ANÀLISI DE POLIOMAVIRUS I ADENOVIRUS HUMANS COM A INDICADORS DE LA CONTAMINACIÓ VÍRICA D'ORIGEN HUMÀ EN AIGUA

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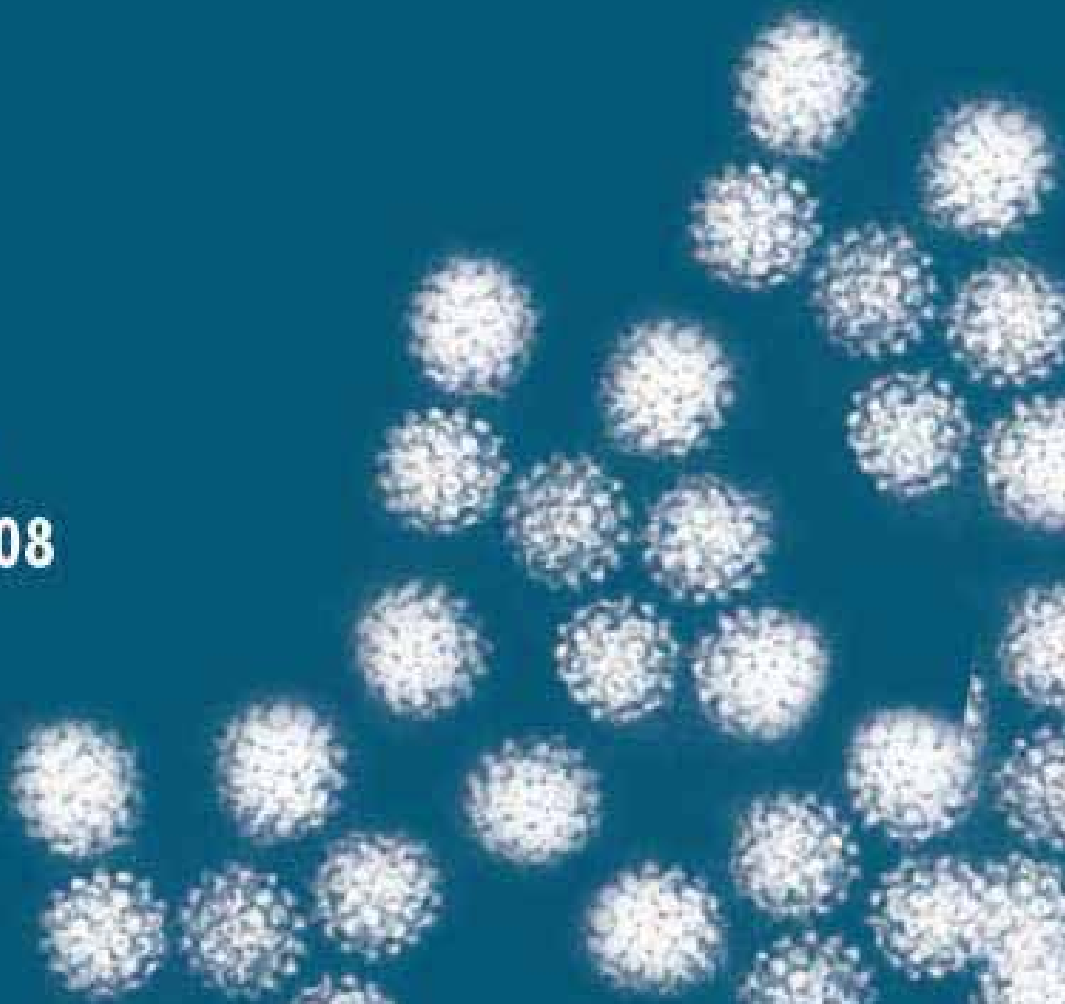

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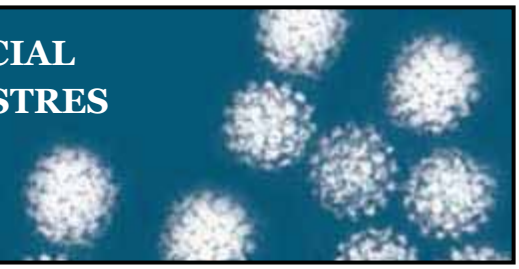
VP de la directora, El doctorand,

Dra. Rosina Gironés Llop Néstor Albiñana Giménez

Barcelona, Setembre 2008

RESULTATS

CAPÍTOL I: DETECCIÓ I POTENCIAL TRANSMISSIÓ DE SV40 EN MOSTRES AMBIENTALS.



Aïllament de SV40 de l'ambient d'una colònia de micos *Cynomolgus* infectats naturalment amb el virus.

S. Bofill-Mas, N. Albinana-Gimenez, P.A. Pipkin, P.D. Minor, R. Girones

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Resum

SV40 (Simian Virus 40) infecta naturalment macacs Asiàtics salvatges i diverses espècies relacionades en captivitat. SV40 pot causar leucoencefalopatia multifocal progressiva (PML) i malalties renals en micos immunocompromesos. S'ha suggerit que SV40 podria tenir un rol en la formació d'alguns tumors cerebrals i lesions al sistema nerviós central en macacs. Aquest virus va ser descobert com a contaminant de les vacunes de la polio que van ser distribuïdes per tot el món, exposant centenars de milions de persones al virus entre 1955 i 1963. Estudis recents sobre la connexió de SV40 i tumors en humans s'han estat acumulant.

El principal objectiu d'aquest treball és l'avaluació de la presència i estabilitat de SV40 en l'ambient i l'anàlisi de les partícules víriques excretades i potencialment transmeses en una comunitat de micos *Cynomolgus* naturalment infectats pel virus. La seroprevalència de les infeccions de SV40 en la comunitat va ser també analitzada. La metodologia aplicada en aquest estudi està basada en la elució de partícules víriques dels sòlids i residus orgànics de les mostres usant tampó glicina. La concentració de les partícules víriques es va fer per ultracentrifugació i l'amplificació genòmica per PCR niada i anàlisi de seqüències. Aquesta metodologia ha estat desenvolupada en estudis previs on aigua residual urbana de diverses àrees de món va ser analitzada per la presència d'adenovirus humans i poliomavirus amb absència de resultats positius per SV40.

L'estabilitat de SV40 en aigua residual a 20°C va ser estimada, amb una t_{90} (temps requerit per la degradació del 90% de les partícules víriques) de 39,9 dies i una t_{99} (temps requerit per la degradació del 99% de les partícules víriques) de 64,7 dies. El material del terra de 4 gàbies on habitaven micos va ser recollit en dos dies diferents. Hem detectat 10^2-10^4 equivalents genòmics de PCR de SV40 per ml de mostra en les dues mostres analitzades de dues de les quatre caixes. En seqüenciar el virus detectat en la colònia es va identificar una soca que presentava regió reguladora arquetípica i

una regió C-terminal del TAg idèntica a la prèviament descrita W17, aïllada als National Institute for Biological Standards and Control, UK (NIBSC) d'una biòpsia de ronyó d'un mico.

Com no hi ha informació prèvia del comportament de SV40 a l'ambient, l'estudi d'aquest virus en mostres ambientals proporciona dades importants sobre el mecanisme de transmissió de SV40 en la població humana.

Resum del Resultats i la Discussió

Les colònies de cria de micos Cynomolgus consisteixen en 24 femelles i 2 mascles. Quan les cries assoleixen 1 Kg i tenen al menys 6 mesos se'ls separa de la colònia de cria i se'ls junta en grups d'aproximadament 20 individus. Els micos juvenils viuen en aquests grups fins que tenen 12 mesos i pesen 1,5 Kg, en aquest moment se'ls aïlla en gàbies en grups més petits (2 o 3 individus). És en aquesta fase on es van recollir les mostres de residus de les caixes.

Es van analitzar mostres ambientals de 4 gàbies de micos naturalment infectats per SV40. Es va detectar SV40 per PCR en les dues mostres de les caixes 3 i 4. La semiquantificació d'aquestes mostres per dilució límit amb PCR niada va mostrar una concentració de 10^4 còpies genòmiques per 4ml de mostra en la caixa 3. Les mostres de la caixa 4 van presentar 10^3 i 10^1 GC per 10µl del concentrat obtingut per elució dels virus presents en mostres heterogènies de restes de menjar i orina. La seqüenciació dels amplicons va confirmar la identitat del virus a les mostres.

Els experiments de serologia en la colònia de micos van mostrar que tots els micos estudiats en aquest treball van ser seropositius amb un alt títol d'anticossos. Això suggereix que la transmissió d'aquests virus i la seroconversió es produeix durant la seva estança als grups juvenils, tal com va suggerir Minor i col. (2003) quan va estudiar la cinètica de seroconversió en la colònia.

S'ha suggerit que la transmissió del virus es produeix per la ruta fecal-oral pels micos en condicions naturals. Sembla ser que els micos s'infecten quan estan en grups grans, probablement per l'increment de probabilitats de contacte entre virus excretats i el nou hoste sense la protecció dels anticossos materns.

La soca aïllada de les mostres de residus de les gàbies va ser denominada UK; les dues regions amplificades (RR i C-terminal de TAg) van ser comparades amb soques prèviament aïllades i va resultar idèntica a la soca W17, aïllada a partir d'una biòpsia de ronyó de mico efectuada al *National Institute for Biological Standards and Control*, UK (NIBSC) (Minor i col. 2001; 2003). Aquesta soca té la regió reguladora amb configuració arquetípica idèntica a algunes soques aïllades de teixits de mico; la regió hipervariable (C-terminal del TAg) va presentar identitat amb soques trobades a tumors humans. Al 1998, Stewart i col. van descriure la similaritat d'alguns aïllats de SV40 obtinguts de mostres tant humanes com de micos, i no hi havia evidència de soques específiques de micos o d'humans.

La regió reguladora de la soca aïllada conté només una còpia de l'*enhancer*. Tot i això, no hi ha informació sobre la possible varietat de soques que podrien estar presents a la colònia (Minor i col. 2003). Segons la informació disponible sobre SV40 en micos es podria esperar gran varietat de soques en un mateix animal, inclús en el teixit renal d'un individu. De tota manera les soques majoritàries excretades en orina, com en el cas de JCPyV i BKPyV en humans, són les d'estructura arquetípica com les que hem trobat en aquest estudi.

S'ha aïllat gran varietat de soques de SV40 en cervell, melsa i cèl·lules mononuclears de sang de micos immunocompromesos (Lednicky i col. 1998), mostrant una mescla d'organitzacions arquetípiques i reorganitzades, éssent la soca arquetípica la predominant en cervell.

La soca que s'ha aïllat en aquest estudi va presentar estabilitat en la regió reguladora, ja que va resultar idèntica abans i després d'experiments d'infecció en cèl·lules CV-1, on va coinfectar amb adenovirus, uns virus molt comuns a l'ambient (Bofill-Mas i col. 2000).

Per comprovar el paper probable de la contaminació ambiental en la transmissió de SV40 de mico a mico en aquesta colònia es van realitzar anàlisis de l'estabilitat de SV40 en aigua residual tot inoculant tres mostres d'aigua residual i un control de PBS i mantenint els contenidors a 20°C. Es va trobar SV40 fins al dia 40 en dues de les mostres i fins al dia 60 en la tercera. Els virus detectats eren virions intactes, ja que tractaments amb DNAsa previs a l'extracció d'àcids nuclèics no van aconseguir eliminar els genomes. La t_{90} estimada de SV40 en aigua residual a 20°C va

ser de 39.9 dies, i la t_{99} de 64.7 dies. En el control de PBS es va detectar SV40 fins al dia 210.

La presència de partícules infeccioses de SV40 en les mostres ambientals de 2 de les 4 gàbies de micos *Cynomolgus* suggereix que aquesta contaminació pot ser una ruta de transmissió del virus. Minor i col (2003) han presentat conclusions similars en estudiar la cinètica de seroconversió dels micos de la colònia. Les soques més abundantment excretades per aquesta colònia van presentar una estructura molt homogènia i estable en la regió reguladora i C-terminal del TAg. Els resultats confirmen l'aplicabilitat dels mètodes utilitzats per la detecció i aïllament de SV40 en mostres ambientals, per tant el fet que no poguéssim detectar SV40 en mostres d'aigua residual en anteriors estudis va ser probablement perquè realment no hi era present en nivells detectables (Bofill-Mas i col. 2000).

Title: Isolation of SV40 from the environment of a colony of cynomolgus monkeys naturally infected with the virus

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Abstract

The presence of SV40 viral particles in the environment of cynomolgus monkeys naturally infected with this virus has been analyzed by testing waste of the cages samples.

SV40 was detected in 2/4 cages tested where mixed infection of SV40 and adenoviruses was observed after inoculation of virions concentrated from cage waste in CV-1 cells. The detected SV40 strains were identical in the regions studied to the strain W17, isolated at NIBSC from a (1/19) monkey kidney biopsy and contains an archetypal regulatory region. The recovery of infectious SV40 virions from the cages provides information about the potential mechanism of transmission of this virus.

Keywords: SV40, environment, cynomolgus, SV40 infection, SV40 stability, SV40 transmission.

Introduction

The discovery of the polyomavirus SV40 is tied to the development and distribution of polio vaccines which were inadvertently contaminated with the virus and distributed worldwide potentially exposing several millions of persons to the virus during 1955 to 1963 (Shah & Nathanson, 1976). SV40 naturally infects a few species of Asiatic macaques, especially Rhesus monkeys (revised in Shah et al., 1971). In rhesus macaques, the virus multiplies readily producing viremia and a high-titered antibody response. In the wild, only a small proportion of the juvenile macaques, but nearly all adults, have SV40 antibodies (Meyers et al., 1962; Shah and Southwick, 1965). This infection appears to be sub-clinical and the virus establishes a long-term latent chronic infection in the kidneys and is excreted in the urine providing an opportunity for environmental contamination and transmission to non-immune monkeys although the exact manner of transmission of SV40 is not known (Hull, 1968). Experimentally, non-immune rhesus monkeys are readily infected by the oral, intranasal and subcutaneous route leading to viremia and viruria. In captivity, SV40 is readily transmitted to other macaques in contact with infected rhesus as is the case for cynomolgus macaques and African green monkeys (Shah and Nathanson, 1976). The presence of SV40 in the brain of immunodeficient monkeys demonstrated that SV40 is neurotropic in addition to being kidney tropic. A role for SV40 in the etiology of some brain tumors and in the development of central nervous system lesions distinct from PML have been suggested (Hurley et al., 1997; Simon et al., 1999). Recently, reports linking SV40 and human tumors have accumulated and the potential role of SV40 in cancer etiology requires serious consideration. SV40 has been associated with mesotheliomas and different types of brain tumors such as ependymomas and choroid plexus carcinomas. In addition to mesotheliomas and brain tumors, SV40 has been associated with other human tumors and non-tumor tissues, including osteosarcomas, non-Hodgkin's lymphomas, AIDS-related lymphomas, peripheral blood cells, sperm fluids and kidney tissue (Arrington & Butel, 2001). In previous studies no SV40 virions have been detected in urban sewage samples containing almost all human polyomaviruses JCV and BKV (Bofill-Mas et al., 2000). The first goal of this study was to evaluate our methods to detect SV40 in the environment by evaluating

the stability of SV40 in sewage samples and by analyzing the waste of a colony of cynomolgus monkeys naturally infected with the virus. A second goal of this study was to characterize the SV40 strains being excreted by the cynomolgus colony studied.

Results

Detection of SV40 in cage waste. The samples from the cages were processed as labeled samples without identification of the specific cages and analyzed in two independent assays. Two different sets of primers that amplify different regions of SV40 genome were used. SV40 was detected by PCR in the two samples collected from cages 3 and 4 when the two regions of the genome were analyzed, the C-terminal of the T-antigen region as well as the regulatory region. We semi-quantified the concentration of viral particles present in the samples by limiting-dilution nested-PCR experiments. The two samples of liquids obtained from cage 3, inhabited by three monkeys, showed approximately 10^4 genome copies/10 µl of viral concentrate which was equivalent to 4 ml of sample while the two samples from cage 4, inhabited by only two monkeys, showed approximately 10^3 and 10^1 genome copies/10 µl respectively in the viral concentrate prepared by elution of the viruses present in the total waste (heterogeneous solid food residues and some liquid excreta) collected from the cages.

The DNA amplicons from the positive samples were sequenced without having any information on previously isolated strains in the colony and confirmed to be SV40. The strain isolated from cages was designated strain UK and was further compared, in the two regions studied, to previously isolated strains and was found to be identical to strain W17, isolated from 1/19 monkey kidney biopsies at NIBSC (Minor et al., 2001; Minor et al., 2003). The SV40 UK strain had been not present in our laboratory before the PCR analysis of the cages waste was carried out. The strain W17 contains an archetypal configuration in its regulatory region and it is identical to SV40 stains 777*, Rh911, Pa-57 (previously isolated from monkey tissues) and to SV40 strains CPC/MEN and cpc (isolated from human tumors) in its hypervariable region, located at the C-terminal of the T-antigen gene regions (Figures 1 and 2).

Neutralization assays. The results obtained in the SV40 antibody screening showed that all the monkeys studied in this issue were seropositive and had a high antibody titer (Table 2).

Cell culture. In order to test the infectivity of the SV40 viral particles detected and to isolate the SV40 viruses excreted by these animals, viral concentrates prepared from the environmental

samples positive for SV40 in the PCR assays were inoculated on CV-1. Cells infected with sample UK8 from cage 3 showed SV40 cytopatic effect (CPE) within 6 days post infection and cells infected with the tenfold dilution of the UK8 viral concentrate showed CPE within 33 days. Nested-PCR assays confirmed the presence of SV40 in the supernatant of the infected cell cultures and the analysis of these supernatants by electron microscopy revealed a co-infection of SV40 and what appeared to be simian adenoviruses. The regulatory region of PCR products obtained from the cell culture were cloned and five clones were sequenced and the sequence of these clones were identical to the previously isolated strain W17 (Minor et al., 2001; Minor et al., 2003).

Stability of SV40 viral particles in sewage at 20ºC. As there is no previous information on the behavior of SV40 in the environment, data on the stability of SV40 virions in the environmental waste might contribute to a better evaluation of their mechanism of transmission. Comparative analysis of the 3 samples of raw sewage from Barcelona and a PBS control, all spiked with SV40 viral particles and kept at 20ºC, showed that SV40 viral particles were detected in all the sewage samples until day 40 and in one of them until day 60. The viruses detected during this time appeared to represent intact virions since treatment with DNAse prior to nucleic acid extraction did not eliminate the viral genomes. In evaluating the stability of SV40 in sewage at 20ºC, we estimated a t_{90} of 39.9 days and t_{99} of 64.7 days. SV40 DNA was detectable in the spiked PBS control until day 210.

Discussion

The breeding colony of cynomolgus colony consists initially of approximately 24 females and 2 males per group. A colony due to natural processes may become occasionally so small that it becomes unviable. When this occurs, those females that are desirable to retain are either introduced into another established colony or, if several depleted colonies exist, they may be amalgamated. Colony founder animals are identified by number alone, while those born in the colony are identified by a sequential letter added to their mother's number. For example animal 394 would be a founder (female) individual, 394A would be the first offspring and 394B the second. 394AA would be the offspring of animal 394A whose second offspring would be 394AB. When the babies are at least six months old and have attained a weight greater than 1kg they are weaned and put together in a gang (groups of about 20 animals) away from the breeding colonies. Juveniles live in this group until such time as they reach a target weight of 1.54 kg and are at least 12 months of age. They are then taken from the gang and caged in small groups of 2 or 3. It is at this stage that cage waste samples were collected. The cages were housed in the same room and no other animals were in the room.

Table 2 shows, as expected, high antibody titers for the monkeys after being in their group of juveniles for few weeks, suggesting that SV40 infection spreads between the monkeys during their stay in the weaned groups and the seroconversion takes place during gang caging as has been suggested by Minor and coworkers (2003) after studying the kinetics of seroconversion of SV40 in the colony. Transmission through the environment by the fecal-oral route has been suggested for monkeys in natural conditions. Most of the juveniles monkeys in the studied colony seem to be infected when are caged with a high number of other monkeys, probably because an increase in the probability of contact between excreted virus and new hosts without the protection of maternal antibodies. At this stadium is when we collected the samples and detected 10^4 genome copies/4 ml of sample from cage 3 and 10^3 and 10^1 genome copies in the two samples prepared by elution of the viruses present in the waste (an undetermined quantity of heterogeneous solid food residues and some liquid excreta) collected from the cage 4.

When analyzing environmental samples from monkey cages, the possibility of contamination of SV40 strains used in the laboratory was carefully analyzed. The blind analysis of the original numbered samples, repeated PCR amplification and isolation of viral particles together with the identity, compared at the end of the study, of the identified sequence, in the C-terminal T-antigen and in the regulatory region, with the previously described W17, support the results we have obtained.

In previous studies carried out at NIBSC, cultures of kidney tissue were set up from 19 seropositive cynomolgu*s* monkeys. One culture established from animal 586BE showed typical SV40-like CPE which evidenced by the isolation of a SV40 strain identified as W17 (Minor et al., 2001; Minor et al., 2003).

The sequence identified in the C-terminal T-antigen variable domain of the strains detected in the cages in these experiments was identical to the sequence of the W17 SV40 strain and also to sequences previously isolated from monkeys and humans (Figure 1 and 2). In 1998, Stewart et al. described the similarity of some SV40 isolates recovered from humans as well as from monkeys and there was no compelling evidence for human-specific or monkey-specific SV40 strains. The sequences of the C-terminal T-antigen of strains Pa-57, Rh911 and CPC/MEN are identical, in the regions studied, to the strain W17/UK although all have independent sources. Stewart et al. (1998) and Newman et al. (1998) also failed to observe tissue-specific differences among the sequences of the SV40 strains recovered from different tissues.

The strain identified in this study has been found to have a single copy of the enhancer in the regulatory region. However, there is little information about the possible variety of SV40 strains that might be present in the colony (Minor et al., 2003). A variety of structures in the regulatory region could be expected in one animal, or even in the kidney tissues of one animal, according to data available on SV40 isolated from monkeys. However, archetypal strains like the ones described in this study could be the most common structure of SV40 strains excreted in urine as is the case for JCV and BKV in humans.

A diversity of SV40 strains isolated from brain, spleen and peripheral blood mononuclear cells have been described in immunocompromised monkeys (Lednicky et al., 1998) showing the

presence of a mixture of diverse archetypal and non-archetypal forms, with archetypal SV40 as the predominant from in the monkey brain tissue. Other descriptions of SV40 strains infecting rhesus monkeys with simian immunodeficiency virus-induced immunodeficiency have been reported by Ilyinskii et al. (1992). SV40 isolated from kidney and brain tissues presented an archetypal structure of the regulatory region as the most common structure in these tissues. Newman et al. (1998) analyzed the sequence of SV40 strains isolated from the urine, kidney and brain of five SIV-infected and one healthy rhesus monkeys. The SV40 strains detected in the urine of the healthy animal presented an archetypal regulatory region as it was the case of the strains detected in the brain and kidney of SIV-infected animals, although two of which presented an archetypal regulatory region variant due to the lack of one 21 bp repeat.

The SV40 strain we identified before and after culture in CV-1 cells showed stability in its regulatory region in the first infection and after confirmative culture. In both cases these cells were coinfected with SV40 and adenoviruses. Adenoviruses are viruses commonly detected in the environment (Bofill-Mas et al, 2000).

To support the probable role of environmental contamination in transmission of SV40 from monkey to monkey in this colony and in other environments we examined the stability of SV40 viral particles in sewage samples at 20ºC. The values we have obtained (t_{90} of 39.9 days and t_{99} of 64.7 days) were intermediate to those obtained for the human polyomaviruses JCV and BKV in previous studies (Bofill-Mas et al., 2001). It must be taken into account when considering SV40 stability in the environment that SV40 DNA was found in SV40 spiked PBS after 210 days at 20ºC.

The presence of infectious SV40 viral particles in the environmental waste from 2/4 cynomolgus monkeys cages that we have studied suggests that this contamination might be a route for the virus to be transmitted. Minor and coworkers (2003) have presented similar conclusions after studying the kinetics of seroconversion of the colony. The most abundant SV40 strains excreted by the colony of cynomolgus monkeys studied presented a very homogeneous and stable genetic structure in the RR and C-terminal T-antigen region. The results confirm the applicability of the methodology developed for the detection and isolation of

SV40 in environmental samples, thus our prior failure to detect SV40 in sewage samples was likely the result of not being present in detectable levels (Bofill-Mas et al, 2000). Studies of the presence of this virus in the environment of humans and macaques which are at this moment been developed by our research group could provide information on the potential mechanism of the transmission of SV40 from monkeys to humans.

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Material and Methods

Cells and positive controls. SV40 DNA strain 776 (Gibco BRL) was used as a positive control. CV-1, an African green monkey cell line, and viral particles of SV40 strain WT 800, were kindly donated by Dr. Azorin from the Institute Juan de la Cierva, CSIC (Consejo Superior de Investigaciones Científicas), Barcelona. Cells were propagated in Eagle's minimal essential medium (EMEM) supplemented with 1% glutamine, 50 μg of gentamicin per ml and 5% (growth medium) or 2% (maintenance medium) of heat-inactivated FBS. The cell line BSC-1 was grown in EMEM supplemented with 10% fetal bovine serum (FBS) buffered with 15mM HEPES. The SV40 strain used in the neutralization assays was the laboratory strain NIBSC isolated in the 1960's (Sangar et al., 1999).

Environmental samples. A total of eight samples were obtained from four different cynomolgus monkey (*Macaca fascicularis*) cages over a 24 hour period (2 samples per cage) at the NIBSC (National Institute for Biological Standards and Control, UK). Samples contained excrements, urine and residues of food. These samples were frozen at –20ºC and shipped to Spain for further analysis.

Serum samples and virus neutralization assay. As part of the general health check, a 2ml blood sample was removed from the monkeys femoral vein and transferred into a suitable container without anti-coagulant. The serum was removed from the clotted blood sample and held at 56ºC for 30 minutes, this reduced or removed any toxic effect to the BSC-1 cells. The serum was then stored at –20ºC until use. The virus dose was established by titration in BSC-1 cells as previously described (Minor et al, 2003).

Concentration of viral particles from environmental samples from monkey's cages. Recovery of viral particles obtained from the monkey cages was carried out in two ways depending on the characteristics of each sample. Liquid samples were processed as sewage as described in previous studies (Puig et al., 1994). Solid samples were dissolved with 4 ml of 0.25 N glycine buffer pH 9.5 using magnetic stirring for 30 min, the suspended solids were separated by centrifugation at 12,000x*g* for 15 min after the addition of 4 ml of 2x PBS. Viruses were pelleted by ultracentrifugation (229,600x*g* for 1 h at 4 ºC), resuspended in 0.1 ml of 1x PBS and stored at -80 ºC.

Stability of SV40 viral particles in sewage. One liter of a raw sewage sample was collected in the area of Barcelona (Spain) in a sterile polyethylene container and kept at 4ºC for less than 8 hours until it was spiked with SV40 WT800 viral particles and divided into three sterile beakers. Beakers were kept in an oven at 20°C. One liter of PBS spiked with SV40 WT800 viral particles was used as a positive control. Aliquots of 40 ml of each sample were concentrated at days 0, 10, 30, 40, 60 and 210 as described in the previous section. The presence of viral particles was evaluated by nested PCR and, to determine if the viral particles remained intact, viral concentrates were treated with DNAse as previously described (Bofill-Mas et al, 2001). The estimated concentration of SV40 viral particles in the samples was carried out by nested PCR limiting dilution assays.

In order to obtain an estimated T_{90} and T_{99} (time required for a 90% and 99% reduction in the viral concentration), we computed a linear regression model with the logarithm of the estimated concentration of viral particles detected by nested-PCR expressed as PCR-genomic equivalents.

The method applied had been previously applied and described when studying the stability of human polyomaviruses JCV and BKV (Bofill-Mas et al, 2001).

Enzymatic amplification and sequencing of the PCR products. Viral nucleic acids were extracted using a procedure that uses guanidinium thiocyanate (GuSCN) and adsorption of the nucleic acids to silica particles (Boom et al., 1990) providing clean nucleic acids for genomic amplification. Ten µl aliquots of the extracted nucleic acids were used in each test. Amplifications were carried out as previously described for SV40 and human polyomaviruses JCV and BKV (Bofill-Mas et al, 2000; Bofill-Mas et al, 2001). Primers and annealing temperatures used in this study are represented in Table 1. The results were analyzed by agarose gel electrophoresis using ethidium bromide staining.

