Nuevas metodologías para el estudio de virus humanos contaminantes del medio ambiente

Byron Tomas Calgua de León

TESIS DOCTORAL

NUEVAS METODOLOGÍAS PARA EL ESTUDIO DE VIRUS HUMANOS CONTAMINANTES DEL MEDIO AMBIENTE

Departamento de Microbiología

Facultad de Biología de la Universidad de Barcelona

Programa de doctorado: Microbiología Ambiental y Biotecnología

Memoria presentada por **Byron Tomas Calgua de León** para optar al grado de *Doctor* por la Universidad de Barcelona

Tesis Doctoral realizada bajo la dirección de la Prof. Rosina Girones Llop, Catedrática en Microbiología del Departamento de Microbiología, Facultad de Biología, Universidad de Barcelona

Directora y Tutora	Autor
Prof. Rosina Girones Llop	Byron Tomas Calgua de León

“Ser cultos para ser libres”

José Martí

(La Habana, Cuba, 1853 – 1895)

"Nunca andes por el camino trazado
pues él te conducirá únicamente a donde otros ya fueron"

Alexander Graham Bell

(Escocia, Reino Unido, 1847 – 1922)

Tesis terminada!!! Esto sin lugar a dudas ha sido posible gracias a muchas personas durante estos años. A continuación mencionaré a los que mi memoria me permita recordar, sin embargo hay muchas personas que me han apoyado, animado y acompañado, a todas ellas muchas gracias aunque no logre mencionarlas.

En primer lugar quiero agradecer a Rosina Girones, indiscutiblemente gracias por aceptarme en tu grupo para hacer la tesis, por tu confianza y por apoyarme en mi progreso profesional durante estos años. En todos estos años, además de ser mi supervisora de tesis, a nivel personal siempre he contado con tus concejos y palabras adecuadas en el momento necesario, muchas gracias !!!

A todos mis colegas del laboratorio 8, que con muchos además nos une una gran amistad, gracias por todos los grandes momentos en el ámbito profesional y personal durante todo este tiempo. Definitivamente con todos vosotros el trabajo siempre fue más agradable e interesante y seguro los echare de menos. Los que ya no están: Néstor, Carlos, Pili, Chus y Annita. Los actuales y nuevos: Plaka, Laura, Marta, Sandra, Silvia, Persi y las Natalias. A todos nuestros visitantes: Marize, Celia, Adriana, Maria Paula, Vannesa, Leticia y Viviana.

A nuestros vecinos, los Araujos, muchas gracias por todos los buenos momentos: Alejandra, Sara, Silvia y Tarik. Igualmente gracias a todos los demás miembros de departamento por todas las charlas y buenos momentos tanto en el pasillo como fuera de la universidad: Alex, Markus, Andrés, las Martas, Aiora, Anna, Nerea, Lucia, Fran, Maru, Míriam, Raquel, Laura Eli+Jordi….. y seguro que muchos más, tanto en la fase I y como en la II. En secretaría muchas gracias a Bea, Macu, Susana, Rosario y Manolo.

También quiero agradeced a todos los titulares por su amabilidad, consejos y charlas, especialmente a Rosa Araujo, Mª José Prieto, José Maria Nieto, Maite Muniesa, Joan Jofre, Francisco Lucena, Anicet Blanch, Cristina Madrid, Albert Bosch, etc.

También quiero agradecer a los coautores de los trabajos presentados en esta tesis, muchas gracias por todo el trabajo compartido: Prof. Jim Pipas y su equipo; Al equipo de Marize en Río de Janeiro, especialmente a Tulio y a todo el equipo del proyecto VIROBATHE.

Gracias a la AECID por otorgarme la beca en el inicio de mi carrera en Barcelona. Este trabajo como muchos más, demuestra que la colaboración entre diferentes países puede dar lugar a pequeños éxitos.

A los amigos, algunos ya mencionados, a otros que están lejos o cerca pero siempre en contacto dándome ánimos y buenos momentos, Christian, Carlos Roman, Mauricio, Alejandro, Toto, Juan Pablo, Claudia Chapeton, Adonay, Katlin, Laura, Liss, Heidy, Mike, Rosa, Yanik…etc.

A nivel personal, muchísimas gracias a toda mi familia, especialmente a mis padres, a mi hermana Ana Lucia y su esposo Kriton, a mis queridos tíos Erwin y Anny, gracias por todo el apoyo, consejos y ánimos. Sin lugar a dudas gracias a vosotros me encuentro en este punto.

Y finalmente un gracias muy muy especial a Maire y Ernesto y al pequeño que viene en camino, siempre han sido el gran motor impulsor en mi vida. Ernesto, tu sonrisa siempre ha sido lo mejor de cada día. Este trabajo va dedicado a ustedes.

ABREVIATURAS

°C	Grados centígrados.
µm	Micrómetros.
ASFV	Asfarvirus.
ASFVLV	Asfarvirus-*like.*
BKPyV	Polyomavirus BK.
BOE	Boletín Oficial del Estado.
CAR	Receptor coxsackievirus-adenovirus (*coxsackievirus-adenovirus receptor*).
CE	Comunidad Europea (*European Community*).
cm²	Centímetro cuadrado.
CPE	Efecto citopático (*Citopatic Effect*).
CV	Coeficiente de variación.
DNA	Ácido desoxirribonucleico (Desoxirribonucleic Acid).
EC	*E. coli.*
EEUU	Estados Unidos de Norteamérica (*United States of America*).
EI	Enterococos intestinales.
et al.	Y colaboradores.
etc.	Etcétera.
FAO	Organización de las Naciones Unidas para la agricultura y la alimentación (*Food and Agriculture Organization of the United Nations*).
FFU	Unidades formadoras de focos (*Foci Forming Units*)
g	Gramos.
GC	Copias genómicas (*Genomic Copies*).
GG	Genogrupo.
h	Horas.
HAdV	Adenovirus humanos.
HPyV	Polyomavirus humano.
IFA	Ensayo de inmunofluorescencia (*immunofluorescence assays*).
JCPyV	Polyomavirus JC.
Kb	Kilobases.

KDa	Kilodaltons.
Kg	Kilogramos.
KIPyV	Polyomavirus KI.
KV	Klassevirus.
L	Litro.
m^3	Metro cúbico.
MCC	Carcinoma de células de Merkel.
MCPyV	Polyomavirus de células Merkel.
mg	Miligramos.
min	Minutos.
mL	Mililitros.
mm	Milímetros.
Mr	Masa molecular relativa.
NaCl	Cloruro de sodio
NaOH	Hidróxido de sodio.
NGS	Nueva generación de tecnología de secuenciación.
nm	Nanómetros.
NoV	Norovirus.
OMS	Organización Mundial de la Salud.
ORF	Pauta de lectura abierta (*Open Reading Frame*).
PA	Ensayo de placas (*Plaque Assay*).
pb	Pares de bases.
PCR	Reacción en cadena de la polimerasa (*Polymerase Chain Reaction*).
PFU	Unidades Formadoras de Placa (*Plaque Forming Unit*).
pH	Potencial de Hidrógeno.
PML	Leucoencefalopatía multifocal progresiva (*Progressive Multifocal Leukoencephalopathy*).
PyV	Polyomavirus.
qPCR	PCR cuantitativa (*Quantitative PCR*).
RNA	Ácido ribonucleico (*Ribonucleic Acid*).
RR	Región reguladora.

RT-PCR	PCR con transcriptasa inversa (*Reverse Transcription PCR*).
RV	Rotavirus.
RVA	Rotavirus grupo A.
S	Siemens.
sAg	Antígeno tumoral T pequeño.
SMFP	Procedimiento de floculación acida de leche descremada (*Skim Milk Flocculation Procedure*).
tAg	Antígeno tumoral T largo.
$TCID_{50}$	Dosis infectiva media de cultivo tisular (*Tissue Culture Infective Dose 50*).
UFC	Unidades Formadoras de Colonia.
USEPA	Agencia de protección ambiental de los EEUU (*United States Environmental Protection Agency*).
UV	Ultravioleta.
WHO	Organización Mundial de la Salud (*World Health Organization*).
WUPyV	Polyomavirus WU.

1. INTRODUCCIÓN GENERAL

1. Introducción general

1.1. Contaminación del medio ambiente por virus humanos patógenos

En los últimos años ha habido un considerable progreso en el mejoramiento del saneamiento del agua a nivel mundial, sin embargo aún se está lejos de cumplir con las metas establecidas en los *Objetivos del Milenio para el Desarrollo*, esto se traduce en que aún hay una gran parte de la población mundial expuesta a agua contaminada y que si no se toman medidas sanitarias adecuadas esta cifra podría incrementarse considerablemente en los próximos años (*Progress on Sanitation and Drinking-Water Report* 2013, WHO/UNICEF). El crecimiento de la población mundial y por lo tanto de las urbanizaciones, lleva implícito el incremento de residuos generados tales como el agua residual urbana, hospitalaria y de origen agrícola. Estos residuos son vertidos al medio ambiente y aunque estos hayan sido tratados previamente en plantas depuradoras, en ambos casos el impacto para el medio ambiente y la salud es negativo. Además, debido a la escasez de recursos de agua de buena calidad, la tendencia a reutilizar e incorporar el agua residual en la sociedad se ha incrementado considerablemente en los últimos años (*Water Sanitation Health*, WHO 2013). Los riesgos para la salud asociados con estas prácticas se han reconocido desde hace tiempo y aunque existen diferentes legislaciones y guías que regulan esta situación, su aplicación a menudo es incompatible con los valores socio-económicos de las regiones donde estas prácticas son más frecuentes (*Water Sanitation Health*, WHO 2013).

El agua residual es la principal fuente de contaminación para el medio ambiente, especialmente en los ríos, lagos, océanos y agua subterránea. Esta situación representa un serio problema para la salud y las autoridades sanitarias. Entre la gran diversidad de microorganismos de origen humano presentes en el agua residual, se encuentran los virus. Los humanos podemos tener contacto frecuente con estas aguas contaminadas a través de diferentes vías (Figura 1). La ruta fecal-oral, es sin lugar a duda la principal vía de transmisión de los virus transmitidos por agua contaminada. Entre otros factores, esto se debe a que los virus entéricos se pueden llegar a excretar en concentraciones tan altas como 10^5 a 10^{11} partículas víricas por gramo de heces en individuos con infecciones agudas, además se sabe también que varios virus son excretados a través de la orina y las heces de individuos sanos (Fong y Lipp, 2005). Recientemente también se ha comenzado a prestar interés por virus que potencialmente podrían excretarse y trasmitirse a través de la piel (La Rosa *et al*., 2013; Spurgeon y Lambert, 2013).

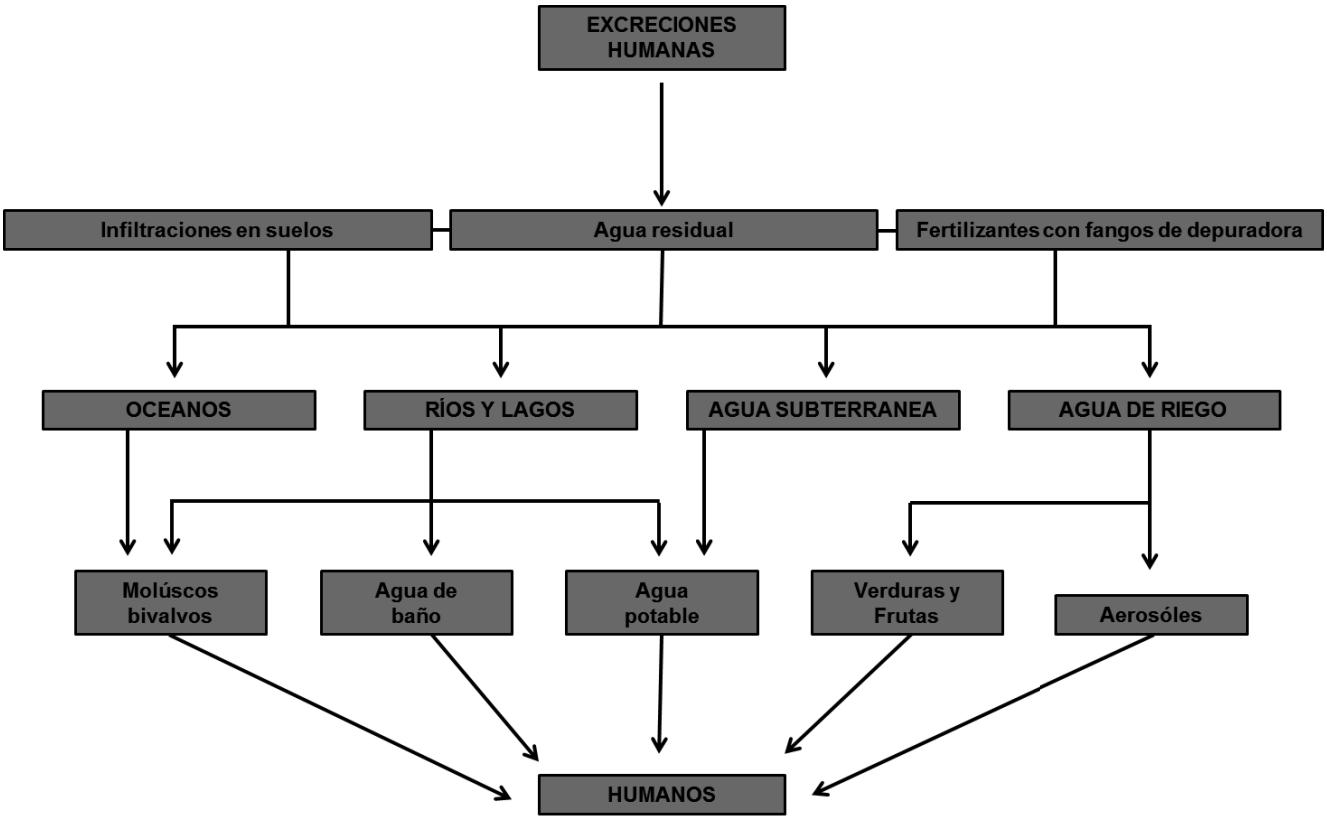


Figura 1. Rutas de transmisión de los virus contaminantes presentes en el medio ambiente. Modificado a partir de Melnick *et al*., 1978

Los principales patógenos víricos transmitidos por agua han sido estudiados ampliamente. Sin embargo, actualmente se tienen nuevos datos de virus emergentes y virus potencialmente nuevos que los humanos podemos excretar al medio ambiente. Esta situación ha motivado la realización de varios estudios relacionados con la mejora y desarrollo de metodologías para la concentración y detección virus, así como también estudios que permitan entender mejor el comportamiento de estos virus en el medio ambiente.

1.2. Virus humanos patógenos transmitidos por el agua contaminada

Los virus son la principal causa de las enfermedades trasmitidas por el agua contaminada (Fong y Lipp, 2005). Este tipo de virus son relativamente estables en los procesos de inactivación de las depuradoras de agua residual y a las condiciones del medio ambiente, como la temperatura y la radiación solar (Carter, 2005). Además estos virus frecuentemente se detectan en agua residual, ríos, agua de mar, de bebida y agua subterránea, cuando presentan contaminación fecal.

Entre los virus transmitidos por agua contaminada se encuentran principalmente los virus entéricos (Tabla 1). Los virus entéricos son excretados en heces de individuos infectados. Entre los más relevantes y frecuentemente reportados en agua contaminada se encuentran los miembros de las familias *Adenoviridae*, *Caliciviridae*, *Hepeviridae*, *Picornaviridae, Polyomaviridae* y *Reoviridae* (Hamza *et al*., 2011; Rodríguez-Lázaro *et al*., 2012; Wong *et al*., 2012). Las enfermedades gastrointestinales son el principal padecimiento negativo para la salud producido por los virus entéricos (Tabla 1), aunque ellos también son responsables de infecciones respiratorias, conjuntivitis, hepatitis, enfermedades del sistema nervioso central e infecciones crónicas (Carter, 2005; La Rosa *et al*., 2012; Rodríguez-Lázaro *et al*., 2012).

Tabla 1. Virus humanos transmitidos potencialmente por agua contaminada. Modificado a partir de Hamza *et al*., 2011

Familia	Genero	Principales enfermedades
Adenoviridae	Mastadenovirus (human adenovirus A–G)	Gastroenteritis, Enfermedades Respiratorias, Conjuntivitis
Astroviridae	Astrovirus humanos	Gastroenteritis
Caliciviridae	Norovirus, Sapovirus	Gastroenteritis
Hepeviridae	Hepevirus (Virus de la hepatitis E)	Hepatitis
Picornaviridae	Hepatovirus (Virus de la hepatitis A)	Hepatitis
	Enterovirus (Enterovirus humanos A-D)	Gastroenteritis, Meningitis, Miocarditis, conjuntivitis
	Kobuvirus (Aichi virus)	Gastroenteritis
Polyomaviridae	Polyomavirus (JC; BK; KI; WU; MC)	Leucoencefalopatía multifocal progresiva, nefritis, neumonía y Carcinoma de las células de Merkel
Reoviridae	Rotavirus (A-C)	Gastroenteritis
	Orthoreovirus (Reovirus)	Gastroenteritis y enfermedades respiratorias
Parvoviridae	Bocavirus (Bocavirus humano)	Gastroenteritis y enfermedades respiratorias
Picobirnaviridae	Picobirnavirus (Picobirnavirus humano)	Gastroenteritis
Coronaviridae	Coronavirus	Gastroenteritis y enfermedades respiratorias
Papillomaviridae	Papillomavirus	Las verrugas genitales y cutáneas, cáncer cervical, otros tipos de cáncer menos comunes

En los últimos años, también nuevos virus humanos patógenos se ha venido reportando en agua contaminada. Por ejemplo, Bofill-Mas *et al*., 2010 describió la presencia de los polyomavirus nuevos KI, WU y de Merkel *Cell* en agua residual. Este último ha sido propuesto como parte del viroma de la piel (Wieland *et al*., 2009; Schowalter *et al*., 2010; Moens *et al*., 2011; Foulongne *et al*., 2012; Spurgeon y Lambert, 2013), sugiriendo que la principal vía de transmisión es a través del contacto con la piel y el agua contaminada. El nuevo picornavirus salivirus/klassevirus asociado a gastroenteritis también se ha descrito en agua residual (Holtz *et al*., 2009; Haramoto *et al*., 2013). Entre los virus recientemente desritos existe un asfarvirus-*like* asociado a pacientes con enfermedades febriles que también se ha detectado en agua residual (Loh *et al*., 2009). Este virus sería el primer miembro de la familia *Asfarviridae* potencialmente asociado a humano. Por otro lado, también se han reportado cepas y variantes emergentes de virus conocidos como los rotavirus, norovirus y adenovirus (Vega y Vinjé, 2011; Komoto *et al*., 2013; Robinson *et al*., 2013).

Fenómenos como la globalización, el crecimiento de la población, el desarrollo de nuevas tecnologías y la evolución genética de los virus, humanos y vectores, han estimulado la aparición de nuevos virus y vías de transmisión emergentes.

1.2.1. Indicadores víricos de contaminación fecal en el medio ambiente

La calidad microbiológica del agua empleada con fines recreativos (mar, río y lagos), es evaluada empleando los indicadores estándar de contaminación fecal: *E. coli* (EC) y enterococos intestinales (EI), según lo indicado en varias legislaciones de agua de baño reconocidas a nivel mundial (WHO, 2003; USEPA, 2004; 2006/160/EC). Sin embargo estos indicadores suelen fallar al predecir la ausencia de virus humanos contaminantes (Gerba *et al*., 1979; Lipp *et al*., 2001). Varios estudios han demostrado que los virus son más estables a las condiciones del medio ambiente y que por lo tanto las concentraciones de los indicadores bacterianos usualmente no se correlacionan con las de los virus (Contreras-Coll *et al*., 2002; Thurston-Enriquez *et al*., 2003; Rzeżutka y Cook, 2004; Miagostovich *et al*., 2008; de Roda Husman *et al*., 2009). Con el propósito de obtener información más certera de la calidad microbiológica del agua, especialmente con relación a los virus, los adenovirus humanos y polyomavirus JC se han propuesto como una nueva generación de indicadores de contaminación fecal (Pina *et al*., 1998; Bofill-Mas *et al*., 2000; McQuaig *et al*., 2006; Miagostovich *et al*., 2008; Wyn-Jones *et al*., 2011; Vieira *et al*., 2012).

1.2.1.1. Adenovirus humanos

Los adenovirus constituyen la familia *Adenoviridae*. Los miembros de esta familia son virus no envueltos, con una estructura icosaédrica y un genoma DNA linear no segmentado de doble cadena. Los adenovirus tienen un diámetro de entre 60-90 nm y un genoma de 30-38 kb que teóricamente codifica a 13 polipéptidos estructurales y 35 proteínas no estructurales. El genoma presenta una proteína de 55 kDa unida covalentemente al extremo 5' de cada una de las cadenas y también secuencias repetidas e invertidas de 100-140 pb en los extremos terminales.

La entrada de los adenovirus a la célula es a través de un proceso de endocitosis activado en dos etapas; interacciones entre la proteína fibra con varios receptores celulares, entre ellos el receptor coxsackievirus-adenovirus (CAR), y la internalización a través de vías de endocitosis mediada por interacciones entre receptores celulares (integrinas) y proteínas víricas (pentonas). El endosoma posteriormente es roto debido a cambios en el pH interno y en la carga neta de la cápside vírica, esto permite que el virión sea liberado al citoplasma y transportado para que el *core* del virión entre al núcleo celular. La transcripción, replicación y maduración de los adenovirus ocurre en el núcleo celular. El ciclo lítico de los genes víricos es un proceso organizado en tres fases nombradas según el momento en que estas son expresadas (Figura 2). En la fase temprana, cinco unidades de transcripción codifican para las proteínas E1A, E1B, E2, E3 y E4, durante la fase intermedia se codifican las proteínas IVa2 y pIX y en la fase tardía se codifican las proteínas L1, L2, L3, L4 y L5. La principal función de las proteínas de la fase temprana es forzar a la célula a entrar a la fase S con el objetivo de promover las condiciones óptimas para la replicación del DNA vírico y suprimir la respuesta antiviral del huésped. Las proteínas de la fase intermedia intervienen en la estabilización de la estructura de la cápside. Mientras que las proteínas de la fase tardía son las proteínas estructurales de las nuevas partículas víricas.

La familia *Adenoviridae* actualmente agrupa a 5 géneros diferentes que infectan a los humanos y a un amplio número de especies animales: *Atadenovirus, Aviadenovirus, Ichtadenovirus, Mastadenovirus* y *Siadenovirus* (*International Committe on Taxonomy of Viruses*, Versión vigente 2012). Los adenovirus humanos (HAdV) pertenecen al género *Mastadenovirus*. Actualmente hay más de 60 tipos de HAdV, agrupados en 7 especies diferentes (A-G), siendo los HAdV D la especie con más miembros (Robinson *et al*., 2013). Las principales enfermedades producidas por HAdV van desde, gastroenteritis (HAdV F), infecciones respiratorias (principalmente HAdV B y C) y conjuntivitis (HAdV B y D). Sin

embargo estos virus también pueden causar otras enfermedades como, cistitis, encefalitis y meningitis. Los HAdV también se han relacionado con algunas transmisiones de primates a humanos (Wevers *et al*., 2011) y también se ha especulado como posible causa de obesidad (Esposito et a., 2012).

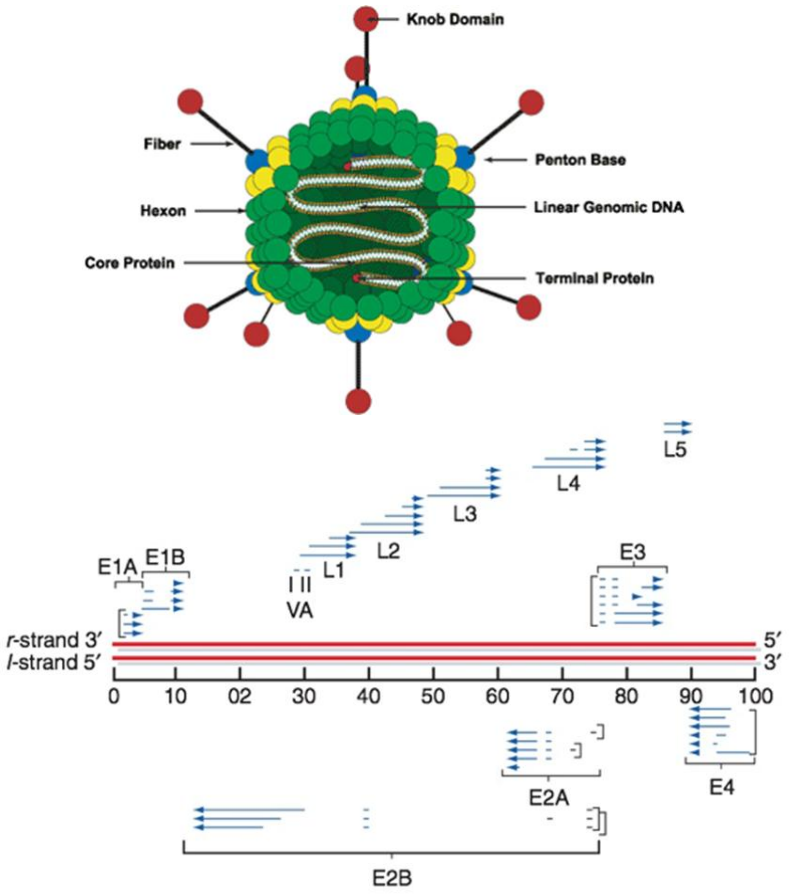


Figura 2. Estructura y mapa de la organización de la transcripción de los adenovirus

Las infecciones por HAdV están extendidas en toda la población en general. Aproximadamente el 60% de las infecciones por HAdV se produce en niños menores de 4 años. El 47-55% de las infecciones pueden ser asintomáticas. Se cree que los HAdV son responsables del 5-10% de las infecciones respiratorias en niños. Después de infecciones agudas por los HAdV, estos se pueden secretar a través de las heces durante meses e incluso años, siendo este hecho probablemente el principal responsable de la diseminación endémica de HAdV a través de la ruta fecal – oral y respiratoria (Carter, 2005). Además algunas infecciones pueden persistir en algunos tejidos en fase latente, como por ejemplo adenoides, tracto intestinal y urinario (Carter, 2005).

Los HAdV son detectados frecuentemente en agua fecalmente contaminada y también han sido identificados en moluscos bivalvos (Pina *et al*. 1998; Formiga-Cruz *et al*., 2002; Carter, 2005). Los HAdV se han reportado consistentemente en casi el 100% de muestras de agua residual de diferentes zonas geográficas analizadas (Bofill-Mas *et al*., 2000; Fumian et., 2013). También se han detectado HAdV en agua de río, lago y mar (Puig *et al*., 1994; Pina *et al*., 1998; Albinana-Gimenez *et al*., 2009; Wyn-Jones *et al*., 2011; Vieira *et al*., 2012). La elevada prevalencia y estabilidad de los HAdV, así como su origen humano, sugieren que estos virus pueden ser utilizados como indicadores de la presencia de virus entéricos en el medio ambiente (Pina *et al*., 1998; Bofill-Mas *et al*., 2000; McQuaig *et al*., 2006; Miagostovich *et al*., 2008; Wyn-Jones *et al*., 2011; Vieira *et al*., 2012).

1.2.1.2. Polyomavirus JC

Los polyomavirus hasta el momento son el único género de la familia *Polyomaviridae* . Sin embargo recientemente el Grupo de Estudio de los Polyomavirus en el Comité Internacional para la Taxonomía de Virus (Johne *et al*., 2011), ha propuesto tres géneros más que incluyan a los polyomavirus de mamíferos (*Orthopolyomavirus* y *Wukipolyomavirus*) y otro de aves (*Avipolyomavirus*). Los miembros de esta familia son virus no envueltos de un diámetro de 40-45 nm, con una estructura icosaédrica y un genoma DNA circular de doble cadena de aproximadamente 5.000 pb que se encuentra súper enrollado a 4 histonas de origen celular (H2A, H2B, H3 y H4).

El genoma de los polyomavirus está dividido en 3 regiones diferentes como se muestra en la figura 3 (Dalianis y Hirsch, 2013): (i) La región control no codificante o región reguladora (RR), que incluye el origen de la replicación, el sitio de inicio de la transcripción y así como también elementos promotores que son múltiples secuencias consenso y redundantes para sitios de unión de proteínas y factores de transcripción. La RR, regula la expresión de los genes de la región temprana y tardía, en concreto, la activación y diferenciación del estado de la célula huésped. Esta región puede variar de tamaño, incluso dentro de una misma especie de polyomavirus, dando origen a pequeñas alteraciones en el tamaño del genoma. (ii) Los genes de la región temprana codifican el antígeno T largo (LTag) y el antígeno T pequeño (STag), los cuales se generan a partir del transcripto principal por *splicing* alternativo. Algunos polyomavirus, como por ejemplo el poliomavirus JC, tienen además otras variantes de la proteína antígeno que se generan igualmente por procesos de *splicing*. Las variantes de la proteína T antígeno son multifuncionales, pueden interactuar con las proteínas y el DNA vírico y del hospedero.

Estas proteínas están involucradas en dirigir la célula huésped hacia la fase S del ciclo celular, con la finalidad de favorecer la replicación vírica, también regulan la transcripción del huésped y del genoma vírico y participan directamente en la replicación del DNA vírico. (iii) Los genes de la región tardía codifican para las proteínas de la cápside (VP1, VP2 y VP3), las cuales son generadas a partir del transcripto primario por *splicing* alternativo y ensambladas en el núcleo para formar la cápside. En esta región el polyomavirus JC también codifica una proteína pequeña no estructural, llamada agnoproteina, la cual pudiera estar involucrada en la interferencia del mecanismo de reparación del DNA de huésped (Bellizzi *et al*., 2013).

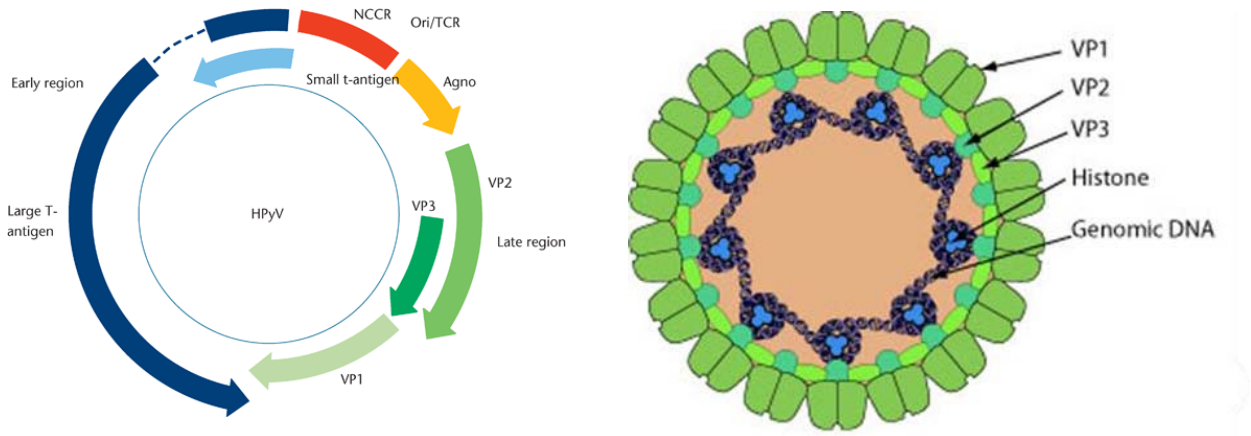


Figura 3. Estructura y mapa de la organización de la transcripción de los polyomavirus

Los polyomavirus incluyen diferentes especies que pueden infectar una gran variedad de vertebrados; aves, roedores, vacunos, simios y humanos. A pesar de que los polyomavirus tienen una estructura y organización genética muy conservada, estos virus tienen una estrecha especificidad de huésped y celular, lo cual está determinado a nivel extracelular, por la ausencia/presencia de receptores específicos y a nivel intracelular, por la presencia/ausencia de factores celulares que permiten coordinar la expresión de los genes víricos y la viabilidad del ciclo de infección vírico (Dalianis y Hirsch, 2013).

La familia *Polyomaviridae* actualmente agrupa a 11 especies de polyomavirus humanos (Tabla 2), de los cuales 6 han sido descubiertos en los últimos 6 años (DeCaprio y Garcea, 2013). En 1971 los polyomavirus BK y JC fueron los primeros polyomavirus humanos descritos. BK fue aislado a partir de la orina de un paciente trasplantado de

riñón, mientras que JC fue identificado como el agente causal de la leucoencefalopatía multifocal progresiva (PML), una enfermedad extraña de desmielinización asociada con afecciones en el sistema inmune. Se considera que los polyomavirus después de una infección primaria, pueden producir infecciones persistentes o latentes asintomáticas con niveles bajos de replicación y de forma permanente. La infección por el polyomavirus JC (JCPyV), puede reactivarse de su fase latente y manifestar síntomas de PML. Este es un proceso complejo y requiere; (i) de una disminución o cambios en el sistema inmune, (ii) un reordenamiento de la región reguladora de JCPyV para incrementar la replicación vírica, (iii) una movilización de JCPyV producido a partir de la activación de infecciones latentes en el riñón y la medula ósea, hacia las células gliales en el cerebro, y (iv) el incremento de activadores transcripcionales y procesos de regulación para la replicación de JCPyV (Major, 2010; Ferenczy et al., 2012). Todos estos procesos pueden ocurrir en algunos casos de inmunosupresión o en terapias que modulan el sistema inmune, por ejemplo en los casos inducidos de este estado en pacientes trasplantados o con tratamientos para la esclerosis múltiple (Major, 2010; Ferenczy et al., 2012; Feltkamp *et al*., 2013).

Tabla 2. Polyomavirus humanos. Modificado a partir de DeCaprio y Garcea, (2013)

Nombre	Abreviación	Año de descubrimiento	Fuente del aislamiento
BK polyomavirus	BKPyV	1971	Orina
JC polyomavirus	JCPyV	1971	Orina, Cerebro
Karolinska Institute polyomavirus	KIPyV	2007	Nasofaringe
Washington University polyomavirus	WUPyV	2007	Nasofaringe
Merkel cell polyomavirus	MCPyV	2008	Lesión en piel
Human polyomavirus 6	HPyV 6	2010	Piel
Human polyomavirus 7	HPyV 7	2010	Piel
Trichodysplasia spinulosa-associated polyomavirus	TSPyV	2010	Lesión en piel
Human polyomavirus 9	HPyV 9	2011	Piel, Sangre, Orina
Malawi polyomavirus	MWPyV	2012	Heces, Verruga
St Louis polyomavirus	STLPyV	2012	Heces

La seroprevalencia de JCPyV analizada en muestras de sangre de individuos sanos indica que un 50-90% de adultos ha tenido contacto con este virus. Se cree que el primer contacto ocurre en un estado de baja inmunidad durante la adolescencia y que la infección por este virus no está asociada a síntomas clínicos. JCPyV detectado en muestras de fluido cerebroespinal de pacientes con PML se caracteriza por presentar una RR altamente variable y reorganizada. En contraste de JCPyV detectado en orina de individuos inmunodeprimidos, donde la RR es arquetípica y se encuentra de forma consistentemente linear. La hipótesis prevalente es que JC con la RR arquetípica es la forma en la cual el virus es transmitido de persona a persona. Según este modelo, el virus con la RR arquetípica se dispersa a través del cuerpo y permanece latente en el riñón y medula ósea. Una vez la región reguladora se reorganiza, el proceso de reactivación de la infección comienza y JCPyV es capaz de producir PML (Major, 2010; Ferenczy et al., 2012).

La vía de transmisión de JCPyV no está definida totalmente, aunque varios estudios sugieren que una vía podría ser por la vía respiratoria o la fecal-oral mediante el consumo de agua fecalmente contaminada (Bofill-Mas *et al*., 2000). Estos virus han sido detectados frecuentemente en agua fecalmente contaminada de diferentes regiones geográficas, su elevada prevalencia y estabilidad así como su origen humano, sugieren que estos virus pueden ser utilizados como indicadores de la presencia de virus entéricos en el medio ambiente (Bofill-Mas *et al*., 2000; Albinana-Gimenez *et al*., 2006, 2009; McQuaig *et al*., 2006; Miagostovich *et al*., 2008; Vieira *et al*., 2012; Fumian *et al*., 2013).

1.2.2. Principales virus causantes de gastroenteritis

Según la OMS alrededor de 2 millones de personas mueren por gastroenteritis en todo el mundo cada año. Los virus son la causa más común de gastroenteritis en todas las edades. Los rotavirus del grupo A han sido la principal causa de gastroenteritis en niños menores de 5 años. Sin embargo la amplia distribución de las vacunas contra rotavirus podría cambiar la distribución de este virus en los próximos años. Actualmente los norovirus son reconocidos como la principal causa de gastroenteritis vírica en todas las edades (Kirby y Iturriza-Gómara, 2012). Los adenovirus humanos de la especie F también están entre los principales agentes de gastroenteritis víricas, dependiendo de la zona geográfica, estos son responsables de 1,4 al 10% de todos los casos (Eckardt y Baumgart, 2011).

1.2.2.1. Norovirus humanos

Los norovirus (NoV) humanos constituyen el género *Norovirus* que pertenece a la familia *Calciviridae*. Los NoV son virus no envueltos de 27-30 nm, con una estructura icosaédrica y con un genoma RNA linear de simple cadena de 7,5 kb de polaridad positiva. El genoma está dividido en tres pautas abiertas de lectura (ORF1-ORF3), que codifican de 5' a 3' para genes no estructurales y estructurales (Figura 4). En el extremo 5' se encuentra una proteína vírica única covalentemente (VPg), mientras que en el extremo 3' hay una cola poli-adenilada (poli-A). El ORF1 se traduce a una poliproteína que es procesada por una proteasa vírica (PRO) que da lugar a 6 proteínas no estructurales diferentes: p48, NTPasa, P22, VPg, una RNA polimerasa dependiente de RNA y la propia proteasa vírica. Estas proteínas intervienen en la copia de RNA (+) a RNA (-), el cual actual de molde para generar RNA subgenómico (RNAsg +). Este RNAsg es traducido principalmente a VP1 que es la proteína mayoritaria de la cápside y en menor cantidad que VP2, estas finalmente se auto ensamblan para generar la partícula vírica (Green *et al*., 2001).

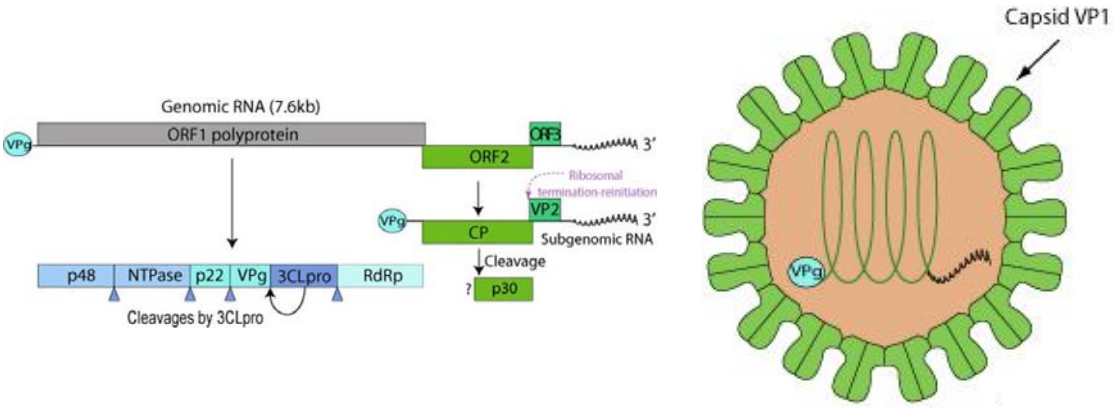


Figura 4. Estructura y mapa de la organización de la transcripción de los norovirus

La familia *Calciviridae* también está constituida por los géneros *Sapovirus* el cual causa gastroenteritis aguda en humanos, así como también por los géneros *Lagovirus*, *Vesivirus* y *Nebovirus,* los cuales no son patógenos humanos. Basándose en la homología de aminoácidos de la principal proteína estructural VP1, los NoV pueden dividirse en al menos 5 genogrupos diferentes (GGI-GGV). Las cepas que infectan humanos se encuentran en los GGI, GGII y GGIV (Figura 5). La transmisión inter-especie de los norovirus no se ha descrito, pero cepas que infectan a porcinos se ubican en el GGII y también cepas en el NoV

GGIV han sido detectadas recientemente como causa de gastroenteritis en caninos, sugiriendo el potencial zoonótico de los NoV. Basados en análisis filogenéticos de VP1, los NoV pueden clasificarse en genotipos, de esta forma al menos 8 genotipos se agrupan en el GGI y 21 en el GGII. Desde el año 2001, los NoV GGII genotipo 4 (NoV GGII.4) han sido reconocidos como la principal causa de brotes gastroenteritis víricas en todo el mundo (Hall *et al*., 2011). Estudios recientes han demostrado que estos virus evolucionan a través del tiempo debido a una serie de mutaciones y procesos de recombinación en VP1, esto permite la evasión de estos virus al sistema inmune de la población (Hall *et al*., 2011).

Los NoV causan gastroenteritis aguda en personas de todas las edades. La enfermedad por lo general comienza después de un periodo de incubación de 12-48 horas y se caracteriza por diarreas sin sangre, vómitos, nausea y dolores abdominales. Los NoV son excretados por las heces hasta durante 4 semanas después de la infección en concentraciones de 10^{10} copias de genomas por gramo de heces (Atmar *et al*., 2008). Sin embargo los NoV no tienen un modelo de cultivo celular convencional que permita estimar la cantidad de virus infecciosos. Por otro lado el 30% de las infecciones por NoV son asintomáticas y aun así los virus son excretados aunque en menores concentraciones (Hall *et al*., 2011).

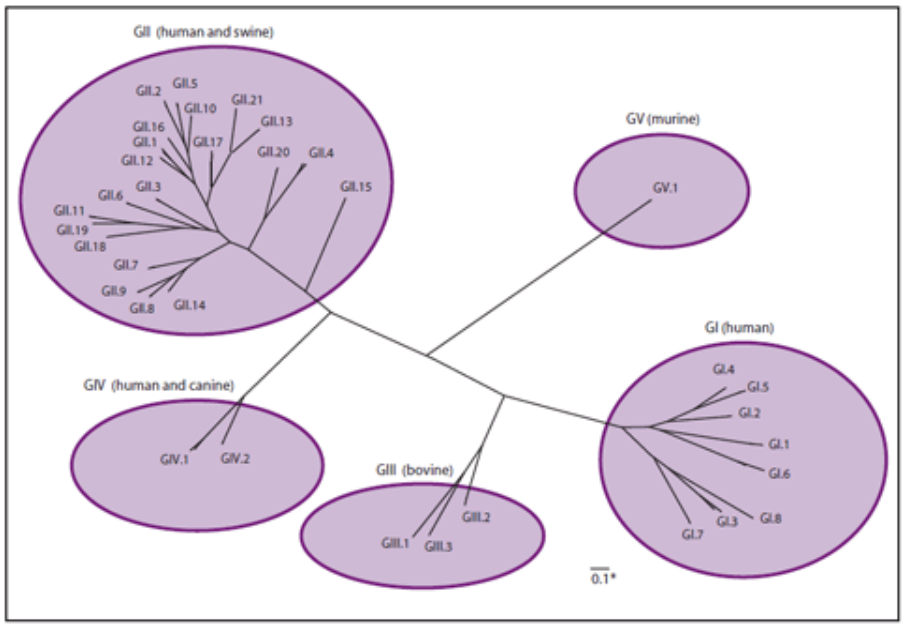


Figura 5. Clasificación de norovirus en 5 genogrupos (I – V) y 35 genotipos según la diversidad de las secuencias de la proteína VP1. Modificado a partir de Hall *et al*. (2011)

Los NoV son extremadamente contagiosos, se ha estimado que la dosis infecciosa mínima es de 18 partículas víricas, sugiriendo aproximadamente 5 millones de dosis infecciosas por gramo de heces durante el pico máximo de excreción (Atmar *et al*., 2008). La trasmisión de NoV puede ocurrir por tres rutas diferentes: persona-persona, alimentos contaminados y agua contaminada. Los avances en los métodos de diagnóstico de NoV están cada vez más disponibles y el papel de estos virus como la principal causa de gastroenteritis esporádica en todas las edades es cada vez más claro. Varios estudios de seroprevalencia han demostrado que la infección por NoV es altamente prevalente en toda la población y con una posible primera exposición durante la niñez. En los Estados Unidos de Norteamérica y Europa, 50% de todos los brotes reportados de gastroenteritis es debido a NoV, confirmando a este virus como la primera cusa de estos padecimientos en la población. Los brotes ocurren durante todo el año, aunque existe un patrón de estacionalidad que se incrementa durante el invierno. En última década los brotes por NoV han sido causados por el GGII.4, mientras que el resto de genotipos fueron responsables de menos del 7%. Análisis retrospectivos con muestras de 1974 a 1991 mostraron que GGII.3 causó 48%, GGII.4 16% y GII.7 14%. Durante 2009 a 2010 la cepa emergente GGII.12 fue asociada con una gran número de brotes en EEUU (Vega y Vinjé, 2011). El uso de agua de baño y agua de bebida contaminada con NoV ha dado lugar diferentes brotes (Hall *et al*., 2011). En estudios recientes se ha detectado NoV GGI y GGII en agua de baño Europeas y de Brasil (Wyn-Jones *et al*., 2011; Vieira *et al*., 2012).

1.2.3. Virus nuevos recientemente descritos

1.2.3.1. Polyomavirus KI, WU y de Merkel *cell*

Desde el año 2005 un total de 9 nuevos polyomavirus humanos se han descrito (Tabla 2). Entre estos se encuentran los polyomavirus KI (KIPyV) y WU (WUPyV), aislados partir de muestras de la nasofaringe en el 2007 por investigadores del *Karolinska Institute* y *Whashington University*, respectivamente (Allander *et al*., 2007; Gaynor et a., 2007). La seroprevalencia de estos virus varía entre 55 a 90% para KI y entre 64 a 97,5% para WU. El tejido específico de KI y WU no se ha establecido aún, no obstante el DNA vírico se ha detectado en sangre, cerebro, sistema nervioso central, pulmón y amígdalas (Van Ghelue *et al*., 2012; Dalianis y Hirsch, 2013; DeCaprio y Garcea, 2013; Feltkamp *et al*., 2013). A la fecha KIPyV y WUPyV no se han asociado con una enfermedad específica o síntoma, aunque secuencias de ambos virus de han detectado en canceres de pulmón y linfomas (Van Ghelue *et al*., 2012; Dalianis y Hirsch, 2013; DeCaprio y Garcea, 2013; Feltkamp *et al*.,

2013). Ambos virus se detectan regularmente en muestras del tracto respiratorio, pero no hay una correlación clara entre KI y WU y una infección respiratoria, por lo general estos virus se han detectado junto a otros virus conocidos (Van Ghelue *et al*., 2012; Dalianis y Hirsch, 2013; DeCaprio y Garcea, 2013; Feltkamp *et al*., 2013).

En el año 2008 Feng y colaboradores describieron la presencia de secuencias víricas en cuatro carcinomas de las células de Merkel (MCC). Los análisis posteriores de estas secuencias víricas mostraron que estas codificaban para un polyomavirus que posteriormente se le nombro como polyomavirus de las células de Merkel (MCPyV). El MCC es un extraño tumor de piel neuroendocrino observado principalmente en personas mayores o pacientes inmunosuprimidos. La prevalencia de MCPyV en MCC es del 40 al 100%. En varias muestras de MCC positivas para MCPyV, el DNA vírico estaba integrado en el genoma de las células del tumor con un patrón idéntico, sugiriendo que la infección por MCPyV y la integración del genoma precedieron a la expansión de la metástasis del cáncer (Spurgeon y Lambert, 2013). Partículas víricas y DNA de MCPyV han sido ampliamente detectados en muestras de piel, lo que refuerza la idea de que este virus sea parte de la microflora normal de la piel. De hecho los eventos iniciales de la replicación de MCPyV se ha visto que ocurren en keratinocitos primarios, los cuales son un tipo de células prominentes de la piel y otros epitelios estratificados (Schowalter *et al*., 2011, Spurgeon y Lambert, 2013). Se ha reportado que un 80% de la población adulta contiene anticuerpos contra MCPyV, por otro lado no se han detectado anticuerpos en muestras prenatales, pero si en infantes durante la primera década de vida, aumentando la seroprevalencia con la edad (Spurgeon y Lambert, 2013). El DNA de MCPyV también se ha detectado en muestras del tracto respiratorio, saliva, tejidos linfáticos, orina y tracto gastrointestinal (Spurgeon y Lambert, 2013). No obstante en estas muestras las concentraciones son notablemente bajas cuando se comparan con las cantidades detectadas en la piel (Spurgeon y Lambert, 2013).

No se ha establecido un ruta de transmisión para los polyomavirus KI, WU y MC, pero los datos disponibles proponen varias posibilidades. La presencia de estos virus en el tracto gastrointestinal y en agua residual sugieren que una ruta puede ser fecal oral (Bofill-Mas *et al*., 2010; Loyo *et al*., 2010; Campello *et al*., 2011), mientras que la presencia en el tracto aerodigestivo supone una vía respiratoria (Spurgeon y Lambert, 2013). En el caso de MCPyV se ha sugerido que se excreta crónicamente a través de la piel en grandes concentraciones y que puede persistir en varias superficies, además los resultados que indican que la diferenciación celular de la piel está relacionada con el ciclo del virus, hacen considerar una ruta de transmisión cutánea (Spurgeon y Lambert, 2013).

1.2.3.2. Salivirus/Klassevirus

Salivirus (por *stool Aichi-like*) es un nuevo miembro de la familia *Picornaviridae* descrito en el año 2009 en Nigeria en heces de infantes con parálisis flácida negativa para poliovirus (Li *et al*., 2009). Paralelamente en el 2009, klassevirus (por *kobu-like viruses associated with stool and sewage*), otro picornavirus es descrito en heces de infantes con gastroenteritis en dos estudios diferentes con muestras de los Estados Unidos de Norteamérica (EEUU) y Australia y en agua residual de Barcelona (Greninger *et al*., 2009; Holtz *et al*., 2009). Genéticamente, salivirus y klassevirus son virus idénticos y de acuerdo con el Grupo Internacional de Estudio de los Picornavirus del Comité Internacional para la Taxonomía de los Virus, a partir de febrero del 2013 ambos virus están clasificados dentro del nuevo género *Salivirus*.

Los salivirus como miembros de la familia *Picornaviridae*, son virus no envueltos icosaédricos de 30nm, con un genoma RNA de simple cadena de polaridad positiva de 7-9 kb. El genoma contiene en ambos extremos una región no traducible (UTR), siendo esta mucho mayor en el 5', el extremo 3' además contiene una región poli-adenilada. El extremo 5' está unido covalentemente a una proteína vírica (VPg). El genoma da lugar a una poliproteína que es proteolíticamente procesada en diferentes proteínas víricas estructurales (1A-1D o VP1-VP4) y no estructurales (2A-2C y 3A-3D).

Los salivirus/klassevirus se han asociado a gastroenteritis víricas, detectándose en muestras de heces de diferentes regiones; Australia, China, EEUU (California, Minnestoa y San Louis), Corea del Sur, Nigeria, Nepal y Dinamarca (Greninger *et al*., 2009; Holtz *et al*., 2009; Li *et al*., 2009; Shan *et al*., 2010; Han *et al*., 2012; Nielsen *et al*., 2013). También se han detectado en Túnez y Nigeria en heces de pacientes con parálisis flácida negativas para poliovirus, (Li *et al*., 2009). La seroprevalencia de estos virus fue del 6,8% de 353 pacientes analizados (Greninger *et al*., 2010).

En muestras ambientales salivirus/klassevirus se detectó por primera vez en muestras de agua residual de Barcelona a través de ensayos de PCR convencional (Holtz *et al*., 2009). También se han detectado por secuenciación en masa usando la plataforma 454 Titamium, en muestras de agua residual de Nepal (Ng *et al*., 2012). Interesantemente en otro estudio empleando también secuenciación en masa pero con la plataforma Illumina HiSeq 2000, se reportó salivirus/klassevirus como el virus RNA más abundante en muestras de agua residual de diferentes zonas de EEUU (Bibby y Peccia, 2013). Recientemente Haramoto *et al*. (2013) describieron un ensayo de RT-qPCR para estos virus, reportando 93% (13 muestras) y 16% (9 muestras) de muestras de agua residual y

río positivas en Japón y en concentraciones máximas de $9,7x10^3$ copias de genomas por mL de muestra.

1.2.3.3. Asfarvirus-*like*

Los asfarvirus (ASFV), son los agentes etiológicos responsables de la fiebre porcina africana. Estos virus miden aproximadamente 200 nm, son virus envueltos e icosaédricos que tienen un genoma lineal DNA de doble cadena de un tamaño entre 170 a 190 kb. Actualmente y de acuerdo con el Comité Internacional de Taxonomía de los Virus (2013), todas las cepas de los asfarvirus están agrupadas en el género *Asfivirus*, el cual es el único género de la familia *Afarviridae*. La fiebre porcina africana es endémica del continente africano y puede estar presente de forman natural en diferentes especies porcinas salvajes sin causar enfermedad. En el ciclo natural del virus está involucrada la transmisión por garrapatas. Hasta el momento se sabe que este es el único virus DNA transmitido por artrópodos (Dixon *et al*. 2013). Brotes de esta patogénesis fuera de África se han reportado previamente en Europa y América Central y del Sur (Dixon *et al*. 2013).

Los datos disponibles sugieren que ASFV no produce infección en humanos. Sin embargo recientemente se han venido reportando la presencia de secuencias relacionadas pero no idénticas a los ASFV en muestras humanas o relacionadas con la actividad humana. Loh *et al.* (2009) reportaron la presencia de un Asfarvirus-*like* en muestras de suero humano en pacientes sanos y con un cuadro clínico de fiebre aguda provenientes de la región del Medio Oriente, además en el mismo estudio se detectaron estas secuencias en muestras de agua residual urbana de Barcelona. Recientemente en el año 2012, Yozwiak *et al* reportaron secuencias de ASFV-like en muestras de suero humano de pacientes con un cuadro febril similar al dengue en Nicaragua. La presencia de secuencias de ASFV-*like* también se ha reportado en muestras de agua del río Missisipi a través de secuenciación en masa (Wan *et al*., 2013) e incluso en agua de mar (Monier *et al*., 2008). La presencia de secuencias de ASFV-*like* sugiere que puede haber más de un miembro dentro de la familia *Asfarviridae* y que son necesarios más estudios para entender mejor la naturaleza de las especies huésped de estas secuencias y de su potencial patogénico. La amplia distribución geográfica de estas secuencias también indica que estos ASFV-*like* persisten en la población humana y el medio ambiente.

1.3. Calidad microbiológica de ríos, lagos y agua costera.

Los ríos, lagos y agua costera pueden emplearse con fines recreativos por la población humana, por esta razón estos tipos de agua pueden clasificarse como agua de baño. En la actualidad, en algunos países o regiones existen legislaciones que regulan la calidad microbiológica del agua de baño (WHO, 2003; USEPA, 2004; 2006/160/EC). Un ejemplo, fue la primera Directiva de la Unión Europea para el agua de baño (76/160/CE), que entró en vigor en 1976. Esta Directiva incluía la detección de los grupos bacterianos; coliformes totales, coliformes fecales, estreptococos fecales y *Salmonella* y como indicador vírico a los enterovirus. En el año 2001 y con la finalidad de incrementar el rigor de la legislación del agua recreacional, la Comisión Europea comenzó el proceso de revisión de la Directiva 76/160/CE. Posteriormente en febrero del 2006, la nueva Directiva para el agua recreacional entró en vigor (2006/07/CE). La actual Directiva, describe el uso de indicadores bacterianos para la evaluación microbiológica del agua, concretamente las bacterias *E. coli* (EC) y los enterococos intestinales (EI). Indiscutiblemente, el cambio en lo referente a los análisis bacterianos, refleja los avances científicos que se han logrado en años recientes, obteniendo una base y resultados científicos más firmes (Kay *et al*., 1994 y Kay *et al*., 2004: Wiedenmann *et al*., 2006).

