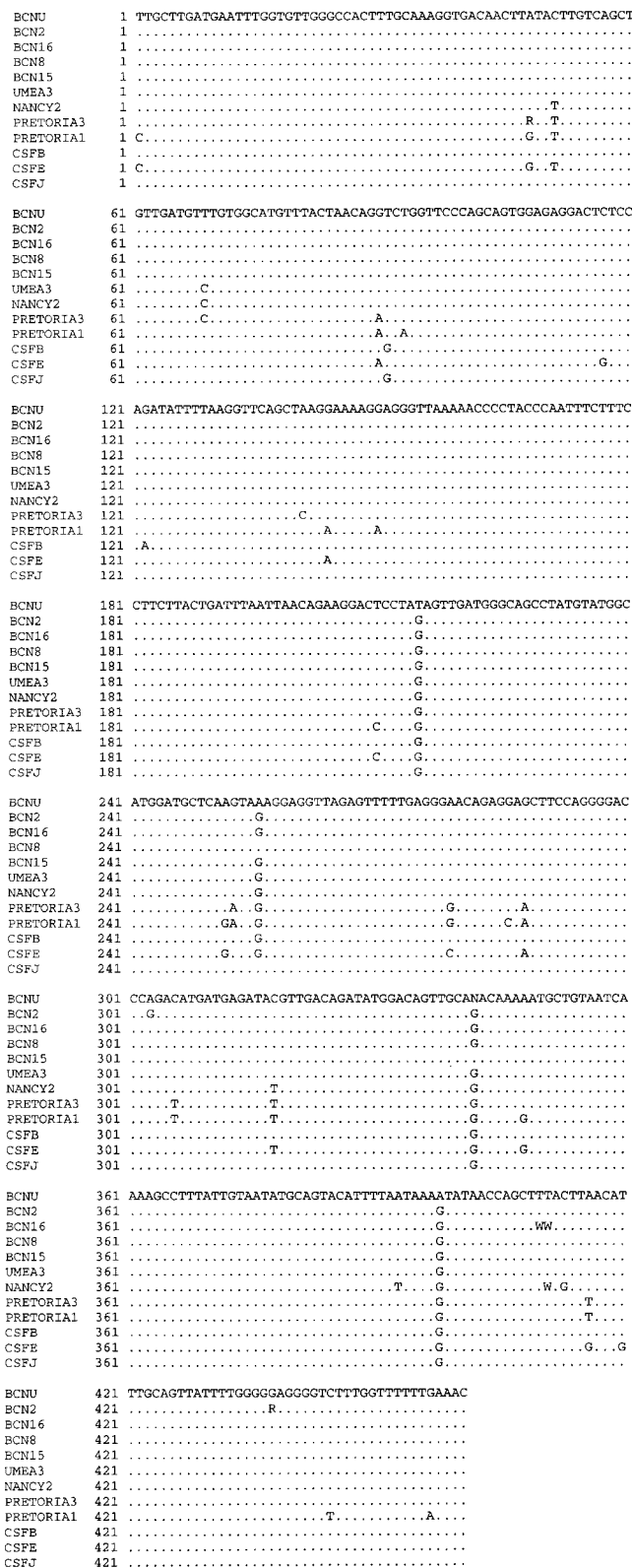


FIG. 2. Sequence alignment of the IG regions of 12 JCV-positive samples. Dots indicate sequence identities. R = A + G; W = A + T.

TABLE 6. Comparison of the sequence of the JCV detected with the IG primers with previously described isolates

Sample	Most closely related isolate(s) <sup>a</sup>		Nucleotide difference from the most closely related strain (no. of nucleotides different/total no. of nucleotides)
	Strain	City and country	
BCNU	UK-2	London, United Kingdom	4/461
	IT-1	Rome, Italy	4/461
BCN2	UK-2	London, United Kingdom	2/461
BCN8	UK-2	London, United Kingdom	1/461
BCN15	IT-1	Rome, Italy	1/461
	UK-2	London, United Kingdom	1/461
BCN16	UK-2	London, United Kingdom	2/461
NANCY2	RS-3	Novosibirsk, Russia	4/461
	G-5	Illertisen, Germany	4/461
	HU-5	Budapest, Hungary	4/461
	CR-5	Prague, Czech Republic	0/461
	SO-3	Welkom, South Africa	1/461
UMEA3	SO-1	Welkom, South Africa	1/461
	MA-4	Nouakchott, Mauritania	6/461
PRETORIA1	MA-5	Nouakchott, Mauritania	6/461
	MA-1	Nouakchott, Mauritania	6/461
	MR-2	Fez-frane, Morocco	6/461
CSFB	UK-2	London, United Kingdom	2/461
CSFE	N-4	Deventer, The Netherlands	3/461
CSFJ	UK-2	London, United Kingdom	2/461

<sup>a</sup> From the work of Kunitake et al. (23).

Ampli Taq DNA polymerase FS (Perkin-Elmer, Applied Biosystems) following the manufacturer's instructions. The results were checked with the ABI PRISM 377 automated sequencer (Perkin-Elmer, Applied Biosystems). The sequences were compared with the GenBank and EMBL databases by using the BLAST program of the National Center for Biotechnology Information. Sequences were aligned by using the BOXSHADE 3.21 program of the EMBNET.CH. GenBank accession numbers of the JCV sequences used for phylogenetic studies are shown in Table 3.

Phylogenetic analysis of the JCV sequenced regions was performed with the nucleic acid maximum likelihood method (dnaml), version 3.572c, included in the PHYLIP software package (16). The phylogenetic tree was visualized with the TREEVIEW 1.5 program (29). The JCV genotypes included in the phylogenetic analysis are described in Table 3.

**Nucleotide sequence accession numbers.** The sequences reported in this paper have been deposited in the GenBank database under accession no. AF119345 to AF119356 (JCV IG region sequences), AF120240 to AF120242 (JCV R region sequences), and AF120243 to AF120247 (BKV VP1 region sequences).

## RESULTS

**Sewage analysis.** The results obtained are shown in Tables 4 and 5 and reflect a very high level of excretion of human Ad, JCV, and BKV and the absence of positive results for SV40. About 93% of the samples collected in Barcelona were positive for human Ad and JCV, and 10 (62.5%) were positive for BKV. Figure 1 shows the agarose gel electrophoresis of one sewage sample positive for human Ads, BKV, and JCV and negative for SV40; small-size bands, nonspecific or made by amplification of combined primers, can be seen in the gel, as they appear frequently in amplified samples. Samples collected in Pretoria (four samples) and Nancy (four samples) showed positive results for all viruses with the exception of SV40. All four samples collected in Umeå were also positive for human Ads and JCV, and three of these four samples were positive for BKV. From a total of 28 sewage samples analyzed, 96% were positive for human Ads and JCV and 77.8% were positive for BKV. Results of the nested-PCR tests and serial dilution experiments are shown in Tables 4 and 5. Very high numbers of JCV particles are observed, with the concentration estimated as between  $10^2$  and  $10^4$  viral particles per 4 ml of the sewage samples analyzed. On the basis of the results obtained in the

serial dilution experiments, the concentrations estimated for human Ad were  $10^1$  to  $10^4$  viral particles and for BKV were  $10^1$  to  $10^3$  BK viral particles in the 4 ml of the sewage samples analyzed.

**Sensitivity of the method.** In the sewage samples supplemented with SV40 viral particles, the viral genomes were detected by nested PCR with an estimated sensitivity of five genomes compared with the stock suspensions that showed a nested-PCR detection unit five times higher (one genome). The applied nested-PCR procedure with SV40 DNA detected one SV40 genome. This is the level of sensitivity of detection of three different strains of human Ad as described in previous studies (34). The results observed with SV40 DNA agree with the sensitivity described previously for the detection of human Ads and enteroviruses in sewage samples (between 10 and 100 viral particles per 4-ml sample).

The number of polyomavirus-like particles counted in a urine sample by electronic microscopy was  $8.7 \times 10^5$ /ml. According to this, the sensitivity observed after nested PCR in the urine sample is at least 5.2 viral particles/4 ml of JCV and at least 520 BKV particles per 4 ml.

According to these results, we estimate the minimum concentration of viruses producing a positive result in the nested-PCR test for sewage samples to be on the order of 10 viral particles.

**Molecular characterization of the viruses detected.** We sequenced 461 nucleotides of the IG region of 12 JCV-positive samples randomly selected: five samples collected in Barcelona (one from a urine sample), two from Pretoria, one from Nancy, and one from Umeå. Three CSF samples from PML patients from different regions in Spain were also sequenced as positive controls.

The sequences analyzed confirmed the specificity of the nested-PCR amplification, since all viral sequences were identified as the expected polyomavirus.

The alignment of the sequences of the JCV obtained is shown in Fig. 2, and the relationship of the detected strains

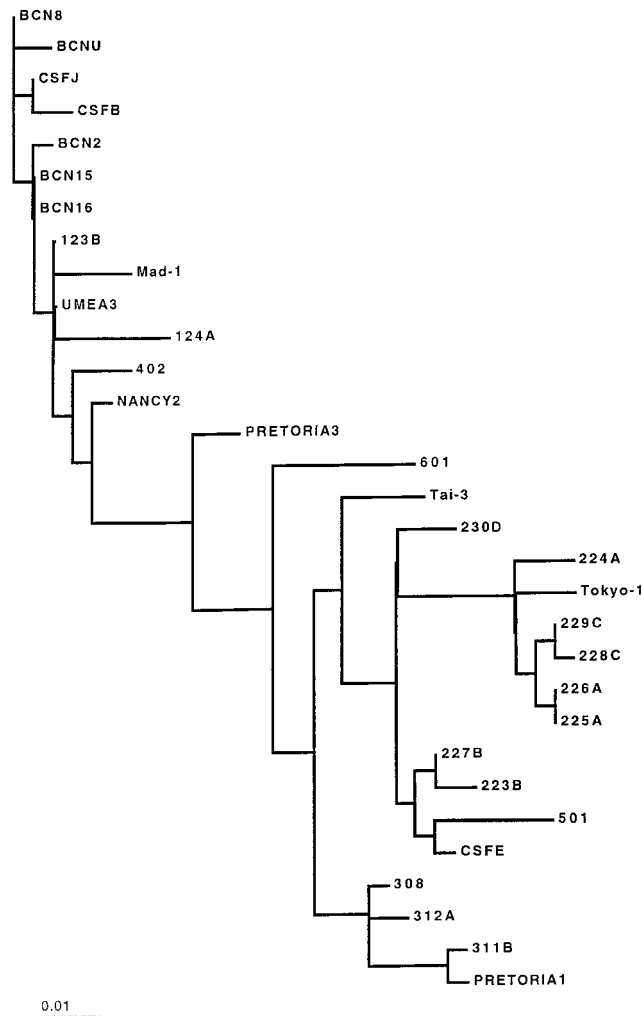


FIG. 3. Phylogenetic analysis of the IG regions of 12 sequenced JCV strains compared with previously described JCV subtypes (Table 3) by the maximum likelihood method.

with the nucleotide sequences of the EMBL and GenBank sequence data banks is shown in Table 6. The results of the maximum likelihood method applied to the phylogenetic analysis are shown in Fig. 3. The analysis of the R region of the JCV shows that all strains detected in sewage and urine samples and sequenced are archetype strains; the JCV obtained from the clinical samples presented specific rearrangements in the regulatory region (Fig. 4).

The nucleotide sequences of positive BKV amplicons from one sample collected in Pretoria, one from Nancy, and three samples from Barcelona were compared. These samples showed between two and four nucleotide differences in a fragment of 248 nucleotides (Fig. 5).

**DISCUSSION**

We are not aware of previously reported data on the presence of human polyomavirus in sewage. In this regard, we have detected high concentrations of JCV and BKV in the sewage of different cities of widely divergent geographical origins and have studied the most prevalent strains of JCV excreted.

For this purpose, we have amplified and sequenced JCV DNA in the region designated the IG region, which encom-



FIG. 4. Sequences of the R regions of JCV from nine analyzed samples. CSFE and CSFB are clinical samples of CSF from two patients. Archetypal sequences are observed in samples BCNU, BCN15, BCN16, BCN8, BCN2, NANCY2, and UMEA3. Underlined nucleotides are identical to the archetypal sequence. Nucleotides in boldface and in italics represent duplications present in these sequences.

passes the 3'-terminal sequences of both T-antigen and VP1 (major capsid protein) genes, since it contains abundant nucleotide variation compared with other regions. These variations have been described as a means of tracing human migrations (5, 39). The strong relationship of the sequenced JCV strains with previously described viral isolates from related populations is remarkable. The sequenced strains described for sewage samples from European countries are strongly related to European subtypes, and the two strains from the Pretoria area are related to African isolates. PRETORIA1 is related to strains isolated from sub-Saharan populations, and PRETORIA3 is more closely related to isolates from Mauritania and North Africa which appear more similar to the European strains, correlating with the relationship of these populations. We also show in this study that the most common JCV strains that are excreted in the areas studied show archetypal R regions, as described for urine samples. In agreement

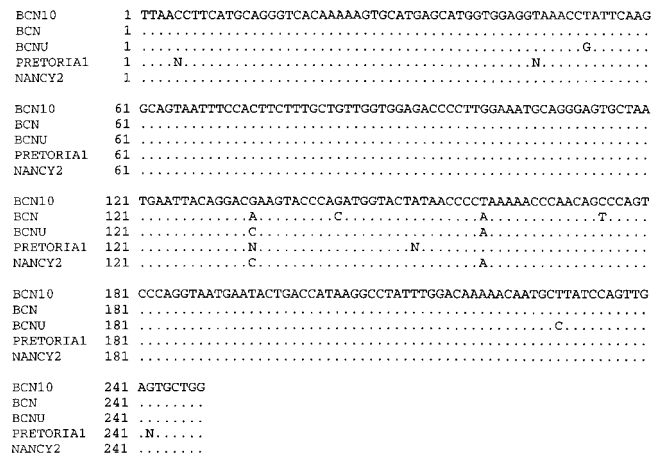


FIG. 5. Sequence alignment of the VP1 regions of five BKV-positive samples. Dots indicate sequence identities.



also with previous studies, the three PML-derived strains detected from CSF samples presented individual rearrangements of these genomic regions (40).

The fact that immunocompetent hosts frequently excrete JCV in urine (21, 36) indicates that renal JCV is not latent under these conditions but replicates to generate progeny that are excreted in urine. Thus, JCV persistence in the kidney may be characterized by continuous viral replication and virus shedding. This would be required for JCV to be transmitted among humans, given that JCV infection has been described as very inefficient (22).

It has also been reported that JCV in urine is infectious, and urine is the most likely source of JCV infection in humans. Although the cells that support JCV replication at portals of entry remain to be identified, some data suggest that tonsil tissue could be a possible site of the initial viral infection (27). BKV seems to circulate independently of JCV in the population. As with JCV, BKV has been detected in kidney but also in tonsils and other tissues as well (35).

The nested amplicons of BKV obtained from four sewage samples and one urine sample were sequenced in order to confirm their identity as BKV. The differences observed in the 248 sequenced nucleotides were around 0.8 to 2% as described in the literature (36). Some indeterminations were observed in nucleotides that showed variability when sequences from the data banks were compared. This is the case for the sample PRETORIA1, suggesting the presence of a mixture of related strains in this sewage sample. Further sequencing studies are required for the identification of the specific BKV genomic subtypes.

In previous studies, we have isolated hepatitis E virus (32), human Ad, and enterovirus (33) from similar sewage samples, and these viruses are infectious. Those polyomaviruses that remain intact as viral particles in sewage may be infectious and could be transmitted to other humans in a fecally polluted environment.

The semiquantitative results for the detection of the polyomavirus indicate the concentration of the viral particles detected. Definitive numbers could be obtained only by quantitative PCR test. The finding of lower titers of BKV than of JCV could be attributable to a lower sensitivity of the PCR amplification and/or to a lower frequency of BKV shedding in immunocompetent persons as described by Shah et al. (36).

We did not detect SV40 in any of these sewage samples. The results indicate that, if SV40 is excreted in human urine or feces, its concentration in the analyzed sewage samples is lower than 10 viral particles in 4 ml of raw sewage.

The high prevalence of human Ad detected in all of these geographical areas is consistent with previous studies carried out in Barcelona, where it was suggested that this parameter could be a good viral indicator of the fecal contamination of human origin in the environment and shellfish (33).

The detection by PCR of polyomaviruses in sewage allows the identification and analysis of the genomes of viral strains that are infecting the population and gives information on the spread, frequency, and distribution of these viruses. These data are also needed for the study of the epidemiology of the related diseases. However, the molecular techniques applied do not give information on the level of infectivity of the strains detected in the sewage samples, and further studies will be needed in order to establish the stability of human polyomavirus in the environment. It is also clear that this approach is likely to provide a method with a higher level of specificity, sensitivity, and speed than isolation of viruses in cell culture. In these experiments, the differences between the nucleotide sequences of the detected genomes and those of the viruses of

our positive controls argue against the possibility of laboratory contamination.

The human polyomavirus has been shown to be present in high concentrations in the sewage of the different geographical areas studied, especially JCV, and its specificity as a human virus may be useful as a marker for fecal pollution of anthropogenic origin. The high level of excretion detected also supports the idea previously described that fecal-oral transmission (including contamination from urine) will probably happen soon in vivo, inside the family or from closely related people and less frequently later in life from other polluted sources.

The procedure that we used to study the presence of viruses in the sewage of a community may be useful as a tool for studying changes in epidemiological patterns of some viral infections and in future studies for the analysis of environmental dynamics.

#### ACKNOWLEDGMENTS

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**Potential Transmission of Human Polyomaviruses through the Gastrointestinal Tract after Exposure to Virions or Viral DNA.**

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**RESUM**

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**1. Objectius**

En l'estudi que es presenta al capítol I es descriu la detecció d'elevades concentracions de poliomavirus humans però no de SV40 en aigües residuals urbanes de 4 zones geogràfiques diferents. Les dades exposades al capítol I són les primeres publicades en relació a la presència de poliomavirus humans a l'ambient i vam creure necessari ampliar aquest estudi a més àrees per fer que els resultat obtinguts prèviament fossin extrapolables. La via de transmissió de JC i BK no es coneix encara, però la detecció d'aquests virus a l'ambient obre noves portes en relació a aquest tema. En aquest moment de l'estudi no coneixíem quina podia ser l'estabilitat d'aquestes partícules víriques a l'ambient ni si el que estàvem detectant eren partícules víriques (PV) senceres o ADN viral. El fet de que aquests virus que s'excreten en l'orina es trobin presents en elevades concentracions en aigües residuals podria implicar una transmissió per la via fecal-oral. Caldria però estudiar la resistència d'aquests virus a l'ambient característic del tracte gastrointestinal (pH àcid) per obtenir dades que fessin plausible aquesta hipòtesi.

Els objectius d'aquest treball van ser:

- Ampliar els punts de mostreig de l'estudi en 3 noves zones : Patras (Grècia), El Caire (Egipte) i Washington D. C. (Estats Units d'Amèrica).
- Ampliar el nombre de mostres analitzades a l'àrea de Barcelona.
- Caracteritzar genèticament les soques de poliomavirus detectades.

- Estudiar l'estabilitat de partícules víriques i/o ADN viral en l'aigua residual.
- Estudiar la presència de poliomavirus humans en mol·luscs bivalves.
- Estudiar l'estabilitat de les partícules de JC i BK a pHs àcids.

## 2. Material i Mètodes

Es van dissenyar iniciadors específics nous per estudiar la regió reguladora (RR) de BK i es van modificar 2 dels iniciadors utilitzats per amplificar la regió VP1 de BK per tal d'augmentar la sensibilitat del mètode de detecció (Taula 1 de l'article). La detecció de poliomavirus en mostres ambientals es va dur a terme de la mateixa manera que en el capítol I. Per tal de detectar poliomavirus en mostres de mol·luscs bivalves es va aplicar un protocol lleugerament modificat que es descriu a la secció de Materials i Mètodes de l'article.

Per a realitzar els estudis d'estabilitat es va modificar la tècnica d'extracció d'àcids nucleics incloent DNasa a l'assaig per tal de diferenciar partícules víriques senceres de l'ADN viral lliure. Els material i mètodes utilitzats es troben descrits detalladament a l'article adjunt.

## 3. Resultats

- 1) Les mostres recol·lectades a Patras, El Caire i Washington D. C. van resultar ser positives per tots els virus analitzats excepte per SV40 (Taula 3 de l'article). La concentració de JC estimada en les mostres va ser de  $10^2$ - $10^4$  PV/ml d'aigua residual. La de BK va ser de  $10^1$ - $10^3$  PV/ml d'aigua residual i la d'AdH de  $10^2$ - $10^3$  PV/ml d'aigua residual. Aquest resultat no difereixen significativament dels obtinguts en les àrees estudiades al capítol I i es poden resumir conjuntament de la següent manera: 51/52 mostres analitzades van ser positives per AdH on s'assolien concentracions de  $10^2$ - $10^3$  PV/ml. Aquesta dada era una indicació clara de que les mostres analitzades contenien contaminació fecal d'origen humà. 50/51 mostres analitzades contenien  $10^2$ - $10^3$  PV/ml de JC i 46/51

contenien  $10^1$ - $10^2$  PV/ml de BK. Cap de les 51 mostres analitzades va ser positiva per SV40.

- 2) De les 10 mostres de mol·luscs bivalves analitzades 7 van ser positives per AdH i 5 van ser positives per JC. Les concentracions d'ambdós virus en les mostres positives van ser de 1-10 PV/g. Totes les mostres van ser negatives per BK i SV40.
- 3) Es va ampliar el nombre de les regions intergèniques (RI) de JC estudiades i es va construir un nou arbre filogenètic amb les soques tipus (Taula 2 de l'article), les descrites al capítol I i les noves soques estudiades (Figura 1 de l'article). Les noves soques estudiades pertanyien al tipus característic de les regions geogràfiques objecte de l'estudi.
- 4) Es va estudiar la regió reguladora (RR) de 4 mostres més d'origen ambiental i es va comprovar que presentaven RR arquetípiques.
- 5) Es van analitzar les seqüències de la RR dels BK presents en 6 mostres d'aigua residual. Tres de les soques estudiades presentaven una estructura arquetípica (WW) i 3 una estructura semblant a l'arquetípica (*archetypal-like* o WWT) (Figura 2 de l'article).
- 6) Es va caracteritzar la regió VP1 dels BK presents en 5 de les mostres, 4 d'ells pertanyien al serotip I, el més comú, mentre que una de les mostres va resultar ser molt semblant, però no idèntica al serotip II de BK (Figura 3 de l'article).
- 7) Les partícules víriques de JC presents en una mostra d'aigua residual van ser estables durant una exposició de 30 minuts a pH 3. L'ADN lliure de JC va ser detectat després de sotmetre aigua residual que contenia JC a un pH de 1 durant 30 minuts i a un pH de 3 durant 90 minuts. Les partícules víriques de BK van resultar ser estables després de patir un tractament a pH 5 durant 30 minuts mentre que l'ADN lliure va ser detectat després de ser sotmès a pH 1 durant 30 minuts.