Direct and tenfold dilutions of the samples were analyzed and the assays for detection of the presence of SV40 in samples from monkey's cages were carried out three times in independent experiments for the confirmation of the results using two different sets of primers that amplify two different regions of the SV40 genome, the regulatory and the C-terminal T-antigen regions. Standard precautions were applied in all the manipulations in order to reduce the probability of sample contamination by amplified DNA molecules. Products obtained after PCR were purified and both strains automatically sequenced as previously described (Bofill-Mas et al, 2000).

Infection of CV-1 cells. Concentrates of the samples positive for SV40 by PCR were diluted 1:10 in PBS and filtered using 0.22 µm low protein binding Durapore syringe driven filters (Millex®-GV, Millipore) in order to eliminate any bacteria remaining in the concentrate.

One hundred µl of the filtrates obtained were used to infect cells plated on 25-cm^2 flasks. PBS was inoculated in cell culture as negative controls. After 1 hour at 37ºC maintenance medium was added. Supernatants were renewed weekly using maintenance medium and stored at –80ºC for further studies. The time course of the infection was examined by observation of CPE. Stored supernatants of different days post-infection were assayed by PCR. Positive results were confirmed by cell culture, cloning and sequencing of the progeny virions.

Cloning of the regulatory region of SV40. The regulatory regions of the SV40 growing in cell culture and present in the supernatant of CV-1 cells were amplified by nested PCR. Bands obtained were purified with the QUIAquick PCR purification kit (QIAGEN, Inc.) Purified bands were cloned as previously done with other polyomaviruses (Bofill-Mas et al, 2001). The inserts were recovered by PCR amplification using primers RA and RA2 (Table 1). Products obtained after the PCR were further analyzed by sequencing using the amplification primers.

Legends

Figure 1. Nucleotide sequences of the T-antigen C-terminal variable domains of strain UK and previously described SV40 strains isolated from monkeys (776, AF316139; 777*, AY271817; Pa-57, M99362; Rh911, AF316140; K661, AF038616; T302, AF168994; H388, AF168993; I508, AF169000; Kid155, M99356; Br54, M99351; H328, AF316141; 6593, AF168995; VA45-54-2, AF156105). W17 was isolated from a kidney biopsy of a member of the cynomolgus colony at NIBSC (Minor et al., 2001). The sequence shown is for the coding strand and numbering is according to SV40 776. Dots indicate identity to 776 strain and dashes indicate deletion or, when at the beginning or the end of sequences indicate that this part of the sequence is not available at GenBank.

Figure 2. Nucleotide sequences of the T-antigen C-terminal variable domains of strain UK and previously described SV40 strains isolated from human tumors (CPC/MEN, AF156108; Ost9, AF168998; ependy, AF136003; cpc1, AF136000; cpc, AF135999; Ost2, AF168996; cpc2, AF136002; gang, AF136001; Ost3, AF168997) and SV40 contaminated polio vaccines (MC-028846B, AF18737; Baylor-1, AF155359). The sequence shown is for the coding strand and numbering is according to SV40 CPC/MEN. Dots indicate identity to CPC/MEN strain and dashes indicate deletion or, when at the beginning or the end of sequences indicate that this part of the sequence is not available at GenBank.

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Anàlisi de mostres d'aigua procedents del nord de la Índia per avaluar la presència i potencial transmissió de SV40 en poblacions humanes.

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Resum

El virus de simi 40 (SV40) es un virus de mico que va ser administrat a poblacions humanes a través de vacunes de la polio contaminades, produïdes en cèl·lules de mico naturalment infectades pel virus. La transmissió zoonòtica és possible però la via encara és desconeguda. L'objectiu d'aquest treball va ser estudiar la transmissió de SV40 al medi ambient al nord de la Índia, on cohabiten els hostes naturals del virus (micos *rhesus*) i la població humana. Es van analitzar trenta mostres d'aigua per SV40 i els poliomavirus humans JC i BK (JCPyV i BKPyV); també es van analitzar els adenovirus humans (HAdV) com a indicadors de contaminació vírica humana. Els HAdVs van ser trobats en el 40% de les mostres, JCPyV en el 20% i BKPyV en el 10%. SV40 va ser trobat en una mostra d'origen humà i de mico, indicant que SV40 podria estar circulant en l'ambient, però no hi ha cap evidència de que estigui sent excretat per la població humana.

La seqüència de la regió reguladora de SV40 de la mostra positiva va presentar una organització arquetípica. La seqüència de la regió C-terminal de TAg va presentar un 100% d'homologia amb la soca W17 prèviament aïllada d'una colònia de micos naturalment infectats, al National Institute for Biological Standards and Control, UK (NIBSC).

Aquest és el primer estudi fet sobre la transmissió de SV40 en el medi ambient. Es necessiten més anàlisis per poder confirmar els resultats trobats en aquest estudi.

Resum dels Resultats i la Discussió

Es van analitzar 30 mostres d'aigua superficial i residual per la presència d'adenovirus humans, poliomavirus humans i SV40. El 40% de les mostres van ser

positives per HAdV. D'acord amb estudis previs, aquests virus són freqüentment excretats per la població humana i es poden trobar en aigua amb contaminació fecal humana (Girones i col. 1995; Pina i col. 1998; Puig i col. 1994). Les mostres provinents de zones rurals es van prendre de rius i estanys freqüentats per colònies de macacs salvatges. Aquesta pot ser la raó de la baixa detecció d'adenovirus humans en aquestes mostres. En canvi, la majoria de mostres provinents de zones urbanes (origen humà) són positives.

En estudis fets per Shah i col. (1997) es confirma que JCPyV i BKPyV són excretats a la orina per individus immunocompetents. El poliomavirus JC va ser trobat en sis mostres (20%) i BK en tres d'aquestes mostres (10%). En estudis previs fets en aigua residual d'àrees geogràfiques molt divergents es va trobar el 98% de mostres positives per JCPyV i el 90% per BKPyV (Bofill-Mas i col. 2000). La baixa detecció dels poliomavirus humans en el present treball pot ser degut al factor dilució produït per la pluja i l'aigua superficial no contaminada.

Totes les mostres positives per JCPyV excepte una van ser també positives per HAdV, i totes les mostres positives per BKPyV ho van ser també per JCPyV. Això confirma l'origen humà de la contaminació i recolza la hipòtesi de la dilució que explica les mostres negatives.

SV40 només es va trobar en una mostra d'origen humà i macac de Bengala Occidental, indicant que el virus podria estar circulant en el medi ambient però no hi ha cap evidència de que sigui excretat per la població humana. Els virus presents en la mostra probablement provenien d'una colònia de micos.

Fins a l'actualitat la prevalència d'infeccions de SV40 en humans no es coneix. Les dades de serologia han estat controvertides. Hi ha estudis recents que hipotetitzen que la infecció pot succeir en nens i adults, però s'ha observat contaminació creuada amb BKPyV en els assajos serològics (Viscidi i Clayman, 2006). S'ha detectat SV40 en teixits normals i neoplàsics en persones massa joves o massa velles per haver estat inoculades amb la vacuna contaminada de la polio (Martini i col. 2007). La transmissió zoonòtica també s'ha descrit en persones que treballen en contacte amb micos infectats durant llarg períodes de temps (Engels i col. 2004; Shah 2006).

Els àcids nuclèics amplificats de la mostra positiva per SV40, tant de la regió reguladora com de la regió C-terminal del TAg, van ser clonats. Cinc clons de cada regió

van ser seqüenciats. Totes les seqüències de la regió C-terminal del TAg van presentar identitat amb la soca W17 aïllada prèviament d'una biòpsia de ronyó de mico al *National Institute for Biological Standards and Control*, UK (NIBSC) (Bofill-Mas i col. 2004), indicant que la mateixa soca detectada a l'ambient circula en una colònia de micos *Cynomolgus*. Totes les seqüències de la regió reguladora van presentar estructura arquetípica. Dissortadament, degut a la baixa concentració de virus a la mostra, no es van poder fer més estudis i per tant els resultats no van poder ésser degudament confirmats.

Es van detectar HAdV, JCPyV i BKPyV en mostres d'aigua superficial i residual de diferents localitzacions del nord de la Índia. Els nivells de detecció han estat més baixos del que s'havia esperat probablement degut a la naturalesa de les mostres i la impossibilitat de controlar el procés de mostreig. Aquesta és la primera aproximació a l'estudi de l'excreció i transmissió de SV40 on la població humana està en contacte amb l'hoste natural del virus. SV40 no es va detectar en mostres amb contaminació exclusivament humana, el que suggereix que els humans no presenten un patró d'excreció similar als poliomavirus humans i no representen un potencial contaminant de l'ambient. No obstant, es necessiten més anàlisis per confirmar els resultats trobats en aquest estudi.

Analysis of water samples in northern India to evaluate the presence and potential transmission of SV40 in human populations

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Abstract

Simian virus 40 (SV40) is a monkey virus that was inadvertently administered to human populations through contaminated polio vaccines produced in SV40 naturally infected monkey cells. Although zoonotic transmission is a possibility, the transmission route is still unknown. The aim of this study was to assess the transmission of SV40 in the environment in northern India, where a natural host (rhesus monkeys) of the virus coexists with the human population. Thirty water samples were analyzed for the presence of SV40, the human polyomaviruses JC and BK (JCPyV and BKPyV) and human adenoviruses (HAdV), which were be used as human viral fecal indicators. HAdV was found in 40% of the water samples tested, JCPyV in 20%, and BKPyV in 10%. SV40 was only found in one sample of human and monkey origin , indicating that the virus could circulate in the environment. SV40 was not detected in fecally contaminated water samples of northern India even though a natural host of this virus is highly abundant in the area.

Introduction

The discovery of SV40 was linked to the development and distribution of polio vaccines between 1955 and 1963. These vaccines, inadvertently contaminated with SV40, were distributed worldwide, potentially exposing several million people to the virus (Shah and Nathanson 1976). It was soon shown that vaccinated children shed infectious SV40 in stool at least 5 weeks after vaccination (Melnick *et al.* 1962) and that antibody titles persisted for up to 3 years after inoculation (Gerber, 1967).

The vaccines had been prepared in primary cell culture of Rhesus monkey kidney cells: the Rhesus monkey is a natural host of SV40 (Shah *et al.* 1971). In rhesus macaques, the virus multiplies producing viremia and a high-titered antibody response. The infection appears to be subclinical; the virus establishes a long-term latent chronic infection in the kidneys and is then excreted in urine (Minor *et al.* 2003). The exact transmission pathway of SV40 is not fully understood although several studies hypothesize that the transmission might be produced via the ingestion of urine (Bofill-Mas *et al.* 2004; Minor *et al.* 2003; Shah and Morrison, 1969).

The presence of SV40 in the brain of immunodeficient monkeys indicates that SV40 is neurotropic in addition to being kidney tropic (Lednicky *et al*. 1998). Reports linking SV40 and human tumors have accumulated and the potential role of this virus in human cancer merits serious consideration. Sequences of SV40 have been found, mainly by PCR methods, in different human cancers including mesothelioma, osteosarcoma, and non-Hodgkin's lymphoma. It has also been found in different lymphoproliferative disorders, and in a variety of childhood brain tumors such as ependymoma and choroids plexus tumors, as well as thyroid, pituitary and parotid gland tumors (reviewed in Martini *et al.* 2007).

In previous studies we reported the presence of the human polyomaviruses JC and BK (JCPyV and BKPyV), and also human adenoviruses (HAdV), in sewage samples from Barcelona (Spain), Nancy (France), Patras (Greece), Umeå (Sweden), Washington D.C. (USA), Pretoria (South Africa) and Cairo (Egypt). Almost all the samples were positive, demonstrating the applicability of these viruses in identifying human fecal contamination. However, SV40 was not found in any of the samples (Bofill-Mas *et al.* 2000, 2001).

The viruses JCPyV and BKPyV, classified in the *Polyomaviridae* family, are human viruses closely related to SV40. Both viruses produce latent and chronic infections that persist indefinitely in individuals. JCPyV is excreted regularly in the urine of healthy individuals (Shah, 1995). However, JCPyV is associated with progressive multifocal leukoencephalopathy (PML), a fatal demyelinating disease that accounts for 4% of deaths of AIDS patients (Berger 2007). Infection with BKPyV is associated with diseases of the urinary tract, which include polyomavirus associated nephropathy (de Bruyn and Limaye, 2004). The pathogenicity of these viruses is commonly associated with immunocompromised states; however, JCPyV has attracted more attention due to the immunosuppressive factor associated with AIDS (Berger *et al.* 1987). It is also proposed in some studies that these viruses are linked with human cancers, in particular the role of JCPyV in colon cancer (Riccardiello *et al.* 2000).

The HAdV are grouped in the Mastadenovirus genus in the Adenoviridae family, which contains 51 human serotypes classified in 6 species (A-F). Some serotypes such as 40 and 41 are commonly associated with gastroenteritis. Others included in subspecies B cause respiratory infections and conjunctivitis (Horwitz, 1995).

A method developed to detect HAdV in the environment was used to analyze viral contamination in sewage, river water, seawater, and shellfish (Puig *et al.* 1994). As

HAdVs are more prevalent and stable than enteroviruses, in both the environment and shellfish (Formiga *et al.* 2003; Pina *et al*. 1998,), their specific detection has been suggested as a molecular index for the presence of human fecal contamination in the environment, water, and food (Pina *et al.* 1998; Puig *et al.* 1994).

The aim of this study was to analyze water samples taken from diverse locations in northern India, where a natural host of SV40 coexists in close proximity to the human population. Additionally, this study aimed to assess the potential transmission of SV40 between humans. It was considered that the detection of SV40 in sewage exclusively of human origin would be sufficient evidence that this virus is circulating in the human population. In this study we also describe the presence of JCPyV and BKPyV in sewage and contaminated surface-water ponds from different areas in northern India. HAdV were used as controls to characterize the level of human contamination of the samples.

Materials and Methods

Viruses. For positive controls, the viruses used were SV40 DNA strain 776 (Gibco BRL) and viral particles of the SV40 strain WT 800, kindly donated by Ferran Azorin from the Institute Juan de la Cierva, Consejo Superior de Investigaciones Científicas, Barcelona, Spain. JCPyV and BKPyV were obtained from the urine of a healthy woman in her thirty-eighth week of pregnancy. The viruses were grown in SVG cells, kindly donated by Dr. Eugene O. Major from the Laboratory of Molecular Medicine and Virology, National Institute of Neurological Disorders and Stroke, NIH (U.S.A). The SVG cells were propagated in Eagle's minimal essential medium (EMEM) supplemented with 1% glutamine, 50 μg of gentamicin per mL, and either 5% (growth medium) or 2% (maintenance medium) heat-inactivated FBS. A strain of HAdV2 that was isolated from a clinical sample was grown on A549 cells. These cells were

propagated in Eagle's minimum essential medium (EMEM) supplemented with 1% glutamine, 50 μg of gentamicin per mL, and either 5% (growth medium) or 2% (maintenance medium) heat-inactivated FBS.

Sample collection. For environmental sample collection, a total of 30 water samples from human or human and macaque origin were collected from different locations in northern India. The sampling locations are detailed in Figure 1. The samples were collected between 2003 and 2004 in sterile 500 ml containers and then frozen at –80°C. The samples were shipped from the study areas to our laboratory in dry ice for analysis.

Concentration of viral particles. The water samples were processed according to methods used in previous studies (Puig *et al.* 1994). Briefly, 42-mL samples were ultracentrifuged (110,000 x*g* for 1h at 4°C) to pellet all of the viral particles together with any suspended material. The sediment was then eluted by mixing it with 3.5 mL of 0.25 N glycine buffer (pH 9.5) on ice for 30 min and the suspended solids were separated by centrifugation at 12,000 x*g* for 20 min after the addition of 3.5 mL of 2x PBS. Viruses were finally pelleted by ultracentrifugation (110,000 *xg* for 1h at 4°C), resuspended in 0.1 mL of PBS, and stored at -80 °C.

Nucleic acid extraction. For nucleic acid extraction the viral nucleic acids were extracted by a procedure described by Boom *et al.* (1990). This procedure uses guanidinium thiocyanate and adsorption of the nucleic acids to silica particles.

Enzymatic amplification. For the detection of the specific viral genomes, 10-μL aliquots of the extracted nucleic acids were used in each test.

Nested-PCR amplifications were carried out as previously described for HAdV, JCPyV, BKPyV and SV40 (Allard *et al.* 2001; Bofill-Mas *et al.* 2000, 2001; Maluquer de Motes *et al.* 2004). The regions targeted were the exon for HAdV, C-terminal T-Ag region for JCPyV and BKPyV and both Regulatory Region and C-terminal T-Ag region for SV40. Standard precautions were applied when performing the PCR assays: the PCR mix was prepared in a DNA-free environment, the DNA was added in a separate location, and positive and negative controls were included in each assay. The results were analyzed by agarose gel electrophoresis with ethidium bromide as a stain.

Virus typification. The SV40 amplicons found in the positive samples, which were obtained from the regulatory and the C-terminal T-Ag region, were cloned and five clones from each region were sequenced. The cloning was performed using the pGEM-TEasy cloning kit (Promega, Madison, WI) following the manufacturer's instructions. The clones were transformed in *E. coli* JM109 and amplified by PCR. The amplicons obtained were purified using the QIAquick PCR purification Kit, following the manufacturer's instructions. Both strands of the purified DNA amplicons were sequenced with the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction V 3.1 kit with AmpliTaq DNA polymerase FS (Applied Biosystems), following the manufacturer's instructions. The results were checked using the ABI PRISM 3700 DNA analyzer (Applied Biosystems). The sequences were compared with those present in GenBank and in the European Molecular Biology Library using the basic BLAST program of the National Center for Biotechnology Information (available from URL: http://www.ncbi.nlm.nih.gov/BLAST/).

Results and Discussion

Thirty samples of waste water and ground water were analyzed to test for human adenoviruses, human polyomaviruses and SV40. The results are shown in Table 1. HAdV was found in approximately 40% of the samples. According to previous studies (Girones *et al.* 1995; Pina *et al.* 1998; Puig *et al.* 1994) these viruses are frequently excreted by humans and are found in water with human fecal contamination. The samples from rural zones were taken from rivers, lakes and ponds frequented by macaque colonies, which may explain the low detection of HAdV in these samples since rural areas have a much lower level of human contamination; HAdV was found in most of the samples from urban areas (human origin) and the occurrence in urban areas was higher than in rural areas.

JCPyV was found in six samples (20%) and BKPyV was found in three of those samples (10%). High proportion of positive samples were obtained in raw sewage samples in widely divergent geographical areas in previous studies (Bofill-Mas *et al.* 2000) In these samples, 98% tested positive for the presence of JCPyV and 90% for BKPyV. The low prevalence of the human polyomaviruses observed in this study may be explained by the abundance of samples taken from superficial water bodies. Although these water bodies cannot be considered sewage, fecal contamination was detected. All JCPyV-positive samples, except one, were also positive for HAdV and all BKPyV-positive samples were also positive for JCPyV. This confirms the human origin of the contamination in the positive samples and supports the dilution hypothesis that explains the negative samples. The three BKPyV-positive samples, taken from urban sewage in large cities, were the most contaminated.

SV40 was only found in one sample from west Bengal, where human and macaque contamination was expected. This indicates that SV40 may be circulating in the

environment but there is no evidence that it is being excreted by the human population. The viruses present in this sample are likely to come from a wild macaque colony present in the area.

To date, the prevalence of SV40 infections in humans is unknown and serological data is conflicting. Recent studies hypothesize that the infection occurs in children and adults; however, cross-contamination with BK in serological assays has also been observed (Viscidi and Clayman, 2006). In some cases, SV40 DNA has been detected in normal and neoplasic tissues in people that are either too young or too old to have been vaccinated with the contaminated polio vaccine. SV40 was also found in the urine and stool of such patients, indicating that haematic, sexual, and orofecal transmission could explain the spread of SV40 by horizontal infection in humans (Martini *et al.* 2007). Additionally, zoonotic transmission has been reported in people who work closely and for long periods of time with infected monkeys (Engels *et al.* 2004; Shah, 1966).

The occurrence of SV40 antibodies was reported in only a small portion of the wild juvenile macaques, however, it was found in nearly all adults (Jones-Engel *et al.* 2006; Meyers *et al.* 1962; Shah, 1965). Previous studies confirm that SV40 is excreted in urine (Minor *et al.* 2003; Shah and Morrison, 1969). In previous studies, our researchers have reported the occurrence of SV40 in environmental samples taken from the cages of naturally infected *cynomolgus* monkeys (Bofill-Mas *et al.* 2004).

SV40 nucleic acids amplified from the regulatory region and the C-terminal T-Ag region from the positive sample were cloned. Five clones from each region were sequenced. All the clones from the C-terminal T-Ag region contained 100% homology with the SV40 strain W17, which was isolated at the National Institute for Biological Standards and Control, UK (NIBSC) from a monkey kidney biopsy (Bofill-Mas *et al.* 2004). This result indicates that the same strain detected in the environment samples,

circulates among *Cynomolgus* monkeys. All the clones of the regulatory region had an archetypal structure. Unfortunately, due to the low concentration of viruses in the sample, further studies could not be performed to appropriately characterize the viruses.

In environmental water samples from different locations in northern India both HAdV and the human polyomaviruses JC and BK were detected. The levels of detection were lower than expected, which was probably due to the nature of the samples and the difficulty in controlling the sampling process. This research was the first of its kind to study the excretion and potential transmission of SV40 between humans in an area where the human population coexists in close proximity to a natural host of the virus. SV40 was not found in samples with human contamination exclusively, wich suggests that the huamsn don't present an excretion pattern similar to the human polyomavirus pattern and that SV40 does not represent a potential environmental contaminant. However, further analysis is required to expand the study and confirm the results.

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Table 1. Nested-PCR results in environmental water samples from different locations in northern India.

Location	Sample #	Origin	HAdV (%)	JCPyV (%)	BKPyV (%)	SV40 (%)
Calcutta	7	Human	3 (43%)	1 (14%)	0	0
West Bengal	4	Human + Macaque	2 (50%)	0	0	1 (25%)
Siligury	2	Human	2 (100%)	0	0	0
Tripura	5	Human + Macaque	0	0	0	0
Varanasi	1	Human + Macaque	0	1 (100%)	0	0
Assam	2	Human	0	0	0	0
Lucknow	1	Human + Macaque	1 (100%)	0	0	0
Lucknow	2	Human	1 (50%)	1 (50%)	0	0
New Delhi	3	Human	1 (33%)	1 (33%)	1(33%)	0
Kanpur	3	Human	2 (66%)	2 (66%)	2 (66%)	0
Total Human	19		9 (48%)	5 (27%)	3 (16%)	0
Total H+M	11		3 (28%)	1 (9%)	0	1 (9%)
Total	30		12 (40%)	6 (20%)	3 (10%)	1 (3%)

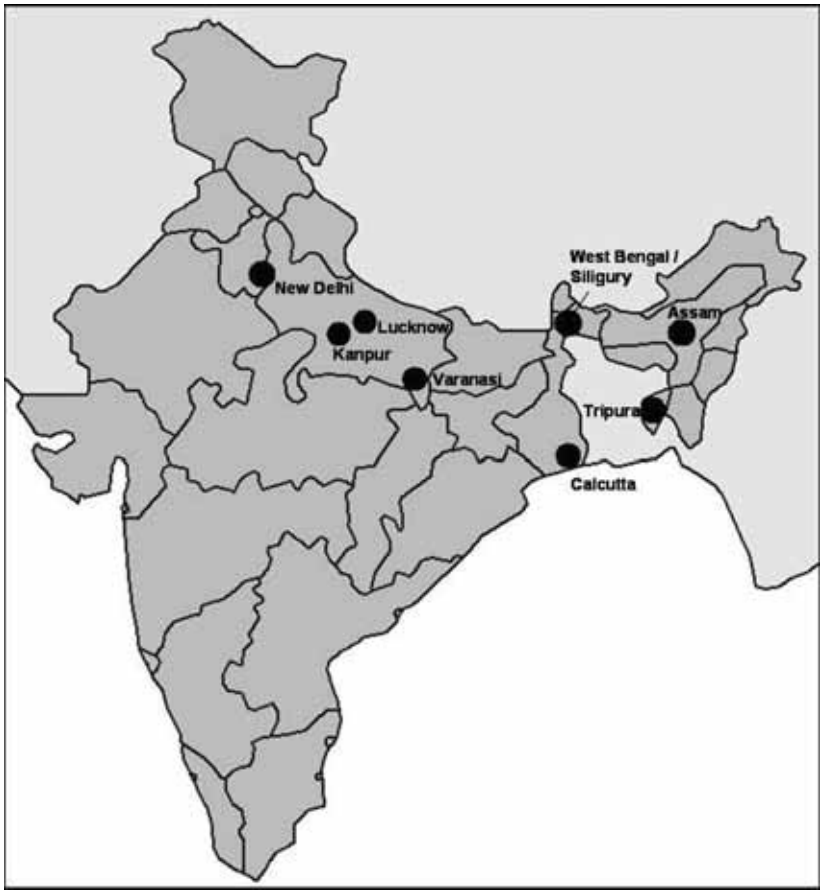

fig. 1. Map of India indicating the sampling points.

CAPÍTOL II: DISTRIBUCIÓ DELS POLIOMAVIRUS I ADENOVIRUS HUMANS EN L'AMBIENT I EN UNA PLANTA POTABILITZADORA.

Distribució de poliomavirus humans, adenovirus i virus de l'hepatitis E a l'ambient i a una planta potabilitzadora d'aigua.

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Resum

Grans quantitats de virus són excretats en les femtes i orina humanes. Aquests virus fins i tot a molt baixes concentracions poden causar malalties quan són ingerits. Alguns d'aquests virus tradicionalment no han estat monitoritzats en termes de malalties transmeses per l'aigua i són considerats patògens emergents, com el virus de l'hepatitis E (HEV) i els poliomavirus JC i BK (JCPyV i BKPyV). La gran prevalència dels adenovirus humans (HAdV) i els poliomavirus, ambdós amb genomes de DNA, en aigua residual d'àrees geogràfiques molt diverses ha suggerit la rellevància d'avaluar aquests virus com a possibles indicadors de contaminació vírica.

Utilitzant tècniques de concentració basades en l'aplicació de filtres electropositius es va fer una primera aproximació a la quantificació de virus en aigua residual, aigua de riu i després del tractament en una planta potabilitzadora que inclou cloració, floculació, ozonització i filtració per carbó activat (GAC). Les mostres d'aigua filtrada per GAC es van recollir abans de la cloració final.

El riu usat com a font d'aigua va presentar una concentració mitja de $2{,}6x10^1$ GC (còpies genòmiques) de JCPyV i $4x10^2$ GC HAdV per litre. Es va observar una eliminació de dos logaritmes (99%) d'HAdV i JCPyV en la planta de tractament d'aigua de beguda. Totes les mostres d'aigua filtrada per GAC contenies HAdV, amb una mitja de 4,3 GC/L. Es van detectar freqüentment soques d'HEV pertanyents al genotip 3 en baixes concentracions en aigua residual urbana i en biosòlids o aigua residual que contenia femta de porcs, però no en les mostres d'aigua de riu estudiades.

La detecció de virus per tècniques moleculars és útil per descriure genèticament els virus emergents en aigua residual i aigua potable de les comunitats. La quantificació de JCPyV i HAdV usant la PCR quantitativa a temps real (qPCR) pot ser útil per avaluar l'eficiència d'eliminació de virus en plantes de tractament d'aigua de

beguda i com a índex de la qualitat virològica de l'aigua i la potencial presència de virus humans.

Resum dels Resultats i la Discussió

En aquest treball es va estudiar la presència d'adenovirus humans, patògens emergents com els poliomavirus humans JC i BK i el virus de l'hepatitis E en aigua superficial i avaluar la seva eliminació en una planta de tractament d'aigües de beguda. Com a indicadors de contaminació fecal animal es van usar els adenovirus porcins.