No obstante, y a pesar de los avances sobre indicadores bacterianos de contaminación fecal, la Directiva vigente no incluye el análisis para la detección de un indicador vírico de contaminación fecal humana. Existen varios datos que confirman que muestras de agua recreacional consideradas aptas para baño según los valores establecidos para los indicadores bacterianos, contenían valores altos de virus entéricos humanos y que por lo tanto estos indicadores estándar suelen fallar en la predicción del riegos de patógenos transmitidos por el agua, incluyendo los virus entéricos (Gerba *et al*., 1979 y Lipp *et al*., 2001). Además, se ha reportado que los niveles de indicadores bacterianos no se correlacionan con los virus, especialmente cuando los indicadores estándar se encuentran en concentraciones bajas (Contreras-Coll *et al*., 2002). También se han obtenido resultados que indican que los virus, en comparación con las bacterias, son notablemente más resistentes a las condiciones del medio ambiente (Thurston-Enriquez *et al*., 2003, Rzezutka y Cook, 2004 y de Roda Husman *et al*., 2009). Los virus entéricos han sido frecuentemente asociados a gastroenteritis adquiridas con el uso de agua recreacional (Sinclair *et al*., 2009). Varios estudios en Europa y EEUU sugieren que estas infecciones se pueden adquirir como resultado de actividades como el canotaje, nado u otro uso recreacional del agua (Medema *et al*., 1995 y Gray *et al*., 1997). Varios brotes de enfermedades asociadas con virus entéricos, como los norovirus y astrovirus, en agua

recreacionales has sido ampliamente descritas (Maunula *et al*., 2004; Hauri *et al*., 2005), sin embargo estos tipos de brotes fácilmente pasan desapercibidos por las autoridades sanitarias, principalmente porque no se logra definir el origen ni el agente causal.

La antigua Directiva como se menciona anteriormente, incluía los enterovirus como parámetro microbiológico a analizar y el valor estipulado era que el 95% de las muestras de 10L analizadas deberían ser negativas para enterovirus (cero unidades formadoras de calvas). El uso de los enterovirus como parámetro microbiológico a evaluar, estaba basado en los estudios y resultados obtenidos por Farrah y Bitton (1990), en los cuales se sugería que entre 1 y 20 virus infecciosos de poliovirus, Coxsackie A o B, eran suficientes para producir infección en humanos. Sin embargo, actualmente se tienen mayores conocimientos de la patogénesis de los enterovirus y existe suficiente evidencia para no relacionar a estos virus con la calidad microbiológica del agua. Estos virus sin lugar a duda son realmente patógenos muy importantes en otros contextos, a pesar de esto la presencia de enterovirus en agua no necesariamente se puede correlacionar con la presencia de otros patógenos víricos como el virus de la hepatitis A (Dubrou *et al*., 1991 y Pina *et al*., 1998).

Tabla 3. Parámetros obligatorios y valores para la clasificación del agua de baño

	Agua continental			
	Calidad			
	Suficiente**	Buena*	Excelente*	Unidad
Enterocos intestinales	330	400	200	UFC o NMP/ mL
E. coli	900	1000	500	
Agua costera y de transición				
Enterocos intestinales	185	200	100	UFC o NMP/ mL
E. coli	500	500	250	

*Con arreglo a la evaluación del percentil 95. **Con arreglo a la evaluación del percentil 90. Tabla modificada a partir del Real Decreto 1341/2007.

Es evidente que además de los indicadores bacterianos estándar, una nueva generación de indicadores víricos de contaminación fecal es necesaria. Como virus candidatos de indicadores virales se han propuesto los adenovirus humanos y los polyomavirus JC (Pina *et al*., 1998; Bofill-Mas *et al*., 2000; McQuaig *et al*., 2006; Miagostovich *et al*., 2008; Wyn-Jones *et al*., 2011; Vieira *et al*., 2012). Estos virus son considerablemente estables en el medio ambiente y excretados por las heces y/u orina específicamente por humanos, los cuales en la mayoría de los casos no presentan síntomas de infección (Enriquez *et al*., 1995; Thurston-Enriquez *et al*., 2003). Además en varios

estudios ambos virus se han detectado en agua fecalmente contaminada (Pina *et al*., 1998, Laverick *et al*., 2004, Lee *et al*., 2004 y Miagostovich *et al*., 2008). El hecho que en la actual Directiva para agua baño no se incluyan los virus, se debe entre otros factores al hecho de que en años anteriores no se contaba con suficiente información sobre indicadores virales, protocolos para su concentración, métodos para su detección molecular y procedimientos para la detección de virus infecciosos.

España como miembro de la Comunidad Europea está obligada a cumplir la regulación Europea 2006/7/CE para agua de baño. Esta Directiva se ha traspuesto a la legislación interna española mediante el Real Decreto 1341/2007 (BOE no 257, 26/10/2007). Esta legislación establece que el número de muestreos bajo condiciones normales debe ser como mínimo 8 por temporada de baño, más una muestra inicial antes de comenzar la temporada, sin embargo bajo algunas circunstancias excepcionales la cantidad de muestro puede ser 4 por temporada de baño. Por otro lado, el plazo máximo entre dos muestreos no debe de ser mayor de 30 días. De acuerdo a la legislación vigente, según los valores detectados de EC y EI en la temporada evaluada y tomando en cuenta también los datos de las tres últimas temporadas, los sitios de baño se pueden clasificar como agua de *Calidad Excelente, Buena, Suficiente o Insuficiente* (Tabla 3).

1.4. Métodos de concentración de virus en agua

Los virus humanos presentes en el medio ambiente como contaminantes, frecuentemente se encuentran en bajas concentraciones, especialmente en agua de mar, río y lago. Por esta razón grandes cantidades de esta agua debe ser tratada para concentrar los virus a un volumen mucho menor. Generalmente para el análisis de virus en agua de baño, los virus presentes en muestras de 10L son concentrados en un volumen final de 10 mL y habitualmente a partir de este concentrado vírico se pueden hacer análisis directos en cultivo celular o extracciones de DNA/RNA vírico para posteriores análisis moleculares. Los protocolos para concentrar estos virus son muy diversos y a través del tiempo estos han ido evolucionando con la principal finalidad de ser más eficientes y sencillos (Tabla 4).

Los virus tienen carga neta y por lo tanto polaridad, debido a esta característica los virus pueden adsorberse a diferentes matrices cargadas. Además los virus se pueden considerar partículas proteicas y por lo tanto tienen una masa molecular relativa ($Mr>10^6$), esto permite concentrarlos por procesos de ultrafiltración o ultracentrifugación.

Basados es estas dos características generales, se han desarrollado una gran variedad de métodos para concentra virus presentes en agua. De acuerdo con Block y Schwartzbrod, (1989) cualquier método debe cumplir con los siguientes criterios:

- Debe ser técnicamente fácil de realizar y en corto tiempo
- Debe recuperar grandes cantidades de virus
- Debe concentrar varios tipos de virus
- Debe tener un ratio de concentración elevado
- No debe ser costoso
- Debe concentrar grandes volúmenes de muestra
- Debe ser repetible (dentro de un laboratorio) y reproducible (entre laboratorios)

Pero además estos métodos también deben permitir concentrar virus infecciosos para poder evaluar si los virus en el medio ambiente realmente permanecen estables y representan un riesgo para la salud humana. Igualmente estos métodos deben ser diseñados para evitar concentrar grandes cantidades de compuestos que inhiban o afecten los análisis moleculares o de cultivo celular para la detección de los virus. A pesar de todas estas recomendaciones, actualmente no hay un método que cumpla con todos estos requerimientos. Existen 3 principalmente enfoques diferentes para la concentración de virus en agua, estos se han empleado durante varios años y también se han ido modificándose o incluso combinándose para mejorar la eficiencia en general (Tabla 4).

Tabla 4. Principales procedimientos para concentrar virus en agua. Modificado a partir de Wyn-Jones y Sellwood, 2001

Técnica	Metodología	Segundo paso de concentración	Volumen (L)
Adsorción/Elución	Membranas electronegativas	Si	1-10
	Membranas electropositivas	Si	1-10
	Cartuchos electropositivos	Si	1-50
	Lana de vidrio	Si	1-1000
Ultrafiltración	Ultrafiltros	Algunas veces	1-10
	Ultrafiltración tangencial	Algunas veces	1-10
Ultracentrifugación	Ultracentrifugación directa	No	<1
	Ultracentrifugación/Centrifugación/Elución	Si	<1

Entre los procedimientos comúnmente empleados para concentrar virus en agua de baño, están los métodos de dos pasos llamados VIRADEL, los cuales están basados en procesos de adsorción/elución (Wallis y Melnick, 1967). En estos métodos los virus se adsorben en un primer paso a través de interacciones electrostáticas a una matriz con carga, posteriormente los virus son separados de esta matriz empleando generalmente *buffers* de elución alcalinos que contienen una solución proteica como el extracto de carne o leche descremada y en muchos casos también contienen glicina (Katzenelson *et al*., 1976; Bitton *et al*., 1979;). Una vez los virus han sido eluídos, estos se encuentran presentes en el *buffer* de elución en un volumen aún demasiado mayor, por lo que un segundo paso de concentración es necesario (Wallis y Melnick, 1967; Katzenelson *et al*., 1976). Este segundo paso de concentración generalmente se lleva a cabo a través de una floculación orgánica bajo condiciones de pH ácido de las proteínas presentes en el *buffer* de elución. Finalmente los virus se adsorben a los flóculos de las proteínas, los cuales son concentrados por centrifugación y disueltos en un volumen mucho menor de un *buffer* fosfato (Katzenelson *et al*., 1976). Las matrices cargadas donde se adsorben los virus pueden tener ser electronegativas o electropositivas. Los virus presentes en el medio ambiente presentan una carga neta negativa, debido a esto los virus pueden adsorberse directamente a una matriz electropositiva. Contrariamente, cuando se emplea una matriz electronegativa, la carga neta del virus debe modificarse a positiva para que el virus se adsorba eficientemente a la matriz electronegativa. Este cambio de la carga neta ocurre cuando el pH de la solución en la cual están presentes los virus es inferior al punto isoeléctrico del virus. Por lo tanto es necesario acondicionar el pH de la muestra cuando se emplean matrices electronegativas, situación que limita procesar volúmenes grandes de muestra. En el caso de usar matrices electropositivas no es necesario ajustar el pH de la muestra y por lo tanto si se pueden concentrar virus a partir de grandes volúmenes. Sin embargo se ha reportado que el uso de matrices electronegativas es más eficiente que las matrices electropositivas (Hsu *et al*., 2007). Las matrices electropositivas o electronegativas son muy diversas en lo referente a su composición y presentación, existen membranas y cartuchos de materiales como la nitrocelulosa, celulosa y fibra de vidrio (Virosorb 1MDS), microfibras de vidrio combinadas con nano fibras de aluminio (NanoCeram®), unas de las matrices más empleada es la lana sodocálcica aceitada de lana de vidrio descrita por Vilagines *et al*. (1993) y que tiene un coste económico considerablemente bajo. Los datos disponibles sobre la recuperación de los métodos VIRADEL son variables y difíciles de comparar ya que en cada estudio se emplearon condiciones específicas. Los protocolos VIRADEL sin lugar a dudas han contribuido a la virología ambiental, sin embargo presentan algunas desventajas; metodológicamente son

técnicas engorrosas de llevar a cabo, esto especialmente porque incluyen dos pasos de concentración y porque generalmente requieren algún tipo de dispositivo para hacer de soporte de la matriz o para que el sistema funcione, evitando de este modo poder procesar varias muestras al mismo tiempo. También al ser técnicas basadas en interacciones electrostáticas (Lukasik *et al*., 2000), se reportado que por ejemplo en agua de mar la eficiencia para adsorber virus es baja, igualmente ahora se sabe más sobre el punto isoeléctrico específico de cada virus y por lo tanto de su carga neta (Michen y Graule, 2010), sugiriendo que cada virus podría tener una eficiencia específica de adsorción a las matrices empleadas en los métodos VIRADEL.

El proceso de floculación orgánica de proteínas es un proceso complejo. En el caso de usar leche descremada, se debe tener en cuenta que la caseína presente en la leche está en forma de una sal cálcica, el caseinato de calcio, que tiene una estructura compleja formando una micela o unidad de solubilización. Entre las moléculas proteicas como la caseína existen diferentes interacciones electrostáticas, por ejemplo entre moléculas similares con la misma carga neta hay fuerza de repulsión que hace que hacen que la caseína se mantenga soluble (Corredig y Dalgleish, 1996, Lucey *et al*., 1996; Demetriades *et al*., 2006). También existen fuerzas van der Waals que tienden a que la caseína sea insoluble formando agregados (flóculos). Por lo tanto en dependencia de que interacción electrostática este favorecida, la caseína se encontrara disuelta o en flóculos. La caseína se mantiene estable mediante fuerzas constantes de repulsión, sin embargo esta estabilidad es extremadamente dependiente del pH y la fuerza iónica (Corredig y Dalgleish, 1996; Lucey *et al*., 1996; Demetriades *et al*., 2006). El punto isoeléctrico de la caseína esta aproximadamente a pH 4,6, valor en el cual la carga neta de esta molécula es negativa (Corredig y Dalgleish, 1996; Lucey *et al*., 1996; Demetriades *et al*., 2006). Mientras que si el pH de la leche se ajusta por debajo del punto isoeléctrico, la carga neta de la caseína cambia a positiva. Cuando el pH de la leche es ajustado a valores cercanos al punto isoeléctrico, los contra-iones de la fase acuosa cubren las cargas de la caseína haciendo que la repulsión electrostática sea débil y que la floculación sea favorecida. En estudios añadiendo NaCl a la suspensión de leche, se ha reportado que la fuerza iónica puede ampliar el efecto del pH ácido sobre la caseína, actuando los contra-iones de sales con las cargas de la caseína (Demetriades *et al*., 2006). Estos datos sugieren que los flóculos de leche tienen la capacidad de adsorber a través de interacciones electrostáticas a partículas cargadas como los virus. Igualmente los virus al ser proteínas podrían flocular conjuntamente con la caseína de la leche. En ambos casos el proceso se incrementa con la presencia de iones.

1.5. Métodos de detección y cuantificación de virus en agua

El estudio de los virus presentes en el agua, además de requerir un proceso de concentración, posteriormente también incluye el análisis para la detección, cuantificación e identificación de los virus estudiados. Estos análisis se realizan principalmente a través de técnicas de cultivo celular o reacciones moleculares como la PCR, aunque la elección de la técnica depende de los requerimientos del estudio.

Los ensayos de cultivo celular tienen la ventaja de que se puede obtener información cuantitativa sobre la presencia de virus infecciosos en la muestra analizada y esto en determinados estudios específicos sobre la evaluación de riesgo para la salud humana es importante. Los protocolos empleados comúnmente son el ensayo PFU (*plaque forming units*) y $TCID_{50}$ (*tissue culture infectious dose* 50 %), ambos protocolos están basados en la observación y cuantificación del efecto citopático (CPE) producido en la células por el virus analizado (Wyn-Jones y Sellwood, 2001; Fong y Lipp, 2005). Entre las principales desventajas de estos ensayos está el hecho de que no todos los virus infecciosos producen CPE y algunos incluso requieren un tiempo muy prolongado para poder observarlo (Ridinger *et al*., 1982; Smith y Gerba, 1982; Teunis *et al*., 2005; Hamza *et al*., 2011). En concentrados víricos de muestras ambientales, muchas veces el CPE no es producido por el virus estudiado sino por otro capaz de infectar la línea celular. También se sabe que estos concentrados víricos pueden contener varios compuestos que son tóxicos para las células, evitando de esta forma obtener una lectura adecuada de los resultados. De forma general entre los inconvenientes del cultivo celular está el hecho de que no todos los virus humanos detectados en el agua tienen una línea celular en la que pueden multiplicarse, por ejemplo los norovirus, que son importantes patógenos responsables de gastroenteritis, no tiene establecido una línea celular convencional para estudios de infectividad (Wyn-Jones y Sellwood, 2001; Fong y Lipp, 2005;). Además en comparación con los ensayos moleculares como la PCR, analizar una muestra por cultivo celular tiene un coste económico elevado (Wyn-Jones y Sellwood, 2001; Fong y Lipp, 2005).

Actualmente, entre las herramientas más rápidas, especificas, sensibles, robustas y económicas, están las técnicas basadas en ensayos de PCR, especialmente la qPCR/RT-qPCR (Girones *et al*., 2010). Sin embargo, estas técnicas *per se* no permiten obtener datos sobre la infectividad de los virus detectados. En los últimos años se han descrito combinaciones entre cultivo celular con PCR o qPCR (Reynolds *et al*., 1996; Hamza *et al*., 2011). Sin lugar a dudas estos últimos han aumentado la especificidad en la detección de

virus infecciosos, pero igualmente tienen algunos inconvenientes en relación al tiempo de obtención de resultados y al coste económico (Hamza *et al*., 2011). Otro tipo de combinación son los ensayos de detección de virus infecciosos en líneas celulares a través inmunofluorescencia, estos ensayos se han empleado con considerable poca frecuencia para la detección de virus en agua. Los trabajos de Smith y Gerba, (1982) y Ridinger *et al*. (1982) fueron de los primeros en publicar la inmunofluorescencia para la detección de rotavirus en agua residual.

1.6. Nuevas metodologías para la identificación de virus en agua

La nueva generación de tecnologías de secuenciación de DNA (NGS) ha permitido grandes avances en el descubrimiento de nuevos virus. En la tabla 5 se muestran las principales tecnologías que actualmente se emplean con más frecuencia (Radford *et al*., 2012). Empleando estas tecnologías se han reportado virus nuevos en diferentes muestras clínicas pero también en diferentes muestras ambientales, como por ejemplo en agua residual, en el artículo presentado en la presente tesis, el cual ha sido el primer artículo sobre el estudio de los virus en agua residual por técnicas de metagenómica (Cantalupo et al., 2011), posteriormente en agua residual también ha habido otros estudios (Tamaki *et al*., 2011; Ng *et al*., 2012;), en océanos (Angly *et al*., 2006; Kristensen *et al*., 2010), ríos y lagos (Djikeng *et al*., 2009; Rodriguez-Brito *et al*., 2010) y en ambientes acuáticos extremos (Schoenfeld *et al*., 2008; Lopez-Bueno *et al*., 2009).

Tabla 5. Principales tecnologías de secuenciación de DNA de nueva generación. Modificado a partir de Radford *et al*., 2012.

Método	Secuenciación	Tamaño de fragmentos generados (pb)
453 Life Science	Pirosecuenciación	400 - 700
SOLiD	Ligazón	50 - 75
Illumina	Terminadores reversibles	25 - 500
Helicos	Terminadores reversibles	25 - 55
PacBio	dNTP fluorescentes	1000
Ion Torrent	Detección de los H^+ liberados	35 - 400

El análisis de los datos generados por las NGS es la etapa más desafiante de estos estudios. Para obtener resultados de alta calidad, las secuencias obtenidas (*reads*) son procesadas con diferentes herramientas bioinformáticas con la finalidad de eliminar cualquier tipo de secuencias inconsistentes y secuencias que no tengan origen vírico. Los *reads* resultantes pueden ser ensamblados para obtener fragmentos de DNA más grandes (*contigs*). Este tipo de ensamblaje se realiza con diferentes algoritmos y programas informáticos y deben seguir parámetros específicos para evitar secuencias quimeras. Los estudios filogenéticos y comparaciones de homología con bases de datos como el *Genbank* se llevan a cabo a partir de los *reads* (especialmente si tiene un tamaño adecuado) o de los *contigs*. Los resultados obtenidos permiten saber si la secuencia pertenece a un virus conocido o si tiene cierto grado de relación, no obstante la mayoría de los estudios sobre virus empleando NSG muestran que la mayoría de las secuencias no se relacionan con las familias taxonómicas víricas descritas actualmente (Figura 6).

La plataforma de NGS, 454 *Life Science* (Roche Diagnostics) está entre las tecnologías comúnmente empleadas. Esta plataforma de forma general, se basa en un protocolo donde el DNA de la muestra es fragmentado y seguidamente se construye una librería añadiendo una serie de adaptadores a cada fragmento, posteriormente el DNA es amplificado a través de una PCR de emulsión y para el proceso de secuenciación, se lleva a cabo una pirosecuenciación optimizada (Figura 7). Esta técnica actualmente permite obtener secuencias de un tamaño medio de 600 pb.

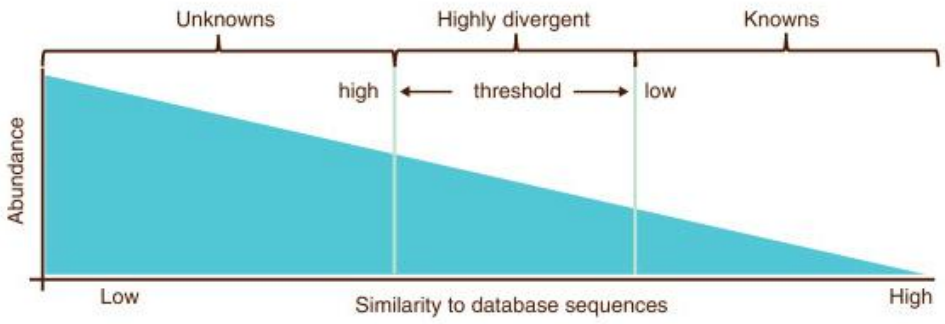


Figura 6. Secuencias con homólogos no detectables en Genbank. Modificada de Mokili *et al*., 2012

Debido a que en los últimos 5 años ha habido un considerable avance en el desarrollo de estas tecnologías, es de suponer que actualmente existen nuevas metodologías desarrollándose. Sin lugar a dudas la preparación de la muestra es un punto clave para obtener datos fiables en estos estudios. En virología ambiental es de suponer que con los métodos de concentración de virus, otros microorganismos como bacterias, hongos y parásitos estén presentes en una considerable menor cantidad, sin embargo esto no se puede garantizar y mejoras en este aspecto no se deben olvidar a la hora de desarrollar nuevas metodologías para la identificación de virus en agua.

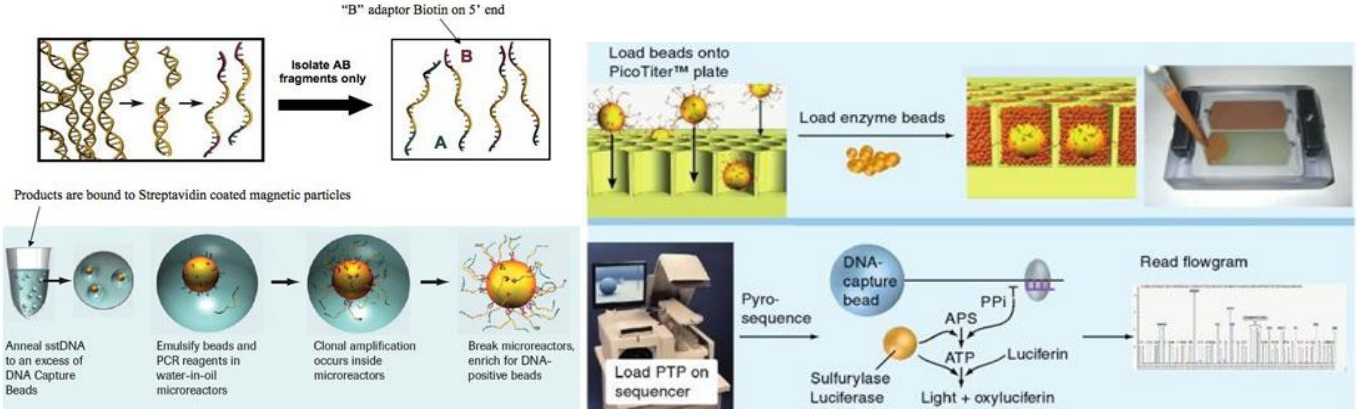


Figura 7. Esquema del protocolo de 454 *Life Science*.

2. OBJETIVOS

Los principales objetivos del presente trabajo de *Tesis Doctoral* se describen a continuación:

1) Desarrollar y validar protocolos eficientes y de bajo coste económico para concentrar virus en agua, con la finalidad de que estos puedan ser implementados en laboratorios de rutina implicados en control microbiológico de la calidad del agua.

2) Definir un ensayo de infectividad sensible y rápido para la cuantificación de adenovirus humanos y polyomavirus JC infecciosos.

3) Evaluar la aplicabilidad de las técnicas desarrolladas y de los parámetros víricos; adenovirus humanos y polyomavirus JC como indicadores de contaminación fecal en el medio ambiente en diferentes áreas geográficas.

4) Evaluar los niveles de contaminación vírica en agua de baño en Europa empleando ensayos de PCR cuantitativa TaqMan®.

5) Analizar la diseminación de virus contaminantes humanos emergentes/nuevos y clásicos en agua de río de diferentes zonas geográficas para evaluar niveles de excreción y estabilidad.

6) Aplicar técnicas de secuenciación de DNA de nueva generación para el estudio del viroma presente en el agua residual urbana de diferentes regiones geográficas.

3. PUBLICACIONES

La presente *Tesis Doctoral* está basada en las siguientes publicaciones:

1. **Calgua B, Mengewein A, Grunert A, Bofill-Mas S, Clemente-Casares P, Hundesa A, Wyn-Jones AP, López-Pila JM, and Girones R**. Development and application of a one-step low cost procedure to concentrate viruses from seawater samples. Journal of Virological Methods, 2008, 153(2):79-83.

2. **Calgua B, Barardi CR, Bofill-Mas S, Rodriguez-Manzano J, and Girones R.** Detection and quantitation of infectious human adenoviruses and JC polyomaviruses in water by immunofluorescence assay. Journal of Virological Methods, 2011, 171(1):1-7.

3. **Bofill-Mas S, Calgua B, Clemente-Casares P, la Rosa G, Iaconelli M, Muscillo M, Rutjes S, de Roda Husman AM, Grunert A, Gräber I, Verani M, Carducci A, Calvo M, Wyn-Jones P, and Girones R.** Quantification of human adenoviruses in European recreational waters. Food and Environmental Virology, 2010, (2):101–109.

4. **Calgua B, Fumian T, Rusinol M, Rodriguez-Manzano J, Mbayed V, Bofill-Mas S, Miagostovich M, and Girones R.** Detection and quantification of classical and emerging viruses in river water by applying a low cost one-step procedure. Water Research, 2013, 47, 2797 - 2810.

5. **Cantalupo PG, Calgua B, Zhao G, Hundesa A, Wier AD, Katz JP, Grabe M, Hendrix RW, Girones R, Wang D, and Pipas JM.** Raw sewage harbors diverse viral populations. MBio, 2011, 2(5). pii: e00180-11.

Universitat de Barcelona

Informe sobre la participación del doctorando en los artículos presentados.

Calgua B, Mengewein A, Grunert A, Bofill-Mas S, Clemente-Casares P, Hundesa A, Wyn-Jones AP, López-Pila JM, and Girones R. Development and application of a one-step low cost procedure to concentrate viruses from seawater samples. Journal of Virological Methods, 2008, 153(2):79-83.

Estos resultados son parte de los objetivos del proyecto Europeo VIROBATHE. El doctorando participó activamente en el desarrollo y aplicación de la metodología descrita en la publicación. El trabajo consistió en la comparación y evaluación de diferentes metodologías para la concentración de virus en aguas recreacionales, en evaluar diferentes metodologías para la extracción de ácidos nucleicos, y en la detección y cuantificación de virus por ensayos moleculares. Como primer autor, el doctorando participó en los análisis de los resultados y la elaboración del manuscrito. El trabajo fue planteando y dirigido por la directora de tesis.

Calgua B, Barardi CR, Bofill-Mas S, Rodriguez-Manzano J, and Girones R. Detection and quantitation of infectious human adenoviruses and JC polyomaviruses in water by immunofluorescence assay. Journal of Virological Methods, 2011, 171(1):1-7.

Como primer autor, el doctorando tuvo a su cargo los principales experimentos relacionados con el diseño, desarrollo y aplicación de la metodología propuesta, así como también el análisis de los resultados y la elaboración del manuscrito. El trabajo del doctorando, incluyó la evaluación de diferentes condiciones para la detección de virus infecciosos presentes en diferentes matrices. Todo el trabajo fue supervisado por la directora de tesis.

Bofill-Mas S, Calgua B, Clemente-Casares P, la Rosa G, Iaconelli M, Muscillo M, Rutjes S, de Roda Husman AM, Grunert A, Gräber I, Verani M, Carducci A, Calvo M, Wyn-Jones P, and Girones R. Quantification of human adenoviruses in European recreational waters. Food and Environmental Virology, 2010, 2:101–109.

El trabajo es parte del proyecto Europeo VIROBATHE, en el manuscrito se describe la ocurrencia de adenovirus humanos en diferentes muestras de aguas recreacionales de Europa. El doctorando desarrollo los experimentos de concentración, detección y cuantificación de los adenovirus humanos en las muestras de mar procedentes de Barcelona. También participó activamente en la cuantificación de los virus en el resto de muestras conjuntamente con la primera autora. Además colaboró en la elaboración del manuscrito y el análisis de los

resultados. Los demás autores pertenecientes a otras instituciones Europeas tuvieron a su cargo entre otras cosas la concentración de los virus en aguas recreacionales, análisis e interpretación de los resultados y revisión del manuscrito. El trabajo fue dirigido por la directora de tesis.

Calgua B, Fumian T, Rusinol M, Rodriguez-Manzano J, Mbayed V, Bofill-Mas S, Miagostovich M, and Girones R. Detection and quantification of classical and emerging viruses in river water by applying a low cost one-step procedure. Water Research, 2013, 47, 2797 - 2810.

Bajo la supervisión de su directora de tesis el doctorando diseñó los estudios para la puesta a punto de la metodología para la concentración de virus en agua de río y coordinó los ensayos inter-laboratorio para la validación de la metodología. También describió las técnicas moleculares para la detección de los virus nuevos, Klassevirus y el Asfavirus-*like* en muestras ambientales. Como primer autor tuvo a su cargo la realización de la mayoría de los experimentos realizados en el laboratorio de la Universidad de Barcelona, el análisis de los resultados y la elaboración del manuscrito.

Cantalupo PG, Calgua B, Zhao G, Hundesa A, Wier AD, Katz JP, Grabe M, Hendrix RW, Girones R, Wang D, and Pipas JM. Raw sewage harbors diverse viral populations. MBio, 2011, 2(5). pii: e00180-11.

El doctorando formó parte del equipo de Barcelona que participó en el estudio dirigido por el Prof. Jim Pipas. Bajo la supervisión de la directora de tesis, el doctorando trabajo en el desarrollo del método de concentración, así como también realizó el análisis de virus con ensayos convencionales de PCR. Además participó activamente en el análisis y discusión de los resultados y también en la revisión y escritura del manuscrito.

Ninguno de los coautores de los manuscritos ha utilizado los datos descritos en estas publicaciones para la elaboración de una *Tesis Doctoral*.

Firmado,

Prof. Rosina Girones Llop

Barcelona, Julio 2013

Universitat de Barcelona

Informe sobre el factor de impacto de los artículos presentado en la *Tesis Doctoral.*

Las publicaciones presentadas por Byron T. Calgua de León en su *Tesis Doctoral* han sido publicadas en revista científicas internacionales relevantes en la línea de investigación en que se ha participado.

Los artículos "***Development and application of a one-step low cost procedure to concentrate viruses from seawater samples***" y "***Detection and quantitation of infectious human adenoviruses and JC polyomaviruses in water by immunofluorescence assay***" fueron publicados en ***Journal of Virological Methods*** en los años 2008 y 2011, presentando las revistas un índice de impacto de 1,93 y 2,01, respectivamente. El artículo ***"Quantification of human adenoviruses in European recreational waters"*** fue publicado en ***Food and Environmental Virology*** en el 2010, año en el cual la revista presentó un índice de impacto de 1,38. El artículo ***"Raw sewage harbors diverse viral populations"*** fue publicado en MBio en el año 2011, con un índice de impacto de 5,31. El último artículo "***Detection and quantification of classical and emerging viruses in river water by applying a low cost one-step procedure***" fue publicado en el 2013 en ***Water Research*** con un índice de impacto de 4,65.

Firmado,

Prof. Rosina Girones Llop

Barcelona, Julio del 2013.

CAPÍTULOS

4. CAPÍTULO I

Desarrollo de nuevas metodologías para el estudio de virus en agua

Resumen Estudio 1:

Development and application of a one-step low cost procedure to concentrate viruses from seawater samples.

Calgua B, Mengewein A, Grunert A, Bofill-Mas S, Clemente-Casares P, Hundesa A, Wyn-Jones AP, López-Pila JM, and Girones R.

Journal of Virological Methods, 2008, 153(2):79-83.

RESUMEN:

La contaminación fecal en el agua costera representa un alto riesgo para la salud humana y por lo tanto es una prioridad de interés en salud pública. Los virus patógenos contaminantes del agua pueden infectar a población que realiza actividades recreacionales o durante el consumo de moluscos bivalvos.

Este estudio describe parte de los resultados obtenidos en el proyecto Europeo VIROBATHE (*Methods for the detection of adenoviruses and noroviruses in European bathing waters*, www.virobathe.org). Entre los objetivos de este proyecto se encontraba el establecimiento de protocolos para la concentración y detección de virus entéricos en agua recreacional de río, lago y marina. En este trabajo se describe una metodología nueva para concentrar virus presentes en 10 L de agua de mar. Este protocolo *one-step* está basado en la floculación orgánica de leche descremada bajo condiciones de pH ácido (SMFP). Bajo estas condiciones los virus se adsorben a los flóculos de leche, posteriormente los flóculos con los virus adsorbidos precipitan por gravedad y finalmente los flóculos con los virus son recuperados y concentrados en *buffer* fosfato. Los virus concentrados pueden ser detectados por técnicas de PCR y cultivo celular.

El protocolo SMFP fue validado en un ensayo inter-laboratorio con muestras de mar previamente contaminadas con adenovirus humanos (HAdV). Los resultados mostraron que aproximadamente un 50% de los virus fueron recuperados empleando esta metodología *one-step*. Entre los objetivos de VIROBATHE, también se incluía un programa de vigilancia para monitorizar la calidad microbiología del agua en diferentes áreas con usos recreacionales. En la presente publicación se describen los resultados obtenidos en

una temporada de baño en un área de la zona costera de Barcelona. Con SMFP se detectaron por PCR anidada (nPCR), HAdV en 7 de 50 muestras analizadas. En estas muestras se identificaron HAdV 41 y 31, además se confirmó por ensayos de cultivo celular que una muestra contenía HAdV 31 infeccioso. De las muestras positivas por nPCR, cuatro se analizaron por qPCR TaqMan®, obteniéndose valores de $1{,}26x10^3$ GC/L ($1{,}96x10^1$ -$3{,}48x10^3$ GC/L). Los valores de los indicadores bacteriológicos, *E. coli* (EC) y enterococos intestinales (EI), en muestras positivas para HAdV cumplían con los descritos en la directiva de aguas recreacionales vigente (2006/07/CE).

Parte de los resultados obtenidos en el estudio, no incluidos en la publicación, se describen en el Anexo1. Esta información se corresponde con los ensayos inter-laboratorio realizados dentro de los objetivos de VIROBATHE, para la evaluación de métodos de concentración de virus en agua recreacional. En los ensayos inter-laboratorio se compararon 3 métodos *two-step* o VIRADEL (Anexo I), dos empleando en un primer paso de concentración una membrana de nitrocelulosa electronegativa y uno lana de vidrio, seguido de un proceso de elución de los virus con un *buffer* alcalino con glicina y extracto de carne o leche descremada y finalmente una segunda concentración a través de una floculación orgánica bajo condiciones de pH ácido, protocolos basado en los trabajos de Bitton *et al.* (1979), Katzenelson *et al*., 1976, Villaginès *et al.* (1993) y Wallis y Melnick (1967).

Según los resultados obtenidos, SMFP es un procedimiento fácil de implementar y estandarizar que no requiere equipo especializado, permite procesar varias muestras simultáneamente y es eficiente cuando se concentran HAdV en agua de mar. La aplicabilidad de SMFP fue demostrada en el estudio de adenovirus en agua de mar de una playa recreacional. En este estudio los valores de los indicadores bacteriológicos, EC y EI, en muestras positivas para HAdV cumplían con los descritos en la directiva de agua de baño vigente en el momento del estudio (EC:< 15 – 61/100 mL y EI:<15 – 77/100 mL), reafirmando que los actuales indicadores bacterianos de contaminación fecal no siempre se correlacionan con la presencia de virus en el medio ambiente y que con las normativa actual se podría estar infravalorando el riesgo de la salud de los bañistas por la presencia de patógenos como los virus.

Journal of Virological Methods 153 (2008) 79–83

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Development and application of a one-step low cost procedure to concentrate viruses from seawater samples

B. Calgua[a], A. Mengewein[b], A. Grunert[b], S. Bofill-Mas[a], P. Clemente-Casares[a], A. Hundesa[a], A.P. Wyn-Jones[c], J.M. López-Pila[b], R. Girones[a,*]

[a] *Department of Microbiology, Faculty of Biology, University of Barcelona, Av. Diagonal 645, 08028 Barcelona, Spain*
[b] *German Environmental Agency (Umweltbundesamt), WörlitzerPlatz 1, 06844 Dessau-Roßlau, Berlin, Germany*
[c] *I.G.E.S., University of Aberystwyth, Aberystwyth SY23 3DB, Wales, UK*

Article history:
Received 9 May 2008
Received in revised form 31 July 2008
Accepted 6 August 2008

Keywords:
Adenovirus
Seawater
Flocculation
Concentration method
QPCR

A B S T R A C T

A novel and simple procedure for concentrating adenoviruses from seawater samples is described. The technique entails the adsorption of viruses to pre-flocculated skimmed milk proteins, allowing the flocs to sediment by gravity, and dissolving the separated sediment in phosphate buffer. Concentrated virus may be detected by PCR techniques following nucleic acid extraction. The method requires no specialized equipment other than that usually available in routine public health laboratories, and due to its straightforwardness it allows the processing of a larger number of water samples simultaneously. The usefulness of the method was demonstrated in concentration of virus in multiple seawater samples during a survey of adenoviruses in coastal waters.

1. Introduction

Surveillance of coastal waters, for recreation, cultivation of shellfish, or other activities with relevance for human health, includes monitoring for faecal pollution. While any pollution might pose a health risk for humans, faecal pollution from human sources presents a particular hazard since it might contain pathogens that specifically infect humans. Although detection of the conventional faecal pollution indicators, *E. coli* and intestinal enterococci, is straightforward, occurrence of bacterial indicators does not necessarily correlate with the presence of viral pathogens that are more stable than bacteria in the environment, nor do bacterial indicators provide information on the potential origin of the contamination. Several workers have reported substantial levels of pathogenic viruses in bathing waters complying with local public health regulations (Gerba et al., 1979; Goyal et al., 1984; Griffin et al., 1999; Jiang et al., 2001; Noble and Fuhrman, 2001). In addition, epidemiological studies carried out to estimate the health risk of swimming in bathing waters have suggested that the gastroenteritis burden of bathers which is attributable to the presence of viruses is detected at concentrations of bacterial indicators well below statutory standards (Griffin et al., 2003; Lipp et al., 2001).

There is therefore a public health requirement for additional parameters that indicate the presence of viruses in bathing waters and shellfish-growing areas more reliably. Human adenoviruses (HAdV) have been proposed to fill this gap both as indicators and as source tracking organisms (Fong and Lipp, 2005; Hundesa et al., 2006), since several studies have reported that HAdV have a high stability under environmental stress, such as UV radiation, temperature, chlorine concentration and pH variation, including sewage treatment procedures (Carter, 2005; Fong and Lipp, 2005; Pina et al., 1998). The levels of HAdV found in sewage match the numbers of the usual faecal indicator *E. coli* (EC), and might outnumber intestinal enterococci (IC) (Bofill-Mas et al., 2006; Wolf, 2005). Therefore the development of a simple efficient method for the concentration and quantitation of HAdV is of high interest in respect of indicators, of instruments for source tracking of faecal pollution, and for carrying out risk assessment studies of polluted coastal waters, which, in the European Union, comprise more than 14,200 out of about 21,000 registered bathing water sites.

Detection of viruses in low or moderately polluted waters calls for the concentration of the viruses from at least several liters of water into a much smaller volume (typically 1000-fold concentration), a procedure that usually includes two consecutive concentration steps (1) electrostatic adsorption of the virus

* Corresponding author. Tel.: +34 93 4039043; fax: +34 93 4039047.
E-mail address: rgirones@ub.edu (R. Girones).

0166-0934/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.jviromet.2008.08.003

particles to a solid matrix followed by their elution with an alkaline proteinaceous solution (eluant, usually skimmed milk or beef extract solution), and (2) precipitation of the proteins to which any viruses are adsorbed from the eluant by lowering the pH ("organic flocculation"), and resuspending the flocculated protein in a small volume of neutral buffer. This somewhat cumbersome procedure hampers significantly larger scale virus-detection programs, not least because of its many steps and the need for several pH adjustments of the sample during concentration.

Following a surveillance investigation for detecting viruses in bathing waters (Virobathe, www.virobathe.org) was observed that two-step procedures worked satisfactorily for freshwaters whereas their performance in recovering HAdV from coastal waters was less satisfactory.

A one-step protocol for the concentration of viruses from coastal waters was therefore developed based on the direct binding of the viruses to pre-flocculated skimmed milk proteins, thereby reducing the number of processing steps. The procedure described do not require the availability of specific equipment as filters or pressure pumps, commonly used for virus concentration from water, and represents a significant reduction in the cost of the assays. In this study details of this protocol are reported and its performance on a large scale study.

2. Materials and methods

2.1. Virus and cell lines

Adenovirus Type 2 NCPV #00213 was used in this study as a positive control of the process. The virus titre was approximately $10^{4.3}$ PFU mL^{-1}. The cell line A549 (ECACC, UK) was used to propagate the virus. A549 cells were grown in 175 cm^2 flasks containing MEM medium (Life Technologies Ltd., Paisley, Scotland) supplemented with 10% (growth medium) or 2% (maintenance medium) heat inactivated foetal bovine serum (Life Technologies Ltd., Paisley, Scotland) and 100 units mL^{-1} penicillin, 10 mg mL^{-1} streptomycin and 2.5 μg mL^{-1} amphotericin B (Fungizone, Life Technologies Ltd., Paisley, Scotland).

2.2. Water samples

Three water types were used in this study. (i) Tap water from the metropolitan area of Berlin, Germany, seeded with raw strained wastewater to which was added, where indicated, artificial sea salts. (ii) Artificial seawater, prepared by adding 33.33 g artificial sea salts (Sigma, Aldrich Chemie GMBH, Steinheim, Germany), per liter of a 1:1 (v/v) mix of distilled and tap water from metropolitan area of Barcelona, which had been treated in a conventional municipal treatment plant. The mix was stirred until the salts were completely dissolved. (iii) Natural seawater from one sampling point in the coast near of metropolitan area of Barcelona collected at least at six meters from the shore and one meter from the surface.

The collection of samples for field studies was carried out based according to ISO 19458 (2006) for the collection of water samples for microbiological analysis. Samples were stored for a maximum of 24 h at 4 °C before being processed.

2.3. Virus concentration by glass wool filtration

Ten liters of either tap water or artificial seawater were spiked with viruses (stock of adenoviruses or strained municipal raw wastewater), and concentrated by a glass wool column based in the study described by Vilaginès et al. (1997). Briefly, viruses in 10 L-water samples were concentrated by adsorption at pH 3.5 to glass wool filters and eluted with beef extract/glycine buffer at pH 9.5 followed by organic flocculation at pH 4.5. The flocs were pelleted by centrifugation at 7000 × *g* for 30 min and the pellet was resuspended in 10 mL of PBS. Nucleic extraction was performed on this suspension as described in Section 2.6.

2.4. Virus concentration by skimmed milk (SM) flocculation procedure

Pre-flocculated skimmed milk solution (1% (w/v)) was prepared by dissolving 10 g skimmed milk powder (Difco) in 1 L artificial seawater and carefully adjusting the pH to 3.5 with 1 N HCl. One hundred mL of this solution were added to each of the previously acidified (pH 3.5) 10 L seawater samples (final concentration of skimmed milk 0.01% (w/v)). Samples were stirred for 8 h at room temperature and flocs were allowed to sediment by gravity for another 8 h. Supernatants were carefully removed using a vacuum pump without disturbing the sediment. The final volume of about 500 mL containing the sediment was transferred to a centrifuge pot and centrifuged at 7000 × *g* for 30 min at 12 °C. Aliquots of the supernatants which had been suctioned previously were used to balance the pots. The supernatant was carefully removed and the pellet resuspended in 8 mL of 0.2 M phosphate buffer at pH 7.5 (1:2, v/v of Na_2HPO_4 0.2 M and NaH_2PO_4 0.2 M). Once the pellet was completely dissolved, phosphate buffer was added to a final volume of 10 mL. The concentrate was stored at −20 °C.

2.5. Detection of bacterial indicators and somatic coliphages

The detection of IC, EC and somatic coliphages was carried out according to ISO 7899-1 (1998), ISO 9308-3 (1998) and ISO 10705-2 (2000), respectively. The most probable number (MPN) technique was used to estimate the number of IC and EC/100 mL of sample according to standard methods and a plaque assay was used to estimate the concentration of somatic phages.

2.6. Nucleic acids extraction from viral concentrates

The NA extraction was performed with a NucleoSpin® RNA Virus F kit (Machery & Nagel, Dueren, Germany), using 1 mL of the viral concentrate, NA being finally eluted in 100 μL of elution buffer, or with a QIAamp Viral RNA kit (Qiagen), extracting 200 μL of viral concentrate in 80 μL of elution buffer. NA extracts were stored at −20 °C until analyzed by PCR.

2.7. Quantitation and detection of HAdV

Quantitation of HAdV genomes by QPCR was based on the assay previously described by Hernroth et al. (2002), which had been previously applied to the detection and quantitation of adenoviruses in shellfish samples (Formiga-Cruz et al., 2002), in river and drinking water (Albinana-Gimenez et al., 2006), in urban sewage, sludge and biosolids (Bofill-Mas et al., 2006). The method is based in a TaqMan® assay and uses two primers and a fluorogenic probe that recognize a fragment of the hexon gene of the HAdV genome. Amplification was performed in a 25-μL reaction mixture containing 10 μL of DNA and 12.5 μL of TaqMan® Universal PCR Master Mix, 0.9 μM of each primer (AdF and AdR) and 0.225 μM of fluorogenic probe AdP1. TaqMan® Universal PCR Master Mix was supplied 2× concentrated and contained AmpliTaq Gold® DNA polymerase, dNTPs with dUTP, optimized buffer components and AmpErase® uracil-*N*-glycosylase. Following activation of the uracil-*N*-glycosylase (2 min, 50 °C) and activation of the AmpliTaq Gold for 10 min at 95 °C, 40 amplification cycles (15 s at 95 °C and 1 min at 60 °C) were performed with an ABI 7700 detector system (Applied Biosystems).

Undiluted and 10-fold dilutions of the extracted DNA were run in duplicate (4 runs/sample). In all quantitative PCRs carried out the amount of DNA was defined as the mean of the data obtained. A non-template control and an amplification control were included in each run. The data produced by QPCR presented low variability in the diverse replicates. Significant variability was only observed in the results of a few undiluted samples probably due to the presence of inhibitors in the reaction, being these values excluded of the quantitation. The detection of HAdV genomes by nested PCR (nPCR) was based on studies by Allard et al. (2001), which has been previously applied to the detection of HAdV in urban sewage and shellfish (Bofill-Mas et al., 2000; Formiga-Cruz et al., 2002, 2005) and in slaughterhouse, river and drinking water (Albinana-Gimenez et al., 2006; Hundesa et al., 2006). For the specific amplification of HAdV genomes, 10 μL aliquots of the extracted nucleic acid were amplified by using a conventional nPCR test for amplifying a region of the HAdV hexon gene.

2.8. Sequencing of PCR products

Products obtained after nPCR were purified with the QIAquick PCR purification kit (Qiagen). 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 (PerkinElmer, 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 3.2 μM concentration. The results were checked using the ABI PRISM 3730 XL automated sequencer (PerkinElmer, Applied Biosystems).

2.9. Quality control of the amplification methods

To reduce the probability of sample contamination by amplified DNA molecules, standard precautions were applied in all manipulations. Separate areas of the laboratory were used for reagents, treatment of samples, and manipulation of amplification products. All assays included negative controls. In all amplifications the mixture included uracil–DNA–glycosylase (UDG) (Invitrogen Corp., Carlsbad, USA) for degradation of amplified material that could contaminate the samples. Undiluted and a 10-fold dilution of the nucleic acid extract were analyzed in order to avoid false negatives because inhibition of the reactions.

3. Results

3.1. Kinetics of adsorption of HAdV to flocculated skimmed milk

Two samples of 500 mL of artificial seawater sample was spiked with adenovirus type 2, and concentrated according to the SM-flocculation procedure. During stirring, six aliquots were collected at different times and the NA in each concentrate was extracted and analyzed by QPCR to determine the HAdV genome concentration. Fig. 1 shows that after 5 h the adsorption is essentially complete.

3.2. Adenovirus recovery after concentration of natural seawater and artificial seawater samples by applying the SM-flocculation procedure. Comparison with the two-step concentration procedure with glass wool and organic flocculation

Three different sets of 10 L-samples were used in these experiments. (i) Five artificial seawater samples were each seeded with HAdV containing 4.04×10^4 GC. (ii) Four natural seawater samples were each seeded with HAdV containing 6.91×10^4 HAdV GC. (iii)

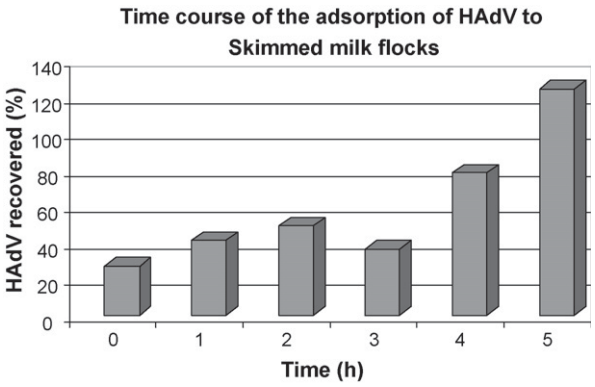


Fig. 1. Kinetics of the adsorption of HAdV to flocculated skimmed milk.

Five natural seawater samples were each seeded with HAdV containing 4.88×10^4 HAdV GC. All samples were concentrated using the SM-flocculation procedure and the concentrates were analyzed by QPCR. Mean values of viral recovery were 52%, 42% and 49%, respectively (Table 1). The NA extractions were preformed with QIAamp Viral RNA kit (Qiagen) for the samples (i) and (ii), and NucleoSpin® RNA Virus F kit (Machery & Nagel, Dueren, Germany) for samples (iii).

For comparison of the SM-flocculation procedure with the two-step procedure, 10 samples of 10 L of artificial seawater were spiked with a suspension of 3.87×10^5 HAdV GC and concentrated them by the glass wool method followed by organic flocculation. HAdV recoveries in the final concentrates were estimated by QPCR after NA extraction with QIAamp Viral RNA kit (Qiagen). The recovery obtained was 0.77% ($(0–3.34) \times 10^3$ GC/10 L).

3.3. Field study

During the summer 2006 bathing season (June–September), 50 samples collected in the coast of the metropolitan area of Barcelona were analyzed by applying the SM-flocculation procedure. The nucleic acid extraction procedure applied was NucleoSpin® virus F kit (Macherey & Nagel) because only low levels of inhibitors were detected. The levels of inhibitors present in the final QPCR assays were evaluated by two procedures. One has been testing decimal dilutions of the spiked samples with HAdV and analyzing the quantity provided by the undiluted and diluted samples. The inhibition was identified if significant differences were observed in the concentration estimated in the respective dilution. Also low concentrations (10^3 and 10^5 GC) of the plasmid used as standard

Table 1
Recovery values of adenoviruses in seawater analyzed by SM-flocculation method

Sample ID	HAdV added GC/10 L	HadV recovered GC/10 L	Mean recovery (%)
AS1[a]	4.04×10^4	1.20×10^4	51.98
AS2[a]		3.53×10^4	
AS3[a]		1.55×10^4	
AS4[a]		2.73×10^4	
AS5[a]		1.49×10^4	
NS1[b]	6.91×10^4	3.33×10^4	42.53
NS2[b]		4.55×10^4	
NS3[b]		3.28×10^4	
NS4[b]		2.56×10^4	
NS5[b]	4.88×10^4	1.68×10^4	49.33
NS6[b]		2.70×10^4	
NS7[b]		4.08×10^4	
NS8[b]		2.16×10^4	
NS9[b]		1.42×10^4	

[a] AS: artificial seawater.
[b] NS: natural seawater.

in QPCR assays were added to three representative samples in the QPCR reaction. The selected procedure for the field studies in the sampling site analyzed in this study was NucleoSpin® RNA Virus F kit (Machery & Nagel, Dueren, Germany) allowing to concentrate 1 mL of the viral concentrate in 100 μL of extracted NA. However seawater analyzed in other sampling sites presented higher concentration of inhibitors for the QPCR reactions and extraction of 200 mL of the viral concentrate using QIAamp viral RNA kit showed to be more efficient (data not shown). The volume of the viral concentrate tested in one PCR assay would correspond to 200 mL of the seawater sample. The samples were collected on 13 different sampling days (3–4 samples per day), two of them after rainfall events. In each sampling day, an extra sample was collected as a control of the process, theses samples were spiked with a suspension of adenoviruses and concentrated under the same conditions as the field samples. Artificial seawater samples were tested as negative control of methodology in each sampling day.

The viral concentrates obtained were analyzed by nPCR and positive samples were further analyzed by QPCR and ICC-PCR. Six of seven positive samples were confirmed by sequencing. The levels of bacteriological indicators (IC and EC) also were tested on each sampling day.

From a total of 50 samples tested for HAdV 7 samples were positive for HAdV using nPCR. Six samples were confirmed by sequencing: 4 samples were identified as HAdV 41 and 2 samples were identified as HAdV 31 with between 90 and 100% of homology with GenBank/EMBL databases entries. One of the positive samples was confirmed to contain infectious HAdV 31 as tested by ICC-PCR (Reynolds et al., 1996). Moreover, 4 samples were quantified by QPCR obtaining mean values of 1.26×10^4 GC/10 L (1.96×10^2 to 3.48×10^4 GC/10 L). The levels of bacteriological indicators were high in the samples collected after rainfall event (mean values/100 mL EC: 1.21×10^4 and IC: 6.25×10^3). However in samples collected during dry weather and found positive for HAdV the values for EC and IC complied with the actual directive for quality of bathing waters (EC: <15–61/100 mL and IC: <15–77/100 mL). The turbidity in the samples was also measured (Hach Ratio/XR Turbidimeter) in each sampling day. The values detected were 1.56–28.00 NTU in normal days and 103.00 NTU in samples collected after rainfall event.