- 8) Vam estimar una  $t_{90}$  (temps requerit per la degradació del 90% de les partícules víriques) i una  $t_{99}$  (temps requerit per la degradació del 99% de les partícules víriques) de 26,7 i 61.5 dies respectivament per les partícules víriques de JC presents en una mostra d'aigua residual mantinguda a 20°C. Per BK la  $t_{90}$  i la  $t_{99}$  obtingudes en el mateix experiment van ser de 53,6 i 96,8 dies respectivament.

Aquest resultat es troben més àmpliament descrits i discutits a les seccions Resultats i Discussió de l'article.

#### **4. Annex. Anàlisi dels poliomavirus humans presents en mostres ambientals del Nord de la Índia**

Posteriorment a la realització de l'estudi descrit en aquest darrer article es van analitzar 23 mostres ambientals del Nord de la Índia: aquestes mostres comprenien aigües residuals, mostres de rius preses en punts d'abocament d'aigües residuals, i mostres d'aigua de pous recol·lectors d'aigua residual situats fora les ciutats i propers a temples. Les mostres van ser processades i analitzades segons el mètode descrit per l'anàlisi d'aigües residuals.

7/23 mostres van resultar ser positives per JC i 3/23 per BK. Com a control de la contaminació fecal que podien presentar les mostres es van analitzar els adenovirus humans (AdH) presents en les mateixes trobant 11/22 mostres positives. Els resultat obtinguts es representen en la Taula annexa A.

La RI de les 7 mostres de JC detectades van ser seqüenciades i amb aquestes seqüències es va construir un nou arbre filogenètic que comprèn soques de JC de totes les àrees geogràfiques estudiades fins al moment (Figura annexa A).

El percentatge de mostres positives per JC i BK és més baix que l'observat en altres zones geogràfiques estudiades. No obstant, cal considerar que el tipus de mostra és també diferent i si s'observen les dades obtingudes en l'anàlisi dels AdH es podria concloure que només un 50% de les mostres presenten contaminació d'origen fecal humà i que la resta de les mostres o bé no en presenten o la concentració de virus en aquestes és massa baixa per ésser detectada probablement degut a un efecte de dilució de les mostres.

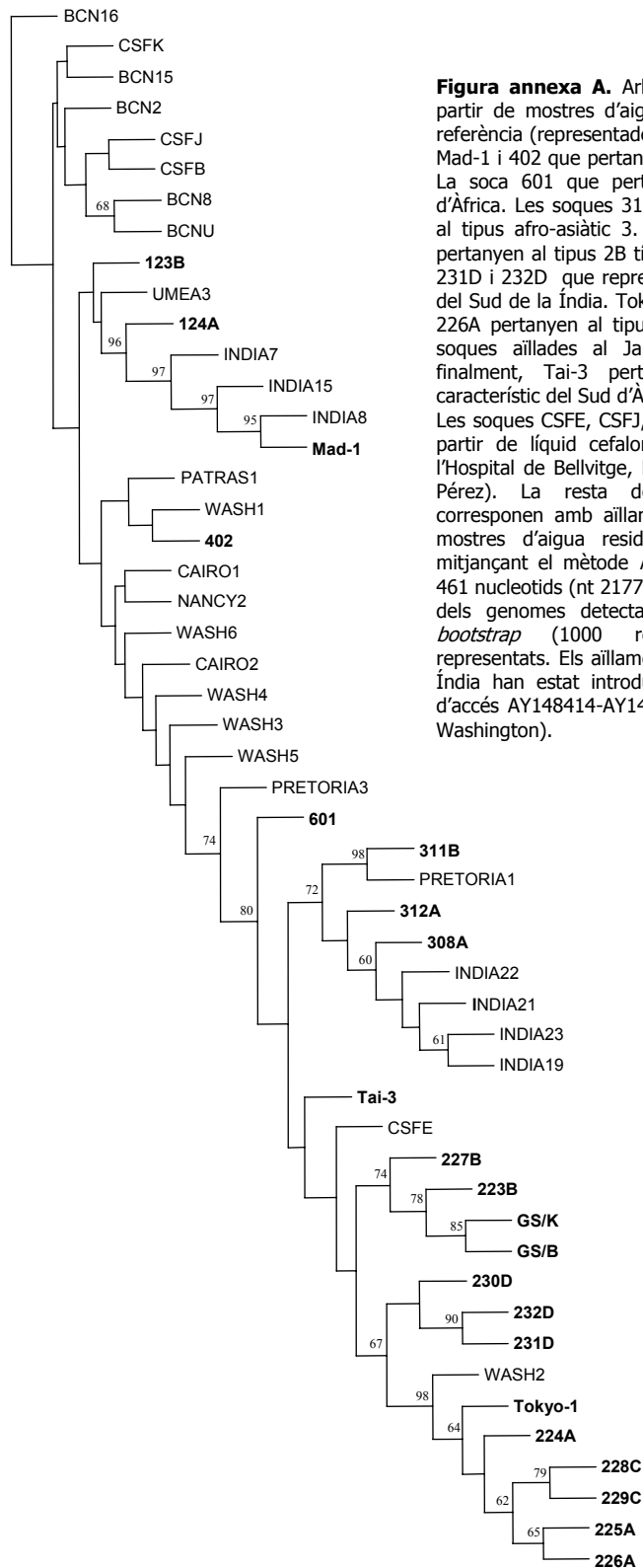
## 5. Conclusions

- 1) Els poliomavirus humans estan presents en un molt alt percentatge i en elevades concentracions en les aigües residuals urbanes de diferents zones geogràfiques d'Europa, Àfrica i Nord Amèrica.
- 2) Mostres ambientals de diferent origen del Nord de la Índia també presenten (en menor percentatge) JC i BK.
- 3) Les soques de JC detectades en l'ambient presenten majoritàriament RR arquetípiques i les seves RI permeten corroborar el seu origen geogràfic.
- 4) Les soques de BK detectades presenten RR arquetípiques o semblants a les arquetípiques i la majoria pertanyen al serotip I.
- 5) Les PV de JC i BK són relativament estables en mostres ambientals i també són relativament estables al pH àcid. Aquestes dades ens van portar a suggerir el tracte gastrointestinal com a possible via d'entrada dels poliomavirus o del seu ADN a l'organisme.

**Taula annex A.** Resultats obtinguts en analitzar mostres ambientals del Nord de la Índia per la presència de AdH, JC i BK. (NT, no testada).

Mostra	Lloc de recol·lecció	Tipus de mostra	Virus detectats		
			AdH	JC	BK
1	Calcuta centre	Aigua residual	-	-	-
2	Calcuta centre	Aigua residual	+	-	-
3	Calcuta centre	Aigua residual	+	-	-
4	Calcuta centre	Aigua residual	+	-	-
5	Calcuta oest	Aigua residual	-	-	-
6	Calcuta oest	Aigua residual	-	-	-
7	Bengala occidental, oest	Pou recol·lector d'aigua residual	+	+	-
8	Bengala occidental, oest	Pou recol·lector d'aigua residual	-	+	-
9	Bengala occidental, centre	Rierol procedent dels temples	+	-	-
10	Bengala occidental, Siligury	Aigua residual	+	-	-
11	Tripura sud	Pou recol·lector d'aigua residual	-	-	-
12	Tripura oest	Aigua residual	-	-	-
13	Tripura, Agartala	Rierols recol·lectors d'aigua residual	-	-	-
14	Tripura, Agartala	Rierols recol·lectors d'aigua residual	-	-	-
15	Banaras	Pou recol·lector d'aigua residual	-	-	-
16	Assam, Guhawati	Riu, punt de descàrrega d'aigua residual	NT	-	-
17	Assam, Guhawati	Riu, punt de descàrrega d'aigua residual	-	+	-
18	Lucknow	Riu Gonoti, punt de descàrrega d'aigua residual	+	-	-
19	Lucknow	Aigua residual	+	+	-
20	Nova Delhi	Riu Jemuna, punt de descàrrega d'aigües residuals i industrials	+	+	+
21	Nova Delhi	Aigua residual	+	+	+
22	Kanpur	Aigua residual	-	-	-
23	Kanpur	Aigua residual	+	+	+





# Potential Transmission of Human Polyomaviruses through the Gastrointestinal Tract after Exposure to Virions or Viral DNA

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**The mechanism of human-to-human transmission of the polyomaviruses JC virus (JCV) and BK virus (BKV) has not been firmly established with regard to possible human exposure. JCV and BKV have been found in sewage samples from different geographical areas in Europe, Africa, and the United States, with average concentrations of  $10^2$  to  $10^3$  JCV particles/ml and  $10^1$  to  $10^2$  BKV particles/ml. Selected polyomavirus-positive sewage samples were further characterized. The JCV and BKV present in these samples were identified by sequencing of the intergenic region (the region found between the T antigen and VP coding regions) of JCV and the VP1 region of BKV. The regulatory region of the JCV and BKV strains found in sewage samples presented archetypal or archetypal-like genetic structures, as described for urine samples. The stability (the time required for a 90% reduction in the virus concentration) of the viral particles in sewage at 20°C was estimated to be 26.7 days for JCV and 53.6 days for BKV. The presence of JCV in 50% of the shellfish samples analyzed confirmed the stability of these viral particles in the environment. BKV and JCV particles were also found to be stable at pH 5; however, treatment at a pH lower than 3 resulted in the detection of free viral DNA. Since most humans are infected with JCV and BKV, these data indicate that the ingestion of contaminated water or food could represent a possible portal of entrance of these viruses or polyomavirus DNA into the human population.**

JC virus (JCV) and BK virus (BKV) are nonenveloped, icosahedral viruses with double-stranded, negatively supercoiled, circular DNA genomes of approximately 5.13 kb. The polyomavirus genome consists of an early region, a late region, and a regulatory region (RR) containing promoters, enhancers, and the replication origin. The genome is transcribed bidirectionally from the origin. It codes for the early region proteins (large T and small t antigens) that regulate the transcription of the late region proteins (VP1, VP2, VP3, and agnoprotein). JCV and BKV are associated primarily with progressive multifocal leukoencephalopathy (PML) and hemorrhagic cystitis, respectively, and a role for these viruses in human cancer has been suggested (23). Both viruses are found at high frequencies throughout most human populations, and their pathogenicity, which is associated primarily with immunocompromised states, has attracted more attention due to the immunosuppression associated with AIDS. As determined by seroconversion, primary infection with BKV occurs early in childhood, while JCV infection occurs slightly more toward adolescence (17, 36, 51). Initial infection is not apparent and rarely causes clinical disease, although respiratory symptoms or urinary tract disease is sometimes found in the case of BKV (18, 21, 37). JCV and BKV can be detected in tonsillar tissue (19, 32), and the hypothesis that the respiratory tract is the primary site of viral infection has been suggested. After the initial infection, the virus disseminates and establishes a persistent infection in renal tissue throughout life (27, 46). The presence of JCV DNA in the upper and lower parts of the

human gastrointestinal tract and particularly in the mucosa of the human colon and colorectal cancers has been recently described (29, 42).

In a previous study, using nested PCR methods that we developed for studying viruses in wastewater (39, 40), we reported the presence of human polyomavirus JCV and BKV DNAs, but not simian virus 40 DNA, in urban sewage (12). In this study, we report the presence of JCV and BKV in sewage from other geographical areas. In addition, we have detected JCV and BKV in shellfish, have evaluated the stability of JCV and BKV virions in the environment, and have begun to determine the genetic characteristics of the strains excreted by populations in different geographical areas. Human adenoviruses (HAd) were used in this study as a control for human contamination of the samples. Also, shellfish were used as biosensors for contamination studies since they filter large volumes of water, concentrating, especially in the digestive tract, any viruses that are present in the water. We believe that viruses excreted in urine and feces are transmitted through what is known as fecal contamination, which includes viruses excreted in feces and urine. Our findings of high levels of JCV and BKV in most sewage samples and the relative stability of these viruses under environmental conditions suggest that the alimentary tract could be an important point of exposure and transmission of these viruses among humans.

## MATERIALS AND METHODS

**Viruses.** BKV and JCV positive controls were obtained from the urine of a healthy woman who had been pregnant for 38 weeks. We also used JCV obtained from cerebrospinal fluid (CSF) samples kindly donated by J. L. Pérez, Microbiology Department, Hospital de Bellvitge, Barcelona, Spain. Adenovirus type 2 (prototype) grown on A549 cells and partially purified was used as a positive control.

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TABLE 1. Oligonucleotide primers used for PCR amplification and sequencing of human polyomaviruses BKV and JCV

Virus type (region)	Position	Primer	Sequence <sup>a</sup>
JCV (IGR)	2062–2087 <sup>b</sup>	EP1A	5'-TGAATGTTGGGTTCTGATCCCACC-3'
JCV (IGR)	2774–2798	EP2A	5'-ACCCATTCTTGACTTTCCTAGAGAG-3'
JCV (IGR)	2099–2124	P1A <sup>d</sup>	5'-CAAGATATTTTGGGACACTAACAGG-3'
JCV (IGR)	2742–2766	P2A <sup>d</sup>	5'-CCATGTCCAGAGTCTTCTGCTTCAG-3'
JCV (IGR)	2511–2536	JCSR	5'-TGATTACAGCATTTTTGTCTGCAAC-3'
JCV (IGR)	2364–2388	JCSL	5'-GGAAGTCCTTCTGTAAATTAATCAG-3'
JCV (RR)	4992–5011	JR1 <sup>e</sup>	5'-CCCTATTCAGCACTTTGTCC-3'
JCV (RR)	428–447	JR2 <sup>e</sup>	5'-CAAACCACTGTGTCTCTGTC-3'
JCV (RR)	5060–5079	JR3 <sup>e</sup>	5'-GGGAATTTCCCTGGCCTTCT-3'
JCV (RR)	298–317	JR4 <sup>e</sup>	5'-ACTTTCACAGAGGCTTACG-3'
BKV (VP1)	1452–1467 <sup>c</sup>	BK1	5'-TATTGCCCCAGGAGGT-3'
BKV (VP1)	2132–2148	BK2	5'-AACATTTTCCCCTCCTG-3'
BKV (VP1)	1762–1781	BK4	5'-AGTAGATTTCCACAGGTTAG-3'
BKV (VP1)	1486–1506	BK6	5'-CCAGGGGCAGCTCCCAAAAAG-3'
BKV (VP1)	1425–1442	BK3	5'-ACTGTAACACCTGCTCTT-3'
BKV (RR)	5024–5043	BR1	5'-CCCTGTTWARRACTTTATCC-3'
BKV (RR)	431–457	BR2	5'-GTAAAGCAGTGTACTTT-3'
BKV (RR)	5083–5102	BR3	5'-ATAGTTTTGCTAGGCCTCAG-3'
BKV (RR)	305–322	BR4	5'-CAACTTTCCTGAAGCTT-3'

<sup>a</sup> W = A or T; R = A or G.

<sup>b</sup> Sequence positions refer to the JCV Mad-1 strain sequence.

<sup>c</sup> Sequence positions refer to the BKV Dunlop strain sequence.

<sup>d</sup> Modified from Kunitake et al. (28).

<sup>e</sup> From Monaco et al. (32).

**Sewage samples.** Twenty-four raw sewage samples from different geographical areas were analyzed. Nine samples (BCN17 to BCN25) were collected from October 1998 to June 2000 in the sewers of Barcelona, Spain. Each sample was collected in a sterile 500-ml polyethylene container, kept at 4°C for less than 8 h until the viral particles were concentrated in phosphate-buffered saline (PBS), and stored at –80°C.

Five samples (G1 to G5) were collected in Patras, Greece, during June and July 1999. Four samples (E1 to E4) were collected in Cairo, Egypt, during July 1999, and six samples (W1 to W6) were collected in Washington, D.C., during December 1999. These samples were collected and shipped, frozen, to Spain, where they were concentrated in PBS and stored at –80°C.

**Shellfish samples.** Six oyster (*Crassostrea gigas*) samples and four mussel (*Mytilus galloprovincialis*) samples were obtained from shellfish-growing in areas with different levels of fecal pollution located in the delta of the Ebro river. Shellfish sample analysis was carried out by following a slightly modified form of the protocol of Pina et al. (39) and Munain-Mujika et al. (33). The digestive diverticulum was the only part of the animals tested in this analysis. These samples were analyzed for the presence of different human viruses, including: hepatitis A virus, enteroviruses, HAd, JCV, and BKV.

**Concentration of viral particles and nucleic acid extraction.** Recovery of viral particles and nucleic acid extraction were carried out as described previously (40). Briefly, 40-ml sewage samples were ultracentrifuged (229,600 × g for 1 h at 4°C) to pellet all of the viral particles together with any suspended material. The sediment was then 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 4 ml of 2× PBS. 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.

Viral nucleic acids were extracted by using a procedure that uses guanidinium thiocyanate and adsorption of the nucleic acids to silica particles (13), providing clean nucleic acids for molecular studies.

**Enzymatic amplification.** Ten-microliter aliquots of the extracted nucleic acids were used in each test, corresponding to a 4-ml sewage sample, 1 g of shellfish, 1 ml of urine, or 10 µl of CSF, depending on the origin of the samples analyzed. Serial tenfold dilutions were also analyzed in order to carry out a semiquantitative analysis of the samples studied by limiting-dilution experiments. Knowing the sensitivity of the detection procedure applied, we could estimate the concentration of the sewage samples by considering the lower decimal dilution positive for the nested-PCR assay.

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<sub>2</sub>, 200 µM each deoxynucleoside triphosphate, 2 U of Ampli Taq DNA polymerase (Perkin-Elmer

Cetus), and the corresponding primers at the corresponding concentrations (25 µM for all polyomavirus amplifications). In all PCR assays, the first cycle of denaturation was carried out for 4 min at 94°C. The conditions for the 29-cycle amplification were as follows: denaturing at 92°C for 60 s, annealing at the corresponding annealing temperature for 60 s, and extension at 72°C for 75 s. All amplifications were completed with 4 min of extension at 72°C. The PCR amplifications of adenovirus genomes were carried out as previously described (12). JCV genomes were amplified by using EP1A and EP2A as external primers and P1A and P2A (modified from Kunitake et al. [28]) as internal primers and an annealing temperature of 59°C in both PCRs. BKV genomes were amplified by using external primers BK1 and BK2 at an annealing temperature of 46°C and internal primers BK4 and BK6 at an annealing temperature of 50°C. All of the primer sequences used in this study are represented in Table 1. The results were analyzed by agarose gel electrophoresis using ethidium bromide as a stain.

**Quality control of the amplification method.** Standard precautions were applied in all of the manipulations in order to reduce the probability of sample contamination by amplified DNA molecules. All of the samples were analyzed twice in independent experiments, and a negative control was added every two samples.

**Characterization of the JCV genomes.** The amplicons obtained from the nested PCR when using primers to amplify the intergenic region (IGR) of nine JCV-positive samples (W1 to W6, E1, E2, and G1) and a positive CSF sample (CSFK) were further characterized by amplifying products of the first-round PCR by using primers P1A-JCSR and P2A-JCSL at an annealing temperature of 55°C. That permitted us to amplify the IGR of JCV in two overlapping fragments. Sequencing of these fragments was carried out by using the same two pairs of primers.

Two JCV-positive samples (W5 and G3) and two samples that tested positive for JCV in a previous study (BCN26 and BCN28) were analyzed by using primers suitable for amplification of the RR. JR1 and JR2 were used in the first-round PCR, and JR3 and JR4 were used in the nested PCR, and in both cases, the annealing temperature was 55°C. These primers have been described by Monaco et al. (32). The amplicons obtained from the nested PCR were further characterized by sequencing with primers JR3 and JR4.

**Characterization of BKV genomes.** Two BKV-positive samples (W2 and W5), other samples that had tested positive for BKV in a previous study (U3, BCN26, and BCN28), and a control urine sample (BCNU) were analyzed by using primers suitable for amplification of the RR, i.e., BR1 and BR2 at 44°C for the first PCR and BR3 and BR4 at 46°C for the nested PCR. We further characterized these samples by sequencing amplicons obtained from the nested PCR by using primers BR3 and BR4.

The amplicons obtained from the first-strand PCR using primers BK1 and

TABLE 2. JCV types used for phylogenetic analysis<sup>a</sup>

Strain(s)	Origin (ethnicity) <sup>b</sup>	GenBank accession no(s).	Reference	Type
Mad-1	United States (C)	J0227	16	1
124	United States (C)	AF015526	4	1
123	United States (C)	AF015227	4	1
Tokyo-1	Japan	AF030085	7	2A
224	United States (H)	AF015529	7	2A
225	United States (NA)	AF015530	7	2A
226	United States (NA)	AF015531	7	2A
223	United States (AA)	AF015532	7	2B
227	United States (C)	AF015533	7	2B
GS/K	Germany	AF004349	30	2B
GS/B	Germany	AF004350	30	2B
228	United States (NA)	AF015534	7	2C
229	United States (C)	AF015535	7	2C
230	United States (AA)	AF015536	7	2C
308	Tanzania	U73500	2	3
312	United States (AA)	U73502	2	3
311	United States (AA)	U73501	2	3
402	United States (C)	AF015528	4	4
601	United States (AA)	AF015537	6	6
Tai-3	Taiwan	U61771	35	7
BCNU-2, -16, -8, -15	Spain	AF119345-49	12	
U3	Sweden	AF119350	12	
F2	France	AF119351	12	
P3, P1	South Africa	AF119352-53	12	
CSFB, -E, -J	Spain	AF119354-56	12	
CSFK	Spain	AF304389	This paper	
G1	Greece	AF303943	This paper	
E1-2	Egypt	AF303944-45	This paper	
W1-3/W4-6	United States	AF303946-48/AF304386-88	This paper	

<sup>a</sup> Strains and genomes used to construct the phylogenetic tree are represented. The geographic origins of the strains are shown. Some data are from the work of Jobes et al. (25).