Es van analitzar mostres d'aigua del riu Ter, aigua del riu Llobregat i aigua filtrada per carbó activat (en el punt previ a la cloració final dins la planta de tractament); també en aquesta planta es van analitzar mostres de fangs de sedimentació. Es van analitzar a més mostres d'aigua residual urbana i mostres de biosòlids procedents de plantes depuradores d'aigua residual d'escorxadors.

La metodologia usada per analitzar mostres d'aigua residual urbana va ser un mètode basat en la ultracentrifugació prèviament descrit pel nostre grup d'investigació (Puig i col. 1994). La metodologia emprada per analitzar mostres d'aigua va ser una combinació de dos mètodes, un basat en l'adsorció dels virus en filtres electropositius (*Manual Methods for virology*, EPA) i a continuació un altre basat en ultracentrifugació i elució en glicina (Puig i col. 1994). La metodologia emprada per concentrar virus a partir de llots de sedimentació i biosòlids d'escorxador també va ser una combinació del mètode EPA (*Manual Methods for virology*, EPA) basat en floculació i a continuació ultracentrifugació (Puig i col. 1994). Els HAdV i els JCPyV van ser quantificats mitjançant la PCR quantitativa a temps real, mentre que els pAdV, BKPyV i HEV van ser analitzats per PCR niada.

L'eficiència de recuperació dels mètodes de concentració va ser avaluada dopant mostres de 50L d'aigua, 100ml de llots de sedimentació de la planta de tractament d'aigua de beguda i 50ml d'aigua residual, amb una quantitat coneguda d'HAdV, de JCPyV i de BKPyV.

Les eficiències de recuperació de virus en aigua van ser de 2-25% per HAdV i de 0,15-0,16% per JCPyV depenent del tipus de mostra. L'eficiència de recuperació dels dos virus en llots de sedimentació va ser similar, però en ambdós casos inferior a l'1%.

L'eficiència de recuperació de BKPyV es va analitzar per dilució límit amb PCR niada i va donar un valor estimat del 2%. El mètode de concentració de virus a partir d'aigua residual va presentar una eficiència de 73% per JCPyV i de 43% per HAdV.

Els nivells trobats de HAdV, JCPyV i BKPyV en aigua residual van ser alts ($1.4x10^7$ GC/L, $2,6x10^6$ GC/L i $1,5x10^6$ GC/L respectivament) i 5 logaritmes més baixos en aigua de riu ($4x10^2$, $2,6x10^1$, $2x10^1$ GC/L respectivament). Tot i això s'ha de tenir en compte que l'eficiència del mètode de concentració usat per aigua de riu és molt menor i per tant estem subestimant la quantificació.

La reducció de la concentració d'HAdV i JCPyV en la planta de tractament d'aigua de beguda va ser de dos logaritmes (99%) fins al punt previ a la cloració final. Les dades van mostrar una major acumulació de virus en els llots de sedimentació. Totes les mostres d'aigua filtrada per GAC van ser positives per HAdV amb una concentració mitja de 4,5 GC/L i el 56% van ser positives per JCPyV amb una concertació mitja d'1,63 GC/L.

BKPyV va ser trobat en totes les mostres d'aigua residual i en el 66% de mostres d'aigua de riu (amb una concentració estimada de 20 equivalents de PCR/L). Cap mostra de llots de sedimentació ni d'aigua filtrada per GAC va ser positiva.

HEV es va trobar en les mostres d'aigua residual i en una mostra de biosòlids d'escorxador, però no es va detectar en aigua de riu o filtrada per GAC ni en els llots. Els adenovirus porcins van ser analitzats en les mostres de biosòlids d'escorxadors i en les mostres d'aigua del riu Ter, ja que és una zona amb molta explotació porcina. Totes les mostres d'aigua i 3 de 4 mostres d'escorxadors van ser positives.

Es van tipificar els adenovirus humans detectats en mostres d'aigua del riu Llobregat i en mostres d'aigua filtrada per GAC. En aigua de riu es va trobar HAdV 41, comunament associat a diarrea aguda en nens, i una seqüència amb el 94% d'homologia amb HAdV 40. En aigua tractada es va trobar HAdV 41, 40 i una seqüència amb un 96% d'homologia amb HAdV B. Es van seqüenciar també els HEV trobats. Les tres seqüències obtingudes van presentar alta homologia amb altres aïllades en previs estudis a Barcelona.

En aquest estudi hem analitzat i quantificat la presència d'adenovirus humans, JCPyV, BKPyV i HEV en mostres ambientals i hem fet una avaluació preliminar de

l'eficiència d'eliminació d'aquests virus en una planta de tractament d'aigua de beguda. Els mètodes aplicats per a la concentració de virus han presentat una eficiència baixa i molt variable, indicant-nos que la concentració dels virus estudiats a les mostres podria ésser molt més alta. S'ha trobat una diferència d'un logaritme entre les concentracions d'HAdV i JCPyV tant a les mostres d'aigua com a les mostres d'aigua residual. Aquesta diferència ja s'havia observat en estudis anteriors usant PCR niada (Bofill-Mas i col. 2000).

A la planta de tractament d'aigües de beguda, l'eficiència d'eliminació dels virus estudiats entre la entrada i el punt previ a la cloració final s'ha determinat en un 99%. Tot i això, assumint la baixa eficiència del mètode de recuperació, encara trobem virus a l'aigua preclorada, que per altra banda presenta absència d'indicadors microbians. Això podria representar un risc per la salut pública si la desinfecció secundària no funciona correctament. S'haurien de fer estudis epidemiològics i de "risk assesment" per tal de definir els valors acceptables de paràmetres vírics que haurien de ser usats pel control microbiològic de l'aigua de beguda.

Els adenovirus humans s'acumulen als llots de sedimentació. Amb l'aplicació d'aquests llots en l'agricultura, els virus podrien ser disseminats i representar una font difusa de contaminació vírica a l'ambient. HEV va ser detectat en aigua residual, confirmant els resultats d'estudis previs que indiquen que aquest virus és persistentment excretat en baixes concentracions al medi ambient per la població d'àrees industrialitzades (Clemente-Casares col. 2007).

El procediment desenvolupat per la concentració i detecció o quantificació de virus va presentar un nivell satisfactori d'especificitat. Encara que els mètodes de concentració necessiten millores i la sensibilitat està limitada per una eficiència baixa i molt variable s'ha de destacar la presència estable d'adenovirus humans i JCPyV en aigua de riu i, en més baixes concentracions, en aigua tractada. La quantificació de JCPyV i d'HAdV per qPCR ha demostrat ser una bona tècnica per avaluar l'eficiència d'eliminació de virus en plantes de tractament d'aigua, com a índex de la qualitat virològica de l'aigua i de la presència de virus humans.

Distribution of human polyomaviruses, adenoviruses and Hepatitis E virus in the environment and in a drinking-water treatment plant

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Abstract

Large numbers of viruses are excreted in human and urine, which even at low concentrations may cause illness when ingested. Some of these viruses are considered emergent viruses, such as Hepatitis E virus (HEV), JC polyomavirus (JCPyV) and BK polyomavirus (BKPyV). The high prevalence of adenoviruses and polyomaviruses, which both show DNA genomes, in sewage from widely divergent areas has suggested the relevance of evaluating these viruses as possible indicators of viral contamination. The concentration of these viruses was analyzed in sewage and river water and after the treatment in a drinking-water treatment plant with chlorination, flocculation, ozonation and granulate active carbon (GAC) filters. Samples of GAC-filtered water were collected before a second chlorination treatment. The river used as a source of fresh water has an average concentration of $3.3x10^1$ JCPyV and $6.1x10^2$ HAdV GC (genome copies)/l. A removal of 2 logarithms (99%) of the viruses was observed in the drinking-water treatment plant for HAdV and JCPyV. All the GAC-filtered water samples studied contained adenoviruses, with a mean value of 4.3 HAdV GC/l. HEV strains belonging to genotype 3 have been frequently detected in low concentrations in urban sewage and in biosolid or sewage containing swine feces, but not in the river water samples studied.

The quantification of JCPyV and HAdV by real-time PCR may be a useful tool for the evaluation of virus removal efficiency in water treatment plants and as an index of the virological quality of water and the potential presence of human viruses.

Introduction

Among the viruses infecting humans, many different types are excreted in high concentrations in the feces of patients with gastroenteritis or hepatitis, and in lower concentrations in the feces or urine of patients with other viral diseases (1). Moreover, viruses are also present in healthy individuals, and thus high viral loads are detected in urban sewage and are regarded as environmental contaminants (2). Some viruses, such as the human polyomaviruses and some adenovirus strains, infect humans during childhood, thereby establishing persistent infections. In the case of many frequent adenoviral respiratory infections, viral particles may continue to be excreted in feces for months or even years afterwards (3).

There is available information about some water-borne pathogens but the improvement in molecular technology for detecting viruses present in water has focused attention on new groups of viruses that could be considered emergent viruses in diverse geographical areas. Technical advances are then most readily associated with the concept of emergent microorganisms, which are defined as newly identified microorganisms, those already existent but characterized by a rapidly increasing incidence and/or geographical ambit, and those for which transmission through food or water has only recently been discovered (4). Several studies have confirmed that infectious diseases related to water are not only a primordial cause of mortality and morbidity worldwide, but also that both the spectrum and incidence of many diseases related to water are increasing (5). Human polyomaviruses, Hepatitis E virus (HEV) and adenoviruses are three groups of viruses which are being detected more often in the environment (2, 6, 7)

JC virus and BK virus are human viruses classified in the *Polyomaviridae* family and have a circular dsDNA genome. Both viruses produce latent infections that persist

indefinitely in individuals and are excreted regularly in urine by healthy individuals (8). JCPyV is associated with progressive multifocal leukoencephalopathy (PML), a fatal demyelinating disease that accounts for 4% of deaths of AIDS patients (9). Infection with BKPyV is associated with diseases of the urinary tract including polyomavirus-associated nephropathy (10). The pathogenicity of these viruses is commonly associated with immunocompromised states, but has attracted more attention due to their AIDS-linked immunosuppression (9). The role of these viruses in human cancer has also been suggested; in particular JCPyV is associated with colon cancer (11).

Previous studies describe the presence of human polyomaviruses, JCPyV and BKPyV in urban sewage of Barcelona (Spain), Nancy (France), Umeå (Sweden), Patras (Greece), Washington D.C. (USA), Pretoria (South Africa) and Cairo (Egypt). Only one of 52 samples proved negative for JCPyV (collected in Barcelona in 1997) in 4 ml of urban sewage analyzed (98% of positive samples), while 90% were positive for BKPyV (6). Moreover, these same samples were positive for human adenoviruses, thereby demonstrating the applicability of molecular assays for human adenoviruses and polyomaviruses in identifying human fecal contamination in widely divergent geographical areas (12).

Hepatitis E virus is a non-enveloped, positive-sense ssRNA, classified in the *Hepeviridae* family (13). Infection by it is a major cause of epidemic and sporadic acute hepatitis in many areas of Asia, Africa and Mexico (14, 15), where it is considered endemic. The disease is self-liming but sometimes has severe complications and a high case-fatality rate, particularly in pregnant women (approximately 20%) (16). North America and Europe have traditionally been considered non-endemic for HEV but recent studies have demonstrated that autochthonous strains circulate among the population. A previous study showed that in Barcelona 43.5% of sewage samples tested

had detectable levels of HEV (7). Infection by different animal HEV strains in swine, wild boar, deer (animals suggested as potential reservoirs) and in chicken has also been shown (17, 18, 19). There is, however, still very little information regarding the presence and distribution of polyomaviruses and HEV in surface water, their resistance to the drinking-water and depuration treatments or their stability in the environment.

Human adenoviruses have linear dsDNA genomes and are grouped in the *Mastadenovirus* genus in the *Adenoviridae* family which contains 51 human serotypes, classified in 6 species (A-F). Some serotypes such as 40 and 41 are commonly associated with gastroenteritis. Others included in subspecies B are responsible for respiratory infections and conjunctivitis (20). In a previous study, a method for the detection of HAdV in environmental samples was developed and used to analyze viral contamination in sewage, river water, seawater and shellfish (21). As HAdVs are more prevalent and stable in the environment and in shellfish than enteroviruses (22), their specific detection has been suggested as a molecular index for the presence of human fecal contamination in the environment, water and food (2).

The general demand for high-quality water has increased the pressure on environmental and public health policies to ensure the microbiological safety of water. Classic microbiologic indicators such as fecal coliform bacteria were established as safety standards to evaluate the removal of fecal contamination in water purification processes although it has been demonstrated that these indicators fail to predict the presence of viruses and protozoans (22, 23, 24, 25).

The aim of this study was to analyze by applying a quantitative PCR procedure the presence of adenoviruses and emergent pathogens such as HEV and human polyomaviruses in source water and their removal in a drinking-water treatment plant. The secondary objective of the study was to evaluate the possible role of adenoviruses

and polyomaviruses as a tool for the control of the viral quality of source and treated water.

Materials and Methods

Viruses. The strain of HAdV2 isolated from a clinical sample was grown on A549 cells propagated in Eagle's minimal essential medium (EMEM) supplemented with 1% glutamine, 50 μg of gentamicin per ml and 5% (growth medium) or 2% (maintenance medium) of heat-inactivated FBS. JCPyV and BKPyV were obtained from the urine of a healthy woman at the 38th week of pregnancy and the viruses were grown in SVG cells kindly donated by Dr. Eugene O. Major from the Laboratory of Molecular Medicine and Virology, National Institute of Neurological disorders and Stroke, NIH (USA). SVG cells were propagated in Eagle's minimal essential medium (EMEM) supplemented with 1% glutamine, 50 μg of gentamicin per ml and 5% (growth medium) or 2% (maintenance medium) of heat-inactivated FBS. Positive control for pAdV was obtained by PCR amplification of a positive swine fecal sample. The amplicon was cloned in pGEM-T Easy vector (Promega, Madison, WI, USA) following manufacturer's instructions and modified by insertion of an extra 8nt sequence. Fecal suspensions obtained from monkeys (*Macaca mulatta*) infected with Hepatitis E virus BCN strain (10% in PBS) were used as positive control for the PCR analysis.

Sample collection. (i) Water samples. Twenty-three water samples (9 from Llobregat river at the drinking-water treatment plant entry point; 9 from GAC-filtered water and 5 from Ter river) were collected and filtered (Zeta Plus MK electropositive filters, AMF Corp., Cuno Division, Meriden Conn., USA) at a rate of 1 l/min. over a period from November 2004 to May 2006. All the filters were kept at 4ºC in sterile containers and

processed within 24 hours of collection. Nine samples of 250 l of water were filtered from the Llobregat River as source water. These samples were analyzed for the presence of HAdV, JCPyV (Real-time PCR), BKPyV and HEV (Nested PCR). Nine samples of 1000 l were filtered after the granular-activated carbon (GAC) filtration step, at the point previous to the final chlorination. The samples were analyzed for the presence of HAdV, JCPyV (Real-time PCR) and BKPyV (Nested PCR). A second river water sampling site was studied; 5 samples (100 l each) were collected and filtered from a river close to an important farming area in Catalonia between December 2004 and May 2006. The Ter River sampling location was selected because of its proximity to animal farms and the probability of its containing contamination from the waste produced by swni on these farms with and from the diffuse contamination caused by biosolids applied to soils in agriculture. The samples were analyzed for the presence of HAdV and JCPyV (Real-time PCR), HEV and for porcine adenoviruses (pAdV) as a possible indicator of animal fecal contamination (26).

(ii) Sludge samples from a drinking-water treatment plant. Five samples of 100 ml of sludge were collected at the drinking-water treatment plant. Sludge samples consisted of a mix of sedimentation sludge and washing water from sand and GAC filters. Samples were kept at 4°C and processed within 24 hours of collection. These samples were analyzed for the presence of HAdV, JCPyV (Real-time PCR), BKPyV and HEV (Nested PCR).

(iii) Biosolid samples from slaughterhouse. Four biosolid samples were collected from a slaughterhouse in Catalonia (Spain). This slaughterhouse processed mainly pigs (>80%) whose biosolid might accumulate HEV strains. The samples were collected after physicochemical flocculation. Samples were kept at 4°C and processed within 24 hours of collection. They were analyzed for the presence of HEV and pAdV.

(iv) Sewage samples. Five sewage water samples (50 ml each) were collected at the inffluent of the wastewater treatment plant 10 Km south of Barcelona. Samples were kept at 4°C and processed within 24 hours of collection. These samples were analyzed for the presence of HAdV, JCPyV (Real-time PCR), BKPyV and HEV (Nested PCR).

Drinking-water treatment plant. The plant is situated in the area of Barcelona, next to the Llobregat River used as source water and has a treatment capacity of $4.5x10^8$ l/day. The raw water is breakpoint pre-chlorinated, and polyelectrolytes and aluminum sulphate are added to the water which is conducted to the sedimentation tanks for flocculation. After sedimentation, the water goes through sand filters, ozonation and GAC filters. Finally, the water is stored in tanks in which it is post-chlorinated. The sludge treatment plant collects particulate material from the sedimentation tanks and the water used for the periodical washing of the filters.

Concentration of virus from water. Recovery of viral particles from sewage was carried out as described elsewhere (21). Briefly, 42-ml sewage samples were ultracentrifuged (110,000 *xg* for 1 h at 4°C) to pellet all of the viral particles together with any suspended material. The sediment was then eluted by mixing it with 3.5 ml of 0.25 N glycine buffer (pH 9.5) on ice for 30 min, and the suspended solids were separated by centrifugation at 12,000 *xg* for 15 min after the addition of 3.5 ml of 2x PBS. Viruses were finally pelleted by ultracentrifugation (110,000 *xg* for 1 h at 4°C), resuspended in 0.1ml of PBS, and stored at -80°C.

The protocol used for recovering viral particles from water samples is a combination of an EPA method (27) with minor modifications and the method based on ultracentrifugation and elution in glycine buffer 0.25 N pH 9.5 developed in previous studies by our research team (21).

Samples were filtered through Zeta Plus MK electropositive filters (AMF Corp., Cuno Division, Meriden Conn., USA), at 1 liter/min in a Millipore peristaltic pump. Viruses were eluted in 900 ml glycine buffer (0.25 N pH 9.5 1% beef extract) (Becton, Dickinson & Co., Sparks MD, USA) by reverse flow using a Millipore peristaltic pump at 400 ml/min for 45 min. For the flocculation, 3% beef extract was added, the pH was adjusted to 3.5 by HCl 5M and was magnetically stirred for 30 min. The sample was centrifuged at 12,800x*g* for 25 min at 4ºC and the pellet was then eluted in 42 ml of PBS. From this point, the sample was treated under the protocol designed for wastewater samples. In this case the pellet was finally eluted in 200 µl PBS and stored at -80°C until nucleic acid extraction. The DNA extracted from 10 µl of the viral concentrate was equivalent to 12.5 l of Llobregat River water, 50 l of GAC-filtered water and 5 l of Ter River water.

Concentration of virus from sludge/ biosolids. The method used for the concentration of viral particles from the sludge collected in the drinking-water treatment plant is a combination of an EPA method (27) and the method based on ultracentrifugation and elution in glycine buffer previously developed by our research group (21). To bind the viral particles to the suspended solids, 1 ml of 0.05M $AlCl_3$ was added, the pH was adjusted to 3.5 with HCl 5M and was stirred for 30 min. with adjustment of the pH whenever necessary. The sample was centrifuged at 5,400x*g* for 15 min at 4ºC and the supernatant was removed. The pellet was eluted in 42 ml glycine buffer pH 9.5 and stirred for 1h. The pH was neutralized with HCl 5M and the sample was centrifuged for 45 min at 38,400*xg* at 4ºC. The supernatant was ultracentrifuged at 110,000x*g* for 1h at 4ºC. The pellet was finally eluted in 200 µl 1x PBS and stored at -80°C until nucleic acid extraction. The DNA extracted from 10 µl of the viral concentrate was equivalent to 5 ml of sludge.

Four biosolid samples were collected in the slaughterhouse; three of them had high water content and were processed following the protocol used for sewage water. One biosolid sample, with less water, was processed following the same protocol but beginning at the elution step with the elution of 1 g of biosolid with 3.5 ml of 0.25 N glycine buffer (pH 9.5). The viral particles were finally eluted with 0.1 ml PBS. The DNA extracted from 10 µl of the viral concentrate was equivalent to 4 and 0.4 ml of slaughterhouse diluted biosolid and 100 and 10 mg of slaughterhouse concentrated biosolid.

Nucleic acid extraction. Viral nucleic acids were extracted by a procedure described by Boom et al. (28). This procedure uses guanidinium thiocyanate and adsorption of the nucleic acids to silica particles. To reduce the presence of PCR inhibitors, a 10-fold dilution of every extraction was also assayed.

Enzymatic amplification. (i) Nested PCR. Nested PCR was used for the specific detection of BKPyV, pAdV and for typification of HAdV-positive samples. Ten-µl aliquots of the extracted nucleic acid and their respective tenfold dilution were used in each test. Amplifications were carried out as described in previous studies using two amplification rounds with 30 cycles each (12, 26, 29). Standard precautions were applied when performing these assays: the PCR mix was prepared in DNA-free environment, the samples were added at a separate location and positive and negative controls were included in each assay.

(ii) Semi-nested RT-PCR. The presence of genomic RNA of HEV was analyzed by semi-nested RT-PCR assay to amplify a fragment within the ORF2 of the genome using the degenerated primers described by Erker et al. (30) and the OneStep RT-PCR kit (QIAGEN GmbH, Inc., Hilden, Germany). Five µl of the extracted nucleic acids or tenfold dilution were tested by RT-PCR in a total volume reaction of 50 µl containing 1

x OneStep QIAGEN Buffer, 2µl of QIAGEN OneStep Enzyme Mix, 400µM of each dNTP, 10 units of ribonuclease inhibitor (Applied Biosystems, Foster City, CA, USA) and 25 pmols of each outer primer (HEVORF2con-a1 and HEVRF2con-s1). After 30 min at 50ºC, the reaction was heated at 95ºC for 15 min, followed by 35 cycles at 94ºC for 20 s, annealing at 55ºC for 30 s, and extension at 72ºC for 20 s. All amplifications were completed with a 10 min, 72ºC extension period. The second-round of amplification was performed with the same conditions described elsewhere (30). Standard precautions were applied when performing these assays. The PCR mix was prepared in DNA-free environment, the samples were added in a separate location and positive and negative controls were added in each assay.

(iii) Real-time quantitative PCR. For the specific detection and quantification of JCPyV and HAdV genomes, 10 µl of the ten-fold and hundred-fold dilutions of every DNA extraction were also assayed; these dilutions were made to avoid amplification inhibition due to the high sensitivity to inhibitors of this assay.

Amplification was performed in a 25-µl reaction mixture with the PCR Master Mix (Applied Biosystems). The reaction contained 10 µl of DNA sample or 10 µl of quantified plasmid DNA, 0.95X TaqMan master mix and the corresponding primers and TaqMan probes at their corresponding concentrations. JCPyV genomes were quantified with 0.5 µM of the primers JE3F and JE3R and 0.15 µM of the fluorogenic probe JE3P described in Pal et al. (31). HAdV genomes were quantified with 0.9 µM of the primers AdF and AdR and 0.225 µM of the AdP1 probe described by Hernroth et al. (32).

Following activation of the Uracil N-glycosilase contained in the core mix (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 with an ABI 7700 sequence detector system (Applied Biosystems). The principle of real-time PCR has been described elsewhere (33).

All samples were run in quadruplicate, and positive and negative controls were included. The amount of DNA was defined as the average of the quadruplicate data obtained.

Standard controls for Real-time PCR. For the generation of standards to use in the Real-Time PCR assays two plasmid constructions were employed: pJCPyV, containing the whole JCPyV genome strain Mad-1 in pBR322 kindly donated by Andrew M. Lewis of the Office of Vaccine Research and Review, CBER, FDA, (USA); pAd41, containing the hexon region of HAdV 41 in pBR322 kindly donated by Dr. Annika Allard of the University of Umeå (Sweden).

E. coli JM109 cells (Promega, Madison, WI, USA) were transformed with the plasmid (pJCPyV or PAdV41). The plasmids were purified from the bacteria using the Plasmid Midi Kit (QIAGEN GmbH, Inc., Hilden, Germany) following the manufacturer's instructions and the DNA obtained was quantified with a Genequant pro (Amersham Biosciences). To reduce the possibility of DNA contamination in the laboratories, the plasmids were linearized with EcoRI (pJCPyV) or NruI (pAd41) (Promega, Madison, WI, USA). The reaction product was purified, quantified again and dilutions 10^{-3} to 10^{7} viral DNA molecules per 10µl were made in TE buffer. The standard dilutions were then aliquoted and stored at –80°C until use.

Efficiency of the methods for the recovery of viruses. To evaluate the efficiency of virus recovery, three samples (50 l each) of river water, three samples (50 l each) of treated water and three samples (100 ml each) of sludge were collected and seeded with HAdV 2, JCPyV and BKPyV. HEV cannot be cultured in cell culture and due to the low quantity of HEV available, seeding with this virus was not possible. Three samples (42 ml each) of sewage water were also collected and seeded with JCPyV and HAdV 2. The water samples were filtered through electropositive filters with a Millipore

peristaltic pump at a flow of approximately 1 liter/min. Both the filters as well as the processing of the sludge and sewage samples are described above. Virus stocks were DNAse I (GE Healthcare UK Ltd., Buckinghamshire, England) treated to eliminate any free DNA. The reactions where performed at a DNAse concentration of 0.2 U/µl following manufacturer's instructions and processed with the samples as described above.

Typification of viruses. In the samples susceptible of containing more viral variability, such as river water and biosolids, the amplicons obtained in a nested-PCR assay were cloned in order to evaluate the potential variants of strains present and 3 clones were analyzed and sequenced. Amplicons obtained from samples with lower concentrations of viruses and expected to present less variability were sequenced directly without cloning. The HAdV amplicons obtained by Nested-PCR from river water samples, and the HEV obtained from the slaughterhouse biosolid were cloned using the pGEM-T Easy cloning kit (Promega, Madison, WI, USA) following manufacturer's instructions, the clones transformed in *E.coli* JM109 and amplified by PCR. HAdV and HEV amplicons from the clones were purified using the QIAquick PCR purification Kit, following the manufacturer's instructions. PAdV detected in river water and HAdV detected in the GAC-filtered water samples were also purified using QIAquick PCR purification Kit. Both strands of the purified DNA amplicons were sequenced with the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction V 3.1 kit with Ampli Taq DNA polymerase FS (Applied Biosystems), following the manufacturer's instructions. The results were checked using the ABI PRISM 3700 DNA analyzer (Applied Biosystems) and the sequences were compared with those present in GenBank and the European Molecular Biology Library by means of the basic BLAST program of

the National Center for Biotechnology Information (available from: URL: http://www.ncbi.nlm.nih.gov/BLAST/).

The HEV sequences reported in this study have been deposited in GenBank under the following accession numbers: DQ400352 for BCN21, DQ400353 for BCN22, DQ400354 for BCN23, DQ400355 for BCN24 and DQ400356 for E5.

Results

Efficiency in the recovery of viruses. The efficiencies of the procedures used for the concentration and recovery of viruses used in the tested samples are shown in Table 1. The results indicate that the range of viral particle recovery in river and treated water varies between 0.15% and 0.16% for JCPyV and between 1% and 12% for HAdV. The recovery efficiency for both viruses from sludge was similar although it was under 1%. The recovery efficiency of BKPyV was analyzed with end-point dilution nested-PCR and was estimated around 2%. The procedure for virus recovery from sewage based in ultracentrifugation showed an efficiency of 73% for JCPyV and 43% for HAdV.

Quantification of viruses in sewage, water and sludge. The concentration of viruses detected is expressed as genome copies (GC) and the results are shown in Table 2. The levels of HAdV, JCPyV and BKPyV are high (average of $1.4x10^7$ GC/l, $2.6x10^6$ GC/l and $1.5x10^6$ respectively) in sewage samples and about 5 logs lower in river water ($4x10^2$ GC/l, $2.6x10^1$ and $2x10^1$ respectively). However the results obtained after the concentration of viruses using electropositive filters and acid flocculation must be considered as underestimated according to the low recovery efficiency of the methods applied.

The results show that the concentration of HAdV and JCPyV in the Llobregat and Ter rivers are very similar.