3.4. Recovery rates of somatic coliphages from tap water and artificial seawater

High concentrations of somatic coliphages are present in urban sewage and can be easily quantified by PFU (Plaque Forming Units) without the use of concentration protocols, for this reason somatic coliphages where used as a model to evaluate the concentration process in fresh and seawater. Ten samples of 9 L of either tap water or artificial seawater were seeded with 1 L of strained municipal raw wastewater. A total of 10 L of sample was immediately concentrated by glass wool filtration (first step) and organic flocculation (second step). The somatic coliphages titre was determined before and after concentration by plaque assay. In the experiments using tap water, the glass wool retained 85% of the phage; however, it retained only 27% of the phage when artificial seawater was used. Recovery values represent the difference between the phage titre after concentration of the samples and the titre detected in the samples before filtration takes place.

4. Discussion

Due to the poor recovery observed when evaluating the retention of HAdV and somatic coliphages in seawater by a two-step procedure, a new methodology for viral concentration in seawater samples has been explored in the present study. The recovery of the one-step procedure was determined to be 52% and 42–49% using QPCR assays for adenoviruses in artificial seawater and natural seawater samples respectively, whereas the glass wool filtration procedure yielded a much poorer recovery of 0.77%. Its poor performance in seawater seems to be a general phenomenon concerning many viruses as shown in the present study, the overall recovery of the heterogeneous somatic phage population present in wastewater also decreased in seawater. This effect is also in agreement with previous studies described by Lukasik et al. (2000). Apparently, the attachment of the viruses to the matrices, which in the first step is of electrostatic nature, is hampered by a high ionic strength and this would explain the low efficiency observed of adsorption-elution methods for the concentration of viruses from seawater (Lukasik et al., 2000). Experiments conducted to determine the time necessary for adenoviruses present in spiked samples to attach to skimmed milk flocs, indicate, as shown Fig. 1, that approximately 5 h of stirring are needed for full attachment. However 8 h to 10 h of stirring are recommended to guarantee a maximum adsorption of viruses, permitting also process the samples overnight.

In preliminary studies for the selection of the nucleic acid extraction protocol diverse NA extraction kits were tested on viral suspensions obtained from cell culture supernatants and viral suspension in seawater with and without skimmed milk. The results indicate that presence of skimmed milk did not produce inhibition for the QPCR assay when using the two described nucleic acid extraction protocols (data not shown). The one-step procedure was evaluated further in a field study carried out in a coastal area near the metropolitan area of Barcelona. Using the developed method adenoviruses were quantified in samples with standard indicators complying with the Directive 2006/07/EC for quality of bathing waters and also in samples collected in rainfall days presenting higher values of bacterial standards. Turbidity was measured and viruses were detected in days presenting the higher turbidity levels 103 NTU but also in days showing the lower levels of turbidity (1.56 NTU). The relevance of these results is that the procedure is a useful tool for detecting contaminant HAdV, even in cases when the bathing site complies with the actual regulation for presence of bacteriological indicators. According to these results, the procedure described in present study would fulfil the conditions for a fitting method for routine public health laboratories: reproducible, reliability, straightforwardness and cost-effectiveness.

Acknowledgments

'This work was partially supported by the EC Contract 513648, Project VIROBATHE; the "Xarxa de Referència de Biotecnologia de Catalunya and the Grup de Recerca Consolidat de la Generalitat de Catalunya 2005SGR00592. We thank David Kay for his contributions in the organization of the project and Jane Sellwood for supplying standard materials in the study. During the development of this study Byron Calgua was a fellow of the MAEC-ECID, Spanish Government (Ministerio de Asuntos Exteriores y Cooperación). We thank the Serveis Científico-Tècnics of the University of Barcelona for the very efficient sequencing services.

References

Albinana-Gimenez, N., Clemente-Casares, P., Bofill-Mas, S., Hundesa, A., Ribas, F., Girones, R., 2006. Distribution of human polyomaviruses, adenoviruses, and hepatitis E virus in the environment and in a drinking-water treatment plant. Environ. Sci. Technol. 23, 7416–7422.

Allard, A., Albinsson, B., Wadell, G., 2001. Rapid typing of human adenoviruses by a general PCR combined with restriction endonuclease analysis. J. Clin. Microbiol. 39, 498–505.

Bofill-Mas, S., Pina, S., Girones, R., 2000. Documenting the epidemiologic patterns of polyomaviruses in human populations by studying their presence in urban sewage. Appl. Environ. Microbiol. 66, 238–245.

Bofill-Mas, S., Albinana-Gimenez, N., Clemente-Casares, P., Hundesa, A., Rodriguez-Manzano, J., Allard, A., Calvo, M., Girones, R., 2006. Quantitation and stability of human adenoviruses and Polyomavirus JCPyV in wastewater matrices. Appl. Environ. Microbiol. 72, 7894–7896.

Carter, M.J., 2005. Enterically infecting viruses: pathogenicity, transmission and significance for food and waterborne infection. J. Appl. Microbiol. 6, 1354–1380.

Fong, T., Lipp, E.K., 2005. Enteric viruses of humans and animals in aquatic environmental: health risks, detection, and potential water quality assessment tools. Microbiol. Mol. Biol. Rev. 69, 357–371.

Formiga-Cruz, M., Tofiño-Quesada, G., Bofill-Mas, S., Lees, D.N., Henshilwood, K., Allard, A.K., Condin-Hansson, A.-C., Hernroth, B.E., Vantarakis, A., Tsibouxi, A., Papapetropoulou, M., Furones, M.D., Girones, R., 2002. Distribution of human viral contamination in shellfish from different growing areas in Greece, Spain, Sweden and the United Kingdom. Appl. Environ. Microbiol. 68, 5990–5998.

Formiga-Cruz, M., Hundesa, A., Clemente-Casares, P., Albiñana-Gimenez, N., Allard, A., Girones, R., 2005. Nested multiplex PCR assay for detection of human enteric viruses in shellfish and sewage. J. Virol. Methods 125, 111–118.

Gerba, C.P., Goyal, S.M., LaBelle, R.L., Cech, I., Bodgan, G.F., 1979. Failure of indicator bacteria to reflect the occurrence of enteroviruses in marine waters. Am. J. Public Health 69, 1116–1119.

Goyal, S.M., Adams, W.N., O'Malley, M.L., Lear, D.W., 1984. Human pathogenic viruses at sewage sludge disposal sites in the Middle Atlantic region. Appl. Environ. Microbiol. 48, 758–763.

Griffin, D.W., Gibson, C.J., Lipp, E.K., Riley, K., Paul, J.H., Rose, J.B., 1999. Detection of viral pathogens by reverse transcriptase PCR and of microbial indicators by standard methods in the canals of the Florida Keys. Appl. Environ. Microbiol. 65, 4118–4125.

Griffin, D.W., Donaldson, K.A., Paul, J.H., Rose, J.B., 2003. Pathogenic human viruses in coastal waters. Clin. Microbiol. Rev. 16, 129–143.

Hernroth, B.E., Conden-Hansson, A.C., Rehnstam-Holm, A.S., Girones, R., Allard, A.K., 2002. Environmental factors influencing human viral pathogens and their potential indicator organisms in the blue mussel, *Mytilus edulis*: the first Scandinavian report. Appl. Environ. Microbiol. 68, 4523–4533.

Hundesa, A., Maluquer de Motes, C., Bofill-Mas, S., Albinana-Gimenez, N., Girones, R., 2006. Identification of human and animal adenoviruses and polyomaviruses for determination of sources of faecal contamination in the environment. Appl. Environ. Microbiol. 72, 7886–7893.

ISO 7899-1, 1998. Water quality—detection and enumeration of intestinal enterococci. Part 1. Miniaturized method (Most Probable Number) for surface and waste water.

ISO 9308-3, 1998. Water quality—detection and enumeration of *Escherichia coli* and coliform bacteria. Part 3. Miniaturized method (Most Probable Number) for the detection and enumeration of *E. coli* in surface and waste water.

ISO 10705-2, 2000. Water quality—detection and enumeration of bacteriophages. Part 2. Enumeration of somatic coliphages. International Organization for Standarization, Geneva, Switzerland.

ISO 19458, 2006. Water quality—Sampling for microbiological analysis. Guidance on planning water sampling regimes, on sampling procedures for microbiological analysis and on transport, handling and storage of samples until analysis begins.

Jiang, S., Noble, R., Chui, W.P., 2001. Human adenoviruses and coliphages in urban runoff-impacted coastal waters of Southern California. Appl. Environ. Microbiol. 67, 179–184.

Lipp, E.K., Jarrell, J.L., Griffin, D.W., Lukasik, J., Jacukiewicz, J., Rose, J.B., 2001. Preliminary evidence for human faecal contamination in corals of the Florida Keys. Mar. Pollut. Bull. 44, 666–670.

Lukasik, J., Scott, T.M., Andryshak, D., Farrah, S.R., 2000. Influence of salts on virus adsorption to microporous filters. Appl. Environ. Microbiol. 66, 2914–2920.

Noble, R.T., Fuhrman, J.A., 2001. Enteroviruses detected by reverse transcriptase polymerase chain reaction from the coastal waters of Santa Monica Bay, California: low correlation to bacterial indicator levels. Hydrobiologia 460, 175–184.

Pina, S., Puig, M., Lucena, F., Cofre, J., Girones, R., 1998. Viral pollution in the environment and in shellfish: human adenovirus detection by PCR as an index of human viruses. Appl. Environ. Microbiol. 64, 3376–3382.

Reynolds, K.A., Gerba, C.P., Pepper, I.L., 1996. Detection of infectious enteroviruses by an integrated cell culture-PCR technique. Appl. Environ. Microbiol. 62, 1424–1427.

Vilaginès, P., Sarrette, B., Champsaur, H., Hugues, B., Dubrou, S., Joret, J.-C., Laveran, H., Lesne, J., Paquin, J.L., Delattre, J.M., Oger, C., Alame, J., Grateloup, I., Perrollet, H., Serceau, R., Sinègre, F., Vilaginès, R., 1997. Round robin investigation of glass wool method for poliovirus recovery from drinking water and sea water. Water Sci. Technol. 35, 445–449.

Wolf, S., 2005, Evaluierung der hygienischen wasserqualität unter besonderer berücksichtigung von bakteriophagen am beispiel eines tagebausees dissertation, University of Dresden, Germany.

Resumen Estudio 2:

Detection and quantitation of infectious human adenoviruses and JC polyomaviruses in water by immunofluorescence assay.

Calgua B, Barardi CR, Bofill-Mas S, Rodriguez-Manzano J, and Girones R.

Journal of Virological Methods, 2011, 171(1):1-7.

RESUMEN:

Los adenovirus humanos (HAdV) y polyomavirus JC (JCPyV) han sido propuestos como una nueva generación de indicadores víricos de contaminación fecal humana en el medio ambiente. Actualmente las técnicas basadas en ensayos de PCR, como PCR anidada (nPCR) y PCR cuantitativa (qPCR) han mostrado ser ensayos sensibles y rápidos para la detección de virus presentes en el medio ambiente. Sin embargo estas técnicas no permiten discriminar entre virus infecciosos y no infecciosos y en algunos estudios específicos es imprescindible determinar la infectividad de los virus detectados. Los ensayos de $TCID_{50}$ y ensayos de unidades formadoras de calvas (PA) han sido frecuentemente empleados para cuantificar los virus infecciosos en diferentes muestras ambientales. No obstante, se han reportado diversas desventajas en ambas técnicas cuando estas son aplicadas a muestras ambientales. Tanto $TCID_{50}$ como PA están basadas en la detección del efecto citopático (CPE) producido por el virus en la línea celular. Se debe tener en cuenta, que aunque los virus analizados produzcan CPE, no todos presentan la misma cinética de replicación, requiriendo en algunos casos periodos prolongados de cultivo y además en muestras ambientales el CPE puede ser producido por otro virus.

En este estudio se describe un protocolo de inmunofluorescencia (IFA) para detectar y cuantificar HAdV y JCPyV. En el desarrollo de IFA descrito, se determinaron las condiciones de cultivo y de replicación del virus, optimizando la cuantificación de las células infectadas. Se determinó que el día óptimo de lectura de IFA para HAdV 2 era de 4 días y para HAdV 41 y JCPyV, 8 días. También se estableció que IFA era específico para detectar virus infecciosos, al obtener resultados negativos cuando se analizaron suspensiones víricas tratadas previamente con temperaturas de 100° C y luz UV.

El ensayo de IF fué comparado con las técnicas de cuantificación $TCID_{50}$ y PA. Estos resultados mostraron que para HAdV2, con IFA se detectó 10 veces más virus infecciosos expresados en FFU (*focus forming unit*)/mL, que PA y $TCID_{50}$, expresados en PFU (*plaque forming unit*)/mL y $TCID_{50}$ unidades/mL, respectivamente. Los resultados o ensayos de $TCID_{50}$ y PA para HAdV 41 y JCPyV, se omitieron debido a que los días necesarios para observar efecto citopático afectaban la interpretación de los resultados finales y el hecho además de que estas cepas no producen calvas.

El ensayo de IF, también se optimizó para muestras ambientales y con este propósito se analizaron concentrados víricos obtenidos a partir de agua de río previamente contaminada con HAdV 2 y HAdVs presentes de forma natural en agua residual urbana. Para evitar posibles efectos de citotoxicidad producidos por diferentes componentes de las matrices ambientales, todos los concentrados víricos obtenidos se trataron con cloroformo y diferentes diluciones fueron analizadas. Bajo estas condiciones se detectaron aproximadamente 10 veces menos HAdV 2 infecciosos, que copias de genomas detectadas en paralelo por qPCR. Mientras que los HAdV infecciosos en agua residual se detectaron en concentraciones 100 veces menores que los valores obtenidos por qPCR. Aproximadamente la misma diferencia observada con los HAdV 2 añadidos artificialmente al agua de rio (1 Log), se obtuvo cuando se cuantificaron HAdV2, HAdV 41 y JCPyV por IFA y qPCR, a partir de suspensiones víricas que fueron tratadas con DNAsa, eliminando posibles genomas víricos libres. Estos resultados confirman que IFA es una técnica sensible y robusta, aplicable a matrices tan complejas como las muestras ambientales.

La infectividad de JCPyV no se analizó en muestras ambientales, ya que comúnmente las cepas excretadas en orina no son cultivables en líneas celulares convencionales. La cepa usada en los ensayos experimentales es JC-Mad4, la cual presenta un reordenamiento en la región reguladora de su genoma. Esta cepa se asilo a partir de un paciente con leucoencefalopatia multifocal progresiva. Mad4 es una cepa modelo adecuado para estudiar el comportamiento de los polyomavirus en el medio ambiente y para validar procesos y tratamientos de inactivación y desinfección.

El ensayo de IF descrito en el presente estudio es una herramienta idónea para la detección de HAdV infecciosos en el medio ambiente. Además permite evaluar la infectividad de HAdV y JCPyV (Mad-4) como virus modelo en estudios de estabilidad y en tratamientos de desinfección. El ensayo de IF tiene una gran especificidad, presenta resultados reproducibles y consistentes, tiene un costo económico adecuado y es una técnica fácil de estandarizar en un laboratorio.

Journal of Virological Methods 171 (2011) 1–7

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Protocols

Detection and quantitation of infectious human adenoviruses and JC polyomaviruses in water by immunofluorescence assay

Byron Calgua[a], Celia Regina Monte Barardi[b], Silvia Bofill-Mas[a], Jesus Rodriguez-Manzano[a], Rosina Girones[a,*]

[a] Department of Microbiology, Faculty of Biology, University of Barcelona, Diagonal 645, 08028 Barcelona, Spain
[b] Department of Microbiology, Immunology and Parasitology, Center of Biological Sciences, Universidade Federal de Santa Catarina, 88040-900 Florianópolis, Santa Catarina, Brazil

Article history:
Received 26 April 2010
Received in revised form 3 September 2010
Accepted 13 September 2010
Available online 21 September 2010

Keywords:
Immunofluorescence assay
Plaque assay
TCID50
qPCR
Cell culture
Adenoviruses
JC polyomavirus

A B S T R A C T

Human adenoviruses (HAdV) and JC polyomaviruses (JCPyV) have been proposed as markers of fecal/urine contamination of human origin. An indirect immunofluorescence assay has been developed to quantify infectious human adenoviruses types 2 and 41 and JC polyomaviruses strain Mad-4 in water samples. The immunofluorescence assay was compared with other quantitative techniques used commonly such as plaque assay, tissue culture infectious dose-50 and quantitative PCR (qPCR). The immunofluorescence assays showed to be specific for the detection of infectious viruses, obtaining negative results when UV or heat-inactivated viruses were analyzed. The assays required less time and showed higher sensitivity for the detection of infectious viral particles than other cell culture techniques (1 $\log_{10}$ more) evaluated. River water samples spiked previously with human adenoviruses and raw sewage samples were also analyzed using the proposed immunofluorescence assay as well as by qPCR. The results show quantitations with 2 $\log_{10}$ reduction in the numbers of infectious viruses compared with the number of genome copies detected by qPCR. The immunofluorescence assay developed is fast, sensitive, specific, and a standardizable technique for the quantitation and detection of infectious viruses in water samples.

1. Introduction

Water quality is impaired by the presence of pathogenic microorganisms derived from treated effluent or untreated sewage that is released into the environment. These pathogens include many types of viruses that infect humans and that are excreted in high concentration in the feces of patients with gastroenteritis (Carter, 2005; IAWPRC, 1983). Some viruses, such as polyomaviruses and some strains of human adenoviruses (HAdV), establish persistent infections, and viral particles may be excreted in feces and/or urine for months and even years (Carter, 2005; Crabtree et al., 1997; Imperiale and Major, 2007; Wadell et al., 1988). HAdV and JC polyomavirus (JCPyV) have been reported in high concentrations in sewage (Bofill-Mas et al., 2000, 2006; Katayama et al., 2004; Pina et al., 1998), river and lake water (Albinana-Gimenez et al., 2009a; Wong et al., 2009), seawater (Calgua et al., 2008; McQuaig et al., 2009) and even drinking water (Albinana-Gimenez et al., 2009b; Hamza et al., 2009; Lambertini et al., 2008). Both DNA viruses are highly stable to environmental conditions (Bofill-Mas et al., 2006; Fong and Lipp, 2005). Several studies have questioned the use of bacterial indicators to predict the occurrence of viruses and have proposed HAdV and JCPyV as indicators of fecal contamination of human origin (Bofill-Mas et al., 2000; Calgua et al., 2008; Formiga-Cruz et al., 2003; Lipp et al., 2001; Pina et al., 1998; Sinclair et al., 2009; Wong et al., 2009).

HAdVs have linear double-stranded DNA and are included in the *Mastadenovirus* genera, in the *Adenoviridae* family (Stewart et al., 1993). HAdVs are grouped in 52 serotypes, which have been divided in 7 species (A–G). Most of the serotypes (main serotypes: 1–7, 14 and 21) cause respiratory diseases, particularly in children. HAdV 40 and 41 are the most important serotypes responsible for gastroenteritis in children (Wold and Horwitz, 2007).

JC polyomavirus is a human virus classified in the *Polyomaviridae* family. This virus produce latent and chronic infections that persist indefinitely in individuals and viral particles are excreted regularly in urine of healthy individuals (Imperiale and Major, 2007). The virus affects a large proportion of the population worldwide; consequently, its presence in water may not represent a significant health risk for most of the population. The pathogenicity of the virus is commonly associated with progressive multifocal leukoencephalopathy (PML) in immunocompromised states and

* Corresponding author. Tel.: +34 93 4021483; fax: +34 93 4039047.
E-mail address: rgirones@ub.edu (R. Girones).

0166-0934/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.jviromet.2010.09.013

attracted new attention due to their reactivation in some patients of multiple sclerosis and other autoimmune diseases treated with immunomodulators (Berger et al., 1987; Yousry et al., 2006). In previous studies, JCPyV was found in 98% of the 52 sewage samples collected from different geographical areas around the world (Bofill-Mas et al., 2000).

DNA-amplified techniques, such as quantitative PCR (qPCR) and nested-PCR (nPCR), are the most sensitive and rapid methods for the detection and quantitation of viruses in environmental samples and are at the present used widely (Haramoto et al., 2007; Pina et al., 1998). However, these techniques detect both infectious and non-infectious viral particles. In some samples, it may be important to analyze the infectivity of the viral particles identified applying techniques with enough sensitivity. Such techniques may be useful for risk assessment studies in a wide range of scenarios, such as exposures to potential contaminated food or water treated previously by disinfection procedures.

Cell culture-based techniques are used to detect and quantify infective viruses from environmental samples, as well as to analyze the viability of viruses after treatment with UV-light, chlorine, temperature, and other conditions (Greening et al., 2002; Reynolds et al., 1996). Used commonly cell culture-based techniques for this purpose include tissue culture infectious dose-50 ($TCID_{50}$), plaque assays, immunofluorescence assays (IFAs) and integrated cell culture-PCR (ICC-PCR). $TCID_{50}$ and plaque assay are classical techniques used to quantify infective viruses in the environment (Bitton et al., 1982; Brashear and Ward, 1982; Jacangelo et al., 2003; Jiang et al., 2009; Melnick et al., 1978). However, not all viruses produce clear cytopathic effect (CPE) or plaques, as occurs with some of the 52 HAdV serotypes. Additionally, not all viruses infect the cell lines used for their detection with the same efficiency. A more recent approach is ICC-PCR, which combines cell culture and PCR or qPCR techniques (Chapron et al., 2000; Dong et al., 2009; Gerrity et al., 2008; Greening et al., 2002; Reynolds et al., 2001; Reynolds, 2004; Rigotto et al., 2005; Shieh et al., 2008). This technique may be costly and exists the possibility to detect the DNA of inactivated viruses inoculated onto cultured cells (Fong and Lipp, 2005). IFA has been used with several types of viruses mainly in the clinical field (Rigonan et al., 1998; Terletskaia-Ladwing et al., 2008). The use of the IFAs for detecting infectious viral particles in environmental samples have been described previously for rotavirus in sewage by Smith and Gerba (1982) and Ridinger et al. (1982).

In the present study, IFAs have been developed for the detection and quantitation of HAdV and JCPyV strain Mad-4 in environmental samples. The capacity of the IFA to quantify infectious HAdV and JCPyV was compared with $TCID_{50}$ and also with plaque assay for HAdV. A relation between values quantified by the IFA and by qPCR was obtained from the analysis of raw sewage and spiked artificially river water samples.

2. Materials and methods

2.1. Cell lines and viral stocks

HAdV types 2 and 41 (provided kindly by Annika Allard, Umeå Universiy, Sweden) were selected because they are among the most prevalent human adenoviruses in the environment. A549 and 293 cell lines (provided kindly by Annika Allard, Umeå Universiy, Sweden) were used for the propagation of these viruses and for the cell culture assays, respectively. JCPyV Mad-4 (provided kindly by Dr. Eugene O. Major, NINDS, National Institutes of Health, MD, USA) was analyzed and propagated in SVG-A cell lines (provided kindly by Dr. Walter Atwood, NINDS, National Institutes of Health, MD, USA).

A549 is an epithelial cell line derived from human lung carcinoma, 293 is an epithelial cell line derived from human kidney tumor transformed with HAdV 5, and SVG-A is a fibroblast cell line subcloned from the original SVG human fetal glial cell line. All cell lines were grown in Earl's minimum essential medium (EMEM) supplemented with 1% glutamine, 50 μg of gentamicin per mL and 10% (growth medium) or 2% (maintenance medium) of heat-inactivated FBS (fetal bovine serum). For the 293 cell line, the maintenance medium also contained 10% of heat-inactivated FBS.

2.2. River water samples

Four 5 L-river water samples were collected and treated to concentrate the viral particles in 6.5 mL of phosphate buffer, following the procedure described previously by Calgua et al. (2008). These samples were spiked previously with HAdV 2. All viral concentrates were analyzed by IFA and qPCR. To remove contaminants for cell culture-based assays chloroform (1:3 v/v) was added to 1 mL of the viral concentrate. The sample containing the chloroform was vortexed for 1 min and centrifuged at $10\,000 \times g$ for 10 min at 4 °C. The clean viral concentrate was recovered carefully and used immediately for IFA or stored at −80 °C for further analysis.

2.3. Sewage samples

A total of seven 42-mL raw sewage samples were collected from different waste water treatment plants (WWTPs) in Spain. Five samples were taken from two WWTPs in the area of Barcelona and two from a WWTP in Valencia. All samples were treated individually in order to concentrate the viruses in a volume of 240 μL of phosphate buffer, following the procedure described by Pina et al. (1998). The sample concentrates were treated with chloroform as described above.

2.4. Antibodies

Three specific antibodies were used in the IFA as primary antibodies: (i) for HAdV 2, a dilution 1:200 of MAB8052 mouse anti-adenovirus monoclonal antibody (Millipore, Chemicon); (ii) for HAdV 41, a dilution 1:10 of 23A11 anti-adenovirus monoclonal antibody produced from a mouse hybridoma, supplied kindly by Dr. Ladwing from the Laboratory Enders&Partner, Stuttgart, Germany (Terletskaia-Ladwing et al., 2008); and (iii) for JCPyV, a dilution 1:10 of PAB597 antibody, supplied kindly by Professor Walter Atwood from Brown University, Providence, USA.

2.5. Immunofluorescence assay (IFA)

An IFA based on that described previously by Barardi et al. (1999) and Smith and Gerba (1982) for human rotavirus was modified for the present study.

To quantify virus present in viral suspensions produced by the cell culture lines, the lines (A549 for HAdV 2, 293 for HAdV 41 and SVG-A for JCPyV strain Mad-4) were incubated overnight in 4-well Lab-Tek II chamber slides (Nagle Nunc International, Naperville, IL) at 37 °C in 5% CO_2 until they reached 90–100% confluence. Growth medium was then discarded and 100 μL of the viral suspension was added to the cell monolayer. Cells were incubated for 90 min at 37 °C in 5% of CO_2. After incubation, the inoculated viral suspension was removed carefully and 500 μL of fresh maintenance medium was added. To quantify HAdVs in sewage or spiked river water sample concentrates, 760 μL of 293 cell suspension (5.00×10^5 cell/mL, suspension prepared in growth medium) was added to each well of the Lab-Tek 8-well chamber slide (Nagle Nunc

International, Naperville, IL), and 240 μL of different dilutions of the sample concentrate was added and mixed in each well. Cells were then incubated overnight at 37 °C in 5% of CO_2. Once cells were attached, the medium was removed carefully and 1 mL of fresh growth medium was added. Infected cells were incubated with HAdV 2 for 4 days, or with HAdV 41 or JCPyV for 8 days at 37 °C in 5% CO_2. After this period, the maintenance medium was removed and the cells were washed with cold 1X PBS (Gibco, Scotland, UK). The cells were fixed with ice-cold absolute methanol for 10 min and PBS was added again for 5 min. Monolayers were incubated with blocking solution (BS), which contained PBS with 1% of BSA (w/v) and 0.05% of Tween (v/v), for 1 h at 37 °C. The BS was removed and cells were stained for 1 h at 37 °C with their corresponding primary antibody diluted previously in BS (100 or 240 μL for 8- or 4-well chambers, respectively). The antibody solution was removed and cells were washed for 15 min at 37 °C with BS. Cells were then stained for 15 min at RT with a 1:100 (v/v) dilution of goat anti-mouse IgG-FITC (Sigma–Aldrich, Steinheim, Germany) in BS (100 or 240 μL for 8- or 4-well chambers, respectively). The IgG antibody was removed and cells were washed with BS for 15 min at 37 °C. Chambers were mounted and drops of UltraCruz™ mounting medium were added (Santa Cruz Biotechnology, Inc.). Finally, cells were observed under an epifluorescence microscopy with UV light.

The IFAs were checked during ten days in order to determine the optimal day to read them. The optimal day for reading IFAs was determined based on the maximum number of cells infected and the minimum number of expanded foci and the minimum number of cells derived from them.

To evaluate the specificity of the assay for infectious viruses, a suspension of 2.7×10^5 GC/mL of HAdV 2 was divided into three aliquots. The first aliquot was treated with UV light (186 mJ/cm^2), the second was incubated at 99 °C for 20 min and the third was stored at 4 °C as a positive control. All aliquots were treated with DNAse and analyzed by qPCR and IFA using MAB8052 antibody.

2.6. Plaque assay

Plaque assay to quantify HAdV 2 was performed on A549 cells grown to 90–100% of confluence in 25-cm^2 bottles (Nagle Nunc International, Naperville, IL). The growth medium was removed and 1 mL of the viral suspension was added to the cell monolayer and left to adsorb for 1 h at 37 °C. After incubation, the inoculated viral suspension was removed and 15 mL of overlay medium (2% FBS maintenance medium and 3% of carboxymethyl cellulose, 1:1 v/v) was added. Infected cells were incubated at 37 °C for 8 days, after which the overlay medium was removed carefully and 10 mL of formalin (1:10 v/v of formaldehyde 34% in distilled water) was added. Cells were then incubated at RT for 1 h, the formalin was removed and 10 mL of crystal violet was added to stain the monolayer. Finally, the crystal violet was removed and the monolayer was washed twice with tap water. All assays were performed in triplicate and negative and positive controls were included.

2.7. Tissue culture infectious dose ($TCID_{50}$)

Given that the strain available of HAdV 41 and JCPyV strain Mad-4 used do not produce plaques, the $TCID_{50}$ assay was chosen to quantify the viral suspension of these viruses. The assay was performed respectively on A549 or SVG-A cells, and $TCID_{50}$/mL values were calculated following the method described by Reed-Muench (Hierholzer and Killington, 1996). A549 or SVG-A cells were grown in 96-well plates (Nagle Nunc International, Naperville, IL) until they reached 90–100% confluence. Viral suspensions were diluted in serial 10-fold dilutions. Growth medium was removed and 100 μL of each dilution of the viral suspension was added (10 wells per dilution) to the cell monolayer and incubated at 37 °C for 1 h. After incubation, the inoculated viral suspensions were removed and 400 μL of maintenance medium was added to each well. Plates were checked every day for 8 days or longer when required.

2.8. Quantitative PCR (qPCR)

Free viral DNA is present in supernatants from infected cell cultures and can be quantified by qPCR simultaneously with DNA derived from lysed virus during nucleic acid extraction. When applying qPCR, in order to remove free DNA and quantify only DNA from potentially infectious viral particles, all viral suspensions were treated with 100 units of DNAse, following manufacturer's instructions (Sigma–Aldrich, Steinheim, Germany).

Before using DNAse, two experiments were performed. First, to check whether that the DNAse worked properly, a known amount of plasmidic DNA was added to PBS and treated with DNAse I. Afterwards the reaction was stopped following manufacturer's instructions. Second, to determine whether residual enzymatic activity remained after stopping the DNAse reactions, DNAse treatment was applied to a PBS sample. A known amount of plasmidic DNA was then added and the reaction was stopped as usual. Nucleic acids were extracted with a QIAamp Viral RNA mini Kit (QIAGEN, Hilden, Germany), using 140 L of viral suspensions or viral concentrates from environmental samples.

HAdV and JCPyV quantitation was based on the assays described previously by Hernroth et al. (2002) and Pal et al. (2006), respectively.

qPCRs used were based on the TaqMan® assay, which uses two primers and a fluorogenic probe that recognizes a specific fragment of the HAdV or JCPyV genome. Amplifications were per-

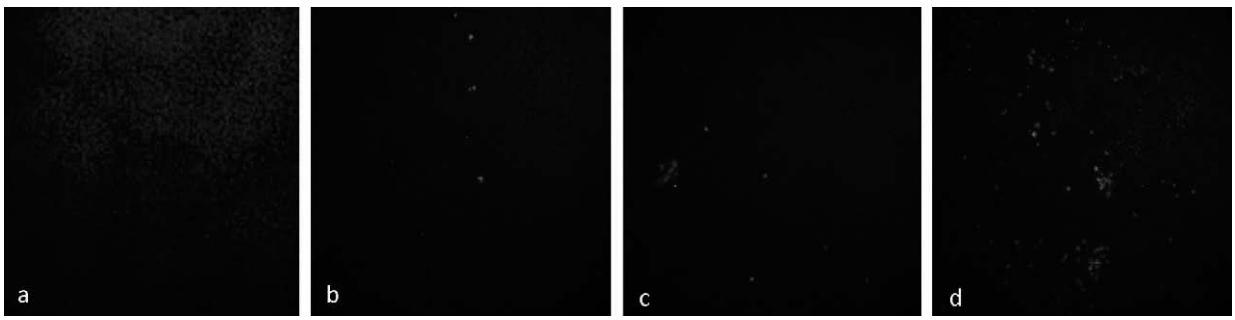


Fig. 1. Infected SVG-A cells with JCPyV strain Mad-4 by IFA. (a) Negative control (b) 4th day of infection, individual infected cells are detected, (c) 8th day of infection, individual infected cells and foci are detected, (d) 11th day of infection, the cells infected from the virus spiked are not distinguished from the cells infect from the foci expanded. Cells stained with anti-mouse-IgG/FITCC and with mountain medium DAPI.

formed in a 25-μL reaction mixture with TaqMan® Environmental Master Mix (Applied Biosystems). The reaction contained 10 μL of a DNA sample, 1X TaqMan® Environmental Master Mix, and the corresponding primers at 0.9 μM and TaqMan® probes at 0.225 μM. TaqMan® Environmental Master Mix was supplied 2× concentrated and contained AmpliTaq Gold® DNA polymerase ultra pure, dNTPs, optimized buffer components. Following activation of AmpliTaq Gold for 10 min at 95 °C, 40 amplification cycles (15 s at 95 °C and 1 min at 60 °C) were performed with an MX3000P sequence detector system (Stratagene). Undiluted DNA, and 10- and 100-fold dilutions of the extracted DNA were run in duplicate (6 runs/sample). In all qPCRs, the amount of DNA in GC/mL was defined as the mean of the data obtained. Non-template controls and non-amplification controls were included in each run. The data produced by qPCR presented low variability in the diverse replicates. However variability was observed in few undiluted samples. This variability may be attributable to the presence of inhibitors in the reaction. Thus, these values were excluded from the measurement.

3. Results

3.1. Immunofluorescence assays

The optimal reading days for the IFAs were day 4 for HAdV 2 and day 8 for HAdV 41 and JCPyV (Fig. 1).

No FFUs (focus-forming unit) were observed when HAdV 2 were treated with UV or kept at 99 °C; however, viral DNA was detected and quantified by qPCR in both cases (data not shown).

3.2. Comparison of qPCR, IFA and $TCID_{50}$ for the quantitation of HAdV 2, HAdV 41 and JCPyV strain Mad-4, and plaque assay for the quantitation of HAdV 2

Two different stocks for HAdV 2, two for HAdV 41 and two for JCPyV were divided into three replicates each one (6 aliquots per virus). All aliquots were used in the comparative analysis.

Three different dilutions of each viral aliquot were tested by IFA on 8-well Lab Teck Chambers and the readings were done on day 4 post-infection (p.i.) for HAdV 2 and day 8 p.i. for HAdV 41 and JCPyV. All IFA were performed in duplicate.

For qPCR analysis, undiluted, 1:10 and 1:100 dilutions of the nucleic acid extraction of each aliquot treated previously with DNAse were analyzed in duplicate.

In $TCID_{50}$ assays, given the long time required to observe a CPE (when observed) when infecting 293cells with HAdV41 and SVG-A cells with JCPyV strain Mad-4 the results of the quantitations were omitted. For the quantitation of HAdV 2 by $TCID_{50}$, 6 consecutive 10-fold dilutions of each aliquot were tested and the results were recorded on day 8 p.i. For the quantitation of HAdV 2 by plaque assay, a 10-fold dilution of each aliquot was analyzed in duplicate using 25-cm^2 flasks and PFU (plaque-forming unit) were counted on day 8 p.i. (the Hep-2 cell line was also tested, though, 293 cells were selected since the reading of the stained cell and the integrity of the monolayer was clearer and more stable, (data not shown))

The comparison of the results of each quantitative technique shows that FFU/mL values obtained by the IFA were approximately 1 $\log_{10}$ less than the qPCR values expressed in GC/mL for HAdV 2, HAdV 41 and JCPyV Mad-4 (Table 1). Furthermore, in the quantitation of HAdV 2 the results obtained by IFA were 1 $\log_{10}$ greater than the obtained by plaque assay and $TCID_{50}$ expressed in PFU/mL and $TCID_{50}$ units/mL, respectively (Table 1).

Table 1
Comparison of different methods for the quantitation of viruses.

Viral suspensions	qPCR[a] (GC/mL)	Quantitative cell culture assays		
		IFA (FFU/mL)	Plaque assay (PFU/mL)	$TCID_{50}$ ($TCID_{50}$ units/mL)
HAdV 2	1.25×10^5	1.15×10^4 (1.00×10^4–1.70×10^4; SD: 2271)	3.44×10^3 (1.00×10^3–6.00×10^3; SD: 1580)	6.17×10^3 (2.63×10^3–7.94×10^3; SD: 3065)
	2.70×10^5	1.60×10^4 (1.35×10^4–1.90×10^4; SD: 1565)	9.52×10^2 (7.80×10^2–1.10×10^3; SD: 1036)	3.75×10^3 (–; SD: 0)
HAdV 41	5.00×10^5	1.40×10^4 (1.20×10^4–2.00×10^4; SD: 2730)	NT	RO
	5.10×10^5	1.70×10^4 (1.10×10^4–2.00×10^4; SD: 2260)		
JCPyV strain Mad-4	2.35×10^5	1.31×10^4 (1.00×10^4–1.90×10^4; SD: 2905)	NT	RO
	5.00×10^5	2.31×10^4 (1.58×10^4–3.50×10^4; SD: 5202)		

[a] qPCR was performed after DNAse treatment in the viral suspensions; SD: standard deviation; NT: no-tested; RO: results omitted.

Table 2
Quantitation and detection of HAdV 2 in spiked river water samples by IFA and qPCR.

Sample	HAdV 2 detected by qPCR (GC/mL[a])	[+]HAdV detected by IFA (FFU/mL)	IFA[b] range (FFU/200mL)	IFA[c] SD
B1	115	8.32	153–180	19.02
B2	133	8.55	172–170	1.41
B3	316	9.57	213–170	30.40
B4	958	7.97	161–158	2.12

[a] qPCR was performed after DNAse treatment in the viral suspension.
[b] The volume of the viral concentrate tested in each well (240 μL) corresponds to 200 mL of the original sample (5 L).
[c] The standard deviation corresponds to the number of FFUs detected in each well, which correspond to the 240 μL analyzed ([+]200 mL of the original samples).
SD: standard deviation.

Table 3
HAdVs detected and quantified in sewage samples by qPCR and IFA.

Sample	HAdV detected by qPCR (GC/mL[a])	Mean values by qPCR (GC/mL)	HAdV[b] detected by IFA FFU/mL	Mean value by IFA (FFU/mL)
AR-GV-180507	1.21×10^3		5.71×10^1	
AR-GV-061106	2.06×10^3		5.47×10^1	
AR-GV-141106	1.04×10^3		5.95×10^1	
AR-GV-190906	5.07×10^3	3.52×10^3	6.42×10^1	6.21×10^1
AR-VLN30-121207	9.93×10^3		5.47×10^1	
AR-VLN32-191207	2.93×10^3		7.61×10^1	
AR-SA-100609	2.41×10^3		6.90×10^1	

[a] qPCR was performed after DNAse treatment in the viral suspension.
[b] The volume analyzed in each well (240 μL) corresponds to 42 mL of original sewage sample. The values shown in the table are represented as mL from the original sample.

3.3. Detection of spiked-infectious adenoviruses in river water samples by IFA

River water samples were spiked with HAdV 2, concentrated and treated first with chloroform and the clarified concentrates with DNAse. Undiluted sample and 1:10 dilutions of viral concentrates were analyzed by duplicate in 4-well Lab Teck Chambers using the MAB8052 antibody. The IFA detected spiked infectious viruses (Table 2). Treatment with chloroform to remove possible contaminants and inhibitors, such as fungi and organic matter, proved effective; however, cytotoxicity was observed when the undiluted viral concentrate was tested. The FFU/mL values were approximately 2 $\log_{10}$ less than the values in GC/mL detected by qPCR (Table 2).

3.4. Detection of infectious human adenoviruses in raw sewage samples by IFA

Sewage samples were concentrated and treated with chloroform and the clarified concentrates with DNAse. Dilutions of the sewage concentrates were analyzed (1:2, 1:10 and 1:100 dilutions) in 4-well Lab Teck Chambers using the 23A11 antibody. The samples also were evaluated by qPCR for HAdV detection (Table 3). The values in FFU/mL obtained by the IFA were approximately 2 $\log_{10}$ less than the values in GC/mL obtained by qPCR (Table 3). The concentrate diluted 1:2 produced cytotoxicity and the results were quantified in the viral concentrate diluted 1:10 and 1:100.

4. Discussion

In this study IFAs have been developed as tools to quantify and detect infectious HAdVs and JCPyV. The assay showed high sensitivity for HAdV in natural and spiked samples.

The optimal day for reading the IFA was determined on the basis of the maximum number of cells infected, the minimum number of expanded foci and the minimum number of cells derived from them. For this purpose, IFAs were monitored from day 1 (day after infection) to day 10 (day of methanol-cell fixation). The criterion used to identify the infected cells were based on the osbervation of the flourescence emitted by these cells. Fluorescence corresponded initially to isolated infected cells, while in late days it corresponded to fluorescent foci. Optimal reading days were then selected when high counts were obtained, identifying only infected cells and isolated infected foci. Furthermore, potential detection of viral proteins of non-infectious viral particles of HAdV 2 by the binding of antibodies was discarded after treatment at 99 °C and treatment with UV since the IFA provided negative results. However, DNA of non-infectious viral particles was still detected by qPCR. This finding implies that the results from the latter do not correlate with infectious viral particles when analyzing the treated viral suspension.

Cell culture techniques for detecting and quantifying viruses include usually cytopathic-effect-related assays, such as plaque assay and $TCID_{50}$. However, in some cases, viruses do not form or require a long time to produce CPE. Thus, some viruses require more than one passage on the cell lines (Birch et al., 1983; Mautner, 2007). The IFA developed detected 1 $\log_{10}$ more infectious viruses for HAdV 2 than the plaque assay and $TCID_{50}$. The differences in the sensitivity of the IFA and plaque assay could be explained by the observation that not all viruses form plaques with equivalent efficiency. It was not possible to validate the $TCID_{50}$ results for HAdV 41 because the time required to produces a CPE in the 293 cell line exceeded that required to maintain cell integrity without further passages of the monolayer. As part of the IFA optimization the cell line HEp-2 was also tested for HAdV 41, although the results were not consistent (data not shown).

Human JCPyV is known to infect only humans and has been proposed as marker of human fecal/urine contamination in the aquatic environment. It is difficult to propagate this virus by cell culture techniques. The regulatory region of JCPyV strain Mad-4 is reorganized and is easier to culture in SVG-A cell line than the archetypical strain excreted commonly in urine. However even the re-arranged PML-type strain as Mad-4 is not able to produce plaques and requires a long time to produce a CPE (the cells in most of the cases require more than one passage). Consequently, the plaque assay and $TCID_{50}$ were not considered for comparison purposes with the IFA. Furthermore, the strains of JCPyV reported in the environment correspond most frequently to archetypical strains, which may not produce infection in the conditions assayed (Bofill-Mas et al., 2000). The Mad-4 strain would be useful for disinfection and validity experiments producing information on the

behavior of JCPyV in environmental conditions and inactivation treatments.

IFAs have been used previously to quantify and detect human rotavirus in sewage samples (Ridinger et al., 1982; Smith and Gerba, 1982). To establish the concentration of infectious viruses present in environmental samples may be required when evaluating risks associated with viral contamination, such as in drinking water or after specific treatments applied to remove viral particles, such as chlorine or UV disinfection treatments. In sewage samples and HAdV 2-spiked river water samples the IFA detected 2 $\log_{10}$ fewer HAdVs in FFU than genomic copies. Considering that 1 $\log_{10}$ was the difference between IFA and qPCR with viral suspensions treated previously with DNAse, a titer of 2 $\log_{10}$ less in comparison with qPCR in environmental samples could be attributed to the presence of some inactivated viral particles or environmental factors affecting the infectious capability of the virus.

The qPCR is a useful tool to detect and quantify viruses in the environment as it produces sensitive and accurate results in a considerably short time. However, in some conditions qPCR data may not correlate with infectious viral particles and thus complementary infection assays are required. The results obtained by qPCR are also useful as a tool for microbial source tracking and for rapid analysis to evaluate the occurrence of viruses in different types of samples (Bofill-Mas et al., 2006; Hundesa et al., 2009a,b).

Techniques to detect and quantify infectious viruses, such as the plaque assay, have been used frequently; however, previous studies have reported disadvantages when applied to environmental samples, for instance false plaques have been attributed to cytotoxic materials or other viruses present in the sample. Also, the viruses to be analyzed in the sample may not have the capacity to produce plaques (Ridinger et al., 1982; Smith and Gerba, 1982). Integrated cell culture (ICC) combined with PCR or qPCR has been applied recently, especially in cases where the viruses do not produce a CPE; however, these methods are costly and may also detect DNA or RNA from inactivated viruses in the inoculum (Fong and Lipp, 2005). The protocol for detection and quantitation infectious viral particles by IFA could be simplified by the use of an image analysis system during the counting of cells and foci.

The results in the present study indicate that the described IFA is highly specific, and has a good cost-effective ratio, representing a suitable tool for infectivity assays for the detection of HAdVs in the environment, also allowing the use of JCPyV strain Mad-4 as a model in stability studies and disinfection treatments.

Acknowledgments

This work was supported by the "Ministerio de Ciencia e Innovación, MICINN" of the Spanish Government (project AGL2008-05275-C03-01/ALI). During the development of this study Jesus Rodriguez-Manzano was a fellow of the Spanish Government (MICINN) and Celia R.M. Barardi was a fellowship from Conselho de Aperfeiçoamento de Pessoal de Ensino Superior, CAPES Process number 3542/07-6. We thank Serveis Científico Tècnics of the University of Barcelona for the help with the microscopy based quantitations. We also thank INIA laboratory in Valencia, (Spain) for providing raw sewage samples.

References

Albinana-Gimenez, N., Clemente-Casares, P., Calgua, B., Huguet, J.M., Courtois, S., Girones, R., 2009a. Comparison of methods for concentrating human adenoviruses, polyomavirus JC and noroviruses in source waters and drinking water using quantitative PCR. J. Virol. Methods 158 (1–2), 104–109.

Albinana-Gimenez, N., Miagostovich, M.P., Calgua, B., Huguet, J.M., Matia, L., Girones, R., 2009b. Analysis of adenoviruses and polyomaviruses quantified by qPCR as indicators of water quality in source and drinking-water treatment plants. Water Res. 43 (7), 2011–2019.

Barardi, C.R.M., Yip, H., Emslie, K.R., Vesey, G., Shanker, S.R., Williams, K.L., 1999. Flow cytometry and RT-PCR for rotavirus detection in artificially seeded oyster meat. Int. J. Food Microbiol. 49, 9–18.

Berger, J.R., Kaszovita, B., Donovan, P.J., Dickinson, G., 1987. Progressive multifocal leukoencephalopathy associated with human immunodeficiency virus infection. Ann. Intern. Med. 107, 78–87.

Birch, C.J., Rodger, S.M., Marshall, J.A., Gust, I.D., 1983. Replication of human rotavirus in cell culture. J. Med. Virol. 11, 241–250.

Bitton, G., Chou, Y.J., Farrah, S.R., 1982. Techniques for virus detection in aquatic sediments. J. Virol. Methods 4 (1), 1–8.

Bofill-Mas, S., Pina, S., Girones, R., 2000. Documenting the epidemiologic patterns of polyomaviruses in human populations by studying their presence in urban sewage. Appl. Environ. Microbiol. 66, 238–245.

Bofill-Mas, S., Albinana-Gimenez, N., Clemente-Casares, P., Hundesa, A., Rodriguez-Manzano, J., Allard, A., Calvo, M., Girones, R., 2006. Quantitation and stability of human adenoviruses and polyomavirus JCPyV in wastewater matrices. Appl. Environ. Microbiol. 72, 7894–7896.

Brashear, D.A., Ward, R.L., 1982. Comparison of methods for recovering indigenous viruses from raw wastewater sludge. Appl. Environ. Microbiol. 3 (6), 1413–1418.

Calgua, B., Mengewein, A., Grünert, A., Bofill-Mas, S., Clemente-Casares, P., Hundesa, A., Wyn-Jones, A.P., López-Pila, J.M., Girones, R., 2008. Development and application of a one-step low cost procedure to concentrate viruses from seawater samples. J. Virol. Methods 153 (2), 79–83.

Carter, M.J., 2005. Enterically infecting viruses: pathogenicity, transmission and significance for food and waterborne infection. J. Appl. Microbiol. 6, 1354–1380.

Chapron, C.D., Ballester, N.A., Fontaine, J.H., Frades, C.N., Margolin, A.B., 2000. Detection of astroviruses, enteroviruses, and adenovirus types 40 and 41 in surface waters collected and evaluated by the information collection rule and an integrated cell culture-nested PCR procedure. Appl. Environ. Microbiol. 66 (6), 2520–2525.

Crabtree, K.D., Gerba, C.P., Rose, J.B., Haas, C.N., 1997. Waterborne adenovirus: a risk assessment. Water Sci. Technol. 35, 1–6.

Dong, Y., Kim, J., Lewis, G.D., 2009. Evaluation of methodology for detection of human adenoviruses in wastewater, drinking water, stream water and recreational waters. J. Appl. Microbiol. 108 (3), 800–809.

Fong, T., Lipp, E.K., 2005. Enteric viruses of humans and animals in aquatic environmental: health risks, detection, and potential water quality assessment tools. Microbiol. Mol. Biol. Rev. 69, 357–371.

Formiga-Cruz, M., Allard, A.K., Conden-Hansson, A.C., Henshilwood, K., Hernroth, B.E., Jofre, J., Lees, D.N., Lucena, F., Papapetropoulou, M., Rangdale, R.E., Tsibouxi, A., Vantarakis, A., Girones, R., 2003. Evaluation of potential indicators of viral contamination in shellfish and their applicability to diverse geographical areas. Appl. Environ. Microbiol. 69 (3), 1556–1563.

Gerrity, D., Ryu, H., Crittenden, J., Abbaszadegan, M., 2008. UV inactivation of adenovirus type 4 measured by integrated cell culture qPCR. J. Environ. Sci. Health A Tox. Hazard Subst. Environ. Eng. 43 (14), 1628–1638.

Greening, G.E., Hewitt, J., Lewis, G.D., 2002. Evaluation of integrated cell culture-PCR (C-PCR) for virological analysis of environmental samples. J. Appl. Microbiol. 93, 745–750.

Hamza, I.A., Jurzik, L., Stang, A., Sure, K., Uberla, K., Wilhelm, M., 2009. Detection of human viruses in rivers of a densly-populated area in Germany using a virus adsorption elution method optimized for PCR analyses. Water Res. 43 (10), 2657–2668.

Haramoto, E., Katayama, H., Oguma, K., Ohgaki, S., 2007. Quantitative analysis of human enteric adenoviruses in aquatic environments. J. Appl. Microbiol. 103, 2153–2159.

Hernroth, B.E., Conden-Hansson, A.C., Rehnstam-Holm, A.S., Girones, R., 2002. Environmental factors influencing human viral pathogens and their potential indicator organisms in the blue mussel. Mytilus edulis: the first Scandinavian report. Appl. Environ. Microbiol. 68 (9), 4523–4533.

Hierholzer, J.C., Killington, R.A., 1996. Quantitation of viruses. In: Mahy, B.W.J., Kangro, O. (Eds.), Virology Methods Manual, 1st ed. Academic Press, San Diego, pp. 25–46.

Hundesa, A., Maluquer de Motes, C., Albiñana-Gimenez, N., Rodriguez-Manzano, J., Bofill-Mas, S., Suñen, E., Girones, R., 2009a. Development of a qPCR assay for the quantification of porcine adenoviruses as a microbial source-tracking tool for swine fecal contamination in the environment. J. Virol. Methods 158, 130–135.

Hundesa, A., Bofill-Mas, S., Maluquer de Motes, C., Rodriguez-Manzano, J., Bach, A., Casas, M., Girones, R., 2009b. Development of quantitative PCR assay for the quantification of bovine polyomavirus as a microbial source-tracking tool. J. Virol. Methods 163, 385–389.

IAWPRC, Study Group on Health Related Water Virology, 1983. Health significance of viruses in water. Water Res. 17, 121–132.

Imperiale, M.J., Major, E.O., 2007. Polyomavirus. In: Knipe, D.M., Howley, P.M. (Eds.), Adenoviruses. Fields Virolog, 5th ed. Lippincott Williams & Wilkins, Philadelphia, PA, pp. 2263–2298.

Jiang, S.C., Han, J., He, J.W., Chu, W., 2009. Evaluation of four cell lines for assays of infectious adenovirus in water samples. J. Water Health. 7 (4), 650–656.

Jacangelo, J.G., Loughran, P., Petrik, B., Simpson, D., McIlroy, C., 2003. Removal of enteric viruses and selected microbial indicators by UV irradiation of secondary effluent. Water Sci. Technol. 47 (9), 193–198.

Katayama, H., Okuma, K., Furumai, H., Ohgaki, S., 2004. Series of surveys for enteric viruses and indicator organisms in Tokyo Bay after an event of combined sewer overflow. Water Sci. Technol. 50, 259–262.

Lambertini, E., Spencer, S.K., Bertz, P.D., Loge, F.J., Kieke, B.A., Borchardt, M.A., 2008. Concentration of enteroviruses, adenoviruses and noroviruses from drinking water with glass wool filters. Appl. Environ. Microbiol. 74 (10), 2990–2996.

Lipp, E.K., Jarrell, J.L., Griffin, D.W., Lukasik, J., Jacukiewicz, J., Rose, J.B., 2001. Preliminary evidence for human faecal contamination in corals of the Florida Keys. Mar. Pollut. Bull. 44, 666–670.

Mautner, V., 2007. Growth and purification of enteric adenovirus type 40. Methods Mol. Med. 130, 145–156.

McQuaig, S.M., Scott, T.M., Lukasik, J.O., Paul, J.H., Harwood, V.J., 2009. Quantification of human polyomaviruses JC Virus and BK Virus by TaqMan quantitative PCR and comparison to other water quality indicators in water and fecal samples. Appl. Environ. Microbiol. 75 (11), 3379–3388.

Melnick, J.L., Gerba, C.P., Wallis, C., 1978. Viruses in water. Bull. World Health Organ. 56 (4), 499–508.

Pal, A., Sirota, L., Maudru, T., Peden, K., Lewis, A.M., 2006. Real-time PCR assays for the detection of virus-specific DNA in samples with mixed populations of polyomaviruses. J. Virol. Methods. 135 (1), 32–42.

Pina, S., Puig, M., Lucena, F., Cofre, J., Girones, R., 1998. Viral pollution in the environment and in shellfish: human adenovirus detection by PCR as an index of human viruses. Appl. Environ. Microbiol. 64, 3376–3382.

Reynolds, K.A., Gerba, C.P., Pepper, I.L., 1996. Detection of infectious enteroviruses by an integrated cell culture-PCR procedure. Appl. Environ. Microbiol. 62 (4), 1424–1427.

Reynolds, K.A., Gerba, C.P., Abbaszadegan, M., Pepper, L.L., 2001. ICC/PCR detection of enteroviruses and hepatitis A virus in environmental samples. Can. J. Microbiol. 47 (2), 153–157.

Reynolds, K.A., 2004. Integrated cell culture/PCR for detection of enteric viruses in environmental samples. Methods Mol. Biol. 268, 69–78.