<sup>b</sup> C, Caucasian; H, Hispanic; NA, Native American; AA, African American.

BK2 of five samples, two positive samples (W3 and BCN17), two other positive samples that tested positive for BKV in a previous study (BCN29, BCN30), and the urine sample used as a control (BCNU), were further amplified in a nested PCR using primers BK2 and BK3 (at an annealing temperature of 46°C), which amplify a VP1 region used for the typing of these viruses (24). The amplicons obtained were sequenced by using BK2 and BK3.

**Sequencing of nested-PCR products.** Products obtained from the nested PCR were purified with the QIAquick PCR purification kit (QIAGEN, Inc.). 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) by following the manufacturer's instructions. The conditions for the 25-cycle sequencing amplification were as follows: denaturing at 96°C for 10 s, annealing at 50°C for 5 s, and extension at 60°C for 4 min. The primers used for sequencing were used at a concentration of 2.5 μM. The results were checked by using the ABI PRISM 377 automated sequencer (Perkin-Elmer, Applied Biosystems). The sequences were compared with the GenBank and EMBL databases by using the basic BLAST program of the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/BLAST/>). Alignments of the sequences were carried out by using the ClustalW program of the European Bioinformatics Institute of the European Molecular Biology Laboratory (<http://www.ebi.ac.uk/clustalw/>).

**Phylogenetic analysis of JCV.** Sequenced regions of JCV were analyzed by using the seqboot and neighbor programs included in the PHYLIP software package (15). The phylogenetic tree constructed by the neighbor-joining (NJ) method and bootstrap analysis was visualized by using the TREEVIEW 1.5 program (38). The JCV isolates used in the phylogenetic analysis are described in Table 2. The NJ method was also applied for analysis of the full sequences of 20 of the strains described in Table 2 (the 20 classified into types and subtypes by Jobes et al. [25]) and the partial 461-nucleotide (nt) sequence used in our analysis.

**Stability of human polyomavirus and HAd particles in sewage.** One liter each of three sewage samples (BCN23, BCN24, and BCN25) was kept in a sterile beaker in a heater at 20°C. One liter of PBS spiked with 84 ml of urine from a

healthy woman pregnant for 38 weeks that was positive for both JCV and BKV was used as a positive control. Aliquots of 40 ml of each sample were analyzed within a 5-month period for the presence of JCV and BKV on days 0, 3, 10, 17, 30, 45, 62, 78, 92, 120, and 150. The presence of HAd was also analyzed on days 30, 60, and 92. The presence of viral particles was evaluated, and in order to find out if the viral particles had remained intact, viral concentrates were treated with DNase before nucleic acid extraction. One aliquot of each sample was treated with DNase with the appropriate buffer in order to destroy free DNA, and the other aliquot was treated with DNase buffer but no DNase for analysis of total DNA under equivalent conditions. Twenty-five microliters of DNase buffer containing Tris-HCl (pH 7.5) at 100 mM, MgCl<sub>2</sub> at 20 mM, bovine serum albumin at 100 μg/ml, 25 μl of viral particles, and 1 μl of DNase I (Amersham Pharmacia Biotech Inc.) at 10,000 U/ml (DNase treatment) or 1 μl of sterile water was mixed before nucleic acid extraction.

In order to obtain the estimated  $t_{90}$  and  $t_{99}$  (times required for 90 and 99% reductions of the viral concentration), we computed a linear regression model with the logarithm of the estimated concentration of viral particles detected by nested PCR expressed as PCR genomic equivalents. More precisely, the model is  $\log y_t = at + \log y_0$ , where  $y_t$  is the mean of the three observed values of  $y$  at time  $t$ ,  $y_0$  is the value of the PCR genomic equivalents at time zero, and  $a$  is the slope of the regression line. The log-transform is suggested by the proportional relationship between the mean and the standard deviation of the PCR genomic equivalents at every time and is strongly supported by inspection of the residuals of the model. However, the usual inferences in linear regression (i.e., significance test of correlation, confidence intervals, etc.) are not applicable because of the known dependence of the observed values of the PCR genomic equivalents in the stability assays. For our purposes, the model attempts to give an approximate (descriptive) approach to  $t_{90}$  and  $t_{99}$ . Inverting the equation described above, estimates of  $t_{90}$  and  $t_{99}$  are computed by substituting for  $y_t$  10 and 1%, respectively, of the observed value of  $y_t$  at time zero ( $t_0$ ).

**Stability of JCV and BKV particles at acidic pHs.** A sewage sample at pH 7 to 7.5 was divided into three aliquots, and their pHs were adjusted to 1, 3, and 5 with 35% HCl. The samples were magnetically stirred at room temperature.

TABLE 3. Summary of results obtained by analysis of 52 sewage samples for the presence of human polyomaviruses

Area (no. of samples)	No. positive/total (no. of viral particles/ml)		
	HAd	JCV	BKV
Barcelona, Spain (16 <sup>a</sup> + 9)	24/25 (10 <sup>1</sup> -10 <sup>2</sup> )	23/24 (10 <sup>1</sup> -10 <sup>2</sup> )	20/25 (1-10 <sup>1</sup> )
Nancy, France (4) <sup>a</sup>	4/4 (10 <sup>1</sup> -10 <sup>2</sup> )	4/4 (10 <sup>2</sup> -10 <sup>3</sup> )	4/4 (1-10 <sup>1</sup> )
Umeå, Sweden (4) <sup>a</sup>	4/4 (1-10 <sup>1</sup> )	4/4 (10 <sup>2</sup> -10 <sup>3</sup> )	4/4 (1-10 <sup>1</sup> )
Pretoria, South Africa (4) <sup>a</sup>	4/4 (10 <sup>2</sup> -10 <sup>3</sup> )	4/4 (10 <sup>1</sup> -10 <sup>2</sup> )	4/4 (10 <sup>1</sup> -10 <sup>2</sup> )
Cairo, Egypt (4)	4/4 (10 <sup>2</sup> -10 <sup>3</sup> )	4/4 (10 <sup>2</sup> -10 <sup>3</sup> )	4/4 (10 <sup>1</sup> -10 <sup>2</sup> )
Patras, Greece (5)	5/5 (10 <sup>2</sup> -10 <sup>3</sup> )	5/5 (10 <sup>2</sup> -10 <sup>3</sup> )	4/4 (10 <sup>2</sup> -10 <sup>3</sup> )
Washington, D.C. (6)	6/6 (10 <sup>2</sup> -10 <sup>3</sup> )	6/6 (10 <sup>3</sup> -10 <sup>4</sup> )	6/6 (10 <sup>2</sup> -10 <sup>3</sup> )
Total (52)	51/52 (10 <sup>2</sup> -10 <sup>3</sup> )	50/51 (10 <sup>2</sup> -10 <sup>3</sup> )	46/51 (10 <sup>1</sup> -10 <sup>2</sup> )

<sup>a</sup> Samples analyzed in a previous study (12).

After 30 min, the pH was neutralized with 1 M NaOH. The samples were then concentrated and treated with DNase or left untreated before nucleic acid extraction. This assay was repeated while keeping the samples at a low pH for 1.30 h.

**Nucleotide sequence accession numbers.** The JCV IGR sequences reported in this paper have been deposited in the GenBank database under accession no. AF303943 to AF303948 and AF304386 to AF304389. The JCV RR sequences obtained in this study are identical to those described by Bofill-Mas et al. (12) (accession no. AF120242; archetypal JCV RR sequence obtained from sewage samples). The BKV RR sequences analyzed have been deposited under accession no. AF356528 to AF356531, and the BKV VP1 region sequences have been deposited under accession no. AF356534 to AF356538.

## RESULTS

### Human polyomaviruses in sewage and shellfish samples.

Viruses excreted by people in their feces or urine are found in urban sewage and seawater with fecal contamination. Since shellfish filter large volumes of seawater, viruses that are present in water accumulate and remain in shellfish digestive tissues. Thus, analysis of the viruses present in urban sewage and shellfish samples will give information about the excretion patterns of polyomaviruses in specific communities. The sensitivity of the nested PCR assay was tested by following the procedure applied in a previous study (11, 12). The observed sensitivity of the nested PCR was 5 JCV genome copies and 50 BKV genome copies. A sensitivity of 1 to 10 HAd particles has been reported previously (40).

The results obtained in the limiting-dilution experiments when analyzing sewage samples collected in the United States, Egypt, Greece, and Spain are shown in Table 3 and reflect a very high level of excretion of JCV, BKV, and HAd. All of the samples from these areas were positive for HAd, JCV, and BKV.

Table 3 also reflects results obtained in a previous study with samples collected in South Africa, France, Sweden, and Spain (12). The total percentage of positive samples and estimated mean values of HAd, JCV, and BKV are represented.

Of the 10 shellfish samples analyzed, 8 were positive for at least one of the human viruses analyzed (HAd or JCV) and the average *Escherichia coli* level of these samples was 40 bacteria/100 g. These data indicates that these samples had been in contact with fecal contamination of human origin. Seven samples were positive for HAd, with a mean concentration of 1 to 10 viral particles/g. Six samples were positive for hepatitis A virus, with mean concentrations of 10 viral particles/g. Five

samples were positive for JCV at concentrations between 1 and 10 viral particles/g. All of the samples tested negative for BKV.

**Characterization of the IGR of JCV.** The IGR of JCV has been studied largely as a tool with which to trace human migrations, since it presents differences corresponding to the different geographic origins of the populations that excrete the viruses.

Sequencing of the IGR of JCV classifies the JCV genomes into at least seven types and a larger number of subtypes (25). Type 1 is found in Europeans, types 2 and 7 are found in Asians, and types 3 and 6 are found in Africans (3, 14, 20); type 6 is found in western and central Africa but not in eastern Africa (3, 14, 20). Types 1, 2, and 3 present two or more subtypes (A, B, C... ). The inhabitants of America present genotypes characteristic of other continents. African Americans present mainly type 3, while European Americans present type 1 and Native Americans present type 2A, which is typical of their northeastern Asian origins (8).

We sequenced 461 nt of the IGR of JCV-positive samples. The consistency of the phylogenetic analysis using the 461-bp sequence of the IGR was evaluated by comparing trees obtained by using this 461-bp sequence with phylogenetic trees obtained by using the full-length JCV sequences described in the GenBank and EMBL data banks. The two types of trees showed significant similarity (data not shown).

The sequences analyzed confirmed the specificity of the nested PCR amplification, since all viral sequences were identified as JCV when specific primers were used. These results also confirmed the absence of cross-contamination, since all of the viral sequences analyzed were different from the positive controls used in the assays.

The phylogenetic tree obtained by the NJ method is shown in Fig. 1. The strains used to construct this tree are represented in Table 2.

**Characterization of the RRs of JCV genomes.** In order to check whether or not the genomes detected presented the archetypal transcriptional control regions (TCRs) excreted by individuals in urine or the rearranged TCRs typically found in the CSF of PML patients, we analyzed the RR sequences from four JCV-positive samples and compared these sequences with sequences previously obtained. All of the sequences obtained from sewage samples and urine samples were identical in these 187 nt and were also identical to the archetypal consensus sequence.

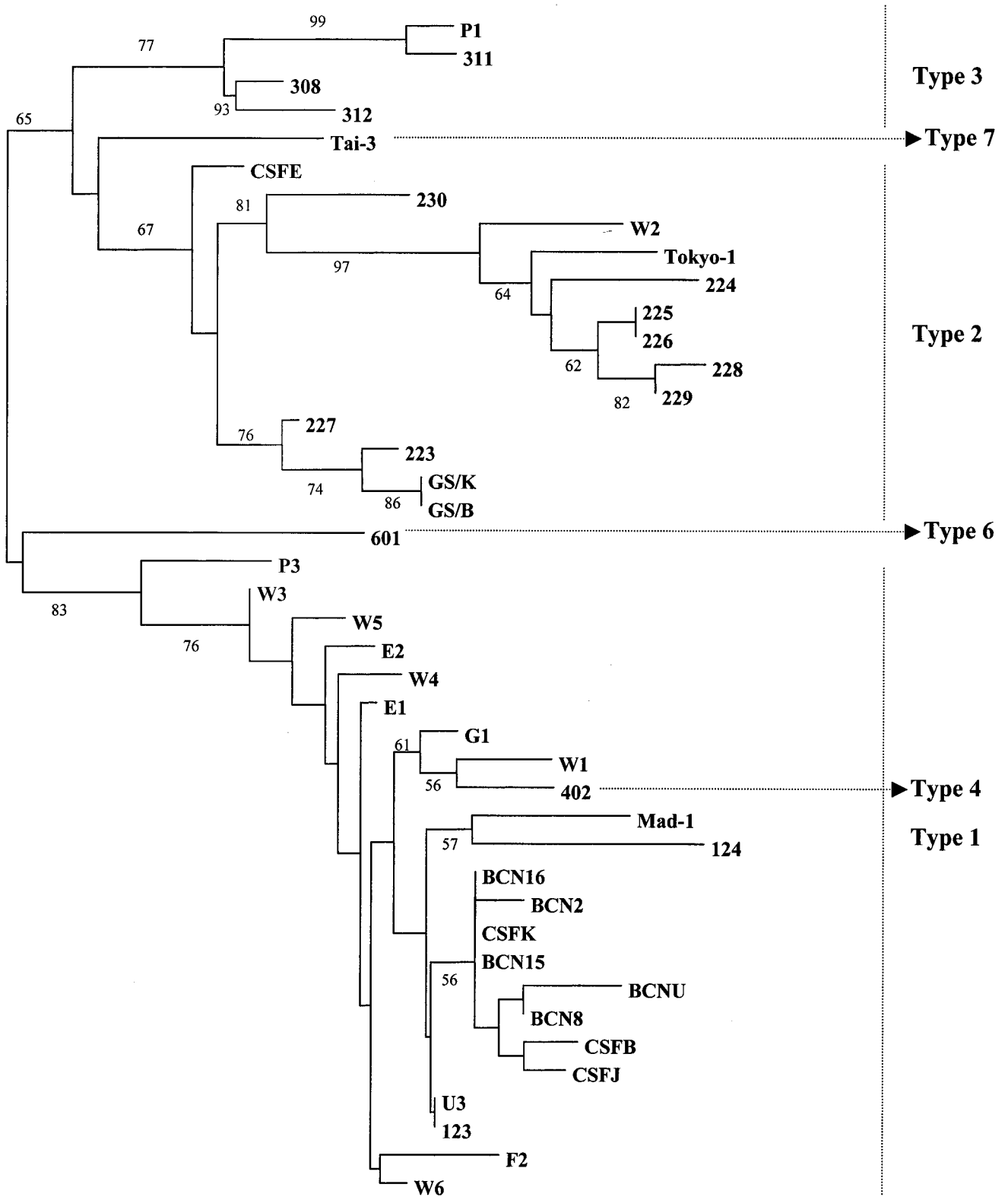


FIG. 1. NJ tree constructed to represent phylogenetic relationships among 42 JCVC sequences (see Table 3) for nt 2177 to 2637 of the IGR. The bootstrap confidence levels obtained for 100 replicates are shown (only significant values are indicated).

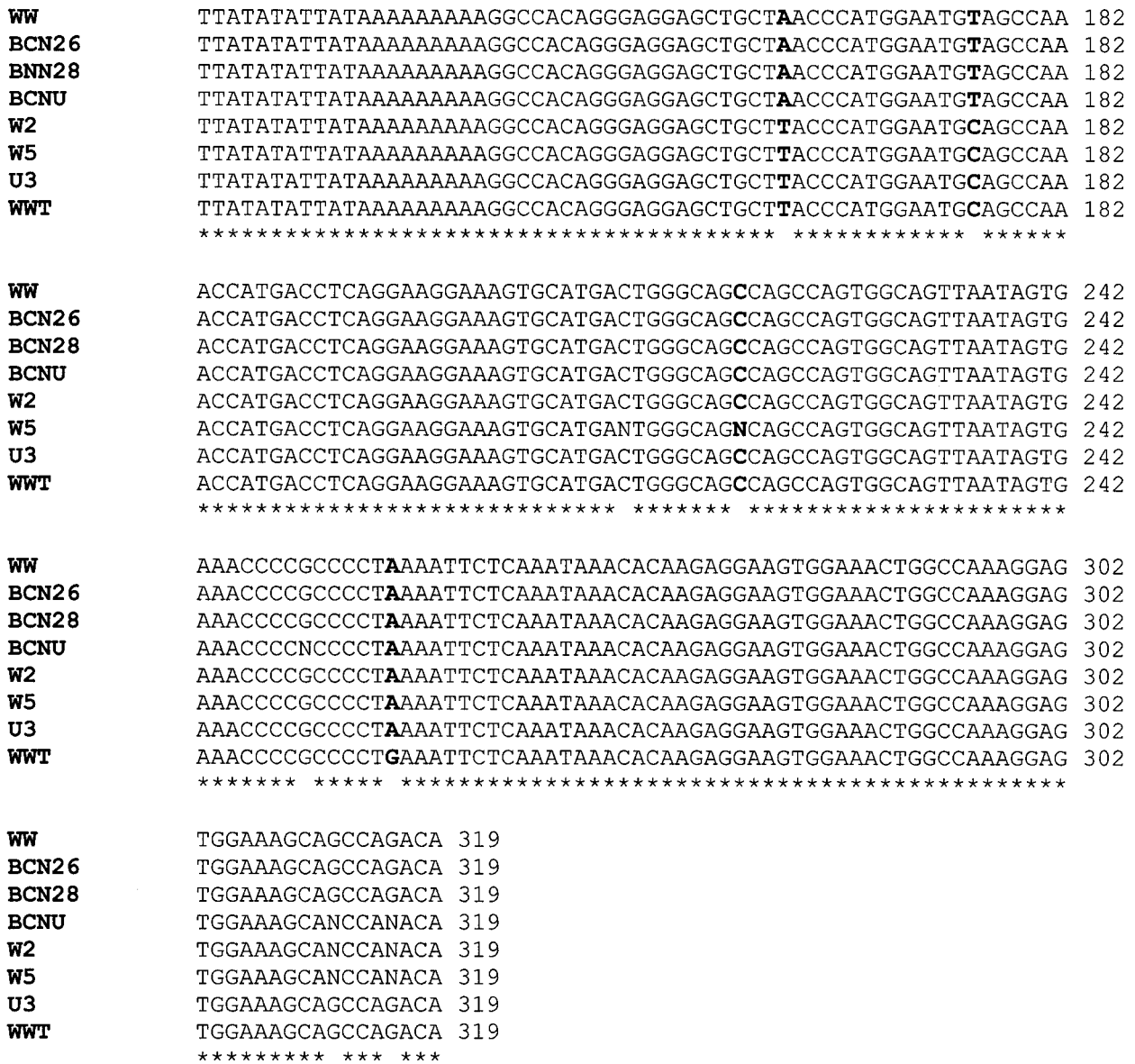


FIG. 2. Annealing of the RRs of some of the BKV-positive samples sequenced (Gardner numbering). Asterisks represent similarity between nucleotides. Bold nucleotides represent sites of diversity in the BKV genome. Archetypal (WW) and archetype-like (WW-T) sequences were also included in the annealing.

**Characterization of BKV genomes.** It has been reported that BKV transcriptional RRs also fall into two groups, archetypal and rearranged, depending on their sequence. We sequenced the RRs of some samples positive for BKV and a urine sample used as a control. BKV studied in sewage and urine presented archetypal (WW) or archetype-like (WW-T) structures of the RR, as previously described (43, 49). Alignments of these sequences are shown in Fig. 2. We could establish two groups of archetypal regions that differed in 2 of 180 nt. Samples from the United States and Sweden were almost identical (with the exception of some undetermined nucleotides) and differed by 1 nt from BKWW-T (nt 256, numbering of the Gardner strain). Sewage samples tested from Barcelona and the urine sample used as a control (from a Spanish individual) were identical in the identified nucleotides (three nucleotides in the urine sam-

ple could not be defined) and identical to BKWW. The relevant consensus elements, such as protein binding sites and regulatory elements, that have been described by Markowitz et al. (31) were conserved in the genomes analyzed. Differences between the two groups were restricted to nucleotides that present polymorphisms (nt 163 and 176).

To begin typing the BKV present in these sewage samples, we sequenced the VP1 region of five BKV-positive samples. Of the five samples sequenced, four presented the characteristics defined by Jin et al. (24) for BKV type 1, the type most frequently detected in human populations. One of the samples collected in Barcelona (BCN29) seemed to be identical to type 2, the second BKV type most frequent in human populations, with the exception of one nucleotide that corresponded to type 1, 3, or 4 but not type 2. Alignments of the sequences detected

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BCN30      TTCTAAACCCAGAAATGGGGGATCCAGATGAAAAACCTTAGGGGCTTTAGTCTAAAGCTA
BCN17      TTCTAAACCCAGAAATGGGGGATCCAGATGAAAAACCTTAGGGGCTTTAGTCTAAAGCTA
BCNU       TTCTAAACCCAGAAATGGGGGATCCAGATGAAAAACCTTAGGGGCTTTAGTCTAAAGCTA
W3         TTCTAAACCCAGAAATGGGGGATCCAGATGAAAAACCTTAGGGGCTTTAGTCTAAAGCTA
BCN29      TTNCTAAACCCAGAAATGGGGGATCCAGATGATAAACCTTAGGGGCTATAGTCTAAAGCTA
** *****

BCN30      AGTGCTGAAAATGACTTTAGCAGTGATAGCCAGAAAAGAAAAAATGCTTCCCTGTTACAGC
BCN17      AGTGCTGAAAATGACTTTAGCAGTGATAGCCAGAAAAGAAAAAATGCTTCCCTGTTACAGC
BCNU       AGTGCTGAAAATGACTTTAGCAGTGATAGCCAGAAAAGAAAAAATGCTTCCCTGTTACAGC
W3         AGTGCTGAAAATGACTTTAGCAGTGATAGCCAGAGAGAAAAAATGCTTCCCTGTTACAGC
BCN29      ACTGCTGAAAATGCCTTTGNCAGTGATAGCCAGACAGAAAAAATGCTTCCCTGTTACAGC
* *****

BCN30      ACAGCAAGAATTCCCCTCCCCAATTTAAATGAGGACCTAA
BCN17      ACAGCAAGAATTCCCCTCCCCAATTTAAATGAGGACCTAA
BCNU       ACAGCAAGAATTCCCCTCCCCAATTTAAATGAGGACCTAA
W3         ACAGCAAGAATTCCCCTNCCCAATTTAAATGAGGACCTAA
BCN29      ACAGCAAGAATTCCCCTCCCCAATTTAAATGAGGACCTAA
*****
    
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FIG. 3. Annealing of the VP1 regions of some of the BKV-positive samples sequenced. Asterisks represent similarity between nucleotides. Bold nucleotides represent sites used for BKV typing.

are presented in Fig. 2. We also observed some indeterminate nucleotides in the polymorphic sites.