The reduction of the concentration HAdV and JCPyV during the treatment in the drinking-water treatment plant is calculated by the difference between the concentration in the river and the concentration in the GAC-filtered water. The data consistently show that there is a two-logarithm reduction, i.e. that there was approximately 99% removal of viruses during the water treatment.

The data also show 3 logs higher concentration of HAdV and JCPyV in the sludge generated in the drinking-water treatment plant than in river water. All the GAC-filtered water samples were positive for HAdV, though with low concentration of viral genomes (4.3 GC/l). JCPyV was at lower concentrations in GAC-filtered water; it was only detected in 56% of the samples. JCPyV followed the same pattern as HAdV but its concentrations were about tenfold lower. All the negative controls performed in these assays proved negative. The positive samples were also confirmed by nested-PCR assays and the sequencing of the amplicons.

BKPyV was detected in this study in all wastewater (average of 10^6 PCR units/l) and in 66% of river water samples ($2x10^1$ PCR units/l). None of the sludge and GAC-filtered water samples was found to be positive for BKPyV.

HEV was found in all (4/4) urban sewage samples but was not detected in river water samples tested or the sludge samples analyzed from the drinking-water treatment plant.

The presence of porcine adenoviruses in the Ter River proved the presence of animal fecal contamination at the sampling site on the river. Porcine adenoviruses was found in 3/4 samples tested and the identity was confirmed by sequencing analysis. The limiting decimal dilution semi-quantification revealed that the concentration was around 10^2 PCR units/l.

Detection of viruses in slaughterhouse biosolids. All the samples were positive for pAdV. One of the four slaughterhouse biosolid samples was positive also for HEV.

Typification of viruses. HAdV nucleic acids amplified from a positive river water sample (River1) were amplified by conventional nested PCR and then cloned in pGEM-T Easy vector. Three clones were sequenced and identified with 100% homology as HAdV type 41, commonly associated with acute diarrhea in children. HAdV DNA from every positive treated water sample was amplified and directly sequenced. Five sequences were identified as HAdV type 41 with 100% homology. The sequence obtained from the sample GAC4 was identified to 96% homology with HAdV B, HAdV 11, 14, 34 and 35.

The sequencing analysis of the PCR products of the sewage samples showed 3 HEV isolates (BCN21, BCN22 and BCN24) that belonged to genotype 3 and were very similar to other isolates identified previously in Barcelona (>90% homology in the amplified fragment). One isolate, BCN23, was related to strains belonging to genotype 1. The amplified region of the HEV genome found in a biosolid sample from the slaughterhouse was cloned and the strain (E5) was very similar to HEV strains isolated previously in Barcelona, i.e. VH3 (95% homology), identified in a patient with acute hepatitis.

Discussion

The sewage treatments used in depuration plants, including biological and physicochemical processes, have significantly reduced the incidence of diseases amongst the population, especially those etiologically related to bacteria; however, protozoa and viruses are more resistant than bacteria to most treatments. Significant concentrations of viruses are detected in the effluent water shed to the environment and

in the biosolids generated in these plants (34, Bofill-Mas et al. submitted for publication). The treated water reaches rivers and other sources of drinking water that could represent a risk of viral infections in the population if efficient drinking-water treatment is not applied and properly controlled before tap water distribution and consumption.

A method for concentration of virus from large volumes of water and from sludge was developed. The virus recovery efficiencies were quite variable and low, although they may be underestimated. Viral stocks from cell culture contain many defective particles and free DNA.

High concentrations of viruses were found in raw urban sewage, $1.4x10^7$ HAdV GC/l and $2.6x10^6$ JCPyV GC/l.

The Llobregat River used as a source of water for the drinking-water treatment plant has a Mediterranean regime, with irregular flow (mean flow of around 20 m^3/sec) and important seasonal changes (including drought and flood) throughout the year and from year to year. The river water at the entrance of the drinking-water treatment plant has maintained the bacterial parameters used as an index of fecal pollution at very similar values through the last 20 years and only coliform bacteria values have been observed to be one logarithm lower in the tested samples than those observed in early data (35). Llobregat and Ter river water samples presented significant levels of fecal contamination. Llobregat river had an average of $4.8x10^4$ *E.coli* most probable number (MPN) / 100ml; Ter river had an average of $5.1x10^3$ *E.coli* MPN/100ml. On the contrary, GAC-filtered water had no detectable *E.coli* or *Enterococci* and only 1.5 most probable number (MPN) of Coliform bacteria /100 ml were found (data not shown).

In this study, we analyzed and quantified the presence of human adenoviruses and the emergent viruses JCPyV, BKPyV and HEV in river water and developed a preliminary

evaluation of the removal efficiency for these viruses in a drinking-water treatment plant. HAdV, JCPyV and BKPyV were found in river water in average concentrations of $5x10^2$, $2x10^1$ GC/L and $2x10^1$ PCR units/l. respectively. This difference of 1 log between the polyomaviruses and the adenoviruses is also seen in the results on the concentration of these viruses in the GAC-filtered water samples. It has to be considered that the recovery efficiencies are quite low and variable, and appear to be lower for the isolation of JCPyV than for HAdV. However the concentration in urban sewage obtained using the ultracentrifugation method support also that JCPyV might be excreted in sewage in about 1-log lower concentration when compared to HAdV. This 1-log difference has also been observed in previous studies using nested PCR (6).

In the drinking-water treatment plant studied, the virus removal observed for human adenoviruses and JCPyV was of two logarithms between raw and GAC-filtered water, removing 99% of the viruses present in the source water. Even assuming the low recovery of the method applied, low concentrations of these viruses are still detected in treated pre-chlorinated water even in absence of fecal bacterial indicators, and could represent a risk for the population if secondary disinfection is not properly applied. Human adenoviruses accumulate in sludge as the higher concentrations found in these samples show. By the application of sludge to agricultural soils, viruses may also be disseminated, which also represents a diffuse source of viral contamination in the environment. Further studies are being developed to analyze more samples and also analyze post-chlorinated water to evaluate the global disinfection process.

HEV was detected in sewage water, confirming the results of previous studies that showed that this virus was consistently present and excreted by the population of industrialized areas (7). The isolates identified belonged mainly to genotype 3, the genotype most frequently found in these areas, including Barcelona. A strain typical of

endemic areas was also detected in urban sewage (genotype 1), which confirmed that pathogens are not limited to specific geographical areas and may, in fact, be easily disseminated. HEV is frequently detected in sewage, although in low concentrations, and the negative results observed in the few river water samples analyzed in this study may easily be due to dilution/inactivation factors producing HEV concentrations under the detection threshold of our method. Its absence or low concentrations in river water didn't allow us to study the removal of this virus during the drinking-water treatments in the plant. According to this, the GAC-filtered water samples were not analyzed for HEV. Further studies are being developed to analyze more samples and, in particular, evaluate the possible presence of HEV in source water contaminated with animal waste, especially from pig farms. The detection in previous studies of HEV in swine fecal samples and, in this study, in biosolids generated in a slaughterhouse where pigs were processed confirm that, though HEV infection of adult pigs is not usual according to the literature, pigs commonly used for consumption are sporadically infected and swine HEV strains. In fact, HEV has been detected in raw pig liver sold in Japan (1.9%) (36) and cases of hepatitis E in humans after ingesting uncooked meat of infected animals have been described (37). The porcine HEV strain identified, E5 isolate, was more like strains infecting humans in Barcelona than like Por1, another swine HEV strain detected previously in the same area (7, 38).

The procedure developed for the concentration and detection or quantification of viruses showed a satisfactory level of specificity which was proved by the confirmation of the expected viral identities by the sequencing analysis of the amplicons. Although sensitivity may be limited by the low and variable recovery efficiencies, it is important to highlight the stable presence of human adenoviruses and JCPyV in river water and, in lower concentrations, in purified water. The results strongly suggest that the specific

quantification of JCPyV and human adenoviruses is a very efficient tool for evaluating the presence of human viral contamination in water and the removal efficiency of viruses in a water purification plant.

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Table 1. Recovery efficiencies of the methods for the concentration of viruses from river water, sludge, GAC-filtered water and waste water.

Sample	JCPyV		Sample	HAdV	
	Quantity	Efficiency		Quantity	Efficiency
Stock[a]	7.68×10^4		Stock[a]	8.6×10^3	
River (GC/ 2.5 l)	1.11×10^2	0.15%	River (GC/ 2.5 l)	2.2×10^3	25%
Sludge (GC/5ml)	1.87×10^2	0.25%	Sludge (GC/5ml)	6.2×10^1	0.72%
GAC (GC/2.5l)	1.28×10^2	0.16%	GAC (GC/2.5l)	1.8×10^2	2%
Stock[b]	2.35×10^3		Stock[b]	1×10^4	
Sewage	1.72×10^3	73%	Sewage	4.4×10^3	43%

[a]Stock results expressed in GC/2.5l river water or GC/5ml sludge.
[b]Stock results expressed in GC/ml waste water.

Table 2. Concentration of HAdV and JCPyV in the samples analyzed. Results expressed in GC/l.

	HAdV[a]		**JCPyV**[a]		**BKPyV**[b]	
	Average (min-max)	% Positive	Average (min-max)	% Positive	Average (min-max)	% Positive
Wastewater	1.4×10^7 (4.71×10^5-2.52×10^7)	100 (5/5)	2.6×10^6 (1.83×10^5-8.9×10^6)	100 (5/5)	1.5×10^6 (10^5 -2×10^6)	100 (5/5)
Ter river water	2.9×10^2 (9.1×10^1-6.9×10^2)	100(5/5)	2.7×10^1 (9.8×10^0–6.3×10^1)	100 (5/5)	NT[c]	NT[c]
Llobregat river water	4×10^2 (1.4×10^1- 1.7×10^3)	89 (8/9)	2.6×10^1 (5.3-6.6×10^1)	100 (9/9)	2×10^1 (10^{-1}- 8×10^1)	66 (4/6)
Sludge	1.9×10^5 (1.3×10^2-7.96×10^5)	100 (5/5)	1.2×10^4	20 (1/5)	0	0 (0/5)
GAC	4.5 (5.6×10^{-2}-1.28×10^1)	100(9/9)	1.36 (4.6×10^{-1}- 5.47)	56 (5/9)	0	0 (0/6)

[a] Results obtained in Real-time quantitative PCR assays.
[b] Results obtained in End-point dilution Nested-PCR assays.
[c]NT: No Tested.

Annex A. Resultats dels análisis microbiològics de les mostres recollides.

Les mostres analitzades per poliomavirus humans, adenovirus humans i el virus de l'hepatitis E van ser analitzades paral·lelament pels indicadors microbiològics de contaminació fecal, els resultats es presenten a la taula A.2.1.

A.1 Materials i mètodes

Els anàlisis bacteriològics de les mostres d'aigua es van fer seguint mètodes prèviament validats i acreditats (ISO 17025), alguns d'ells basats en estàndards ISO: pel contatge en placa d'heteròtrofs (aerobis totals) a 22ºC es va usar el "Water Plate Count Agar" (ISO) (Oxoid Inc., Basingstoke, UK); per la quantificació de bactèries coliforms i *E.coli*, el Colilert-18 Quantitray (Idexx Inc. Westbrook, ME, USA); Pels enterococs, l'agar Sklanetz & Barley + Bilis-esculina (Oxoid); per *Clostridium perfringens* TSC Agar (Oxoid) amb el fluorogen MUP (Merck & Co., Inc. Whitehouse Station, NJ, USA) i per Aeromonas i Pseudomonas l'agar GSC (Merck).

A.2 Resultats

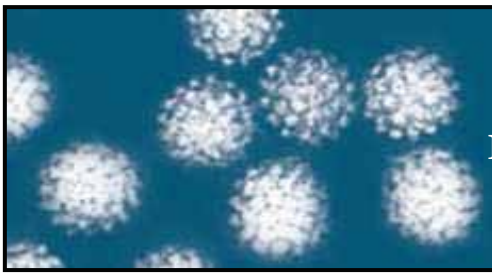
Taula A.2.1 Valors mitjans dels paràmetres microbiològics de les mostres d'aigua de riu i d'aigua filtrada per carbó.

	Aigua Crua Llobregat	**Aigua Filtrada per GAC**	**Aigua Crua Ter**
Aerobis 22°C (ufc/ml)	5.38×10^5	$1{,}9 \times 10^3$	$1{,}9\ 0x\ 10^5$
Aerobis 37°C (ufc/ml)	2.04×10^5	$6{,}53 \times 10^2$	NT
Coliforms totals (ufc/100ml)	9.01×10^4	1.5×10^0	$4{,}66 \times 10^4$
Coliforms fecals (ufc/100ml)	8.77×10^4	<1	NT
E. Coli (ufc/100ml)	4.81×10^4	<1	$5{,}17 \times 10^3$
Enterococs (ufc/100ml)	1.05×10^4	<1	$1{,}66 \times 10^2$
Clostridis sulfit-red. (ufc/100ml)	6.94×10^3	$3{,}33 \times 10^{-1}$	NT
C. prefringens (ufc/100ml)	6.33×10^3	$3{,}33 \times 10^{-1}$	$1{,}03 \times 10^3$
Aeromonas (ufc/100ml)	2.94×10^6	$8{,}29 \times 10^2$	$3{,}50 \times 10^4$
Pseudomonas (ufc/100ml)	1.55×10^6	$4{,}01 \times 10^3$	$2{,}76 \times 10^4$

NT: No testat.

A.3 Interpretació

Les mostres d'aigua tant del riu Llobregat com del riu Ter van presentar nivells significatius de contaminació fecal. Pel contrari, l'aigua filtrada per carbó (GAC filtered) va presentar absència o molt baixa contaminació microbiana. Cap de les mostres d'aigua filtrada per carbó va presentar nivells detectables de coliforms fecals ni *E. coli*. Com ja ha estat mencionat a l'article, les mostres filtrades per carbó activat van presentar contaminació vírica, confirmant la manca de correlació entre els indicadors de contaminació bacterians i el vírics.



CAPÍTOL III: MÈTODES DE CONCENTRACIÓ DE VIRUS A PARTIR DE MOSTRES D'AIGUA DE DIFERENTS VOLUMS.

Avaluació de mètodes per la concentració i quantificació d'adenovirus humans i del poliomavirus JC en aigua.

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Resum

Grans quantitats de virus són excretades a l'ambient en femtes i orina i poden causar malalties quan són ingerits. Alguns són considerats patògens emergents, com el poliomavirus humà JC (JCPyV). La gran prevalència d'adenovirus humans (HAdV) i poliomavirus en aigua residual d'àrees geogràfiques molt divergents ha suggerit la rellevància d'avaluar aquests virus com a possibles indicadors de contaminació vírica d'origen humà.

L'objectiu d'aquest estudi va ser avaluar l'eficiència de recuperació de cinc mètodes de concentració de virus en aigua. Per portar-ho a terme es van dopar dos volums representatius d'aigua de beguda (10 i 50 litres) amb una concentració coneguda d'HAdV, JCPyV i norovirus (NoV). Aquestes mostres van ser processades per cinc mètodes de concentració diferents i els resultats testats per PCR quantitativa.

En mostres de 10L, els cartutxos d'ultrafiltració van presentar bones recuperacions per ambdós virus (5,06% per HAdV i 18,7% per JCPyV), però van demostrar ser inaplicables en aigua de riu amb terbolesa moderada. La llana de vidre amb pre-acidificació de la mostra va presentar recuperacions similars: 4,18% per HAdV i 4,36% per JCPyV. Per mostres de 50 litres, el mètode que va presentar la millor recuperació i aplicabilitat va ser la filtració per llana de vidre (1,21% per HAdV i 13,72% per JCPyV).

També en van comparar dos kits d'extracció d'àcids nuclèics; es va seleccionar un mètode que permet extreure els àcids nuclèics d'1 ml de concentrat víric.

Per determinar quin hauria de ser el volum idoni d'una mostra d'aigua de riu es van analitzar quatre volums diferents sense dopar (42ml, 1L, 10L, 50L). La comparació

va mostrar que la millor detecció amb bona sensibilitat es produïa en analitzar un litre d'aigua (1,5 x 10^4 HAdV còpies genòmiques (GC)/L i 2,8x10^3 JCPyV GC/L).

S'han avaluat i adaptat mètodes simples de concentració basats en la adsorció/elució de virus en llana de vidre, aquests mètodes presenten baix cost i bona eficiència de recuperació de virus a partir d'aigua superficial i grans volums d'aigua de beguda en comparació amb altres mètodes testats. El volum de mostra òptim d'aigua d'un riu amb nivells significatius de contaminació fecal que rep aigua tractada d'un gran nombre de plantes de tractament d'aigua residual, per l'avaluació dels nivells dels indicadors de contaminació vírica proposats es d'un litre.

Resum dels Resultats i la Discussió

Es van comparar quatre mètodes per concentrar virus a partir de mostres d'aigua. Per avaluar la seva eficiència de recuperació es van dopar mostres de 10 i 50 litres d'aigua amb concentracions conegudes d'adenovirus humans, *Poliomavirus JC* i *Norovirus* i es van processar amb els diferents mètodes. La quantificació de la recuperació es va fer amb PCR quantitativa.

Per mostres de 10 litres el mètode que va presentar millors recuperacions va ser el mètode 3 basat en ultrafiltració (5,6% per HAdV i 18,7% per JCPyV). Aquest mètode permet la concentració de fins a 30 litres d'aigua en un volum molt petit (300µl), però pot ser aplicat només en mostres amb molt baixa terbolesa perquè els filtres es colmaten fàcilment i no va permetre la recuperació de *Norovirus*. El mètode 1, basat en l'adsorció dels virus en una matriu de llana de vidre amb una pre-acidificació prèvia de la mostra va presentar bones recuperacions (4,18% per HAdV, 4,26% per JCPyV i 3,4% per NoV) i per tant va ser seleccionat per l'anàlisi de virus en mostres de fins a 10 litres.

En mostres de 50 litres, el mètode 4 (filtres electropositius) va presentar recuperacions molt baixes (0,016% per HAdV i 0,374% per JCPyV), similars a les trobades en estudis previs usant filtres electropositius (Albinana-Gimenez i col. 2006). Aquest volum de mostra va ser escollit per representar mostres de grans volums (>100 litres), que són els que es necessita analitzar de mostres on s'espera molt baixa concentració de virus, com l'aigua de beguda. El mètode 1 no va ser avaluat en aquestes mostres, degut a la impossibilitat d'ajustar el pH en mostres de grans volums. El

mètode 2, essent idèntic al 1 però sense la pre-acidificació de la mostra, va presentar millors recuperacions (1,21% per HAdV i 13,72% per JCPyV).

En altres estudis obtenen majors recuperacions usant mètodes similars, per exemple, Vilaginès i col. (1993) va trobar eficiències de recuperació del 62-75% per enterovirus en aigua de beguda. La quantificació dels virus es va efectuar per comptatge de partícules infectives en cultiu cel·lular. Usar mètodes de comptatge per PFUs pot sobreestimar l'eficiència de recuperació dels mètodes de concentració degut a la disgregació de cúmuls de partícules víriques després de la dilució de l'inòcul en la mostra, que representaria un increment del nombre de PFUs quantificats en la concentració. Quan s'usa qPCR per la quantificació de virus, es conten els àcid nuclèics, tant de les partícules víriques infectives com de les defectuoses, inclús petites quantitats de DNA lliure que podrien estar presents en les suspensions virals usades per dopar.

S'ha de tenir en compte que la majoria de les soques de JCPyV presents a l'ambient presenten regió reguladora arquetípica i creixen molt ineficientment en cultiu cel·lular (Bofill-Mas i col. 2000). Per tant, la qPCR no és només el mètode més sensible per la quantificació de virus sinó que de vegades també és l'únic del que disposem per alguns virus com JCPyV i NoV.

En estudis recents fets per Lanbertini i col. (2008) s'ha fet servir llana de vidre per concentrar mostres d'aigua de beguda i qPCR per la seva quantificació. Les eficiències que van trobar van ser de 70% per *Poliovirus*, 14% per *Coxsackievirus* B5, 19% per *Echovirus* 18, 21% per *Adenovirus* 41 i 29% per *Norovirus*.

S'ha observat freqüentment inhibició de reaccions d'amplificació degut a substàncies presents a les mostres, com àcids húmics i metalls (Abbaszadegan i col. 1999, Donaldson i col. 2002). A més, els mètodes 1, 2 i 4 requereixen per eluir els virus dels filtres l'ús d'extracte de carn, el qual potencialment pot inhibir la amplificació per PCR (Abbaszadegan i col. 1993). S'ha observat anteriorment que usant diferents mètodes d'extracció d'àcids nuclèics s'obtenen eficiències molt divergents depenent de la matriu de la mostra; aquest pas té una gran influència en la quantificació de l'eficiència de recuperació dels virus quan s'usen mètodes moleculars. Per millorar la recuperació de virus tot disminuint al màxim el límit de detecció es van comparar dos kits d'extracció d'àcids nuclèics. El kit A va presentar sempre millor recuperació tot i que sempre en el mateix logaritme que el kit B. El kit A permet l'extracció d'àcids nuclèics a partir de 140µl de concentrat, el kit B permet l'extracció a partir d'1 ml de

concentrat, o sigui que encara que el kit A té una eficiència unes 2,6 vegades millor que el kit B, aquest últim permet disminuir el límit de detecció concentrant més volum de mostra. Tot i això els kits no van poder eliminar del tot els inhibidors i es van haver d'analitzar dilucions 10^{-1} i 10^{-2} de les extraccions.

Per determinar quin hauria de ser el volum idoni de mostra d'aigua del riu Llobregat, usat com a matèria prima de la planta potabilitzadora de Sant Joan Despí, es va comparar l'anàlisi de quatre volums de mostra sense dopar. Volums d'1 l, 10 l i 50 l van ser processats amb el mètode 1 (llana de vidre i pre-acidificació) i 42 ml van ser processats amb el mètode 5 (ultracentrifugació). Les dades mostren que a més volum analitzat, menor és la concentració detectada dels virus, probablement degut a que a més volum processat, més volum d'inhibidors es concentra. La ultracentrifugació té alta eficiència de recuperació, un 70% per *Poliovirus* en aigua residual (Pina i col. 1998), però molt poques de les mostres d'aigua de riu van ser positives per qPCR. Les mostres de 10 i 50 l van presentar força inhibició a la qPCR. El volum que millors resultats va presentar va ser d'1 litre, donat que va ser el volum que va permetre la major quantificació de virus ($1{,}54x10^4$ GC/L per HAdV i $2{,}75x10^3$ GC/L per JCPyV) i el mateix límit de detecció que les mostres de 10 l ($1{,}25x10^2$ GC/L), ja que la menor inhibició permetia detectar virus a una dilució inferior.

En resum, s'han comparat diversos mètodes de concentració de virus a partir de mostres d'aigua. Aquest estudi forma la part inicial un projecte que inclou el mostreig durant un any de diverses plantes de tractament d'aigua potable de Catalunya. Els mètodes usats en aquest projecte van ser seleccionats depenent de la seva eficiència de recuperació de virus, aplicabilitat i cost, tenint en conte les propietats de les mostres a analitzar. Aquests mètodes presenten bones eficiències de recuperació i són reproduïbles a un cost molt baix. Els mètodes desenvolupats per la concentració d'indicadors vírics humans (adenovirus humans i *Poliomavirus JC*) i patògens emergents (*Norovirus*) usant llana de vidre i qPCR són fàcils d'estandaritzar i poden ser eines valuoses pel control de la contaminació vírica en aigua crua i l'eficiència d'eliminació de virus en plantes de tractament d'aigua de beguda.

Comparison of methods for quantifying human adenoviruses, polyomavirus JC and noroviruses in source and drinking water.

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Abstract

Human adenovirus and JC polyomavirus have been suggested as viral indicators of fecal contamination in water. This study compares concentration and nucleic acid extraction methods and defines a protocol for quantifying human adenoviruses (HAdV), JC polyomavirus (JCPyV) and noroviruses (NoV) in source and drinking water. River water samples and spiked tap water samples were used to evaluate virus recovery, applying qPCR in the case of five concentration methods.

For 10 L samples ultrafiltration cartridges in the applied conditions produced acceptable recoveries for HAdV and JCPyV (5.06% HAdV, 18.7% JCPyV) but they delivered poor efficiencies for noroviruses and proved to be inapplicable in high-volume and river-water samples with medium turbidity. The glass wool method with pre-acidification presented recoveries of 4.18% HAdV, 4.36% JCPyV and 3.4% for NoV. For 50 L samples the method that presented the highest recovery efficiency and applicability was glass wool filtration, with recoveries of 1.21% HAdV and 13.72% JCPyV. Comparing different sample volumes of a river used as source water showed that the highest numbers of viruses were quantified when lower volumes (1 L) were tested (1.5×10^4 HAdV genome copies (GC)/L and 2.8×10^3 JCPyV GC/L).

The developed methods are easy to standardize and may be highly valuable tools for the control of viral contamination in source water and for the evaluation of the efficiency of virus removal in drinking water treatment plants.

Keywords: Drinking Water, Polyomavirus, Adenovirus, Norovirus, Concentration Methods, qPCR.

1. Introduction

Water quality, and therefore human health, is affected by the presence of pathogenic microorganisms derived from sewage discharged into the environment. Among these pathogens there are many types of viruses infecting humans which are excreted in high concentrations in the feces of patients with gastroenteritis (IAWPCR, 1983; Koopmans and Duizer, 2004) and even in the feces and urine of healt5hy people. These pathogens can be found in urban sewage and are regarded as environmental contaminants (Pina et al. 1998). Some viruses, such as human polyomaviruses and some strains of human adenoviruses (HAdV), infect humans during childhood and cause persistent infections. After acute infection, some HAdV types may be shed in stool for months or even years, causing their endemic spread to other individuals via the fecal-oral route (Wadell et al. 1988).

Not only are infectious diseases related to water a leading cause of mortality and morbility worldwide, but also both the spectrum and incidence of some water-related diseases are increasing (WHO, 2003). Human polyomaviruses and HAdVs are being detected more often in the environment and have been suggested as an index of viral contamination of human origin (Bofill-Mas et al. 2000; Pina et al. 1998; Puig et al. 1994).

JC polyomavirus (JCPyV) is a human virus classified in the *Polyomaviridae* family, producing latent and chronic infections that persist indefinitely in individuals and viral particles are excreted regularly in urine by healthy individuals (Shah, 1995). JCPyV is associated with progressive multifocal leukoencephalopathy (PML), a fatal demyelinating disease that accounts for 4% of deaths in AIDS patients (Berger et al.1987). The pathogenicity of the virus is commonly associated with immunocompromised states but has attracted most attention due to AIDS-linked

immunosuppression. (Berger et al. 1987). JCPyV has also been associated in some studies with human cancer such as colon cancer (Enam et al. 2002; Hori et al. 2005). In previous studies JCPyV was found in 98% of the 52 sewage samples collected from widely disparate geographical areas around the world (Bofill-Mas et al. 2000), and it also has been found in river water used as a water source in a drinking water treatment plant (DWTP) (Albinana-Gimenez et al. 2006). Moreover the samples were also positive for HAdV, demonstrating the applicability of molecular assays to detect HAdV and JCPyV in the identification of human fecal contamination (Bofill-Mas et al. 2001).

Noroviruses (NoVs) are single-stranded RNA viruses which form the genus *Norovirus* within the *Caliciviridae* family and are a major cause of acute viral gastroenteritis worldwide. Transmission through food and water are the major exposure routes (Koopmans et al. 2002). Genetically, noroviruses are a highly diverse group (Phan et al. 2007) in which most of the human-infecting members are distributed in two genogroups and about 19 genotypes (Vinjé et al. 2004).

Human adenoviruses are members of the genus *Mastadenovirus* in the *Adenoviridae* family, which comprises 51 serotypes classified in 6 species (A-F). They have double-stranded linear DNA and a non-enveloped icosahedral shell that has fiber-like projections from each of its 12 vertices (Stewart et al.1993). Some serotypes, such as 40 and 41, are unique in being responsible for most cases of Ad-associated gastroenteritis in children. Others, in subspecies B, are responsible for 5-10% of childhood respiratory diseases and conjunctivitis (Wold and Horwitz, 2007).