Ridinger, D., Spendlove, R., Barnett, B.B., George, D.B., Roth, J., 1982. Evaluation of cell lines and immunofluorescence and plaque assay procedures for quantifying reoviruses in sewage. Appl. Environ. Microbiol. 43 (4), 740–746.

Rigonan, A.S., Mann, L., Chonmaitree, T., 1998. Use of monoclonal antibodies to identify serotypes of enterovirus isolates. J. Clin. Microbiol. 36 (7), 1877–1881.

Rigotto, C., Sincero, T.C., Simoes, C.M.O., Barardi, C.R.M., 2005. Detection of adenoviruses in shellfish by means of conventional PCR, nested-PCR and integrated cell culture PCR (ICC/PCR). Water Res. 39 (2–3), 297–304.

Shieh, Y.C., Wong, C.I., Krantz, J.A., Hsu, F.C., 2008. Detection of naturally occurring enteroviruses in waters using direct RT-PCR and integrated cell culture-RT-PCR. J. Virol. Methods. 149 (1), 184–189.

Sinclair, R.G., Jones, E.L., Gerba, C.P., 2009. Viruses in recreational water-borne disease outbreaks: a review. J. Appl. Microbiol. 10 (6), 1769–1780.

Smith, E., Gerba, C.P., 1982. Development of a method for detection of human rotavirus in water and sewage. Appl. Environ. Microbiol. 43 (6), 1440–1450.

Stewart, P.L., Fuller, S.D., Burnett, R.M., 1993. Difference imaging of adenovirus-bridging the resolution gap between X-ray crystallography and electron-microscopy. EMBO J. 12, 2589–2599.

Terletskaia-Ladwing, E., Meier, S., Hahn, R., Leinmüller, M., Schneider, F., Enders, M., 2008. A convenient rapid culture assay for the detection of enteroviruses in clinical samples: comparison with conventional cell culture and RT-PCR. J. Med. Microbiol. 57, 1000–1006.

Wadell, G., Allard, A., Svensson, L., Uhnoo, I., 1988. Enteric adenoviruses. In: Farthing, M. (Ed.), Viruses and The Gut. Proceedings of the Ninth BSG·SK&F International Workshop. Swan Press Ltd., London, UK, pp. 71–78.

Wold, W.S.M., Horwitz, M.S., 2007. Adenoviruses. In: Knipe, D.M., Howley, P.M. (Eds.), Fields Virology, 7th ed. Lippincott Williams & Wilkins, Philadelphia, PA, pp. 2395–2436.

Wong, M., Kumar, L., Jenkins, T.M., Xagoraraki, I., Phankumar, M.S., Rose, J.B., 2009. Evaluation of public health risks at recreational beaches in Lake Michigan via detection of enteric viruses and a human-specific bacteriological marker. Water Res. 43 (4), 1137–1149.

Yousry, T.A., Major, E.O., Ryschkewitsch, C., Fahle, G., Fischer, S., Hou, J., Curfman, B., Miszkiel, K., Mueller-Lenke, N., Sanchez, E., Barkhof, F., Radue, E.W., Jäger, H.R., Clifford, D.B., 2006. Evaluation of patients treated with natalizumab for progressive multifocal leukoencephalopathy. N. Engl. J. Med. 354 (9), 924–933.

5. CAPÍTULO II

Virus humanos indicadores y patógenos emergentes en agua

Resumen Estudio 3:

Quantification of human adenoviruses in European recreational waters

Bofill-Mas S, **Calgua B**, Clemente-Casares P, la Rosa G, Iaconelli M, Muscillo M, Rutjes S, de Roda Husman AM, Grunert A, Gräber I, Verani M, Carducci A, Calvo M, Wyn-Jones P, and Girones R.

Food and Environmental Virology, 2010, 2:101–109.

RESUMEN:

La presencia de microrganismos patógenos en agua de baño contaminada con residuos fecales representa un problema en salud pública y económico, especialmente en áreas donde la economía se basa en el turismo. La Directiva Europea de agua de baño (2006/7/EC), tiene la finalidad de proteger la salud de la población en Europa. En esta Directiva, desde un punto de vista microbiológico, la evaluación de la calidad del agua recreacional se basa en análisis de los indicadores fecales de origen bacteriano, *E. coli* (EC) y enterococos intestinales (EI). Sin embargo, en varios estudios se ha demostrado que los virus son más estables a las condiciones del medio ambiente y que por lo tanto las concentraciones de los indicadores bacterianos usualmente no se correlacionan con las de los virus. En varios estudios en nuestro grupo se han propuesto los adenovirus humanos y polyomavirus JC como una nueva generación de indicadores de contaminación fecal.

En la presente publicación, se describen resultados obtenidos en el desarrollo del proyecto Europeo VIROBATHE (www.viobathe.com). Con la finalidad de evaluar la aplicabilidad de HAdV para evaluar la contaminación fecal de agua recreacional en Europa; se aplicó un protocolo de qPCR TaqMan® para cuantificar HAdV en 132 muestras colectadas en 24 puntos recreacionales diferentes de agua marina y no marina de nueve países Europeos. Las muestras analizadas en el presente estudio fueron positivas para HAdV por nPCR y forman parte del programa de vigilancia microbiológica de agua recreacional incluido en VIROBATHE, en el cual participaron 15 laboratorios diferentes. De las 132 muestras, en 80 se cuantificaron HAdV con un valor medio de 3,3 x 10^2 copias de genomas por 100 mL, siendo HAdV 41 el serotipo más prevalente de las muestras tipificadas. Los análisis estadísticos mostraron una relación linear no homogénea entre las

concentraciones de HAdV y EC, EI o fagos somáticos, especialmente cuando se analizaban todos los datos en conjunto. Se obtuvieron algunas correlaciones significativas entre HAdV y al menos otro indicador cuando los valores individuales de alguno de los laboratorio se consideraron. Para concentrar los virus en agua marina y no marina se emplearon métodos basados en el uso de membranas de nitrocelulosa o lana de vidrio, eluyendo los virus con un *buffer* alcalino de leche descremada y extracto de carne, respectivamente. En Barcelona se empleó SMFP (ANEXO I). Los resultados obtenidos demuestran que es factible el análisis cuantitativo de virus en agua de baño en Europa y también el hecho de que el uso de HAdV proporciona información complementaria a los estándares bacterianos, especialmente con relación a la presencia de virus patógenos.

Food Environ Virol (2010) 2:101–109
DOI 10.1007/s12560-010-9035-4

ORIGINAL PAPER

Quantification of Human Adenoviruses in European Recreational Waters

Sílvia Bofill-Mas · Byron Calgua · Pilar Clemente-Casares · Giuseppina La Rosa · Marcello Iaconelli · Michele Muscillo · Saskia Rutjes · Ana Maria de Roda Husman · Andreas Grunert · Ingeburg Gräber · Marco Verani · Annalaura Carducci · Miquel Calvo · Peter Wyn-Jones · Rosina Girones

Received: 19 December 2009 / Accepted: 19 May 2010 / Published online: 11 June 2010

Abstract The presence of human adenoviruses (HAdV) in recreational water might cause disease in the population upon exposure. HAdV detected by PCR could also serve as indicators of the virological water quality. In order to assess the applicability of HAdV to the evaluation of the faecal contamination in European bathing waters, a real-time quantitative PCR assay was used for the quantification of HAdV in 132 samples collected from 24 different recreational marine and freshwater sites in nine European countries. Selected samples presenting positive nested PCR results for HAdV were analyzed using quantitative PCR and 80 samples from a total of 132 produced quantitative results with mean values of 3.2×10^2 per 100 ml of water, being human adenovirus 41 the most prevalent serotype between the samples where adenovirus was typified. HAdV were quantified in samples from all 15 surveillance laboratories. Statistical analysis showed no homogeneous linear relation between HAdV and *E. coli*, intestinal enterococci or somatic coliphages concentrations in the tested samples when considering all the data together. Significant correlations between HAdV and at least one of the other indicators were observed only when data from individual laboratories were considered. The quantification of HAdV may provide complementary information in relation to the use of bacterial standards in the control of water quality in bathing water.

Keywords Adenoviruses · Quantitative real-time PCR · Bathing waters · Recreational waters · Seawater · Freshwater

Introduction

The presence of pathogenic microorganisms in faecally polluted recreational waters produces a perceived public

S. Bofill-Mas (✉) · B. Calgua · P. Clemente-Casares · R. Girones
Department of Microbiology, Faculty of Biology, University of Barcelona, Av. Diagonal 645, 08028 Barcelona, Spain
e-mail: sbofill@ub.edu

G. La Rosa · M. Iaconelli · M. Muscillo
Environment and Primary Prevention Department, National Institute of Health Istituto Superiore di Sanità (ISS), Viale Regina Elena 299, 00161 Rome, Italy

S. Rutjes · A. M. de Roda Husman
Laboratory for Zoonoses, National Institute for Public Health and the Environment (RIVM), Centre for Infectious Disease Control, Antonie van Leeuwenhoeklaan 9, 3721 MA Bilthoven, The Netherlands

A. Grunert · I. Gräber
German Environmental Agency (Umweltbundesamt), WörlitzerPlatz 1, 06844 Dessau-Roßlau, Berlin, Germany

M. Verani · A. Carducci
Laboratory of Hygiene and Environmental Virology, Department of Biology, University of Pisa, Via S. Zeno 35/39, Pisa, Italy

M. Calvo
Department of Statistics, Faculty of Biology, University of Barcelona, Av. Diagonal 645, 08028 Barcelona, Spain

P. Wyn-Jones
I.G.E.S., University of Aberystwyth, Aberystwyth SY23 2DB, UK

health and economic problem, especially in countries which depend strongly on tourism. The European Bathing Water Directive (2006/7/EC) came into force in March 2006 to protect the health of the European bathers. The adequacy of using bacteria as indicators of the microbial water quality has been questioned since viruses and protozoan cysts have shown to be more resistant to treatment and disinfection processes commonly applied in sewage treatment plants (Tree et al. 2003). However, the new Directive does not include the analysis of viruses as one of the microbiological parameters listed. Article 14 of the Directive highlights a special interest on scientific, analytical and epidemiological developments relating to bathing water quality including those in relation to viruses, and encourages the report of these developments.

Human adenoviruses (HAdV) have been proposed as indicators of the presence of human viral pathogens in the environment (Puig et al. 1994). HAdV have been shown to be more prevalent than enteroviruses in water and shellfish (Pina et al. 1998), to be highly stable in the environment (Bofill-Mas et al. 2006) and highly resistant to UV radiation (Gerba et al. 2002; Thurston-Enriquez et al. 2003). Moreover, adenoviruses have been included in the U.S. Environmental Protection Agency's contaminant candidate list (EPA CCL) and have been documented to be the second most significant cause of viral outbreaks in recreational waters (Sinclair et al. 2009).

Adenoviruses contain a double-stranded DNA genome of approximately 35,000 bp. They may be excreted in faeces for months or years following infection and may cause both enteric illness and respiratory and eye infections (Crabtree et al. 1997). Infection may be caused by consumption of contaminated water or food as well as by inhalation of aerosols during water recreation (Sinclair et al. 2009).

HAdV have previously been detected in environmental samples by PCR-based techniques (Pina et al. 1998, Bofill-Mas et al. 2006; Xagoraraki et al. 2007, Albinana-Gimenez et al. 2009). Occurrence of HAdV in river and coastal waters has been recently reviewed by Jiang (2006). Although quantitative real-time PCR (qPCR) methods for the quantification of some HAdV serotypes in diverse environmental samples have been recently described (Bofill-Mas et al. 2006; Choi and Jiang, 2005; Dong et al. 2010; Haramoto et al. 2007; He and Jiang, 2005; Jiang et al. 2005; Van Heerden et al. 2005a; Xagoraraki et al. 2007), to our knowledge, quantitative data on the occurrence of HAdV in European recreational waters has only been reported in one European country (Muscillo et al. 2008).

In this study, a real-time quantitative PCR assay (qPCR) was used for the quantification of HAdV in fresh and marine recreational waters of nine different European countries. The assay (Hernroth et al. 2002; Bofill-Mas et al. 2006) has previously demonstrated sensitive detection of the wide diversity of serotypes and has been used for the detection of HAdV in shellfish samples from divergent geographical areas (Formiga-Cruz et al. 2002) as well as for the monitoring of viral removal efficiency in a drinking-water treatment plant (Albinana-Gimenez et al. 2009), and for the detection and quantification of HAdV in different wastewater matrices (Bofill-Mas et al. 2006).

In this study, developed as part of the VIROBATHE project [a European Union Research Framework 6 funded project aimed at the evaluation of the feasibility of trans-European analysis of viruses in recreational waters (www.virobathe.org)], a total of 132 fresh water and seawater samples collected from 24 different recreational sites in nine different European countries was analyzed for the presence of HAdV and the concentration of these viruses was estimated by qPCR. Recreational waters evaluated include inland and marine waters used for a wide range of activities including whole-body water contact sports, such as swimming, surfing and slalom canoeing as well as non-contact sports, such as fishing, walking, bird watching and picnicking. To evaluate the potential role of HAdV as an indicator of faecal viral contamination, the potential correlation between the HAdV genome copy numbers and bacterial and bacteriophage levels in these samples was also evaluated.

Materials and Methods

Environmental Samples

In the bathing season of 2006, 10-l water samples were collected at approximately weekly intervals during the bathing season by 15 different laboratories from nine European countries (see Table 1 for a detailed list of countries), according to ISO 19458 2006. Samples were collected from 24 different sites representing typical seawater as well as freshwater bathing sites in the European Union. Samples were collected at least at 6 m from the shore and 1 m from the surface. Samples were processed within 24 h after collection.

A 10-l sample of artificial seawater or freshwater was used as negative control of the concentration step and an extra sample, spiked with HAdV2 virus grown on A549 cells, was processed as a positive control of the concentration process.

Viral Strains

To confirm the applicability of the assay, a collection of supernatants obtained from adenovirus-infected cell

Table 1 Mean HAdV GC values observed per 100 ml of bathing water collected from different sites (countries)

Samples	Bacteria and phages			HAdV		
Site number (country)	*E. coli* (CFU/100 ml)	Intestinal enterococci (CFU/100 ml)	Somatic coliphages (CFU/100 ml)	+qPCR/+nPCR[a]	HAdV range (GC/100 ml)	HAdV mean value (GC/100 ml)
Marine						
2 (Italy)	<15–30	<15	50–100	17/24	10.1–6482	1.8E+03
8 (UK)	400	108	1414	2/3	30.9–45.8	3.8E+01
9 (Spain)	2418	2418	4122	4/10	44.5–384	1.4E+02
13 (Italy)	473	263	31	7/9	10.2–13640	2.0E+03
14 (Portugal)	1310	252	NT	3/10	2.3–94.8	3.3E+01
15 (Cyprus)	46	77	NT	0/1		
Total seawater				33/57		9.1E+02
Freshwater						
3 (UK)	2319	25008	750	4/8	4.8–213.3	7.4E+01
4 (The Netherlands)	179	46	430	3/14	43.2–89.8	6.4E+01
5 (Italy)	45	538	333	3/3	3–95.6	5.0E+01
6 (Germany)	9982	1511	2131	9/10	1.7–133.6	4.3E+01
7 (France)	12606	1791	1115	9/10	29.8–228.1	6.5E+01
10 (Germany)	3590	106	675	5/10	50.8–298.	1.1E+02
11 (Germany)	1392	1455	1733	3/6	0.6–63.2	2.2E+01
12 (Poland)	284	0	7.2	7/10	3.3–47.2	4.2E+00
16 (UK)	495585	9389	6788	4/4	13.1–202.8	7.4E+01
Total freshwater				47/75		5.6E+01
Total marine + freshwater				80/132		3.2E+02

Mean values of *E. coli*, intestinal enterococci and somatic coliphages per 100 ml of water are also shown

[a] Number of positive QPCR samples out of total of analyzed nested PCR positive samples

NT non tested

cultures from routine clinical analysis comprising representative serotypes of HAdV species A (31), B (3, 7, 7b, 35), C (1, 2, 6), D (37) and F (40, 41) were tested using the qPCR protocol.

During the study, the sensitivity of the qPCR assay applied in the different laboratories was tested by analyzing a commercial quantified suspension of HAdV5 DNA (ABI, Advanced Biotechnologies Incorporated. Columbia, MD, USA). This strain was purchased as an interlaboratory calibration of the quantification technique used since every single laboratory could purchase the quantified product in equivalent storage conditions and use it as a quality control for the sensitivity of the qPCR assays.

Bacteriological Analysis

Escherichia coli (EC) and intestinal enterococci (IE) levels present in the samples were determined by Bio-Rad miniaturized methods using culture in liquid media (most probable number) for the detection and enumeration of EC (ISO 9308-3 1998) and enterococci (ISO 7899-1 1998).

Bacteriophage Analysis

Somatic coliphage titres were determined following the double agar layer procedure as described in ISO 10705-2 2000.

Concentration of Viral Particles from Seawater Samples

Recovery of viral particles from 10-l seawater samples was performed using either a procedure based on the use of cellulose nitrate membrane filters (Wallis and Melnick 1967a, b) and virus elution with glycine-skimmed milk buffer as described in (Bitton et al. 1979a, b) or a method based on a one-step concentration of viruses by direct flocculation with skimmed milk (Calgua et al. 2008).

Concentration of Viral Particles from Freshwater Samples

Recovery of viral particles from 10 l of fresh water was performed by applying a procedure based on the use of

glass wool columns and elution with glycine-beef extract buffer as described previously (Vilagines et al. 1993).

Nucleic Acid Extraction

Experiments were conducted in order to evaluate the most efficient extraction method for every concentration protocol used (data not shown). Nucleic acids were extracted from 5-ml sample concentrates using NucliSense® reagents (Biomeriéux, Boxtel, The Netherlands). For the seawater samples concentrated by the methodology described by Calgua et al. (2008), NucleoSpin RNA virus F (Macherey & Nagel, Germany), was used for extraction of nucleic acids. Nucleic acids were frozen until further qPCR analysis.

Extracted viral nucleic acids were transported frozen when the qPCR assays were performed in a laboratory distant from the laboratory collecting and processing the samples.

Construction of qPCR Standards

The DNA concentrations of plasmid pBR322 containing the HAdV 41 hexon sequence (kindly donated by Dr. Annika Allard, University of Umeå, Sweden) was estimated using Genequant pro (Amersham Biosciences). Ten micrograms of each DNA were linearized with BamHI, purified with the QIAquick PCR purification kit (QIAGEN, Inc.), quantified again and serially diluted such that 10 µl of the sample contained 10^0, 10^1, 10^2, 10^3, 10^4, 10^5 and 10^6 copies of DNA.

The stability of the standard DNA suspension was evaluated in 3 different eluents: DNA eluted with RNAse-free distilled water, Tris–EDTA, and the elution buffer provided in the NucliSens® kit from Biomerieux (Biomérieux, Boxtel, The Netherlands). Aliquots were kept at 4 and −80°C for 3 h and 2 weeks and variations on Ct values were analyzed by applying the qPCR as described. The stability of standard suspensions resuspended in TE buffer was also evaluated by repeated analysis after more than 2 weeks of storage at 4 and −80°C.

qPCR assay for the quantification of HAdV DNA

Samples previously identified to be positive by nested PCR (nPCR) analysis using the primers developed by Allard et al. (2001) were analyzed by qPCR. The assay applied in this study was been described by Hernroth et al. (2002) and is based in the amplification of the HAdV hexon gene. Amplifications were performed in a 25-µl reaction mixture containing 10 µl of DNA and 15 µl of TaqMan® Universal PCR Master Mix (Applied Biosystems) containing 0.9 µM of each primer (AdF and AdR) and 0.225 µM of fluorogenic probe (AdP1) for HAdV detection. TaqMan® Universal PCR Master Mix was supplied in a 2× concentration and contained AmpliTaq Gold® DNA polymerase, dNTPs with dUTP, ROX as passive reference, optimized buffer components and AmpErase® uracil-*N*-glycosylase.

Following activation of the uracil-*N*-glycosylase (2 min, 50°C) and activation of the AmpliTaq Gold for 10 min at 95°C, 40 cycles (15 s at 95°C and 1 min at 60°C) were performed in the detection system currently used in every laboratory: Stratagene Mx3000P, ABI Sequence Detection System 7000 and LightCycler 480.

Neat and a tenfold dilution of the DNA suspensions were run in duplicate (4 runs/sample) for analyzing environmental samples whereas each dilution of standard DNA suspensions (from 10^0 to 10^6) was run in triplicate. In all qPCRs, the amount of DNA was defined as the mean of the data obtained. Standard precautions were applied in all assays, including separate areas for the different steps of the protocol and addition of non-template control (NTC) and non-amplification control (NAC) to each run. The presence of enzymatic inhibitors in the samples was evaluated by adding 10^4 GC of target DNA as an external control to the environmental samples assayed.

Sequence Analysis of the PCR Products Obtained by nPCR

The amplicons obtained after nPCR assays of HAdV were purified using the QIAquick PCR purification kit (QIAGEN, Inc.). Purified DNA was directly sequenced with the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction kit version 3.1 with Ampli Taq® DNA polymerase FS (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 and extension at 60°C for 4 min. The nested primers nehex3deg and nehex4deg described by Allard et al. (2001) were used for sequencing ast a concentration of 0.05 µM.

The results were checked using the ABI PRISM 377 automated sequencer (Perkin-Elmer, Applied Biosystems). The sequences were compared with the GenBank and the EMBL (European Molecular Biology Library) using the basic BLAST program of the NCBI (The National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/BLAST/). Alignments of the sequences were carried out using the ClustalW program of the EBI (European Bioinformatics Institute of the EMBL, http://www.ebi.ac.uk/clustalw/).

Statistical Analysis

In order to measure the correlation between HAdV genome copy numbers with the three other quantitative biological indicators (*E. coli* (EC), intestinal enterococci (IE) and somatic coliphages (SC) a synthetic approach based on a linear model was applied, simultaneously taking into account the possible effects of the water type and the laboratory. The variables were first transformed by using the $\log(x + 1)$ function. With all the quantitative variables transformed, the model included the following sources of variation: (1) *type of water*, a fixed factor with two levels (marine or fresh), denoted by α in the equation below, (2) *laboratory*, a *nested* factor to water type, according to the ANOVA terminology, and denoted by β in the equation, and (3), the *interaction* between the *laboratory* and the *covariate* included in the model (EC, or IE or SC). This latter parameter is denoted by γ. We thus have three separate models including in each one a different covariate. For the three models, the following generic equation is applied:

$$y_{ijk} = \mu + \alpha_i + \beta_{j(i)} + \gamma_{j(i)} x_{ijk} + e_{ijk}$$

In the equation, y_{ijk} is the log transform of HAdV, x_{ijk} the log transform of the biological indicator considered (EC, or IE or SC), and e_{ijk} is the error term of the linear model. The sub indexes denote that the data correspond to the k sample in the j laboratory on the i water type. In the ANOVA literature, it is a classical model which allows testing on several groups the equality of the slopes of a linear relation between two variables.

Notice that if the ANOVA table shows the interaction γ term to be statistically significant, it must be interpreted that the slopes of the linear relation between x and y are different for some laboratories. If such is the case, the analysis must be conducted separately on each laboratory's data to estimate the linear relation between the variables. That is, the model must be reduced to the ordinary simple linear regression, splitting the full data set into several subsets corresponding to each laboratory:

$$y_{ijk} = \beta + \gamma x_{ijk} + e_{ijk}$$

All the statistical tests were computed using the statistical package SPSS 15.0.1 (SPSS Inc., Chicago, IL, USA).

Results

Specificity and Sensitivity of the qPCR

The assay was selected for the quantification of HAdV in bathing waters because it was shown previously that the sensitivity of this assay was significantly higher than that obtained by other qPCR assays (Bofill-Mas et al. 2006).

The specificity of the assay was confirmed with a wide range of strains isolated by cell cultures from approximately 100 clinical samples. Serotypes of human adenovirus species A (adenovirus 31), B (3, 7, 7b, 35), C (1, 2, 6), D (37) and F (40, 41) were quantified by applying the HAdV qPCR assay here described. High concentrations of human polyomaviruses JCPyV and BKPyV, commonly present in urban sewage samples, were not detected by using the HAdV qPCR assay (data not shown).

The sensitivity of the assay was estimated to be 1–10 genome copies (GC) based on the data obtained in 20 different HAdV qPCR runs using synthetic plasmid DNA and the quantification of the commercial quantified suspension of HAdV5 DNA (ABI, Advanced Biotechnologies Incorporated. Columbia, MD, USA). A fluorescent signal was obtained in 90% of the runs when analyzing 10^0 GC according to spectrophotometrical measurements of standards. Thus, the sensitivity of the assay was confirmed to be between 1 and 10 GC for HAdV 5. The commercial standard was used as an intra laboratory control in all the laboratories performing qPCR analysis.

Stability of the DNA used as Standard

To guard against the degradation of the qPCR standard DNA, stability was determined following storage for 3 h at 4 and −80°C in one of: molecular grade water, TE, or Biomérieux kit elution buffer. No significant differences were observed after storage of the DNA with the different eluents at −80°C for 3 h and 2 weeks. Ct values showed differences between different eluents and between different temperatures lower than 1 Ct. Moreover, during the study aliquots of plasmids resuspended in TE were kept at 4°C for more than 2 weeks and no differences in the Ct values were observed during qPCR reactions.

Virus recovery efficiency from water samples

HAdV2 virus preparations were used to spike positive control samples before concentration and nucleic acid extraction in order to quantify the recovery efficiency of the methods used.

The concentration method applied to determine the recovery of viruses from freshwater samples (glass wool concentration) showed an efficiency ranging from 6 to 81.5%. Two different concentration protocols had been applied to marine samples: a nitrocellulose negatively charged membrane filter-based method showed highly variable recoveries ranging between 1.9 and 35.4% whereas one-step concentration by skimmed milk flocculation showed recoveries of 42.5–52.0% as described by Calgua et al. (2008).

Quantification of HAdV in Recreational Waters

A total of 132 nPCR HAdV positive seawater and freshwater samples were analyzed by the qPCR assay in different laboratories. The results obtained are summarized as mean values of all samples tested at each collection site (Table 1).

Eighty out of 132 samples (60.6%) tested positive with a mean value of 3.2×10^2 GC/100 ml of water. The percentage of positive samples was similar in both types of bathing water tested: 59.6% for marine and 61.3% for freshwater samples and mean values were 9.1×10^2 (3.3×10^1–2.0×10^3) and 5.6×10^1 GC/100 ml (4.2×10^0–1.1×10^2) of marine and freshwater, respectively.

Forty-seven samples were further typed by nPCR and sequenced: HAdV serotypes 12, 19, 31, 40 and 41 sequences were obtained with Ad41 being the serotype most commonly found.

Correlation of HAdV genome Copy Numbers with Bacteria or/and Bacteriophage Titres

The relation between HAdV and the other microbiological parameters observed was highly variable and the statistical analysis of the data showed no significant correlation between the numbers of HAdV, bacterial standards and somatic coliphages analyzed.

For every covariate analyzed, Table 2 shows strong evidence against equality of the slopes (P values < 0.05). There was also strong evidence against equality on the mean of HAdV detected by the laboratories (P values <0.05), but not in the water type (P values >0.05). The analysis of the residuals (not shown) confirms the adequacy of the log-transformation on the variables. Because the laboratory origin has significant effects on the slopes of the model for the three covariates (*E. coli*, IE, SC) the samples were analyzed separately. The linear regression analysis showed a significant linear relation between HAdV and the different variables tested in four laboratories (Table 3). Two laboratories presented significant correlation between HAdV and *E. coli*, IE and SC concentrations while one laboratory presented significant correlations between HAdV and *E. coli* concentrations. One laboratory showed a negative correlation that could be related to a non-recent contamination event, however, it should be considered that the number of samples in this specific site is limited (24).

Table 2 P values corresponding to the different ANOVA tests

Source of variation	*E. coli*	IE	SC
Water type	0.497	0.048	0.108
Laboratory	<0.001	0.001	0.007
Covariate slope	0.001	0.015	0.031
R^2	0.486	0.432	0.417

Each column corresponds to a model including one covariate

Table 3 Laboratories showing significant linear relations between HAdV concentration and other microbiological parameters studied in the samples tested

Laboratory code	*E.coli*	IE	SC
Laboratory 2	−0.46 (0.037)		
Laboratory 4	0.62 (0.025)	0.65 (0.015)	0.66 (0.015)
Laboratory 9	0.72 (0.020)	0.69 (0.029)	0.72 (0.021)
Laboratory 10	0.74 (0.015)		

Correlation coefficient and P value (in parentheses) of the linearity test are shown

Discussion

In order to have rapid quantitative information on the level of viral contamination in the recreational waters studied, a standardized quantitative real-time PCR assay was applied to the specific quantification of HAdV in recreational water samples. Cell culture assays, though providing quantitative information on infectivity have a very high cost and take several days to produce a result. Moreover, not all HAdV produce a distinct cytopathic effect in culture. The study presented was part of the VIROBATHE project that had as its main objective of evaluating the feasibility of trans-European analysis of viruses in recreational waters. The samples analyzed were selected on the basis of the results obtained by nPCR during a surveillance study including 15 European participant laboratories from nine different countries. The overall objective of this study was to evaluate the applicability of the quantification of HAdV by qPCR as an index of the presence of human faecal contamination in European recreational waters.

HAdV were detected and quantified in both marine and freshwater collection sites including sites that, according to the European Bathing Water Directive (2006/7/EC), would be classified as bathing sites with good or excellent water quality, indicating that these are not free of the presence of HAdV DNA. However, it should also be acknowledged that although HAdVs are known to be more stable than bacterial standards in the environment, especially in sea water and in most water treatments (Calgua et al. 2008; Albinana-Gimenez et al. 2009), the presence of viral DNA does not necessarily indicate the presence of infectious viruses. However, as part of the VIROBATHE project, the infectivity of HAdV was evaluated in some representative samples by ICC-PCR (Dong et al. 2010) and infectious HAdV were recovered from collection sites of laboratories 4, 10 and 13 (Fig. 1).

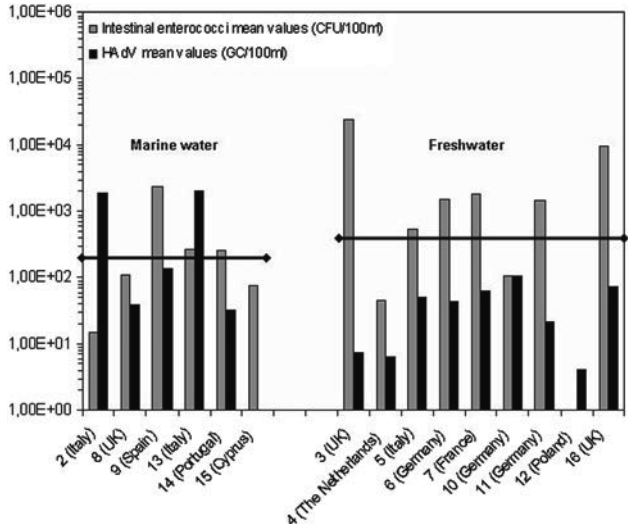


Fig. 1 Comparison between mean value of IE and HAdV GC per 100 ml of water in the studied sites. *B* indicate the maximum level of IE per each type of water (coastal and transitional or inland) required for good quality waters (based upon a 95-percentile evaluation) as established in the European Bathing Water Directive (2006/7/EC)

Standardization of the qPCR assay described was straightforward. Frozen nucleic acid extractions from overseas laboratories were transported without major problems during this study, and the DNA used as standard in the qPCR assays was also shown to retain high stability under different storage conditions.

The percentage of positive samples from the total number of samples collected in the study could not be evaluated, since qPCR was done on samples which had already tested positive by nPCR. However, as expected, high variability in the percentage of positivity has been observed in other studies (Van Heerden et al. 2003, 2005b; Miagostovich et al. 2008; Verheyen et al. 2009).

The methods applied in this study represent low cost methods with acceptable values of recovery efficiencies, for marine samples concentrated by nitrocellulose membranes (1.9–35.4%), while alternative concentration methods by flocculation with skimmed milk showed more homogeneous recoveries (42.5–58%). Variable recoveries ranging from 6–81.5% for freshwater sample concentrated by using glass wool were obtained.

Not only some of the previously positive samples by nPCR were negative for qPCR but also some samples which had previously tested negative by nPCR produced positive results by qPCR (data not shown). Observed differences between nPCR and qPCR may be due to several factors such as small differences in sensitivity of qPCR and nPCR, different responses to enzymatic inhibition between qPCR and nPCR. qPCR because reduce the manipulation of the sample compared to nPCR and is less prone to PCR contaminants than conventional nPCR.

It should be also considered that when HAdV are present in concentrations near the limit of detection of the technique the analysis of different replicates may show different results.

Enzymatic inhibition has been observed by other authors when applying qPCR to environmental samples (e.g. Jiang 2006). In our hands, enzymatic inhibition had been observed when applying the assay to samples with higher level of contamination (Bofill-Mas et al. 2006) and also in this study was observed in some of the sites studied in the undiluted sample. This inhibition is not inherent to qPCR as it has also been observed during this study when analyzing these samples by conventional nPCR techniques. Future efforts should be conducted to decrease enzymatic inhibition of samples to be tested by qPCR.

Different HAdV serotypes have been observed in positive qPCR, with HAdV 41 being the most commonly isolated serotype. The high prevalence of HAdV 41 in the samples studied is in accordance with what has been previously reported (Haramoto et al. 2007; Xagoraraki et al. 2007).

Statistical analysis evaluating potential correlations between the numbers of HAdV obtained in the study and the observed concentrations of IE, *E. coli* and SC in the tested samples showed no homogeneous linear relation between HAdV and the other variables when considering all the data.

The analysis of the linear model showed that the water type had no significant effects on the HAdV concentration measured. It shows also that the linear relation between HAdV and the other variables is not homogeneous across the laboratories and separate linear regressions show that only in three laboratories (4, 9 and 10) there is a significant correlation coefficient between HAdV and at least one of the covariates.

The qPCR methodology applied appears to be a technology feasible to standardise and to be repeatable in routine laboratories. The HAdV qPCR assay provides a quantitative estimation of the presence and sources of faecal contamination in the water and should be considered as a molecular index providing complementary information for the control of water quality in bathing water.

Acknowledgements The study here described was part of the Project VIROBATHE, a European Union Research Framework 6 funded project (Contract No. 513648) aimed at the rapid detection of viruses in recreational waters (www.virobathe.org). We thank all VIROBATHE participants for their collaboration providing us with samples to be analyzed in this study. The authors thank David Kay for his support as coordinator of VIROBATHE. During the development of this study Byron Calgua de León was a fellow of the MAEC-AECID, Spanish Government (Ministerio de Asuntos Exteriores y Cooperación).We thank Serveis Científico Tècnics of the University of Barcelona for the sequencing of PCR products.

References

Albinana-Gimenez, N., Miagostovich, M. P., Calgua, B., Huguet, J. M., Matia, L., & Girones, R. (2009). Analysis of adenoviruses and polyomaviruses quantified by qPCR as indicators of water quality in source and drinking-water treatment plants. *Water Research, 43*(7), 2011–2019.

Allard, A., Albinsson, B., & Wadell, G. (2001). Rapid typing of human adenoviruses by a general PCR combined with restriction endonuclease analysis. *Journal of Clinical Microbiology, 39*(2), 498–505.

Bitton, G. B., Feldberg, B. V., & Farrah, S. R. (1979a). Concentration of enterovirus from seawater and tapwater by organic flocculation using nonfat dry milk and casein. *Water, Air, and Soil Pollution, 12*, 187–195.

Bitton, G. B., Charles, M. J., & Farrah, S. R. (1979b). Virus detection in soils: A comparison of four recovery methods. *Canadian Journal of Microbiology, 25*(8), 874–880.

Bofill-Mas, S., Albinana-Gimenez, N., Clemente-Casares, P., Hundesa, A., Rodriguez-Manzano, J., Allard, A., et al. (2006). Quantification and stability of human adenoviruses and polyomavirus JCPyV in wastewater matrices. *Applied and Environmental Microbiology, 72*, 7894–7896.

Calgua, B., Mengewein, A., Grunert, A., Bofill-Mas, S., Clemente-Casares, P., Hundesa, A., et al. (2008). Development and application of a one-step low cost procedure to concentrate viruses from seawater samples. *Journal of Virological Methods, 153*(2), 79–83.

Choi, S., & Jiang, S. C. (2005). Real-time PCR quantification of human adenoviruses in urban rivers indicates genome prevalence but low infectivity. *Applied and Environmental Microbiology, 71*(11), 7426–7433.

Crabtree, K. D., Gerba, C. P., Rose, J. B., & Haas, C. N. (1997). Waterborne adenovirus: A risk assessment. *Water Science and Technology, 35*, 1–6.

Dong, Y., Kim, J., & Lewis, G. D. (2010). Evaluation of methodology for detection of human adenoviruses in wastewater, drinking water, stream water and recreational waters. *Journal of Applied Microbiology, 108*, 800–809.

Formiga-Cruz, M., Tofino-Quesada, G., Bofill-Mas, S., Lees, D. N., Henshilwood, K., Allard, A. K., et al. (2002). Distribution of human virus contamination in shellfish from different growing areas in Greece, Spain, Sweden, and the United Kingdom. *Applied and Environmental Microbiology, 68*(12), 5990–5998.

Gerba, C. P., Gramos, D. M., & Nwachuku, N. (2002). Comparative inactivation of enteroviruses and adenovirus 2 by UV light. *Applied and Environmental Microbiology, 68*, 5167–5169.

Haramoto, E., Katayama, H., Oguma, K., & Ohgaki, S. (2007). Quantitative analysis of human enteric adenoviruses in aquatic environments. *Journal of Applied Microbiology, 103*(6), 2153–2159.

He, J. W., & Jiang, S. C. (2005). Quantification of enterococci and human adenoviruses in environmental samples by real-time PCR. *Applied and Environmental Microbiology, 71*(5), 2250–2255.

Hernroth, B. E., Conden-Hansson, A. C., Rehnstam-Holm, A. S., Girones, R., & Allard, A. K. (2002). Environmental factors influencing human viral pathogens and their potential indicator organisms in the blue mussel, *Mytilus edulis*: The first Scandinavian report. *Applied and Environmental Microbiology, 68*(9), 4523–4533.

ISO 7899-1, 1998 ISO 7899-1. (1998). *Water quality—detection and enumeration of intestinal enterococci. Part 1. Miniaturized method (most probable number) for surface and waste water*. Geneva: International Organization for Standardization.

ISO 9308-3, 1998 ISO 9308-3. (1998). *Water quality—detection and enumeration of Escherichia coli and coliform bacteria. Part 3. Miniaturized method (most probable number) for the detection and enumeration of E. coli in surface and waste water*. Geneva: International Organization for Standardization.

ISO 10705-2, 2000 ISO 10705-2, (2000). Water quality—detection and enumeration of bacteriophages. Part 2. Enumeration of somatic coliphages. Geneva: International Organization for Standardization.

ISO 19458, 2006 ISO 19458, (2006). *Water quality—Sampling for microbiological analysis. Guidance on planning water sampling regimes, on sampling procedures for microbiological analysis and on transport, handling and storage of samples until analysis begins*. Geneva: International Organization for Standardization.

Jiang, S. C. (2006). Human adenoviruses in water: Occurrence and health implications: A critical review. *Environmental Science and Technology, 40*(23), 7132–7140.

Jiang, S., Dezfulian, H., & Chu, W. (2005). Real-time quantitative PCR for enteric adenovirus serotype 40 in environmental waters. *Canadian Journal of Microbiology, 51*, 393–398.

Miagostovich, M. P., Ferreira, F. F., Guimarães, F. R., Fumian, T. M., Diniz-Mendes, L., Luz, S. L., et al. (2008). Molecular detection and characterization of gastroenteritis viruses occurring naturally in the stream waters of Manaus, central Amazonia, Brazil. *Applied and Environmental Microbiology, 74*(2), 375–382.

Muscillo, M., Pourshaban, M., Iaconelli, M., Fontana, S., Di Grazia, A., Manzara, S., et al. (2008). Detection and quantification of human adenoviruses in surface waters by nested PCR, TaqMan real-time PCR and cell culture assays. *Water, Air, and Soil Pollution, 191*, 83–93.

Pina, S., Puig, M., Lucena, F., Jofre, J., & Girones, R. (1998). Viral pollution in the environment and in shellfish: Human adenovirus detection by PCR as an index of human viruses. *Applied and Environmental Microbiology, 64*(9), 3376–3382.

Puig, M., Jofre, J., Lucena, F., Allard, A., Wadell, G., & Girones, R. (1994). Detection of adenoviruses and enteroviruses in polluted waters by nested PCR amplification. *Applied and Environmental Microbiology, 60*, 2963–2970.

Sinclair, R, G., Jones, E. L., & Gerba, C. P. (2009). Viruses in recreational water-borne disease outbreaks: A review. *Journal of Applied Microbiology, 107*, 1769–1780.

Thurston-Enriquez, J. A., Haas, C. N., Jacangelo, J., & Gerba, C, P. (2003). Chlorine inactivation of adenovirus type 40 and feline calicivirus. *Applied and Environmental Microbiology, 69*, 3979–3985.

Tree, J. A., Adams, M. R., & Lees, D. N. (2003). Chlorination of indicator bacteria and viruses in primary sewage effluent. *Applied and Environmental Microbiology, 69*(4), 2038–2043.

Xagoraraki, I., Kuo, D. H., Wong, K., Wong, M., & Rose, J. B. (2007). Occurrence of human adenoviruses at two recreational beaches of great lakes. *Applied and Environmental Microbiology, 73*(24), 7874–7881.

Van Heerden, J., Ehlers, M. M., Heim, A., & Grabow, W. O. (2005a). Prevalence, quantification and typing of adenoviruses detected in river and treated drinking water in South Africa. *Journal of Applied Microbiology, 99*(2), 234–242.

Van Heerden, J., Ehlers, M. M., Vivier, J. C., & Grabow, W. O. (2005b). Risk assessment of adenoviruses detected in treated drinking water and recreational water. *Journal of Applied Microbiology, 99*(4), 926–933.

Van Heerden, J., Ehlers, M. M., Van Zyl, W. B., & Grabow, W. O. (2003). Incidence of adenoviruses in raw and treated water. *Water Research, 37*, 3704–3708.

Verheyen, J., Timmen-Wego, M., Laudien, R., Boussaad, I., Sen, S., Koc, A., Uesbeck, A., Mazou, A. F., & Pfister, H. (2009).

Detection of adenoviruses and rotaviruses in drinking water sources used in rural areas of Benin, West Africa. *Applied and Environmental Microbiology, 75*(9), 2798–2801.

Vilagines, P., Sarrette, B., Husson, G. P., & Vilagines, R. (1993). Glass wool for virus concentration at ambient water pH level. *Water Science and Technoogy, 27*, 199–306.

Wallis, C., & Melnick, J. L. (1967a). Concentration of viruses on membrane filters. *Journal of Virology, 1*, 472–477.

Wallis, C., & Melnick, J. L. (1967b). Concentration of viruses from sewage by adsorption onto Millipore membranes. *Bulletin of the World Health Organization, 36*, 219–225.

Resumen Estudio 4:

Detection and quantification of classical and emerging viruses in river water by applying a low cost one-step procedure

Calgua B, Fumian T, Rusinol M, Rodriguez-Manzano J, Mbayed V, Bofill-Mas S, Miagostovich M, and Girones R.

Water Research, 2013, 47, 2797 - 2810.

RESUMEN:

En los últimos años se ha venido reportando la presencia de virus o cepas emergentes, e incluso una gran variedad de virus nuevos en diferentes tipos de agua.

En este estudio se describe la adaptación de SMFP (método descrito para agua de mar en el Estudio 1), para aplicarlo en agua de río y su utilización para el estudio de virus patógenos clásicos y emergentes en agua de río de Barcelona y Río de Janeiro.

El método de SMFP para agua de río se validó en ensayos inter- e intra- laboratorio llevados a cabo en Barcelona y Río de Janeiro. Para adaptar el método de SMFP, se determinó que la conductividad de la muestra debía ser ≥ 1,5 mS, de no ser así, este parámetro debía ajustarse añadiendo sales de agua de mar artificial hasta obtener valores mayores que 1,5 mS. Los resultados de validación de SMFP mostraron que este método recuperaba aproximadamente el 50 % de los virus añadidos artificialmente a las muestras (adenovirus humanos, polyomavirus JC, norovirus y rotavirus). Además estos resultados fueron homogéneos, mostrando un coeficiente de variación menor al 50%.

Tres grupos de virus fueron analizados en agua de río después de validar SMFP; (i) virus nuevos recientemente descritos: klassevirus (KV), un asfarvirus-*like* (ASFLV) y los polyomavirus Merkel *cell* (MCPyV), KI (KIPyV) y WU (WUPyV); (ii) virus responsables de gastroenteritis: norovirus geno-grupo II (Nov GGII) y rotavirus humanos (RV), y (iii) los indicadores víricos de contaminación fecal: adenovirus humanos (HAdV) y polyomavirus JC (JCPyV). La detección de los virus se realizó por ensayos de nPCR/qPCR TaqMan®. Para ASFLV y KV se diseñaron oligonucleótidos específicos y ensayos de nPCR y RT-nPCR, respectivamente. Tanto en Barcelona como Río de Janeiro, todas las muestras fueron

positivas para HAdV y JCPyV (12/12). En Barcelona solamente se detectó NoV GGII y MCPyV en 5/6 y 3/6 muestras, respectivamente. Mientras que en Río de Janeiro se detectó, NoV GGII, KV, ASFLV y MCPyV en 2/6, 2/6, 1/6 y 3/6 muestras, respectivamente. Por otro lado, RV solamente se analizó en Río de Janeiro, detectándose en 4 de 6 muestras.

Los resultados mostraron que SMFP es un método eficiente para para concentrar virus DNA o RNA en muestras de río. Además es un método fácil de implementar y estandarizar. La información de la presencia de los virus estudiados, ha contribuido a expandir los conocimientos de la distribución de estos en diferentes zonas geográficas así como su persistencia en el medio ambiente.

Available online at www.sciencedirect.com

SciVerse ScienceDirect

journal homepage: www.elsevier.com/locate/watres

Detection and quantification of classic and emerging viruses by skimmed-milk flocculation and PCR in river water from two geographical areas

Byron Calgua [a], *Tulio Fumian* [b], *Marta Rusiñol* [a], *Jesus Rodriguez-Manzano* [a], *Viviana A. Mbayed* [c], *Silvia Bofill-Mas* [a], *Marize Miagostovich* [b], *Rosina Girones* [a,*]

[a] *Department of Microbiology, Faculty of Biology, University of Barcelona, Av. Diagonal 643, Barcelona 08028, Spain*
[b] *Laboratory of Comparative and Environmental Virology, Oswaldo Cruz Institute, Avenida Brasil 4365, Rio de Janeiro, Brazil*
[c] *Laboratory of Virology, Faculty of Pharmacy and Biochemistry, University of Buenos Aires, Junín 956, Buenos Aires, Argentina*

ARTICLE INFO

Article history:
Received 24 September 2012
Received in revised form
16 February 2013
Accepted 21 February 2013
Available online 13 March 2013

Keywords:
Emerging virus
Polyomavirus
Merkel cell polyomavirus
Klassevirus
Asfarvirus-like virus
Rotavirus
Norovirus
Adenovirus
River water
Concentration method
Viral indicator

ABSTRACT

Molecular techniques and virus concentration methods have shown that previously unknown viruses are shed by humans and animals, and may be transmitted by sewage-contaminated water. In the present study, 10-L river-water samples from urban areas in Barcelona, Spain and Rio Janeiro, Brazil, have been analyzed to evaluate the viral dissemination of human viruses, validating also a low-cost concentration method for virus quantification in fresh water. Three viral groups were analyzed: (i) recently reported viruses, klassevirus (KV), asfarvirus-like virus (ASFLV), and the polyomaviruses Merkel cell (MCPyV), KI (KIPyV) and WU (WUPyV); (ii) the gastroenteritis agents noroviruses (NoV) and rotaviruses (RV); and (iii) the human fecal viral indicators in water, human adenoviruses (HAdV) and JC polyomaviruses (JCPyV). Virus detection was based on nested and quantitative PCR assays. For KV and ASFLV, nested PCR assays were developed for the present study. The method applied for virus concentration in fresh water samples is a one-step procedure based on a skimmed-milk flocculation procedure described previously for seawater. Using spiked river water samples, inter- and intra-laboratory assays showed a viral recovery rate of about 50% (20–95%) for HAdV, JCPyV, NoV and RV with a coefficient of variation $\leq$50%. HAdV and JCPyV were detected in 100% (12/12) of the river samples from Barcelona and Rio de Janeiro. Moreover, NoV GGII was detected in 83% (5/6) and MCPyV in 50% (3/6) of the samples from Barcelona, whereas none of the other viruses tested were detected. NoV GGII was detected in 33% (2/6), KV in 33% (2/6), ASFLV in 17% (1/6) and MCPyV in 50% (3/6) of the samples from Rio de Janeiro, whereas KIPyV and WUPyV were not detected. RV were only analyzed in Rio de Janeiro and resulted positive in 67% (4/6) of the samples. The procedure applied here to river water represents a useful, straightforward and cost-effective method that could be applied in routine water quality testing. The results of the assays expand our understanding of the global distribution of the viral pathogens studied here and their persistence in the environment.

* *Corresponding author*. Tel.: +34 93 402 1483; fax: +34 93 403 9047.
E-mail address: rgirones@ub.edu (R. Girones).
0043-1354/$ – see front matter
http://dx.doi.org/10.1016/j.watres.2013.02.043

1. Introduction

Microbiological pollution in water represents a health risk for human populations. Many viral infectious diseases are transmitted by consumption of or contact with water contaminated with sewage (Fong and Lipp, 2005). The discharge of untreated or even treated sewage into the aquatic environment is well known as the main cause of fecal pollution in water. The treatments commonly applied for wastewater depuration do not guarantee the absence of viral pathogens (Gantzer et al., 1998; Pusch et al., 2005; van den Berg et al., 2005; Bofill-Mas et al., 2006; Fumian et al., 2010).

Among the most frequently detected human viruses in water samples are the well-known groups of gastroenteric viruses: rotaviruses (RV) and noroviruses (NoV), together with the proposed human viral indicators (Puig et al., 1994; Pina et al., 1998; Bofill-Mas et al., 2000): human adenoviruses (HAdV) and JC polyomavirus (JCPyV). Furthermore, recent studies have shown that new and emerging viruses may also be present in water contaminated with sewage, such as: the new polyomaviruses Merkel cell, KI and WU (MCPyV, KIPyV and WUPyV); the new picornavirus klassevirus (KV); and an asfarvirus-like virus (Bofill-Mas et al., 2000; Miagostovich et al., 2008; Hotlz et al., 2009; Loh et al., 2009; Bofill-Mas et al., 2010b; Lodder et al., 2010; Wyn-Jones et al., 2011).

Rotavirus species A is considered the leading cause of severe diarrhea in children worldwide and according to the WHO, RV-diarrhea results in approximately half a million deaths and 2.4 million hospitalizations in developing countries each year (Parashar et al., 2009). RV are ubiquitous, they will have infected virtually all children by the time they reach 5 years of age regardless of socioeconomic status or geography; they are environmentally stable; and they are spread via direct or indirect contact with infected individuals (Schael et al., 2009). NoV are the leading cause of food-borne disease outbreaks worldwide; it is estimated that they cause 80–95% of all cases of gastroenteritis globally and may soon eclipse RV as the most common cause of severe pediatric gastroenteritis (Patel et al., 2008; Koo et al., 2010). NoVs are the major cause of sporadic outbreaks of infectious gastroenteritis and occasionally lead to hospitalization (Glass et al., 2009). Outbreaks tend to be most common in closed populations, such as childcare centers and cruise ships, and tend to involve children past infancy as well as adults (Khan and Bass, 2010; Glass et al., 2009). NoV are divided into five genogroups based on the phylogenetic analysis of the viral capsid (VP1) gene, and further subdivided into genetic clusters called genotypes. Genogroups I (GGI), II (GGII) and IV (GGIV) are the human strains (Glass et al., 2009; Koo et al., 2010). Despite this diversity, only a few strains, primarily those of genogroup II and genotype 4 (GGII.4), have been responsible for the majority of cases and outbreaks of food-borne infections in recent years (Barreira et al., 2010; Ferreira et al., 2010; Bull and White, 2011; Prado et al., 2011).

The DNA viruses HAdV and JCPyV have been proposed as human fecal/urine indicators in the environment (Puig et al., 1994; Pina et al., 1998; Bofill-Mas et al., 2000). They are ubiquitous as they are excreted by a high percentage of the human population. Several studies have reported an elevated prevalence of HAdV and JCPyV in water samples from different geographical areas (Bofill-Mas et al., 2000; Albinana-Gimenez et al., 2006; McQuaig et al., 2006; Miagostovich et al., 2008; McQuaig et al., 2009; Wyn-Jones et al., 2011). Although current policies concerning water quality include the use of bacterial indicators *E. coli* and intestinal enterococcus to evaluate microbiological water quality, various studies have shown that bacterial levels do not always correlate with viral presence. This is particularly so when the concentrations of fecal bacterial indicators are low and is probably due to the high environmental stability of HAdV and JCPyV (Brownell et al., 2007; Colford et al., 2007; Calgua et al., 2008; Wyn-Jones et al., 2011). HAdV are grouped into 7 species (A–G), which have been widely reported to cause a broad range of clinical manifestations including respiratory tract infection, acute conjunctivitis, cystitis, gastroenteritis, and systemic infections. Antibodies against JCPyV were detected in over 80% of humans worldwide (Weber et al., 1997) and consequently their presence in water may not represent a significant health risk for most of the population. The pathogenicity of JCPyV is commonly associated with progressive multifocal leukoencephalopathy (PML) in immunocompromised states and has attracted attention due to its reactivation in some patients with multiple sclerosis and other autoimmune diseases when treated with immunomodulators (Berger and Major, 1999; Yousry et al., 2006). The kidneys and bone marrow are sites of chronic and latent infection with JCPyV, which is also excreted in the urine of healthy individuals and patients with PML (Kitamura et al., 1990; Koralnik et al., 1999).

MCPyV, KIPyV and WUPyV are novel viruses that have only recently been reported (Allander et al., 2007; Gaynor et al., 2007; Feng et al., 2008). Similarly to JCPyV, infection by these three viral agents is widespread among the human population (Babakir-Mina et al., 2009). They persist in a latent state in an unidentified body location and they can reactivate in a setting of immune suppression due to immunosuppressive drugs or other medical conditions (Babakir-Mina et al., 2009). KIPyV and WUPyV have been detected in the respiratory tract, suggesting that they might play a role in at least a subset of pneumonia infections in immunocompromised patients (Babakir-Mina et al., 2009). Moreover, they have been detected in various types of samples, including blood, feces, plasma and the tonsils (Babakir-Mina et al., 2009). Although KIPyV has been detected in lung cancer patients, only MCPyV has been strongly associated with being the primary human oncogenic polyomavirus candidate (Feng et al., 2008; Foulongne et al., 2008; Babakir-Mina et al., 2009), and has been found to be monoclonally integrated into the genome of Merkel cell carcinomas (Feng et al., 2008). Interestingly it has been suggested that MCPyV forms part of the skin microbiome in humans (Wieland et al., 2009; Schowalter et al., 2010; Moens et al., 2011; Foulongne et al., 2012). KIPyV, WUPyV and MCPyV have also been found in sewage samples, with MCPyV being detected most frequently (Bofill-Mas et al., 2010a,b), which could mean that it is more prevalent in silent infections or that it is a virus that is highly excreted.