**Stability of human polyomaviruses and HAd particles in sewage.** There is no previous information on the behavior of human polyomaviruses in the environment. Data on the stability of these viral particles in the environment could contribute to a better evaluation of their mechanism of transmission. By analyzing the three samples of raw sewage from Barcelona (BCN23, BCN24, and BCN25) and the control (PBS spiked with urine positive for both BKV and JCV), which were all kept at 20°C for 150 days, we found that both BKV and JCV were detected until day 92. The viruses detected during this time appeared to represent intact virions, since treatment with DNase prior to nucleic acid extraction did not eliminate the viral genomes (Fig. 3). The presence of HAd DNA was also analyzed at days 30, 45, and 92 by using DNase. HAd viral particles were detected at days 30 and 45, but disrupted (DNase-sensitive) virions were obtained at day 92.

In evaluating the stability of JCV in sewage at 20°C, we estimated a  $t_{90}$  of 26.7 days and a  $t_{99}$  of 61.5 days. For BKV, the estimated  $t_{90}$  was 53.6 days and the estimated  $t_{99}$  was 96.8 days. These data are approximate and were calculated in accordance with the regression line obtained (Fig. 4).

**Stability of JCV and BKV particles at acidic pH.** Data on the resistance of polyomaviruses to acidic pH could be useful when considering the gastrointestinal tract as a possible route of transmission of these viruses. Viruses detected in sewage may be ingested through contaminated water or food. After analyzing human polyomaviruses present in a sewage sample treated at pHs 1, 3, 5, and 7 for 30 min, we could detect the DNAs of both JCV and BKV at all of these pHs, including pH 1. When we used DNase before nucleic acid extraction, we found that BKV DNA detected at pHs 1 and 3 was free DNA, implying that the BKV particles were disrupted at these pHs. Free JCV DNA was also found after 90 min at pH 3. However,

after 30 min at pH 3, the viral particles appeared to be intact in half of the assays carried out (Fig. 5).

DISCUSSION

All of the populations in the different geographical areas that we sampled excreted a high number of human polyoma-

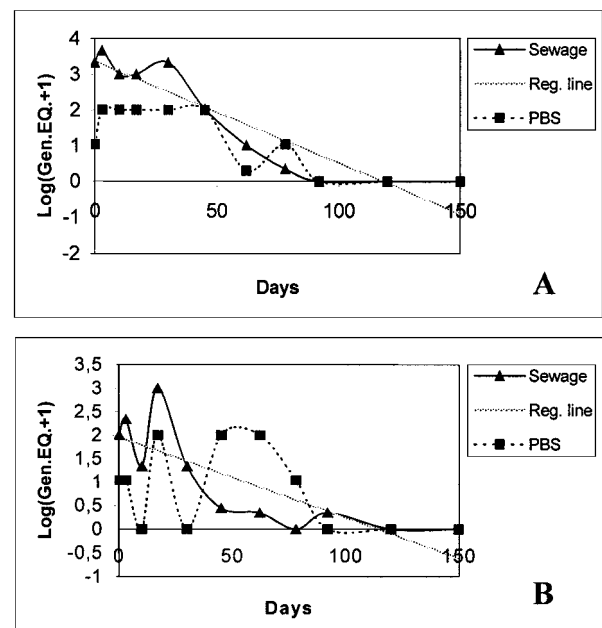


FIG. 4. Stability of JCV (A) and BKV (B) in sewage samples. The regression (Reg.) line, the transformed average number of genome equivalents (Gen.EQ+1) detected by nested PCR in the three samples (sewage), and the number of genome equivalents of a spiked PBS control are represented.



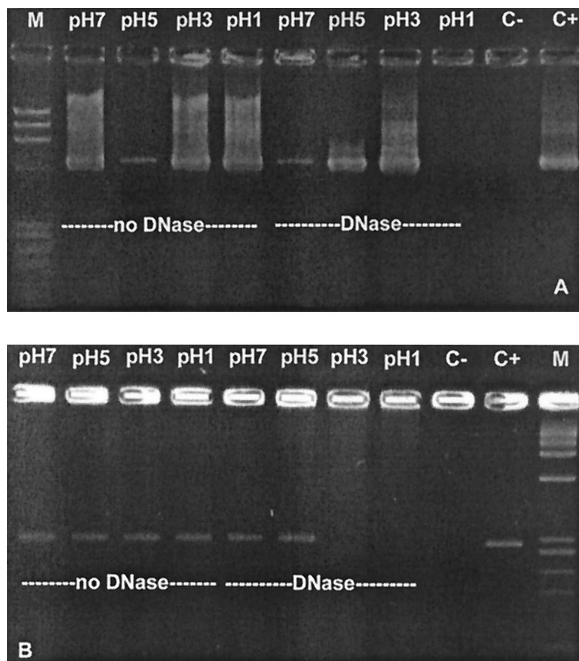


FIG. 5. Electrophoretic gel showing the bands obtained by analysis of a sewage sample containing JCV (A) and BKV (B) after exposure to different pHs for 30 min. The pH assayed and also the absence or presence of DNase treatment are indicated. Lanes M contained molecular size standards. C-, negative control; C+, positive control.

viruses. These high levels of human polyomaviruses in urban sewage probably came from urine, since JCV has been detected in 20 to 80% of adult urine samples, depending on age (26) and ethnic group (8). Our finding of a higher concentration of JCV than BKV in sewage agrees with a study on the genomic sequences of JCV and BKV in 176 urine samples by Shah et al. (47). In that study, 37% JCV and 5% BKV viruria was observed and immunosuppression was associated with a higher frequency of BKV viruria.

The primers applied in this study have proven to be specific, sensitive, and able to detect a wide variety of JCV strains. Sequences obtained from the IGRs of 22 JCV genomes were analyzed in phylogenetic studies, and the clusters obtained were used for classification of the viral genomes. The amplified sequences of 461 nt include the 3' end of VP1, a noncoding short region of about 69 nt, and the 3' end of the large T antigen. Hatwell et al. (22) found that this region encloses a large number of sequence differences between strains and that these sequences are useful for comparing JCV genomes of diverse origins. Most of the samples studied presented sequences closely related to European types. Only one genome detected in sewage was found to be closely related to Asian strains, i.e., sample W2, from sewage collected in the Washington, D.C., area. This isolate was classified as type 2A, typical of the northeastern Asian origin of Native Americans. A sequence detected in sewage from South Africa was the only one classified as JCV type 3. Samples G1 and W1, collected in Greece and the United States, respectively, were closely related to strain 402, the prototype of previously described type 4, which is closely related to type 1. The genotypes studied appear to be some of the most abundant genotypes in the

population. However, a study of the diversity of types excreted in a specific population would require multiple samples of sewage and cloning and sequencing of the diverse genomes that could be amplified.

The JCV strains obtained around the world fall into distinct types and have been described as markers that may be useful in tracing ancient and modern human migrations (1, 2, 9, 48, 52).

Like JCV, BKV also appears to have two different RR structures, archetypal and rearranged (34, 43, 44). When BKV samples were analyzed to determine whether they contained archetypal regions, we found two different types of archetypal regions similar to those defined as BKWW (archetypal) and BKWW-T (archetypal-like) by Sundsfjord et al. (49) among naturally occurring BKV strains.

The BKV genomes from a few samples were typed by sequencing of the VP1 region (24), and with one exception, all of them were found to be of genomic subtype I, which has been described as the BKV type most frequently detected in the human population.

The high prevalence of human polyomaviruses observed in urban sewage suggests that humans are being exposed at a high frequency to JCV and BKV through the digestive tract when ingesting water and foods, such as vegetables or shellfish, previously exposed to fecal contamination. Passage of the contaminated food through the digestive tract could produce infection in the intestinal epithelium or lymphoid cells if JCV and BKV were as resistant to the proteolytic enzymes of the digestive tract as are other nonenveloped viruses that are excreted. The alimentary tract was proposed by Sundsfjord et al. (50) as a portal of infection by JCV and BKV. After studying nasopharyngeal aspirates in children with respiratory infections and saliva from immunodeficient and immunocompetent adult patients, only BKV DNA (but no infectious BKV) was detected in 2 of 201 nasopharyngeal aspirates. However, other routes of infection by these viruses have not been ruled out, since tonsil tissue has also been suggested as a possible site of initial JCV infection (32). This possibility may also be considered as potentially complementary to entry at the intestinal level because swallowed material passes the mouth and nasopharynx and viruses transmitted through the oral-fecal route are often able to multiply in the throat. Human polyomaviruses are relatively stable at acidic pH, and when ingested with food, viruses may be protected, as has been shown for other intestinal pathogens. Gastric secretion is known to be about pH 1, although the total pH of the stomach is highly variable, depending on the ingested material and the stimuli influencing the secretions. The contents of the stomach always have a pH higher than 4 when they pass into the duodenum. We have observed intact JCV particles after treatment at pH 5 for 90 min and also after treatment at pH 3 for 30 min. The stability of these viral particles was confirmed by treatment with DNase. Viruses could enter humans through the digestive tract by infecting intestinal epithelia, by pinocytic mechanisms in the cells, or through M cells in Peyer's patches. Although archetypal JCV is highly difficult to grown in cell cultures, further studies are currently under way to evaluate the potential infectivity of the excreted polyomaviruses in the gastrointestinal tract.

Moreover, there are other aspects that need to be considered. It has been shown that foreign DNA ingested with food is not completely degraded in the gastrointestinal tract in an

animal model and a study by Schubert et al. (45) suggests that transport of foreign DNA through the intestinal wall and Peyer's patches to peripheral blood leukocytes and into several organs can occur. According to this information, free polyomavirus DNA, either completely or partially digested, could also enter the human body through the gastrointestinal tract. JCV DNA has been described in the epithelium of the upper and lower parts of the gastrointestinal tract (42), and although it has been not proven, this could be a direct consequence of the frequent ingestion of polyomaviruses and the intake of viral particles or viral DNA by the oral route.

JCV strains fall into two groups, designated archetypal and rearranged, based on the structure of their TCRs. The TCR of archetypal JCV contains a single copy of the promoter and enhancer, while the rearranged strains contain deletions and duplications in this region (5, 10, 52, 53). The archetypal strains can be isolated from normal individuals and immunocompromised patients and are believed to be the viruses that spread throughout the population and establish persistent infections; however, this has not been firmly established. The rearranged strains found in PML patients appear to be derived from the archetypal strains which can be isolated from the same individuals (53). All of the JCV genomes amplified from the sewage samples and the urine sample contained identical archetypal structures in the RR. In contrast, the CSF samples from PML patients used as a positive control in these experiments (CSFE and CSFB) presented rearranged genomes (12) with duplicated regions, including the binding sites for a variety of transcription factors. Recently, Ricciardiello et al. (41) have described the presence of Mad-1 strains in the human colon. It is necessary to analyze the source of these strains, which may have originated from viremia associated with reactivation of persistent infections, from infection by the small percentage of excreted strains containing tandem repeats sporadically identified in urine (6), or from rearrangements in the RR of archetypal strains.

Analysis of the polyomaviruses excreted by the human population by studying the viral nucleic acids extracted from urban sewage provides information on the status of these viruses in the environment, the potential transmissibility of polyomaviruses and polyomavirus genes, and the characteristics and diversity of the viral strains excreted by humans in different geographic locations.

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**Analysis of the Excreted JCV Strains and their Potential Oral Transmission.**

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Journal of Neurovirology, acceptat, en procés de publicació.

**RESUM**

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**1. Objectius**

Tal i com es descriu als capítols I i II, JC és un virus molt prevalent en mostres ambientals i per tant en la població humana. JC és el causant de la PML, malaltia letal en un 4% de pacients de SIDA, i a més a més, s'ha relacionat amb tumors de cervell i amb el càncer de colon. La seva via de transmissió és encara desconeguda i s'ha postulat que JC es podria transmetre per via respiratòria o oral.

Es creu que les soques de JC que presenten regions reguladores (RR) arquetípiques són les que circulen a la població i que les RR reorganitzades, que es creu s'originarien a partir de formes arquetípiques en l'organisme, són les causants de patologies. A més a més, s'ha descrit que les formes arquetípiques de JC i BK no creixen en cultiu cel·lular mentre que les formes reorganitzades sí que ho fan.

Els objectius principals d'aquest estudi es van centrar en estudiar la capacitat infectiva de soques de JC amb RR reorganitzades i soques amb RR arquetípiques.

També es va estudiar la possible excreció de soques amb RR reorganitzades a l'ambient com a potencial origen de noves infeccions a la població.

Els objectius concrets d'aquest treball van ser:

- Estudiar la infectivitat d'una soca tipus PML (Mad-4) en condicions que mimetitzen les existents al tracte gastrointestinal (pH àcid i presència de proteases).
- Estudiar la possible presència de soques amb RR reorganitzades en mostres d'aigua residual.
- Estudiar la infectivitat dels JC i BK arquetípics presents en una mostra d'orina en cèl·lules SVG (cèl·lules glials fetals humanes), COS-7 (cèl·lules de ronyó de

mico verd africà transformades amb l'AgT de SV40) i CaCo-2 (cèl·lules humanes de càncer de colon).

- Avaluat la infectivitat de JC arquetípics i amb RR reorganitzades (Mad-4) en cèl·lules CaCo-2.

## 2. Material i Mètodes

Es van utilitzar cèl·lules SVG, COS-7 i CaCo-2 per estudiar diversos aspectes de la infectivitat de JC. Per una banda es va treballar amb una mostra d'orina que contenia JC i BK arquetípics i per una altra amb una soca prototip de soca amb RR reorganitzada, Mad-4, tractada amb pH àcid i proteasa (tripsina). La infectivitat es va avaluar mitjançant observació de l'efecte citopàtic (CPE), assaigs d'hemaglutinació, hibridació *in situ*, PCR i microscòpia electrònica.

Per tal d'estudiar la possible presència de soques de JC amb reorganitzacions a la RR en aigua residual es van clonar els amplicons obtinguts a l'analitzar 2 de les mostres. Els materials i mètodes utilitzats es troben descrits detalladament a l'article adjunt.

## 3. Resultats

- 1) No es van observar diferències en les cèl·lules SVG infectades amb la soca Mad-4 tractada i sense tractar amb 10 µg/ml de tripsina. Mad-4 sotmès a pH 3 durant 1 hora va infectar cèl·lules SVG tot i que es va observar un descens en el títol víric de la progènie obtinguda (3 logaritmes inferior).
- 2) Mad-4 no va infectar cèl·lules CaCo-2 en condicions normals ni després de ser preactivat amb tripsina, tractament que s'utilitza sovint per cultivar virus que infecten a través de la ruta gastrointestinal.
- 3) Els JC arquetípics presents en una mostra d'orina d'una dona sana embarassada van infectar cèl·lules SVG (però no COS-7) després de més de 45 dies d'infecció (Figures 2, 3 i 4 de l'article). Els virus produïts en la infecció van

resultar ser idèntics als presents en la mostra original. Els BK arquetípics presents en menys concentració en la mostra, també van infectar les cèl·lules eficientment.

- 4) 17/20 clons obtinguts després de clonar els amplicons de RR dels JC presents en una mostra d'aigua residual de Barcelona presentaven una RR arquetípica mentre que en 3/20 es van observar petites diferències com canvis en un sol nucleòtid (nt), delecions d'un nt i una duplicació de 33 nt (Figura 1a de l'article). 12/20 clons obtinguts de la mateixa manera a partir d'una mostra d'aigua residual de Washington D. C. presentaven RR arquetípiques mentre que 7/20 clons presentaven petites variacions respecte a l'estructura arquetípica que consistien en canvis d'un sol nt i petites delecions (Figura 1b de l'article). Un dels clons presentava una estructura reorganitzada de la RR similar a la de Mad-4 (Figura 1c de l'article).

Aquest resultat es troben més àmpliament descrits i discutits a les seccions Resultats i Discussió de l'article.

#### **4. Annex. Clonació de la regió reguladora dels JC presents en dues mostres d'aigua residual urbana**

Posteriorment a la realització de l'estudi descrit en aquest darrer article es va clonar la RR dels JC detectats per PCR en 2 mostres més d'aigua residual urbana de les mateixes àrees: Washington D.C. (EUA) i Barcelona.

Els resultats obtinguts a l'analitzar els 10 clons procedents de clonar la RR dels JC presents a la mostra de Washington van ser els següents:

- 2/10 clons presentaven estructures idèntiques a l'arquetípica.
- 2/10 clons presentaven 1 nt diferent a aquesta estructura i eren diferents entre ells.
- 1/10 clons presentava 2 nt diferents a l'estructura arquetípica.
- 4/10 presentaven una estructura similar a la de Mad-4, variant un parell dels clons en 1 nt i sent idèntics entre ells mentre que un altre parell de clons diferien 2 nt respecte Mad-4 i eren també idèntics entre ells. Aquests

clons són similars al descrit a l'article adjunt a aquest capítol que va ser obtingut a partir d'una mostra d'aigua residual de la mateixa àrea geogràfica.

- Un dels clons analitzats presentava una estructura reorganitzada, en principi similar a Mad-4 (que presenta deleció de les seccions *b* i *d* de la RR, duplicació parcial de la secció *a* i duplicació total de *c* i *e*), però amb una duplicació extra del segment *a-c-e* que apareix duplicat una vegada en la soca Mad-4.

La meitat dels clons analitzats presentaven doncs una estructura *archetypal-like* mentre que l'altra meitat presentaven una estructura reorganitzada de la RR. Els clons obtinguts es representen a les Figures annexes A i B.

Els resultats obtinguts a l'analitzar els 10 clons procedents de clonar la RR dels JC presents a la mostra de Barcelona van ser els següents:

- 4/10 clons presentaven seqüències idèntiques a l'arquetípica.
- 1/10 clons presentava 2 nt diferents a la seqüència arquetípica.
- 5/10 clons presentaven 1 nt diferent a l'estructura arquetípica i una deleció de 14 nt a la secció *c* de la RR. Aquests 5 clons eren idèntics entre ells. Una seqüència amb el mateix tipus de deleció va ser introduïda al GenBank per Chang D. i Wang M. l'any 1999 amb títol "Infecció viral per JC en un trasplantament de ronyó".

Tots els clons analitzats a partir d'aquesta mostra presentaven doncs una estructura arquetípica o *archetypal-like*.

Els resultats obtinguts es troben representats a la Figura annexa C.

## 5. Conclusions

- 1) El tractament amb tripsina no influeix significativament en l'eficiència de la infecció de Mad-4 en cèl·lules SVG mentre que un tractament a pH 3 provoca una disminució d'aquesta. Mad-4 no infecta la línia cel·lular CaCo-2 en les condicions experimentals assajades, i si infecta COS-7.
- 2) Després de clonar els amplicons de la RR de 4 mostres d'aigua residual (2 de Washington i 2 de Barcelona) que contenien JC, es van observar soques amb RR arquetípiques o amb petites variacions de l'estructura arquetípica (*archetypal-like*) a les 4 mostres estudiades i soques amb reordenacions d'aquesta (tipus PML) només als clons analitzats a partir de mostres d'aigua residual obtingudes a Washington D.C. (EUA).
- 3) Partícules víriques de JC i BK amb una estructura arquetípica de la RR van produir una infecció productiva en cèl·lules SVG. Aquesta és la primera descripció, fins al moment, de soques arquetípiques d'ambdós virus que infecten cultiu cel·lular.



## Analysis of the Excreted JCV Strains and their Potential Oral Transmission

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JC virus particles have been detected in urban sewage of divergent geographical areas.

In this study we evaluate the genetic characteristics and the infective capabilities of the JCV strains in relation to the potential oral transmission of JCV in the population. JCV strains excreted in urine and detected in sewage have been described as presenting archetypal structure of the regulatory region of the viral genome. The regulatory region of JCV viral particles detected in two urban sewage samples have been cloned and characterized. From a total of forty clones tested, thirty-nine presented archetypal-like regulatory regions while one of the clones analyzed presented a tandem repeated structure.

Archetypal strains present in the urine of a pregnant woman were able to infect SVG cells producing infectious virions as demonstrated by confirmative cell culture, electron microscopy and *in situ* DNA hybridization. This is the first description of archetypal JCV productive infection of SVG cells.

SVG cells were also successfully infected with Mad-4 JCV viral particles subjected to pH 3 for one hour at 37°C and to 10 µg/ml of trypsin in the same conditions. A decrease in the viral progeny production was observed when Mad-4 was subjected to acidic pH. Mad-4 did not produce any detectable infection in the enteric cell line CaCo-2.

The oral route could represent a significant route of transmission of JCV infections since JCV virions have demonstrated relative resistance in the environment and to some of the conditions present in the gastrointestinal tract. The archetypal strains commonly detected in the environment may be implicated in the transmission of JCV among the population. Sporadic infection with strains presenting tandem repeated structures may have implications in pathogenicity.

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## Introduction

JCV (JC virus), one of the members of the *Polyomaviridae* family and the etiologic agent of progressive multifocal leukoencephalopathy (PML), infects a high percentage of the human population producing persistent infections in the kidney (Shah et al, 1995). The infection in childhood is usually asymptomatic, leading to the production of specific antibodies (Taguchi et al, 1982). The virus has also been detected in the lung and liver (Grinnell et al, 1983), in gastrointestinal tissues (Laghi et al, 1999), in lymphoid cells of PML patients (Houff et al, 1988) and in peripheral blood leukocytes of non-PML AIDS patients (Dorries et al, 1994; Tornatore et al, 1994; Dubois et al, 1996; Dubois et al, 1997). When JCV was found in lymphoid preparations after bone marrow transplantation, Schneider et al. (1993) suggested the lymphoid tissue as another site of viral latency with the lymphocytes implicated for trafficking the viruses throughout the body. JCV lytically infects oligodendrocytes causing PML a fatal demyelinating disease that affects immunocompromised people primarily those affected by AIDS. Aproximately 4% of AIDS patients develop PML (Berger et al, 1987).