In previous studies sensitive methods for the detection of HAdV in sewage and shellfish have been developed using molecular techniques (Formiga-Cruz et al. 2002; Pina et al. 1998; Puig et al. 1994). In addition, high-cost methods have been applied in previous studies to detect adenoviruses and polyomaviruses in river water and drinking water

(Albinana-Gimenez et al. 2006), achieving low recovery efficiencies. Quantitative detection based on qPCR, however, is the method of choice for the quick and sensitive assessment of viruses in water matrices. The main objectives of this study are to assess the efficiency of concentration methods using molecular techniques and to define an easy, cost-effective procedure that can be standardized for the simultaneous quantification of human adenoviruses, JCPyV and noroviruses.

Suitable methods to concentrate viruses from water must fulfill a number of criteria (Wyn-Jones and Sellwood, 2001). They must: (i) be technically easy to accomplish in a short time; (ii) have a high virus recovery rate; (iii) concentrate a large range of viruses; (iv) provide a small volume of concentrate; (v) not be costly; (vi) be capable of processing large volumes of water; and (vii) be repeatable (within a laboratory) and reproducible (between laboratories). Current virus monitoring methods, under the USEPA's Information Collection Rule (ICR), are based on the total culturable virus assay enumerated by most-probable number (TCVA-MPN) in cell culture (Jiang, 2006), but the advancement of PCR-based technologies has significantly shortened the detection time and improved the sensitivity of detecting viruses, such as HAdV, JCPyV and NoV, that cannot be grown in standard cell lines (Albinana-Gimenez et al. 2006; Bofill-Mas et al. 2006; Haramoto et al. 2005; Pina et al. 1998; Puig et al. 1994; Van Heerden et al. 2005).

Human adenoviruses and human polyomaviruses have been proposed in previous studies in our group as potential indicators of contamination of human origin (Bofill-Mas et al. 2000; Hundesa et al. 2006; Pina et al.1998). Also, porcine adenovirus and bovine polyomavirus have been suggested as microbial source tracking tools for detecting water contamination of animal origin (Hundesa et al. 2006; Maluquer de Motes et al. 2004).

We have chosen two approaches to the concentration method: adsorption-elution (using glass wool or electropositive filters) and ultrafiltration. In addition, we have used qPCR to quantify HAdV, JCPyV and noroviruses. The aim of this study has been to compare the selected methods and develop standardizable protocols in order to maximize simultaneous virus recovery while minimizing the time, cost and detection limits.

2. Materials and methods.

2.1 Viruses.

The HAdV2 strain isolated from a clinical sample was grown on A549 cells propagated in Eagle's minimum essential medium (EMEM) supplemented with 1% glutamine, 50 µg of gentamicin per ml, and 5% (growth medium) or 2% (maintenance medium) of heat-inactivated FBS. JCPyV was obtained from the urine of a healthy woman in the 38th week of pregnancy, then grown in SVG cells, which had kindly been donated by Dr. Eugene O. Major from the Laboratory of Molecular Medicine and Virology, National Institute of Neurological Disorders and Stroke, NIH (USA). SVG cells were propagated in Eagle's minimal essential medium (EMEM) supplemented with 1% glutamine, 50 µg of gentamicin per ml, and 5% (growth medium) or 2% (maintenance medium) of heat-inactivated FBS. Lastly, NoV genogroup II was obtained from a clinical fecal sample from gastroenteritis patients.

2.2 Spiking experiments to compare methods.

Two sample volumes were selected to represent the standard sampling volumes: 10 liters, used frequently in recreational water analysis, and 50 liters, representing higher volumes. For spiking experiments, water samples were prepared with equal volumes of

tap water and deionized water. In each case, 5 or 25 L of tap water were collected in a plastic container and 5 or 25 ml of 1.8% sodium thiosulphate was added; the solution was then mixed thoroughly and let stand for several minutes to ensure that any chlorine present in the sample was neutralised. Finally, 5 and 25 L of deionized water were added to reach a final volume of 10 or 50 L. To evaluate virus recovery efficiencies all the water samples were seeded with known quantities of HAdV 2, JCPyV and NoV GII calculated using qPCR.

2.3 Sample collection.

Water samples from the Llobregat River were collected in order to define the most suitable sampling volume to quantify the viruses selected for study. River water samples of 42ml, 1L, 10L and 50L were collected in plastic containers. All the samples were processed within 24 hours of collection.

2.4 Concentration methods.

Comparisons were made of concentration methods applicable to volumes up to roughly 10 liters of water and methods applicable to higher volumes. In the latter case, 50 liters of water were tested. The methods can be described as follows:

Method 1. Glass-wool columns with pre-acidification of the water sample. The samples were pre-acidified to pH 3.5, then filtered at 1L/min through a column with 10g of compacted glass wool (Panreac) previously washed with HCl, NaOH and tap water. The columns were eluted by gravity flow using 200ml of 0.05M glycine buffer containing 3% beef extract at pH 9.5 ± 0.2. The eluate was flocculated at pH 3.5 ± 0.2 and centrifuged at 7000 xg for 30 minutes. The pellet was resuspended in 5 ml PBS and stored at -80°C until analysis.

Method 2. The same as method 1 but without pre-acidification of the sample. These two methods are partially based on the one described by Vilaginès et al. (1993).

Method 3. Ultrafiltration cartridges (Infilco Degremont, France). Samples were filtered through the ultrafiltration module (previously treated with 0.1% BSA) at 0.5 L/min. The filter was eluted twice, adding NaOH 1mM and shaking for 30 minutes (Rajal et al. 2007). The eluate was neutralized and filtered again through a 40-Kd pore diameter filter. The residue of 300µl was then stored at -80°C until analysis.

Method 4. Electropositive cartridges. This method is based on a method described by Sobsey and Glass (1980) and later modified (Albinana-Gimenez et al. 2006). In short, samples were filtered through Zeta Plus MK electropositive filters (AMF Corp., Cuno Division, Meriden, CT, USA), at 1 L/min in a Millipore peristaltic pump. Viruses were eluted in 900 ml of glycine buffer (0.25 N pH 9.5 1% beef extract) (Becton, Dickinson & Co., Sparks, MD) by reverse flow using a Millipore peristaltic pump at 400 ml/min for 45 min. The secondary concentration step was organic flocculation with 3% beef extract, adjusting the pH to 3.5 using 5 M HCl and magnetically stirring for 30 min. The sample was centrifuged at 12 800 xg for 25 min at 4 °C, and the pellet was then eluted in 5ml PBS and stored at -80°C until analysis.

For the concentration of small volumes (40ml) of river water presenting significant levels of fecal contamination, a method that has been developed to study viruses in urban sewage was also tested:

Method 5. This method is described in Puig et al. (1994) and Bofill-Mas et al. (2006). In short, 40-ml river water samples were ultracentrifuged (110 000 xg for 1 h at 4 °C) to pellet all of the viral particles together with any suspended material. The sediment was then eluted by mixing it with 3.5 ml of 0.25 N glycine buffer (pH 9.5) on ice for 30 min, and the suspended solids were separated by centrifugation at 12 000 xg for 20 min after

the addition of 3.5 ml of 2 x PBS. Lastly, the viruses were pelleted using ultracentrifugation (110 000 xg for 1 h at 4 °C), resuspended in 0.1 ml of PBS, and stored at -80 °C.

2.5 Nucleic acid extraction.

Two procedures were compared for extracting nucleic acids from the concentrated viruses. The two methods were Nucleospin RNA Virus F (Macherey-Nagel) and QIAamp Viral RNA Mini Kit (QIAGEN) and they were used according to manufacturer instructions.

2.6 Enzymatic Amplification.

(i) Quantitative Real-Time PCR (qPCR). For the specific detection and quantification of JCPyV and HAdV genomes, 10 µL of neat, 10-fold and 100-fold dilutions of every DNA extraction were tested; these dilutions were made to detect and reduce amplification inhibition due to the potential presence of inhibitory substances that may interfere with the qPCR. Amplification was performed in a 25-µL reaction mixture with PCR Master Mix (Applied Biosystems). The reaction contained 10 µL of a DNA sample or 10 µL of a quantified plasmid DNA, 1x TaqMan master mix, and the corresponding primers and TaqMan probes. JCPyV genomes were quantified with 0.5 µM of the primers JE3F and JE3R and 0.15 µM of the fluorogenic probe JE3P described in Pal et al. (2006). HAdV genomes were quantified with 0.9 µM of the primers AdF and AdR and 0.225 µM of the AdP1 probe, as described by Hernroth et al. (2002). Following activation of the uracil N-glycosylase contained in the core mix (2 min at 50 °C) and activation of the AmpliTaq Gold for 10 min at 95 °C, 40 cycles (15

sec at 95 °C and 1 min at 60 °C) were performed with an MX3000P sequence detector system (Stratagene).

(ii) Quantitative Real-Time RT-PCR (qRT-PCR). For the specific detection and quantification of NoV GII genomes, 5 µl of the DNA extraction and of their 10-fold dilution were assayed; these dilutions were made in order to avoid amplification inhibition due to the assay's high sensitivity to inhibitors. Amplification was performed in a 25-µL reaction mixture with the QuantiTect Probe RT-PCR kit (QIAGEN). The reaction contained 5 µL of an RNA sample or 5 µL of a quantified plasmid cDNA, 1x QuantiTect mix, 1x QuantiTect RT mix, 1µM of the primers JJV2F and COG2R and 0.1 µM of the probe RING2-TP, as described by Jothikumar et al. (2006). Following retrotranscription (30 min at 50°C) and activation of the HotStarTaq (15 min at 95°C), 45 cycles (10 sec at 95°C, 20 sec at 55°C, 15 sec at 72°C) were performed with an MX3000P sequence detector system (Stratagene).

The principle of real-time PCR has been described elsewhere (Heid et al. 1996). All samples were run in quadruplicate, and positive and negative controls were included. The amount of DNA was defined as the average of the duplicate data obtained.

2.7 qPCR Standards.

For the generation of standards to use in the real-time PCR assays, three plasmid constructions were employed: pJCPyV, containing the whole JCPyV genome strain Mad-1 in pBR322, which had kindly been donated by Andrew M. Lewis of the Office of Vaccine Research and Review, CBER, FDA, (USA); pAd41, containing the hexon region of HAdV 41 in pBR322, kindly donated by Dr. Annika Allard of the University of Umeå (Sweden); and pNoV, containing the ORF1-ORF2 junction in pTrueBlue, kindly donated by Dr. Vinjé and Dr. Jothikumar from the CDC (Atlanta, GA, USA). E.

coli JM109 cells (Promega, Madison, WI. USA) were transformed with the plasmid (pJCPyV, pAdV41 or pNoV). The plasmids were purified of the bacteria using the Plasmid Midi Kit (QIAGEN GmbH, Inc., Hilden, Germany) following the manufacturer's instructions and the DNA obtained was quantified with a GeneQuant pro (Amersham Biosciences). To reduce the possibility of DNA contamination in the laboratories, the plasmids were linearized with EcoRI (pJCPyV and pNoV) or NruI (pAd41) (Promega, Madison, WI). The reaction product was purified and quantified again, and dilutions of 10^{-2}-10^{7} viral DNA molecules per 10 µL were made in TE buffer. The standard dilutions were then aliquoted and stored at -80 °C until use.

3. Results and discussion

3.1 Efficiency in the recovery of viruses in 10-liter samples.

Three methods were compared by means of spiking experiments repeated between 5 and 10 times and the results are shown in Table 1, expressed as a % of the genome copies spiked and recovered, using quantification by qPCR. NoVs were detected in two of the 10 L samples processed with method 1, and the average recovery was 3.4%. The recovery of NoV using the other methods was < 0.95%. NoV is an unculturable virus and it is difficult to obtain large concentrations to perform this kind of experiment.

Other studies have reported higher recovery efficiencies using similar methods. For example, Vilaginès et al. (1993) reported recovery efficiencies of 62-75% of enteroviruses in tap water. In that study the quantification of the viruses was performed by cell culture. Using PFU methods, observed recoveries are higher than when using qPCR methods (data not shown), probably due to the desegregation of clumped viral particles after the dilution of the viral inoculum in the water. That would represent an increase in the number of PFU quantified in the spiked water samples relative to the

original viral suspensions. When using qPCR as the quantitative method for viruses, the quantification of total viral NA also includes also potential non-infectious viral particles or even small quantities of free viral DNA that might be present in the viral suspensions produced from cultured cells. qPCR is not only the quickest and most sensitive method for virus quantification, but also the only one available for many viral types, including JCPyV and NoV. It must be taken into account that most of the JCPyV strains found in the environment present archetypal regulatory regions and would grow very inefficiently in cell culture (Bofill-Mas et al. 2000).

Recent studies have described the use of glass wool filtration to concentrate viruses from tap and ground water and the use of qPCR to quantify them; Lambertini et al. (2008) found recovery efficiencies of 8-28% for *Adenovirus* 41 and 16-45% for *Norovirus*. The differences between the described recovery efficiencies can be related to the dissimilar origin of the samples used in the study and also to the fact that the recovery from the method in our study is calculated directly in relation to the number of genome copies found in the cell culture supernatant used to seed the samples. By contrast, the recoveries in Lambertini et al. refer to the number of genome copies quantified in an unseeded sample concentrate, ensuring that the viruses were in the same eluate matrix when determining the numerator and denominator of the percent recovery calculation.

For the 10-liter samples the method that presented the highest recovery was method 3, based on ultrafiltration. This method allows concentrating up to 30 liters in a very small volume (300µl) but it can only be applied in samples with very low turbidity, because the filters can easily become clogged and, as currently applied, do not enable the recovery of spiked noroviruses. In these samples, method 1, based on glass wool

columns including the pre-acidification step, was the selected method for the simultaneous analysis of all viruses in volumes of up to 10 liters.

3.2 Efficiency in the recovery of viruses in 50-liter samples.

Two methods were compared by means of spiking experiments repeated 5 times. The results are shown in Table 1, expressed as a % of the genome copies spiked and recovered, using quantification by qPCR. Method 4 presented very low efficiencies, which were similar to the efficiencies observed in previous studies using electropositive filters (Albinana-Gimenez et al. 2006), although viruses have been detected using this method. The mean recovery of JCPyV by method 4 is 0.374%, but only one of the 5 replicates was positive (1.87%), while the recovery from the other four was lower than 0.0006%. A volume of 50 liters was chosen to represent large-volume samples, which must be expected to present zero or very low concentrations of the viruses, such as drinking water. Method 1 was not evaluated in these samples due to the impossibility of performing the sample conditioning in large volumes of water. Method 2, being the same as method 1, using glass wool columns but without the pre-acidification of the sample, was the best choice for concentrating viruses from large volumes of water.

As method 3 (using ultrafiltration cartridges) could not be applied to large volumes of water, we considered it preferable to unify methods for all the volumes. Methods 1 and 2, following the same principle, were selected to be used in environmental samples and all further analyses refer to them.

3.3 Nucleic acid extraction comparison.

Inhibition of amplification reactions by substances present in the samples, such as humic acids and metals, has frequently been observed in water samples (Abbaszadegan

et al. 1999; Donaldson et al. 2002). Furthermore, methods 1, 2 and 4 require beef extract to elute the viruses from the filters and the extract is known potentially to inhibit PCR amplification (Abbaszadegan et al. 1993). It has previously been observed that widely divergent efficiencies are obtained from different nucleic acid extraction protocols depending on the matrix of the viral concentrate (data not shown), and that this step has a critical influence on quantifying the recovery efficiency of viruses when using quantitative molecular methods. To improve the recovery of the viruses by minimizing the inhibition of enzymatic amplification, two nucleic acid extraction kits were compared with the seeded samples. Results are shown in Table 2.

Fifteen viral concentrates from the spiking analyses were tested with the two NA extraction protocols. The results are shown in table 2. The ratio indicates how many times one kit produced higher efficiencies than the other. The ratio is always >1 so the QiaAmp Viral RNA Mini Kit (kit A) presents higher recovery, however all the quantification results are in the same log. Kit A makes it possible to extract 140µl of virus concentrate to achieve a final extract of 80 µl. Nucleospin RNA Virus F (kit B) makes it possible to extract 1 ml of virus concentrate to a final volume of 100µl. As a result, although the detection is on average 2.6 times lower, it permits lowering the detection limit by analyzing a greater volume of the sample. In highly contaminated water samples, kit A may be recommended. To increase the probability of positive samples when analyzing treated or drinking water, kit B will be useful to decrease the detection limit.

These kits, however, fail to eliminate the PCR inhibitions completely. To eliminate the inhibition, 10-fold and 100-fold dilutions of every nucleic acid extraction were also analyzed, as reported in previous studies (Albinana-Gimenez et al. 2006).

3.4 Sample volume comparison.

The objective of developing and comparing these methods is to apply them in surveys of source water and in drinking water treatment plants (DWTPs), such as the plant surveyed in a previous study (Albinana-Gimenez et al. 2006). That plant uses the Llobregat River as source water. The Llobregat has a Mediterranean regime, irregular flow, important seasonal changes (including drought and flood) and high turbidity. To determine which Llobregat River water-sample volume would be the most suitable to analyze, taking into account virus detection and sample handling, five replicates of four different volumes of unseeded river water were processed using methods 1 and 5. The results are shown in Table 3. The data show that the higher the volume analyzed, the lower the concentration found, probably because suspended materials and inhibitors are concentrated with viral particles as well. Method 5, using ultracentrifugation and elution procedures developed for the study of urban sewage, has proved to be very efficient in these types of samples, allowing the detection of a high concentration of viruses in the river water samples tested. Ultracentrifugation has high recovery efficiencies, around 70% for polioviruses (Pina et al. 1998) in urban sewage. However, a very low percentage of the river water samples was positive in the qPCR amplification and, because the sample volume was the lowest, the detection limit was the highest (1.2 x 10^5 GC/L). Viral concentrates from 10-liter and 50-liter samples presented high inhibition in the qPCR, so 100-fold dilutions had to be analyzed to find positive results and they showed the lowest detection limits. The detection limits were 1.25 x 10^2 GC/L for 10L and 2.5 x 10^1 GC/L for 50L. In the specific source water analyzed, the highest quantification efficiency was obtained by analyzing one-liter samples, showing the same detection limit as the 10L samples (1.25 x 10^2 GC/L), because positive results could be found in the 10-fold dilution. HAdV and JCPyV have been found in Llobregat

River water in concentrations that were two logs higher than the ones found in previous studies using other methods (Albinana-Gimenez et al. 2006).

4. Conclusions.

Several methods of virus concentration from water have been compared. This study is the initial part of a larger investigation that includes a one-year survey of several control points in DWTPs. The methods to be used in the project were chosen based on their recovery efficiency, applicability, feasibility and cost, taking into account the properties of the samples to be analyzed. The methods present acceptable and reproducible recovery efficiencies at very low cost. The developed methods for the quantification of human viral indicators (human adenoviruses and JCPyV) and emergent pathogens (noroviruses) using glass wool-based protocols and qPCR are easy to standardize and may be very valuable tools for the control of viral contamination in source water and the efficiency of virus removal in drinking water treatment plants. The methods defined for the quantification of JCPyV and human adenovirus will also represent a useful protocol for microbial source tracking studies, as human and animal adenoviruses and polyomaviruses have been suggested as specific microbial source tracking tools in identifying and quantifying contamination of human/porcine/bovine origin in water.

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Table 1. Recovery efficiencies of methods for concentration of viruses from fresh water.

			HAdV		JCPyV		NoV	
Vol.	**Method**	**Sample #**	**Recov.**	**Range**	**Recov.**	**Range**	**Recov.**	**Range**
10 L	1 Glass wool+ pre-acid.	10	4.18%	2.02-6.91%	4.36%	1.23-9.48%	3.4%	0-18.6%
	2 Glass wool	5	0.95%	0.7-1.36%	2.82%	1.71-3.65%	<0.95%	-
	3 Ultrafiltration	5	5.06%	3.18-6.02%	18.7%	13.36-32.98%	<0.95%	-
50 L	2 Glass wool	5	1.21%	0.71-1.57%	13.72%	9.51-17.57%	<0.95%	-
	4 Electropos. filters	5	0.016%	0.007-0.02%	0.374%	<0.0006 -1.87%	<0.95%	-

Table 2. Comparison of two nucleic acid extraction kits. Concentrations represented as mean values of 5 samples tested in parallel.

	HAdV			JCPyV		
	A (GC/L)[a]	**B (GC/L)[b]**	**Ratio A/B**	**A (GC/L)[a]**	**B (GC/L)[b]**	**Ratio A/B**
10 L Method 1	$4.47x10^6$	$1.40x10^6$	3.19	$1.16x10^4$	$6.31x10^3$	1.84
10 L Method 2	$7.87x10^5$	$2.86x10^5$	2.75	$7.21x10^3$	$2.32x10^3$	3.11
50 L Method 2	$5.62x10^5$	$3.28x10^5$	1.71	$3.53x10^4$	$1.12x10^4$	3.16
Mean			2.55			2.70

[a] QIAamp viral RNA Mini Kit (QIAGEN). [b] Nucleospin RNA virus F (Macherey-Nagel).

Table 3. Comparison of different volumes of Llobregat River water. Concentrations represented as mean values of 5 samples tested in parallel.

		HAdV		JCPyV	
Volume	**Method**	**Mean concentration (GC/L)**	**Std Dev.**	**Mean concentration (GC/L)**	**Std Dev.**
42 ml	5	$1.18x10^4$	$8.33x10^3$	$4.62x10^3$	$8.10x10^3$
1 L		$1.53x10^4$	$8.22x10^3$	$2.75x10^3$	$2.36x10^3$
10 L	1	$3.49x10^3$	$1.68x10^3$	$3.70x10^2$	$2.93x10^2$
50 L		$3.46x10^2$	$1.59x10^2$	$5.16x10^1$	$3.04x10^1$

CAPÍTOL IV: ANÀLISI DE LA PRESÈNCIA D'ADENOVIRUS HUMANS I POLIOMAVIRUS JC EN PLANTES POTABILITZADORES.

Anàlisi d'adenovirus i poliomavirus per qPCR com a indicadors de qualitat de l'aigua en plantes de tractament d'aigua de beguda.

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Water Research, pendent de publicació.

Resum

La gran prevalència d'adenovirus humans i poliomavirus JC en aigua residual procedent d'àrees geogràfiques molt diverses mostra la importància d'avaluar aquests virus com a potencials indicadors de contaminació vírica d'origen humà. La PCR quantitativa proporciona una forma ràpida i sensible de quantificar els virus presents en aigua, per això podria ser aplicada eficientment al control de la qualitat de l'aigua i l'avaluació de la eficiència d'eliminació de virus en plantes de tractament d'aigua de beguda.

S'han estudiat tres plantes de tractament d'aigua de beguda per definir la seva qualitat en relació a la presència de patògens vírics i l'eficiència dels tractaments aplicats. La planta 1 està situada en una àrea urbana i inclou sedimentació, ozonització i filtració per carbó activat (GAC); la planta 2 està situada en una àrea urbana i usa desinfecció per ultraviolats (UV) i filtració per membranes; la Planta 3 està situada en una àrea rural i inclou sedimentació i filtració per GAC. Totes les plantes també inclouen tractaments de cloració.

Un 90% de les mostres d'aigua de riu van presentar resultats positius per HAdV (10^1 - 10^4 Còpies Genòmiques (GC)/L) analitzant mostres d'un litre durant un període d'un any i un 48% van ser positives per JCPyV (10^0 - 10^3 GC/L). Es van quantificar concentracions més baixes dels dos virus en diferents passos dels tractaments de les plantes en absència d'indicadors bacterians. Les eficiències d'eliminació de virus van ser superiors a 5 logaritmes en les plantes 1 i 3 i van poder quantificar-se com a majors de 2 logaritmes a la planta 2. Tot i això, tres mostres d'aigua postclorada de les plantes 2 i 3 (11%) van ser positives per HAdV per qPCR, però no van presentar infectivitat en cultiu cel·lular. Els genomes virals detectats en aquestes mostres van presentar alta homologia amb HAdV2.

Mètodes simples basats en l'adsorció/elució de virus en llana de vidre aplicats a mostres d'aigua de riu i mostres de gran volum proporcionen una recuperació eficient de virus a baix cost. La quantificació de JCPyV i HAdV per qPCR és útil per l'avaluació de l'eficiència d'eliminació de virus en plantes de tractament d'aigua de beguda, per la identificació de punts crítics de control i com a índex de la qualitat virològica de l'aigua. Encara que la infectivitat no està garantida quan s'usa qPCR en aigua tractada amb processos de desinfecció, la qualitat de l'aigua d'entrada i l'eficiència d'eliminació de virus pot ser eficientment quantificada.

Resum dels Resultats i la Discussió.

Grans quantitats de virus són excretades a l'ambient i en rius usats com a font d'entrada a plantes potabilitzadores. En estudis previs s'han detectat alts nivells de contaminació vírica en aigua residual i plantes de tractament d'aigua residual (Albinana-Gimenez i col. 2006, Bofill-Mas i col. 2000, Bofill-Mas i col. 2006, He i Jiang 2005, Katayama i col. 2008, Pina i col. 1998). A l'àrea geogràfica estudiada aquí, els nivells d'HAdV trobats en aigua residual urbana van ser de $3{,}8x10^7$ HAdV GC/L i $3{,}4x10^7$ JCPyV GC/L. Els nivells trobats en efluents de plantes de tractament d'aigua residual van ser de $8{,}08x10^4$ HAdV GC/L (Bofill-Mas i col. 2006). Els efluents de les plantes de tractament d'aigua residual són sovint conduïts a rius que després són usats com a font d'aigua per les plantes potabilitzadores. Aquesta càrrega vírica podria representar un risc d'infeccions per la població si no s'apliquen i es controlen adequadament els tractaments de potabilització. Els HAdV han estat descrits com a estables a l'ambient i són resistents a alguns tractaments de potabilització (Albinana-Gimenez i col. 2006). L'estabilitat dels HAdV i JCPyV ha demostrat ésser molt alta quan ha estat quantificada per qPCR (Bofill-Mas i col. 2006).

En aquest estudi s'ha quantificat JCPyV i HAdV per qPCR en mostres de diversos punts en tres plantes de tractament d'aigua de beguda de Catalunya. El mètode de concentració usat presenta millors eficiències de recuperació que els usats en estudis previs (Albinana-Gimenez i col. 2006). Conseqüentment la concentració de còpies genòmiques trobada és significativament superior a la concentració trobada a la mateixa localització en aquest estudi previ. Els resultats obtinguts estan representats a les Taules 1, 2 i 3.

Totes les mostres d'aigua de riu (entrada) de la planta 1 van ser positives per HAdV, amb una concentració mitjana de $1{,}24 \times 10^4$ GC/L; 44% de les mostres van ser

positives per JCPyV amb una concentració mitjana de 7,4 x 10^2 GC/L. Els resultats obtinguts en aigua de riu a la planta 3 són similars, amb un 83% de mostres positives per HAdV (9,24 x 10^3 GC/L) i un 50% positives per JCPyV (1,3 x 10^3 GC/L). Les mostres d'aigua crua de la planta 2 van presentar nivells mes baixos de contaminació, ja que provenien d'aqüífers subterranis. Nivells similars de virus van ser trobats al llarg de tot el tractament d'aquesta planta.

S'han realitzat anàlisis dels indicadors bacterians estàndard a les tres plantes. La planta 1 va presentar nivells significatius de contaminació bacteriana a l'aigua d'entrada: 3,4 x 10^4 ufc/100ml coliforms totals, 2,1 x 10^3 ufc/100ml *E.coli*, 2,5 x 10^3 ufc/100ml *C.perfringens*, 4,4 x 10^2 ufc/100ml enterococs i 2,8 x 10^5 ufc/ml heteròtrofs. Els contatges van disminuir dràsticament en la mostra d'aigua filtrada per sorra, amb positius esporàdics, excepte els heteròtrofs que es van poder detectar en concentracions cada vegada menors durant tot el procés. A la planta 2 es van trobar mostres positives esporàdiques amb baixos contatges durant tot el procés. A la planta 3 es van trobar nivells de contaminació bacteriana a l'aigua d'entrada més alts que a la planta 1: 7,5 x 10^4 ufc/100ml coliforms totals, 6,7 x 10^3 ufc/100ml *E.coli*, 5,2 x 10^3 ufc/100ml *C.perfringens*, 7,3 x 10^3 ufc/100ml enterococs i 5,1 x 10^4 ufc/ml heteròtrofs. Es va observar un logaritme de reducció després de la sedimentació.