The proposed new picornavirus KV was identified by deep-sequencing in stool samples from Australia and the USA, and its presence was confirmed in urban sewage from Barcelona

by PCR (Holtz et al., 2009). Phylogenetic analysis shows that KV is most closely related to the Aichi virus in the genus *Kobuviru*, a known cause of food-borne gastroenteritis in humans (Holtz et al., 2009; Greninger et al., 2009). KV has also been reported in South Korea, China and the USA (Northern California and Missouri), and in all cases it was associated with gastroenteritis in infants (Greninger et al., 2010; Han et al., 2010; Shan et al., 2010). Deep-sequencing also detected an asfarvirus-like virus in human serum from the Middle East and urban sewage from Barcelona (Loh et al., 2009). The *Asfarviridae* family (single double-stranded DNA) comprises a single genus with only one previously reported species, the asfarvirus, which primarily infects swine, leading to African swine fever. It is considered endemic to sub-Saharan Africa, but has been introduced to countries in Europe, South America and the Caribbean. Phylogenetic analyses show that ASFLV sequences are most closely related to the asfarvirus but are highly divergent from known asfarviruses (ASFV) strains. Therefore ASFLV is considered to be derived from at least one novel virus in the *Asfarviridae* family (Loh et al., 2009). Although ASFV is not known to infect humans even where the virus is endemic in pigs, identification of ASFLV in serum from multiple human patients suggests that human infection might occur.

The presence and concentration of viral pathogens in wastewater may vary according to the wastewater treatments, geographical area, season, and the hygiene and sanitary conditions. The use of new approaches in molecular detection such as viral metagenomics studies of stools (Finkbeiner et al., 2008), urban sewage (Cantalupo et al., 2011) and water matrices (Rosario et al., 2009) indicate that the number of viruses reported to date is tiny compared to the results of the new studies.

The recovery of viruses from water samples such as river water, seawater and groundwater, where fecal contamination could be low or moderate, requires the concentration of viruses from several liters of sample into a much smaller volume. Probably the most frequently used procedures to concentrate viruses are the two-step methods based on adsorption–elution protocols with a second concentration step, commonly by organic flocculation with beef extract. Those methods include the use of electropositive or negative nitrocellulose membranes or cartridges, glass wool and fiber glass (Sobsey et al., 1973; Vilaginès et al., 1993; Pallin et al., 1997; Lambertini et al., 2008; Albinana-Gimenez et al., 2009). Albinana-Gimenez et al. (2009) reported that glass wool columns are more efficient than the electropositive filters tested in the study; they recovered HAdV (1.21%) and JCPyV (13.7%) by qPCR from 50 L of fresh water. Lambertini et al. (2008), also using a glass wool method, obtained viral recoveries of 70%, 21% and 29%, for poliovirus, adenovirus 41 and norovirus respectively. Haramoto et al. (2004) and Katayama et al. (2002) described two-step methods using electronegative membranes, an inorganic elution with 1 M NaOH and finally a second concentration step using Centripep. They reported viral recoveries for poliovirus of around 90%. Calgua et al. (2008) describe a one-step concentration method based on organic flocculation with skimmed milk to concentrate viruses from 10 L of seawater and reported a viral recovery of 50% for HAdV by qPCR. The protocol using skimmed-milk flocculation presented good recoveries from seawater and lower intra-laboratory variability than other common procedures (Girones et al., 2010), it is also more simple, has a lower cost and is a useful protocol for the routine analysis of large numbers of samples.

In the present study, human viruses grouped into emerging viruses (KIPyV, WUPyV, MCPyV, KV and ASFLV), classical gastroenteritis agents (NoV, RV) and human viral fecal indicators (HAdV and JCPyV) were detected in river water samples from two different geographical areas with very different hydrological and climate conditions (Barcelona, Spain and Rio de Janeiro, Brazil). The procedure initially reported to concentrate viral particles from seawater (Calgua et al., 2008) was adapted and validated for use with a wide range of fresh water matrices and viruses of public health interest.

2. Materials and methods

2.1. Virus

Viruses for use in the recovery assays were initially isolated from clinical samples and were as follows: HAdV 2 (originally provided by Annika Allard, Umeå University, Sweden, and in Brazil kindly provided by Dr. José Paulo Leite, LVCA, Fiocruz, Brazil), JCPyV strain Mad-4 (originally provided by Dr. Eugene O. Major, NINDS, National Institutes of Health, MD, USA), NoV GGII (fecal samples kindly provided by Annika Allard, Umeå University, Sweden and by Dr. José Paulo Leite, LVCA, Fiocruz, Brazil) and RVA G1P[8] (fecal samples provided by Dr. José Paulo Leite, LVCA, Fiocruz, Brazil). HAdV 2 was also used as a control. HAdV 2 and JCPyV Mad-4 were cultured in A549 (epithelial cell line derived from human lung carcinoma) and SVG-A cells (fibroblast cell line subcloned from the original SVG human fetal glial cell line), respectively. The cell lines were grown in Earle's minimum essential medium (EMEM) supplemented with 1% glutamine, 50 μg of gentamicin/mL and 10% (growth medium) or 2% (maintenance medium) of heat-inactivated fetal bovine serum.

2.2. Water samples

For validation assays of the virus concentration method, approximately forty 5-L river samples, 20 in each laboratory were used. In order to analyze viral contamination in field samples from two geographical areas, six 10-L river water samples were collected over one month and analyzed in each laboratory for the selected viruses. River water samples (5 and 10 L) were collected from two different geographical areas: the Llobregat river in Barcelona, Spain, a Mediterranean area; and the Macacos and Fairas Timbó rivers in the urban area of Rio de Janeiro, Brazil. Samples from Barcelona and Rio de Janeiro were collected on two different days in March in every location. The selected sampling site in Barcelona corresponds with a source of water at the entrance to a drinking water treatment plant. Moreover, upstream from the point there are more than 30 sewage treatment plants that discharge secondary effluents into the river. The Llobregat river has a flow rate of 16.9 m^3/s and is 170 km long. Samples from Rio de Janeiro were also collected in March. Both Brazilian rivers, the Macacos and Farias Timbó, receive domestic sewage

discharge from urbanized areas in Rio de Janeiro. The Macacos river, which is less polluted, flows through the Tijuca rainforest and into Rodrigo de Freitas Lagoon to the south of the city. The Farias Timbó river is a highly polluted river that flows through the greater metropolitan and slum area of the city of Rio de Janeiro, in a northerly direction. It receives a high load of untreated domestic sewage discharge. It flows into Cunha channel, and finally in Guanabara Bay, in the Atlantic Ocean.

Samples were collected according to ISO 19458 (2006). Water samples for microbiological analysis were stored for a maximum of 24 h at 4 °C before being processed. Samples collected in Barcelona showed turbidity and conductivity values between 6.18 and 44.5 NTU and 588-1360 mS respectively; while the samples from Rio de Janeiro had values of 1 NTU and between 490 and 830 mS.

2.3. Virus concentration by skimmed milk (SM) flocculation procedure

Water samples with high levels of organic matter (by simple observation) such as leaves, algae or sand, were left to settle for two hours and the clear water was then transferred to a new container to start the concentration protocol. The conductivity of all the samples was measured before starting the virus concentration protocol, and samples with conductivity $\leq$1.5 mS were conditioned by adding artificial sea salts (Sigma, Aldrich Chemie GMBH, Steinheim, Germany) to obtain values $\geq$1.5 mS.

The river water samples were then concentrated based on a procedures described previously by Calgua et al. (2008) and Bofill-Mas et al. (2011). Briefly, once the samples were conditioned, a pre-flocculated 1% (w/v) skimmed milk solution (PSM) was prepared by dissolving 10 g skimmed milk powder (Difco, Detroit, MI, USA) in 1 L of artificial seawater at pH 3.5 (Sigma, Aldrich Chemie GMBH, Steinheim, Germany). The sample was then carefully acidified to pH 3.5 by adding HCl 1 N. The PSM was added to each of the previously conditioned samples until the final concentration of skimmed milk in the sample was 0.01% (w/v). Samples were stirred for 8 h at room temperature and flocs were allowed to form sediment by gravity for another 8 h. The supernatant was carefully removed using a vacuum pump without disturbing the sediment. The final volume of about 500 mL containing the sediment was transferred to a centrifuge tube and centrifuged at 7000 × *g* for 30 min at 12 °C. The supernatant was carefully removed and the pellet dissolved in phosphate buffer (1:2, v/v of Na_2HPO_4 0.2 M and NaH_2PO_4 0.2 M) at pH 7.5, at a ratio of 1 mL of phosphate buffer per 1 L of concentrated sample. The viral concentrate was stored at −80 °C. When necessary, an aliquot of the clarified phase of PSM was used to balance the centrifuge pots.

2.4. Validation of SM-flocculation procedure for detecting viruses in river water

In order to validate the use of the SM-flocculation procedure in river water, assays to evaluate the reproducibility and repeatability of viral recovery were performed in two laboratories in different geographical areas: Barcelona and Rio de Janeiro. A total of approximately 40 samples were tested, 20 in each laboratory. Each laboratory used two sets of ten 5-L river water samples, each set having been collected on different days and then mixed together. Based on the recovery assays described by Lambertini et al. (2008), sets of ten samples were divided into three groups as follows. (i) Six samples to test viral recovery. These samples were spiked at the same time with viral suspensions of HAdV 2, JCPyV Mad-4, NoV GGII in Barcelona and HAdV 2, RV and NoV GGII in Río de Janeiro. (ii) Three non-spiked samples were treated to concentrate the viral particles, after which the viral concentrates were spiked as above. The idea here was to extract nucleic acids and quantify viral genomes under the same conditions as the spiked samples in (i). These conditions allow to be averted false estimates of viral recovery due to nucleic acid extractions and qPCR quantification, and therefore the values obtained (Fig. 1) were taken as the reference spiked viral quantity (i.e. 100% recovery). (iii) One sample was used to analyze the endogenous viruses present in the set of samples, and the values obtained together with the samples in groups (i) and (ii) were used to estimate viral recovery.

2.5. Nucleic acid extractions

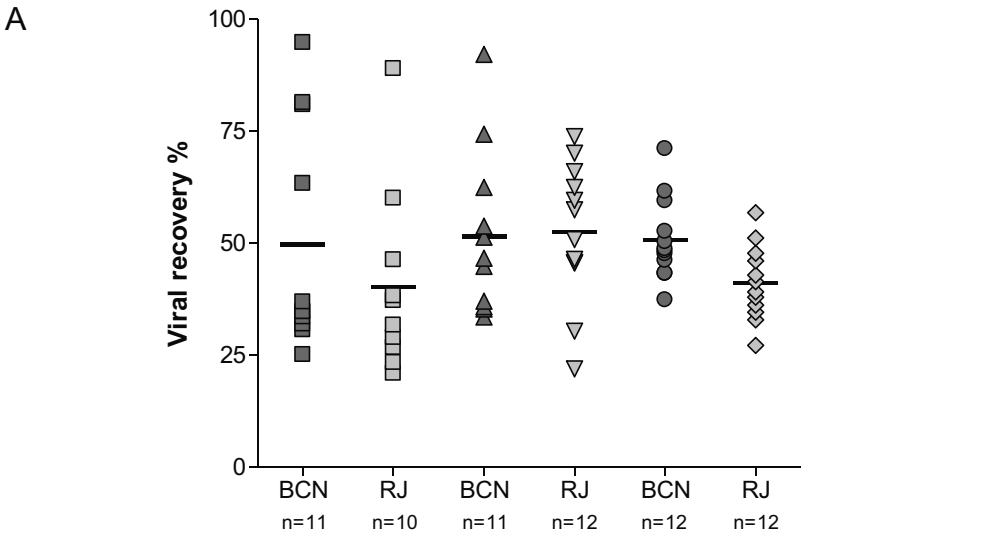
Nucleic acids (DNA and RNA) were extracted using the QIAamp Viral RNA kit (Qiagen, Valencia, USA) according to the manufacturer's instructions, using 140 μL of viral concentrate or viral suspension and eluting the resulting nucleic acid extraction in 80 μL of elution buffer. Nucleic acid extractions were analyzed immediately or stored at −80 °C until further analysis.

2.6. Enzymatic detection and amplification of viruses

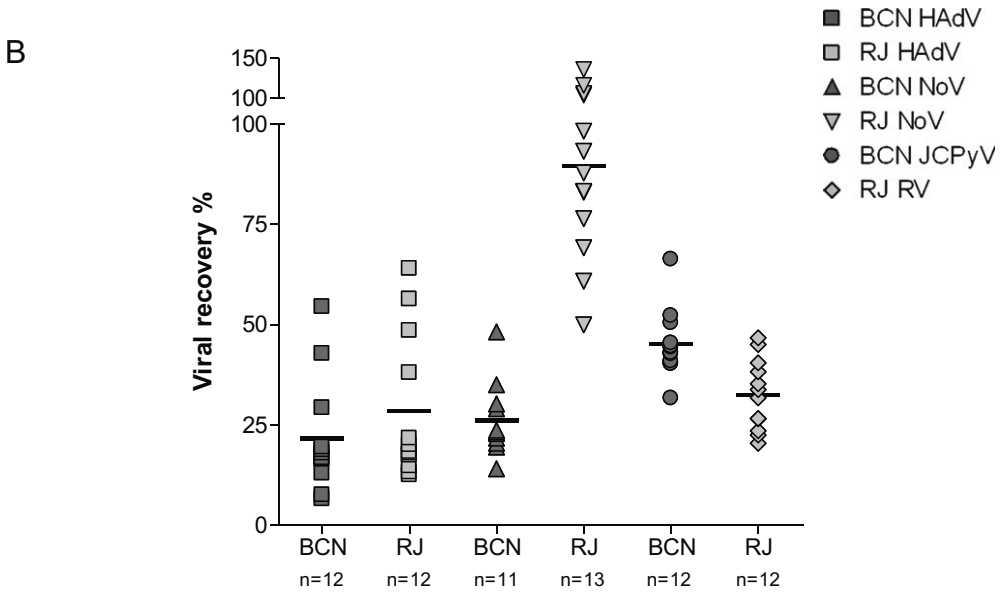
Based on previously reported sequences and their specificity against related viruses, oligonucleotides for ASFLV and KV were designed for nested PCR (nPCR) and reverse transcription (RT)-nPCR, respectively (Table 1). For RNA viruses, the first rounds of enzymatic amplifications were performed using OneStep RT-PCR Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. While in the first round of DNA amplification, 40 μL of amplification mix contained: PCR Buffer 1×, $MgCl_2$ 1.5 mM, 250 μM of each dNTP, 0.5 μM of the specific primer for the virus analyzed, and 4 units of Taq Gold DNA polymerase (Applied Biosystems, Foster, CA, USA). In the first round of either PCR or RT-PCR, 10 μL of undiluted nucleic acid extract and a 10-fold dilution was analyzed.

In the second round of enzymatic amplification, 2 μL of the product obtained in the first round was added to 48 μL of amplification mix containing a set of specific primers for each virus and the same reagent composition described above. The amplification conditions were as follows: 95 °C for 10 min, 30 cycles of 94 °C for 60 s, annealing temperature for 60 s, and 72 °C for 60 s, and finally 7 min at 72 °C.

Nested PCR, (RT)-nPCR and quantitative PCR assays for the other viruses were performed according to previous studies (Tables 2 and 3) in which they were applied to environmental samples such as river water, groundwater, seawater, sewage and drinking water (Bofill-Mas et al., 2000; Bofill-Mas et al., 2003; Albinana-Gimenez et al., 2006; Bofill-Mas et al., 2006;



Input viruses set I:	$1.81x10^6$	$1.66x10^8$	$1.70x10^8$	$1.99x10^5$	$4.61x10^7$	$3.34x10^8$
Input viruses set II:	$5.96x10^6$	$8.52x10^7$	$1.21x10^8$	$1.89x10^5$	$3.34x10^7$	$2.35x10^8$
Mean recovered viruses set I:	$1.12x10^6$	$7.03x10^7$	$7.66x10^7$	$8.32x10^4$	$2.31x10^7$	$1.48x10^8$
Mean recovered viruses set II:	$2.38x10^6$	$3.16x10^7$	$7.18x10^7$	$1.18x10^4$	$1.72x10^7$	$9.25x10^7$



Input viruses set I:	$8.08x10^6$	$2.61x10^8$	$2.63x10^8$	$8.60x10^4$	$4.95x10^7$	$3.76x10^8$
Input viruses set II:	$1.01x10^7$	$1.81x10^8$	$3.20x10^8$	$1.42x10^5$	$3.94x10^7$	$2.40x10^8$
Mean recovered viruses set I:	$1.73x10^6$	$7.00x10^7$	$7.68x10^7$	$8.31x10^4$	$2.31x10^7$	$1.00x10^8$
Mean recovered viruses set II:	$3.39x10^6$	$5.50x10^7$	$7.22x10^7$	$1.18x10^5$	$1.73x10^7$	$1.45x10^8$

Fig. 1 – Inter-and-intra laboratory assays to evaluate the viral recovery of virus concentration procedure by qPCR. (A) viral recovery when results from spiked-viral concentrates were defined as 100% of input virus. (B) Viral recovery when results from viral suspensions were defined as 100% of input virus. Tables show the concentrations (GC/mL) of viruses added and recovered for each set. Values in the columns correspond with assays for each laboratory described in the graphic above.

Albinana-Gimenez et al., 2009; Calgua et al., 2008; Bofill-Mas et al., 2010a; Wyn-Jones et al., 2011; Fumian et al., 2011; Lambertini et al., 2008). Each qPCR assay applied contained a set of specific primers and a TaqMan®-fluorogenic probe.

The nucleic acids from the samples were analyzed undiluted, 10- and when necessary 100-fold diluted. Each sample was run in duplicate (4–6 runs/sample). In all qPCRs or RT-qPCRs, the amount of DNA or RNA in GC/mL was defined as the mean of the data obtained. Non-template and inhibition controls were included in each run. The inhibition controls were extra aliquots of the nucleic acids extracted from one sample with standard DNA added.

2.7. *Sequencing products*

Products obtained after nPCR were purified using the QIAquick PCR purification kit (Qiagen, Valencia, USA). Both strands of the purified DNA amplicons were sequenced with

Table 1 – Primers designed for molecular detection of klassevirus (KV) and asfarvirus-like virus (ASFLV).

Virus	Primer	Sequence 5′–3′	Region on the genome	Position[b]
KV	LG0119[a] (1st round PCR)	GCTAACTCTAATGCTGCCACC	VP	1933–1953
	KV-VP-R (1st round PCR)	GAGGTCCAGGTCAAGTTCC	Amplicon size	2319–2337
	KV-VPn-F (2nd round PCR)	GAAGGACTCCACAACTATTGG	404pb1st round PCR (primers T_a: 55 °C)	1997–2017
	KV-VPn-R (2nd round PCR)	CATAGAAAGCTGAGTCAATAGG	123pb2nd round PCR (primers T_a: 55 °C)	2099–2120
	LG0118[a] (1st round PCR)	ATGGCAACCCTGTCCCTGAG	3D	6795–6814
	KV-3D-R (1st round PCR)	TCCAGAACACGACCAGGTTGG	Amplicon size	7177–7197
	KV-3Dn-F (2nd round PCR)	GATACAAGCAATTGTAGTCG	402pb1st round PCR (primers T_a: 60 °C)	6940–6959
	KV-3Dn-R (2nd round PCR)	TAGACCAGACATTAGAGAAGG	157pb2nd round PCR (primers T_a: 58 °C)	7077–7097
ASFLV	ASFLV-Pol-F (1st round PCR)	GAATTGAAGGATCTAATGAAACC	Polymerase	10–32
	ASFLV-Pol-R (1st round PCR)	GGCAGGAAGATCCACATGAAC	Amplicon size	320–340
	ASFLV-Pol-nF (2nd round PCR)	GCGGCTATCAATTGAATCCC	330pb1st round PCR (primers T_a: 62 °C)	50–69
	ASFLV-Pol-nR (2nd round PCR)	CGGCCAATACAATATTCAACTCG	195pb2nd round PCR (primers T_a: 58 °C)	223–245

a Primer from Holtz et al. (2009).
b According to GenBank sequence GQ184145 for KV and FJ957909 for ASFL.

the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction kit with Ampli Taq® DNA polymerase FS (PerkinElmer, Applied Biosystems, Foster, CA, USA) following the manufacturer's instructions. The results were analyzed using the ABI PRISM 3730 XL automated sequencer (PerkinElmer, Applied Biosystems).

2.8. *Phylogenetic studies*

The Merkel cell virus nucleotide sequences introduced in this work (corresponding to the VP2/VP3-VP1 junction) were analyzed with representative sequences of human polyomaviruses obtained from GenBank. Codon-based alignments

Table 2 – Sequences of primers used to detect viruses in water by nPCR assays.

Virus	Target region	Primers 5′–3′ (position)	Reference
HAdV	Hexon protein[a]	Hex1deg: GCCSCARTGGKCWTACATGCACATC (18858–18882) Hex2deg: CAGCACSCCICGRATGTCAAA 19138–19158) neHex3ded: GCCCGYGCMACIGAIACSTACTTC (18931–18954) neHex4deg: CCYACRGCCAGIGTRWAICGMRCYTTGTA (19077–19102)	Allard et al., 2001
JCPyV	Regulatory region[b]	JR1: CCCTATTCAGCACTTTGTCC (4992–5011) JR2: CAAACCACTGTGTCTCTGTC (428–447) JR3: GGGAATTTCCCTGGCCTCCT (5060–5079) JR4: ACTTTCACAGAAGCCTTACG (297–317)	Bofill-Mas et al., 2001
NoV GGII	RdRp[c]	JV12Y: ATACCACTATGATGCAGAYTA (4279–4299) JV13Y: TCATCATCACCATAGAAIGAG (4878–4858) Ni-R: AGCCAGTGGGCGATGGAATTC (4515–4495)	Vennema et al., 2002
RV-A	VP6[d]	VP6F: GACGGVGCRACTACATGGT (747–766) VP6R: GTCCAATTCATNCCTGGTTGG (1126–1106) VP6NF: GCWAGAAATTTTGATACA (867–884) VP6NR: GATTCACAAACTGCAGA (1005–1021)	Iturriza-Gomara et al., 2002 and Gallimore et al., 2006
MCPyV	VP1/2/3[e]	MC1c: GAATTAACTCCCATTCTTGGATTCA (4228–4252) MC2c: TTGGCTTCTTCCTCTGGTACT (4492–4472) MC3c: ATTTGGGTAATGCTATCTTCTCC (4264–4286) MC3c: GGATATATTTCTCCTGAATTACA (4461–4439)	Bofill-Mas et al., 2010a,b
KIPyV	VP1[f]	KI1: GCTGCTCAGGATGGGCGTGA (1684–1704) KI2: CAGKGTTCTAGGGTCTCCTGGT (2061–2043) KI3: GTTGCTTGTTGTACCTCTAG (1899–1918) KI4: AATTGTATAGGTAGTTGGGCCT (2088–2067)	Bofill-Mas et al., 2010a,b
WUPyV	VP1[g]	WU1: CCCACAAGAGTGCAAAGCCTTC (1730–1750) WU2: AGGCACAGTACCATTGGTTTTA (2234–2213) WU3: AGTTTTGGTGCTTCCTKTSC (2044–2063) WU4: TACAGTATACTGAGCAGGC (22072118)	Bofill-Mas et al., 2010a,b

Position according to GenBank virus sequence.
a (DQ315364.2).
b (Frisque et al., 1984).
c (AF356599).
d (Iturriza-Gomara et al., 2002).
e (EU375803).
f (EF127906).
g (EF444549).

Table 3 – Sequences of primers and probes used to quantify viruses in water by qPCR assays.

Virus	Target region	Primers and probes 5′–3′ (position)	Reference
HAdV	qPCR (hexon protein)[a]	AdF: CWTACATGCACATCKCSGG (17629–17647) AdR: CRCGGGCRAAYTGCACCAG (17679–17697) AdP: FAM-CCGGGCTCAGGTACTCCGAGGCGTCCT-BHQ1 (17650–17676)	Hernroth et al., 2002
JCPyV	qPCR (Large T antigen)[b]	JE3F: ATGTTTGCCAGTGATGATGAAAA (4339–4317) JE3R: GGAAAGTCTTTAGGGTCTTCTACCTTT (4251–4277) JE3P: FAM-AGGATCCCAACACTCTACCCCACCTAAAAAGA-BHQ1 (4313–4482)	Pal et al., 2006
NoV GGII	qPCR (ORF1-ORF2)[c]	JJV2F: CAAGAGTCGATGTTTAGGTGGATGAG (5003–5028) COG2R: TCGACGCCATCTTCATTCACA (5080–5100) RING2: FAM-TGGGAGGGCGATCGCAATCT-BHQ1 (5048–5067)	Johtikumar et al., 2006
RV-A	qPCR (NSP3)[d]	NSP3f: ACCATCTWCACRTRACCCTCTATGAG (963–988) NSP3r: GGTCACATAACGCCCCTATAGC (1028–1049) NSP3p: FAM-AGTTAAAAGCTAACACTGTCAAA-MGB (995–1017)	Zeng et al., 2008

Position according to GenBank virus sequence.
a (DQ315364.2).
b (NC_001699.1).
c (X86557).
d (X81436).

of nucleotide sequences were determined using Prankster software (Löytynoja and Goldman, 2005) and edited using the Bioedit v7.0.9.0 program (Hall, 1999). A maximum likelihood (ML) phylogenetic tree was obtained using PhyML software v3.0 (Guindon et al., 2010) with the substitution model estimated by the jModelTest software v0.1.1 (Posada, 2008) according to the Akaike Information Criterion (AIC). The robustness of the phylogenetic grouping was evaluated by bootstrap analysis using ML (1000 replicates) and the PhyML software.

2.9. Statistical analysis

Analysis of variance (one- and two-way ANOVA tests) was used to evaluate differences between recovery rates through intra- and inter-laboratory assays. The Shapiro–Wilks and Bartlett tests were used to test for normality and homogeneity of variance in the ANOVA procedures. *P*-values of <0.05 were considered significant. The statistical analysis was performed using R software version 2.14.1 (Verzani, 2004; R, 2008).

3. Results

3.1. Inter- and intra-laboratory variability of viral recovery values for SM flocculation procedure for detecting viruses in river water

Values of intra- and inter-laboratory variability in the viral recovery of HAdV 2 and NoV GGII showed low variability according with values described for virus concentration methods by Calgua et al. (2013), with mean values of 50% (25–95% [mean: 1.80×10^6; 1.50×10^6–1.72×10^6 GC/mL]; SD = 24.21; coefficient of variation [CV] = 48.47%) and 41% (21–89% [mean: 5.48×10^7; 5.26×10^7–1.48×10^8 GC/mL]; SD = 20.70; CV = 51.44%) for HAdV, and 52% (34–74% [mean: 7.45×10^7; 5.70×10^7–1.26×10^8 GC/mL]; SD = 18.45; CV = 35.78%) and 53% (22–73% [mean: 1.01×10^5; 4.30×10^4–1.40×10^5 GC/mL]; SD = 15.59; CV = 29.68%) for NoV, in Barcelona and Rio de Janeiro, respectively (Fig. 1). The mean recovery of JCPyV in Barcelona was 51% (38–71% [mean: 2.02×10^7; 1.25×10^7–3.28×10^7 GC/mL]; SD = 9.26; CV = 18.23%), and for RV tested in Rio de Janeiro the mean recovery was 41% (27–57% [mean: 1.18×10^8; 6.38×10^7–1.90×10^8 GC/mL]; SD = 8.33; CV = 20.24%); both recovery values showed low variability (Fig. 1a). Whereas that the recovery values estimated by the quantitation from the raw data directly using the viral suspension (Fig. 1b) were 22% (7–54% [mean input: 9.45×10^6; mean recovered: 2.06×10^6; 7.58×10^5–4.80×10^6 GC/mL]; SD = 14.11; CV = 65,9%) and 29% (12–64% [mean input: 2.21×10^8;mean recovered: 6.25×10^7; 2.27×10^7–1.16×10^8 GC/mL]; SD = 18.24; CV = 63,77%) for HAdV, and 26% (14–48% [mean input; 2.91×10^8; mean recovered: 7.47×10^7; 4.50×10^7–1.27×10^8 GC/mL]; SD = 9.25; CV = 35.31%) and 89% (50–135% [mean input: 1.14×10^5; mean recovered: 1.02×10^5; 4.29×10^4–1.17×10^5 GC/mL]; SD = 23.14; CV = 25.87%) for NoV, in Barcelona and Rio de Janeiro, respectively. For JCPyV the values were 45% (66–31% [mean input: 4.44×10^7; mean recovered: 2.02×10^7; 1.25×10^7–3.28×10^7 GC/mL]; SD = 8.43; CV = 18.54%) and for RV 32.59% (20–47% [mean input: 3.08×10^8; mean recovered: 2.02×10^7; 7.68×10^7–1.76×10^8 GC/mL]; SD = 8.86; CV = 27.19%).

3.2. Distribution of viruses in river water

To evaluate the viral contamination in the geographical areas studied and during March, six 10-L river water samples per laboratory were treated to concentrate the viruses. A list of the viruses detected in each laboratory is given in Table 4. The average values given were calculated only from the positive samples.

3.2.1. Human fecal viral indicators HAdV and JCPyV

HAdV and JCPyV were detected in 100% of the samples analyzed in Barcelona (6/6) and Rio de Janeiro (6/6). In Barcelona the mean concentration of HAdV and JCPyV was 6.43×10^3 GC/L (1.99×10^3–1.18×10^4 GC/L) and 1.05×10^4 GC/L (4.40×10^3–1.49×10^4 GC/L), respectively (Table 4). In Rio de

Table 4 – Virus detected in river water from Barcelona and Rio de Janeiro.

Site	River water sample	Viruses analyzed								
		HAdV GC/L	JCPyV GC/L	NoV GGII GC/L	RV GC/L	MCPyV nPCR	KIPyV nPCR	WUPyV nPCR	KV nPCR	ASFLV nPCR
BCN	BCN1a0309	7.90×10^3	9.40×10^3	1.27×10^3	NT	–	–	–	–	–
	BCN2a0309	1.10×10^4	1.21×10^4	–	NT	+	–	–	–	–
	BCN3a0309	1.18×10^4	1.49×10^4	2.93×10^3	NT	+	–	–	–	–
	BCN4b0309	1.99×10^3	4.40×10^3	1.04×10^4	NT	+	–	–	–	–
	BCN5b0309	2.48×10^3	1.21×10^4	1.47×10^5	NT	–	–	–	–	–
	BCN6b0309	3.46×10^3	9.94×10^3	8.95×10^4	NT	–	–	–	–	–
RDJ	RJN1a09[a]	7.11×10^4	1.58×10^2	8.57×10^3	2.70×10^4	–	–	–	–	–
	RJN2a09[a]	1.47×10^4	1.07×10^3	6.76×10^3	1.63×10^4	+	–	–	–	–
	RJN3b09[a]	1.59×10^3	1.98×10^4	–	–	+	–	–	+	–
	RJN4b09[a]	3.98×10^4	2.97×10^4	–	–	+	–	–	+	–
	RJN5b09[b]	1.11×10^4	2.82×10^3	–	7.29×10^2	–	–	–	–	+
	RJN6b09[b]	7.13×10^2	2.71×10^3	–	1.11×10^3	–	–	–	–	–

NT: Not tested.
a Farias Timbó river.
b Macacos River river.

Janeiro the mean concentrations of HAdV and JCPyV were 2.31×10^4 GC/L (7.13×10^2–7.11×10^4 GC/L) and 9.38×10^3 GC/L (1.58×10^2–2.97×10^4 GC/L), respectively (Table 4). Four out of the six positive qPCR results for HAdV were sequenced and using the BLAST tool. It identified three samples as HAdV 41 and one samples as HADV 40, showing a similarity of about 95–99% over 99–98% of sequence coverage. For JCPyV, 3/6 samples were sequenced and the strains identified by BLAST showed the expected archetypical structure in the regulatory region (Bofill-Mas et al., 2001).

3.2.2. *Emerging viruses KIPyV, WUPyV, MCPyV, KV and ASFLV*

As shown in Table 4, MCPyV was detected in 50% of the river samples from Barcelona (3/6) and Río de Janeiro (3/6). KIPyV and WUPyV were not detected in Barcelona or Rio de Janeiro. KV and ASFLV were detected in 33% (2/6) and 16% (1/6), respectively, of the samples from Rio de Janeiro and were not detected in river water from Barcelona. The sequence analysis using BLAST showed a similarity of about 95–99% over 96–98% of sequence coverage with the corresponding target sequences present in GenBank.

3.2.3. *Common gastroenteritis viral agents NoV GGII and RV*

The results for NoV and RV are also presented in Table 4. NoV GGII were detected at a concentration of 5.02×10^4 GC/L (1.27×10^3–1.47×10^5 GC/L) in 83% (5/6) of the samples from Barcelona. In Rio de Janeiro NoV were detected in 33% (2/6) of the river samples with a mean value of 7.66×10^3 GC/L. In Rio de Janeiro RV were detected and quantified in 67% (4/6) of the river samples at a concentration of 1.13×10^3 GC/L (7.29×10^2–2.70×10^4 GC/L). Selected positive samples for NoV GGII from Barcelona (4/5) were sequenced and the results as analyzed using BLAST showed that three samples were NoV GGII.4 and one was NoV GGII.12. Positive samples of NoV from Rio de Janeiro were not sequenced. Sequence analysis for RVA showed 99% similarity with RVA genotype I2, which is generally grouped with RVA genotype G2P.

3.3. *Phylogenetic studies*

In order to characterize MCPyV, the PCR amplicons were sequenced and phylogenetically analyzed along with reference sequences for the human polyomaviruses. The sequences clearly grouped with the Merkel cell cluster, with high bootstrap support (Fig. 2a). Further analysis of the MC cluster showed that the sequences reported in this work are divergent from previously reported sequences. While BCN 4 was the most divergent sequence, the sequences from Río de Janeiro formed a cluster (with low bootstrap support) with viral genomes from clinical specimens from the United States and Japan (Fig. 2b).

3.4. *Statistical studies*

The results show that there is no statistically significant difference between the results of viral recoveries obtained by the two laboratories in the intra- or inter-laboratory assays. In the intra-laboratory assay, (one-way ANOVA) the *P*-values obtained were 0.895 in Barcelona (HAdV, NoV and JCPyV) and 0.118 in Rio de Janeiro (HAdV, NoV and RV), whereas in the inter-laboratory assay (two-way ANOVA) the *P*-values for HAdV and NoV II (viruses analyzed in both laboratories) were 0.479 and 0.692, respectively. No statistically significant differences were observed due to the day of the analysis (*P*-value >0.05). The data were normally distributed (Shapiro–Wilks test, *P*-value >0.05) and homoscedastic (Bartlett's test, *P*-value >0.05).

4. Discussion

The presence of human viruses in rivers is due to contamination from urban sewage and the stability of the viruses in response to environmental conditions. Here, the occurrence of new and emerging viruses (MCPyV, KIPyV, WUPyV, KV and ASFLV), gastroenteritis-related viruses (NoV GGII and RV) and

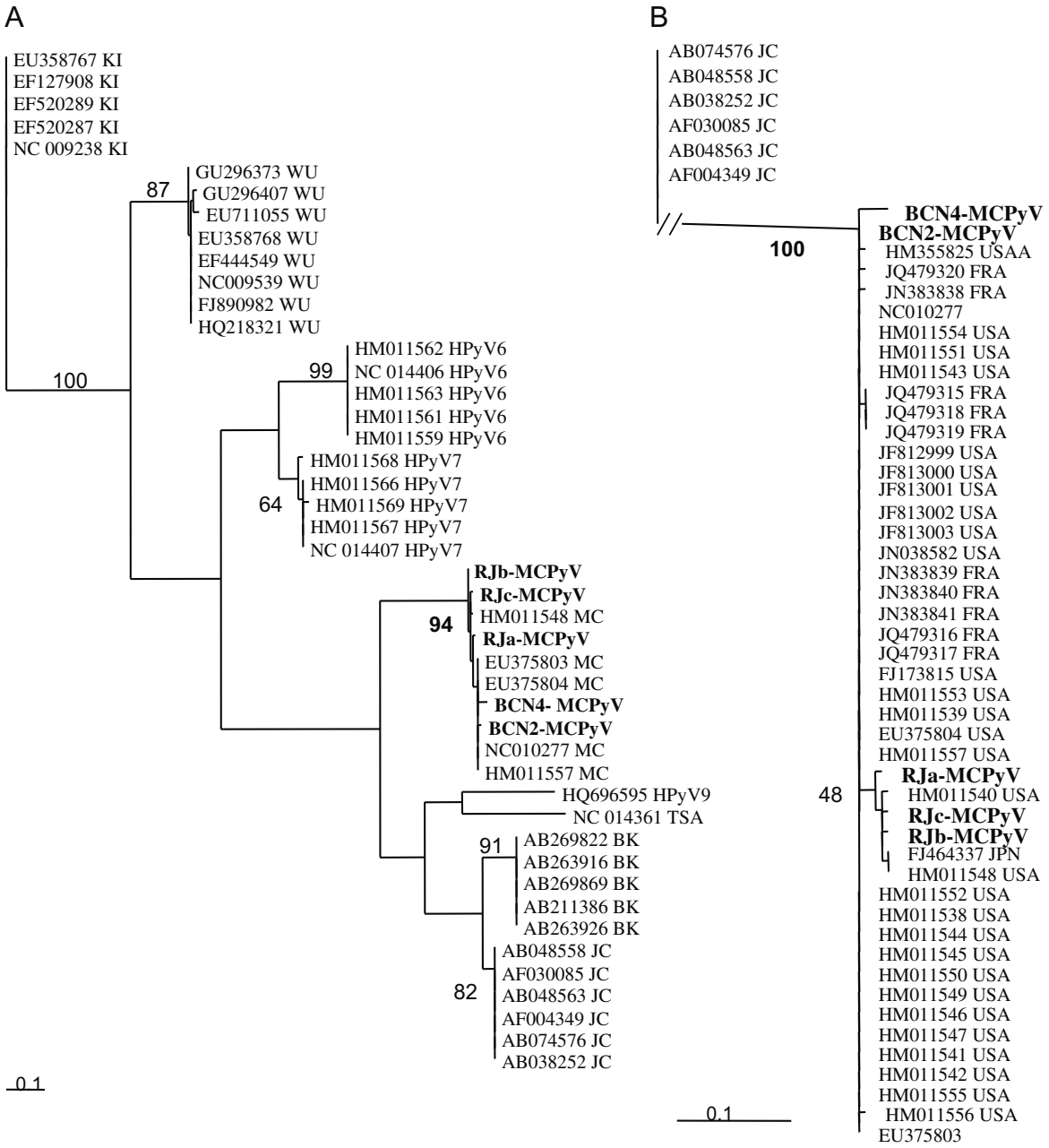


Fig. 2 – Maximum likelihood phylogenetic trees constructed at the VP2/VP3-VP1 junction of viral genomes from distinct human polyomaviruses (A) and MCPyVs (B) Sequences reported in this work are shown in bold letters. Sequences from GenBank are indicated by their accession numbers. Bootstrap values are given for the relevant groups. JCPyV was used as the outgroup in B. USA: United States; FRA: France; JPN: Japan; BCN: Barcelona, Spain; RJ: Río de Janeiro, Brazil.

viral indicators (HAdV and JCPyV) were studied in Barcelona and Rio de Janeiro, two different geographical contexts. Six samples per laboratory were analyzed during the month of March. The field samples analyzed represent specific information showing the level of viral contamination in the same period in two very different geographical areas, however, they may not accurately reflect the viral contamination in other periods of the year.

RT-nPCR and nPCR assays were designed to detect KV and ASFLV, by applying a set of specific primers for each virus. Although the samples of river water from Barcelona tested negative for these viruses in the present study, previous tests on sewage from Barcelona have confirmed the presence of KV and ASFLV by conventional PCR and deep-sequencing, respectively (Holtz at al., 2009; Loh at al., 2009). The sporadic presence of KV and ASFLV in river water was confirmed by the tests conducted in Rio de Janeiro, where 2/6 and 1/6 positive results were obtained for KV and ASFLV, respectively. This data represents the first report of the presence of these viruses in river water.

Of the new polyomaviruses studied here, only MCPyV was detected in Barcelona (3/6) and Rio de Janeiro (3/6), suggesting that this virus is stable in both sets of environmental conditions, probably similar to other human polyomaviruses such as BK and JCPyV. Interestingly, recent results describe MCPyV as an important member of the skin microbiota and so this virus could be shed from healthy humans via the skin (Wieland et al., 2009; Schowalter et al., 2010; Moens et al., 2011; Foulongne et al., 2012). The data described above strongly suggest that an important transmission route for MCPyV may be via water. Bofill-Mas et al. (2010a,b) reported a prevalence of 29% (2/7) for MCPyV in river water, and a much higher prevalence in urban sewage 89% (8/9). The positive detection of MCPyV in Rio de Janeiro represents the first data on the presence of this virus in Brazil. The identity of the MCPyV sequences was confirmed by the phylogenetic analysis. The high divergence of the sequences reported in this work, compared with the viral genomes for which full-length sequences are available in GenBank, could reflect the diversity of sequences from distinct geographical locations, since most of the GenBank genomes come from the United States and France. However, it should also be considered that the sequences from the environmental samples could represent the consensus of multiple viral genomes that are co-circulating in the population and have been discharged into the environment. Despite the low bootstrap support, the clustering of the Brazilian sequences is noteworthy and deserves further study.

Norovirus GGII was the most prevalent genogroup detected in many studies, especially genotype II.4 (Barreira et al., 2010; Ferreira et al., 2010; Wyn-Jones et al., 2011; Victoria et al., 2010; Bull and White, 2011; Prado et al., 2011). Previous studies showed a prevalence of 96.3% (104/108) for NoV GGII.4 in stool samples from patients with gastroenteritis in Río de Janeiro (Ferreira et al., 2010), while in a European study, a total of 1410 samples of water at popular recreational locatins (rivers and seawater) were positive for NoV: 6.2% for GGII and 3.5% for GGI (Wyn-Jones et al., 2011). Based on these data, detection of NoV was only focused on GGII in the present study. The qPCR results showed a greater prevalence of NoV GGII in Barcelona 83% (5/6) than in Rio de Janeiro 33% (2/6), this is probably related to the seasonal epidemiology of these viruses, with higher numbers during periods of lower temperatures. In the current study, the temperature in Barcelona at the time of sample collection was lower than in Rio de Janeiro, both in March 2009. The samples from Rio de Janeiro were not sequenced, however, selected samples from Barcelona (4/5) were sequenced and three samples were identified as NoV GGII.4, which is, as described above, the predominant genotype detected in many studies. One river water sample presented the emerging novel NoV GGII.12 (Vega and Vinjé, 2011). Rotaviruses was quantified and detected in 67% (4/6) of the samples from Rio de Janeiro. The qPCR assays used do not discriminate between pathogenic and vaccine derived strains, however, the strains detected should be considered as pathogenic viruses since previous studies (Fumian et al., 2011) have shown that vaccine strains are not detected in urban wastewater from Rio de Janeiro.

The standard fecal indicators, *E. coli* and enterococci, are used to monitor fecal pollution in accord with public health regulations related to the quality of river water, groundwater and seawater (WHO, 2003; USEPA, 2004, 2006/160/EC). Nevertheless, the occurrence of bacterial indicators does not necessarily correlated with the presence of viral pathogens, which are more stable than bacteria in the environment (de Roda Husman et al., 2009), and does not provide information on the potential origin of the contamination. Some studies have reported substantial levels of enteric viruses in water that complies with regulations regarding the levels of bacterial fecal indicators (Brownell et al., 2007; Colford et al., 2007; Calgua et al., 2008; Wyn-Jones et al., 2011). To overcome this lack of correlation, several studies have proposed the use of HAdV and JCPyV as human fecal indicators (Pina et al., 1998; Bofill-Mas et al., 2000; Albinana-Gimenez et al., 2006; McQuaig et al., 2006; Miagostovich et al., 2008; McQuaig et al., 2009; Wyn-Jones et al., 2011). In the present study, 100% (12/12) of the samples were positive for both viral these proposed indicators, HAdV and JCPyV, and in concentrations similar to those found in previous studies (Bofill-Mas et al., 2000; Albinana-Gimenez et al., 2006; McQuaig et al., 2006; Miagostovich et al., 2008; , McQuaig et al., 2009; Wyn-Jones et al., 2011). Although the number of samples is not high, these data support the stable distribution of both viruses in different geographical areas and the fact that their presence might be an accurate indication of human fecal contamination, and therefore also indicates the potential presence of other pathogenic viruses, such as the viruses detected in the present study.

The SM-flocculation procedure is based on the adsorption of the viruses to the flocs of skimmed milk. This concentration procedure was previously developed to concentrate viruses from seawater (Calgua et al., 2008). Fresh water and seawater differ in conductivity; fresh water having a conductivity of 40–2000 μS/m and seawater having a conductivity of 4500–5000 μS/m, which may affect adsorption of viral particles to flocs. Using a pre-flocculated skimmed milk solution, and artificial seawater either undiluted or in serial 10-fold dilutions, the effect of conductivity on the flocs and therefore viral recovery was assessed (data not shown). Based on the results, any water sample with levels of conductivity $\leq$1.5 mS/cm should be conditioned by adding e.g. artificial sea salt (Sigma, Aldrich Chemie GMBH, Steinheim, Germany), to reach conductivity values $\geq$1.5 mS/cm, prior to concentrating the viruses using the skimmed-milk flocculation procedure. During validation assays, to avoid underestimation of viral recovery caused by the specific composition of the concentrates from the water matrices (samples), viral recovery was estimated using as a reference, data on viruses quantified in concentrated water matrices following direct spiking of the viruses into the concentrate, similar to the procedure described by Lambertini et al. (2008). The endogenous virus strains of those used in the recovery assays (HAdV 2, JCPyV, NoV GGII and RV) were subtracted from the values of the viruses recovered in the recovery assays, the concentration of endogenous viruses were 10^2–10^4 GC/L (data non show), low values in comparison with the spiked viruses. Validation assays were performed with the water matrices analyzed in the study and no inhibition problems were observed in the 10-fold diluted samples. Non-template and inhibition controls were included in each run. The inhibition controls were extra aliquots of the nucleic acids extracted from one sample with

spiked standard DNA. Nucleic acid extractions of all samples were analyzed undiluted and 10-fold diluted and although low levels of inhibition were observed in some assays with undiluted samples, robust results were obtained in the 1:10 dilution. Intra- and inter-laboratory assays showed about 50% viral recovery for the viruses tested, values similar to the recoveries obtained by the method based on organic flocculation of skimmed milk for HAdV in seawater reported by Calgua et al. (2008). Available data of viral recovery values of different methods described in the literature may be difficult to compare. Concentration and quantification protocols (infectivity cell culture assays or qPCR), volume and type of water matrix used in the study have a strong influence on final results. Katayama et al. (2002), reported a concentration method (electronegative cellulose membrane and elution with NaOH), for seawater based on two-step procedures, which showed a viral recovery of poliovirus about 82–95% obtained by plaque assays when they concentrate 50 mL–5 mL (viruses 10-fold concentrated), and 62% when 1 L of sample was concentrate to 5 mL. Wyn-Jones et al. (2011) described mean recoveries of HAdV using PFU and a protocol with glass wool filtration with elution using beef extract and flocculation of 57.1% (range 34.2–78%). Recoveries using qPCR for the quantification of adenoviruses were described using 10 L samples of freshwater by Albinana-Gimenez et al. (2009) with a protocol based on ultrafiltration in the range between 3 and 6%. Girones et al. (2010) showed a comparison of concentration methods for fresh and seawater for HAdV, where a low variability was observed with an one-step protocol for seawater, while high variability was observed with two step glass wool and electronegative-nitrocellulose membranes. The method for river water, similarly to the method for seawater (Calgua et al., 2008), does not require specialized equipment and would fulfill the conditions for a fitting method for routine public health laboratories: reproducibility, reliability, straightforwardness and cost-effectiveness.

In the present study, the inter-laboratory assays validated the use of the low-cost one-step procedure described above for the analysis of viruses in river water, detecting important viral pathogens such as RV, NoV and HAdV, as well as new and emerging viruses that are potentially transmitted through water, and confirming the global distribution of the proposed human viral indicators: HAdV and JCPyV.

5. Conclusions

- This study is the first description of the recently described viruses ASFLV and KV in river water and the first report of the presence of MCPyV in the environment in Brazil.
- The presence of MCPyV in rivers in Rio de Janeiro (3/6 samples) and Barcelona (3/6 samples) demonstrates that these viruses are abundantly excreted by the human population in different geographical areas.
- The RT-nPCR and nPCR developed here for the detection of KV and ASFLV, respectively, are specific molecular assays which could be applied in future clinical or environmental studies.
- The viral indicators HAdV and JCPyV are useful as markers of human fecal/urine contamination in water from diverse geographical areas since they show a high worldwide prevalence and stable concentrations.
- The results obtained in the inter- and intra-laboratory assays support the applicability of the one-step virus concentration procedure reported here as a routine protocol for virus quantification and for improving control of the microbiological quality of both seawater and fresh water.
- Gastroenteritis viruses such as NoV and RV, which are of great importance as pathogens in the regions studied, were quantified using the method described, and show highly variable concentrations in river samples in accordance with the reported epidemiology of these viruses.

Acknowledgments

This work was partially supported by the Spanish Government “Ministerio de Educación y Ciencia” (projects AGL2008-05275-C01/ALI and AGL2011-30461-C02-01). During the study Marta Rusiñol was a fellow of the Catalan research agency “AGAUR” (FI-DGR) in Spain. This work was also financially sponsored by the Brazilian National Council for Scientific and Technological Development (CNPq) and by the IOC/Fiocruz/Ministry of Health, Brazil. The study was conducted under the scope of the activities of Fiocruz as a collaborating center of PAHO/WHO for Public and Environmental Health.

REFERENCES

Albinana-Gimenez, N., Clemente-Casares, P., Calgua, B., Huguet, J.M., Courtois, S., Girones, R., 2009. Comparison of methods for concentrating human adenoviruses, polyomavirus JC and noroviruses in source waters and drinking water using quantitative PCR. Journal of Virological Methods 158 (1–2), 104–109.

Albinana-Gimenez, N., Clemente-Casares, P., Bofill-Mas, S., Hundesa, A., Ribas, F., Girones, R., 2006. Distribution of human polyomaviruses, adenoviruses, and hepatitis E virus in the environment and in a drinking-water treatment plant. Environmental Science & Technology 40 (23), 7416–7422.

Allander, T., Andreasson, K., Gupta, S., Bjerkner, A., Bogdanovic, G., Persson, M.A.A., Dalianis, T., Ramqvist, T., Andersson, B., 2007. Identification of a third human polyomavirus. The Journal of Virology 81, 4130–4136.

Allard, A., Albinsson, B., Wadell, G., 2001. Rapid typing of human adenoviruses by a general PCR combined with restriction endonuclease analysis. Journal of Clinical Microbiology 39 (2), 498–505.

Babakir-Mina, M., Ciccozzi, M., Campitelli, L., Aquaro, S., Lo Coco, A., Perno, C.F., Ciotti, M., 2009. Identification of the novel KI Polyomavirus in paranasal and lung tissues. Journal of Medical Virology 81, 558–561.

Barreira, D.M., Ferreira, M.S., Fumian, T.M., Checon, R., de Sadovsky, A.D., Leite, J.P., Miagostovich, M.P., Spano, L.C., 2010. Viral load and genotypes of noroviruses in symptomatic and asymptomatic children in Southeastern Brazil. Journal of Clinical Virology 47 (1), 60–64.

Berger, J.R., Major, E.O., 1999. Progressive multifocal leukoencephalopathy. Seminars in Neurology 19 (2), l193–1200.