The development of brain tumors in experimental animals has been described (ZuRhein, 1983; ZuRhein et al, 1987; Krynska et al, 1999) and JCV has also been reported to transform cells in culture (Theile et al, 1990). A role for JCV in brain tumors has been recently suggested (Rencic et al, 1996; Boldorini et al, 1998; Khalili et al, 1999; Del Valle et al, 1999; Imperiale et al, 2000; Calderelli-Stefano et al, 2000). Although there is no direct evidence for tumorigenesis of JCV in the human CNS, as some articles had reported (Herbarth et al, 1998; Del Valle et al, 2001) Del Valle et al. (2001) have recently suggested re-evaluating the role of JCV in the pathogenesis of human brain tumors after detecting JCV DNA in 69% of brain tumors analyzed. JCV has also been related to human colorectal cancer (Laghi et al, 1999; Ricciardiello et al, 2000) and to chromosomal instability in the denominated rogue cells (Neel et al, 1996).

More than a 80% of the adult human population present antibodies against JCV (Padget et al, 1973). The virus persists in the kidney and is excreted in urine, indicating that the kidney is a site of virus multiplication. Viruses excreted in urine present an archetypal regulatory region (RR) characterized by presenting the 23-bp and 66-bp sections (Yogo et al, 1990) usually deleted in the strains present in the brain and cerebrospinal fluid of PML patients that commonly present tandem repeated structures in

the RR. It is thought that these hyper-variable forms are derived from the archetypal type (Ault et al, 1993; Iida et al, 1993). The rearrangements occurring in tandem repeated structures have inferred that JCV has the capability of infecting cell culture with extended cell tropism (Yogo et al, 1990; Yogo et al, 1991; Iida et al, 1993).

Human primary fetal glial cell cultures (PFGC) efficiently support the growth of JCV presenting tandem repeated structures in the RR (Padget et al, 1971). Hara et al. (1998) reported the growth of archetypal JCV after infection of COS-7 cells with urine. To our knowledge, there have not been any more descriptions of successful replication of archetypal JCV after infecting cell culture. The route of infection of JCV has not been defined.

In a previous study we described the detection of high concentrations of JCV in sewage samples and suggested the potential transmission of JCV through the oral route. We described that the viruses excreted presented the archetypal structure in their RR (Bofill-Mas et al, 2000). The JC viral particles detected were significantly stable in sewage samples ( $T_{90}$  at 26.7 days) and relatively stable to acidic pH treatment (Bofill-Mas et al, 2001). In the present study we have evaluated the infectivity in SVG cells of Mad-4 JCV viral particles treated with acidic pH and with trypsin. We also have studied the capability of infection of JCV archetypal strains from urine and the characteristics of the RR present in urban sewage and potential transmission to the population.

## Results

*Cloning of the RR of JCV strains detected in sewage sample containing JCV.* In previous studies, direct sequencing of the bands obtained by nested-PCR revealed that the most abundant type of JCV strains excreted presented the archetypal structure of the RR (Bofill-Mas et al, 2000; Bofill-Mas et al, 2001). In the present study, we have cloned the RR of the JCV from sewage samples in two different geographical areas in order to evaluate the diversity of JCV strains present in urban sewage which potentially infect the population. After sequencing 40 recombinant clones obtained by cloning the amplicons of the RR of the two samples, we determined that 17/20 clones from the Barcelona (Spain) sample were identical in the 235 nt analyzed and identical also in the archetypal regulatory region structure. In 3/20 clones we observed differences in the archetypal structure. Clone BCN2.32 presented a deletion of one A at the  $A_8$  tract, as previously reported in JCV clones generated from colon cancer samples

(Ricciardiello et al, 2001). Clone BCN2.5 presented a tandem duplication of a 33 nt sequence (nucleotides 210 to 242 and 243 to 275) in block *f* (Ault et al, 1993) of the RR as previously described (Jensen et al, 2001 and articles cited there). Clone BCN2.27 presented a single change in nucleotide 181. These variations are summarized in Figure 1a.

A total of 12/20 clones obtained from Washington D.C (USA) sample were identical to the archetypal structure. 7/20 clones presented little variations consisting in single changes and small deletions. These variations are represented in Figure 1b. Clone USA2.41 presented a tandem repeated structure of the RR differing in one nucleotide with the sequence described for Mad-4 (Figure 1c).

*Infection of SVG, COS-7 and CaCo-2 cells with urine samples containing JCV viral particles.* In this study, we have assayed the potential infectivity of two urine samples containing JCV viral particles in several cell lines and at long term. The concentration of JCV and BKV (BK virus) present in urine samples was estimated by limiting-dilutions PCR assays. BCNU1 and BCNU2 presented  $10^4$  JCV viral particles per ml while BCNU1 presented also  $10^3$  BKV viral particles per ml. By day 30 post-infection, SVG cells infected with urine BCNU1 did not exhibit any obvious CPE (cytopathic effect) or positive result by HA (hemagglutination test) when compared with the positive control (Mad-4) that presented clear CPE by day 30 post-infection being positive for HA (1:25,600). JCV is a cell-associated virus, with small amounts of virus shed into the cell culture medium. These results may also indicate that the quantity of virions, if produced, was too low to be detected by this assay. Stored supernatants were further analyzed by ultracentrifugation, nucleic acid extraction and PCR. Although negative results were obtained when analyzing supernatants of day 30 post-infection, positive results were obtained for days 45 and 70 post-infection with a significant increase of the viral titer between days 45 and 70 as determined by limiting-dilution PCR assays (Figure 2). The RR and intergenic region (IGR) of the viruses grown in cell culture were sequenced and found to be identical to the sequences previously obtained and reported from JCV present in urine sample BCNU1 (AF119345 and AF120242 in Bofill-Mas et al, 2000). An 80-cm<sup>2</sup> SVG flask was infected with 300  $\mu$ l of the supernatant of day 45 post-infection. After observation of CPE at day 15 together with PCR amplification and sequencing of the regulatory and intergenic regions, we confirmed the results previously obtained. Analysis of the supernatants with transmission electron microscopy (EM)

demonstrated the presence of polyomavirus-like particles with a diameter of 37-38 nm (Figure 3). This data is consistent with the description of JC and BK virions of 38-44 nm diameter reported by Frisque (Frisque, 1986). Analysis of the supernatant also revealed that archetypal BKV had replicated in SVG cells, although there was one logarithm less of BKV DNA than JCV DNA. The sequence of BKV grown in cell culture was identical to the one obtained from the urine sample. Further confirmation of the infection of SVG cells was obtained by observing positive cells when assaying *in situ* hybridizations of SVG cells grown on cover slides and infected with the supernatant analyzed by EM during 7 days (Figure 4).

COS-7 and CaCo-2 cells infected with BCNU1 did not present any CPE or positive HA or PCR results for a period of 2 months. PCR revealed that Mad-4 positive control had infected COS-7 cells without presenting CPE while there was no evidence of infection of Mad-4 in CaCo-2 cells. SVG cells infected with BCNU2 did not present any CPE or HA or PCR positive results within a period of 3 months. Negative and positive controls corresponded to previous results.

*Infection of SVG cells with JCV-Mad-4 stock treated with trypsin and acid pH.* As we hypothesized in a previous study (Bofill-Mas et al, 2001), the transmission of JCV, that remains still unknown, could occur via the gastrointestinal tract. That would require that viral particles were relatively resistant to proteolytic enzymes and acid pH, conditions present in the gastrointestinal environment. Although archetypal JCV present in a urine sample replicate in SVG cells after two months of infection, the stability of JC viral particles at acidic pH or trypsin was evaluated using JCV Mad-4 strain in order to produce results in experiments with shorter periods of incubation. SVG cells were infected with Mad-4 as a control, Mad-4 subjected to pH3 for 1 hour at 37°C and Mad-4 subjected to 10  $\mu$ g/ml of trypsin for 1 hour at 37°C. Frozen supernatants from days 3 and 13 were assayed by PCR obtaining negative results at day 3 for the 3 stocks assayed and positive results at day 13 for all the stocks. The virus concentration in the cell cultures was estimated by PCR limiting-dilution assays. The results were similar in those cells infected with Mad-4 and Mad-4 subjected to trypsin treatment and were 3 logarithms lower in the cells infected with Mad-4 subjected to pH 3 treatment.

*Infection of CaCo-2 cells with JCV-Mad-4 stock with and without pre-treatment with trypsin.* Trypsin has been extensively used as pre-treatment for some viruses infecting intestinal

cells. We tested the effect of pre activating JCV viral particles with trypsin in the infection of JCV-Mad-4 viral particles in CaCo-2 intestinal cells. Negative results were obtained for CPE and PCR of the cell culture supernatants 45 days after infection. With these results, together with other results obtained in the infection assays in normal conditions described above, we conclude that JCV Mad-4 did not replicate in CaCo-2 cells under normal conditions or after trypsin pre-activation.

## Discussion

The human gut is frequently exposed to JCV or JCV viral DNA (Bofill-Mas et al, 2001). JCV, which is excreted in urine and found in urban sewage and commonly presents an archetypal structure in the regulatory region (Bofill-Mas et al, 2000) as observed by direct sequencing of the JCV isolated in urban sewage.

Some of the studies based on the expression of reporter genes indicated that archetypal JCV exhibited significant activity (Sock et al, 1996; Ault, 1997). A study by Hara et al. (1998) reported that archetypal JCV produced efficient infection in COS-7 cell culture describing some variations in the sequence of the regulatory region of cultured JCV not observed in the archetypal strains cultured in SVG cells and described in this study. We report in this study, that archetypal strains which are present in the urine of a healthy pregnant woman have demonstrated infectivity. Pregnancy is the most common condition of altered immunocompetence that has been linked to the activation of polyomaviruses. A low level of neutralizing antibodies in urine may be required for assessing the infectivity of the urine excreted strains. It is known that anti-BKV and anti-JCV neutralizing antibodies are shed in urine and coat the viral particles (reviewed in Knowles, 2001).

We can not rule out the possibility that co-infection with BK facilitated growth of JCV in SVG cells. However, it should be considered that BK was present in the urine sample in a titre one logarithm lower than JC. The estimated quantity of JC and BK viral particles present in one ml of urine and used in the infection was of  $10^4$  and  $10^3$  respectively. The low number of virus present in the sample reduces the possibility of co-infection of BKV and JCV to a highly improbable event which is consistent with the higher titer of JCV produced in the infected cell culture in comparison with BKV.

The archetype strains did not show infection in COS-7 or CaCo-2 cells. A review from Jensen et al. (1999) provides an extensive list describing permissive and semi-permissive cells that

support virus replication. Although a derivative of PHFG cells (e.g., SVG cells) and also tonsillar stromal cells support moderate production of infectious virions after infection with strains presenting tandem repeated structures in the RR, further studies are required to evaluate the natural cell population responsible for the initial site of infection in humans.

It has been hypothesized that tonsils could be a route of entry. Oral transmission may involve both tonsil and gastrointestinal tissues. Viruses that infect gastrointestinal tissues often multiply in the throat. The results of stability to 10 µg/ml of trypsin and to pH 3 for 1 hour also support this hypothesis, although after the treatment with acidic pH a decrease in the progeny titer was observed. When ingested with food, JCV or JCV DNA may be protected due to the variable pH in the stomach. The ingestion of JCV from contaminated food, water or fomites could lead to the entry of polyomavirus into the human organism. The digestive tract is also exposed to polyomaviral DNA. Free JCV DNA was detected in sewage treated at pH 1 for 30 minutes (Bofill-Mas et al, 2001). It has been reported that the DNA does not completely degrade in the gastrointestinal tract (Schubert et al, 1997). The oral route of transmission has also been proposed by other authors (Walker et al, 1986; Sunforjd et al, 1994; Ricciardiello et al, 2000) and, in addition, has also been suggested for the closely related polyomaviruses BKV and SV40 (Bofill-Mas et al, submitted for publication).

One of the objectives of the present study was to analyze the JCV strains involved in the transmission of infection in the population. The archetypal configuration is highly conserved among JCV strains isolated from urine around the world (Agostini et al, 1999). This structure can be modified by both point mutations and small deletions or duplications. Some of these minor changes, found in PML patients, can be related to immunodeficiency or AIDS (Agostini et al, 1995; Kitamura et al, 1994). Agostini et al. reported the finding, in urine, of 7/48 archetypal sequences containing minor changes such as short deletions, unusual mutations or atypical duplications (Agostini et al, 1998). The percentage of sequences containing minor changes correlates with our results. These minor changes have also been reported in urine samples from non-immunocompromised individuals (Yogo et al, 1990) and in immunosuppressed renal transplant patients (Yogo et al, 1991; Kitamura et al 1994).

The tandem repeated strain detected in 1/20 clones from a sewage sample collected in Washington differs only one nucleotide with Mad-4.

The Taq polymerase error rate is 1/50 Kb and although the possibility of introducing PCR errors in a short fragment during the amplification reaction is very small, this possibility can not be ruled out. Confirmation of the presence of tandem repeated strains in the environment will require further studies analysing higher number of samples.

It has been suggested that tandem repeated strains evolved from an initial infection of archetypal strains after rearrangement in lymphocytes which produce extended cell tropism, these strains could be related to a higher level of pathogenicity and PML disease after immunosuppression (Yogo et al, 2001). We could hypothesize that primary infection would occur through archetypal strains, however, potential implication which is related with pathogenicity need to be evaluated for co-infection or re-infection with tandem repeated strains.

### Material and Methods

*Viruses.* JCV Mad-4 (Padgett et al, 1976) viral particles grown on SVG cells that were subsequently partially purified were utilized as a positive control.

*Cells.* SVG cell line, a cell line established by immortalization of human fetal brain cells with an original defective mutant of SV40 (Major et al, 1985), were propagated in Eagle's minimal essential medium (EMEM) supplemented with 1% glutamine, 50 µg of gentamicin per ml and 10% (growth medium) or 2% (maintenance medium) of heat-inactivated fetal bovine serum (FBS).

COS-7 cell line, a derivative of CV-1, an African green monkey cell line transformed with an origin-defective mutant of SV40, (Gluzman, 1981) was obtained from ATCC. COS-7 were propagated in Dulbecco's modified minimal essential medium (DMEM) supplemented with 1% glutamine, 50 µg of gentamicin per ml and 10% (growth medium) or 2% (maintenance medium) of heat-inactivated FBS.

CaCo-2 cells, derived from a human colon carcinoma, were propagated in EMEM supplemented with 1% glutamine, 1% non-essential amino acids (NEAA), 50 µg of gentamicin per ml and 10% (growth medium) or 2% (maintenance medium) of heat-inactivated FBS.

*Concentration of viral particles from urine samples.* Sample BCNU1 was obtained from a healthy-38-week-pregnant woman and sample BCNU2 was obtained from a healthy individual. Cells and viruses present in 120 ml of urine were

concentrated by ultracentrifugation for 1 hour at 4°C and 229,600xg, re-suspended in 1 ml of phosphate-buffered-saline (PBS) and, after a nucleic acid extraction (Boom et al, 1990), urine samples were tested by nested-PCR which contained JC (both samples) and BK (BCNU1) viruses as described below. Viruses were resuspended in PBS and stored at -80°C for further experiments.

*Concentration of viral particles from sewage samples.* A sewage sample (BCN) was collected in the sewers of Barcelona (Spain) in February 2001. The collection in a sterile 500-ml polyethylene container, was kept at 4°C for less than 8 hours until the viral particles were concentrated in PBS and stored at -80°C. One sample from the area of Washington D.C (USA) was collected in December 1999 and shipped frozen to Spain.

Recovery of viral particles was carried out as described in previous studies (Puig et al, 1994; Pina et al, 1998). Briefly: 40 ml of sewage sample were ultracentrifuged (229,600xg for 1 h at 4 °C) to pellet all the viral particles together with any suspended material. 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 12,000xg for 15 min after the addition of 4 ml of 2x PBS. Viruses were pelleted by ultracentrifugation (229,600xg for 1 h at 4 °C), re-suspended in 0.1 ml of 1x PBS and stored at -80 °C.

*Nucleic acid extraction.* Viral nucleic acids were extracted using a procedure that applies guanidinium thiocyanate (GuSCN) and adsorption of the nucleic acids to silica particles (Boom et al, 1990) providing clean nucleic acids for genomic amplification.

*Enzymatic amplification.* Ten µl aliquots of the extracted nucleic acids were used in each test. 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<sub>2</sub>, 200 µM of each dNTP, 2 units of Ampli Taq®DNA polymerase (Perkin-Elmer Cetus) and the corresponding primers at 25 µM. Throughout the PCR assays, the first cycle of denaturalization 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 at the corresponding annealing temperature (AT) for 60 s, and extension at 72°C for 75 s. All amplifications were completed with a 4 min, 72°C extension period. Primers and ATs used in this study are represented in Table 1. The results were analyzed by agarose gel

electrophoresis using ethidium bromide stain. Standard precautions were applied in all the manipulations in order to reduce the probability of sample contamination by amplified DNA molecules.

*Sequencing of PCR products.* Products obtained after PCR were purified with the QUIAquick PCR purification kit (QIAGEN, Inc.). 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) following the manufacturer's instructions. The conditions for the 25-cycle sequencing amplification were: denaturing at 96°C for 10 s, annealing at 50°C for 5 s and extension at 60°C for 4 min. Primers for sequencing were used at 2.5 µM concentration. The results were checked using the ABI PRISM 377 automated sequencer (Perkin-Elmer, Applied Biosystems).

#### *Hemagglutination assays (HA).*

Hemagglutination assays using human type O erythrocytes were carried out as previously described (Neel et al, 1996). Briefly: cell culture supernatants were centrifuged for 15 min at 10,000xg. Pellets were re-suspended in EMEM 10% DOC (sodium salt deoxycholic acid monohydrate 0.25%) and kept at 37°C for 10 min. Fifty µl of each sample were serially diluted at 1:2 in Asever's buffer in a 96-well plate. Fifty µl of human type O red blood cells (0.5% in Asever's buffer) were added to each well. Plates were covered and kept at 4°C for 90 min. The HA titer was expressed as the reciprocal of the final dilution which agglutinated the erythrocytes.

*In situ hybridization.* In situ hybridization was carried out as previously described (Askamit et al, 1985). Briefly, SVG cells grown on coverslips were washed in PBS and fixed for 30 minutes in 4% paraformaldehyde at room temperature. The cells were dehydrated in serial ethanol washes, hydrolyzed in HCl, washed in Triton-X, subjected to limited protein digestion with pronase, washed in glycine buffer and dehydrated in serial ethanol washes. The cells were hybridized with 25 µl of a probe mixture containing 10% dextran sulfate, 50% formamide, 0.4 mg/ml calf thymus DNA, 2 µg/ml biotinylated JCV DNA probe (ENZO Biochem) and 2x SSC (300mM sodium chloride and 30 mM sodium citrate). Probe and cellular DNA were denatured by incubation at 85°C for 10 minutes. Hybridization was performed at 37°C overnight. Cells were then washed with 2X SSC for 2 minutes with PBS 0.1% Triton-X and in

PBS for 2 and 3 minutes respectively. Detection of the biotinylated probe was carried out immediately using streptavidine-biotin-horseradish peroxidase kit (Detek I-hrp, ENZO, Biochem.). A fresh solution of diaminobenzidine tetrahydrochloride (DAB) was used as a chromogen. Cells were then washed in 2X SSC, counterstained with hematoxylin, dehydrated and mounted.

*Electron microscopy.* Four ml of cell culture supernatant were concentrated by ultracentrifugation. Five µl of the concentrate were deposited on copper grids coated with Formvar film and carbon that were negatively stained with 2% PTA (phosphotungstic acid) pH 7.0 for 1 min. Particles were viewed with a Hitachi H600AB electron microscope.

*Cloning of the regulatory region of the JCV present in a sewage sample.* Sewage samples collected in Spain and USA were analyzed for the presence of JCV after concentration of the viral particles and nucleic acid extraction. The primers used in the nested-PCR were JR1, JR2, JR3 and JR4 (Monaco et al, 1998) that amplifies the RR of JCV. The band obtained after analyzing the results in a 3% agarose gel electrophoresis using ethidium bromide as a stain was directly ligated into a pGEM®-T EasyVector (pGEM®-T EasyVector System II, Promega Co.) in accordance with the manufacturer's instructions. Ligation was used to transform competent *Escherichia coli* JM109 competent cells (Promega Co.) that were plated on LB plates supplemented with ampiciline 100µg/ml, 0.5mM IPTG and X-Gal 80µg/ml. Transformed bacterial clones were detected by blue/white screening. In twenty of the clones obtained from each sample the insert was recovered by PCR amplification using primers JR3 and JR4. Products obtained after the PCR were further analyzed by sequencing using the amplification primers.

*Infection of SVG, COS-7 and CaCo-2 cells with urine samples containing JCV viral particles.* Five-hundred µl of concentrated urines BCNU1 and BCNU2 were filtered using 0.22 µm low protein binding durapore syringe driven filters (Millex®-GV, Millipore) in order to eliminate any bacteria or cells remaining in the urine concentrate.

One-hundred µl of the filtrate BCNU1 were used to infect SVG, CaCo-2 and COS-7 cells plated on 25-cm<sup>2</sup> flasks. Samples were also diluted 1:10 and dilutions were used to infect another set of the 3 cell types. One-hundred µl of the filtrate BCNU2, and its respective 1:10 dilution, were used to infect SVG cells plated on 25-cm<sup>2</sup> flasks.

Negative (100 µl of PBS) and positive controls (100 µl of a 1:1000 dilution of a JCV-Mad-4 stock containing 1024 HA/50µl) were also assayed. After 1 hour at 37°C maintenance medium was added. Supernatants were renewed weekly using maintenance medium (2% FBS) and stored at -80°C for further studies. After one month of culturing, cells were passed at a split ratio 1:2. The time course of the infection was examined by observation of cytopathic effect (CPE) and hemagglutination tests (HA). Stored supernatants of different days post-infection were ultracentrifuged at 229,600 xg for 1 h at 4°C and pellets were re-suspended in 100 µl of PBS and a nucleic acid extraction and a subsequent PCR using primers JR1, JR2 was carried out. Confirmative cell culture and additional PCR and sequencing of the IGR and RR of the progeny virions were investigated for evidence of infection. Electron microscopy and in situ hybridization were also assayed as further described.