La reducció de les concentracions de JCPyV i HAdV quantificats com a còpies genòmiques durant el tractament en les plantes ha estat calculat per la diferència entre la concentració en aigua crua i la concentració a l'aigua de beguda. Per HAdV, la planta 1 va presentar una reducció de més de 5,13 logaritmes entre l'aigua d'entrada i la de sortida i 5,13 logaritmes entre la d'entrada i l'aigua filtrada per GAC. Només una mostra d'aigua filtrada per GAC va ser positiva per HAdV. Per JCPyV la reducció va ser de >5 logaritmes a l'aigua de sortida i de 3,51 logaritmes entre l'aigua d'entrada i la filtrada per GAC. La mostra filtrada per GAC positiva per HAdV va ser testada en cultiu cel·lular però no va produir infecció en cèl·lules A549.

El tractament d'ozonització aplicat sembla que té poc efecte en la quantificació de genomes vírics, tot i que es va observar un 98,8% d'eliminació de bactèries heteròtrofes. L'ozó és un agent molt oxidant i una alternativa efectiva al clor per la reducció de patògens a l'aigua, però té alguns desavantatges com la seva curta vida mitja, la necessitat de ser generat *in situ*, la seva corrosivitat i la seva toxicitat (AWWA 1995). El tractament dissenyat a la planta 1, si és correctament aplicat garanteix 0,1-0,2 mg/L d'ozó residual a les cambres d'ozonització on l'aigua i roman durant 15-20

minuts, encara que el sistema pot patir parades ocasionals. Diversos estudis descriuen la inactivació efectiva de bactèries i virus amb ozó. Herbold i col. (1989) va descriure reduccions del 93,2% d'*E.coli* en tractaments d'ozó de 0,1 mg/L a 20ºC, del 93,2% d'Hepatitis A a concentracions d'ozó de 0,18 mg/L i del 98,95% de Poliovirus 1 a 0,02 mg/L. Les concentracions d'ozó requerides per inactivar diferents microorganismes són altament dependents del propi microorganisme; poden haver-hi diferències de resistència fins i tot dins la mateixa família. Estudis més recents (Thurston-Enriquez i col. 2005) descriuen un 76% d'eliminació d'HAdV 40 en 0,01 mg d'ozó per litre. Tots aquests estudis es van realitzar amb aigua tamponada sense demanda d'ozó i els virus van ser quantificats per cultiu cel·lular; en canvi en el present estudi el DNA dels virus va ser quantificat en mostres consistents en una mescla d'aigua subterrània i aigua filtrada per sorra. No es descarta la possibilitat de que la major part dels virus fossin inactivats durant la ozonització però les partícules i el DNA quedés relativament intacte. La tendència d'aquests virus a agregar-se no hauria de ser oblidada, els agregats podrien protegir algunes partícules víriques dels tractaments desinfectants. No es va identificar partícules víriques de HAdV infectives en cultiu cel·lular el que suggereix que els genomes quantificats podrien correspondre a virus no infectius.

En aquest cas la reducció més important de virus es va observar després de la filtració per sorra, però probablement el tractament més efectiu és la cloració efectuada a la entrada de la planta.

La planta 2 va presentar nivells més baixos de contaminació. L'aigua subterrània que usa te menys probabilitats de ser contaminada, encara que les filtracions o el contacte amb aqüífers contaminats podrien ser la causa de trobar baixos nivells de contaminació fecal trobats a les mostres. L'eliminació global calculada per HAdV va ser de més de 2 logaritmes, però hi va haver una mostra positiva d'aigua de sortida o sigui que en el pitjor dels casos la reducció va ser de 0,67 logaritmes. Per JCPyV la reducció va ser de més de 2 logaritmes. Els resultats de la quantificació de virus en aquesta planta eren inesperats, ja que la planta usa sistemes de nanofiltració i osmosi reversa. D'acord amb la literatura, cap partícula vírica, ni tan sols DNA podria travessar aquest tipus de membranes però en canvi es troben baixos nivells de virus en totes les fases dels tractaments. Una possible explicació de la detecció esporàdica de virus després d'aquests tractaments és que les membranes o la instal·lació a la planta pot tenir petites fugues. Mi i col. (2004) van estudiar la retenció del bacteriòfag MS2 en membranes d'osmosi reversa. La retenció de les membranes era del 99,9995% però baixava a 99,95-99,99% quan s'usaven membranes amb integritat compromesa. També

hi ha estudis sobre nanofiltració: Yokoyama i col. (2004) van testar l'eliminació de virus amb membranes de 35 nm de diàmetre de porus usant parvovirus humà B19, el virus de l'encefalomiocarditis humà i el parvovirus porcí, tots ells entre 24 i 30 nm de diàmetre. Van trobar que els virus agreguen en presència de certs aminoàcids i podien ésser retinguts a les membranes. Aquests virus són més petits que els HAdV (90 nm) i JCPyV (42-45 nm), usats en aquest estudi. Les membranes usades a la planta 2 tenen un diàmetre de porus de 10 a 30 nm, per tant els virus no haurien de ser capaços de travessar. És probable, com hem comentat anteriorment, que la instal·lació tingués petites fugues o les membranes petites imperfeccions o fissures que podrien deixar passar una petita quantitat de virus. Les concentracions trobades a la planta 2 són al límit de detecció; la eliminació global es baixa, ja que es van trobar mostres positives a llarg de tot el tractament.

Les concentracions de virus trobats a la planta 3 van ser similars als de la planta 1. Els alts nivells de contaminació bacteriana i vírica a l'entrada poden ser explicats pel fer que la planta està situada uns centenars de metres riu avall de l'efluent d'una planta de tractament d'aigua residual i a la mateixa llera. L'eliminació d'HAdV va ser major de 5 logaritmes, però dues mostres d'aigua tractada van ser positives per tant, en el pitjor dels casos la reducció va ser de 4,94 logaritmes. Cap mostra tractada va ser positiva per JCPyV, la seva eliminació va ser major a 5 logaritmes. Els tractaments més efectius en aquesta planta van ser les dues cloracions, una a l'entrada de la planta i l'altra després dels filtres de GAC.

Tres mostres d'aigua tractada de les plantes 2 i 3 van ser positives per HAdV. La caracterització d'aquests virus va donar un 98% d'homologia amb HAdV2 però no van produir infecció en els cultius cel·lulars assajats, indicant que els virus van ser inactivats en fases prèvies del tractament. Un tractament desinfectant pot afectar l'eficiència de tractaments més endavant en el procés. La presència de sòlids suspesos incrementa la resistència dels microorganismes a la desinfecció, per tant una baixa eficiència en eliminar la terbolesa de l'aigua pot disminuir l'eficiència de posteriors tractaments de desinfecció.

Tres plantes de tractament d'aigua de beguda de la zona de París van ésser també mostrejades i analitzades amb èxit, demostrant que el mètode pot ser fàcilment aplicat en altres laboratoris.

Es van trobar consistentment virus a mostres de totes les plantes amb absència o molt baixa concentració d'indicadors, recolzant estudis previs sobre la manca de correlació entre indicadors vírics i bacterians (Albinana-Gimenez i col. 2006, Formiga-Cruz i col. 2003, Gerba i col. 1979, Lipp i col. 2001, Tree i col. 2003). La concentració d'HAdV detectada al riu és equivalent a la concentració observada de *E.coli* i enterococs en moltes mostres, recolzant l'ús dels adenovirus humans com a indicadors d'eliminació de contaminació en plantes de tractament d'aigua de beguda.

En estudis previs en altres plantes de tractament d'aigua de beguda, Ali i col. (2004) van estudiar la presència d'enterovirus per cultiu cel·lular i HAV, HEV i Norovirus per RT-PCR. Es van detectar virus en tots els passos dels tractaments però no a l'aigua tractada. Les eficiències d'eliminació en enterovirus per cultiu van ser de >1 i >2 logaritmes. Abbaszadegan i col. (2008) van realitzar estudis en una planta pilot que incloïa sedimentació i filtració per sorra. La eliminació d'HAdV després de la filtració per sorra va ser de 1,4 logaritmes. En el present estudi hem trobat eficiències més altes després de la filtració per sorra, però hem de tenir en compte que l'aigua filtrada per sorra a la planta 1 havia estat preclorada i que les eficiències trobades a Ali i col. i a Abbaszadegan i col. van ser calculades usant virus infecciosos en cultiu cel·lular.

La qPCR és una tècnica molt sensible i produeix ràpidament dades quantitatives sobre la presència de genomes vírics. Els resultats negatius són un fort indicador de l'eliminació eficient de virus en plantes de tractament d'aigua de beguda, però resultats positius en aigua de beguda requereixen l'avaluació de la seva capacitat infectiva abans de poder calcular acuradament el risc associat. S'han aplicat amb èxit mètodes simples de concentració basats en l'adsorció/elució de virus en llana de vidre en tres plantes de tractament d'aigua de beguda de Catalunya. La quantificació de JCPyV i HAdV amb qPCR ha provat ésser útil per avaluar l'eficiència d'eliminació de virus en plantes de tractament i per la identificació de punts crítics de control i com a índex de la qualitat virològica de l'aigua d'entrada.

Analysis of adenoviruses and polyomaviruses quantified by QPCR as indicators of water quality in source and drinking water-treatment plants

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Abstract.

Three drinking water-treatment plants were analyzed for the presence of human adenoviruses (HAdV) and JC polyomavirus (JCPyV), previously suggested as viral contamination indicators, in order to define their water quality in relation to the presence of viral pathogens and the efficiency of the treatments applied.

The 90% of the river water samples had positive results of HAdV (10^1 – 10^4 Genome Copies (GC)/L); and 48%, of JCPyV (10^0-10^3 GC/L). Lower concentrations of HAdV and JCPyV were found in different treatment steps of the plants in absence of bacterial standards. Virus removal efficiencies were higher than 5 logs in Plants 1 and 3 and could be quantified as >2 logs in plant 2. However, three post-chlorinated samples from plants 2 and 3 (11%) were found to be positive for HAdV by qPCR, but did not show infectivity in the cell cultures assayed. Simple methods based on the adsorption/elution of viruses from glass wool give low-cost and efficient virus recovery from source water and large-volume water samples. Quantification of JCPyV and HAdV using qPCR is useful for evaluating virus removal efficiency in water treatment plants, identification of Hazard analysis and Critical Control Points (HACCP) and as a molecular index of the virological quality of water. Though infectivity is not guaranteed when using qPCR techniques in water treated with disinfection processes, the quality of source water, where viruses have proved to have infective capabilities and the removal efficiency of viral particles may be efficiently quantified.

Keywords: Drinking Water, Polyomavirus, Adenovirus, Norovirus, qPCR.

Introduction

Infected individuals shed large quantities of viral particles in feces and urine and many viruses transmitted by the fecal-oral route are widely prevalent in the environment (IWAPCR, 1983). Viruses such as JC polyomavirus and some human adenovirus strains establish persistent infections and viral particles may be shed in feces for months or even years (Shah, 1995; Wadell et al., 1988). Thus, high viral loads are detected in sewage (Bofill-Mas et al., 2000; Katayama et al., 2008; Pina et al., 1998) and lower concentrations in river water (Albinana-Gimenez et al., 2006; Choi and Jiang, 2005; Haramoto et al., 2005), sea water (Calgua et al., 2008; Haramoto et al., 2007) and even drinking water (Lambertini et al., 2008; Van Heerden et al., 2005). Several studies confirm that infectious water-related diseases are not only a primordial cause of mortality and morbidity worldwide, but also that both the spectrum and incidence of many water-related diseases are increasing (WHO, 2003).

The general demand for high-quality water has increased the pressure on environmental and public health policies to ensure the microbiological safety of water. To reduce human health risk from waterborne and water-related illness, water quality standards are established by the WHO and are adopted by most nations worldwide. Classic microbiological indicators such as fecal coliform and enterococcus are the most commonly used indicators to evaluate the removal of fecal contamination in water purification processes. However, the adequacy of these bacteria for indicating the occurrence and concentration of human viruses and protozoa cysts has been questioned in recent years (Formiga-Cruz et al., 2003; Lipp et al., 2001; Tree et al., 2003).

There is information available about some water-borne pathogens, but the improvement in molecular technology for detecting viruses in water has focused attention on new groups of viruses that could in general terms be considered emergent pathogens in

diverse geographical areas (Bofill-Mas et al., 2000; Pina et al., 1998). Human Polyomavirus JC and human adenoviruses are two groups of viruses that are being detected more often in the environment by molecular methods (Albinana-Gimenez et al., 2006; Bofill-Mas et al., 2000; Haramoto et al., 2007; Pina et al., 1998) and have been suggested as indicators of viral contamination of human origin (Bofill-Mas et al., 2000; Pina et al., 1998).

JC polyomavirus (JCPyV) is a human virus classified in the *polyomaviridae* family that persists indefinitely in individuals and is excreted regularly in urine by healthy individuals (Shah, 1995). The initial infection occurs in childhood and is not apparent, (Major et al., 1992). The virus infects a large number of the population worldwide and, consequently, its presence in water causes no health risk for most of the population. The pathogenicity of the virus is commonly associated with immunocompromised states but has attracted more attention due to AIDS-linked immunosuppression (Berger et al., 1987). JCPyV is associated with progressive multifocal leukoencephalopathy (PML), a fatal demyelinating disease (Berger et al., 1987). Some laboratories have suggested an association between JCPyV and human cancer such as colon cancer (Enam et al., 2002; Hori et al., 2005). In previous studies, JCPyV was found in 98% of 52 sewage samples from widely diverse geographical areas around the world (Bofill-Mas et al., 2000), as well as in river water and in a drinking water-treatment plant (DWTP) (Albinana-Gimenez et al., 2006). The samples were also positive for HAdV, demonstrating that molecular assays to detect HAdV and JCPyV could be useful in identification of human fecal contamination (Bofill-Mas et al., 2001).

Human adenoviruses (HAdV) are members of the genus *Mastadenovirus* in the *Adenoviridae* family, which comprises 51 serotypes classified in 6 species (A-F). They have double-stranded linear DNA and a non-enveloped icosahedral shell with fiber-like

projections from each of the 12 vertices (Stewart et al., 1993). Human adenovirus infections occur worldwide, but many of them run their course asymptomatically. The most common HAdVs (Ad1, 2 and 5) infect 40-60% of children (Brandt et al., 1969). Some serotypes such as 40 and 41 are unique in being responsible for most cases of Ad-associated gastroenteritis in children. Others included in subspecies B are responsible for 5-10% of childhood respiratory diseases and conjunctivitis (Wold and Horwitz, 2007). In previous studies, methods for the molecular detection of HAdV in sewage, shellfish, river water and drinking water have been developed (Albinana-Gimenez et al., 2006; Cho et al., 2000; Formiga-Cruz et al., 2002; Lee et al., 2004; Pina et al., 1998; Puig et al., 1994; Van Heerden et al., 2005).

At a previous stage of this study, several concentration methods were compared and two methods based on adsorption-elution in glass wool were selected (Albinana-Gimenez et al., submitted for publication). At the present stage, 3 DWTPs were selected and several control points in each purification process were surveyed during a one-year period.

The aim of this study was to analyze the levels of HAdV and JCPyV by applying sensitive concentration methods in water and quantifying virus removal efficiency in several drinking water-treatment plants, using quantitative PCR protocols. An overall goal is also to evaluate the applicability of HAdV and JCPyV quantified by qPCR as a tool for the control of the viral quality of source and treated water after a diversity of treatments.

Materials and methods

Sample collection. A total of 144 samples were collected over a period between January 2007 and March 2008 in three drinking water-treatment plants. The volume

analyzed of raw river water samples from plant 1 and 3 was 1 liter and the volume filtered of sedimented water sample from plant 3 was 10 liters. Small volume samples (1 or 10L) were collected, kept at 4°C, filtered and processed within 24h. Large volume samples (400-2500 L) were filtered at the sampling point, the filters kept at 4°C and processed within 24h of collection.

Drinking water-treatment plants. Drinking water-treatment plant 1 is situated in the Barcelona area, next to a river used as source water, and has a treatment capacity of $4.5x10^5$ m^3/day. The raw water is breakpoint pre-chlorinated, and polyelectrolytes and aluminum sulphate are added to the water running into the sedimentation tanks for flocculation. After sedimentation, the water goes through sand filters. At this point there is an intake of ground water. Then the mixed water goes through ozonation and granular activated carbon (GAC) filters. Finally, the water is stored in tanks in which it is post-chlorinated. Samples were collected at the intake point (raw water), after sand filtration, at the intake of ground water, after ozonation, after GAC filtration and after post-chlorination (finished).

DWTP 2 has a treatment capacity of $1.8x10^4$ m^3/day. The source water for this plant comes from three wells in the area. The plant has three parallel treatment lines. In the first line the water goes through filter cartridges, UV treatment and a final membrane filtration. In the other two lines the water goes through filter cartridges, UV treatment and reverse osmosis. The three lines converge in a tank where the water is post-chlorinated. Samples were collected at the ground water intake of both lines (raw), after filtration cartridges of both lines (filtered), after nanofiltration in line 1, after reverse osmosis in line 2 and after post-chlorination (finished).

DWTP 3 is situated in a rural farming area, next to a river used as source water and has a treatment capacity of $1.4x10^4$ m^3/day. The plant intake is situated about hundred

meters downstream from a wastewater treatment plant effluent. The raw water is breakpoint pre-chlorinated and polyelectrolytes are added to the water running into the sedimentation tanks for flocculation. After sedimentation, the water goes through GAC filters and is finally stored in tanks where it is post-chlorinated. Samples were collected at the intake (raw), after sedimentation, after GAC filtration and after post-chlorination (finished).

Bacteriological analysis. The bacteriology of the samples from the rivers (Ter and Llobregat) and from the drinking water-treatment plants was analyzed by previously validated and accredited (ISO 17025) methods, some of them based on ISO Standards: for Heterotrophic Plate Count (aerobic bacteria) at 22ºC ,Water Plate Count Agar (ISO) (Oxoid Inc., Basingstoke, UK); for Coliform bacteria and *E.coli*, Colilert-18 Quantitray (Idexx Inc. Westbrook, ME, USA); for Enterococci, Sklanetz&Barley Agar + Bile-esculine Agar (Oxoid); for *Clostridium perfringens*, TSC Agar (Oxoid) with the fluorogen MUP (Merck & Co. Inc., Whitehouse Station, NJ, USA) and for Aeromonas and Pseudomonas group, GSP Agar (Merck).

Concentration of viruses from water and nucleic extraction. Two variations of the same method were used when processing different volumes. These methods were selected from previous studies (Albinana-Gimenez et al., submitted for publication) and are based on methods originally described by Vilaginès et al. (1993). Small volume samples (1-10L) were pre-acidified to pH 3.5, then filtered at 1L/min through a column with 10g of compacted glass wool (Panreac) previously washed with HCl, NaOH and tap water. The columns were eluted by gravity flow of 200ml of glycine buffer, 0.05M 3% beef extract pH 9.5 ± 0.2. The eluate was flocculated at pH 3.5 ± 0.2 and centrifuged 7,000 *xg* for 30 minutes. The pellet was resuspended in 5 ml PBS and stored at -80°C until analysis. Large volume samples (>15L) were processed following the

same protocol, but without pre-acidification. Nucleic acids were extracted using Nucleospin RNA virus F (Macherey-Nagel). As samples from DWTP 3 had inhibition problems, a second serial extraction was performed with NucliSens magnetic extraction reagents (Biomerieux, The Netherlands).

Enzymatic Amplification. (i) Nested-PCR. Nested-PCR was used for the typification of HAdV-positive samples. Ten-µL aliquots of the extracted nucleic acid and their respective tenfold dilution were used in each test. Amplifications were carried out as described in previous studies, using two amplification rounds with 30 cycles each (Bofill-Mas et al., 2001). Standard precautions were taken when performing the PCR assays: the PCR mix was prepared in a DNA-free environment, the samples were added at a separate location, and positive and negative controls were included in each assay.

(ii) Quantitative Real-Time PCR (qPCR). For the specific detection and quantification of JCPyV and HAdV genomes, 10 µL of the 10-fold and 100-fold dilutions of every DNA extraction were also assayed. These dilutions were made to avoid amplification inhibition due to this assay's high sensitivity to inhibitors. Amplification was performed in a 25-µL reaction mixture with the PCR Master Mix (Applied Biosystems). The reaction contained 10 µL of a DNA sample or 10 µL of a quantified plasmid DNA, 1X TaqMan master mix, and the corresponding primers and TaqMan probes at their corresponding concentrations. JCPyV genomes were quantified with 0.5 µM of the primers JE3F and JE3R and 0.15 µM of the fluorogenic probe JE3P described in Pal et al. (2006). HAdV genomes were quantified with 0.9 µM of the primers AdF and AdR and 0.225 µM of the AdP1 probe described by Hernroth et al. (2002). Following activation of the uracil N-glycosilase contained in the core mix (2 min at 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 with an MX3000P sequence detector system (Stratagene). All

samples were run in quadruplicate, and positive and negative controls were included. The amount of DNA was defined as the average of the quadruplicate data obtained. Inhibition controls were analyzed too: known quantities of viral genomes (10^3 GC/reaction) were added to reactions with sample.

Cell culture infection. Infection assays of HAdV found in finished water, GAC-filtered water samples (Plants 1 and 3) and Nanofiltered and reverse-osmosis water samples (plant 2) were performed on A549 cell line. A549 is an epithelial cell line from human lung carcinoma and was propagated in Earl's minimum essential medium (EMEM) supplemented with 1% glutamine, 50 µg of gentamicin per mL and 5% (growth medium) or 2% (maintenance medium) of heat-inactivated FBS. The assays were performed in duplicate and neat and ten-fold dilution of every sample's concentrate was inoculated. Culture media were removed from the cell culture flasks. Two 85cm^2 cell culture flasks were inoculated with 500µl of the concentrate and three 25cm^2 cell culture flasks were inoculated with 100µl of the ten-fold dilution of the concentrate in PBS. A negative control 25cm^2 flask was inoculated with PBS. Adsorption was performed at 37°C for 1h. The flasks were washed twice and fresh media added. One of the 25cm^2 flasks was frozen at -80°C as T0 and the rest were incubated at 37°C for two weeks and then frozen at -80°C.

Typification of human adenoviruses. Amplicons obtained from HAdV-positive treated water samples were purified using the QIAquick PCR purification Kit, following the manufacturer's instructions. Both strands of the purified DNA amplicons were sequenced with the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction V 3.1 kit with AmpliTaq DNA polymerase FS (Applied Biosystems), following the manufacturer's instructions. The results were checked using the ABI PRISM 3700 DNA analyzer (Applied Biosystems), and the sequences were compared with those present in

GenBank and the European Molecular Biology Library by means of the basic BLAST program of the National Center for Biotechnology Information (available from URL: http://www.ncbi.nlm.nih.gov/BLAST/).

Results

Bacteriological analyses. *Plant 1.* Raw river water had significant concentrations of bacterial indicators with average values of 3.4 x 10^4 total coliforms cfu/100ml, 2.1 x 10^3 *E.coli* cfu/100ml, 2.5 x 10^3 *C. perfringens* cfu/100ml, 4.4 x 10^2 *Enterococci* cfu/100ml and 2.8 x 10^5 Heterotrophic bacteria cfu/ml. The counts decreased drastically after sand filtration, with sporadic low counts found in the following treatments. However, decreasing Heterotrophic plate counts were observed during all the treatment.

Plant 2. Negative results and sporadic low counts (~ 10^0 cfu/100ml) of all bacterial indicators were found throughout the process. In the case of heterotrophic bacteria, 10^1 – 10^2 cfu/ml were found in all the steps of the process, showing no reduction.

Plant 3. Raw river water had higher concentrations of bacterial indicator than plant 1, with average values of 7.5 x 10^4 total coliforms cfu/100ml, 6.7 x 10^3 *E.coli* cfu/100ml, 5.2 x 10^3 *C. perfringens* cfu/100ml, 7.3 x 10^3 *Enterococci* cfu/100ml and 5.1 x 10^4 Heterotrophic bacteria cfu/ml. One log reduction in the bacterial counts was seen after the sedimentation. GAC-filtered water had no bacterial indicators, except 1.5 x 10^2 Heterotrophic bacteria cfu/ml. No finished water sample had any positive standard bacterial parameter.

Quantification of viruses in three drinking water-treatment plants. The concentration of virus detected is expressed as genome copies (GC) per L, and the results are shown in Tables 1, 2, and 3. All the river water samples from plant 1 were

positive for HAdV in 1 liter analyzed in this study, with a mean concentration of $1.24x10^4$ GC/L; and 44% were positive for JCPyV, with a mean concentration of $7.4x10^2$ JCPyV GC/L. The results of river water obtained in plant 3 are similar, with 83% of the samples positive for HAdV ($9.24x10^3$ GC/L) and 50% positive for JCPyV ($1.3x10^3$ GC/L).

The raw water samples from plant 2 had lower levels of viral contamination, as expected in ground water. Similar levels of viral contamination were found throughout the treatment and higher reduction was observed after the final chlorination.

The reduction of the concentrations of HAdV and JCPyV quantified as genome copies during treatment in the drinking water-treatment plants is calculated by the difference between the concentration in raw water and the concentration in finished water (figures shown in Tables 1, 2 and 3). Data from plant 1 indicate that the reduction in HAdV is more than 5.13 logs for finished water, and 5.13 logs between raw water and GAC-filtered water; for JCPyV, the reduction is >5 logs for finished water, and 3.51 logs between raw water and GAC-filtered water. Data from plant 2 show that low quantities were seen throughout the treatment plant and the reduction is quantified as a reduction of HAdV: > 2 logs in most of the cases. One finished water sample was positive, with removal efficiency occasionally quantified as decreasing to 0.85 logs. The removal efficiency of JCPyV reaches >2 logs. Data from plant 3 show that the reduction of HAdV through the process reaches >5 logs, but occasionally (as the two positive finished water samples show) it decreases to 4.94 logs; for JCPyV, efficiency is >5 logs. HAdV genomes were detected in one finished water sample from plant 2 and two finished water samples from plant 3 at low concentrations ($6.11x10^0$ GC/L, $1.26x\ 10^0$ GC/L and $4\ x10^{-2}$ GC/L, respectively). No finished water samples from DWTP 1 were found to be positive however, HAdV was found in 22.2% of the GAC-filtered water

samples. JCPyV was not found in any of the finished water samples, but was found in one sample from GAC-filtered water in plant 1 and 5 samples from GAC-filtered water in plant 3.

Infection assays. The three positive finished water samples from plants 2 and 3, one GAC-filtered water sample from plant 1, three GAC-filtered water samples from plant 3, one nanofiltered water sample and one water sample after reverse osmosis from plant 2, were inoculated in A549 cell culture. No CPE was observed at 10 days post-infection and no DNA amplification was detected when PCR was performed in the culture supernatants.

Characterization of viruses. HAdV nucleic acids from the positive finished water sample concentrates were amplified by conventional nested-PCR and sequenced. All the sequences had 98% homology and were characterized as HAdV 2.

Discussion

High viral loads are being shed in the environment and in rivers used as source water by drinking water-treatment plants. In previous studies, levels of human viral contamination in sewage and wastewater treatment plants were analyzed (Albinana-Gimenez et al., 2006; Bofill-Mas et al., 2000; Bofill-Mas et al., 2006; He and Jiang, 2005; Katayama et al., 2008; Pina et al., 1998). All these studies reported high concentrations of viruses in sewage. During this study, in the geographical area studied here, the levels of HAdV found in urban sewage were $3.8x10^7$ HAdV GC/L and $3.4x10^7$ JCPyV GC/L. The levels found in the effluent of a wastewater treatment plant with conventional secondary treatment were $8.08x10^4$ HAdV GC/L (Bofill-Mas et al., 2006). Effluents of wastewater treatment plants are commonly discharged into rivers that are

used as source water in drinking water-treatment plants. This viral load could represent a risk of infections in the population if efficient drinking water treatment is not applied and properly controlled before tap water distribution and consumption. HAdV have been described as stable in the environment and highly resistant to UV disinfection (Thompson et al., 2003). The stability of HAdV and JCPyV in sewage has been calculated by qPCR to be very high, showing t_{90} of 60.9 and 63.9 and t_{99} of 132.3 and 127.3 days for HAdV and JCPyV, respectively (Bofill-Mas et al., 2006).