Bofill-Mas, S., Hundesa, A., Calgua, B., Rusiñol, M., de Motes, C.M., Girones, R., 2011. Cost-effective method for microbial source

tracking using specific human and animal viruses. The Journal of Visualized Experiments 3 (58), e2820. http://dx.doi.org/10.3791/2820.
Bofill-Mas, S., Pilar Clemente-Casares, P., Major, E.O., Curfman, B., Girones, R., 2003. Analysis of the excreted JC virus strains and their potential oral transmission. Journal of Neurovirology 9 (4), 498–507.
Bofill-Mas, S., Albinana-Gimenez, N., Clemente-Casares, P., Hundesa, A., Rodriguez-Manzano, J., Allard, A., Calvo, M., Girones, R., 2006. Quantification and stability of human adenoviruses and polyomavirus JCPyV in wastewater matrices. Applied and Environmental Microbiology 72 (12), 7894–7896.
Bofill-Mas, S., Calgua, B., Clemente-Casares, P., la Rosa, G., Iaconelli, M., Muscillo, M., Rutjes, S., de Roda Husman, A.M., Grunert, A., Gräber, I., Verani, M., Carducci, A., Calvo, M., Wyn-Jones, P., Girones, R., 2010a. Quantification of human adenoviruses in European recreational waters. Food and Environmental Virology 2 (2), 101–1009.
Bofill-Mas, S., Formiga-Cruz, M., Clemente-Casares, P., Calafell, F., Girones, R., 2001. Potential transmisión of human polyomaviruses through the gastrointestinal tract after exposure to virions or viral DNA. Journal of Virology 75, 10290–10299.
Bofill-Mas, S., Pina, S., Girones, R., 2000. Documenting the epidemiologic patterns of polyomaviruses in human populations by studying their presence in urban sewage. Applied and Environmental Microbiology 66 (1), 238–245.
Bofill-Mas, S., Rodriguez-Manzano, J., Calgua, B., Carratala, A., Girones, R., 2010b. Newly described human polyomaviruses Merkel cell, KI and WU are present in urban sewage and may represent potential environmental contaminants. Virology Journal 7 (1), 141.
Brownell, M.J., Harwood, V.J., Kurz, R.C., McQuaig, S.M., Lukasik, J., Scott, T.M., 2007. Confirmation of putative stormwater impact on water quality at a Florida beach by microbial source tracking methods and structure of indicator organism populations. Water Research 41 (16), 3747–3757.
Bull, R.A., White, P.A., 2011. Mechanisms of GII.4 norovirus evolution. Trends in Microbiology 19 (5), 233–240.
Calgua, B., Rodriguez-Manzano, J., Hundesa, A., Suñen, E., Calvo, M., Bofill-Mas, S., Girones, R., 2013. New methods for the concentration of viruses from urban sewage using quantitative PCR. Journal of Virological Methods 187 (2), 215–221.
Calgua, B., Mengewein, A., Grunert, A., Bofill-Mas, S., Clemente-Casares, P., Hundesa, A., Wyn-Jones, A.P., López-Pila, J.M., Girones, R., 2008. Development and application of a one-step low cost procedure to concentrate viruses from seawater samples. Journal of Virological Methods 153 (2), 79–83.
Cantalupo, P.G., Calgua, B., Zhao, G., Hundesa, A., Wier, A.D., Katz, J.P., Grabe, M., Hendrix, R.W., Girones, R., Wang, D., Pipas, J.M., 2011. Raw sewage harbors diverse viral populations. MBio 2 (5), 1–11.
Colford, J.M., Wade, T.J., Schiff, K.C., Wright, C.C., Griffith, J.F., Sandhu, S.K., Burns, S., Sobsey, M., Lovelace, G., Weisberg, S.B., 2007. Water quality indicators and the risk of illness at beaches with nonpoint sources of fecal contamination. Epidemiology 18 (1), 27–35.
de Roda Husman, A.M., Lodder, W.J., Rutjes, S.A., Schijven, J.F., Teunis, P.F., 2009. Long-term inactivation study of three enteroviruses in artificial surface and groundwaters using PCR and cell culture. Applied and Environmental Microbiology 75 (4), 1050–1057.
Directive 2006/7/EC of the European Parliament and of the Council of 15 February 2006 concerning the management of bathing water quality and repealing Directive 76/160/EEC.
Feng, H., Shuda, M., Chang, Y., Moore, P.S., 2008. Clonal integration of a polyomavirus in human Merkel cell carcinoma. Science 319, 1096–1100.
Ferreira, M.S., Victoria, M., Carvalho-Costa, F.A., Vieira, C.B., Xavier, M.P., Fioretti, J.M., Andrade, J., Volotão, E.M., Rocha, M., Leite, J.P., Miagostovich, M.P., 2010. Surveillance of norovirus infections in the state of Rio De Janeiro, Brazil 2005-2008. Journal of Medical Virology 82 (8), 1442–1448.
Finkbeiner, S.R., Allred, A.F., Tarr, P.I., Klein, E.J., Kirkwood, C.D., Wang, D., 2008. Metagenomic analysis of human diarrhea: viral detection and discovery. Plos Pathogens 4 (2), e1000011.
Fong, T.T., Lipp, E.K., 2005. Enteric viruses of humans and animals in aquatic environments: health risks, detection and potential water quality assessment tools. Microbiology and Molecular Biology Reviews 69 (2), 357–371.
Foulongne, V., Sauvage, V., Hebert, C., Dereure, O., Cheval, J., Gouilh, M.A., Pariente, K., Segondy, M., Burguière, A., Manuguerra, J.C., Caro, V., Eloit, M., 2012. Human skin microbiota: high diversity of DNA viruses identified on the human skin by high throughput sequencing. PLoS One 7 (6), e38499.
Foulongne, V., Kluger, N., Dereure, O., Brieu, N., Guillot, B., Segondy, M., 2008. Merkel cell polyomavirus and Merkel cell carcinoma, France. Emerging Infectious Diseases 14 (9), 1491–1493.
Frisque, R.J., Bream, G.L., Cannella, M.T., 1984. Human polyomavirus JC virus genome. Journal of Virology 51 (2), 458–469.
Fumian, T.M., Gagliardi Leite, J.P., Rose, T.L., Prado, T., Miagostovich, M.P., 2011. One year environmental surveillance of rotavirus specie A (RVA) genotypes in circulation after the introduction of the Rotarix® vaccine in Rio de Janeiro, Brazil. Water Research 45, 5755–5763.
Fumian, T.M., Guimarães, F.R., Pereira Vaz, B.J., da Silva, M.T., Muylaert, F.F., Bofill-Mas, S., Girones, R., Leite, J.P., Miagostovich, M.P., 2010. Molecular detection, quantification and characterization of human polyomavirus JC from waste water in Rio De Janeiro, Brazil. Journal of Water and Health 8 (3), 438–445.
Gallimore, C.I., Taylor, C., Gennery, A.R., Cant, A.J., Galloway, A., Iturriza-Gomara, M., Gray, J.J., 2006. Environmental monitoring for gastroenteric viruses in a pediatric primary immunodeficiency unit. Journal of Clinical Microbiology 44 (2), 395–399.
Gantzer, C., Maul, A., Audic, J.M., Schwartzbrod, L., 1998. Detection of infectious enteroviruses, enterovirus genomes, somatic coliphages and Bacteroides fragilis phages in treated wastewater. Applied and Environmental Microbiology 64, 4307–4312.
Gaynor, A.M., Nissen, M.D., Whiley, D.M., Mackay, I.M., Lambert, S.B., Wu, G., Brennan, D.C., Storch, G.A., Sloots, T.P., Wang, D., 2007. Identification of a novel polyomavirus from patients with acute respiratory tract infections. Plos Pathogens 3, 595–604.
Girones, R., Ferrús, M.A., Alonso, J.L., Rodriguez-Manzano, J., Calgua, B., Corrêa Ade, A., Hundesa, A., Carratala, A., Bofill-Mas, S., 2010. Molecular detection of pathogens in water-the pros and cons of molecular techniques. Water Research 44 (15), 4325–4339.
Glass, R.I., Parashar, U.D., Estes, M.K., 2009. Norovirus gastroenteritis. The New England Journal of Medicine 361 (18), 1776–1785.
Greninger, A.L., Holtz, L., Kang, G., Ganem, D., Wang, D., De Risi, J.L., 2010. Serological evidence of human klassevirus infection. Clinical and Vaccine Immunology 17 (10), 1584–1588.
Greninger, A.L., Runckel, C., Chiu, C.Y., Haggerty, T., Parsonnet, J., Ganem, D., De Risi, J.L., 2009. The complete genome of klassevirus – a novel picornavirus in pediatric stool. Virology Journal 6, 82.
Guindon, S., Dufayard, J.F., Lefort, V., Anisimova, M., Hordijk, W., Gascuel, O., 2010. New algorithms and methods to estimate

maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Systematic Biology 59 (3), 307–321.
Hall, T., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acid Symposium Series 41, 95–98.
Han, T.H., Kim, C.H., Chung, J.Y., Park, S.H., Hwang, E.S., 2010. Klassevirus infection in children, South Korea. Emerging Infectious Diseases 16 (10), 1623–1625.
Haramoto, E., Katayama, H., Ohgaki, S., 2004. Detection of noroviruses in tap water in Japan by means of a new method for concentrating enteric viruses in large volumes of freshwater. Applied and Environmental Microbiology 70, 2154–2160.
Hernroth, B.E., Conden-Hansson, A.C., Rehnstam-Holm, A.S., Girones, R., Allard, A.K., 2002. Environmental factors influencing human viral pathogens and their potential indicator organisms in the blue mussel, Mytilus edulis: the first Scandinavian report. Applied and Environmental Microbiology 68 (9), 4523–4533.
Holtz, L.R., Finkbeiner, S.R., Zhao, G., Kirkwood, C.D., Girones, R., Pipas, J.M., Wang, D., 2009. Klassevirus 1, a previously undescribed member of the family Picornaviridae, is globally widespread. Virology Journal 6, 86.
ISO 19458, 2006. Water Quality–Sampling for Microbiological Analysis. Guidance on Planning Water Sampling Regimes, on Sampling Procedures for Microbiological Analysis and on Transport, Handling and Storage of Samples until Analysis Begins.
Iturriza Gómara, M., Wong, C., Blome, S., Desselberger, U., Gray, J., 2002. Molecular characterization of VP6 genes of human rotavirus isolates: correlation of genogroups with subgroups and evidence of independent segregation. Journal of Virology 76 (13), 6596–6601.
Jothikumar, N., Lowther, J.A., Henshilwood, K., Lees, D., Hill, V.R., Vinjé, J., 2006. Rapid and sensitive detection of noroviruses by using TaqMan-Based One-Step Reverse Transcription-PCR assays and application to naturally contaminated shellfish samples. Applied and Environmental Microbiology 71 (4), 1870–1875.
Katayama, H., Shimasaki, A., Ohgaki, S., 2002. Development of a virus concentration method and its application to detection of enterovirus and Norwalk virus from coastal seawater. Applied and Environmental Microbiology 68, 1033–1039.
Khan, M.A., Bass, D.M., 2010. Viral infections: new and emerging. Current Opinion in Gastroenterology 26 (1), 26–30.
Kitamura, T., Aso, Y., Kuniyoshi, N., Hara, K., Yogo, Y., 1990. High incidence of urinary JC virus excretion in non-immunosuppressed older patients. Journal of Infectious Diseases 161, 1128–1133.
Koo, H.L., Ajami, N., Atmar, R.L., DuPont, H.L., 2010. Noroviruses: the leading cause of gastroenteritis worldwide. Discovery Medicine 10 (50), 61–70.
Koralnik, I.J., Boden, D., Mai, V.X., Lord, C.I., Letvin, N.L., 1999. JC virus DNA load in patients with and without progressive multifocal leukoencephalopathy. Neurology 52, 253–260.
Lambertini, E., Spencer, S.K., Bertz, P.D., Loge, F.J., Kieke, B.A., Borchardt, M.A., 2008. Concentration of enteroviruses, adenoviruses, and noroviruses from drinking water by use of glass wool filters. Applied and Environmental Microbiology 74 (10), 2990–2996.
Lodder, W.J., van den Berg, H., Rutjes, S.A., de Roda Husman, A.M., 2010. Presence of enteric viruses in source waters for drinking water production in the Netherlands. Applied and Environmental Microbiology 76 (17), 5965–5971.
Loh, J., Zhao, G., Presti, R.M., Holtz, L.R., Finkbeiner, S.R., Droit, L., Villasana, Z., Todd, C., Pipas, J.M., Calgua, B., Girones, R., Wang, D., Virgin, H.W., 2009. Detection of novel sequences related to african Swine Fever virus in human serum and sewage. The Journal of Virology 83 (24), 13019–13025.
Löytynoja, A., Goldman, N., 2005 26. An algorithm for progressive multiple alignment of sequences with insertions. Proceedings of the National Academy of Sciences of the United States of America 102 (30), 10557–10562.
McQuaig, S.M., Scott, T.M., Harwood, V.J., Farrah, S.R., Lukasik, J.O., 2006. Detection of human-drived fecal pollution in environmental waters by use of a PCR-based human polyomavirus assay. Applied and Environmental Microbiology 72 (12), 7567–7574.
McQuaig, S.M., Scott, T.M., Lukasik, J.O., Paul, J.H., Harwood, V.J., 2009. Quantification of human polyomaviruses JC Virus and BK Virus by TaqMan quantitative PCR and comparison to other water quality indicators in water and fecal samples. Applied and Environmental Microbiology 75 (11), 3379–3388.
Miagostovich, M.P., Ferreira, F.F., Guimarães, F.R., Fumian, T.M., Diniz-Mendes, L., Luz, S.L., Silva, L.A., Leite, J.P., 2008. Molecular detection and characterization of gastroenteritis viruses occurring naturally in the stream waters of Manaus, central Amazonia, Brazil. Applied and Environmental Microbiology 74 (2), 375–382.
Moens, U., Ludvigsen, M., Van Ghelue, M., 2011. Human Polyomaviruses in Skin Diseases. Pathology Research International, 123491.
Pal, A., Sirota, L., Maudru, T., Peden, K., Lewis, A.M., 2006. Real-time PCR assays for the detection of virus-specific DNA in simples with mixed populations of polyomaviruses. Journal of Virological Methods 135 (1), 32–42.
Pallin, R., Wyn-Jones, A.P., Place, B.M., Lightfoot, N.F., 1997. The detection of enteroviruses in large volume concentrates of recreational waters by the polymerase chain reaction. Journal of Virological Methods 67, 57–67.
Parashar, U.D., Burton, A., Lanata, C., Boschi-Pinto, C., Shibuya, K., Steele, D., Birmingham, M., Glass, R.I., 2009. Global mortality associated with rotavirus disease among children in 2004. Journal of Infection Disease 1, 200.
Patel, M.M., Widdowson, M.A., Glass, R.I., Akazawa, K., Vinjé, J., Parashar, U.D., 2008. Systematic literature review of role of noroviruses in sporadic gastroenteritis. Emerging Infectious Diseases 14 (8), 1224–1231.
Pina, S., Puig, M., Lucena, F., Jofre, J., Girones, R., 1998. Viral pollution in the environment and shellfish: human adenovirus detection by PCR as an index of human viruses. Applied and Environmental. Microbiology 64, 3376–3382.
Posada, D., 2008. jModelTest: phylogenetic model averaging. Molecular Biology and Evolution 25, 1253–1256.
Prado, T., Silva, D.M., Guilayn, W.C., Rose, T.L., Gaspar, A.M., Miagostovich, M.P., 2011. Quantification and molecular characterization of enteric viruses detected in effluents from two hospital wastewater treatment plants. Water Research 45 (3), 1287–1297.
Puig, M., Jofre, J., Lucena, F., Allard, A., Wadell, G., Girones, R., 1994. Detection of adenoviruses and enteroviruses in polluted waters by nested PCR amplification. Applied and Environmental Microbiology 60, 2963–2970.
Pusch, D., Oh, D.Y., Wolf, S., Dumke, R., Schröter-Bobsin, U., Höhne, M., Röske, I., Schreier, E., 2005. Detection of enteric viruses and bacterial indicators in German environmental waters. Archives of Virology 150 (5), 929–947.
R: A Language and Environment for Statistical Computing, 2008. R Development Core Team, ISBN 3-900051-07-0. http://www.R-project.org.
Rosario, K., Nilsson, C., Lim, Y.W., Ruan, Y., Breitbart, M., 2009. Metagenomic analysis of viruses in reclaimed water. Environmental. Microbiology 11, 2806–2820.
Schael, I.P., Gonzalez, R., Salinas, B., 2009. Severity and age of rotavirus diarrhea, but not socioeconomic conditions, are

associated with rotavirus seasonality in Venezuela. Journal of Medical Virology 81, 562–567.
Schowalter, R.M., Pastrana, D.V., Pumphrey, K.A., Moyer, A.L., Buck, C.B., 2010. Merkel cell polyomavirus and two previously unknown polyomaviruses are chronically shed from human skin. Cell Host & Microbe 7 (6), 509–515.
Shan, T., Wang, C., Cui, L., Yu, Y., Delwart, E., Zhao, W., Zhu, C., Lan, D., Dai, X., Hua, X., 2010. Picornavirus salivirus/klassevirus in children with diarrhea, China. Emerging Infectious Diseases 16 (8), 1303–1305.
Sobsey, M.D., Wallis, C., Henderson, M., Melnick, J.L., 1973. Concentration of enteroviruses from large volumes of water. Applied Microbiology 26 (4), 529–534.
USEPA, 2004. Water Quality Standards for Coastal and Great Lakes Recreation Waters, vol. 69. p. 220.
van den Berg, H., Lodder, W., van der Poel, W., Vennema, H., de Roda Husman, A.M., 2005. Genetic diversity of noroviruses in raw and treated sewage water. Research in Microbiology 156, 532–540.
Vega, E., Vinjé, J., 2011. Novel GII.12 norovirus strain, United States, 2009–2010. Emerging Infectious Diseases 17 (8), 1516–1518.
Vennema, H., de Bruin, E., Koopmans, M., 2002. Rational optimization of generic primers used for Norwalk-like virus detection by reverse transcriptase polymerase chain reaction. Journal of Clinical Virology 25 (2), 233–235.
Verzani, J., 2004. Using R for Introductory Statistics. Chapman & Hall/CRC.
Victoria, M., Guimarães, F.R., Fumian, T.M., Ferreira, F.F., Vieira, C.B., Shubo, T., Leite, J.P., Miagostovich, M.P., 2010. One year monitoring of norovirus in a sewage treatment plant in Rio de Janeiro, Brazil. Journal of Water and Health 8 (1), 158–1565.
Vilaginès, P., Sarrette, B., Husson, G., Vilaginès, R., 1993. Glass wool for virus concentration at ambient water pH level. Water Science and Technology 27, 299–306.
Weber, T., Trebst, C., Frye, S., Cinque, P., Vago, L., Sindic, C.J., Schulz-Schaeffer, W.J., Kretzschmar, H.A., Enzensberger, W., Hunsmann, G., Luke, W., 1997. Analysis of the systemic and intrathecal humoral immune response in progressive multifocal leukoencephalopathy. Journal of Infection Diseases 176, 250–254.
WHO, 2003. Guidelines for Safe Recreational Water Environments. In: Coastal and Freshwaters, vol. 1. World Health Organisation, Geneva, 219.
Wieland, U., Mauch, C., Kreuter, A., Krieg, T., Pfister, H., 2009. Merkel cell polyomavirus DNA in persons without merkel cell carcinoma. Emerging Infectious Diseases 15 (9), 1496–1498.
Wyn-Jones, A.P., Carducci, A., Cook, N., D'Agostino, M., Divizia, M., Fleischer, J., Gantzer, C., Gawler, A., Girones, R., Höller, C., de Roda Husman, A.M., Kay, D., Kozyra, I., López-Pila, J., Muscillo, M., Nascimento, M.S., Papageorgiou, G., Rutjes, S., Sellwood, J., Szewzyk, R., Wyer, M., 2011. Surveillance of adenoviruses and noroviruses in European recreational waters. Water Research 45 (3), 1025–1038.
Yousry, T.A., Major, E.O., Ryschkewitsch, C., Fahle, G., Fischer, S., Hou, J., Curfman, B., Miszkiel, K., Mueller-Lenke, N., Sanchez, E., Barkhof, F., Radue, E.W., Jager, H.R., Clifford, D.B., 2006. Evaluation of patients treated with natalizumab for progressive multifocal. The New England Journal of Medicine 354, 924–933.
Zeng, S.Q., Halkosalo, A., Salminen, M., Szakal, E.D., Puustinen, L., Vesikari, T., 2008. One-step quantitative RT-PCR for the detection of rotavirus in acute gastroenteritis. Journal of Virological Methods 153, 238–240.

6. CAPÍTULO III

Aplicación de nuevas técnicas de secuenciación en masa para el estudio del viroma del agua residual urbana

Resumen Estudio 5:

Raw sewage harbors diverse viral populations

Cantalupo PG, **Calgua B**, Zhao G, Hundesa A, Wier AD, Katz JP, Grabe M, Hendrix RW, Girones R, Wang D, and Pipas JM.

MBio, 2011, 2(5). pii: e00180-11.

RESUMEN:

Actualmente existen cerca de 3.000 virus secuenciados completamente, los cuales se agrupan en 84 familias diferentes. La presencia de virus no se limita solamente a organismos celulares, también se detectan virus en el medio ambiente producto de la excreción de organismos infectados. Estudios metagenómicos en océanos, lagos árticos, muestras de heces humanas y otros entornos, sugieren que los virus conocidos se pueden detectar en localizaciones imprevistas y que además existen un gran número de virus aún sin caracterizar en la naturaleza.

En este estudio se reportan los resultados de un análisis metagenómico de los virus presentes en agua residual. Entre los primeros ensayos realizados en la presente publicación, se encuentran los relacionados con el diseño de un método de concentración de virus que permitiera procesar 10 L de muestra para concentrar la mayor cantidad de virus presentes en agua residual. Una vez establecido el protocolo de concentración, se procedió a concentrar virus en muestras de 10 L de agua residual urbana, en Adís Abeba (Etiopía), Pittsburgh (EU) y Barcelona (España).

La diversidad vírica de agua residual se analizó empleando técnicas de secuenciación de DNA de nueva generación con la plataforma 454 GS FLX-Titatium. En las muestras se identificaron 234 virus conocidos, incluyendo 17 que infectan a humanos. También se detectaron virus que infectan plantas, insectos, algas así como bacteriófagos. Todos estos virus representan a 26 familias taxonómicas, que incluyen virus DNA de simple cadena, DNA de doble cadena, RNA de cada positiva y RNA de doble cadena. También se detectaron secuencias víricas que podrían representar un total de 51 de las 84

familias conocidas, haciendo del agua residual urbana el metagenoma vírico más diverso estudiado hasta ahora.

La presencia virus humanos también se analizó por técnicas convencionales de PCR/qPCR. Entre estos virus se detectaron HAdV y Klassevirus que también fueron detectados dentro de los 17 virus humanos identificados por secuenciación en masa. JCPyV se detectó solamente por qPCR. Igualmente los virus de la hepatitis A y E y un asfarvirus-*like* fueron detectados en estas muestras solamente por nPCR convencionales.

Este es el primer artículo científico publicado sobre el análisis de agua residual urbana por técnicas de secuenciación de nueva generación. Entre los resultados se describe el agua residual como el medio con mayor diversidad vírica estudiada hasta el momento. Los análisis bioinformáticos de los *contigs* (construcciones de secuencias de mayor tamaño), construidos a partir de los *reads* (secuencias obtenidas de la plataforma de secuenciación y tratadas con diferentes programas informáticos para eliminar secuencias inconsistentes), fueron realizados por el equipo de la Universidad de Pittsburgh.

RESEARCH ARTICLE

Raw Sewage Harbors Diverse Viral Populations

Paul G. Cantalupo,[a] Byron Calgua,[b] Guoyan Zhao,[c] Ayalkibet Hundesa,[b] Adam D. Wier,[a] Josh P. Katz,[a] Michael Grabe,[a] Roger W. Hendrix,[a] Rosina Girones,[b] David Wang,[c] and James M. Pipas[a]

Department of Biological Sciences, University of Pittsburgh, Pittsburgh, Pennsylvania, USA[a]; Department of Microbiology, Faculty of Biology, University of Barcelona, Barcelona, Spain[b]; and Departments of Molecular Microbiology and of Pathology and Immunology, Washington University School of Medicine, St. Louis, Missouri, USA[c]

ABSTRACT **At this time, about 3,000 different viruses are recognized, but metagenomic studies suggest that these viruses are a small fraction of the viruses that exist in nature. We have explored viral diversity by deep sequencing nucleic acids obtained from virion populations enriched from raw sewage. We identified 234 known viruses, including 17 that infect humans. Plant, insect, and algal viruses as well as bacteriophages were also present. These viruses represented 26 taxonomic families and included viruses with single-stranded DNA (ssDNA), double-stranded DNA (dsDNA), positive-sense ssRNA [ssRNA(+)], and dsRNA genomes. Novel viruses that could be placed in specific taxa represented 51 different families, making untreated wastewater the most diverse viral metagenome (genetic material recovered directly from environmental samples) examined thus far. However, the vast majority of sequence reads bore little or no sequence relation to known viruses and thus could not be placed into specific taxa. These results show that the vast majority of the viruses on Earth have not yet been characterized. Untreated wastewater provides a rich matrix for identifying novel viruses and for studying virus diversity.**

IMPORTANCE **At this time, virology is focused on the study of a relatively small number of viral species. Specific viruses are studied either because they are easily propagated in the laboratory or because they are associated with disease. The lack of knowledge of the size and characteristics of the viral universe and the diversity of viral genomes is a roadblock to understanding important issues, such as the origin of emerging pathogens and the extent of gene exchange among viruses. Untreated wastewater is an ideal system for assessing viral diversity because virion populations from large numbers of individuals are deposited and because raw sewage itself provides a rich environment for the growth of diverse host species and thus their viruses. These studies suggest that the viral universe is far more vast and diverse than previously suspected.**

Received 10 August 2011 **Accepted** 18 August 2011 **Published** 4 October 2011

Citation Cantalupo PG, et al. 2011. Raw sewage harbors diverse viral populations. mBio 2(5):e00180-11. doi:10.1128/mBio.00180-11.

Editor Michael Imperiale, University of Michigan Medical School

Address correspondence to James M. Pipas, pipas@pitt.edu.

Viruses are everywhere. On Earth, every species of bacteria, archaea, fungi, plants, worms, insects, and animals is likely to harbor numerous viruses. The presence of viruses is not limited to sites within cellular organisms; extracellular virions are also found in the environment. Oceans, rivers, lakes, and air all contain virions released from infected hosts. Every time we touch another human or pet, and often when we have contact with a contaminated environment, we are exposed to microbes, including viruses. Metagenomic studies of the oceans (1–6), arctic lakes (7), stool samples (8–14), and other environments (15–19) suggest that known viruses are found in unsuspected locations and that a large number of uncharacterized viruses exist in nature.

How big is the viral universe and how many types of viruses exist? Current views of viral diversity are shaped by the analysis of about 3,000 fully sequenced viral genomes representing 84 viral families (20). Recently, powerful metagenomic strategies in which all viruses present in an environmental or clinical sample are detected by sequencing virion-associated nucleic acids have been developed (21). Metagenomic approaches allow simultaneous comparisons of many genomes from multiple taxa, including those viruses that cannot be cultured. We are using metagenomics to explore the virus populations in diverse biomes and unique niches throughout the world. For our initial studies, we sought an environment, raw sewage (untreated wastewater), that we hypothesized would harbor a high diversity of viruses.

Raw sewage represents the effluence of society. Human waste from thousands of individuals is deposited into collection systems that terminate at a common point, the wastewater treatment plant. Pathogens excreted into urban sewage reflect the infections that have been transmitted in the population (22) and would include the viral pathogens that are transmitted through fecally contaminated water or food (23, 24). The implementation of current regulations on wastewater treatments has significantly reduced the levels of microbiological contamination. However, human viruses are still widely disseminated in water and the environment through discharges of untreated and treated sewage (25, 26) to river catchments and to coastal water, water reuse in food irrigation, and shellfish production (27). This mixture of water, human and animal wastes, and plant material forms a special ecosystem supporting insect, rodent, and plant populations as well as both prokaryotic and eukaryotic microorganisms. Viruses are associated with the biological wastes deposited into sewage as well as with all the species growing in sewage, making untreated wastewater an ideal environment for exploring viral diversity. In fact,

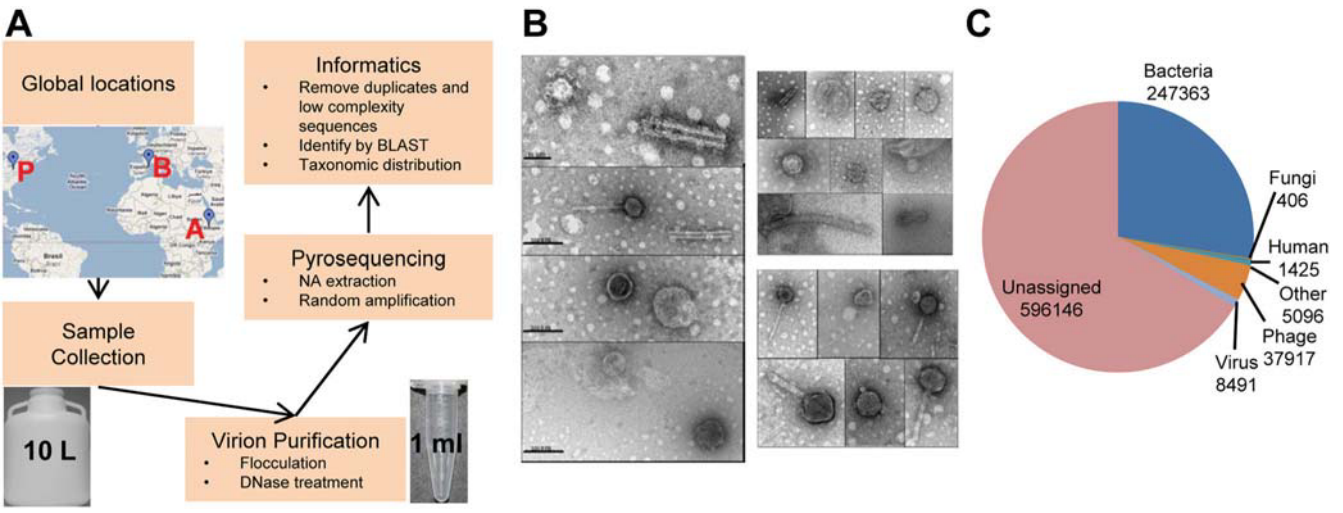


FIG 1 Raw sewage contains diverse viruses. (A) Raw sewage was obtained from three cities (P, Pittsburgh, Pennsylvania, United States; B, Barcelona, Spain; A, Addis Ababa, Ethiopia) on three different continents. Virion populations were concentrated by organic flocculation (31). Raw sewage metagenomes were obtained through pyrosequencing, and the sequences are classified by subsequent bioinformatic methods. 10 L, 10 liters; NA, nucleic acid. (Reproduced from Google—Map data ©2011 Geocentre Consulting, MapLink, Tele Atlas.) (B) Examination of raw sewage by electron microscopy reveals a diversity of virion morphologies. All black bars represent 100 nm, except the top bar, which represents 50 nm. (C) Total nucleic acid (DNA and reverse-transcribed RNA) was sequenced and binned according to taxa based on BLAST searches. Most sequences found within virions do not match the sequences in public databases.

many studies have shown that multiple types of viruses can be found in raw sewage (28–30). Here we report the results of a metagenomic survey of viruses present in raw sewage.

RESULTS

Untreated wastewater was collected from three different locations: (i) Pittsburgh, Pennsylvania, United States; (ii) Barcelona, Spain; and (iii) Addis Ababa, Ethiopia (Fig. 1A). Electron microscopy confirmed the presence of numerous different virion morphologies in the samples (Fig. 1B). Virions were concentrated and purified by organic flocculation and DNase treatment (31). In order to capture the genomes of both DNA and RNA viruses, total nucleic acids were isolated from each sample and reverse transcribed followed by deep sequencing. This resulted in a total of 897,647 high-quality reads (approximately 278 megabases) from all three samples (see Table S1 in the supplemental material). Each individual read was then compared to databases by a series of BLAST searches and binned according to taxa (Fig. 1C). A total of 8,491 sequence reads were most closely related to eukaryotic viruses, while 37,917 were most closely related to bacteriophages. About 27% of the sequence reads (247,363) were identified as bacterial, a number consistent with other metagenomic studies. Since these sequences were obtained from a purification scheme designed to enrich for virions, the putative bacterial sequences most likely represent either prophage genes misannotated as bacterial or gene transfer agents (GTAs) (8, 15, 19, 32, 33). Most sequences (596,146) showed no sequence relation to any known sequences in the databases and thus are most likely to be derived from novel, uncharacterized viruses. Further analysis of the bacterial and unassigned sequences is described in the supplementary material.

Raw sewage contains many known viruses from a diversity of hosts. We further partitioned the individual reads that have a significant BLAST hit (see Materials and Methods) to eukaryotic viruses or phages into two categories: known and novel. We arbitrarily defined known sequences as those that are related to a viral genome listed in the NCBI taxonomy database with ≥80% sequence identity over ≥95% of the length of the sequence read. By these criteria, 3,027 reads were deemed to be derived from known viruses. The remaining sequences were binned as novel viruses and are discussed below. Analysis of the sequences identified as known viruses demonstrates that our methods detected diverse types of viruses. We detected 234 known viruses. Members of 26 different families, including those with double-stranded DNA (dsDNA), single-stranded DNA (ssDNA), positive-sense ssRNA [ssRNA(+)], and dsRNA genomes, and those with either enveloped or nonenveloped virions were found, making raw sewage the most diverse viral biome examined thus far (Fig. 2A; see Table S2 in the supplemental material).

Like other biomes that have been studied, the virome of raw sewage is dominated by bacteriophages. Of the 46,408 high-quality reads that matched viruses in the databases at this time, 37,917 (~80%) were related to bacteriophages. These viruses included members of 13 virus families, but members of five families dominated the population. The five families were the *Microviridae* (37%), *Siphoviridae* (24%), *Myoviridae* (17%), *Podoviridae* (14%), and *Inoviridae* (3%). These bacteriophage families are associated with 24 bacterial host species, but over half of the reads are related to bacteriophages that infect enterobacteria or lactococci (Fig. 2C). The bacteriophage sequences binned as novel viruses outnumbered those that matched bacteriophage genomes in GenBank databases by 30:1.

Most of the known eukaryotic virus reads (90.9%) found in raw sewage were derived from plant viruses (Fig. 2B). This is not surprising, given that plant viruses dominate the viral communities present in human stool samples and that they have been detected in a number of aquatic biomes (13, 29). Roughly 85% of the sequence reads classified as known viruses were derived from 18 different species of the family *Virgaviridae*. Many other types of plant viruses were found; they included members of the *Alphaflexiviridae*, *Betaflexiviridae*, *Bromoviridae*, *Closteroviridae*, sobe-

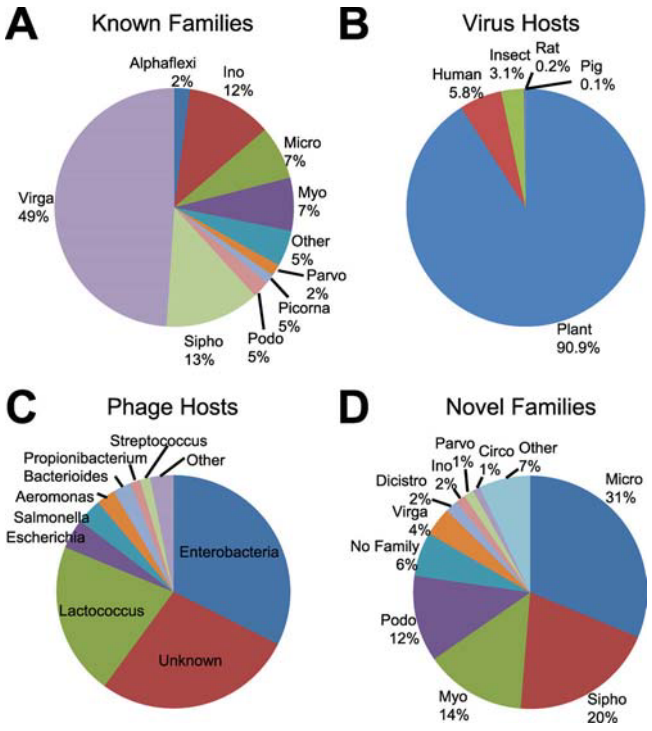


FIG 2 Raw sewage contains many known and novel viruses. (A) Known sequences ($n = 3,027$) identified by BLAST are related to many different viral families. Families with <1% abundance were collapsed into the "Other" category. Only the prefixes of family names are shown (e.g., Virga for *Virgaviridae*). (B) Distribution of the hosts of the known eukaryotic virus reads ($n = 1,748$). Plant, human, and insect viruses are abundant in raw sewage. (C) Distribution of the hosts of the known bacteriophage reads ($n = 1,279$). (D) Novel sequences ($n = 43,381$) identified by BLAST are related to many different virus families. Families with <1% abundance were collapsed into the "Other" category. See Table S6 for a list of families and hosts in the "other" category.

movirus, *Tombusviridae*, and *Tymoviridae*. A large number of insect virus reads (3.1%), including those that infect cockroaches, flies, and mosquitoes, were present in all three samples. Insect viruses most likely are present because some insect viruses also infect plants and because wastewater transmission lines can harbor large insect populations. These viruses included members of the *Dicistroviridae*, *Iridoviridae*, *Nodaviridae*, and *Parvoviridae* families. We also identified several viruses of rodents, including strains closely related to a newly identified rat hepatitis E virus (see Fig. S1 and Table S3 in the supplemental material) (34).

We detected 17 viruses known to infect humans in the three sewage samples (Table 1). These viruses included human adenovirus, a well-studied indicator of human fecal contamination (35, 36), as well as a number of known human pathogens, including astroviruses, Norwalk virus, and members of the family *Picornaviridae*, such as Aichi virus and parechoviruses. We also detected the newly discovered klassevirus (37). The relatively newly characterized human bocavirus and picobirnaviruses were also present. We also detected human papillomavirus 112 (data not shown) and the newly discovered human polyomavirus 6 (see Fig. S1 and Table S3 in the supplemental material) (38). Both of these viruses are tropic for skin, suggesting that viruses from human skin as well as stools find their way into sewage, possibly through excretion in urine as is the case for human polyomaviruses. Despite the large number of viruses detected, the current depth of sequencing was not sufficient to detect all viruses known experimentally to be present in the samples. For example, no sequences related to the human polyomavirus JC virus (JCV) were found, even though its presence in the samples was established by PCR (Table 2).

TABLE 1 Human viruses present in raw sewage

Family	Species	Genome
Adenoviridae	Human adenovirus 41	dsDNA
Astroviridae	Astrovirus MLB1	ssRNA(+)
	Human astrovirus 1	ssRNA(+)
Caliciviridae	Norwalk virus	ssRNA(+)
	Sapporo virus	ssRNA(+)
Papillomaviridae	Human papillomavirus 112	dsDNA
Parvoviridae	Adeno-associated virus	ssDNA
	Human bocavirus 2	ssDNA
	Human bocavirus 3	ssDNA
Picobirnaviridae	Human picobirnavirus	dsRNA
Picornaviridae	Aichi virus	ssRNA(+)
	Human klassevirus 1/Salivirus NG-J1	ssRNA(+)
	Human parechovirus 1	ssRNA(+)
	Human parechovirus 3	ssRNA(+)
	Human parechovirus 4	ssRNA(+)
	Human parechovirus 7	ssRNA(+)
Polyomaviridae	Polyomavirus HPyV6	dsDNA

Raw sewage contains many novel viruses. Next, we examined the 43,381 sequence reads that represent novel viruses according to our criteria (see Table S4 in the supplemental material). Figure 3 shows the distribution of these sequences by identity to known viruses in the GenBank databases. The outer ring represents the group of sequences with >90% identity to the reference genome in the top BLAST hit. The internal rings indicate sequences with decreasing identity, binned by 10% intervals. The area of each colored circle is proportional to the number of sequence reads that match the reference genome at a given percent identity in that location for that virus family. The color of the circle indicates the location from which the sequence was obtained. For some virus families, such as the *Virgaviridae*, nearly all of the sequence reads matched known viruses. In other cases, such as the *Picornaviridae* and *Parvoviridae*, some of the sequences matched recognized viruses, but the majority were only distantly related to known members of these families. In most cases (i.e., *Circoviridae*, *Phycodnaviridae*, *Microviridae*, and *Siphoviridae*), nearly all of the sequence reads were derived from putatively novel viruses. Thus, greater than 90% of the sequence reads that could be aligned to known viruses represent sequences from novel viruses that have not been described previously. The novel viruses in the samples show enormous diversity, falling into 51 different viral families (Fig. 2D; see Table S4 in the supplemental material).

Next, we assembled the virus sequence reads and aligned them to a common GenBank reference genome (Fig. 4). In these fragment recruitment plots, assembled sequences belonging to a particular virus family were aligned to GenBank reference genomes for that virus family. Then, the sequence relations of the common regions were compared to each other and to known members of the viral taxon using standard phylogenetic methods.

For example, Fig. 4A shows the four assembled sequences that align to the same region of the human bocavirus genome. Phylogenetic analysis of these sequences suggests that they each repre-

TABLE 2 Detection of classical and emerging viruses in urban sewage by PCR assays

Virus analyzed[a]	PCR type	Virus detected[b] in urban sewage sample from:		
			Addis Ababa, Ethiopia	
		Barcelona, Spain	Sample 1	Sample 2
Human adenovirus	Real time	10,100 GC/ml	10.3 GC/ml	802 GC/ml
JC polyomavirus	Real time	18.3 GC/ml	178 GC/ml	734 GC/ml
Human hepatitis E virus	Nested	−	+	−
Human hepatitis A virus	Nested	−	+	+
Klassevirus 1	Nested	+	+	+
Asfarvirus like-virus	Nested	−	+	−

[a] See Materials and Methods for references for each PCR.

[b] GC, genome copies; −, not detected; +, detected. The volume of sample analyzed in 10 μl of extracted nucleic acid was 33.33 ml for the sewage sample from Barcelona, Spain, and the volume was 43.75 ml for the samples from Addis Ababa, Ethiopia.

sent a different novel bocavirus. Figure 4C shows a similar analysis of 11 sequences that align to the human picobirnavirus genome. Picobirnaviruses are dsRNA viruses whose genome consists of two segments. Five assembled sequences aligned to a common region of genomic segment 1, while six aligned to segment 2. Again, phylogenetic analysis suggests the presence of 5 or 6 novel picobirnaviruses. Fragment recruitment plots also suggested the presence of at least three different novel viruses related to the human pathogen Aichi virus (Fig. 4B), and multiple novel viruses related to the dicistroviruses (see Fig. S2 in the supplemental material). In addition, a large number of novel circovirus-like genomes were identified (see Table S4 in the supplemental material; also data not shown). Circoviruses are a family of viruses with a single-stranded circular DNA genome that have been shown to be present in animal, bird, and human feces as well as raw sewage (29, 39).

A novel member of the *Inoviridae* is abundant in raw sewage worldwide. The initial assignment of sequence reads to viral taxa was accomplished by BLAST searches. We performed two additional computational steps to confirm our conclusions regarding virus diversity in raw sewage. First, we subjected selected assembled sequences to genetic signature analysis (GSA), a manual sequence analysis procedure in which the sequence reads and open reading frames (ORFs), contained within the reads, were examined for the presence of eukaryotic and prokaryotic genetic signatures such as promoters, factor binding sites, polyadenylation and splice signals, and ribosome binding sites. GSA also included a close examination of the sequence alignments that led to the taxonomic assignment of each sequence. These steps led to the reassignment of some of the sequences to different taxa. The most striking example of a misassignment uncovered by GSA is that of non-A, non-B hepatitis virus. The large number of sequence reads related to this virus suggested that it was among the most abundant eukaryotic viruses present in raw sewage, a result confirmed by PCR (Fig. 5C). This virus was originally isolated from stool samples from hepatitis patients and thus potentially was of great interest (40, 41).

We assembled 794 reads that had at least 80% identity to non-A, non-B hepatitis virus (GenBank accession no. X53411) with phrap (http://www.phrap.org), using the default parameters. The assembly produced a 4,818-bp contig (named WW-nAnB). Initial alignments with the sequence deposited in GenBank under accession no. X53411 (X53411 sequence) showed that WW-nAnB assembled as a circle. After we edited the contig to put it in the same orientation as in the X53411 sequence, we aligned it to the X53411 sequence with BLASTN, and the resulting dot matrix plot is shown in Fig. 5A. At the 5′ end of the contig, there were two small insertions of 20 and 38 bp with respect to the X53411 sequence. There was also a 250-bp deletion in WW-nAnB with respect to the X53411 sequence at nucleotide position 1542. We identified homologs of the four ORFs in the X53411 sequence. We discovered several positions in the sequence of WW-nAnB that disrupted the reading frame of three ORFs compared to homologous ORFs in the X53411 sequence. Additionally, there was an ambiguous base in one position. To determine the correct nucleotide sequence of these positions, targeted regions of the genome were resequenced by Sanger sequencing of PCR products, and appropriate corrections were made to the WW-nAnB sequence. Sanger sequencing gave unambiguous resolution to the uncertainties in the original sequence, in particular correcting all the apparent frameshift errors, which brought the ORF structures of the X53411 sequence and WW-nAnB into agreement (Fig. 5B). We further confirmed the presence of non-A, non-B hepatitis virus in the virion preparations using specific PCR primers targeted to the open reading frame 4 (ORF4) sequence of the X53411 sequence. Forty-five cycles of PCR were performed on different virion preparations from five samples of raw sewage. The expected 373-bp PCR product appeared in all virion preparations (Fig. 5C). Sequencing of the PCR products revealed some nucleotide variation, suggesting the presence of different variants of non-A, non-B hepatitis virus in raw sewage. Also, the phylogenetic relationships among the sequences revealed that they are more similar to each other than to the X53411 sequence.

There are no reports on the properties of non-A, non-B hepatitis virus beyond the original report of the genomic sequence (41), and it has not been classified into any formal taxonomic group. Our attempts to identify some of the sequence signals typically found in a virus infecting eukaryotic hosts, such as promoters and poly(A) addition sequences, were not successful. However, we did find strong evidence of prokaryotic transcription and translation signals, including sigma70-like promoters and Shine-Dalgarno (SD) translation initiation sequences. We found convincing SD sequences appropriately positioned at the beginnings of three of the four ORFs annotated in the X53411 sequence. For the fourth ORF (ORF2), there is no SD sequence upstream from the AUG start codon annotated in the X53411 sequence. However, there is an excellent SD sequence upstream of that position, appropriately located for an initiation codon 90 bases upstream from the annotated start codon in the ORF2 reading frame in both genomes. Therefore, we suggest that this is the correct start site for translation of this gene. This initiation codon is AUG in the WW-

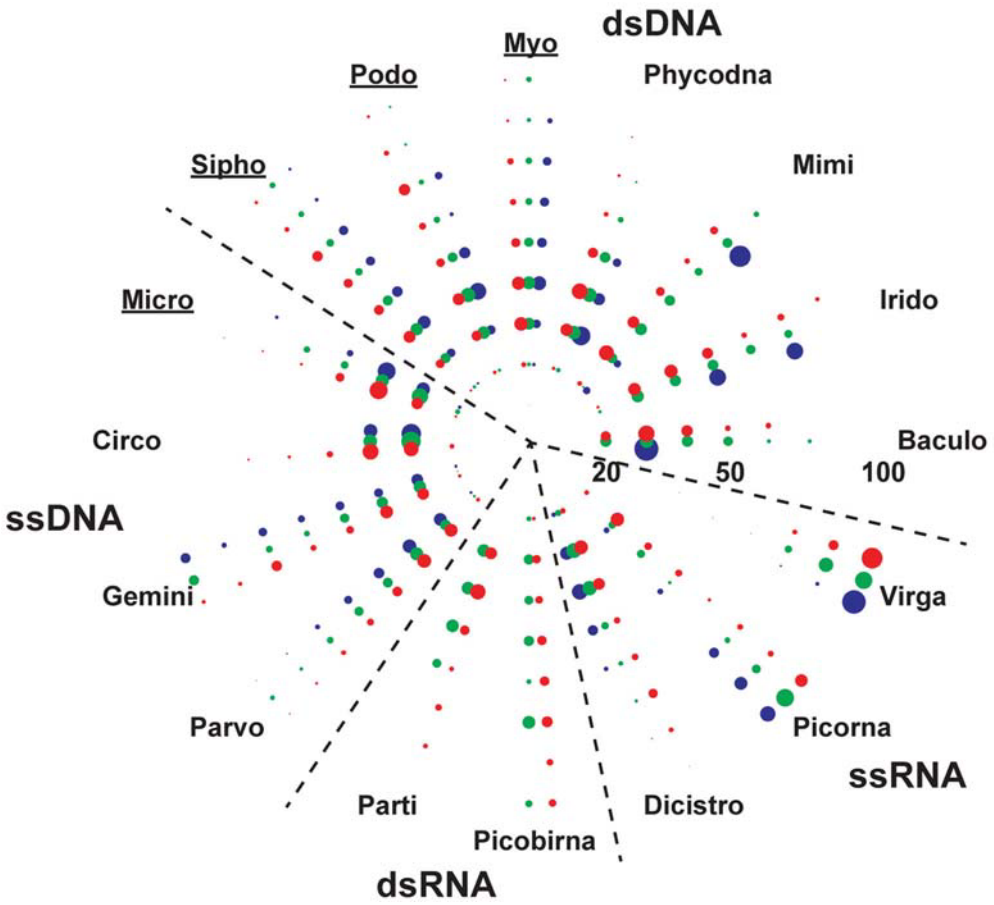


FIG 3 Most virus-related pyrosequencing reads found in raw sewage represent previously unknown viruses. Diversity plot of selected viral families (only the prefix of a family name is shown) are organized by genomic content (dsDNA, ssDNA, ssRNA, and dsRNA). The four phage families are underlined. The rings of the plot represent bins of increasing percent identity (20%, 50%, and 100% are marked for orientation) relative to the GenBank reference sequence as identified by the top BLAST hit. The area of each circle is proportional to the number of virus family reads in that location. The geographic locations from which the sequence reads were obtained are indicated by color: blue, Addis Ababa, Ethiopia; green, Barcelona, Spain; red, Pittsburgh, PA, USA.

nAnB sequence but GUG in the X53411 sequence. GUG start codons are found rather commonly in prokaryotic sequences but virtually never in eukaryotic sequences. In addition to the four large ORFs, we have identified four additional small putative genes located in the spaces between the larger ORFs, based on appropriately positioned SD sequences and good coding potential (Fig. 5D).

We probed the public databases with the predicted protein sequences from WW-nAnB, and the results are reported in the supplemental material. On the basis of the size of the genome, the sequence matches obtained, and other features of the sequence described in the supplemental material, we believe that WW-nAnB (and the non-A non-B hepatitis virus with GenBank accession no. X53411) are members of the *Inoviridae* family of bacteriophages. The *Inoviridae* family contains the filamentous phages, of which the best-characterized examples are the *Escherichia coli* phages f1, fd, and M13. Figure 5D compares the genome map of WW-nAnB to those of 3 well-characterized filamentous phages.

Deep sequencing of virion-associated nucleic acids suggests the presence of large numbers of uncharacterized viruses. Most of our analysis has focused on the 46,408 sequence reads that could be assigned to one of the existing 84 viral taxa. However, over 247,000 reads were binned as bacteria, and nearly 600,000 reads were not related to sequences in genomic databases (Fig. 1C). The bacterial sequences in the samples could represent bacteria that escaped the virion enrichment methods, gene transfer agents (33), or prophage genes (8, 15, 19, 32). Microscopic examination of the virion preparations used for deep sequencing did not reveal any bacterial contamination. Still, we cannot rule out the possibility that a small amount of bacterial DNA remains in the virion preparations. Furthermore, the amount of sequences binned as bacteria in our study is consistent with the results of several other metagenomic studies (1, 8, 12, 18, 19, 29). It is likely that these sequences either represent GTAs or bacterial genes present in bacteriophage transducing particles or they are in fact bacteriophage genes. Thus, novel bacteriophages are likely included among these bacterial sequences.

A majority of the high-quality sequence reads obtained in this study were binned as "unassigned" because they did not significantly match sequences present in the current databases. These sequences most likely represent uncharacterized viruses that are not related to or are very distantly related to the 3,000 or so known viruses. Examination of some of the assembled unassigned sequences revealed ORF patterns consistent with members of the

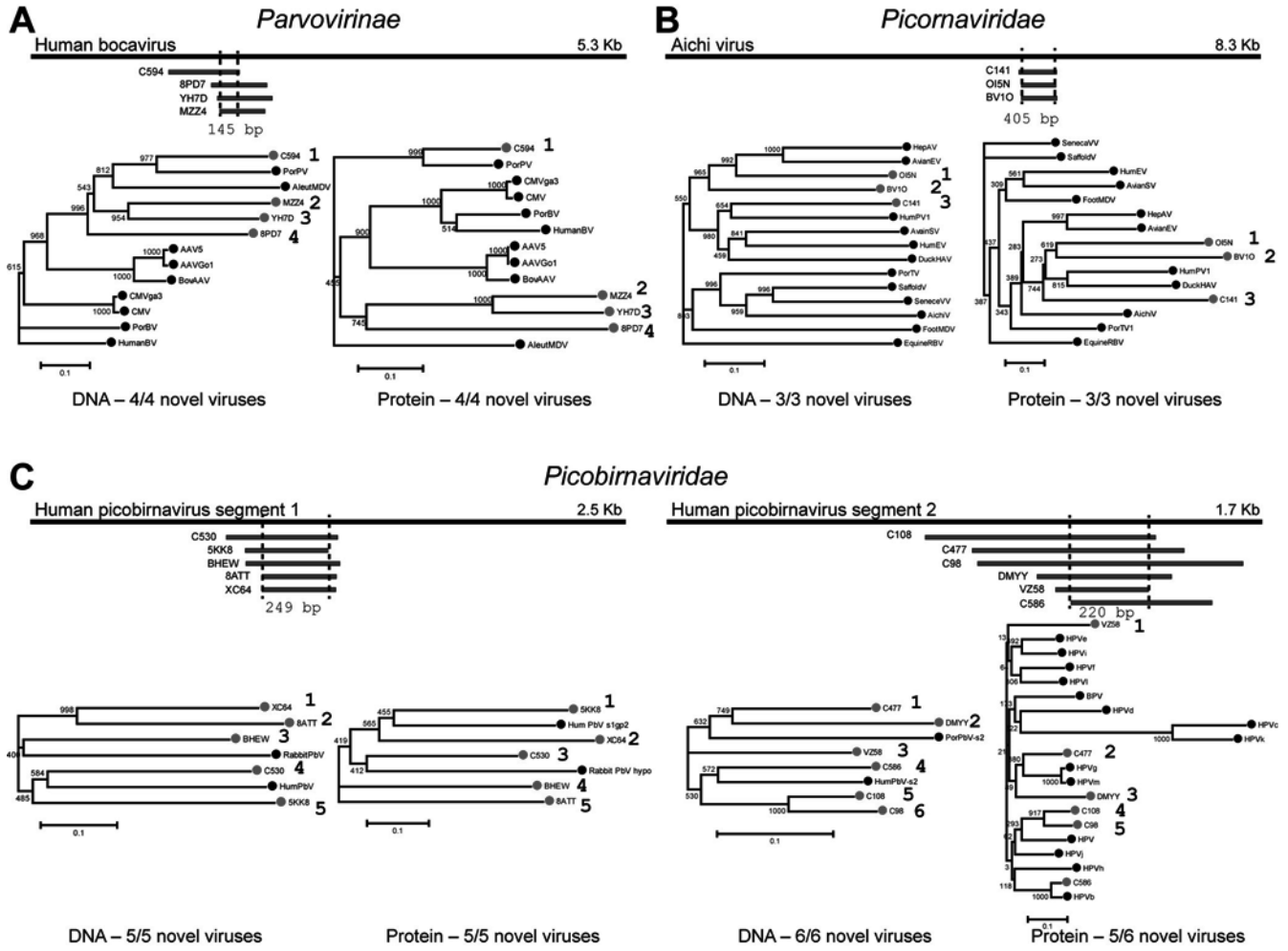


FIG 4 Novel virus analysis from selected virus families. (A to C) A selected set of novel assembled sequences from three different virus families that overlapped each other on a representative genome from each family was aligned with ClustalW2. The nucleotide alignment is shown graphically in the fragment recruitment plot (top) with vertical black broken lines marking the common alignment region against a selected reference genome from the virus family. Each assembled sequence was translated, and the resulting ORFs were aligned with ClustalW2. DNA and protein neighbor-joining (NJ) phylogenetic trees were constructed from homologous positions without any gaps. Metagenomic sequences (red circles) and GenBank sequences (black circles) are indicated. Metagenomic sequences that are labeled with a number represent different novel virus species in the raw sewage. (A) For the *Parvovirinae*, 4 novel assembled sequences were aligned with 9 selected reference *Parvovirinae* genomes, and the nonstructural (NS) gene from each genome was used for the protein alignment. (B) For the *Picornaviridae*, 3 novel assembled sequences were aligned with 12 selected reference *Picornaviridae* genomes, and the polyprotein from each genome was used for the protein alignment. Alignment is in the P-loop NTPase domain of the 2C ATPase mature peptide of the polyprotein. (C) For the *Picobirnaviridae*, for segment 1 (left), 5 novel assembled sequences were aligned with the 2 reference segment 1 sequences in GenBank (human and rabbit), and the segment 1 ORF from each genome was used for the protein alignment. For segment 2 (right), 6 novel assembled sequences were aligned with the 2 reference segment 2 sequences in GenBank (human and porcine) and 14 RdRp ORFs (13 human and 1 bovine) was used for the protein alignment. See Fig. S3 for the ORF alignments. Virus abbreviations: PorPV, porcine parvovirus; AleutMDV, aleutian mink disease virus; AAV5, adeno-associated virus 5; BovAAV, bovine AAV; CMV, canine minute virus; PorBV, porcine bocavirus; HepAV, hepatitis A virus; AvianEV, avian encephalomyelitis virus; HumPV1, human parechovirus 1; DuckHAV, duck hepatitis A virus; PorTV, porcine teschovirus; SaffoldV, Saffold virus; AichiV, Aichi virus; FootMDV, foot-and-mouth disease virus; EquineRBV, equine rhinitis B virus; SenecaVV, Seneca Valley virus; Rabbit PbV, rabbit picobirnavirus; HumPbV, human picobirnavirus; BPV, bovine picobirnavirus.

Microviridae and other bacteriophage taxa (data not shown). Furthermore, approximately 355,000 metagenomic reads did not assemble into multiread contigs, suggesting a high degree of sequence diversity. If we assume that all individual sequence reads binned as unassigned represent novel viruses, then novel viruses (596,146 + 43,381 = 639,527) outnumber those binned as known viruses (3,027) by a ratio of over 200:1. On the other hand, if none of the unassigned sequences represent novel viruses but rather are derived from other taxa (bacteria, etc.), then the ratio (43,381/3,027) of novel to known viral sequence reads is approximately 10:1. In any event, our data demonstrate that known viruses represent a small fraction of the viral universe.