*Infection of SVG cells with JCV-Mad-4 treated with trypsin and acid pH.* A JCV-Mad-4 stock containing approximately 10<sup>5</sup> PV/ml was aliquoted into three different tubes. Ten µg/ml of trypsin (Gibco) were added to one of the tubes, another tube was treated with diluted HCl until it reached pH 3 and the third tube was kept as a control. Tubes were incubated at 37°C for 60 minutes. FBS was added to the tube treated with trypsin in order to inactivate the enzyme. The tube treated with HCl was neutralized with diluted NaOH. We infected 25-cm<sup>2</sup> SVG flasks with 100 µl of each of the three stocks. Negatives controls were also assayed. After 1 hour at 37°C, maintenance medium was added, renewed periodically and supernatants frozen for further studies. The time course of the infection was monitored by observation of CPE and PCR of the supernatants.

*Infection of CaCo-2 cells with JCV-Mad-4 treated with trypsin.* An experiment identical to the one described above was carried out using CaCo-2 cells and Mad-4 viral particles treated with trypsin. The time course of infection was followed by observation of CPE and PCR of the ultracentrifuged supernatants.

*Nucleotide sequence accession numbers.* Sequences for the Barcelona clones reported in this paper have been deposited in the GenBank database under accession no. AY083211-AY083213. Sequences for the Washington D.C clones have been deposited under accession no. AF532778-AF532779 and AF533308-AF533313.

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**1a**

<b>archetypal</b>	GCCTCGGCCTCCTGTATATATAAAAAAAGGGAAGGTAGGGAGGAGCTGGCTAAAACTGGATGGCTGCCAGCCAAGCATGAGCTCATACTAGG	94
BCN2.32	.....-.....	93
BCN2.5	.....	94
BCN2.27	.....	94
<b>archetypal</b>	GAGCCAACCGAGTGACAGCCAGAGGGAGCCCTGGCTGCATGCCACTGGCAGTTATAGTAAAACCCCTCCCATAGTCCTTAATCACAAGTAAACA	188
BCN2.32	.....	187
BCN2.5	.....	188
BCN2.27	.....G.....	188
<b>archetypal</b>	AAGCACAGGGGAAGTGGAAGCAGCCAGGGGAACATGTTTTGCGAGCCAGAGC-----TGTTTTG	249
BCN2.32	.....	248
BCN2.5	.....GCAGCCAAGGGAACATGTTTTGCGAGCCAGAGC.....	282
BCN2.27	.....	249
<b>archetypal</b>	GCTTGTACCAGCTGGCCAT	269
BCN2.32	.....	268
BCN2.5	.....	302
BCN2.27	.....	269

**1b**

<b>archetypal</b>	GCCTCGGCCTCCTGTATATATAAAAAAAGGGAAGGTAGGGAGGAGCTGGCTAAAACTGGATGGCTGCCAGCCAAGCATGAGCTCATACTA	92
USA2.42	.....	92
USA2.16	.....G.....	92
USA2.46	.....G.....	92
USA2.66	.....	92
USA2.39	.....	92
USA2.34	.....	92
USA2.43	.....	92
<b>archetypal</b>	GGGAGCCAACCGAGTGACAGCCAGAGGGAGCCCTGGCTGCATGCCACTGGCAGTTATAGTAAAACCCCTCCCATAGTCCTTAATCACAAGTA	184
USA2.42	.....	184
USA2.16	.....	184
USA2.46	A.....	184
USA2.66	.....	184
USA2.39	..A.....	184
USA2.34	.....	184
USA2.43	.....G.....	184
<b>archetypal</b>	AACAAAGCACAGGGGAAGTGGAAGCAGCCAAGGGAACATGTTTTGCGAGCCAGAGCTGTTTTGGCTTGTACCAGCTGGCCAT	269
USA2.42	.....G.....	269
USA2.16	.....G.....	269
USA2.46	.....	269
USA2.66	.....A.....	269
USA2.39	.....	269
USA2.34	.....	267
USA2.43	.....G.....	264

**1c USA2.41**

GCCTCGGCCTCCTGTATATATAAAAAAAGGGAAGGGATGGCTGCCAGCCAAGCATGAGCTCATACTAGGGAGCCAACCA  
**GCTa**ACAGCCAGTAAACAAAGCACAAGGGGAAGGGATGGCTGCCAGCCAAGCATGAGCTCATACTAGGGAGCCAACCAGC  
**Ta**ACAGCCAGTAAACAAAGCACAAGGGGAAGTGGAAGCAGCCAAGGGAACATGTTTTACGAGCCAGAGCTGTTTTGGCTT  
**GTCACCAGCTGGCCAT**



**Archetypal**

GCCTCGGCCTCCTGTATATATAAAAAAAGGGAAGGTAGGGAGGAGCTGGCTAAAACTGGATGGCTGCCAGCCAAGCATGAGCTCATA  
**CCTAGGGAGCCAACCAGCTg**ACAGCCAGAGGGAGCCCTGGCTGCATGCCACTGGCAGTTATAGTAAAACCCCTCCCATAGTCCTTAATC  
**ACAAGTAAACAAAGCACAAGGGGAAGTGGAAGCAGCCAAGGGAACATGTTTTGCGAGCCAGAGCTGTTTTGGCTTGTACCAGCTG**  
**GCCAT**



**Figure 1.** Structure of the regulatory region of archetypal-like clones detected in sewage from Spain (a) and USA (b) and a tandem repeated structure detected in sewage from USA (c). The archetypal conformation is represented in the upper line of the figure. Numbering system is that of Frisque et al. (1984). Dots represent identities while dashes represent deletions. Figure 1a, shadowed area represent the nucleotides repeated in the 33bp insertion. Figure 1c, from the early side, sections a to f (a b c d e f) following the ori. Archetypal structure presents sections b and d while clone USA2.41 present a tandem repeated structure with a partial duplication of section a in the second 98 bp repeat. Bold lower case nucleotides represent those typically specific of archetypal or tandem repeated structures. USA2.41 differs one nucleotide with JCV Mad-4, this nucleotide is represented with an arrow and is also different in the archetypal structure.

## Analysis of the excreted JCV strains and their potential oral transmission

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Table 1. Oligonucleotide primers used for PCR amplification and sequencing of JCV

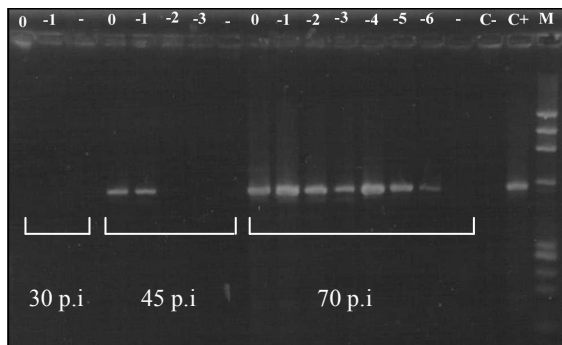
Virus type (region)	Position	Primers	Reaction	AT <sup>a</sup>	Sequence
JCV (IGR) <sup>a</sup>	2062-2087 <sup>b</sup>	EP1A	First PCR	59°C	5'-TGAATGTTGGGTTCTGATCCCACC-3'
JCV (IGR)	2774-2798	EP2A	First PCR	59°C	5'-ACCCATTCTTGACTTTCCTAGAGAG-3'
JCV (RR) <sup>a</sup>	4992-5011	JR1 <sup>c</sup>	First PCR	53°C	5'-CCCTATTCAGCACTTTGTCC-3'
JCV (RR)	428-447	JR2 <sup>c</sup>	First PCR	53°C	5'-CAAACCACTGTGTCTCTGTC-3'
JCV (RR)	5060-5079	JR3 <sup>c</sup>	Nested	53°C	5'-GGGAATTTCCCTGGCCTCCT-3'
JCV (RR)	298-317	JR4 <sup>c</sup>	Nested	53°C	5'-ACTTTCACAGAAGCCTTACG-3'
BKV (RR)	5024-5043 <sup>d</sup>	BR1	First PCR	44°C	5'-CCCTGTTWARRACTTTATCC-3' <sup>a</sup>
BKV (RR)	431-457	BR2	First PCR	46°C	5'-GTAAAGCAGTGGTACTTT-3'

<sup>a</sup> IGR= Intergenic region; RR= Regulatory region; AT= Annealing temperature; W= A or T; R= A or G

<sup>b</sup> The sequence positions are referred to the JCV-Mad1 sequence

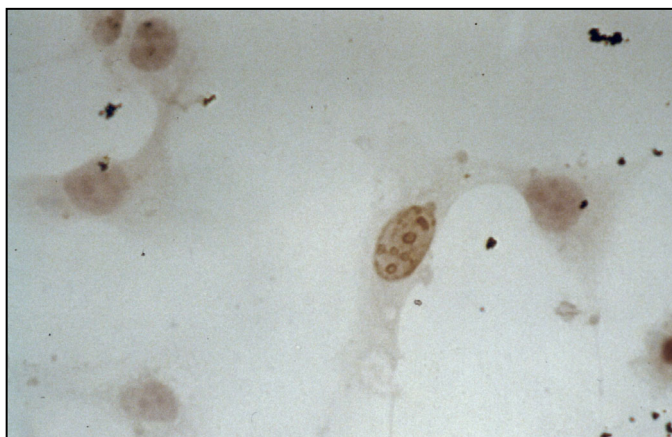
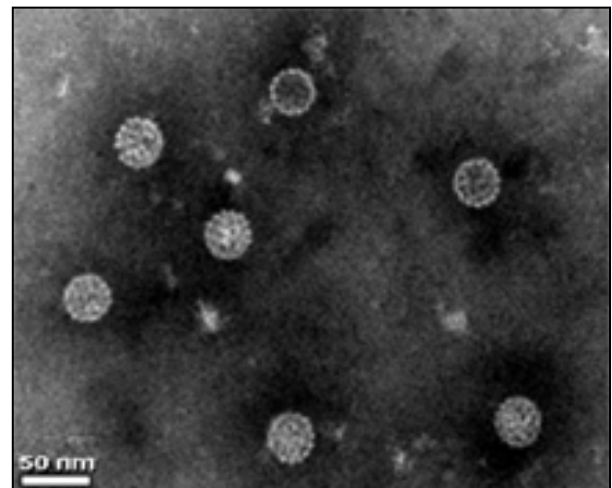
<sup>c</sup> From Monaco et al. (1998)

<sup>d</sup> The sequence positions are referred to the BKV Dunlop strain sequence.




**Figure 2.** Agarose gel showing the limiting-dilution nested-PCR amplifications of the JCV DNA present in the concentrated supernatants of SVG cell cultures infected with urine sample BCNU1 at different stages of infection (days 30, 45 and 70 post-infection). Negative cell culture controls, PCR negative and positive controls and molecular weight marker ( $\Phi$ X174 Hae III digested) are also showed. The bands observed (582 bp) are the ones corresponding to the regulatory region of JCV obtained by amplification with JR1 and JR2.

**Figure 3.** Electron micrograph of JCV present in the supernatant of a SVG cell culture as the result of an infection with a urine sample (BCNU1) containing archetypal JCV. A scale bar (50 nm) and 38 nm virions consistent with polyomaviruses are observed.




**Figure 4.** Identification of JC virus DNA in the nuclei of SVG cells infected with viruses isolated from urine of a pregnant woman using in situ DNA hybridization. A positive signal is demonstrated by the development of a brown precipitate in the nucleus of the cell which viral DNA is replicating (photomicrograph 40x).


  
 Mad4 : GCCTCGGCTCCTGTATATAA AAAAAAAGGGAAGGGATGGCTGCCAGCCAAGCATGAGCTCATACCTAGGGAAGCCAACCAGCTAACAGCCAGTAAACAAGCACAAGGGG : 111
   
 WAS10 : GCCTCGGCTCCTGTATATAA AAAAAAAGGGAAGGGATGGCTGCCAGCCAAGCATGAGCTCATACCTAGGGAAGCCAACCAGCTAACAGCCAGTAAACAAGCACAAGGGG : 111
   
 WAS6 : GCCTCGGCTCCTGTATATAA AAAAAAAGGGAAGGGATGGCTGCCAGCCAAGCATGAGCTCATACCTAGGGAAGCCAACCAGCTAACAGCCAGTAAACAAGCACAAGGGG : 111
   
 WAS2 : GCCTCGGCTCCTGTATATAA AAAAAAAGGGAAGGGATGGCTGCCAGCCAAGCATGAGCTCATACCTAGGGAAGCCAACCAGCTAACAGCCAGTAAACAAGCACAAGGGG : 111
   
 WAS5 : GCCTCGGCTCCTGTATATAA AAAAAAAGGGAAGGGATGGCTGCCAGCCAAGCATGAGCTCATACCTAGGGAAGCCAACCAGCTAACAGCCAGTAAACAAGCACAAGGGG : 111
   
 WAS4 : GCCTCGGCTCCTGTATATAA AAAAAAAGGGAAGGGATGGCTGCCAGCCAAGCATGAGCTCATACCTAGGGAAGCCAACCAGCTAACAGCCAGTAAACAAGCACAAGGGG : 111


  
 Mad4 : AAGGGATGGCTGCCAGCCAAGCATGAGCTCATACCTAGGGAGCCAACCAGCTAACAGCCAGTAAACAAGCACAAGG----- : 188
   
 WAS10 : AAGGGATGGCTGCCAGCCAAGCATGAGCTCATACCTAGGGAGCCAACCAGCTAACAGCCAGTAAACAAGCACAAGG GGAAGGGATGGCTGCCAGCCAAC TTTGAGCTCAT : 222
   
 WAS6 : AAGGGATGGCTGCCAGCCAAGCATGAGCTCATACCTAGGGAGCCAACCAGCTAACAGCCAGTAAACAAGCACAAGG----- : 188
   
 WAS2 : AAGGGATGGCTGCCAGCCAAGCATGAGCTCATACCTAGGGAGCCAACCAGCTAACAGCCAGTAAACAAGCACAAGG----- : 188
   
 WAS5 : AAGGGATGGCTGCCAGCCAAGCATGAGCTCATACCTAGGGAGCCAACCAGCTAACAGCCAGTAAACAAGCACAAGG----- : 188
   
 WAS4 : AAGGGATGGCTGCCAGCCAAGCATGAGCTCATACCTAGGGAGCCAACCAGCTAACAGCCAGTAAACAAGCACAAGG----- : 188


  
 Mad4 : -----GGAAGTGAAAAGCAGCCAAGGGAACATGTTTTGCGAGCCAGAGCTGTTTTGGCTTGTCCACCAGCTG : 254
   
 WAS10 : ACCTAGGGAGCCAACCAGCTAACAGCCAGTAAACAAGCACAAGG GGAAGTGAAAAGCAGCCAAGGGAACATGTTTTGCGAGCCAGAGCTGTTTTGGCTTGTCCACCAGCTG : 333
   
 WAS6 : -----GGAAGTGAAAAGCAGCCAAGGGAACATGTTTTGCGAGCCAGAGCTGTTTTGGCTTGTCCACCAGCTG : 254
   
 WAS2 : -----GGAAGTGAAAAGCAGCCAAGGGAACATGTTTTGCGAGCCAGAGCTGTTTTGGCTTGTCCACCAGCTG : 254
   
 WAS5 : -----GGAAGTGAAAAGCAGCCAAGGGAACATGTTTTGCGAGCCAGAGCTGTTTTGGCTTGTCCACCAGCTG : 254
   
 WAS4 : -----GGAAGTGAAAAGCAGCCAAGGGAACATGTTTTGCGAGCCAGAGCTGTTTTGGCTTGTCCACCAGCTG : 254


  
 Mad4 : GCCATGGTTCTTCGCCAGCTGTCACGTAAGG----- : 285
   
 WAS10 : GCCATGGTTCTTCGCCAGCTGTCACGTAAGG----- : 364
   
 WAS6 : GCCATGGTTCTTCGCCAGCTGTCACGTAAGG----- : 285
   
 WAS2 : GCCATGGTTCTTCGCCAGCTGTCACGTAAGG----- : 285
   
 WAS5 : GCCATGGTTCTTCGCCAGCTGTCACGTAAGG----- : 285
   
 WAS4 : GCCATGGTTCTTCGCCAGCTGTCACGTAAGG----- : 285

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**Figura annexa B.** Anellament de les 5 seqüències reorganitzades obtingudes després de clonar la RR dels JC detectats en una mostra d'aigua residual de l'àrea de Washington D.C. (EUA). A la línia superior es representa la seqüència de la soca Mad-4. Els nucleòtids en negre representen les identitats mentre que els que apareixen en blanc representen nucleòtids diferents. Les barres de color indiquen les diferents seccions de la RR que apareixen en aquestes seqüències tal i com es descriu a la llegenda.

Arquetipic : GCCTCGGCCTCCTGTATATATAAAAAAAGGGAAGGTAGGGAGGAGCTGGCTAAAACGGATGGCTGCCAGCCAAGCATGAGCTCATACCTAGGGAGCCAACCAGCTG : 108  
 WAS9 : GCCTCGGCCTCCTGTATATATAAAAAAAGGGAAGGTAGGGAGGAGCTGGCTAAAACGGATGGCTGCCAGCCAAGCATGAGCTCATACCTAGGGAGCCAACCAGCTG : 108  
 WAS1 : GCCTCGGCCTCCTGTATATATAAAAAAAGGGAAGGTAGGGAGGAGCTGGCTAAAACGGATGGCTGCCAGCCAAGCATGAGCTCATACCTAGGGAGCCAACCAGCTG : 108  
 WAS7 : GCCTCGGCCTCCTGTATATATAAAAAAAGGGAAGGTAGGGAGGAGCTGGCTAAAACGGATGGCTGCCAGCCAAGCATGAGCTCATACCTAGGGAGCCAACCAGCTG : 108  
 WAS3 : GCCTCGGCCTCCTGTATATATAAAAAAAGGGAAGGTAGGGAGGAGCTGGCTAAAACGGATGGCTGCCAGCCAAGCATGAGCTCATACCTAGGGAGCCAACCAGCTG : 108  
 WAS8 : GCCTCGGCCTCCTGTATATATAAAAAAAGGGAAGGTAGGGAGGAGCTGGCTAAAACGGATGGCTGCCAGCCAAGCATGAGCTCATACCTAGGGAGCCAACCAGCTG : 108

Arquetipic : ACAGCCAGAGGGAGCCCTGGCTGCATGCCACTGGCAGTTATAGTGAAACCCTCCCATAGTCCTTAATCACAAGTAAACAAGCACAAGGGGAAGTGGAAAAGCAGCCA : 216  
 WAS9 : ACAGCCAGAGGGAGCCCTGGCTGCATGCCACTGGCAGTTATAGTGAAACCCTCCCATAGTCCTTAATCACAAGTAAACAAGCACAAGGGGAAGTGGAAAAGCAGCCA : 216  
 WAS1 : ACAGCCAGAGGGAGCCCTGGCTGCATGCCACTGGCAGTTATAGTGAAACCCTCCCATAGTCCTTAATCACAAGTAAACAAGCACAAGGGGAAGTGGAAAAGCAGCCA : 216  
 WAS7 : ACAGCCAGAGGGAGCCCTGGCTGCATGCCACTGGCAGTTATAGTGAAACCCTCCCATAGTCCTTAATCACAAGTAAACAAGCACAAGGGGAAGTGGAAAAGCAGCCA : 216  
 WAS3 : ACAGCCAGAGGGAGCCCTGGCTGCATGCCACTGGCAGTTATAGTGAAACCCTCCCATAGTCCTTAATCACAAGTAAACAAGCACAAGGGGAAGTGGAAAAGCAGCCA : 216  
 WAS8 : ACAGCCAGAGGGAGCCCTGGCTGCATGCCACTGGCAGTTATAGTGAAACCCTCCCATAGTCCTTAATCACAAGTAAACAAGCACAAGGGGAAGTGGAAAAGCAGCCA : 216

Arquetipic : AGGGAACATGTTTTGCGAGCCAGAGCTGTTTTGGCTTGTCACCAGCTGGCCATGGTTCTTCGCCAGCTGTCACGTAAGG : 295  
 WAS9 : AGGGAACATGTTTTGCGAGCCAGAGCTGTTTTGGCTTGTCACCAGCTGGCCATGGTTCTTCGCCAGCTGTCACGTAAGG : 295  
 WAS1 : AGGGAACATGTTTTGCGAGCCAGAGCTGTTTTGGCTTGTCACCAGCTGGCCATGGTTCTTCGCCAGCTGTCACGTAAGG : 295  
 WAS7 : AGGGAACATGTTTTGCGAGCCAGAGCTGTTTTGGCTTGTCACCAGCTGGCCATGGTTCTTCGCCAGCTGTCACGTAAGG : 295  
 WAS3 : AGGGAACATGTTTTGCGAGCCAGAGCTGTTTTGGCTTGTCACCAGCTGGCCATGGTTCTTCGCCAGCTGTCACGTAAGG : 295  
 WAS8 : AGGGAACATGTTTTGCGAGCCAGAGCTGTTTTGGCTTGTCACCAGCTGGCCATGGTTCTTCGCCAGCTGTCACGTAAGN : 295

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→ agnoproteïna

**Figura annexa A.** Anellament de les 5 seqüències arquetípiques o semblants a l'arquetípica obtingudes després de clonar la RR dels JC detectats en una mostra d'aigua residual de l'àrea de Washington D.C. (EUA). A la línia superior es representa la seqüència d'una soca arquetípica. Els nucleòtids en negre representen les identitats mentre que els que apareixen en blanc representen nucleòtids diferents. Les barres de color indiquen les diferents seccions de la RR que apareixen en aquestes seqüències tal i com es descriu a la llegenda.