In this study, HAdV and JCPyV were quantified by qPCR in several treatment steps of three drinking water-treatment plants in North-eastern Spain. The concentration method used gave better recovery efficiencies than other methods used (Albinana-Gimenez et al., 2006). Consequently, the GC concentration quantified is significantly higher than the concentration observed in the same location in the previous study, with levels of viruses in raw river water of 1.21×10^4 HAdV GC/L and 7.4×10^2 JCPyV GC/L, as against 4×10^2 HAdV GC/L and 2.6×10^1 JCPyV GC/L in plant 1. The use of electropositive filters, as described in Albinana-Gimenez et al. (2006), was proved less efficient than the glass wool filtration protocol used in this study.

Plant 1 showed >5.13 logs of HAdV removal between raw and finished water and 5.13 logs of virus removal between raw and GAC-filtered water. Only one of the GAC-filtered water samples was positive. For JCPyV, >5 logs of virus removal was observed between raw and finished water and 3.51 logs between raw and GAC-filtered water. The ozonation treatment applied seems to have little effect on the quantification of viral genome copies, although 98.8% elimination of heterotrophic bacteria was observed (data not shown). Ozone is a very strong oxidizing agent and an effective alternative to chlorine for pathogen reduction in water, but has some disadvantages such as its short half-life, its need to be generated on-site, its corrosivity and its toxicity (AWWA 1995).

The treatment designed in plant 1, if correctly applied, guarantees 0.1-0.2 mg/L residual ozone in the ozonation chambers where water remains an average of 15-20 minutes, although the systems can suffer occasional shutdowns. Several studies report effective inactivation of indicator bacteria and viruses by ozone. Herbold et al. (1989) reported 93.2% reduction of *E. coli* in ozone treatment of 0.1 mg/L at 20ºC; 93.2% reduction of Hepatitis A virus (HAV) at 0.18 mg/L; and 98.95% reduction of Poliovirus 1 at 0.02 mg/L. The ozone concentration needed to inactivate different microorganisms is highly dependant on the microorganism itself. There may be differences in resistance even between two viruses of the same family. More recent studies by Thurston-Enriquez et al. (2005) report 76% elimination of HAdV 40 with 0.01 mg/L. These studies were performed in ozone-demand-free buffered water and the viruses were quantified by cell culture infection, whereas in the present study total viral DNA was quantified in samples consisting of a mix of ground water and sand-filtered water. It is possible that most of the viruses were inactivated during the ozonation treatment, but the particles and the DNA remained relatively intact. However, the tendency of these viruses to aggregate should not be forgotten: the aggregates could protect some viral particles from the action of disinfectant treatments. Although ozone can also cause DNA damage (Ito et al., 2005), our finding no infective HAdV particles in GAC-filtered water supports the hypothesis that the viral genomes quantified may correspond to non-infectious viruses.

In the present case, the highest virus removal was found after sand filtration, but the most effective treatment would probably be the breakpoint chlorination performed at the intake of the plant. However, other problems arise when chlorine is added to highly contaminated water, such as production of trihalomethanes and other toxic compounds that would need to be also considered when a DWTP is designed.

Plant 2 had lower levels of virus contamination. Since it uses ground water, this source water is less likely to be fecally contaminated, although filtrations or contact with contaminated aquifers may be related to the presence of low levels of fecal contamination in the samples. The virus quantification results in this plant were quite unexpected, as the plant uses treatments such as membrane nanofiltration and reverse osmosis. Although, according to the literature, no viral particle or even DNA fragments can pass through these kinds of filters; as shown in Table 2, low virus loads after all the treatments in the plant. One possible explanation for the sporadic detection of viruses after these treatments is that the membranes or installations in the plant may have small leaks. Mi et al. (2004) studied the retention of MS2 bacteriophage through reverse-osmosis membranes. The virus rejection with intact membranes was >99.9995% but decreased to 99.95-99.9 % when membranes with compromised integrity were used. There are also studies of virus removal by nanofiltration. Yokoyama et al. (2004) tested the virus removal of nanofiltration membranes (35 nm pore diameter) with human parvovirus B19, human encephalomyocarditis virus (EMC) and porcine parvovirus (PPV), all of them between 24-30nm, under diverse conditions. They found that viruses aggregate in the presence of certain kinds of amino acids and thus can be removed by nanofiltration; but that, in the absence of amino acids, there was no removal. These viruses are smaller than HAdV (90 nm) and JCPyV (42-45nm) used in the present study. The membranes used in plant 2 have a pore range of 10-30nm, so viruses should not be able to pass through them. It is probable, as commented above for reverse osmosis, that the installation has microleaks and/or the membranes have imperfections or small fissures that could let through a small quantity of viral particles. The viral concentrations observed in plant 2 are at the limit of detection and the overall removal

efficiency quantified is low, since sporadic positive samples are identified throughout the treatment.

The virus concentrations in plant 3 were similar to those in plant 1. The high levels of viruses found in raw river water can be explained by there being a wastewater treatment plant effluent a few meters upstream and on the same bank. The HAdV removal efficiency calculated was higher than 5 logs, but two of the finished water samples were positive at low concentrations. Thus, in the worst case, removal efficiency decreases to 4.94 logs. No finished water samples were positive for JCPyV; the removal efficiency calculated is >5 logs. The most effective treatments in this plant were again the two chlorinations, one at the intake of the plant and the other after GAC filtration.

Three finished water samples were positive for HAdV in plants 2 and 3, but the viruses found did not produce infection in the cell culture assayed. The performance of a treatment unit can affect the efficiency of downstream treatment units. The presence of suspended solids increases the resistance of most microbes to disinfection (LeChevalier and Au, 2004). Therefore, failure in the removal efficiency of turbidity or particles can decrease the inactivation efficiency of disinfection processes.

Three water treatment plants from the Paris area were also sampled and analyzed successfully with the method developed in this project, demonstrating that the method can easily be applied in other laboratories (data not shown).

Viruses were consistently found in samples from all the plants with absence or very low concentration of bacterial indicators, supporting previous studies on the lack of correlation between bacterial and viral indicators (Albinana-Gimenez et al., 2006; Formiga-Cruz et al., 2003; Gerba et al., 1979; Lipp et al., 2001; Tree et al., 2003). The concentration of human adenoviruses detected in river water was equivalent to the

concentration observed for *E. coli* and *Enterococci* in many samples, which supports the use of adenoviruses as indicators of virus removal in drinking water-treatment plants.

Although a significant reduction of viral GC quantified by qPCR was observed after chlorination, human Adenovirus 2 was found in post-chlorinated water from plants 2 and 3, indicating that this virus is highly resistant to water treatments. However, no infective HAdV particles were found in these samples or in GAC-filtered water, suggesting that the viruses could be inactivated during the treatment.

In previous studies of several DWTPs in Egypt, Ali et al. (2004) studied the presence of enteroviruses by plaque infectivity assay in BGM, HAV, HEV and of Norovirus by RT-PCR. Viruses were detected at every step of the treatment, but none was detected in post-chlorinated water. Removal efficiencies of >1 log and >2 logs were observed for the infecting enteroviruses. Abbaszadegan et al. (2008) performed studies in a pilot DWTP, including sedimentation and sand filtration evaluating bacterophages as viral surrogates to study adenovirus and Feline Calicivirus removal. The removal efficiencies obtained for HAdV were 1.4 logs after sand filtration. In the present study higher removal efficiencies after sand filtration were observed, but included chlorination. Another important difference is that the efficiencies found in Ali et al. and Abbaszadegan et al. were calculated using infectious viral particles in cell culture.

qPCR techniques are highly sensitive and produce quick quantitative data on the presence of viral genomes. Negative qPCR results are a strong indicator of efficient virus removal in drinking water-treatment plants, but positive results in finished water where disinfectants have been used would require their infection capacity to be tested before the risk associated could be realistically evaluated. Simple methods based on adsorption-elution of viruses in glass wool were successfully used to survey the virological quality of the water at three drinking water-treatment plants in North-eastern

Spain. Quantification of JCPyV and HAdV using qPCR proved useful for evaluating virus removal efficiency in drinking water-treatment plants, identifying HACCP (Hazard analysis critical control points) and as a molecular index of the virological quality of source water, including identification of microbial fecal contamination of human origin.

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Table 1. Concentration of HAdV and JCPyV in plant 1.

Sample	Vol. (L)	HAdV				JCPyV			
		% positive	gc/L	Std Dev.	Reduct.[a]	% positive	gc/L	Std Dev.	Reduct.[a]
Raw	1	100 (9/9)	$1.24x10^{4}$	$1.60x10^{4}$	-	44.4 (4/9)	$7.40x10^{2}$	$1.10x10^{3}$	-
Sand-filtered	426	55.6 (5/9)	$7.02x10^{0}$	$1.56x10^{1}$	3.24	22.2 (2/9)	$2.03x10^{-2}$	$5.70x10^{-2}$	4.56
Ground water	796	44.4 (4/9)	$2.05x10^{0}$	$3.87x10^{0}$	-	44.4 (4/9)	$1.43x10^{0}$	$3.82x10^{0}$	-
Ozonated	508	44.4 (4/9)	$8.70x10^{0}$	$1.58x10^{1}$	3.15	44.4 (4/9)	$8.24x10^{0}$	$1.57x10^{1}$	1.95
GAC-filtered	655	11.1 (1/9)	$9.10x10^{-2}$	$2.37x10^{-1}$	5.13 (>5.13)	11.1 (1/9)	$2.35x10^{-1}$	$7.05x10^{-1}$	3.51 (>4)
Finished	980	0 (0/9)	ND[b]	ND[b]	>5.13	0 (0/9)	ND[b]	ND[b]	>5

[a] Accumulative elimination efficiency expressed in log(10). In brackets: potential efficiency without the sporadic positive sample. [b] ND: Not detected

Table 2. Concentration of HAdV and JCPyV in plant 2.

Sample	Vol. (L)	HAdV				JCPyV			
		% positive	gc/L	Std Dev.	Reduct.[a]	% positive	gc/L	Std Dev.	Reduct.[a]
Raw line 1	1261	66.7 (4/6)	$7.36x10^{0}$	$1.73x10^{1}$	-	50.0 (3/6)	$2.46x10^{0}$	$3.76x10^{0}$	-
Filtered Line 1	1479	16.7 (1/6)	$1.20x10^{-1}$	$2.93x10^{-1}$	1.78	50.0 (3/6)	$2.81x10^{0}$	$5.49x10^{0}$	0
Nanofiltered Line 1	1659	33.3 (2/6)	$3.61x10^{0}$	$8.78x10^{0}$	0.3	50.0 (3/6)	$1.01x10^{0}$	$1.53x10^{0}$	0.38
Raw Line 2	1607	50.0 (3/6)	$3.45x10^{0}$	$4.46x10^{0}$	-	50.0 (3/6)	$1.84x10^{0}$	$2.79x10^{0}$	-
Filtered Line 2	2100	66.7 (4/6)	$4.21x10^{0}$	$4.64x10^{0}$	0	66.7 (4/6)	$1.47x10^{0}$	$1.85x10^{0}$	0.09
Osmosis Line 2	1845	50.0 (3/6)	$1.24x10^{0}$	$2.85x10^{0}$	0.44	16.7 (1/6)	$5.00x10^{-1}$	$1.23x10^{0}$	0.56
Finished	1285	16.7 (1/6)	$1.02x10^{0}$	$2.49x10^{0}$	0.67 (>2)	0 (0/6)	ND[b]	ND[b]	>2

[a] Accumulative elimination efficiency expressed in log(10). In brackets: potential efficiency without the sporadic positive sample. [b] ND: Not detected

Table 3. Concentration of HAdV and JCPyV in plant 3.

Sample	Vol. (L)	HAdV				JCPyV			
		% positive	gc/L	Std Dev.	Reduct.[a]	% positive	gc/L	Std Dev.	Reduct.[a]
Raw	1	83.3 (10/12)	$9.24x10^{3}$	$1.35x10^{4}$	-	50.0 (6/12)	$1.30x10^{3}$	$3.00x10^{3}$	-
Sedimented	10	16.7 (2/12)	$9.58x10^{1}$	$2.96x10^{2}$	1.98	50.0 (6/12)	$1.74x10^{2}$	$2.72x10^{2}$	0.87
GAC-filtered	926	58.3 (7/12)	$2.21x10^{1}$	$5.03x10^{1}$	2.62	45.5 (5/12)	$2.19x10^{0}$	$5.45x10^{0}$	2.77
Finished	1099	16.7 (2/12)	$1.07x10^{-1}$	$3.63x10^{-1}$	4.94 (>5)	0 (0/12)	ND[b]	ND[b]	>5

[a] Accumulative elimination efficiency expressed in log(10). In brackets: potential efficiency without the sporadic positive sample. [b] ND: Not detected

Annex B. Resultats dels anàlisis microbiològics i fisico-químics de les mostres recollides.

Les mostres analitzades per poliomavirus humans i adenovirus humans van ser analitzades paral·lelament pels indicadors microbiològics clàssics i els paràmetres físico-químics. En els següents apartats es resumeixen els resultats obtinguts en les tres plantes estudiades.

B.1 Materials i mètodes.

Els anàlisis bacteriològics de les mostres d'aigua es van fer seguint mètodes prèviament validats i acreditats (ISO 17025), alguns d'ells basats en estàndards ISO: pel contatge en placa d'heteròtrofs (aerobis totals) a 22ºC es va usar el "Water Plate Count Agar" (ISO) (Oxoid Inc., Basingstoke, UK); per la quantificació de bactèries coliforms i *E.coli*, el Colilert-18 Quantitray (Idexx Inc. Westbrook, ME, USA); Pels enterococs, l'agar Sklanetz & Barley + Bilis-esculina (Oxoid) i per *Clostridium perfringens* TSC Agar (Oxoid) amb el fluorogen MUP (Merck & Co., Inc. Whitehouse Station, NJ, USA).

Els anàlisis físico-químics van ser realitzats en el punt de mostreig en el cas de la temperatura i la conductivitat, amb un conductímetre prèviament calibrat. El pH, la turbidesa i el clor lliure van ser mesurats el mateix dia del mostreig.

B.2 Resultats

B.2.1 Planta 1

Resultats dels paràmetres microbiològics i físico-químics de la planta 1. La procedència de les mostres està indicada en anglès per millorar la concordança amb l'article i evitar confusions.

Taula B.2.1.1. Valors mitjans dels paràmetres microbiològics de les mostres de la planta 1.

Mostra	Coliforms totals (ufc/100ml)	E. coli (ufc/100ml)	Clostridium p. (ufc/100ml)	Enterococs (ufc/100ml)	aerobis totals 22°C (ufc/ml)
Raw	$3,8 \times 10^4$	$2,1 \times 10^3$	$2,5 \times 10^3$	$4,4 \times 102$	$2,8 \times 10^5$
Sand filtered	1×10^0	<1	3×10^0	<1	$6,3 \times 10^3$
Ground water	2×10^0	<1	<1	<1	$1,7 \times 10^1$
Ozonizated	<1	<1	1×10^0	<1	$7,5 \times 10^1$
GAC filtered	1×10^0	<1	<1	<1	$2,1 \times 10^3$
Finished	<1	<1	<1	<1	2×10^0

Taula B.2.1.2. Valors mitjans dels paràmetres fisico-químics de les mostres de la planta 1.

Mostra	Temp. (°C)	pH	Conductivitat (µS/cm)	Turbidesa (UT)	Clor lliure (mg/L)
Raw	17,1	8,1	1662	50,29	NT
Sand filtered	15,6	7,3	1690	1,78	0,27
Ground water	17,7	7,0	1801	0,16	NT
Ozonizated	17,0	7,4	1746	0,75	NT
GAC filtered	16,8	7,2	1752	1,03	NT
Finished	17,5	7,2	1715	0,43	0,98

NT: No testat

B.2.2 Planta 2

Resultats dels paràmetres microbiològics i físico-químics de la planta 2. La procedència de les mostres està indicada en anglès per millorar la concordança amb l'article i evitar confusions.

Taula B.2.2.1. Valors mitjans dels paràmetres microbiològics de les mostres de la planta 2.

Mostra	Coliforms totals (ufc/100ml)	E. coli (ufc/100ml)	Clostridium p. (ufc/100ml)	Enterococs (ufc/100ml)	aerobis totals 22°C (ufc/ml)
Raw line 1	1×10^0	<1	<1	<1	$7,5 \times 10^1$
Filtered line 1	2×10^0	<1	<1	<1	$7,9 \times 10^1$
Nanofilt. line 1	<1	<1	<1	<1	$1,6 \times 10^3$
Raw line 2	<1	<1	<1	<1	$1,1 \times 10^2$
Filtered line 2	3×10^0	<1	<1	<1	$6,7 \times 10^2$
Osmosis line 2	<1	<1	<1	<1	$2,3 \times 10^2$
Finished	<1	<1	<1	<1	$1,3 \times 10^1$

Taula B.2.2.2. Valors mitjans dels paràmetres fisico-químics de les mostres de la planta 2.

Mostra	Temp. (°C)	pH	Conductivitat (µS/cm)	Turbidesa (UT)	Clor lliure (mg/L)
Raw line 1	19,1	7,6	1311	0,28	NT
Filtered line 1	17,5	7,2	1454	0,14	NT
Nanofilt.line 1	21,3	6,3	70	0,15	NT
Raw line 2	17,2	6,9	1290	0,22	NT
Filtered line 2	17,4	6,9	1348	0,22	NT
Osmosis line 2	18,7	6,5	33	0,22	NT
Finished	18,9	7,4	159	0,47	0,86

NT: no testat

B.2.3 Planta 3

Resultats dels paràmetres microbiològics i físico-químics de la planta 3. La procedència de les mostres està indicada en anglès per millorar la concordança amb l'article i evitar confusions.

Taula B.2.3.1. Valors mitjans dels paràmetres microbiològics de les mostres de la planta 3.

Mostra	Coliforms totals (ufc/100ml)	E. coli (ufc/100ml)	Clostridium p. (ufc/100ml)	Enterococs (ufc/100ml)	aerobis totals 22°C (ufc/ml)
Raw	$7,5 \times 10^4$	$6,7 \times 10^3$	$5,2 \times 10^3$	$3,7 \times 10^3$	$5,1 \times 10^4$
Sedimented	$2,7 \times 10^3$	$2,2 \times 10^2$	$1,1 \times 10^3$	$1,6 \times 10^2$	$5,1 \times 10^3$
GAC filtered	<1	<1	2×10^0	<1	$1,5 \times 10^2$
Finished	<1	<1	<1	<1	<1

Taula B.2.3.2. Valors mitjans dels paràmetres fisico-químics de les mostres de la planta 3.

Mostra	Temp. (°C)	pH	Conductivitat (µS/cm)	Turbidesa (UT)	Clor lliure (mg/L)
Raw	15,7	8,0	316	21,75	NT
Sedimented	16,5	8,0	340	1,47	NT
GAC filtered	16,2	7,9	343	0,63	NT
Finished	16,7	7,9	348	0,52	0,56

NT: no testat

B.3 Interpretació

L'aigua crua de riu (raw) tant de la planta 1 com de la 3 va presentar nivells significatius de contaminació fecal, similars als trobats en el capítol anterior.

A la planta 1 es veu una disminució dràstica de la contaminació en l'aigua filtrada per sorra (sand filtered), aquesta aigua ha passat per una pre-cloració, per una sedimentació i una filtració per sorra. En fases posteriors del procés de potabilització els nivells dels indicadors van desaparèixer excepte en casos esporàdics. En el cas dels aerobis es va observar una important davallada dels nivells després de la filtració per sorra, deguda a la cloració inicial, la decantació i la filtració per sorra; també es va observar una forta davallada després de la ozonització, demostrant que aquest procés funciona correctament i els aerobis totals són sensibles a les concentracions d'ozó residual usades. S'observa un significatiu augment d'aerobis totals a la mostra d'aigua filtrada per carbó; aquest augment ha estat descrit anteriorment i és degut a l'efecte bioacumulador dels filtres de carbó activat (Galofré i col. 2004). Les grans quantitats de matèria orgànica retingudes als filtres afavoreixen una lleu multiplicació dels microorganismes que hi romanen. Pel que fa a les dades físico-químiques es pot observar que el riu Llobregat te una alta terbolesa amb un pH d'aproximadament 8. Durant el procés de potabilització el pH s'estabilitza al voltant de 7, la conductivitat es manté i la terbolesa baixa.

L'aigua crua provinent dels dos pous d'entrada a la planta 2 van presentar nivells molt baixos de contaminació bacteriana. Excepte casos esporàdics, els únics indicadors trobats van ser els aerobis totals a 22ºC que van presentar nivells més elevats degut probablement al biofilm present a les canonades dels punts de mostreig. Pel que fa a les dades físico-químiques es pot observar una clara disminució de la conductivitat després de la nanofiltració i la òsmosi inversa, degut a que gran part dels ions que conté l'aigua es queden retinguts en aquests processos. El pH es manté durant tot el procés, com també es manté la terbolesa amb un petit augment a la mostra post-clorada (finished) deguda a la precipitació d'òxids de ferro i manganès.

La planta 3 va presentar nivells significatius d'indicadors bacterians a l'aigua d'entrada (raw) que van anar disminuint fins que van pràcticament desaparèixer a l'aigua filtrada per carbó (GAC filtered). El riu Ter presenta una menor turbidesa i una menor conductivitat que el riu Llobregat. El pH es manté a 8 durant tot el procés de potabilització. Cal remarcar que la concentració de clor lliure a la mostra tractada

(Finished) va ser sensiblement menor que a les plantes 1 i 2, fet que potser va afavorir la possibilitat de trobar virus en aquestes mostres.

En l'aigua de riu, la concentració d'adenovirus humans i d'indicadors bacterians com *E.coli*, clostridis sulfit reductors i enterococs és similar, situant-se en el mateix ordre. Aquest fet reforça el paper dels adenovirus humans com a indicadors vírics de contaminació fecal d'origen humà.

Annex C. Anàlisi quantitatiu del risc microbiològic (QMRA).

C.1 Introducció

L'anàlisi del risc en relació a l'aigua de beguda es realitza degut a una sèrie de raons (Percival i col. 2000):

- Per predir el pes d'una malaltia transmesa per l'aigua en una comunitat, tan en situació de brot epidèmic com en situació normal. Això ajuda a determinar l'impacte de les millores en l'aigua de beguda en la salut i com a mecanisme que impulsa cap a una millora de la qualitat.
- Per ajudar a determinar estàndards microbiològics per aigua de beguda que representin nivells tolerables de malalties a la població.
- Per identificar la opció més rendible i menor cost per reduir el risc per la salut als consumidors.
- Per ajudar a determinar el tractament òptim de l'aigua que equilibri risc microbiològic amb risc químic degut a productes secundaris dels desinfectants.
- Per proporcionar un marc conceptual que ajudi a individus i organitzacions comprendre la naturalesa del risc en l'aigua de beguda i com aquest risc es pot minimitzar.

El risc pot ser definit senzillament com "la possibilitat de pèrdua, dany o lesió". Aquesta definició inclou dos conceptes separats: La probabilitat d'un succés i la severitat d'aquest succés. Aquests dos conceptes són il·lustrats a la taula C.1.1, aquest model ajuda a prioritzar les accions que s'han de prendre davant determinat risc.

Taula C.1.1. Classificació bidimensional del risc (extret de Hunter i col. 2003).

Severitat	**Probabilitat del succés**	
	Baixa probabilitat de dany sever (prioritat immediata)	Alta probabilitat de dany sever (atenció urgent)
	Baixa probabilitat de dany suau (ignorar o baixa prioritat)	Alta probabilitat de dany suau (prioritat intermèdia)

Tot i la simplicitat d'aquest model bidimensional, el processos que permeten el càlcul o la quantificació del risc poden diferir molt. Moltes decisions basades en el risc són subjectives o semi-quantitatives. Inclús quan els anàlisis de risc es presenten de manera numèrica aparentment objectiva sovint estan basats en assumpcions que són subjectives. Un dels problemes més grans d'aquest tipus d'estudis és la qualitat i els nivells d'incertesa en les dades (Macgill i col. 2001).

L'aproximació que fa el QMRA al risc difereix de les aproximacions epidemiològiques ja que aquest últim busca mesurar els nivells reals de malaltia en la població mentre que el primer intenta calcular el risc a partir del que se sap, o es pot inferir sobre la concentració de patògens en aigua de beguda i la infectivitat d'aquests patògens.

Determinar la exposició al patogen és el primer pas per l'avaluació matemàtica del risc microbiològic. L'objectiu es determinar els possibles patògens presents, les dosis consumides i les característiques dels patògens que definiran el resultat. Tot això es formalitza en quatre passes clau (Taula C.1.2).

Taula C.1.2. Passes per la realització d'un anàlisi quantitatiu del risc microbiològic (Hunter i col. 2003)

Pas	Objectiu
Formalització del problema i identificació del perill.	Descriure el marc ambiental i els patògens rellevants que puguin causar efectes en la salut humana.
Anàlisis dosi-resposta	Trobar relacions apropiades entre la exposició al patogen i la infecció o malaltia (d'estudis epidemiològics).
Anàlisi de l'exposició	Determinar la mida i naturalesa de les poblacions exposades a cada patogen per ruta, quantitat i durada de l'exposició.
Caracterització del risc	Integrar la informació de la exposició i la dosi-resposta, expressar el risc per la salut pública, tenint en compte la variabilitat i la incertesa de les estimacions.

En el present estudi, hem escollit els adenovirus humans, molt resistents a l'ambient i als processos de potabilització. Els HAdV 1, 2 i 5 infecten del 40 al

60% dels nens als 10 anys (Brandt i col. 1969). Els serotips 40 i 41 estan considerats la segona causa de gastroenteritis en infants, després dels rotavirus.

C.2 Materials i mètodes

C.2.1 Anàlisi de l'exposició.

L'exposició va ser calculada a partir de la concentració d'HAdV a l'aigua crua. Per analitzar l'exposició de la població al patogen (Pas 3), hem d'estimar la dosi de virus que diàriament entra en contacte amb un individu de la població i es pot calcular de la següent manera (Teunis i col. 1996):

$$D = C \times \frac{1}{R} \times I \times 10^{-DR} \times V \times N \qquad [1]$$

On:

C= Concentració de microorganismes patògens a l'aigua (pot ser aigua de beguda o aigua no tractada).

R= Recuperació del mètode de concentració de virus.

I= Fracció dels patògens detectats que són capaços d'infectar la població.

DR=Eficiència d'eliminació dels processos de potabilització (DR=0 si treballem amb aigua de beguda).

V= Consum diari d'aigua d'un individu (en litres).

N= Fracció de les còpies genòmiques corresponents a virus infecciosos.

Taula C.2.1.1. Paràmetres del model per les tres plantes.

Parametres	Planta 1	Planta 2	Planta 3
R (%)	4,18%	1,21%	4,18%
I	1	1	1
DR	5,13	0,64	4,94
V (litres)	1	1	1
N (%)	1%	1%	1%

Per realitzar aquest estudi hem usat els valors de concentració de virus trobats en les mostres d'aigua crua de les tres plantes estudiades en aquest capítol. El valor que hem assignat a les variables és el següent:

C: mitja de la concentració de virus trobada a la mostra a estudiar. Els valors negatius, per sota del límit de detecció de la tècnica emprada, s'han considerat com a concentració 0.

R: Eficiència de recuperació del mètode de concentració emprat. S'han usat els valors indicats al capítol III.

I: Viabilitat. En aquest estudi ens hem posat en el pitjor dels casos, és a dir que una sola partícula vírica és capaç de produir infecció i que tota la població és susceptible al virus.

DR: Eficiència d'eliminació dels processos de tractament. Com hem treballat amb dades d'aigua crua hem usat per cada planta els valors màxims d'eliminació que hem pogut calcular.

V: Consum diari. No hi ha dades específiques sobre el consum diari d'aigua a l'àrea estudiada. En molts estudis de risc usen el consum diari de 2 litres. En el present estudi hem considerat que en la nostra àrea és molt poc probable que la població consumeixi 2 litres diaris d'aigua de l'aixeta sense tractar de cap manera. Per tant hem estimat el consum d'**1** litre diari per persona.