Finally, we compared the high-quality sequence reads from our experiment with sequences detected in other metagenomic studies, including reclaimed wastewater (29), human feces (8, 11, 14), and three marine environments (1, 2, 19). Since several of the metagenomes consisted of individual reads, we used CD-HIT (using the same parameters as performed on the raw sewage metagenome) to remove duplicate reads. For this comparison, we performed a BLASTN search using the 897,647 high-quality raw

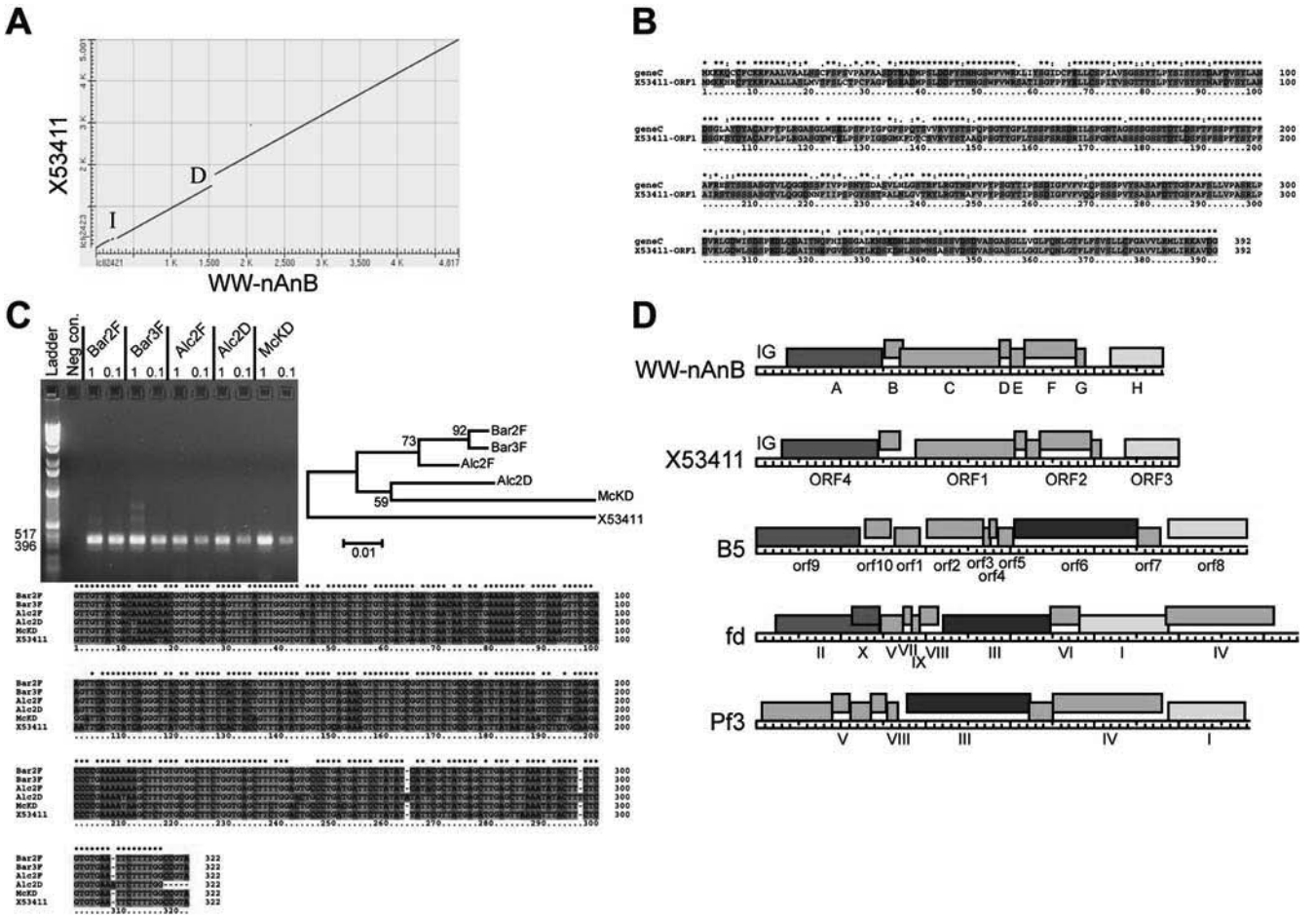


FIG 5 An assembled genome of non-A, non-B hepatitis virus from raw sewage shows that it belongs to the *Inoviridae* family. (A) BLASTN alignment of WW-nAnB and the non-A, non-B hepatitis virus with GenBank accession no. X53411 (X53411 sequence) is displayed as a dot matrix plot. The WW-nAnB sequence and the X53411 sequence run 5′ to 3′ on the *x* axis and *y* axis, respectively. The positions of the insertions (I) and deletion (D) are labeled. (B) Protein alignment of the X53411 ORF1 with the corrected gene C sequence from WW-nAnB. Identical amino acids (*), highly similar amino acids (:), and amino acids with low similarity (.) are indicated. (C) Forty-five cycles of PCR were performed with 0.1 and 1 μl of five different virion preparations from raw sewage. Shinola was used as a negative control (Neg. Con.). PCR products were visualized by EtBr on a 1.5% agarose gel. DNA ladder sizes are indicated in base pairs. The specific PCR product bands (373 bp) were excised and sequenced. The resulting nucleotide sequences were aligned (shown at the bottom of the panel), and a bootstrapped phylogenetic tree was generated based on the alignment (top right corner of panel). (D) WW-nAnB belongs to the *Inoviridae* family of bacteriophages. The genomic organization of WW-nAnB compared to non-A, non-B hepatitis virus (GenBank accession no. X53411), *Propionibacterium* phage phiB5 (B5) (GenBank accession no. AF428260), enterobacterial phage fd (GenBank accession no. J02451), and bacteriophage Pf3 (GenBank accession no. M19377) is shown. Unlabeled X53411 ORFs are homologous to the similarly located ORFs in WW-nAnB. DNA replication initiation proteins are shown in red, assembly proteins with an ATPase domain are shown in yellow, absorption proteins are shown in blue, and all other identified ORFs are shown in green. Each tick mark in the ruler below each genome represents 100 bp. IG, noncoding intergenic region.

sewage reads as the query sequences against each metagenome. We applied an E-value cutoff of 1e − 5 to score a significant match. We found that only a small number of sequences detected in each of these metagenomes were significantly related (see Table S5 in the supplemental material). The metagenome most closely related to raw sewage is the monozygotic twin feces metagenome (11). A total of 486,392 unique sequences were obtained in the twin study of which 40,594 (8.3%) showed a significant match to 17.3% (155,083) of the raw sewage sequence reads. Similarly, about 12.2% and 9.9% of the sequences we identified in raw sewage were similar to sequences from the human gut microbiome and reclaimed water, respectively. Other metagenomes harbored fewer viral sequences similar to those found in raw sewage. In total, these observations emphasize the vastness of viral diversity among different biomes.

DISCUSSION

The International Union for Conservation of Nature lists nearly 1.8 million species of living organisms on Earth. Each of these species is likely to harbor multiple types of viruses uniquely adapted to proliferate in the cellular environment they provide. However, only about 3,000 viruses have been identified thus far, suggesting that our knowledge of the viral universe is limited to a tiny fraction of the viruses that exist. Pioneering studies in viral metagenomics have led to advances in methods for capturing virus particles, sequencing their nucleic acids, and in the computational analysis of metagenomic data (21, 42). The results of metagenomic studies of the viromes present in oceans, lakes, human gut and stool samples, and reclaimed wastewater are consistent with the notion that large numbers of uncharacterized viruses exist in nature.

We performed a metagenomic survey of the viruses present in three samples of untreated wastewater obtained from three different continents. After steps to remove bacteria and other relatively large particles, virus particles were concentrated by organic flocculation and treated with DNase. Virion-associated nucleic acids were extracted and reverse transcribed so as to include both RNA and DNA genomes in the subsequent deep sequencing steps. Although each of the three samples was sequenced separately, we pooled these data for the purposes of this study. Computational methods were then used to assign each sequence read to specific taxa and to determine whether the sequence represented a previously characterized (known) virus recorded in the GenBank databases. This approach detected 234 known viruses. However, the vast majority of genomes present in the samples represent novel viruses. Representatives of 51 viral families were detected, making raw sewage the most diverse viral biome examined thus far.

Despite the large number of known and novel viruses detected, not all viruses present in the samples were detected by our methods. For example, JC virus (JCV), a human polyomavirus frequently associated with fecal/urine contamination was not detected by deep sequencing, although PCR experiments indicated its presence. This suggests that our data underestimate the number of viruses present in the samples. One reason viruses present in the sample could fail to be detected is that their abundance is below the resolution of sequencing. For example, JC polyomavirus is present in samples of raw sewage from Barcelona, Spain, at 18 genome copies (GC)/ml, but human adenovirus, which is represented by 20 sequencing reads in the raw sewage metagenome, is present at 10,100 GC/ml (Table 2). In this case, deeper sequencing of the sample will reveal additional viruses.

The probability of detecting a particular virus in a complex environmental sample such as untreated wastewater is directly proportional to the number of observable virions of species *i* in the sample (N_i^{obs}). This value changes in time according to the differential equation shown below, with the right hand side being a function of five time-dependent variables.

$$\frac{dN_i^{obs}}{dt} = \left(\underbrace{\phi_i}_{deposition} + \underbrace{\kappa_i}_{production} - \underbrace{\delta_i}_{decay} \right) \underbrace{\varepsilon_i}_{recovery} \underbrace{\beta_i}_{detection}$$

First is the rate with which virus particles are deposited in the sample. In the case of raw sewage, virus particles enter the sample in the form of human and animal feces and urine, plant material from domestic and agricultural areas, as well as from insects and rodents found in the sewer system (ϕ_i). Second, new virus particles are created by the infection of host species growing in the sewage (κ_i). Raw sewage provides a rich environment for the growth of bacteria, rotifers, amoeba, and fungi, and as these organisms become infected, the resulting progeny viruses will be shed into the sample. The accumulation of virus particles in sewage via deposition and infection is balanced by the physical decay of virions (δ_i). All three of these parameters are dependent on time and thus will vary during different times of day, in different seasons, and in different climates. Finally, the probability of detection is a function of both the efficiency of virion recovery (ε_i) from the sample and the efficiency of detection (β_i). For example, the use of CsCl gradients to purify virions eliminates certain types of viruses either because they do not band in the selected density range or because they are disrupted by CsCl. Similarly, the methods used to isolate and amplify viral nucleic acids can eliminate or favor certain genome types. No one method efficiently recovers and detects all types of virions, and thus, a complete survey of viral diversity will require a combination of approaches.

A key step in metagenomic analysis is the assignment of individual sequence reads or assembled sequences to viral taxa. Each individual read or assembled sequence should represent the nucleic acid present in an individual virion, and thus, a single viral species. Generally, this taxon assignment is accomplished by a BLAST search with the E value being the arbiter of taxon assignment with most metagenomic studies using the top BLAST hit to identify and classify sequence reads. In this study, we divided the taxonomic classification of sequence reads into three steps. First, the broad binning of sequences into those related to viruses, bacteria, or other major taxa was based on BLAST scores. Second, known viruses were identified on the basis of nucleotide identity through the entire sequence read with a viral genome listed in the GenBank database. However, it is still possible that some novel viruses might be classified as a known virus. For example, bacteriophages exhibit high levels of horizontal gene transfer generating a mosaic of genome types (43–45). Since metagenomic studies seldom yield enough sequence data to assemble an entire genome, it is possible that some of the viruses classified as known are actually chimeras where only a portion of the genome matches the GenBank reference sequence. Finally, the remaining sequences representing potentially novel viruses were manually examined to confirm their taxonomic assignment. This manual analysis revealed numerous ambiguities and in some cases errors in taxon assignments. Some errors in taxon assignments resulted from misannotations of databases. In other cases, the correct viral taxon could not be ascertained because homologs of viral genes exist in multiple viral taxa.

We are using metagenomics to explore viral diversity in a number of different biomes. To begin these studies, we wanted to examine environments where viral concentrations and diversity are relatively high. In this regard, we hypothesize that the highest concentrations of viruses will be found where there is a high density of host species and that viral diversity will correspond to the biodiversity of host species. Urban sewage has been selected as a unique example of a matrix with high concentrations of highly diverse viruses. Urban sewage is a virus-rich matrix because humans excrete waste materials from the diverse food consumed, especially plants that are known to be very rich in viruses, and the bacterial and viral members of the human microbiota and common viral infections. The matrix we analyze includes the excreted virome plus the external input from insects, rodents, and other inhabitants of the urban sewerage system as well as bacteria growing in the wastewater. We have not attempted to measure the relative numbers of different viral species present in the sample. Nor have we sampled sewage in different seasons or in different climates or performed an extensive study of different geographic locations, all of which are likely to influence the dynamics of viral populations. These issues await future studies.

Finally, we point out that while untreated wastewater is a rich source of novel viruses, it is still a limited one. The diversity of host species that occupy this ecosystem is limited by its unique chemical composition. Earth is rich with many disparate biomes, each harboring a multitude of host species and their viruses. The exploration of the viral universe has only just begun.

MATERIALS AND METHODS

Sample collection sites. Untreated wastewater was obtained from three locations: (i) Pittsburgh, Pennsylvania, United States; (ii) Barcelona, Spain; and (iii) Addis Ababa, Ethiopia. The Pittsburgh wastewater treatment plant (WWTP) provides services to approximately 1 million people in the city and many surrounding communities. The Barcelona WWTP is located on the south coast of Spain. The Barcelona WWTP receives wastewater from six towns with an approximate total population of 172,000 inhabitants. The WWTP treats the raw wastewater from domestic origin as well as treated wastewater from industries. The Addis Ababa WWTP services a city that contains approximately 3 million inhabitants. Data on the volume of raw sewage that is treated by the WWTPs are not available.

Enrichment of virion populations from untreated wastewater. Untreated wastewater (5 liters) was collected from the WWTP in Pittsburgh, PA, in December 2009 and was stored at 4°C for 2 h prior to processing. Similarly, 10 liters of untreated wastewater was collected from the WWTP in Barcelona, Spain, in September 2008 and stored for 2 h at 4°C before processing. Two samples (10 liters each) were collected from the WWTP in Addis Ababa, Ethiopia, in June 2009 and processed on-site. In this case, the virion concentrates were stored frozen prior to viral nucleic acid isolation.

Virions were concentrated from wastewater samples by organic flocculation based on the procedure previously described (31). Briefly, 100 ml preflocculated skim milk solution (pH 3.5) was added to 10 liters acidified raw sewage (pH 3.5) and mixed for 8 h. Flocculants were allowed to settle and then centrifuged. The flocculated viral concentrate was resuspended in 15 ml phosphate buffer (1:2 [vol/vol] mixture of 0.2 M Na_2HPO_4 and 0.2 M NaH_2PO_4) and then eluted in 30 ml of 0.25 M glycine (pH 9.5) for 45 min at 4°C by slow agitation with vortexing. Suspended solids were separated by low-speed centrifugation at 7,500 × g for 30 min at 4°C, and the high pH of the supernatant was stabilized by adding 20 ml of 2× phosphate buffer. Virions present in the supernatant were concentrated by ultracentrifugation at 100,000 × *g* for 1 h at 4°C and resuspended in phosphate buffer.

Nucleic acid preparation and 454 sequencing. Aliquots (100 μl) of the virion concentrates from Addis Ababa, Ethiopia, Pittsburgh, Pennsylvania, and Barcelona, Spain, were treated with DNase to remove nonvirion-associated DNA. One thousand units (10 μl) of DNase (catalog no. EN0523; Fermentas) and 10 μl of the supplied 10× reaction buffer were added to each sample and incubated at 37°C for 1 h. Virion nucleic acid was purified from the DNase-treated samples and 100 μl of untreated Barcelona virus preparation using the Qiagen DNeasy blood and tissue kit (catalog no. 69504) using the manufacturer's protocol (46) except that elution was performed with 30 μl of distilled H_2O (dH_2O).

To enable subsequent detection of both RNA and DNA viruses, total virion-associated nucleic acid from each sample was reverse transcribed and amplified as previously described (47, 48). Briefly, RNA templates were reverse transcribed using PrimerA (5′-GTTTCCCAGTCACGATANNNNNNNNN) containing a 17-nucleotide specific sequence followed by 9 random nucleotides for random priming. Sequenase (United States Biochemical) was used for second-strand cDNA synthesis and for random-primed amplification of DNA templates using PrimerA. Each sample was then subjected to 40 cycles of PCR amplification using PrimerB with a bar code (5′-XXXXXXGTTTCCCAGTCACGATA) for the Barcelona samples or PrimerB without the bar code for the Pittsburgh and Addis Ababa samples using the following program: 30 s at 94°C, 30 s at 40°C, 30 s at 50°C, and 60 s at 72°C. The bar code is a unique 6-nucleotide sequence (indicated by "X") at the 5′ end of PrimerB. PrimerB is complementary to the 17-nucleotide sequence that was incorporated by PrimerA. The amplified material was visualized on an agarose gel as a final quality control step and was sequenced at the Washington University Genome Sequencing Center on the 454 GS FLX titanium platform (454 Life Sciences) according to the manufacturer's instructions.

Sequence annotation. Raw sequence reads were trimmed to remove bar codes and PrimerB sequences. CD-HIT (49) was used to remove redundant sequences. Sequences were clustered on the basis of 95% identity over 95% sequence length, and the longest sequence from each cluster was picked as the representative sequence. Then, unique sequences were masked by RepeatMasker (http://www.repeatmasker.org). If a sequence did not contain a stretch of at least 50 consecutive non-"N" nucleotides or if greater than 40% of the total length of the sequence is masked, it was removed from further analysis (i.e., "filtered"). These preprocessing steps resulted in 897,647 high-quality sequences which were sequentially compared against (i) the human genome using BLASTN; (ii) GenBank nt database using BLASTN; (iii) GenBank nr database using BLASTX; and (iv) the NCBI viral genome database (ftp://ftp.ncbi.nlm.nih.gov/refseq/release/viral/) using TBLASTX. The nt and nr databases were downloaded on 29 May 2009, and the viral genome database was downloaded on 12 August 2010. Minimal E-value cutoffs of 1e − 10 for BLASTN and 1e − 5 for BLASTX or TBLASTX were applied. Sequences were phylotyped as human, mouse, fungal, bacterial, phage, viral, or other based on the identity of the top BLAST hit. Sequences without any significant hit to any of the databases were placed in the "unassigned" category. All virus and phage sequences were further classified into families using the taxonomic information from the top BLAST hit.

A second annotation analysis (Bar-v1) was performed with the Barcelona raw sequence reads only. The reads were trimmed to remove any bar code and PrimerB sequences. CD-HIT was used to remove redundant sequences. Sequences were clustered on the basis of 98% identity over 98% sequence length, and the longest sequence from each cluster was picked as the representative sequence. Then, unique sequences were masked using RepeatMasker and processed as described above to generate a high-quality set of reads. The high-quality Barcelona sequences (n = 680,295) were sequentially compared against (i) the human genome using BLASTN, (ii) GenBank nt database using BLASTN and TBLASTX, and (iii) the NCBI viral genome database using TBLASTX. Minimal E-value cutoffs of 1e − 10 for BLASTN and 1e − 5 for TBLASTX were applied. Sequences were phylotyped and classified as described above.

Sequence assembly. Using the high-quality Pittsburgh, Addis Ababa, and Barcelona reads (from Bar-v1 annotation analysis), sequences identified as eukaryotic viruses regardless of the source of isolation were separately assembled into contigs using phrap (version 1.090518; http://www.phrap.org) at 95% nucleotide identity by using the command line option "-penalty -19." The phrap singlets and contig files were merged to create an assembled set of virus sequences (n = 2,782). The assembled sequences were sequentially annotated by (i) BLASTN and then by TBLASTX versus the GenBank nt database and (ii) TBLASTX against the viral genome database using an E-value cutoff of 1e − 5. Sequences with no significant hit were classified as "unassigned." Sequences were binned into families using the taxonomic information from the top BLAST hit.

A full assembly of the 897,647 high-quality Pittsburgh, Addis Ababa, and Barcelona reads and quality scores was done with phrap at 95% nucleotide identity. The phrap singlets and contig files were merged to create a set of assembled sequences (n = 476,960).

Sequence alignments. Nucleotide and protein sequences were aligned with ClustalW2 using default parameters. Bootstrap neighbor-joining (NJ) trees (1,000 iterations) were constructed using homologous positions that do not contain any gaps.

Electron microscopy. Samples were observed with a transmission electron microscope Tecnai SPIRIT (FEI Company, Eindhoven, The Netherlands) working at an acceleration voltage of 120 kV. Images were acquired with a MegaviewIII camera and digitized with the iTEM program, both from Soft Imaging System (SIS).

Wastewater non-A non-B hepatitis virus analysis. The Pittsburgh, Addis Ababa, and Barcelona reads (from Bar-v1 annotation analysis) that had at least 80% identity to non-A, non-B hepatitis virus (n = 794) were assembled using phrap with default parameters. Virions were purified from five different samples of raw sewage. PCR was performed with 0.1

and 1 μl of virion preparations using GoTaq (Promega) under the following conditions: initial denaturation, 5 min at 94°C; 45 cycles, with 1 cycle consisting of 1 min at 94°C, 1 min at 54°C, and 75 s at 72°C; a final extension step of 7 min at 72°C. The primers (forward [5′-GATGCAGGAAGGTCACGAAT] and reverse [5′-ACGGCCAAAAGAATTCACAC]) were designed to ORF4 of non-A, non-B hepatitis virus (GenBank accession no. X53411). PCR products were resolved on a 1.5% agarose gel and stained with ethidium bromide. PCR bands were excised and sequenced using the forward primer. Sequences were aligned with ClustalW2 and a bootstrapped NJ tree was constructed using MEGA4.

Molecular detection of viruses in wastewater by PCR. Extractions of viral nucleic acids from the Addis Ababa and Barcelona samples used in the present metagenomic study were analyzed to detect classical and emerging viruses (Table 2) by nested PCR (nPCR) and quantitative PCR (qPCR) TaqMan assays. The viruses analyzed were human strains of hepatitis E viruses (HEV), hepatitis A (HAV), klassevirus I (KV) (37), asfarvirus-like virus (ASFLV) (50), human adenoviruses (HAdV), and JC polyomavirus. The protocols used are based on previous studies (22, 51–53; B. Calgua et al., submitted for publication).

For the detection of HPyV6 polyomavirus and rat HELV (see Table S3 in the supplemental material), urban sewage samples were collected in Barcelona, Spain. Viruses from 42 ml of each untreated wastewater sample were concentrated in 100 μl of PBS by applying a virus concentration procedure based on ultracentrifugation and elution with glycine-alkaline buffer as described previously (36). Nucleic acids from the viral concentrates were extracted using the QIAamp viral RNA minikit (catalog no. 522906; Qiagen). Nested primers for the VP1 region of HPyV6 were designed for nested PCR (nPCR) assays based on the NCBI reference sequence with accession no. NC_014406. For the detection of rat HELV, a nested set of primers for the ORF1 region was designed on the basis of the sequence obtained in the present metagenomic study (6AIF). For reverse transcription, a Qiagen OneStep RT-PCR kit (catalog no. 210212) was used according to the manufacturer's instructions. The first and second round of enzymatic amplification for both viruses (DNA/RNA) were performed as follows. In the first round of enzymatic amplification, 10 μl of the undiluted and a 10-fold dilution of the extracted nucleic acids was analyzed. The amplification mixture (40 μl) contained 1× PCR buffer, 1.5 mM $MgCl_2$, 250 μM each deoxynucleoside triphosphate (dNTP), 0.5 μM of each specific primer for each virus, and 4 U of TaqGold DNA polymerase (Applied Biosystems). In the second round of enzymatic amplification, 2 μl of the product obtained in the first round was added to 48 μl of amplification mix, containing a set of specific primers for each virus and the same reagent composition described above. The PCR conditions for the first and second rounds were as follows: 10 min at 95°C; 30 cycles, with 1 cycle consisting of 60 s at 94°C, 60 s at 52°C for HPyV6 or 60 s at 56°C for rat HELV, and 60 s at 72°C; a final extension step of 7 min at 72°C.

Accession numbers. The sequence of WW-nAnB was submitted to GenBank (JN402401), and the raw sewage metagenome was deposited in the Sequence Read Archive (SRA040148).

ACKNOWLEDGMENTS

We thank Alcosan for proving samples of untreated wastewater from Pittsburgh, PA. We thank Justin Brown and W. Mark Pipas for providing Shinola.

We are grateful to the University of Pittsburgh School of Arts and Sciences for providing seed funds for this project.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00180-11/-/DCSupplemental.

Text S1, DOCX file, 0.1 MB.
Figure S1, PDF file, 0.3 MB.
Figure S2, PDF file, 0.4 MB.
Figure S3, PDF file, 0.4 MB.
Table S1, XLSX file, 0.1 MB.
Table S2, XLSX file, 0.1 MB.
Table S3, XLSX file, 0.1 MB.
Table S4, XLSX file, 8.6 MB.
Table S5, XLSX file, 0.1 MB.
Table S6, XLSX file, 0.1 MB.

REFERENCES

1. **Angly FE, et al.** 2006. The marine viromes of four oceanic regions. PLoS Biol. **4**:e368.
2. **Breitbart M, et al.** 2002. Genomic analysis of uncultured marine viral communities. Proc. Natl. Acad. Sci. U. S. A. **99**:14250–14255.
3. **Culley AI, Lang AS, Suttle CA.** 2006. Metagenomic analysis of coastal RNA virus communities. Science **312**:1795–1798.
4. **Monier A, Claverie JM, Ogata H.** 2008. Taxonomic distribution of large DNA viruses in the sea. Genome Biol. **9**:R106.
5. **Rusch DB, et al.** 2007. The Sorcerer II Global Ocean Sampling expedition: northwest Atlantic through eastern tropical Pacific. PLoS Biol. **5**:e77.
6. **Williamson SJ, et al.** 2008. The Sorcerer II Global Ocean Sampling expedition: metagenomic characterization of viruses within aquatic microbial samples. PLoS One **3**:e1456.
7. **López-Bueno A, et al.** 2009. High diversity of the viral community from an Antarctic lake. Science **326**:858–861.
8. **Breitbart M, et al.** 2003. Metagenomic analyses of an uncultured viral community from human feces. J. Bacteriol. **185**:6220–6223.
9. **Finkbeiner SR, et al.** 2008. Metagenomic analysis of human diarrhea: viral detection and discovery. PLoS Pathog. **4**:e1000011.
10. **Holtz LR, Finkbeiner SR, Kirkwood CD, Wang D.** 2008. Identification of a novel picornavirus related to cosaviruses in a child with acute diarrhea. Virol. J. **5**:159.
11. **Reyes A, et al.** 2010. Viruses in the faecal microbiota of monozygotic twins and their mothers. Nature **466**:334–338.
12. **Victoria JG, et al.** 2009. Metagenomic analyses of viruses in stool samples from children with acute flaccid paralysis. J. Virol. **83**:4642–4651.
13. **Zhang T, et al.** 2006. RNA viral community in human feces: prevalence of plant pathogenic viruses. PLoS Biol. **4**:e3.
14. **Kurokawa K, et al.** 2007. Comparative metagenomics revealed commonly enriched gene sets in human gut microbiomes. DNA Res. **14**:169–181.
15. **Bench SR, et al.** 2007. Metagenomic characterization of Chesapeake Bay virioplankton. Appl. Environ. Microbiol. **73**:7629–7641.
16. **Djikeng A, Kuzmickas R, Anderson NG, Spiro DJ.** 2009. Metagenomic analysis of RNA viruses in a fresh water lake. PLoS One **4**:e7264.
17. **Li L, et al.** 2010. Bat guano virome: predominance of dietary viruses from insects and plants plus novel mammalian viruses. J. Virol. **84**:6955–6965.
18. **Schoenfeld T, et al.** 2008. Assembly of viral metagenomes from Yellowstone hot springs. Appl. Environ. Microbiol. **74**:4164–4174.
19. **Vega Thurber RL, et al.** 2008. Metagenomic analysis indicates that stressors induce production of herpes-like viruses in the coral *Porites compressa*. Proc. Natl. Acad. Sci. U. S. A. **105**:18413–18418.
20. **Fauquet CM, Mayo MA, Maniloff J, Desselberger U, Ball LA.** 2005. Virus taxonomy: classification and nomenclature of viruses. Elsevier Academic, San Diego, CA.
21. **Thurber RV, Haynes M, Breitbart M, Wegley L, Rohwer F.** 2009. Laboratory procedures to generate viral metagenomes. Nat. Protoc. **4**:470–483.
22. **Pina S, et al.** 2001. Genetic analysis of hepatitis A virus strains recovered from the environment and from patients with acute hepatitis. J. Gen. Virol. **82**:2955–2963.
23. **Rodriguez-Manzano J, et al.** 2010. Analysis of the evolution in the circulation of HAV and HEV in eastern Spain by testing urban sewage samples. J. Water Health **8**:346–354.
24. **Puig M, et al.** 1994. Detection of adenoviruses and enteroviruses in polluted waters by nested PCR amplification. Appl. Environ. Microbiol. **60**:2963–2970.
25. **Bofill-Mas S, et al.** 2006. Quantification and stability of human adenoviruses and polyomavirus JCPyV in wastewater matrices. Appl. Environ. Microbiol. **72**:7894–7896.
26. **Bofill-Mas S, et al.** 2010. Newly described human polyomaviruses Merkel cell, KI and WU are present in urban sewage and may represent potential environmental contaminants. Virol. J. **7**:141.
27. **Formiga-Cruz M, et al.** 2002. Distribution of human virus contamination

in shellfish from different growing areas in Greece, Spain, Sweden, and the United Kingdom. Appl. Environ. Microbiol. **68**:5990–5998.
28. **Blinkova O, et al.** 2009. Frequent detection of highly diverse variants of cardiovirus, cosavirus, bocavirus, and circovirus in sewage samples collected in the United States. J. Clin. Microbiol. **47**:3507–3513.
29. **Rosario K, Nilsson C, Lim YW, Ruan Y, Breitbart M.** 2009. Metagenomic analysis of viruses in reclaimed water. Environ. Microbiol. **11**: 2806–2820.
30. **Symonds EM, Griffin DW, Breitbart M.** 2009. Eukaryotic viruses in wastewater samples from the United States. Appl. Environ. Microbiol. **75**:1402–1409.
31. **Calgua B, et al.** 2008. Development and application of a one-step low cost procedure to concentrate viruses from seawater samples. J. Virol. Methods **153**:79–83.
32. **Kristensen DM, Mushegian AR, Dolja VV, Koonin EV.** 2010. New dimensions of the virus world discovered through metagenomics. Trends Microbiol. **18**:11–19.
33. **Casjens S.** 2003. Prophages and bacterial genomics: what have we learned so far? Mol. Microbiol. **49**:277–300.
34. **Johne R, et al.** 2010. Novel hepatitis E virus genotype in Norway rats, Germany. Emerg. Infect. Dis. **16**:1452–1455.
35. **Albinana-Gimenez N, et al.** 2009. Analysis of adenoviruses and polyomaviruses quantified by qPCR as indicators of water quality in source and drinking-water treatment plants. Water Res. **43**:2011–2019.
36. **Pina S, Puig M, Lucena F, Jofre J, Girones R.** 1998. Viral pollution in the environment and in shellfish: human adenovirus detection by PCR as an index of human viruses. Appl. Environ. Microbiol. **64**:3376–3382.
37. **Holtz LR, et al.** 2009. Klassevirus 1, a previously undescribed member of the family Picornaviridae, is globally widespread. Virol. J. **6**:86.
38. **Schowalter RM, Pastrana DV, Pumphrey KA, Moyer AL, Buck CB.** 2010. Merkel cell polyomavirus and two previously unknown polyomaviruses are chronically shed from human skin. Cell Host Microbe **7**:509–515.
39. **Li L, et al.** 2010. Multiple diverse circoviruses infect farm animals and are commonly found in human and chimpanzee feces. J. Virol. **84**: 1674–1682.
40. **Seelig R, et al.** 1988. Hepatitis non-A, non-B-associated substance in stool from patients with posttransfusion and sporadic hepatitis. Immun. Infekt. **16**:85–90. (In German.)
41. **Burckhardt J, Seelig R, Calvo-Riera F, Seelig HP.** 1988. A hepatitis non-A, non-B-associated substance in the feces—identification and cloning of a partially double-stranded circular DNA. Immun. Infekt. **16**:91–96. (In German.)
42. **Wooley JC, Godzik A, Friedberg I.** 2010. A primer on metagenomics. PLoS Comput. Biol. **6**:e1000667.
43. **Lawrence JG, Hatfull GF, Hendrix RW.** 2002. Imbroglios of viral taxonomy: genetic exchange and failings of phenetic approaches. J. Bacteriol. **184**:4891–4905.
44. **Hendrix RW, Hatfull GF, Smith MC.** 2003. Bacteriophages with tails: chasing their origins and evolution. Res. Microbiol. **154**:253–257.
45. **Juhala RJ, et al.** 2000. Genomic sequences of bacteriophages HK97 and HK022: pervasive genetic mosaicism in the lambdoid bacteriophages. J. Mol. Biol. **299**:27–51.
46. **Qiagen.** 2006. DNeasy blood & tissue handbook, p. 25–27. Qiagen, Hilden, Germany.
47. **Wang D, et al.** 2002. Microarray-based detection and genotyping of viral pathogens. Proc. Natl. Acad. Sci. U. S. A. **99**:15687–15692.
48. **Wang D, et al.** 2003. Viral discovery and sequence recovery using DNA microarrays. PLoS Biol. **1**:E2.
49. **Li W, Godzik A.** 2006. Cd-hit: a fast program for clustering and comparing large sets of protein or nucleotide sequences. Bioinformatics **22**: 1658–1659.
50. **Loh J, et al.** 2009. Detection of novel sequences related to African swine fever virus in human serum and sewage. J. Virol. **83**:13019–13025.
51. **Erker JC, Desai SM, Mushahwar IK.** 1999. Rapid detection of hepatitis E virus RNA by reverse transcription-polymerase chain reaction using universal oligonucleotide primers. J. Virol. Methods **81**:109–113.
52. **Hernroth BE, Conden-Hansson AC, Rehnstam-Holm AS, Girones R, Allard AK.** 2002. Environmental factors influencing human viral pathogens and their potential indicator organisms in the blue mussel, *Mytilus edulis*: the first Scandinavian report. Appl. Environ. Microbiol. **68**: 4523–4533.
53. **Pal A, Sirota L, Maudru T, Peden K, Lewis AM, Jr..** 2006. Real-time, quantitative PCR assays for the detection of virus-specific DNA in samples with mixed populations of polyomaviruses. J. Virol. Methods **135**:32–42.

7. DISCUSIÓN GENERAL

La contaminación del medio ambiente, principalmente de ríos, lagos, playas y agua subterránea, que habitualmente se utilizan para actividades recreacionales, cultivos de moluscos, sistemas riego en la agricultura o como fuentes de agua para el consumo humano, es un tema de gran preocupación en términos de salud pública y de gran importancia económica y de legislación. Si se considera la distribución de la población humana, especialmente en zonas cercanas a estos tipos de agua, es indiscutible que estos ecosistemas tienen un considerable riesgo de contaminación que proviene de agua residual de origen humano e incluso de mataderos de animales y de fertilizantes de origen animal empleados en la agricultura. La mayoría de los estudios y datos disponibles sobre contaminación y calidad microbiológica del agua, hacen especial referencia a la contaminación de origen fecal, es bien conocido que una gran cantidad de microorganismos son excretados en la orina y la heces, entre éstos, los virus. Definitivamente la ruta fecal-oral es la vía responsable de la mayoría de las infecciones víricas asociadas al agua. Existen importantes patógenos transmitidos por el agua contaminada, sin embargo, en la actualidad la calidad microbiológica del agua es controlada mediante estándares bacterianos. Actualmente no existe un protocolo estandarizado en las legislaciones para el estudio de virus en muestras ambientales, ni protocolos estándar para el estudio de la infectividad de estos virus.

El presente trabajo de *Tesis Doctoral* describe el desarrollo de nuevas metodologías para la concentración y detección de virus en agua, la utilización de indicadores víricos para evaluar la contaminación fecal, así como también datos sobre la presencia de virus emergentes y virus potencialmente nuevos. El trabajo consta de tres *Capítulos* que engloban cinco *Estudios* diferentes. En la presente sección de *Discusión general* los resultados presentados en cada *Estudio* se agruparan de forma común en diferentes secciones para una mejor comprensión.

Nuevas metodologías para la concentración de virus en agua (Estudio 1, 3 y 5).

Los métodos aplicados en estudio de virus en agua como agentes contaminantes del medio ambiente generalmente involucra dos etapas: (i) Debido a que los virus se pueden encontrar en pocas concentraciones en ríos, océanos, lagos u otras matrices similares, estos virus se deben concentrar en un volumen mucho menor, (ii) y una vez concentrados los virus, estos se deben analizar empleando técnicas suficientemente sensibles que permitan detectarlos, cuantificarlos e identificarlos. Generalmente estos análisis se llevan a cabo por técnicas basadas en cultivo celular y/o moleculares relacionadas con la PCR.

La presencia de virus patógenos en el agua de baño no es vigilada de forma rutinaria por las autoridades sanitarias correspondientes. A diferencia de los indicadores bacterianos, *E. coli* (EC) y enterococos intestinales (EI), ninguna de las principales legislaciones sobrecalidad de agua (WHO, 2003; USEPA, 2004; 2006/160/EC), incluye actualmente el estudio de virus. Probablemente, entre las razones principales este la falta de una metodología estándar, tanto para concentrar los virus como para su detección.

En los últimos años se han venido realizando varios estudios para poder comprender mejor el comportamiento de los virus como contaminantes en el medio ambiente y se han logrado también avances en las técnicas para la concentración y detección de virus en agua (Cashdollar y Wymer, 2013). Entre estos estudios esta VIROBATHE (www.virobathe.org), un proyecto de la Unión Europea dentro del programa *Framework 6*, que entre sus objetivos contempló tareas de comparación de métodos y la demostración de que su uso era factible para la vigilancia rutinaria de virus entéricos en el agua recreacional. Entre los resultados de este proyecto, se obtuvieron los descritos en el Estudio 1 del presente trabajo. En este estudio se describe el desarrollo de una metodología *one-step* para la concentración de virus en agua de mar, basada en la floculación orgánica de leche descremada bajo condiciones acidas (SMFP). Esta metodología aplicada a muestras de 10L de agua de mar contaminada artificialmente con adenovirus humanos 2 (HAdV 2) y análisis de qPCR TaqMan®, mostró una recuperación media del 50% de estos virus. Además, se realizó un estudio intra-laboratorio donde se comparó SMFP y tres protocolos VIRADEL (*two-step)* para concentrar HAdV 2 añadidos artificialmente a agua mar (Anexo I).

En el ensayo intra-laboratorio no se obtuvieron resultados homogéneos y si una baja eficiencia para concentrar HAdV 2 cuando se emplearon los protocolos *two-step*, 1-16 % de virus recuperados, mientras que con SMFP se obtuvieron valores poco variables y una recuperación vírica del 50% (Anexo I). Anteriormente se ha reportado que la gran cantidad de sales presentes en agua de mar podría interferir entre las interacciones electroestáticas de los virus con las matrices empleadas en los métodos VIRADEL (Lukasik *et al*., 2000). Datos obtenidos en un estudio de Lambertini *et al*. (2008), confirmaron que el pH de la muestra podría influir en la carga neta del virus y por lo tanto en la eficiencia de adsorción de los virus a matrices como la lana de vidrio. Esto podría ser una desventaja importante en los métodos basados en procesos de adsorción/elución. Actualmente se tienen mejores conocimientos del punto isoeléctrico y del comportamiento de la carga neta de cada virus, por ejemplo Michen y Graule (2010), en su revisión dan información

sobre la gran variedad de puntos isoeléctricos no solo entre diferentes tipos de virus sino también entre diferentes cepas de un mismo tipo de virus.

Con los resultados positivos obtenidos con SMFP en agua de mar, en estudios posteriores se procedió a adaptar SMFP para concentrar virus en agua de río (Estudio 3). Los resultados obtenidos inicialmente mostraron que el proceso de floculación en agua de río era similar, aunque menos eficiente comparado con el agua de mar (datos nos mostrados). El agua de mar y el agua de río difieren en sus valores de conductividad entre otros factores; el agua de mar tiene valores entre 4.500 – 5000 mS/m, mientras que el agua de río puede tener valores por debajo del 10µS/m o valores más altos, dependiendo especialmente del tipo de sedimento del río. Entre los estudios para la adaptación de SMFP para agua de río, se realizaron ensayos empleando diluciones seriadas 1:10 de agua de mar para evaluar el proceso de floculación, determinándose que el proceso de floculación estaba relacionado con la conductividad y que este parámetro fisicoquímico en la muestra debería tener un valor ≥ 1,5 mS/m para que la floculación ocurriera de forma adecuada. Estos datos están de acuerdo con otros estudios en donde se describe que la fuerza iónica favorece la floculación de la leche (Demetriades *et al*., 2006). Aunque se evaluaron diferentes reactivos para incrementar la conductividad, los mejores resultados se obtuvieron cuando se añadieron sales de agua de mar artificial. Se definió que para agua de río el protocolo SMFP era el mismo que para agua de mar, sin embargo se estableció que si era necesario, la conductividad de la muestra debe ajustarse añadiendo sales de mar artificial hasta obtener un valor ≥1,5 mS/m.

Posteriormente y con la finalidad de validar la metodología para agua de río, se realizaron ensayos inter-e intra-laboratorio en un estudio en colaboración con el grupo de Virología Ambiental y Comparada, del Instituto Osvaldo Fiocruz (Río de Janeiro, Brasil) dirigido por la Dr. Marize Miagostovich. En estos estudios, se emplearon muestras de 10L de agua de río artificialmente contaminadas con HAdV 2 y Norovirus (NoV) GGII para los ensayos inter-laboratorio, mientras que para los ensayos intra-laboratorio en Barcelona y Brasil se utilizó además el polyomavirus JC (JCPyV) y Rotavirus (RV), respectivamente. Para cada virus estudiado se recuperó un 50% y los análisis estadísticos revelaron que no hay diferencias significativas entre los resultados obtenidos en Barcelona y Río de Janeiro. Además, se demostró que con SMFP se pueden concentrar virus DNA y RNA con equivalente eficiencia.

Actualmente es difícil comparar la eficiencia de métodos de concentración de virus en agua descritos en la literatura. La gran variedad de métodos utilizados para la

cuantificación de virus (cultivo celular o PCR), el tipo de agua y el volumen de los ensayos, influyen considerablemente en el resultado final. Como se recomienda en algunos trabajos, podría ser más interesante que en el desarrollo de metodologías para concentrar virus en agua, se incluyan ensayos de comparación con otros métodos, ensayos de validación multi-laboratorio y criterios unificados en los controles de proceso, que faciliten la interpretación de los resultados (Cashdollar y Wymer, 2013).

Los métodos SMFP para agua de río y agua de mar desarrollados en el presente trabajo, además de mostrar buenas recuperaciones víricas (50%), son métodos con resultados reproducibles y consistentes ya que muestran un coeficiente de variación aproximadamente ≤ al 50%. Como se puede observar en la literatura sobre métodos de concentración de virus en agua, estos trabajos generalmente describen únicamente valores de recuperación de virus y de desviación estándar y realmente son escasos los estudios que incluyen el coeficiente de variación (CV) como parámetro a evaluar. De forma general, el coeficiente de variación es un parámetro que permite evaluar como varían los resultados de un mismo proceso. Recientemente se ha propuesto que el CV es un parámetro que se debe incluir cuando se evalúan métodos de concentración de virus en agua y que su valor debe ser inferior al 50% para que los resultados se consideren reproducibles (Calgua *et al*., 2013). En 1970, Nupen comparó dos métodos de concentración de virus en agua residual (n=11), ambos combinados con $TCDI_{50}$ o *plaque assay* (PA) para la detección de poliovirus. En estos estudios, Nupen reportó para ambos métodos una recuperación de virus similar (aprox. 40%), y coeficientes de variación por lo general por debajo del 50% cuando los poliovirus se analizaban con $TCID_{50}$. Sin embargo, Nupen obtuvo un CV del 82%, cuando analizó los poliovirus concentrados con PA. Este resultado es interesante, ya que la metodología empleada para la detección de los virus concentrados puede influir claramente en los resultados finales. Los resultados obtenidos a partir de SMFP para agua de río y mar, se han obtenido a partir de ensayos basados en qPCR TaqMan®, donde en cada reacción existe una curva estándar (cada punto por triplicado), que muestra significativamente poca variabilidad y una correlación bastante estable muy cercana a 1. Esto indica que la detección molecular de estos virus no introduce ninguna variación en los resultados del procedimiento de concentración de virus.

De acuerdo con los resultados obtenidos en el desarrollo de los protocolos SMFP para concentrar virus en agua de mar y río, metodológicamente son procedimientos *one-step* sencillos y eficientes, que no requieren equipo sofisticado, ni incluyen procesos de filtración o elución, por lo tanto son metodologías fáciles de estandarizar, de bajo coste

económico y adecuadas para ser implementadas en laboratorios de rutina relacionado con el análisis microbiológico del agua.

En el Estudio 5 también se ha desarrollado un método para concentrar virus en agua residual, pero con la finalidad de realizar un estudio específico sobre el viroma en este tipo de muestras. A diferencia de lo descrito anteriormente, este método incluye diferentes pasos y no necesariamente es metodología que se pueda emplear de rutina. El método está basado en concentrar los virus presentes en 10 L de agua residual por SMFP, obteniendo un concentrado vírico de 30 mL. Posteriormente los virus presentes en 15 mL de esta suspensión son concentrados en un volumen final de 2 mL, por un protocolo basado para 42 mL de agua residual descrito previamente por Pina *et al*. (1998). Para evaluar la presencia de virus, se analizó la concentración de HAdV por qPCR, obteniéndose resultados del orden de 10^4 GC/mL, los cuales son similares a los obtenidos en estudio previos en el mismo tipo de muestra (Bofill *et al*., 2006; Rodriguez-Manzano *et al*., 2012; Calgua *et al.,* 2013). Usualmente 50 mL de agua residual es suficientes para detectar virus como HAdV (Bofill-Mas *et al*., 2006; Rodriguez-Manzano *et al*., 2012; Calgua *et al*., 2013), sin embargo en este estudio en concreto se buscaba concentrar grandes cantidades de virus, incluso aquellos que podrían estar en bajas concentraciones. Los resultados de los análisis metagenómicos son comentados más adelante.

Detección y cuantificación de virus infecciosos en agua (Estudio 2).

El estudio de los virus en el medio ambiente, requiere que una vez concentrados los virus en un volumen mucho menor, estos sean detectados, cuantificados e identificados. Actualmente, entre las herramientas más rápidas, especificas, sensibles, robustas y económicas, están las técnicas basadas en ensayos de PCR, especialmente la qPCR/RT-qPCR (Girones *et al*., 2010). Sin embargo, estas técnicas *per se* no permiten obtener datos sobre la infectividad de los virus detectados. En estudios específicos sobre la valoración de riesgo, los datos de infectividad de los virus analizados son indispensables.

En el Estudio 2, se describe un ensayo de inmunofluorescencia (IFA) para detectar HAdV y JCPyV-Mad4. También se compararon otras técnicas para cuantificar virus infecciosos como PA y $TCID_{50}$. Inicialmente se estableció el día óptimo de lectura de los resultados de IFA, basándose en la detección del máximo número de células infectadas y el mínimo número de focos expandidos. Bajo estos criterios se determinó que el día óptimo de lectura de IFA para HAdV 2 era de 4 días y para HAdV 41 y JCPyV, 8 días.

Conjuntamente se determinó que el ensayo de IF era específico para la detección de virus infecciosos, esto después de obtener resultados negativos al cuantificar suspensiones víricas previamente tratadas con luz UV y temperaturas de 100° C, mientras que si se obtuvieron resultados cuantitativos por qPCR.

En los resultados de comparación entre IFA, PA y $TCID_{50}$ para HAdV2, se puede observar como con IFA se detectó 10 veces más virus infecciosos expresados en FFU (*focus forming units*)/mL, que PA y $TCID_{50}$, expresados en PFU (*plaque forming units*)/mL y $TCID_{50}$ unidades/mL, respectivamente. No se realizaron ensayos de PA para HAdV 41 y JCPyV-Mad4 ya que estas cepas no producen calvas. Los ensayos de PA y $TCID_{50}$ se basan en la detección de efecto citopático en las células (CPE), sin embargo en cada muestra pueden haber virus que se repliquen sin necesidad de producir lisis celular, también podrían haber virus que produzcan infecciones ligeramente tardías que más adelante pudieran dar origen a un CPE, pero que en el momento del ensayo no se puedan detectar (Ridinger *et al*., 1982; Smith y Gerba, 1982; Teunis *et al*., 2005; Hamza *et al*., 2011). No obstante en los casos anteriores los virus infecciosos si se pueden detectar por IFA, ya que es un método que se basa en el uso de anticuerpos específicos para detectar proteínas víricas y no en la observación del CPE, esto podría ser una de las principales razones de que IFA sea más sensible que PA y $TCID_{50}$.

El ensayo de IF descrito también se aplicó en muestras ambientales para detectar HAdV. Con este propósito, concentrados víricos obtenidos a partir de agua de río previamente contaminada con HAdV 2 y concentrados de agua residual sin dopar fueron analizados por IFA. Para evitar posibles efectos de citotoxicidad producidos por diferentes componentes de las matrices ambientales, todos los concentrados víricos obtenidos se trataron con cloroformo y fueron analizadas diferentes diluciones. Bajo estas condiciones se detectaron aproximadamente 10 veces menos HAdV 2 infecciosos, que copias de genomas detectadas en paralelo por qPCR. Mientras que los HAdV infecciosos en agua residual se detectaron en concentraciones 100 veces menores que los valores obtenidos por qPCR. Aproximadamente la misma diferencia observada con los HAdV 2 añadidos artificialmente al agua de rio (1 Log), se obtuvo cuando se cuantificaron partículas víricas de HAdV2, HAdV 41 y JCPyV a partir de suspensiones víricas por IFA y qPCR con tratamientos con DNAsa. Estos resultados confirman que IFA es una técnica sensible y robusta, ya que se pueden detectar aproximadamente los mismos virus infecciosos presentes en suspensiones víricas obtenidas a partir de cultivo celular como también los virus añadidos en una matriz tan compleja como las muestras ambientales. Por ensayos de

nPCR y secuenciación se identificó HAdV 40 y 41 (datos nos mostrados) en las muestras de río.

La infectividad de JCPyV no se analizó en muestras ambientales ya que la cepa excretada en orina presenta una estructura arquetípica en la región reguladora de su genoma y no se replica bajo las condiciones ensayadas (Bofill-Mas *et al*., 2000). JCPyV-Mad4 tienen la región reguladora en un estado reorganizado y puede replicarse en las células SVG-A. No obstante, esta cepa es un modelo adecuado para estudiar el comportamiento de los polyomavirus en el medio ambiente y para validar procesos y tratamientos de inactivación y desinfección (de Abreu Corrêa *et al*., 2012).

Las técnicas de PA y $TCID_{50}$ son metodologías útiles para cuantificar virus infecciosos, sin embargo los datos obtenidos en el Estudio 2, confirman algunas desventajas para ambas técnicas, especialmente cuando se analizan virus en muestras ambientales. El ensayo de IF descrito, es más sensible y representa una herramienta adecuada para evaluar la infectividad de HAdVs en el medio ambiente y JCPyV-Mad4 como virus modelo en procesos de desinfección. Como hemos descrito anteriormente, la infectividad de los virus ha venido evaluándose a través de cultivo celular, e incluso en los últimos años se han descrito combinaciones con PCR o qPCR (Reynolds *et al*., 1996; Hamza *et al*., 2011). Sin lugar a dudas estos últimos han aumentado la especificidad en la detección de virus infecciosos, pero igualmente tienen algunos inconvenientes en relación al tiempo de obtención de resultados y al coste económico (Hamza *et al*., 2011). Otro problema en el estudio de la infectividad del virus, es que no todos los virus son capaces de replicarse en cultivo celular. Con la finalidad de superar todas estas desventajas se han descrito varias metodologías prometedoras, como tratamientos con protestas y nucleasas para evaluar la integridad del virus (Nuanualsuwan y Cliver, 2002; Hamza *et al*., 2011) y modelos matemáticos y PCR/qPCR para evaluar el daño en el DNA vírico (Hamza *et al*., 2011; Pecson *et al*., 2011), entre otros.

Los adenovirus humanos como herramienta para evaluar la calidad microbiológica del agua (Estudio 1, 3 y 4).

Los indicadores estándar de contaminación fecal, *E. coli* (EC) y enterococos intestinales (EI) son utilizados para evaluar la contaminación fecal en el agua, según varias legislaciones reconocidas a nivel mundial (WHO, 2003; USEPA, 2004; 2006/160/EC). Existe suficiente evidencia científica que demuestra que los virus son más estables a las condiciones del medio ambiente y que por lo tanto las concentraciones de los indicadores

bacterianos usualmente no se correlacionan con las de los virus (Contreras-Coll *et al*., 2002; Thurston-Enriquez *et al*., 2003; Rzeżutka y Cook, 2004; Miagostovich *et al*., 2008; de Roda Husman *et al*., 2009). Con el propósito de obtener información más certera de la calidad microbiológica del agua, especialmente con relación a los virus, los adenovirus humanos y polyomavirus JC se han propuesto como una nueva generación de indicadores de contaminación fecal (Pina *et al*., 1998, Bofill-Mas *et al*., 2000; McQuaig *et al*., 2006; Miagostovich *et al*., 2008; Wyn-Jone *et al*., 2011).

Entre los objetivos del proyecto VIROBATHE, también se incluyó un programa de vigilancia de la calidad microbiológica de agua recreacioanal durante la temporada de baño en Europa. En dicho programa se analizaron HAdV como potenciales indicadores víricos de contaminación fecal humana y los indicadores bacterianos EC y EI según la Directiva Europea 2006/160/EC para agua de baño. Los virus se analizaron en muestras de 10 L recolectadas semanalmente. En el Estudio 1, se muestran los resultados del análisis de 50 muestras del área costera de Barcelona, de las cuales 7 fueron positivas para HAdV por nPCR y qPCR. En estas muestras los indicadores bacterianos EC y EI se detectaron en concentraciones que cumplían con los valores descritos en la Directiva 2006/07/CE. También se detectaron HAdV en muestras tomadas después de un periodo de lluvia intenso, en donde los indicadores bacterianos mostraron concentraciones elevadas. En 4 de las muestras positivas se identificó HAdV 41 y en las otras dos HAdV 31. Además, empleando ensayos de infectividad (ICC-PCR), en una de las muestras se identificaron HAdV 31.

El proyecto VIROBATHE involucraba la participación de 16 laboratorios Europeos. En el Estudio 3 se describen los resultados cuantitativos de HAdV obtenidos por qPCR TaqMan® en muestras de 24 áreas de baño (mar, río y lago), localizados en 9 de los países participantes. Se obtuvieron resultados cuantitativos HAdV, en 80 de 132 muestras previamente positivas para HAdV por nPCR obtenidas durante la temporada de baño 2006. Los análisis estadísticos no mostraron una correlación linear homogénea entre los valores de HAdV y los valores de EC, EI, especialmente cuando todos los valores se analizan conjuntamente. Los resultados obtenidos en el programa de vigilancia de agua de baño de VIROBATHE, similar que en otros estudios, podrían confirmar que los valores de los indicadores bacterianos no se pueden relacionar con la ausencia de virus, sugiriendo que los virus humanos contaminantes persisten más en el medio ambiente que las bacterias entéricas. Por otro lado, el ensayos de qPCR TaqMan® para cuantificar HAdV han demostrado ser fáciles de estandarizar y de implementar en laboratorios de rutina relacionados con los análisis de la calidad del agua.