Arquetipic : GCCTCGGCTCCTGTATATATAAAAAAAGGGAAGGTAGGGAGGAGCTGGCTAAAACGGATGGCTGCCAGCCAAGCATGAGCTCATACCTAGGGAGCCAACCAGC : 106  
 BCN25 : GCCTCGGCTCCTGTATATATAAAAAAAGGGAAGGTAGGGAGGAGCTGGCTAAAACGGATGGCTGCCAGCCAAGCATGAGCTCATACCTAGGGAGCCAACCAGC : 106  
 BCN14 : GCCTCGGCTCCTGTATATATAAAAAAAGGGAAGGTAGGGAGGAGCTGGCTAAAACGGATGGCTGCCAGCCAAGCATGAGCTCATACCTAGGGAGCCAACCAGC : 106  
 BCN12 : GCCTCGGCTCCTGTATATATAAAAAAAGGGAAGGTAGGGAGGAGCTGGCTAAAACGGATGGCTGCCAGCCAAGCATGAGCTCATACCTAGGGAGCCAACCAGC : 106  
 BCN7 : GCCTCGGCTCCTGTATATATAAAAAAAGGGAAGGTAGGGAGGAGCTGGCTAAAACGGATGGCTGCCAGCCAAGCATGAGCTCATACCTAGGGAGCCAACCAGC : 106  
 BCN15 : GCCTCGGCTCCTGTATATATAAAAAAAGGGAAGGTAGGGAGGAGCTGGCTAAAACGGATGGCTGCCAGCCAAGCATGAGCTCATACCTAGGGAGCCAACCAGC : 106  
 BCN2 : GCCTCGGCTCCTGTATATATAAAAAAAGGGAAGGTAGGGAGGAGCTGGCTAAAACGGATGGCTGCCAGCCAAGCATGAGCTCATACCTAGGGAGCCAACCAGC : 106  
 BCN13 : GCCTCGGCTCCTGTATATATAAAAAAAGGGAAGGTAGGGAGGAGCTGGCTAAAACGGATGGCTGCCAGCCAAGCATGAGCTCATACCTAGGGAGCCAACCAGC : 106  
 BCN18 : GCCTCGGCTCCTGTATATATAAAAAAAGGGAAGGTAGGGAGGAGCTGGCTAAAACGGATGGCTGCCAGCCAAGCATGAGCTCATACCTAGGGAGCCAACCAGC : 106  
 BCN9 : GCCTCGGCTCCTGTATATATAAAAAAAGGGAAGGTAGGGAGGAGCTGGCTAAAACGGATGGCTGCCAGCCAAGCATGAGCTCATACCTAGGGAGCCAACCAGC : 106  
 BCN17 : GCCTCGGCTCCTGTATATATAAAAAAAGGGAAGGTAGGGAGGAGCTGGCTAAAACGGATGGCTGCCAGCCAAGCATGAGCTCATACCTAGGGAGCCAACCAGC : 106

Arquetipic : TGACAGCCAGAGGGAGCCCTGGCTGCATGCCACTGGCAGTTATAGTGAACCCCTCCATAGTCCTTAATCACAAGTAAACAAAGCACAAGGGGAAGTGGAAAGCA : 212  
 BCN25 : TGACAGCCAGAGGGAGCCCTGGCTGCATGCCACTGGCAGTTATAGTGAACCCCTCCATAGTCCTTAATCACAAGTAAACAAAGCACAAGGGGAAGTGGAAAGCA : 212  
 BCN14 : TGACAGCCAGAGGGAGCCCTGGCTGCATGCCACTGGCAGTTATAGTGAACCCCTCCATAGTCCTTAATCACAAGTAAACAAAGCACAAGGGGAAGTGGAAAGCA : 212  
 BCN12 : TGACAGCCAGAGGGAGCCCTGGCTGCATGCCACTGGCAGTTATAGTGAACCCCTCCATAGTCCTTAATCACAAGTAAACAAAGCACAAGGGGAAGTGGAAAGCA : 212  
 BCN7 : TGACAGCCAGAGGGAGCCCTGGCTGCATGCCACTGGCAGTTATAGTGAACCCCTCCATAGTCCTTAATCACAAGTAAACAAAGCACAAGGGGAAGTGGAAAGCA : 212  
 BCN15 : TAAACAGCCAGAGGGAGCCCTGGCTGCATGCCACTGGCAGTTATAGTGAACCCCTCCATAGTCCTTAATCACAAGTAAACAAAGCACAAGGGGAAGTGGAAAGCA : 212  
 BCN2 : TGTAGCCAGAGGGAGCCCTGGCTGCATGCCACTGGCAGTTAT-----CATAGTCCTTAATCACAAGTAAACAAAGCACAAGGGGAAGTGGAAAGCA : 198  
 BCN13 : TGTAGCCAGAGGGAGCCCTGGCTGCATGCCACTGGCAGTTAT-----CATAGTCCTTAATCACAAGTAAACAAAGCACAAGGGGAAGTGGAAAGCA : 198  
 BCN18 : TGTAGCCAGAGGGAGCCCTGGCTGCATGCCACTGGCAGTTAT-----CATAGTCCTTAATCACAAGTAAACAAAGCACAAGGGGAAGTGGAAAGCA : 198  
 BCN9 : TGTAGCCAGAGGGAGCCCTGGCTGCATGCCACTGGCAGTTAT-----CATAGTCCTTAATCACAAGTAAACAAAGCACAAGGGGAAGTGGAAAGCA : 198  
 BCN17 : TGTAGCCAGAGGGAGCCCTGGCTGCATGCCACTGGCAGTTAT-----CATAGTCCTTAATCACAAGTAAACAAAGCACAAGGGGAAGTGGAAAGCA : 198

Arquetipic : GCCAAGGGAACATGTTTTGCGAGCCAGAGCTGTTTTGGCTTGTACCAGCTGGCCATGGTTCTTCGCCAGCTGTCACGTAAGG : 295  
 BCN25 : GCCAAGGGAACATGTTTTGCGAGCCAGAGCTGTTTTGGCTTGTACCAGCTGGCCATGGTTCTTCGCCAGCTGTCACGTAAGG : 295  
 BCN14 : GCCAAGGGAACATGTTTTGCGAGCCAGAGCTGTTTTGGCTTGTACCAGCTGGCCATGGTTCTTCGCCAGCTGTCACGTAAGG : 295  
 BCN12 : GCCAAGGGAACATGTTTTGCGAGCCAGAGCTGTTTTGGCTTGTACCAGCTGGCCATGGTTCTTCGCCAGCTGTCACGTAAGG : 295  
 BCN7 : GCCAAGGGAACATGTTTTGCGAGCCAGAGCTGTTTTGGCTTGTACCAGCTGGCCATGGTTCTTCGCCAGCTGTCACGTAAGG : 295  
 BCN15 : GCCAAGGGAACATGTTTTGCGAGCCAGAGCTGTTTTGGCTTGTACCAGCTGGCCATGGTTCTTCGCCAGCTGTCACGTAAGG : 295  
 BCN2 : GCCAAGGGAACATGTTTTGCGAGCCAGAGCTGTTTTGGCTTGTACCAGCTGGCCATGGTTCTTCGCCAGCTGTCACGTAAGG : 281  
 BCN13 : GCCAAGGGAACATGTTTTGCGAGCCAGAGCTGTTTTGGCTTGTACCAGCTGGCCATGGTTCTTCGCCAGCTGTCACGTAAGG : 281  
 BCN18 : GCCAAGGGAACATGTTTTGCGAGCCAGAGCTGTTTTGGCTTGTACCAGCTGGCCATGGTTCTTCGCCAGCTGTCACGTAAGG : 281  
 BCN9 : GCCAAGGGAACATGTTTTGCGAGCCAGAGCTGTTTTGGCTTGTACCAGCTGGCCATGGTTCTTCGCCAGCTGTCACGTAAGG : 281  
 BCN17 : GCCAAGGGAACATGTTTTGCGAGCCAGAGCTGTTTTGGCTTGTACCAGCTGGCCATGGTTCTTCGCCAGCTGTCACGTAAGG : 281

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- D
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- F

→ agnoproteïna

**Figura annexa C.** Anellament de les 10 seqüències arquetípiques o semblants a l'arquetípica obtingudes després de clonar la RR dels JC detectats en una mostra d'aigua residual de l'àrea de Barcelona. A la línia superior es representa la seqüència d'una soca arquetípica. Els nucleòtids en negre representen les identitats mentre que els que apareixen en blanc representen nucleòtids diferents. Les barres de color indiquen les diferents seccions de la RR que apareixen en aquestes seqüències tal i com es descriu a la llegenda.



**Capítol IV. Detecció de SV40 en mostres ambientals.**

Detection of SV40 in the Environment of a Naturally Infected Colony of *Cynomolgus* Monkeys.

Sílvia Bofill-Mas, Néstor Albiñana-Giménez, Pamela A. Pipkin, Phil D. Minor, Rosina Girones.

En procés de preparació.

**RESUM**

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**1. Objectius**

SV40 infecta micos *rhesus* que habiten a l'Àsia i en alguns casos altres espècies de micos criats en captivitat. La infecció és normalment benigna i en casos d'immunosupressió pot ocasionar PML i deficiències renals. També s'han descrit tumors de cervell i lesions del sistema nerviós central diferents de la PML. SV40 va ser, com ja s'ha comentat a la introducció, un contaminant de les vacunes de la polio distribuïdes entre 1955 i 1963. Des de llavors s'ha descrit la presència de SV40 en diferents tipus de tumors en humans sobretot en mesoteliomes, tumors de cervell, osteosarcomes i limfomes. En els treballs exposats als capítols I i II no es va detectar SV40 en aigües residuals urbanes de diferents zones geogràfiques. Aquests resultats ens van fer concloure que SV40 no circulava a les poblacions estudiades o si ho feia era en concentracions molt baixes que el nostre mètode no podia detectar o bé que SV40 no es comportava com els poliomavirus humans i no era excretat a l'orina i/o femta dels individus infectats. En aquest estudi es va avaluar l'estabilitat de SV40 a l'aigua residual i es va estudiar l'excreció de SV40 per part d'una colònia de micos *cynomolgus* criats en captivitat naturalment infectats amb SV40. Es va valorar l'excreció, presència, i estabilitat de les partícules víriques de SV40 presents en la femta, orina i restes de menjar de les gàbies on habiten els primats objecte de l'estudi.

Els objectius d'aquest treball van ser:

- Avaluar l'estabilitat de partícules víriques de SV40 en aigua residual.
- Avaluar la seropositivitat per SV40 dels micos objecte de l'estudi.

- Estudiar la presència de SV40 en l'ambient on residia la colònia de micos.
- Estudiar quines eren les soques de SV40 que circulaven en la colònia.

## 2. Material i Mètodes

Es van recol·lectar 2 mostres de dies diferents de 4 gàbies. Es van concentrar les partícules víriques (PV) presents en les mostres per ultracentrifugació, elució de la matèria orgànica en tampó glicina pH 9,5 i una segona ultracentrifugació. En alguns casos en els que les mostres recol·lectades eren sòlides es va dur a terme una variació del protocol tal i com es descriu a la secció de Material i Mètodes de l'article adjunt. Es va dur a terme una extracció d'àcids nucleics mitjançant el mètode de Boom i col. (1990). L'amplificació dels àcids nucleics es va fer per PCR niada i semiquantificació de la concentració vírica per assaigs de dilució límit. Els amplicons obtinguts van ser seqüenciats tal i com es descriu a la secció Material i Mètodes de l'article.

## 3. Resultats

- 1) La  $t_{90}$  i la  $t_{99}$  de partícules víriques de SV40 en aigua residual a 20°C van resultar ser de 39.9 i 64.7 dies respectivament.
- 2) Tots els individus de la colònia objecte d'estudi eren seropositius per SV40 (Taula 2 de l'article).
- 3) En les dues mostres de 2/4 gàbies estudiades es van detectar  $10^2$ - $10^4$  partícules víriques de SV40/ml.
- 4) La soca detectada en les 4 mostres positives va ser sempre la mateixa. Es va seqüenciar la regió reguladora i la regió hipervariable (C-terminal de l'AgT) de les soques detectades i les seqüències obtingudes ens van fer concloure que la soca detectada era idèntica a la soca que s'havia aïllat anteriorment a partir de

biòpsies de ronyó de la colònia de micos (SV40 W17). La soca detectada presentava una regió reguladora (RR) d'estructura arquetípica.

Aquest resultat es troben més àmpliament descrits i discutits a les seccions Resultats i Discussió de l'article.

#### 4. Conclusions

- 1) Després d'estudiar l'estabilitat de partícules víriques de SV40 en aigua residual s'ha calculat una  $t_{90}$  de 39.9 dies i una  $t_{99}$  de 64.7 dies. SV40 sembla ser doncs un virus relativament estable en l'ambient.
- 2) Tots els individus de la colònia estudiada eren seropositius pel virus.
- 3) SV40 va ser excretat i detectable, segons el mètode aplicat, per la meitat dels individus que constituïen la colònia.
- 4) La soca excretada pel micos *cynomolgus* era idèntica a la que s'havia aïllat a partir de la biòpsia de ronyó d'uns dels individus de la colònia (W17).
- 5) SV40 és excretat pels micos *cynomolgus* infectats i és relativament estable en el medi ambient. Si SV40 causa infeccions en humans similars a les que causa en micos o a les causades pels poliomavirus humans, SV40 hauria de ser detectable en mostres ambientals.

## Detection of SV40 in the environment of a naturally infected colony of *Cynomolgus* monkeys

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AND ROSINA GIRONES<sup>1</sup>

SV40 (Simian Virus 40) naturally infects Asiatic free-living macaques and several related species in captivity. SV40 can cause progressive multifocal leukoencephalopathy (PML) and renal disease in immunocompromised monkeys. A role for SV40 in some brain tumors and central nervous system lesions distinct from PML in macaques have been suggested. SV40 was a contaminant of poliovaccines which were distributed worldwide exposing hundreds of millions of persons to the virus during 1955 to 1963. Recently, reports linking SV40 and human tumors have accumulated.

The main objective of this work is the evaluation of the presence and stability of SV40 in the environment and the analysis of the SV40 viral particles excreted and potentially transmitted in a community of *Cynomolgus* monkeys naturally infected by SV40. The seroprevalence of SV40 infection in the community was also analyzed. The methodology applied is based in the elution of viral particles from the solids and organic residues of the samples by using glycine buffer, concentration of viral particles by ultracentrifugation and genomic amplification by nested PCR assays and sequencing analysis. This methodology has been developed in previous studies where urban sewage samples collected in divergent geographical areas were analyzed for its presence of adenoviruses and polyomaviruses with absence of positive results for SV40 in the urban sewage studied.

The stability of SV40 in sewage samples at 20°C, was estimated as having a  $t_{90}$  (time required for degradation of 90% of viral particles) of 39.9 days and a  $t_{99}$  (time required for degradation of 99% of viral particles) of 64.7 days. The material from the floor of 4 cages where monkeys were inhabiting was collected in two different days. We have detected  $10^2$ - $10^4$  SV40 PCR genomic equivalents per ml of sample in the two samples analyzed from two of the four cages tested. After sequencing the SV40 detected in the colony, a strain which presented an archetypal regulatory region and a C-terminal T antigen region identical to previously described W17 strain, was identified.

Since there is no previous information on the behaviour of SV40 in the environment, the study of SV40 in environmental samples provides information on the potential mechanism of the transmission of SV40 to the human population.

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## Introduction

SV40 is a member of the *Polyomaviridae* family. Its closest relatives are the two human polyomaviruses JCV and BKV with which SV40 shares about 69% genomic similarity at the nucleotide level. The discovery of SV40 is tied to the development and distribution of polio vaccines which were inadvertently contaminated and distributed worldwide potentially exposing hundreds of millions of persons to the virus during 1955 to 1963 (Shah & Nathanson, 1976). SV40 presents a naked icosahedral capsid of about 45 nm diameter surrounding a molecule of double stranded, circular, covalently closed and super coiled DNA of approximately 5000 bp.

SV40 naturally infects free-living macaques in India, Japan, Taiwan and Vietnam (revised in Shah et al., 1971). SV40 has no known hosts in the USA or Europe (Shah, 2000). In rhesus the virus multiplies readily producing viremia and a high-titered antibody response but the infection is completely benign and the virus establishes a long term latent chronic infection of the kidneys and is excreted in the urine providing an opportunity for transmission to non-immunes (Hull, 1968). All these characteristics suggest a long-established association between SV40 and macaques. In captivity, several related species such as *Cynomolgus* macaque and African green monkeys are easily infected.

SV40 can cause widespread infection in immunocompromised monkeys being detected in several organs. The presence of SV40 in the brain of immunodeficient monkeys demonstrated that SV40 is neurotropic in addition to being kidney tropic. The presence of the virus in spleen and peripheral blood suggests that it may spread by haematogenous routes (Butel & Lednicky, 1999). The infection is benign as SV40 pathology has been observed only in immunocompromised monkeys in which the virus can cause progressive multifocal leukoencephalopathy (PML) (Gribble et al., 1975) and renal disease (Horvath et al., 1992). A role of SV40 in some brain tumors in macaques have been suggested (Hurley et al., 1997). It also has been suggested that SV40 could lead to the development of central nervous system lesions distinct from PML (Simon et al., 1999).

Since 1974, when Soriano et al. (1974) reported for first time an association between SV40 and human cancer, reports linking SV40 and human tumors have accumulated and the potential role of SV40 in cancer etiology requires serious consideration. SV40 has been associated to mesotheliomas and different types of brain

tumors as ependymomas and choroids plexus carcinomas. In addition to mesotheliomas and brain tumors, SV40 has been associated with other human tumors and non-tumor tissues, including osteosarcomas, non-Hodgkin's lymphomas, AIDS-related lymphomas, peripheral blood cells, sperm fluids and kidney tissue (Arrington & Butel, 2001).

In previous studies, we evaluated the potential transmission and excretion of SV40 in human populations analyzing urban sewage samples collected in divergent geographical areas for the presence of human polyomaviruses and SV40 (Bofill-Mas et al., 2000). These studies have shown a high prevalence of the human polyomaviruses and absence of SV40 or concentrations lower than 5 SV40 viral particles per ml of sewage, the estimated limit of sensitivity for the assay (Bofill-Mas et al., 2000). Being able to detect and study SV40 strains excreted in urban sewage would provide a unique opportunity to evaluate and monitor over time those strains that are prevalent in specific geographic areas.

A diversity of SV40 strains have been described in diverse tissues of normal and immunocompromised monkeys (Lednicky et al., 1998) although there is no information available on the genetic characteristics of the excreted strains in naturally infected monkey populations. The main objective of this work is the evaluation of the presence of SV40 in the environment and the characterization of the SV40 strain excreted and potentially transmitted in a community of *Cynomolgus* monkeys naturally infected by SV40.

## Material and Methods

*Cells and positive controls.* SV40 DNA strain 776 (Gibco BRL) was used as a positive control. Viral particles of SV40 strain WT 800, were kindly donated by Dr. Azorin from the Institute Juan de la Cierva, CSIC (Consejo Superior de Investigaciones Científicas), Barcelona. The SV40 strain used in the neutralization assays was the laboratory strain NIBSC isolated in the 1960's (Sangar et al., 1999). The cell line BSC-1 was grown in EMEM supplemented with 10% fetal bovine serum (FBS) buffered with 15mM HEPES.

*Monkeys' breeding.* The breeding colonies consist initially of approximately 24 females and two males per group. Once established, a colony is not added to and diminishes in size through natural wastage. Occasionally, a colony may become so small that it becomes unviable.

When this occurs those females that it is considered desirable to retain are either introduced into another established colony or, if several depleted colonies exist, they may be amalgamated. Colony founder animals are identified by number alone, while those born in the colony are identified by a sequential letter added to their mother's number. For example animal 394 would be a founder (female) individual, 394A would be the first offspring and 394B the second. 394AA would be the offspring of animal 394A whose second offspring would be 394AB. When the babies are at least six months old and have attained a weight greater than 1kg they are weaned, taken and put together in a gang (groups of about 20 animals) away from the breeding colonies, and it is after few weeks of being in the gang when seroconversion usually takes place. They live in this group until such time as they reach a target weight of 1.54 kg and 12 months of age. They are then taken from the gang and caged in small groups of 2 or 3. It is at this stage that the samples have been collected. The cages were in the same room as each other and no other animals were in the room.

*Collection of serum samples.* As part of the general health check programme a 2ml blood sample was removed from the femoral vein and transferred into a suitable container without coagulant. The serum was removed from the clotted blood sample and held at 56°C for 30 minutes; this reduced or removed any toxic effect to the BSC-1 cells. The serum was then stored at -20°C until use.

*Virus neutralization assay.* The virus dose was first established by titration as follows: virus was diluted in growth medium (MEM + antibiotics + 10% FBS + 1.5% 1M HEPES + 2% sodium bicarbonate buffer) in half log steps from  $10^{-1.0}$  to  $10^{-6.5}$  and 50 µl was transferred to the wells of a Falcon Microtest 96-well plate. Two hundred µl of BSC-1 cell suspension containing  $2.5 \times 10^5$  cells/ml was added to each well. The plate was incubated at 37°C and read at 10 days for microscopic evidence of cytopathic effect (CPE). Antibody screening was carried out as follows. Seventy µl of growth medium was placed in each well of a Falcon 3072 microtitre plate and 10 µl of heat inactivated serum was added, making a 1 in 8 dilution used for screening purposes. Twenty five µl was added to each well of one plate as a serum control and 25 µl of SV 40 virus at a concentration of 1000 TCID<sub>50</sub>/ml was added to the duplicate plate. Both plates were sealed and incubated at 35°C for 1 hour. BSC-1 cells were trypsinised and a cell suspension containing

$1.5 \times 10^5$  cells/ml was prepared. Two hundred µl of this cell suspension was added to each well. The plates were sealed and incubated at 35°C for 10 days after which the cultures were read microscopically for typical SV40 CPE. Those sera found to be positive were subsequently titrated. A hyper immune rabbit antiserum (kindly provided by Professor J. Butel from the Department of Molecular Virology and Microbiology, Baylor College of Medicine, Houston, Texas, USA) was titrated as a control in each assay. The titer of this antiserum in the present assay was 1 in 40,000, comparable to that found by Professor Butel, thus confirming the sensitivity of the assay.

*Collection of environmental samples.* A total of eight samples were obtained from four different monkey cages over two 24 hours periods from the NIBSC (National Institute for Biological Standards and Control, UK) and numbered. Samples contained excrements and rests of food and were frozen at -20°C and shipped to Spain for further analysis.

*Concentration of viral particles from environmental samples.* Recovery of viral particles from monkey's environment samples was carried out in two different ways depending on the characteristics of each sample. Liquid samples were processed as described in previous studies (Puig et al, 1994; Pina et al, 1998). Briefly: 40 ml of sample was ultracentrifuged (229,600xg for 1 h at 4 °C) to pellet all the viral particles together with any suspended material. 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 12,000xg for 15 min after the addition of 4 ml of 2x PBS. Viruses were pelleted by ultracentrifugation (229,600xg for 1 h at 4 °C), resuspended in 0.1 ml of PBS (phosphate buffered saline) and stored at -80 °C.

Solid samples were eluted with 4 ml of 0.25 N glycine buffer pH 9.5 using magnetic stirring for 30 min, the suspended solids were separated by centrifugation at 12,000xg for 15 min after the addition of 4 ml of 2x PBS. Viruses were pelleted by ultracentrifugation (229,600xg for 1 h at 4 °C), resuspended in 0.1 ml of 1x PBS and stored at -80 °C.

*Nucleic acid extraction.* Viral nucleic acids were extracted using a procedure that uses guanidinium thiocyanate (GuSCN) and adsorption of the nucleic acids to silica particles (Boom et al, 1990) providing clean nucleic acids for genomic amplification.

**Enzymatic amplification.** Ten  $\mu\text{l}$  aliquots of the extracted nucleic acids were used in each test. Amplifications were carried out in a 50  $\mu\text{l}$  reaction mixture containing 10 mM Tris-HCl (pH 8.3 at 25°C), 50 mM KCl, 1.5 mM  $\text{MgCl}_2$ , 200  $\mu\text{M}$  of each dNTP, 2 units of Ampli Taq®DNA polymerase (Perkin-Elmer Cetus) and the corresponding primers at 25  $\mu\text{M}$ . Throughout the PCR assays, the first cycle of denaturalization 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 at the corresponding annealing temperature for 60 s, and extension at 72°C for 75 s. All amplifications were completed with a 4 min, 72°C extension period. Primers and ATs used in this study are represented in Table 1. The results were analyzed by agarose gel electrophoresis using ethidium bromide stain. Direct and tenfold dilutions of the samples were analyzed and the assays were carried out three times in independent experiments for the confirmation of the results using two different sets of primers that amplify two different regions of the SV40 genome. Standard precautions were applied in all the manipulations in order to reduce the probability of sample contamination by amplified DNA molecules.

**Sequencing of PCR products.** Products obtained after PCR were purified with the QUIAquick PCR purification kit (QIAGEN, Inc.). 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) following the manufacturer's instructions. The conditions for the 25-cycle sequencing amplification were: denaturing at 96°C for 10 s, annealing for 5 s at 50°C when sequencing the C-terminal T-antigen region and at 46°C when sequencing the regulatory region, and extension at 60°C for 4 min. Primers for sequencing were used at 2.5  $\mu\text{M}$  concentration. The results were checked using the ABI PRISM 377 automated sequencer (Perkin-Elmer, Applied Biosystems).

**Stability of SV40 viral particles in sewage.** Three different sewage samples and a PBS positive control were spiked with SV40 800WT viral particles and kept in sterile bakery into a heater at 20°C. Aliquots of 40 ml of each sample were analyzed within a 7-month period for the presence of SV40 on days 0, 10, 20, 30, 40, 60 and 210.

In order to obtain an estimated  $t_{90}$  and  $t_{99}$  (time required for a 90% and 99% reduction in the viral concentration), we computed a linear

regression model with the logarithm of the estimated concentration of viral particles detected by nested-PCR expressed as PCR-genomic equivalents.

## Results and Discussion

Three samples of raw sewage from Barcelona and a PBS control sample, all spiked with SV40 viral particles, were kept at 20°C for 7 months. SV40 was detected until day 40. The stability of SV40 in sewage at 20°C, was evaluated and a  $t_{90}$  of 39.9 days and  $t_{99}$  of 64.7 days were observed. These data are approximated and have been calculated according to the regression line obtained (Figure 1).

The results obtained in the SV40 antibody screening of the monkeys subjected to this study show that all monkeys are seropositive and present in general a high antibody titer as represented in Table 2. The monkeys usually seroconverted after being for few weeks in the gangs, suggesting that the infection spread between the monkeys during the stay in the weaned groups (data not shown).

Samples from the monkey's environment were processed labeled by numbers without identification of the specific cages and analyzed initially in blind experiments with further confirmation in two independent assays. Two different sets of primers that amplify two different regions of SV40 genome were analyzed. We obtained positive results for the two samples collected from cages 3 and 4 in the three experiments carried out. The DNA amplified bands obtained in the nested-PCR were sequenced and confirmed to be SV40 and to be identical between them. Both, the carboxy-terminal region of the T antigen and the regulatory region, of the positive samples were sequenced. We semi-quantified the concentration of viral particles present in the samples by limiting-dilution nested-PCR experiments. The two samples obtained from cage 3 where three monkeys are inhabiting presented abundance of liquid and showed approximately  $10^4$  viral particles/10  $\mu\text{l}$  of viral concentrate equivalent to 4 ml of sample while the two samples from cage 4, with only two monkeys inhabiting, presented principally solid residues and showed approximately  $10^3$  and  $10^1$  viral particles/10  $\mu\text{l}$  respectively in the viral concentrate prepared by elution of the viruses present in the waste (solid food rests and some liquid excreta) collected from the sample.

SV40 is a natural infection of a few species of Asiatic macaques: the rhesus (*Macacca mulatta*), the Japanese macaque (*Macacca fuscata*) and the Formosan rock macaque

(*Macacca cyclopiis*). The bonnet macaque of India (*Macacca radiata*) and the *Cynomolgus* macaque of East Asia (*Macacca fascicularis*) seem to be free of SV40. In the wild, only a small proportion of the juvenile rhesus, but nearly all adults, have SV40 antibodies (Meyers et al., 1962; Shah and Southwick, 1965). Experimentally, non-immune rhesus monkeys are readily infected by the oral, intranasal and subcutaneous routes and viremia and viruria occur in infected animals. Virus may persist in the kidneys although the exact manner of transmission is not known, the most likely possibility is that the viruses excreted in the urine is transmitted to susceptible monkeys by the respiratory or oral route. Infection is apparently completely harmless for these natural hosts producing latent, chronic infections. In captivity, SV40 is readily transmitted to other macaques in contact with infected rhesus, as it is the case for *Cynomolgus* macaques (Shah & Nathanson, 1976) and as it may be the case for the monkey colony studied. It has been established that the C-terminal T-antigen variable domain is genetically stable, and that this region can serve as a means of identifying and tracking the transmission of viral strains (Lednicky & Butel, 1997b). In previous studies, a unique isolate of SV40 was obtained from one of the 25 kidneys of the monkeys in the colony analyzed by isolation in cell culture. This strain was isolated at NIBSC and was named W17. The difficulty of recovery virus from the kidneys of SV40 infected monkeys has been previously described since the virus does not seem to be present in every kidney cell or even in a large percentage of them and some monkeys with negative biopsies presented viruria (Ashkenazi and Melnick, 1962). The sequence identified in the C-terminal T antigen variable domain of the strains detected was found to be identical to W17 SV40 strain (Minor et al., 2001). Strain W17 was sent to the laboratory in Barcelona where it was sequenced again in order to confirm the results previously obtained at NIBSC. The similitude of the sequences in the C-terminal T antigen region isolated by the two different laboratories more than one year apart would support the reliability of the results and it is in agreement with the genetic stability described for the C-terminal T antigen domain of the diverse SV40 strains. Strain W17 has been described as having a single copy of the enhancer in the regulatory region as it was obtained when analyzing the regulatory region of the SV40 detected in the cages of the infected primates.

The possibility of contamination of a strain used in the laboratory was carefully analyzed and the

analysis of the original numbered samples, repeated PCR amplification and isolation of viral particles together with the fact that we have obtained identical sequences to W17, a strain that was not in the laboratory during the analysis of the samples, support the reliability of the results.

There is not a clear description in the scientific literature of the genetic structure of the SV40 regulatory region excreted by the natural host, the Asian rhesus macaques, or the related species as *Cynomolgus* monkeys presenting infections by SV40 of similar characteristics. A diversity of SV40 strains isolated from brain, spleen and peripheral blood mononuclear cells have been described in immunocompromised monkeys by Lednicky et al. (1998) analyzing the regulatory region and showing the presence of a mixture of diverse archetypal and non-archetypal forms, with archetypal SV40 as the predominant form in the monkey brain tissue. This fact contrast with the structure of the regulatory region described for JCV, a closely related human polyomavirus, in the human brain tissues and in CSF (cerebrospinal fluids) of PML patients, a fatal demyelinating disease caused by JCV in immunosuppressed individuals especially those affected by AIDS, which is characterized by presenting tandem repeats in the regulatory region. Low-passage stocks of SV40 strains Baylor and VA45-54 isolated from type 2 Sabin polio vaccine in AGMK cells and from uninoculated grivet monkey kidney cells respectively presented a mixture of archetypal and strains containing a large repeated segment within the enhancer region (Lednicky & Butel, 1997a).

Like JCV, SV40 virions that are excreted, may present a different structure in their regulatory regions when compared with the regulatory regions present in cell-culture adapted strains of SV40 (Lednicky & Butel, 1997a) or strains described in tumors or other tissues (Lednicky et al., 1998). However, in the case of JCV, the dominant populations of JCV excreted in human urine exhibits an archetypal regulatory region and are very difficult to grow in cell culture. In contrast, tandem repetitions and deletions are the dominant structures in the genomes of JCV virions in cell-culture adapted strains and in the virions in cerebrospinal fluid samples from patients with PML. The available information also seems to suggest that grow rates of SV40 strains in BSC-1 cells are not determined by the number of enhancer elements (Minor et al., 2001).

Other descriptions of SV40 strains infecting rhesus monkeys with simian immunodeficiency virus-induced immunodeficiency have been reported by Ilyinskii et al. (1992). SV40 isolated



from kidney and brain tissues presented an archetypal structure of the regulatory region as the most common structure in these tissues. Although strains identified in the diverse tissues of immunocompromised monkeys may not be representative of the viruses excreted by healthy animals. The SV40 viral particles excreted in the colony of *Cynomolgus* analyzed presented a high level of stability in the C-terminal T antigen and regulatory regions since these two cages presented identical strains as the dominant viral genome present in the environment and it is probably the dominant strain isolated from kidney tissue in the colony.

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**Table 1.** Oligonucleotide primers used for PCR amplification and sequencing of SV40.

Virus type (region)	Position	Primers	Reaction	AT <sup>a</sup>	Sequence
SV40 (Tag) <sup>a</sup>	2609-2631 <sup>b</sup>	SV1A	First left	55°C	5'-TGTGAAATTTGTGATGCTATGTC-3'
SV40 (Tag)	3036-3058	SV	First right		5'-AGTTTGCTCAAAGTATTCAGAGC-3'
SV40 (Tag)	2746-2766	SV5 <sup>c</sup>	Nested left	46°C	5'-CTACARATGTGRTATGGCTGA-3' <sup>a</sup>
SV40 (Tag)	2980-3000	SVN	Nested right		5'-GTTTAGTTTGTTCAGTGTATCA-3'
SV40 (RR) <sup>a</sup>	336-358	RA3 <sup>d</sup>	First left	59°C	5'-GCGTGACAGCCGGCGCAGCACCA-3'
SV40 (RR)	5119-5142	RA4 <sup>d</sup>	First right		5'-GTCCATTAGCTGCAAAGATTCCTC-3'
SV40 (RR)	245-266 <sup>d</sup>	RA1 <sup>d</sup>	Nested left	55°C	5'-AATGTGTGTCAGTTAGGGTGTG-3'
SV40 (RR)	5195-5218	RA2 <sup>d</sup>	Nested right		5'-TCCAAAAAGCCTCCTCACTACTT-3'

<sup>a</sup> Tag= C-terminal T-antigen region; RR= Regulatory region; AT= Annealing temperature; R= A+G.

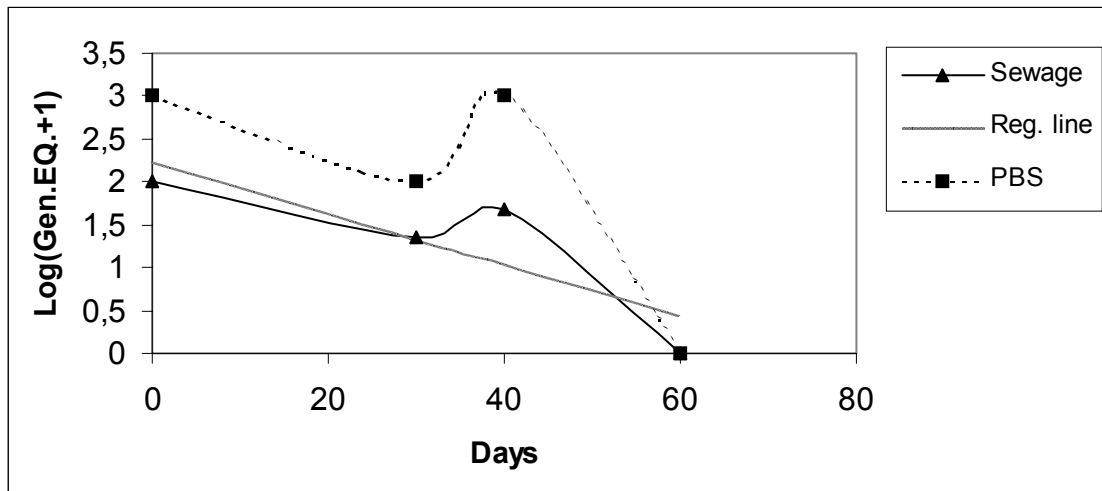
<sup>b</sup> The sequence positions are referred to SV40 strain 776.

<sup>c</sup> Modified from Lednicky et al. (1998).

<sup>d</sup> From Lednicky and Butel. (1997a).

**Table 2.** Results obtained in the virus neutralization assays showing that all the monkeys studied were seropositive for SV40. The number of the animals inhabiting in each of the cages studied and data related to its age, birth colony and gang number are also represented.

Group	Animal	Age	Birth colony	Gang number	Anti-SV40 titer
<b>1</b>	518HC	1.5 years	C63	B240	>8192
	555FBB	1.3 years	C56	B240	>8192
<b>2</b>	020ME	1.3 years	C63	B242	>8192
	436FE	1.1 years	C25	B240	4096
	895H	1.1 years	C48	B242	8192
<b>3</b>	394AD	1.1years	C37	B242	>8192
	930H	1.0 years	C48	B243	8192
	019GD	1.4 years	C58	B243	>8192
<b>4</b>	115GH	1.3 years	C57	B242	8192
	N30C	1.5 years	C64	B240	4096



**Figure 1.** Stability of SV40 strain 800 WT viral particles in a sewage samples. The regression line (Reg. Line), the transformed average number of genome equivalents (Gen.EQ+1) detected by nested PCR in the 3 samples (sewage), and the number of genome equivalents of a spiked PBS control are represented.

## Conclusions generals

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- S'ha desenvolupat un protocol d'elevada sensibilitat i especificitat per a la detecció dels poliomavirus humans i SV40 en mostres ambientals.
- S'han detectat poliomavirus humans (JC i BK) en un elevat percentatge, i en elevades concentracions, en mostres d'aigua residual de diverses àrees geogràfiques d'Europa, Àfrica i Estat Units i també en mostres ambientals del Nord de la Índia, sent JC el virus humà més prevalent en l'ambient dels estudiats fins al moment.
- Els poliomavirus humans són relativament estables en aigua residual a 20°C i a tractaments amb pH àcid.
- La seqüenciació de la regió intergènica (RI) dels JC detectats ha permès corroborar l'origen geogràfic de les mostres analitzades i construir un arbre filogenètic que correlaciona els genotips detectats amb la composició ètnica de les poblacions estudiades.
- Tot i que la majoria dels JC excretats en l'orina presenten una configuració arquetípica de la RR, un percentatge dels JC presents en aigües residuals presenten estructures semblants a l'arquetípica (*archetypal-like*) i en alguns casos, com és el de 2 mostres d'aigua residual dels Estats Units, s'observa presència de JC amb RR reorganitzades. Caldrà que la patogenicitat d'infeccions, coinfeccions o reinfeccions causades per soques amb RR reorganitzades (tipus PML) sigui avaluada en futurs estudis.
- Els BK presents en aigües residuals pertanyen majoritàriament al serotip I i presenten RR arquetípiques o semblants a l'estructura arquetípica.
- Els BK i JC arquetípics presents en una mostra d'orina d'una dona embarassada han infectat efectivament la línia cel.lular SVG.

- El tractament amb pH àcid i tripsina d'una soca control (Mad-4) no destrueix la capacitat infectiva del virus tot i que es va observar un descens en la progènie vírica després de sotmetre les partícules víriques a pH 3 durant 1 hora.
- SV40 és excretat per micos *Cynomolgus* naturalment infectats amb el virus i es detecta, mitjançant el protocol dissenyat, en mostres ambientals recol·lectades a les gàbies on aquests habiten
- SV40 presenta una estabilitat en aigua residual a 20°C superior a la que presenta JC però no ha estat detectat en cap de les mostres d'aigua residual analitzades. Si SV40 està present en aquestes mostres, la concentració en que s'hi troba ha de ser inferior a 5 PV/ml que és el límit de detecció de la tècnica dissenyada.

## Discussió

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### **Presència dels poliomavirus humans JC i BK en aigües residuals urbanes.**

Els resultats obtinguts després d'analitzar mostres d'aigua residual urbana de diferents zones geogràfiques, reflexen elevats nivells d'excreció de JC i BK per part de la població humana. No s'observen diferències significatives en els nivells d'excreció dels poliomavirus humans entre diferents zones tot i que les mostres recol·lectades a Washington D.C (EUA) presenten les concentracions més elevades. Les mostres recol·lectades a la Índia presenten un percentatge de positivitat baix, cal tenir en compte que aquestes mostres es diferencien de la resta de mostres analitzades doncs no són estrictament, i com ja s'ha explicat amb anterioritat, mostres d'aigua residual urbana.

Els resultats obtinguts en el conjunt de les mostres es correlacionen perfectament amb els elevats nivells de positivitat (20-80%) detectats en orines d'individus adults de diferents àrees geogràfiques (Kitamura i col., 1994; Agostini i col., 1997). Com ja havia estat prèviament descrit, les dades obtingudes demostren una més freqüent excreció en orina de JC que de BK. Els JC presents al ronyó es repliquen i són excretats tan per individus immunosuprimits com per individus immunocompetents (Shah i col., 1997). L'excreció de BK podria seguir el mateix patró tot i que sembla estar més lligada a períodes d'immunosupressió o/i embaràs que JC.

### **Estabilitat i capacitat infectiva de les partícules víriques de BK i JC detectades en mostres d'aigua residual. Implicacions en la seva via de transmissió.**

Les partícules víriques de JC i BK van resultar ser relativament estables a pHs àcids. Després d'estudiar l'efecte del pH àcid i la tripsina en la infectivitat de la soca de JC Mad-4, vam concloure que tot i que la infectivitat d'aquestes partícules es veia minvada per l'efecte del pH àcid, aquest no inhibia cert nivell d'infectivitat de Mad-4 en la línia cel·lular SVG.

Un model de transmissió dels poliomavirus humans podria implicar una replicació dels virus al teixit limfoid associat amb la faringe i el tracte gastrointestinal prèvia entrada a l'organisme per la via oral. Un nivell baix de virèmia podria venir



seguit d'una colonització secundària i sistèmica i de l'establiment d'infeccions persistents del ronyó i probablement d'altres teixits.

En els treballs descrits en aquesta tesi hem demostrat que els JC i BK arquetípics presents en l'orina d'una dona embarassada van infectar eficientment cèl·lules SVG. S'ha observat excreció en orina de soques reorganitzades de JC, aquelles que s'han relacionat amb patogènia. La presència d'aquest tipus de soques a l'ambient obre la necessitat d'avaluar la patogenicitat que representa la infecció, coinfecció o reinfecció amb aquestes soques que es multipliquen de manera més eficient, sent les que es relacionen amb les principals patologies associades a JC (PML i càncer).

L'elevada prevalença de JC i BK a l'orina i a mostres d'aigua residual urbana i altres mostres ambientals suggereix que l'aigua, aliments o fomites contaminats podrien actuar com a fonts d'infecció i com a vehicles de la transmissió de JC.

S'ha detectat ADN poliomaviral en teixits gastrointestinals (Ricciardiello i col., 2000). Cal considerar que l'ADN no és degradat completament quan s'ingereix amb l'aliment. S'ha provat que en animals d'experimentació, l'ADN ingerit pot assolir leucòcits perifèrics i d'altres òrgans (Schubbert i col., 1997). Els virions degradats o no infecciosos podrien ser una font d'ADN poliomaviral i representar un mecanisme addicional d'entrada de gens virals a l'organisme i, fins i tot, una via d'infecció ja que l'ADN poliomaviral ha resultat ser infecciosos en experiments de transfecció de cèl·lules (Hara i col., 1998).

#### **SV40. Un poliomavirus humà?**

Tot i que SV40 sembla ser un virus relativament estable en el medi ambient com s'ha demostrat mitjançant assaigs d'estabilitat de partícules víriques de SV40 en aigua residual i presència de SV40 en gàbies de micos *Cynomolgus* naturalment infectats amb el virus, SV40 no ha estat detectat en el conjunt de les aigües residuals estudiades. Això implicaria la no existència de partícules víriques de SV40 en aquestes mostres o l'existència de SV40 en concentracions molt baixes, indetectables mitjançant el mètode desenvolupat.

Cal considerar que un petit percentatge de la població humana presenta títols d'anticossos neutralitzants per SV40 tot i que s'ha suggerit que aquesta neutralització podria ser el resultat d'una reacció creuada amb anticossos contra d'altres poliomavirus (Shah, 1972).

Per tant, si SV40, tal i com ha estat suggerit per diversos autors, infecta teixits humans, el tipus d'infecció que causa en l'home ha de ser diferent a la que causen els altres poliomavirus humans i a la que SV40 causa en micos.

**A**

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