En el estudio 4, también se ha evaluado la presencia de HAdV y JCPyV como indicadores de contaminación fecal. En este estudio se analizaron 12 muestras en Río de Janeiro (6) y Barcelona (6), obteniéndose en todas las muestras resultados cuantitativos positivos de ambos indicadores víricos. Aunque en estas muestras no se analizaron indicadores bacterianos, si se detectaron además otros virus en las muestras (resultados comentados en la siguiente sección). HAdV y JCPyV se han detectado también en casi un 100% de las muestras analizadas en otros estudios, demostrando que ambos virus se excretan por la mayoría de la población de forma estable y que su presencia está relacionada con otros virus humanos contaminantes (Bofill-Mas *et al*., 2000; Albinana-Gimenez *et al*., 2006, 2009; McQuaig *et al*., 2006; Miagostovich *et al*., 2008; Vieira *et al*., 2012; Fumian *et al*., 2013).

Virus clásicos, emergentes y potencialmente nuevos presentes en agua de río y agua residual (Estudio 4 y 5).

En varios estudios se ha demostrado que el agua juega un papel fundamental en la diseminación de virus humanos patógenos. Entre los virus frecuentemente detectados en agua contaminada están los adenovirus, norovirus, rotavirus, astrovirus, hepatitis A y algunos miembros del genero enterovirus (Sinclair *et al*., 2009). Sin embargo, en los últimos años se ha venido reportando la presencia de virus o cepas emergentes, e incluso una gran variedad de virus nuevos en diferentes tipos de agua (Rosario *et al*., 2009; Bofill-Mas *et al*., 2010; La Rosa *et al*., 2012). Sin lugar a dudas fenómenos como el crecimiento de la población, el cambio climático y la gran movilidad de mercancías y personas debido a la globalización, han influido en estos hechos. El incremento y el avance en el desarrollo de nuevas tecnologías, también nos han permitido obtener datos más amplios sobre el comportamiento y presencia de los virus en el medio ambiente.

En el Estudio 4, se evaluó la presencia en agua de río de virus clásicos responsables de gastroenteritis y virus emergentes/nuevos previamente descritos en otros estudios. Con este propósito dos laboratorios localizados en diferentes zonas geográficas (Laboratorio de Virus Contaminantes de Agua y Alimentos, Barcelona, España y Laboratorio de Virología Ambiental y Comparada, Río de Janeiro, Brasil) analizaron muestras de río provenientes de sus respectivas ciudades. Los virus fueron concentrados por SMFP después de validar en el mismo estudio el método para agua de río.

Como virus clásicos responsables de gastroenteritis se analizaron: norovirus (NoV) GGII y rotavirus (RV), el primero en ambos laboratorios y RV solamente en Río de Janeiro.

NoV GGII se detectó y cuantifico en 5 de 6 muestra analizadas en Barcelona, mientras que en Rio de Janeiro en 2 de 6 muestras. Tomando en cuenta que las muestras fueron tomadas en invierno en Barcelona y en verano en Río de Janeiro, la estacionalidad de NoV reportada por algunos autores podría ser la razón de la diferencia en el número de muestras positivas entre ambas regiones (Mounts *et al*., 2000; Lopman *et al*., 2004). Basándose en estudios previos donde el GGII fue más prevalente que GGI, especialmente el genotipo II.4, se decidió solamente analizar el GGII de NoV (Barreira *et al*., 2010; Ferreira *et al*., 2010; Bull y White, 2011; Wyn-Jones *et al*., 2011). Las muestras positivas de Barcelona fueron las únicas que se secuenciaron, tres se correspondieron con NoV GGII.4, e interesantemente una se caracterizó como NoV GGII.12, la cual es una cepa emergente que en EU fue responsable del 16% de los brotes reportados en 2009 para norovirus (Vega y Vinjé, 2011). Los rotavirus se detectaron en 4 de 6 muestras, y aunque la RT-qPCR empleada no discrimina entre la cepas patogénicas y la vacunal, datos previos indicaron que la cepas vacúnales no se detectan en agua residual de Río de Janeiro (Fumian *et al*., 2011). También se detectaron y cuantificaron HAdV en todas las muestras de ambas regiones, identificándose HAdV 40 y 41. Indiscutiblemente la especie HAdV F es un agente responsable de gastroenteritis clásico, sin embargo en el presente estudio HAdV se agrupó dentro de los indicadores víricos de contaminación fecal con los resultados comentados en el apartado anterior.

Entre los virus emergentes/nuevos también se analizaron: asfavirus-*like* (ASVLV) y Klassevirus (KV). Para el estudio de estos virus se diseñaron oligonucleótidos y protocolos específicos de nPCR y RT-nPCR, respectivamente. Las muestras en Barcelona fueron negativas para ambos virus. Mientras que en Río de Janeiro, ASFVLV y KV se detectaron en, 1 de 6 y 2 de 6 muestras, respectivamente. Estos datos representan el primer reporte de la presencia de estos virus en agua de río. Klassevirus ha sido asociado a casos de gastroenteritis en infantes de diferentes zonas geográficas (Greninger *et al*., 2010; Han *et al*., 2010; Shan *et al*., 2010). Este virus previamente ha sido detectado en agua residual de Barcelona, (Holtz at al., 2009), y recientemente en Japón (Haramoto *et al*. 2013), e interesantemente en un estudio de metagenómica en agua residual de EEUU, KV es reportado como el virus RNA más abundante (Bibby y Peccia, 2013). Los resultados obtenidos en el Estudio 4, confirman que KV es un virus que podría estar globalmente distribuido. Es evidente que las autoridades sanitarias deberían tomar interés en KV, ya que este virus podría ser responsable de importantes casos de gastroenteritis sin agente causal identificado hasta ahora. Sobre ASFLV no se han publicados muchos datos, aunque recientemente secuencias similares fueron detectadas en muestras de pacientes que presentaban una enfermedad febril tropical desconocida (Yozwiak *et al*., 2012), y

recientemente en agua del río Missisipi (Wan *et al*., 2013). Sin lugar a duda la detección en humanos de secuencias relacionadas con los asfarvirus resulta al menos inquietante.

Los polyomavirus recientemente descritos, Merkell *cell*, KI WU, (Allander *et al*., 2007; Gaynor *et al*., 2007; Feng *et al*., 2008), también fueron agrupados dentro de los virus emergentes/nuevos analizados en el Estudio 4. De estos polyomavirus solamente MCPyV se detectó en Barcelona y en Río de Janeiro, en 3 de 6 muestras de cada ciudad, respectivamente. Estos resultados sugieren que MCPyV, igual que otros polyomavirus como como JC y BK, podrían ser estables a las condiciones del medio ambiente. Resultados similares fueron obtenidos por Bofill-Mas *et al*. (2010), en cuyo trabajo se reportó la presencia de MCPyV, KIPyV y WUPyV en agua residual y MCPyV en el mismo río en Barcelona. Interesantemente, en recientes estudios se describe a MCPyV como un importante miembro del viroma de la piel en humanos, pudiéndose secretar por la piel de individuos sanos (Wieland *et al*., 2009; Schowalter *et al*., 2010; Moens *et al*., 2011; Foulongne *et al*., 2012; Spurgeon y Lambert, 2013). Considerando estos datos, los resultados obtenidos en el presente estudio pudieran sugerir que el agua juega un papel importante en la transmisión de MCPyV. Por otro lado, los análisis filogenéticos de MCPyV reflejan una posible diversidad de secuencias según la localización geográfica, y especialmente en la distribución de las secuencias de Brasil, esto es notable y merece mayores estudios en el futuro. Es necesario contar con más datos de la vía de transmisión de MCPyV y de su diversidad y epidemiología molecular. Estos resultados representan los primeros datos de MCPyV en el medio ambiente de Brasil.

Los resultados en el Estudio 4, han sido obtenidos a partir de 6 muestras de cada ciudad, colectadas en dos días diferentes durante marzo 2009. Si bien es cierto, los datos no representan la prevalencia de estos virus durante un periodo de tiempo prolongado, no obstante se ha obtenido información de la presencia de estos virus en agua de río de dos continentes diferentes, permitiendo entender un poco más la distribución global de estos virus en el medio ambiente.

Como se ha mencionado anteriormente, el desarrollo de nuevas tecnologías, también ha permitido estudiar más a fondo los virus. Entre estas tecnologías, la nueva generación de plataformas de secuenciación de DNA ha tenido un papel fundamental en estudios que han permitido confirmar la presencia de virus nuevos en diferentes matrices (Finkbeiner *et al*., 2008; Rosario *et al*., 2009; Ng *et al*., 2012).

En el Estudio 5, se analizó la diversidad vírica de agua residual, empleando tecnicas de secuenciación en masa con la plataforma 454 GS FLX-Titatium. Las muestras

de 10 L de agua residual urbana fueron recolectadas en Adís Abeba (Etiopía), Pittsburgh (EEUU) y Barcelona (España). Como se ha descrito anteriormente en la sección de métodos de concentración de la presente *Discusión general*, en este estudio se diseñó un protocolo para concentrar los virus presentes en 10 L de muestra, en aproximadamente 2 mL.

Las secuencias obtenidas en estos ensayos de secuenciación, fueron sometidas a diferentes análisis informáticos para eliminar secuencias indeterminadas y redundantes, obteniendo un total de 897.647 secuencias consistentes. Se descarta que entre estas secuencias hayan resultados de DNA libre presente en las muestras, ya que el concentrado vírico se trató con DNAsa previamente a la extracción de ácidos nucleicos.

En una primera clasificación de los resultados, las 897.647 secuencias fueron agrupadas por su grado de homología con secuencias presentes en bases de datos utilizando diferentes herramientas de BLAST. Los resultados permitieron relacionar 8.491 (casi un 1%) secuencias con virus que infectan a eucariontes y 37.917 (4%) secuencias con bacteriófagos. Mientras que 247.363 (27%) correspondieron con secuencias bacterianas, resultados que son similares a otros estudios metagenómicos (Breitbar *et al*., 2013). Parte de este 27% de secuencias se podría corresponder con la presencia de GTAs (*gen transfer agent*) y con bacteriófagos, especialmente si consideramos que se utilizó una metodología para concentrar virus y que las observaciones a través de microscopia electrónica de los concentrados víricos no revelaron la presencia de bacterias, aunque por supuesto no se descarta la presencia de de bajas cantidades bacterias. Por otro lado, la mayoría secuencias 596.146 (66%), no tuvo relación con las secuencias presentes en las bases de datos actuales, por lo que podría ser posible que muchas de ellas se correspondan con secuencias de virus potencialmente nuevos que no se puedan asociar a las familias taxonómicas descritas al día de hoy.

En una siguiente etapa, se clasificaron las secuencias víricas en secuencias de virus conocidos y secuencias de virus nuevos. Esta clasificación se definió con el criterio de que si la secuencia analizada presentaba una identidad ≥ 80% sobre un tamaño ≥ 95% con la secuencia del GenBank, esta secuencia se consideraba que pertenecía a un virus conocido, de lo contrario se consideraba un virus potencialmente nuevo asociado a alguna de las familias víricas existentes. Bajo este criterio se identificaron un total 3.027 secuencias que se agrupan en 243 virus conocidos, incluyendo 17 virus que infectan a humanos. Estos virus representan a 26 familias taxonómicas de las 84 conocidas y se corresponden con virus DNA de simple cadena, DNA de doble cadena, RNA de cada positiva y RNA de doble

cadena. Estos resultados se obtuvieron a partir de las secuencias víricas [**46.408** = 8.491 (virus de eucariontes) + 37.917 (bacteriófagos)]. Los 17 virus que infectan a humanos se corresponden con las siguiente 8 familias: *Adenoviridae*, *Astroviridae*, *Calciviridae*, *Papillomaviridae*, *Picobirnabiridae*, *Picornaviridae* y *Polyomaviridade.* En otro estudio similar en agua residual de diferentes continentes realizado posteriormente, Ng *et al*. (2012), reportaron 7 familias de virus humanos, entre ellas 5 similares a las obtenidas en el presente estudio (***Astroviridae, Calciviridae***, *Hepaviridae*, ***Parvoviridae, Picobirnabiridae***, ***Picornaviridae*** y *Reoviridae*). El resto de estas secuencias [**43.381** = 46.408 (secuencias virus eucariontes y bacteriofagos) - 3.027 (secuencias de virus conocidos)], no fueron asignadas a virus conocidos bajo el criterio de identidad establecido, sin embargo estas presentaban cierto grado de homología con 51 familias de virus descritas. Estos resultados sugieren que estas secuencias podrían corresponder a virus potencialmente nuevos que pueden asignarse como miembros de alguna de las familias descritas al día de hoy.

La presencia virus humanos también se analizó por técnicas convencionales de PCR/qPCR. Entre estos virus se detectaron HAdV y Klassevirus que también fueron detectados dentro de los 17 virus humanos identificados por secuenciación en masa. Es importante considerar, que pesar de que estas técnicas de secuenciación en masa son potentes, los resultados obtenidos podrían incluso desestimar la presencia de otros virus que frecuentemente se detectan en agua residual. Por ejemplo, JCPyV si pudo detectarse por qPCR. Igualmente, los virus de la hepatitis A y E y un asfarvirus-*like* fueron detectados en estas muestras solamente por nPCR convencionales.

Considerando que 43.381 secuencias podrían pertenecer a virus potencialmente nuevos y que estos se podrían asociar a 51 familias de virus conocidas, se demuestra que el agua residual urbana sin tratamiento representa el más basto metagenoma vírico estudiado hasta ahora. Estudios posteriores han venido confirmando estos datos (Tamaki *et al*., 2011; Ng *et al*., 2012)

En la publicación correspondiente al Estudio 5, se han realizado análisis más complejos con los resultados obtenidos. Sin embargo, en el presente trabajo de *Tesis Doctoral* solo se presentarán los datos descritos anteriormente. Entre los resultados no presentados están los análisis filogenéticos de los *contigs* (construcciones de secuencias de mayor tamaño), construidos a partir de los *reads* (secuencias cortas consistentes obtenidas de la plataforma de secuenciación y comentadas anteriormente).

Sin lugar a dudas la presencia de virus humanos contaminantes en agua representa un riesgo serio para la salud humana. Los casos de gastroenteritis, hepatitis (A y E), infecciones respiratorias y sus respectivos responsables víricos excretados en las heces y orina, han venido siendo el principal foco de interés de las autoridades sanitarias. Sin embargo también se debe empezar a tomar especial interés por virus humanos que pueden ser excretados por otras vías, como la piel, así como también por otros tipos de patogénesis, como el caso de virus asociados a carcinomas. En esta línea, en el presente trabajo se confirma la presencia del polyomavirus MCPyV, con potencial oncogénico, en agua de río de dos continentes diferentes y también se reportan secuencias del papilomavirus 112 en agua residual, el cual pare ser también un virus potencialmente oncogénico (Ekström *et al*., 2010). Recientemente, La Rosa *et al*. (2013) han reportado la presencia de varios papilomavirus en agua residual, incluyendo especies cancerígenas de alto riesgo.

Muchos virus patógenos son altamente estables en el medio ambiente como contaminantes y esto representa un riego importante para la salud de la población. Es necesario incrementar nuestros conocimientos científicos, sobre virus emergentes y definir el uso de indicadores adecuados para evaluar y controlar la contaminación humana. También es necesario contar con metodologías validadas y estandarizadas para la concentración y posterior detección de virus en el medio ambiente. Finalmente es el momento de desarrollar y aplicar nuevas protocolos relacionadas con la nueva generación de tecnologías de secuenciación para el estudio de virus presentes en el medio ambiente, esto con la finalidad de disponer de información global sobre los virus que hasta el momento se conocen, los virus emergentes y virus desconocidos hasta el día de hoy. En la presente *Tesis Doctoral* se pretendió abordar los temas anteriormente descritos.

8. CONCLUSIONES

Los objetivos desarrollados en la presente *Tesis Doctoral* han dado lugar a una serie de resultados publicados, las principales conclusiones de estos trabajos se detallan a continuación:

1) Se desarrolló y validó un protocolo *one-step* basado en la floculación orgánica de leche descremada bajo condiciones de pH ácido (SMFP) para concentrar virus en agua de mar y agua de río.

2) Los protocolos SMFP en estudios intra- e inter- laboratorio mostraron resultados homogéneos y valores altos de recuperación vírica (50% y CV≤ 50%), siendo más eficientes que los métodos VIRADEL comparados (para HAdV 2 en agua de mar). Además con SMFP se concentran diferentes tipos de virus DNA y RNA con la misma eficiencia.

3) Se desarrolló un protocolo de inmunofluorescencia combinado con cultivo celular (IFA) para detectar adenovirus y polyomavirus JC infecciosos. El protocolo de IFA fue más sensible y robusto que $TCID_{50}$ y *plaque assay*, cuando estas técnicas se compararon.

4) Se ha demostrado en un estudio Europeo que el análisis cuantitativo de virus en agua de baño empleando ensayos de PCR cuantitativa TaqMan® es factible para cuantificar adenovirus humanos, produciendo información rápida y específica de gran utilidad en el control microbiológico del agua

5) Las metodologías desarrolladas o validadas para concentrar y detectar virus en agua; SMFP, IFA y PCR cuantitativa, son técnicas sensibles, robustas y de coste económico aceptable, fáciles de estandarizar y que se pueden implementar fácilmente en laboratorios de rutina relacionados con el análisis microbiológico del agua.

6) En este trabajo se describe por primera vez la presencia de klassevirus y un asfarvirus-*like* en agua de río, también los resultados obtenidos representan el primer reporte del polyomavirus Merkel *cell* en el medio ambiente de Brasil.

7) El agua residual es el bioma vírico más diverso estudiado hasta ahora, detectándose por estudios metagenómicos; 234 virus conocidos y una gran cantidad de secuencias que representan virus potenciales nuevos que pudieran asignarse a 51 familias víricas de las 84 conocidas.

9. BIBLIOGRAFÍA

-A-

Albinana-Gimenez N, Clemente-Casares P, Bofill-Mas S, Hundesa A, Ribas F, Girones R. Distribution of human polyomaviruses, adenoviruses, and hepatitis E virus in the environment and in a drinking-water treatment plant. *Environ Sci Technol*. 2006, 1;40(23):7416-22.

Albinana-Gimenez N, Miagostovich MP, Calgua B, Huguet JM, Matia L, Girones R. Analysis of adenoviruses and polyomaviruses quantified by qPCR as indicators of water quality in source and drinking-water treatment plants. *Water Res*. 2009, 43(7):2011-9.

Allander T, Andreasson K, Gupta S, Bjerkner A, Bogdanovic G, Persson MA, Dalianis T, Ramqvist T, Andersson B. Identification of a third human polyomavirus. *Journal of Virology*. 2007, 81: 4130–4136.

Angly FE, Felts B, Breitbart M, Salamon P, Edwards RA, Carlson C, Chan AM, Haynes M, Kelley S, Liu H, Mahaffy JM, Mueller JE, Nulton J, Olson R, Parsons R, Rayhawk S, Suttle CA, Rohwer F. The marine viromes of four oceanic regions. *PLoS Biol*. 2006, 4(11), e368.

-B-

Barreira DM, Ferreira MS, Fumian TM, Checon R, de Sadovsky AD, Leite JP, Miagostovich MP, Spano LC. Viral load and genotypes of noroviruses in symptomatic and asymptomatic children in Southeastern Brazil. *Journal of Clinical Virology*. 2010, 47 (1), 60–64.

Bellizzi A, Anzivino E, Rodio DM, Palamara AT, Nencioni L, Pietropaolo V. New Insights on Human Polyomavirus JC and Pathogenesis of Progressive Multifocal Leukoencephalopathy. *Clin Dev Immunol*. 2013, 2013:839719.

Bibby K, Peccia J. Identification of viral pathogen diversity in sewage sludge by metagenome analysis. *Environ Sci Technol.* 2013, 47(4):1945-51.

Bitton G, Charles MJ, Farrah SR. Virus detection in soils: a comparison of four recovery methods. *Can J Microbiol.* 1979, 25(8):874-80.

Block JC, Schwartzbrod L. Viruses in Water Systems. Detection and Identification. *New York: VCH Publishers*, 1989.

Bofill-Mas S, Albinana-Gimenez N, Clemente-Casares P, Hundesa A, Rodriguez-Manzano J, Allard A, Calvo M, Girones R. Quantification and stability of human adenoviruses and polyomavirus JCPyV in wastewater matrices. *Appl Environ Microbiol*. 2006, 72(12):7894-6.

Bofill-Mas S, Pina S, Girones R. Documenting the epidemiologic patterns of polyomaviruses in human populations by studying their presence in urban sewage. *Appl Environ Microbiol*. 2000, 66(1):238-45.

Bofill-Mas S, Rodriguez-Manzano J, Calgua B, Carratala A, Girones R. Newly described human polyomaviruses Merkel cell, KI and WU are present in urban sewage and may represent potential environmental contaminants. *Virol J.* 2010, 28;7:141.

Bull RA, White PA. Mechanisms of GII.4 norovirus evolution. *Trends in Microbiology*. 2011 19 (5), 233–240.

-C-

Calgua B, Rodriguez-Manzano J, Hundesa A, Suñen E, Calvo M, Bofill-Mas S, Girones R. New methods for the concentration of viruses from urban sewage using quantitative PCR. *J Virol Methods.* 2013, 187(2):215-21.

Campello C, Comar M, D'Agaro P, Minicozzi A, Rodella L, Poli A. A molecular case-control study of the Merkel cell polyomavirus in colon cancer. *J Med Virol*. 2011, 83(4):721-4. doi: 10.1002/jmv.22004.

Carter MJ. Enterically infecting viruses: pathogenicity, transmission and significance for food and waterborne infection. *J Appl Microbiol*. 2005, 98(6):1354-80.

Cashdollar JL, Wymer L. Methods for primary concentration of viruses from water samples: a review and meta-analysis of recent studies. *J Appl Microbiol*. 2013, 115(1):1-11.

Contreras-Coll N, Lucena F, Mooijman K, Havelaar A, Pierz V, Boque M, Gawler A, Höller C, Lambiri M, Mirolo G, Moreno B, Niemi M, Sommer R, Valentin B, Wiedenmann A, Young V, Jofre J. Occurrence and levels of indicator bacteriophages in bathing waters throughout Europe. *Water Res.* 2002 Dec, (20):4963-74.

Corredig M, Dalgleish DG. Effect of temperature and pH on the interactions of whey proteins with casein micelles in skim milk. *Food Research International*. 1996, 29(1), 49–55.

-D-

Dalianis T, Hirsch HH. Human polyomaviruses in disease and cancer. *Virology*. 2013, 15;437(2):63-72.

de Abreu Corrêa A, Carratala A, Barardi CR, Calvo M, Girones R, Bofill-Mas S. Comparative inactivation of murine norovirus, human adenovirus, and human JC polyomavirus by chlorine in seawater. *Appl Environ Microbiol*. 2012, 78(18):6450-7.

de Roda Husman AM, Lodder WJ, Rutjes SA, Schijven JF, Teunis PF. Long-term inactivation study of three enteroviruses in artificial surface and groundwaters, using PCR and cell culture. *Appl Environ Microbiol*. 2009, 75(4):1050-7.

DeCaprio JA, Garcea RL. A cornucopia of human polyomaviruses. *Nat Rev Microbiol.* 2013, 11(4):264-76.

Demetriades K, Coupland JN, McClements DJ. Physical Properties of Whey Protein Stabilized Emulsions as Related to pH and NaCl. Journal of Food Science. 2006, (62), 2, 342-347.

Dixon LK, Chapman DA, Netherton CL, Upton C. African swine fever virus replication and genomics. *Virus Res*. 2013, 173(1):3-14.

Djikeng A, Kuzmickas R, Anderson NG, Spiro DJ. Metagenomic analysis of RNA viruses in a fresh water lake. *PLoS One.* 2009, 4(9),e7264.

Dubrou S, Kopecka H, Lopez-Pila JM, Marechal J, Prevot J. Detection of hepatitis A virus and other enteroviruses in wastewater and surface water samples by gene probe assay. *Water Science and Technology.* 1991, 24 (2), 267–272.

-E-

Eckardt AJ, Baumgart DC. Viral gastroenteritis in adults. *Recent Pat Antiinfect Drug Discov*. 2011, 6(1):54-63.

Ekström J, Forslund O, Dillner J. Three novel papillomaviruses (HPV109, HPV112 and HPV114) and their presence in cutaneous and mucosal samples. *Virology.* 2010, 397(2):331-6.

Esposito S, Preti V, Consolo S, Nazzari E, Principi N. Adenovirus 36 infection and obesity. *J Clin Virol*. 2012, 55(2):95-100.

-F-

Farrah SR, Bitton G. 1990. Bollag Jean-Marc, Arthur Douglas Stotzky, G. Peterson, H. George (Eds.), Soil Biochemistry, 0824782321, vol. 6CRC.

Feltkamp MC, Kazem S, van der Meijden E, Lauber C, Gorbalenya AE. From Stockholm to Malawi: recent developments in studying human polyomaviruses. *J Gen Virol.* 2013 , 94, (3):482-96.

Feng H, Shuda M, Chang Y, Moore PS. Clonal integration of a polyomavirus in human Merkel cell carcinoma. *Science*. 200, 319(5866):1096-100.

Ferenczy MW, Marshall LJ, Nelson CD, Atwood WJ, Nath A, Khalili K, Major EO. Molecular biology, epidemiology, and pathogenesis of progressive multifocal leukoencephalopathy, the JC virus-induced demyelinating disease of the human brain. *Clin Microbiol Rev*. 2012, 25(3):471-506.

Ferreira MS, Victoria M, Carvalho-Costa FA, Vieira CB, Xavier MP, Fioretti JM, Andrade J, Volotão EM, Rocha M, Leite JP, Miagostovich MP. Surveillance of norovirus infections in the state of Rio De Janeiro, Brazil 2005-2008. *Journal of Medical Virology*. 2010, 82 (8): 1442–1448.

Fong TT, Lipp EK. Enteric viruses of humans and animals in aquatic environments: health risks, detection, and potential water quality assessment tools. *Microbiol Mol Biol Rev*. 2005, 69(2):357-71.

Formiga-Cruz M, Tofiño-Quesada G, Bofill-Mas S, Lees DN, Henshilwood K, Allard AK, Conden-Hansson AC, Hernroth BE, Vantarakis A, Tsibouxi A, Papapetropoulou M, Furones MD, Girones R. Distribution of human virus contamination in shellfish from

different growing areas in Greece, Spain, Sweden, and the United Kingdom. *Appl Environ Microbiol*. 2002, 68(12):5990-8.

Foulongne V, Sauvage V, Hebert C, Dereure O, Cheval J, Gouilh MA, Pariente K, Segondy M, Burguière A, Manuguerra JC, Caro V, Eloit M. Human skin microbiota: high diversity of DNA viruses identified on the human skin by high throughput sequencing. *PLoS One*. 2012, 7(6):e38499.

Fumian TM, Leite JP, Rose TL, Prado T, Miagostovich MP. One year environmental surveillance of rotavirus specie A (RVA) genotypes in circulation after the introduction of the Rotarix® vaccine in Rio de Janeiro, Brazil. *Water Res.* 2011, 45(17):5755-63.

Fumian TM, Vieira CB, Leite JP, Miagostovich MP. Assessment of burden of virus agents in an urban sewage treatment plant in Rio de Janeiro, Brazil. *J Water Health*. 2013, 11(1):110-9.

-G-

Gaynor AM, Nissen MD, Whiley DM, Mackay IM, Lambert SB, Wu G, Brennan DC, Storch GA, Sloots TP, Wang D. Identification of a novel polyomavirus from patients with acute respiratory tract infections. *PLoS Pathog*. 2007, 4;3(5):e64.

Gerba CP, Goyal SM, LaBelle RL, Cech I, Bodgan GF. Failure of indicator bacteria to reflect the occurrence of enteroviruses in marine waters. *Am J Public Health*. 1979, 69(11):1116-9.

Girones R, Ferrús MA, Alonso JL, Rodriguez-Manzano J, Calgua B, Corrêa Ade A, Hundesa A, Carratala A, Bofill-Mas S. Molecular detection of pathogens in water--the pros and cons of molecular techniques. *Water Res*. 2010, 44(15),4325-39.

Gray JJ, Green J, Gallimore C, Lee JV, Neal K, Brown DWG. Mixed genotype SRSV infections among a party of canoeists exposed to contaminated recreational water. *Journal of Medical Virology*. 1997, 52, 425–429.

Green KY, Chanock RM, Kapikian AZ. Human Caliciviruses. D.M. Knipe, P.M. Howley (Eds.) et al., Fields virology (4th ed), vol. 1-Lippincott Williams and Wilkins, Philadelphia (2001), pp. 841–874.

Greninger AL, Holtz L, Kang G, Ganem D, Wang D, DeRisi JL. Serological evidence of human klassevirus infection. *Clin Vaccine Immunol*. 2010, 17(10):1584-8.

Greninger AL, Runckel C, Chiu CY, Haggerty T, Parsonnet J, Ganem D, DeRisi JL. The complete genome of klassevirus - a novel picornavirus in pediatric stool. *Virol J*. 2009 18;6:82.

-H-

Hall AJ, Vinjé J, Lopman B, Park BW, Yen C, Gregoricus N, Parashar U. Updated Norovirus Outbreak Management and Disease Prevention Guidelines (CDC). *Recommendations and Reports*. 2011 60, 3.

Hamza IA, Jurzik L, Überla K, Wilhelm M. Methods to detect infectious human enteric viruses in environmental water samples. *Int J Hyg Environ Health*. 2011, 214(6):424-36.

Han TH, Kim CH, Chung JY, Park SH, Hwang ES. Klassevirus infection in children, South Korea. *Emerg Infect Dis*. 2010, 16(10):1623-5.

Haramoto E, Kitajima M, Otagiri M. Development of a reverse transcription-quantitative PCR assay for detection of salivirus/klassevirus. *Appl Environ Microbiol*. 2013, 79(11):3529-32.

Haramoto E, Otagiri M. Prevalence and genetic diversity of klassevirus in wastewater in Japan. *Food Environ Virol*. 2013, 5(1):46-51.

Hauri AM, Schimmelpfennig M, Walter-Domes M, Letz A, Diedrich S, Lopez-Pila J, Schreier E. An outbreak of viral meningitis associated with a public swimming pond. *Epidemiology and Infection*. 2005, 133 (2), 291–298.

Holtz LR, Finkbeiner SR, Zhao G, Kirkwood CD, Girones R, Pipas JM, Wang D. Klassevirus 1, a previously undescribed member of the family Picornaviridae, is globally widespread. *Virol J*. 2009, 24;6:86

Hsu BM, Chen CH, Kung CM, Wan MT, Shen SM. Evaluation of enterovirus recovery in surface water by different adsorption and elution procedures. *Chemosphere*. 2007, 66(5):964-9.

-J-

Jiang SC. Human adenoviruses in water: occurrence and health implications: a critical review. *Environ Sci Technol*. 2006, 1; 40(23):7132-40.

Johne R, Buck CB, Allander T, Atwood WJ, Garcea RL, Imperiale MJ, Major EO, Ramqvist T, Norkin LC. Taxonomical developments in the family Polyomaviridae. *Arch Virol*. 2011, 156(9):1627-34.

-K-

Katzenelson E, Fattal B, Hostovesky T. Organic flocculation: an efficient second-step concentration method for the detection of viruses in tap water. *Appl Environ Microbiol*. 1976, 32(4):638-9.

Kay D, Bartram J, Prüss A, Ashbolt N, Wyer MD, Fleisher JM, Fewtrell L, Rogers A, Rees G. Derivation of numerical values for the World Health Organization guidelines for recreational waters. *Water Res*. 2004, 38(5):1296-304.

Kay D, Fleisher JM, Salmon RL, Jones F, Wyer MD, Godfree AF, Zelenauch-Jacquotte Z, Shore R. Predicting likelihood of gastroenteritis from sea bathing: results from randomised exposure. *Lancet*. 1994, 344(8927):905-9.

Kirby A, Iturriza-Gómara M. Norovirus diagnostics: options, applications and interpretations. *Expert Rev Anti Infect Ther*. 2012, (4):423-33.

Komoto S, Maeno Y, Tomita M, Matsuoka T, Ohfu M, Yodoshi T, Akeda H, Taniguchi K. Whole genomic analysis of a porcine-like human G5P[6] rotavirus strain isolated from a child with diarrhoea and encephalopathy in Japan. *J Gen Virol.* 2013, 94(7):1568-75.

Kosulin K, Hoffmann F, Clauditz TS, Wilczak W, Dobner T. Presence of adenovirus species C in infiltrating lymphocytes of human sarcoma. *PLoS One.* 2013, 6;8(5):e63646.

Kristensen DM, Mushegian AR, Dolja VV, Koonin EV. New dimensions of the virus world discovered through metagenomics. *Trends Microbiol*. 2010, 18, 11–19.

-L-

La Rosa G, Fratini M, Accardi L, D'Oro G, Della Libera S, Muscillo M, Di Bonito P. Mucosal and cutaneous human papillomaviruses detected in raw sewages. *PLoS One*. 2013, 8(1):e52391.

La Rosa G, Fratini M, della Libera S, Iaconelli M, Muscillo M. Emerging and potentially emerging viruses in water environments. *Ann Ist Super Sanita.* 2012, 48(4):397-406.

Laverick MA, Wyn-Jones AP, Carter MJ. Quantitative RT-PCR for the enumeration of noroviruses (Norwalk-like viruses) in water and sewage. *Letters in Applied Microbiology*. 2004, 39, 127–136

Lee C, Lee SH, Han E, Kim SJ. Use of cell culture-PCR assay based on combination of A549 and BGMK cell lines and molecular identification as a tool to monitor infectious adenoviruses and enteroviruses in river water. *Applied and Environmental Microbiology*. 2004, 70, 6695–6705.

Li L, Victoria J, Kapoor A, Blinkova O, Wang C, Babrzadeh F, Mason CJ, Pandey P, Triki H, Bahri O, Oderinde BS, Baba MM, Bukbuk DN, Besser JM, Bartkus JM, Delwart EL. A novel picornavirus associated with gastroenteritis. *J Virol.* 2009, 83(22):12002-6.

Lipp EK, Farrah SA, Rose JB. Assessment and impact of microbial fecal pollution and human enteric pathogens in a coastal community. *Mar Pollut Bull*. 2001, 42(4):286-93.

Loh J, Zhao G, Presti RM, Holtz LR, Finkbeiner SR, Droit L, Villasana Z, Todd C, Pipas JM, Calgua B, Girones R, Wang D, Virgin HW. Detection of novel sequences related to african Swine Fever virus in human serum and sewage. *J Virol.* 2009, 83(24):13019-25.

López-Bueno A, Tamames J, Velázquez D, Moya A, Quesada A, Alcamí A. High diversity of the viral community from an Antarctic lake. *Science.* 2009, 326(5954), 858-61.

Lopman B, Vennema H, Kohli E, Pothier P, Sanchez A, Negredo A, Buesa J, Schreier E, Reacher M, Brown D, Gray J, Iturriza M, Gallimore C, Bottiger B, Hedlund KO, Torvén M, von Bonsdorff CH, Maunula L, Poljsak-Prijatelj M, Zimsek J, Reuter G, Szücs G, Melegh B, Svennson L, van Duijnhoven Y, Koopmans M. Increase in viral gastroenteritis outbreaks in Europe and epidemic spread of new norovirus variant. *Lancet*. 2004, 363(9410):682-8.

Loyo M, Guerrero-Preston R, Brait M, Hoque MO, Chuang A, Kim MS, Sharma R, Liégeois NJ, Koch WM, Califano JA, Westra WH, Sidransky D. Quantitative detection of

Merkel cell virus in human tissues and possible mode of transmission. *Int J Cancer*. 2010, 126(12):2991-6.

Lucey JA, Gorry C, O'Kennedy B, Kalab M, Tan-Kinita R, Fox PF. Effect of acidification and neutralization of milk on some physico-chemical properties of casein micelles. *Inter Dairy J*. 1996, (6);3, 257–272

Lukasik J, Scott TM, Andryshak D, Farrah SR. Influence of salts on virus adsorption to microporous filters. *Appl Environ Microbiol*. 2000, 66(7):2914-20.

-M-

Major E. O. Progressive multifocal leukoencephalopathy in patients on immunomodulatory therapies. *Annu Rev Med*. 2010, 61, 35–47.

Maunula L, Kalso S, von Bonsdorff CH, Pönkä A. Wading pool water contaminated with both noroviruses and astroviruses as the source of a gastroenteritis outbreak. *Epidemiology and Infection*. 2004, 132 (4), 737–743.

McQuaig SM, Scott TM, Harwood VJ, Farrah SR, Lukasik JO. Detection of human-derived fecal pollution in environmental waters by use of a PCR-based human polyomavirus assay. *Appl Environ Microbiol*. 2006, 72(12):7567-74.

Medema GJ, I.A. van Asperen IA, Kokman-Houweling JM, Nooitgedagt A, van de Laar MJW, Havelaar AH. The relationship between health effects in triathletes and microbiological quality of freshwater. *Water Science and Technology*. 1995, 31 (5–6), 19–26.

Melnick JL, Gerba CP, Wallis C. Viruses in water. *Bull World Health Organ.* 1978, 56(4):499-508.

Miagostovich MP, Ferreira FF, Guimarães FR, Fumian TM, Diniz-Mendes L, Luz SL, Silva LA, Leite JP. Molecular detection and characterization of gastroenteritis viruses occurring naturally in the stream waters of Manaus, central Amazonia, Brazil. *Appl Environ Microbiol.* 2008, 74(2):375-82.

Michen B, Graule T. Isoelectric points of viruses. *J Appl Microbiol*. 2010, 109(2), 388-97.

Moens U, Ludvigsen M, Van Ghelue M. Human polyomaviruses in skin diseases. *Patholog Res Int*. 2011, 2011:123491.

Mokili JL, Rohwer F, Dutilh BE. Metagenomics and future perspectives in virus discovery. *Curr Opin Virol*. 2012, 2(1):63-77.

Monier A, Claverie JM, Ogata H. Taxonomic distribution of large DNA viruses in the sea. *Genome Biol.* 2008, 9(7):R106.

Mounts AW, Ando T, Koopmans M, Bresee JS, Noel J, Glass RI. Cold weather seasonality of gastroenteritis associated with Norwalk-like viruses. *J Infect Dis.* 2000, 181 Suppl 2:S284-7.

-N-

Ng TF, Marine R, Wang C, Simmonds P, Kapusinszky B, Bodhidatta L, Oderinde BS, Wommack KE, Delwart E. High variety of known and new RNA and DNA viruses of diverse origins in untreated sewage. *J Virol*. 2012, 86(22):12161-75.

Nielsen AC, Gyhrs ML, Nielsen LP, Pedersen C, Böttiger B. Gastroenteritis and the novel picornaviruses aichi virus, cosavirus, saffold virus, and salivirus in young children. *J Clin Virol*. 2013, 57(3):239-42.

Nuanualsuwan S, Mariam T, Himathongkham S, Cliver DO. Ultraviolet inactivation of feline calicivirus, human enteric viruses and coliphages. *Photochem Photobiol*. 2002, 76(4):406-10.

-P-

Pecson BM, Ackermann M, Kohn T. Framework for using quantitative PCR as a nonculture based method to estimate virus infectivity. *Environ Sci Technol.* 2011, 45(6):2257-63.

Pina S, Puig M, Lucena F, Jofre J, Girones R. Viral pollution in the environment and in shellfish: human adenovirus detection by PCR as an index of human viruses. *Appl Environ Microbiol.* 1998, 64(9):3376-82.

Puig M, Jofre J, Lucena F, Allard A, Wadell G, Girones R. Detection of adenoviruses and enteroviruses in polluted waters by nested PCR amplification. *Appl Environ Microbiol.* 1994, 60(8):2963-70.

-R-

Radford AD, Chapman D, Dixon L, Chantrey J, Darby AC, Hall N. Application of next-generation sequencing technologies in virology. *J Gen Virol*. 2012, 93(Pt 9):1853-68.

Reynolds KA, Gerba CP, Pepper IL. Detection of infectious enteroviruses by an integrated cell culture-PCR procedure. *Appl. Environ. Microbiol.* 1996, 62 (4), 1424–1427.

Ridinger D, Spendlove R, Barnett BB, George DB, Roth J. Evaluation of cell lines and immunofluorescence and plaque assay procedures for quantifying reoviruses in sewage. *Appl. Environ. Microbiol.* 1982, 43 (4), 740–746.

Robinson CM, Singh G, Lee JY, Dehghan S, Rajaiya J, Liu EB, Yousuf MA, Betensky RA, Jones MS, Dyer DW, Seto D, Chodosh J. Molecular evolution of human adenoviruses. *Sci Rep.* 2013, (9);3, 1812.

Rodriguez-Brito B, Li L, Wegley L, Furlan M, Angly F, Breitbart M, Buchanan J, Desnues C, Dinsdale E, Edwards R, Felts B, Haynes M, Liu H, Lipson D, Mahaffy J, Martin-Cuadrado AB, Mira A, Nulton J, Pasić L, Rayhawk S, Rodriguez-Mueller J, Rodriguez-Valera F, Salamon P, Srinagesh S, Thingstad TF, Tran T, Thurber RV, Willner D, Youle M, Rohwer F. Viral and microbial community dynamics in four aquatic environments. *ISME J*. 2010, 4(6):739-51.

Rodríguez-Lázaro D, Cook N, Ruggeri FM, Sellwood J, Nasser A, Nascimento MS, D'Agostino M, Santos R, Saiz JC, Rzeżutka A, Bosch A, Gironés R, Carducci A, Muscillo

M, Kovač K, Diez-Valcarce M, Vantarakis A, von Bonsdorff CH, de Roda Husman AM, Hernández M, van der Poel WH. Virus hazards from food, water and other contaminated environments. *FEMS Microbiol Rev.* 2012, 36(4):786-814.

Rodriguez-Manzano J, Alonso JL, Ferrús MA, Moreno Y, Amorós I, Calgua B, Hundesa A, Guerrero-Latorre L, Carratala A, Rusiñol M, Girones R. Standard and new faecal indicators and pathogens in sewage treatment plants, microbiological parameters for improving the control of reclaimed water. *Water Sci Technol*. 2012, 66(12):2517-23.

Rosario K, Nilsson C, Lim YW, Ruan Y, Breitbart M. Metagenomic analysis of viruses in reclaimed water. *Environ Microbiol.* 2009, 11(11):2806-20.

Rzezutka A, Cook N. Survival of human enteric viruses in the environment and food. *FEMS Microbiol Rev*. 2004, 28(4):441-53.

-S-

Schoenfeld T, Patterson M, Richardson PM, Wommack KE, Young M, Mead D. Assembly of viral metagenomes from yellowstone hot springs. *Appl Environ Microbiol*. 2008, 74(13), 4164-74.

Schowalter RM, Pastrana DV, Buck CB. Glycosaminoglycans and sialylated glycans sequentially facilitate Merkel cell polyomavirus infectious entry. *PLoS Pathog.* 2011, 7(7):e1002161.

Schowalter RM, Pastrana DV, Pumphrey KA, Moyer AL, Buck CB. Merkel cell polyomavirus and two previously unknown polyomaviruses are chronically shed from human skin. *Cell Host Microbe*. 2010, 25;7(6):509-15.

Shan T, Wang C, Cui L, Yu Y, Delwart E, Zhao W, Zhu C, Lan D, Dai X, Hua X. Picornavirus salivirus/klassevirus in children with diarrhea, China. *Emerg Infect Dis*. 2010, 16(8):1303-5.

Sinclair RG, Jones EL, Gerba CP. Viruses in recreational water-borne disease outbreaks: a review. *Journal of Applied Microbiology*. 2009, 107 (6), 1769–1780.

Smith E, Gerba CP. Development of a method for detection of human rotavirus in water and sewage. *Appl. Environ. Microbiol*. 1982, 43 (6), 1440–1450.

Spurgeon ME, Lambert PF. Merkel cell polyomavirus: a newly discovered human virus with oncogenic potential. *Virology.* 2013, 5;435(1):118-30.

-T-

Tamaki H, Zhang R, Angly FE, Nakamura S, Hong PY, Yasunaga T, Kamagata Y, Liu WT. Metagenomic analysis of DNA viruses in a wastewater treatment plant in tropical climate. *Environ Microbiol*. 2012, 14(2):441-52.

Teunis PF, Lodder WJ, Heisterkamp SH, de Roda Husman AM. Mixed plaques: statistical evidence how plaque assays may underestimate virus concentrations. *Water Res.* 2005, 39(17), 4240-50.

Thurston-Enriquez JA, Haas CN, Jacangelo J, Riley K, Gerba CP. Inactivation of feline calicivirus and adenovirus type 40 by UV radiation. *Appl Environ Microbiol*. 2003, 69(1):577-82.

-V-

Van Ghelue M, Khan MT, Ehlers B, Moens U. Genome analysis of the new human polyomaviruses. *Rev Med Virol*. 2012, 22(6):354-77.

Vega E, Vinjé J. Novel GII.12 norovirus strain, United States, 2009-2010. *Emerg Infect Dis.* 2011, 17(8):1516-8.

Vieira CB, Mendes AC, Guimarães FR, Fumian TM, Leite JP, Gaspar AM, Miagostovich MP. Detection of enteric viruses in recreational waters of an urban lagoon in the city of Rio de Janeiro, Brazil. *Mem Inst Oswaldo Cruz*. 2012, 107(6):778-84.

Vilaginès P., Sarrette B, Husson G, Vilaginès R. Glass wool for virus concentration at ambient water pH level. *Water Science and Technology.* 1993, 27, 299-306.

-W-

Wallis C, Melnick JL. Concentration of viruses on membrane filters. *Journal of Virology*. 1967, 1, 472-477.

Wan XF, Barnett JL, Cunningham F, Chen S, Yang G, Nash S, Long LP, Ford L, Blackmon S, Zhang Y, Hanson L, He Q. Detection of African swine fever virus-like sequences in ponds in the Mississippi Delta through metagenomic sequencing. *Virus Genes*. 2013, 46(3):441-6.

Wevers D, Metzger S, Babweteera F, Bieberbach M, Boesch C, Cameron K, Couacy-Hymann E, Cranfield M, Gray M, Harris LA, Head J, Jeffery K, Knauf S, Lankester F, Leendertz SA, Lonsdorf E, Mugisha L, Nitsche A, Reed P, Robbins M, Travis DA, Zommers Z, Leendertz FH, Ehlers B. Novel adenoviruses in wild primates: a high level of genetic diversity and evidence of zoonotic transmissions. *J Virol.* 2011, 85(20):10774-84.

WHO, UNICEF. Progress on Sanitation and Drinking-Water Report 2013. ISBN: 9789241505390.

Wiedenmann A, Krüger P, Dietz K, López-Pila JM, Szewzyk R, Botzenhart K. A randomized controlled trial assessing infectious disease risks from bathing in fresh recreational waters in relation to the concentration of Escherichia coli, intestinal enterococci, Clostridium perfringens, and somatic coliphages. *Environ Health Perspect*. 2006, 114(2):228-36.

Wieland U, Mauch C, Kreuter A, Krieg T, Pfister H. Merkel cell polyomavirus DNA in persons without merkel cell carcinoma. *Emerg Infect Dis.* 2009, 15(9):1496-8.

Wyn-Jones AP, Carducci A, Cook N, D'Agostino M, Divizia M, Fleischer J, Gantzer C, Gawler A, Girones R, Höller C, de Roda Husman AM, Kay D, Kozyra I, López-Pila J, Muscillo M, Nascimento MS, Papageorgiou G, Rutjes S, Sellwood J, Szewzyk R, Wyer M. Surveillance of adenoviruses and noroviruses in European recreational waters. *Water Res*. 2011, 45(3):1025-38.

Wyn-Jones AP, Sellwood J. Enteric viruses in the aquatic environment. *J Appl Microbiol*. 2001, 91(6):945-62.

-Y-

Yozwiak NL, Skewes-Cox P, Stenglein MD, Balmaseda A, Harris E, DeRisi JL. Virus identification in unknown tropical febrile illness cases using deep sequencing. *PLoS Negl Trop Dis*. 2012, 6(2):e1485.

10. OTRAS PUBLICACIONES

A continuación se detallan otras publicaciones no incluidas en la *Tesis Doctoral.*

1. Rodriguez-Manzano J, Hundesa A, **Calgua B**, Carratala A, Maluquer de Motes C, Rusinol M, Moresco V, Ramos AP, Calvo M, Barardi CRM, Bofill-Mas S, Girones R. Failure to control viral contamination in molluscan shellfish applying current depuration treatments. Submitted for publication 2012.

2. **Calgua B**, Rodriguez-Manzano J, Hundesa A, Suñen E, Calvo M, Girones R. 2013. New methods for the concentration of viruses in urban sewage using quantitative PCR. *J Viro Methods*, 187 (22), 215-221.

3. Rodriguez-Manzano J, Alonso JL, Ferrús MA, Moreno Y, Amorós I, **Calgua B**, Hundesa A, Guerrero-Latorre L, Carratala A, Rusinol M, Girones R. 2012. Standard and new faecal indicators and pathogens in sewage treatment plants, microbiological parameters for improving the control of reclaimed water. *Water Sci Technol*, 66 (12), 2517-2523.

4. Bofill S, Hundesa A, **Calgua B**, Rusinol M, Maluquer de Motes C, Girones R. 2011. Cost-effective method for microbial source tracking using specific human and animal viruses. *J Vis Exp,* (58), e2820, DOI: 10.3791/2820.

5. Guerrero-Latorre L, Carratala A, Rodríguez-Manzano J, **Calgua B**, Hundesa A, Girones R. 2011. Environmental study in two settlements based in Eastern Chad from Darfur's conflict: analysis of Human Adenovirus and Hepatitis A and E Virus in water sources. *J Water Health*, 9 (3), 515-524.

6. Girones R, Ferrús MA, Alonso JL, Rodriguez-Manzano J, **Calgua B**, de Abreu Corrêa A, Hundesa A, Carratala A, Bofill-Mas S. 2010. Molecular detection of pathogens in water – the pros and cons of molecular techniques. *Water Res,* 44 (15), 4325-4339.

7. Bofill-Mas S, Rodriguez-Manzano J, **Calgua B**, and Girones R. 2010. Newly described human polyomaviruses Merkel Cell, KI and WU are present in environmental samples showing excretion by human populations. *Virol J,* 28 (7), 141.

8. Loh J, Zhao G, Presti RM, Holtz LR, Finkbeiner SR, Droit L, Villasana Z, Todd C, Pipas JM, **Calgua B**, Girones R, Wang D, Virgin HW. 2009 Detection of novel sequences related to Swine Fever virus in human serum and sewage. *J Virol,* 38 (24), 13019-13025.

9. Albinana-Gimenez N, Clemente-Casares P, **Calgua B**, Huguet JM, Courtois S and Girones R. 2009. Comparison of methods for concentrating human adenoviruses, polyomavirus JC and noroviruses in source waters and drinking water using quantitative PCR. *J Virol Methods,* 158 (1-2), 104-109.

10. Albinana-Gimenez N, Miagostovich MP, **Calgua B**, Huguet JM, Matia L and Girones R. 2009. Analysis of adenoviruses and polyomaviruses quantified by qPCR as indicators of water quality in source and drinking-water treatment plants. *Water Res,* 43 (7), 2011-2019.

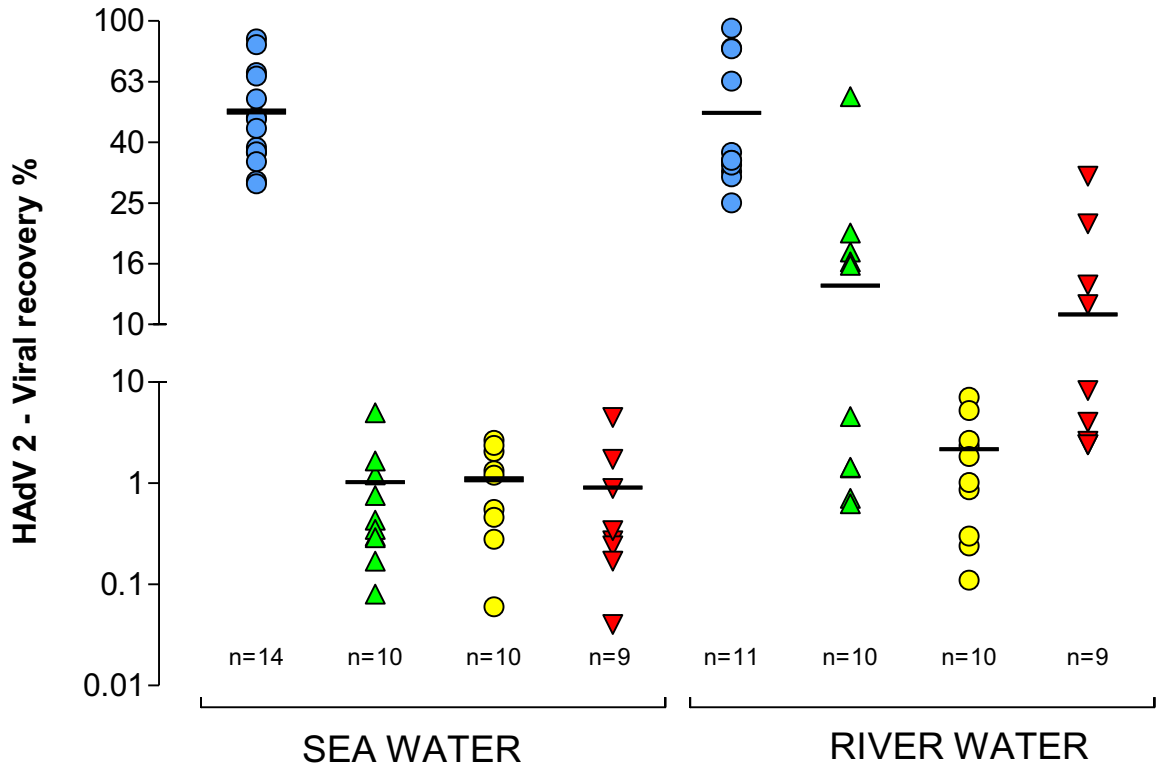
10. ANEXO

ANEXO.

CONDICIONES EXPERIMENTALES:

Cuatro métodos diferentes para concentrar virus en muestras de 10 L de agua marina artificial o natural y agua fresca (excepto para SMFP para agua de río que se realizó durante otro periodo), fueron comparados bajo las mismas condiciones. Todas las muestras fueron contaminadas con suspensiones víricas de adenovirus humanos 2 de concentraciones conocidas y finalmente los virus recuperados en cada método, fueron cuantificados empleando qPCR TaqMan®.

Figura. Comparación de cuatro métodos de concentración para concentrar HAdV 2 en agua marina y fresca.



Mean	50,37	1,02	1,09	0,90	49,82	13,42	2,17	10,78
Coefficient of variation %	37.84	144.74	89.60	159.52	50.59	126.70	107.06	91.65

METHODS:

- Skim milk organic flocculation procedure.
- Nitrocellulose electronegative membranes and glycine 0.25 M pH 9.5 – skim milk buffer.
- Nitrocellulose electronegative membranes and glycine 0.25 M pH 9.5 – beef extract buffer.
- Glass-wool and glycine 0.25 M pH9.5-beef extract buffer.

Modificado a partir de Girones *et al.*, 2010.