N: En els nostres estudis hem comparat la quantificació de les còpies genòmiques (GC) per qPCR i la quantificació de unitats formadores de placa (PFU) corresponents a virus infectius. Aquests estudis han mostrat que aproximadament un 1% de les GC representen una unitat formadora de clapa, infectant en un cultiu ce·lular apropiat, tot i que no es una comparació acurada ja que les partícules víriques formen agregats i per tant la quantificació de la infectivitat per PFU està subestimada. En el present estudi hem usat el valor **0,01**.

C.2.2 Caracterització del risc

Per l'anàlisi del risc d'infecció hem escollit el model exponencial que segueix la següent equació:

$$P_{i/d} = 1 - \exp(-rD) \qquad [2]$$

On D= dosi del patogen i r= paràmetre dosi-resposta.

Si $P_{i/d(t)}$ és el risc diari al dia t i assumint que són estocàsticament independents, el risc anual per una o més infeccions P_y pot ser expressat com:

$$P_y = 1 - \prod_{t=1}^{365} \left(1 - P_{i/d}(t)\right) \quad [3]$$

Un model usat sovint en anàlisi de risc considera que $P_{i/d(t)}$ és constant al llarg de l'any (Haas i col. 1999). Si prenem $P_{i/do}$ com el risc diari comú per tots els dies, la fórmula [3] es pot simplificar fins a:

$$P_y = 1 - \left(1 - P_{i/do}\right)^{365} \quad [4]$$

C.2.3 Anàlisi de la sensibilitat

L'anàlisi de la sensibilitat és l'estudi de cóm la variació en les dades de sortida d'un model matemàtic poden ser adjudicats a diferents causes de la variació de les dades d'entrada del model. L'anàlisi de sensibilitat es va efectuar per determinar la influència del paràmetre dosi-resposta (r) en el risc d'infecció diari i anual. El paràmetre r és la proporció del patogen que realment produeix infecció. Aquest valor és el més difícil d'estimar, ja que no hi ha estudis de dosi-resposta d'adenovirus fets en humans. Hi ha un estudi realitzat per Crabtree i col. (1997) en el que van usar una r de 0,4172, però aquest valor es va determinar *in vitro*. Tot i això els valors de r determinats per diferents laboratoris poden diferir immensament. Per exemple per Norovirus, Masago i col. (2006) van estimar una r *in vitro* de 0,0069-0,00069 mentre que Teunis i col. (2008) han fet estudis dosi-resposta en poblacions humanes i han determinat que el valor de r hauria de ser de 0,5. Així doncs, a falta de més dades sobre els adenovirus humans, en el present estudi hem preferit donar els resultats en funció d'un rang de r entre 0,0005 i 0,5.

C.2.4 Anàlisi de la variabilitat i de la incertesa

S'ha usat un procediment de estàndard de bootstrap no paramètric per calcular numèricament la incertesa de les mesures del risc diari. El nivell de confiança del 95% pel risc diari va ser determinat usant el mètode estàndard "bootstrap percentile method" i el "accelerated and bias corrected method" (Davison i Hinkley, 1997; Manly, 2007) amb n=2000 iteracions. Aquests

intervals van ser computats amb els valors de risc obtinguts quan la fórmula [2] s'aplica a la concentració de cada mostra, amb els paràmetres R, I, DR, V i N prenent els valors especificats a la taula C.2.1.1.

Per l'estimació del risc anual s'ha remostrat la distribució empírica del risc diari per cada planta i cada valor de r, per obtenir 365 valors de risc simulats. Aplicant la fórmula [3] en aquests valors s'ha obtingut un valor *Monte Carlo* de risc anual P_y. La computació de P_y es repetida n=2000 vegades. Finalment, els màxims i els mínims que limiten el 95% dels valors centrals de la distribució del P_y *Monte Carlo* s'han obtingut computant les percentils 2,5% i 97,5% dels 2000 valors simulats.

També s'han considerat dos procediments simples d'estimació més pel risc anual, ambdós usant la fórmula [4]. El primer pren $P_{i/do}$ com la mitja dels riscs diàris. La segona obté les estimacions inferiors i superiors del risc anual prenent respectivament $P_{i/do}$ com els límits de confiança inferiors i superiors del risc diari. S'ha de fer notar que les estimacions inferiors i superiors de P_y no són els límits de l'interval de confiança i probablement s'està subestimant i sobreestimant respectivament els límits reals.

Totes els càlculs estadístics han estat realitzats usant el *R environment language* (R Foundation for Statistical Computing, 2007), versió 2.7.0, carregant addicionalment el *boot package* (Canty i Ripley, 2008) pel bootstrap dels intervals de confiança.

C.3 Resultats i discussió

Taula C.3.1 Risc diari per la Planta 1.

r	Mitjana	Desv. est.	Mínim	Màxim	bpr inf	bpr sup	bca inf	bca sup
0,0005	0,00001	0,00001	0,00000	0,00004	0,00000	0,00002	0,00000	0,00002
0,001	0,00002	0,00003	0,00000	0,00009	0,00001	0,00004	0,00001	0,00005
0,01	0,00022	0,00028	0,00000	0,00087	0,00007	0,00041	0,00009	0,00048
0,05	0,00110	0,00141	0,00000	0,00437	0,00037	0,00207	0,00047	0,00238
0,08	0,00176	0,00226	0,00000	0,00698	0,00059	0,00330	0,00075	0,00380
0,1	0,00220	0,00282	0,00000	0,00871	0,00073	0,00412	0,00094	0,00475
0,15	0,00329	0,00422	0,00000	0,01304	0,00110	0,00618	0,00140	0,00711
0,2	0,00438	0,00561	0,00000	0,01735	0,00146	0,00822	0,00187	0,00946
0,25	0,00547	0,00700	0,00000	0,02164	0,00183	0,01026	0,00234	0,01180
0,3	0,00656	0,00838	0,00000	0,02591	0,00219	0,01229	0,00280	0,01413
0,35	0,00764	0,00976	0,00000	0,03017	0,00256	0,01431	0,00327	0,01646
0,4	0,00872	0,01113	0,00000	0,03440	0,00292	0,01632	0,00373	0,01876
0,4172	0,00909	0,01160	0,00000	0,03586	0,00304	0,01702	0,00389	0,01955
0,5	0,01087	0,01385	0,00000	0,04282	0,00365	0,02033	0,00465	0,02332

Taula C.3.2 Risc diari per la Planta 2.

r	Mitjana	Desv. est.	Mínim	Màxim	bpr inf	bpr sup	bca inf	bca sup
0,0005	0,00051	0,00076	0,00000	0,00201	0,00009	0,00114	0,00014	0,00140
0,001	0,00102	0,00152	0,00000	0,00402	0,00019	0,00229	0,00029	0,00280
0,01	0,01009	0,01492	0,00000	0,03950	0,00188	0,02250	0,00286	0,02751
0,05	0,04766	0,06870	0,00000	0,18249	0,00935	0,10482	0,01415	0,12750
0,08	0,07320	0,10351	0,00000	0,27559	0,01488	0,15932	0,02240	0,19298
0,1	0,08911	0,12441	0,00000	0,33168	0,01850	0,19259	0,02784	0,23255
0,15	0,12540	0,16959	0,00000	0,45365	0,02719	0,26629	0,04118	0,31891
0,2	0,15739	0,20626	0,00000	0,55335	0,03582	0,32818	0,05368	0,38936
0,25	0,18576	0,23605	0,00000	0,63486	0,04424	0,38045	0,06426	0,43997
0,3	0,21111	0,26030	0,00000	0,70150	0,05246	0,42479	0,07305	0,48758
0,35	0,23391	0,28008	0,00000	0,75597	0,06049	0,46257	0,08177	0,52492
0,4	0,25455	0,29628	0,00000	0,80051	0,06832	0,49493	0,09092	0,55584
0,4172	0,26121	0,30115	0,00000	0,81386	0,07098	0,50498	0,09426	0,56563
0,5	0,29059	0,32062	0,00000	0,86667	0,08344	0,54705	0,10979	0,60998

Taula C.3.3 Risc diari per la Planta 3.

r	Mitjana	Desv. est.	Mínim	Màxim	Bpr inf	bpr sup	bca inf	bca sup
0,0005	0,00001	0,00002	0,00000	0,00006	0,00000	0,00002	0,00001	0,00003
0,001	0,00003	0,00004	0,00000	0,00013	0,00001	0,00005	0,00001	0,00006
0,01	0,00025	0,00037	0,00002	0,00129	0,00009	0,00049	0,00011	0,00058
0,05	0,00127	0,00185	0,00011	0,00643	0,00044	0,00244	0,00055	0,00291
0,08	0,00202	0,00296	0,00017	0,01027	0,00070	0,00390	0,00088	0,00464
0,1	0,00253	0,00370	0,00021	0,01283	0,00088	0,00487	0,00110	0,00580
0,15	0,00379	0,00553	0,00032	0,01918	0,00132	0,00729	0,00166	0,00867
0,2	0,00504	0,00735	0,00042	0,02549	0,00175	0,00970	0,00221	0,01152
0,25	0,00629	0,00916	0,00053	0,03176	0,00219	0,01209	0,00275	0,01436
0,3	0,00753	0,01096	0,00063	0,03799	0,00262	0,01447	0,00331	0,01720
0,35	0,00877	0,01275	0,00074	0,04418	0,00306	0,01684	0,00385	0,02000
0,4	0,01000	0,01452	0,00085	0,05033	0,00349	0,01920	0,00440	0,02279
0,4172	0,01043	0,01513	0,00088	0,05244	0,00364	0,02001	0,00459	0,02377
0,5	0,01246	0,01804	0,00106	0,06251	0,00436	0,02388	0,00550	0,02835

Taula C.3.4 Risc anual per la Planta 1.

r	mitja rd	bpr. inf.	bpr. sup.	2,5 remostratge	97,5 remostratge
0,0005	0,00401	0,00134	0,00752	0,00352	0,00451
0,001	0,00801	0,00267	0,01499	0,00704	0,00899
0,01	0,07726	0,02637	0,14018	0,06818	0,08684
0,05	0,33085	0,12505	0,52985	0,29823	0,36386
0,08	0,47402	0,19243	0,70091	0,43224	0,51220
0,1	0,55195	0,23443	0,77872	0,50762	0,59720
0,15	0,69980	0,33009	0,89574	0,65356	0,74295
0,2	0,79873	0,41376	0,95083	0,75701	0,83702
0,25	0,86498	0,48696	0,97678	0,82933	0,89553
0,3	0,90936	0,55098	0,98902	0,87939	0,93351
0,35	0,93911	0,60700	0,99481	0,91600	0,95800
0,4	0,95907	0,65601	0,99754	0,94019	0,97340
0,4172	0,96429	0,67141	0,99810	0,94806	0,97715
0,5	0,98147	0,73641	0,99945	0,97070	0,98927

Taula C.3.5 Risc anual per la Planta 2.

r	mitja rd	bpr, inf,	bpr, sup,	2,5 remostratge	97,5 remostratge
0,0005	0,17027	0,03382	0,34155	0,14954	0,19192
0,001	0,31142	0,06649	0,56632	0,27678	0,34527
0,01	0,97527	0,49718	0,99975	0,96092	0,98589
0,05	1,00000	0,96752	1,00000	1,00000	1,00000
0,08	1,00000	0,99579	1,00000	1,00000	1,00000
0,1	1,00000	0,99890	1,00000	1,00000	1,00000
0,15	1,00000	0,99996	1,00000	1,00000	1,00000
0,2	1,00000	1,00000	1,00000	1,00000	1,00000
0,25	1,00000	1,00000	1,00000	1,00000	1,00000
0,3	1,00000	1,00000	1,00000	1,00000	1,00000
0,35	1,00000	1,00000	1,00000	1,00000	1,00000
0,4	1,00000	1,00000	1,00000	1,00000	1,00000
0,4172	1,00000	1,00000	1,00000	1,00000	1,00000
0,5	1,00000	1,00000	1,00000	1,00000	1,00000

Taula C.3.6 Risc anual per la Planta 3.

r	mitja rd	bpr, inf,	bpr, sup,	2,5 remostratge	97,5 remostratge
0,0005	0,00462	0,00160	0,00890	0,00395	0,00531
0,001	0,00922	0,00321	0,01772	0,00790	0,01063
0,01	0,08848	0,03159	0,16364	0,07694	0,10193
0,05	0,37043	0,14823	0,59042	0,32961	0,40900
0,08	0,52279	0,22632	0,76000	0,46903	0,57135
0,1	0,60319	0,27433	0,83187	0,55024	0,65276
0,15	0,74961	0,38163	0,93082	0,70029	0,79627
0,2	0,84182	0,47295	0,97147	0,79638	0,87960
0,25	0,89996	0,55068	0,98820	0,86162	0,92984
0,3	0,93665	0,61687	0,99511	0,90861	0,95803
0,35	0,95984	0,67324	0,99797	0,93953	0,97537
0,4	0,97452	0,72125	0,99916	0,95892	0,98578
0,4172	0,97820	0,73607	0,99937	0,96472	0,98795
0,5	0,98970	0,79702	0,99985	0,98095	0,99502

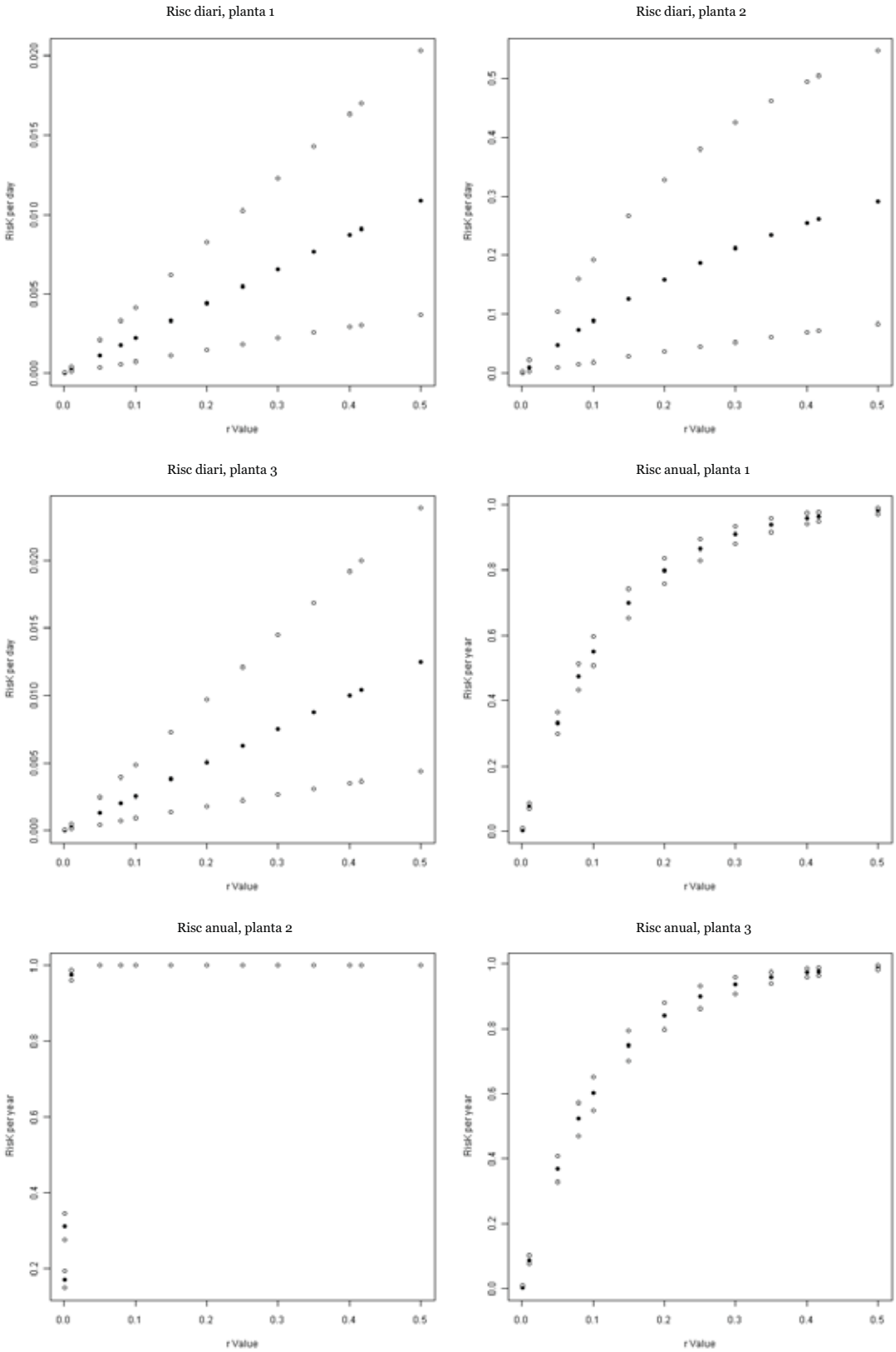


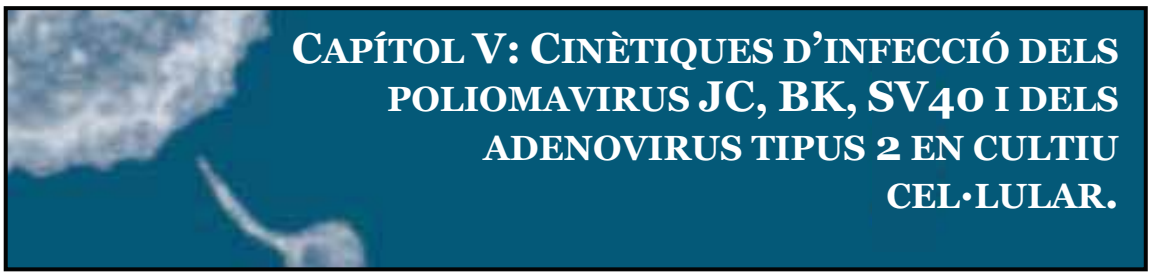
Figura C.3.1 Gràfics dels riscs diaris i anuals de les plantes de tractament.

L'anàlisi quantitatiu del risc microbiològic és un camp d'investigació encara molt nou, i hi ha moltes incògnites que encara no s'han pogut determinar. En el present cas s'ha decidit fer una primera aproximació al QMRA d'adenovirus en aigua de beguda presuposant el pitjor dels casos en aquells paràmetres dels quals no tenim un valor empíric. Ja que no podem calcular quina proporció de virus són infecciosos, hem assumit que totes les partícules víriques són viables. No obstant hem considerat que només un 1% de les còpies genòmiques representen partícules infeccioses (N). A més, hem assumit que tota la població humana és susceptible a les infeccions d'aquests virus. Hi ha molt pocs estudis epidemiològics d'HAdV, tot i això hi ha algunes dades serològiques. Els anticossos contra els Adenovirus 1, 2 i 5 estan presents en el 40-60% de nens (Brandt i col. 1969). Els HAdV més prevalents són els de l'espècie F (HAdV 40 i 41) i són considerats la segona causa de gastroenteritis en nens. Estudis de serologia indiquen que més del 50% de la població de nens entre 6 i 8 anys tenen anticossos contra aquests virus (Wadell, 1999). Aquestes dades serològiques van ser obtingudes a partir de nens, es molt probable que la seroprevalència de la població adulta sigui molt superior. Per tant gran part de la població ja està immunitzada contra aquests virus i aquest estudi està encarat cap a aquella part de la població que és susceptible d'infecció. La falta d'estudis fiables sobre la dosi mínima infecciosa dels adenovirus (r) ens ha portat a fer un estudi comparatiu amb un rang determinat de valors per aquest paràmetre, tot i això, hi ha un valor r per adenovirus que apareix a la bibliografia (Crabtree i col. 1997) igual a 0,4172.

Tenint en compte les consideracions esmentades i el fet que és una primera aproximació a l'estudi del risc, els resultats obtinguts indiquen que la probabilitat d'infecció en un dia si consumim aigua d'una planta que usa aigua de riu significativament contaminada (~10^4 GC HAdV/L) i amb una capacitat d'eliminació de virus de 5 logaritmes (per exemple la planta 1) és del 0,91 % ±1,16%; si consumim aigua d'una planta amb tractaments més simples, que usa aigua de riu significativament contaminat (~10^4 GC HAdV/L) i té una capacitat d'eliminació de virus de 5 logaritmes (per exemple la planta 3) el risc és molt semblant: 1,04% ± 1,51% en canvi si consumim aigua d'una planta que usa aigua subterrània moderadament contaminada (1-10 GC HAdV/L) amb una capacitat d'eliminació de 2 logaritmes (per exemple la planta 2) el risc augmenta molt

(26,1% ± 30%). Els intervals de confiança bootstrap pel mètode percentil són similars als obtinguts amb el "accelerated and bias corrected method" en el rang de valors de r considerat. També s'ha de tenir en compte que no es el mateix la probabilitat d'infecció i la probabilitat de malaltia, ja que moltes infeccions cursen asimptomàticament.

Considerant per tant que tota la població és susceptible a la infecció per aquest virus i que el consum diari és d'un litre, pel risc anual, els diferents mètodes d'estimació són consistents i mostren un alt risc d'infecció per valors superiors a 0,05. S'ha de notar que els valors de concentració de virus van ser obtinguts principalment en els mesos de primavera i estiu, i tots els mètodes usats per estimar el risc anual assumeixen que aquestes mostres són representatives de tot l'any. Per una planta com tipus 1 el risc anual d'infecció és del 96% ± 67%, per una planta tipus 2 és del 100% i per una planta tipus 3 és del 97% ± 73% pel valor de r de 0,4172 utilitzat prèviament a la bibliografia i que correspondria a individus de la població susceptibles al virus.



CAPÍTOL V: CINÈTIQUES D'INFECCIÓ DELS POLIOMAVIRUS JC, BK, SV40 I DELS ADENOVIRUS TIPUS 2 EN CULTIU CEL·LULAR.

Anàlisi de la cinètica d'infecció i coinfecció dels poliomavirus JCPyV, BKPyV i SV40 i de l'adenovirus humà tipus 2 en diverses línies cel·lulars.

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Resum

Els poliomavirus humans JC (JCPyV) i BK (BKPyV) infecten un gran nombre d'humans a tot el món. Aquests virus comparteixen un 75% d´homologia en el seu DNA i poden ser classificats en soques arquetípiques i reorganitzades depenent de l'estructura de la seva regió reguladora (TCR). Els poliomavirus i adenovirus humans estan àmpliament distribuïts en la població; són excretats en femta i orina i se'ls ha proposat com a indicadors de contaminació fecal d'origen humà a l'aigua i a l'ambient. JCPyV és l'agent causal de la leucoencefalopatia multifocal progressiva (PML), una malaltia neurodegenerativa que afecta individus en estats d'immunodeficiència.

Hi ha manca d'informació sobre sistemes eficients de cultiu i sobre la seva cinètica d'infecció d'aquests virus. Aquesta informació és necessària per profunditzar l'estudi dels aspectes biològics i clínics de les infeccions. Per això s'han analitzat corbes d'infecció de JCPyV i BKPyV i de coinfecció amb HAdV2 i SV40 utilitzant mètodes de qPCR. Els resultats mostren que, en les millors condicions assajades, HAdV2 incrementa el seu DNA en 2,5 logaritmes, arribant a una fase estacionària a les 48 hores post-infecció en cèl·lules A549; JCPyV Mad-4 incrementa aproximadament 2 logaritmes en 120 hores en cèl·lules SVG i BKPyV Dunlop incrementa 3 logaritmes en 150 hores en cèl·lules HNK-1. HAdV2 no va mostrar cap interacció amb JCPyV Mad-4 en cèl·lules de càncer de colon sota les condicions analitzades. Encara que la infecció de BKPyV arquetípica en cèl·lules SVG va demostrar ésser molt ineficient, la coinfecció de JCPyV Mad-4 i BKPyV arquetípic en aquestes cèl·lules va disminuir significativament el temps necessari per assolir la màxima replicació del DNA de JCPyV.

La transmissió de JCPyV entre la població és molt ineficient; tot i que s'excreten grans quantitats, la població tendeix a seroconvertir-se anys després de la infecció amb BKPyV. Els resultats observats estan limitats per les dificultats per aconseguir models experimentals apropiats pel cultiu de poliomavirus humans. Tot i això, recolzen la

hipòtesi de que la infecció inicial de BKPyV afavoreix la multiplicació de JCPyV i l'establiment d'infeccions persistents per part d'aquest últim.

Resum dels Resultats i la Discussió

En l'organisme humà, el poliomavirus JC es replica només en un nombre limitat de tipus cel·lulars, incloent oligodendròcits. El mateix tropisme cel·lular s'observa in vitro i s'ha atribuït a factors de transcripció específics requerits per la trans-activació dels promotor primerenc de JCPyV. Tot i això s'han descrit infeccions persistents als ronyons i conseqüentment els virus són excretats en la orina (Eash i col. 2006). Els diversos serotips d'HAdV creixen amb eficiència variable en diverses línies cel·lulars com BGMK, CaCo-2, HeLa, Hep-2, A549 i 293 Human Embryonic Kidney (HEK) (Jiang, 2006).

Per estudiar la infecció i la replicació de DNA d'HAdV en condicions òptimes vam realitzar una corba de creixement d'HAdV2 en cèl·lules A549 que són permissives per la infecció d'aquest virus. La figura 1A mostra que la infecció és molt eficient, el DNA s'incrementa 2,5 logaritmes en 48 hores post-infecció arribant als 3 logaritmes en 120 hores. Es va observar efecte citopàtic (CPE) a les 70 hores post-infecció. La figura 1A també mostra la infecció d'HAdV2 en cèl·lules CaCo-2. El virus infecta menys eficientment amb un creixement exponencial d'aproximadament 2 logaritmes arribant a la fase estacionària després de 200 hores de la infecció. Es va observar CPE a les 96 hores post-infecció. Es estudis previs s'ha comprovat que JCPyV Mad-4 no fa infecció productiva en cèl·lules CaCo-2 (Bofill-Mas i col. 2003).

Hi ha estudis que descriuen l'activació de JCPyV en cultius no permissius mitjançant un virus "helper" o facilitador (Heilbronn i col. 1993; Winklhofer i col. 2000). S'han descrit també interaccions entre poliomavirus humans i adenovirus (Miyamura i Takemoto, 1979). Una hipòtesi és que els HAdVs actuen com a "helpers" en la infecció de JCPyV a l'epiteli intestinal, proporcionant pistes sobre el lloc de la infecció primària d'aquests virus en l'organisme humà.

La coinfecció d'HAdV2 i JCPyV Mad-4 en CaCo-2 es mostra a la Figura 1B. HAdV2 presenta una cinètica similar a quan infecta sol, creixent exponencialment 2 logaritmes i arribant a la fase estacionària a les 200 hores. Mad-4 no presenta cap increment de còpies genòmiques sinó una tendència a reduir el seu nombre. Les còpies genòmiques de JCPyV detectades a les 250 hores post-infecció probablement corresponen a les que s'hi van introduir al principi de l'experiment. La tècnica usada

per la detecció no ens permet saber si les partícules víriques van ser internalitzades o es van quedar adsorbides a la superfície cel·lular. Aquests resultats suggereixen que HAdV2 no actua com a activador de la replicació de JCPyV en les condicions experimentals estudiades.

La cinètica de replicació de la soca reorganitzada Mad-4 de JCPyV va ser definida en cèl·lules glials fetals humanes (SVG). La figura 2 mostra que Mad-4 infecta eficientment aquesta línia cel·lular, amb un increment de còpies genòmiques de 2 logaritmes en 120 hores post-infecció. La cinètica de JCPyV arquetípic també es mostra a la figura 2, no es va observar cap increment durant tot el període de l'experiment. Les variants arquetípiques de JCPyV són molt difícils de cultivar en línies cel·lulars. Hara i col. (1998) va descriure el cultiu d'una soca arquetípica de JCPyV en cèl·lules Cos-7, que contenen el TAg de SV40. En el present estudi JCPyV arquetípic no va presentar replicació en cèl·lules SVG suggerint que no hi ha infecció productiva o que aquesta requeriria més temps. Bofill-Mas i col (2003) van descriure la infecció productiva de JCPyV arquetípic després de 40 dies d'infecció en cèl·lules SVG.

Les variants reorganitzades de JCPyV poden ser cultivades en cèl·lules glials i algunes línies de cèl·lules B (Atwood i col. 1992). S'ha suggerit que la restringida capacitat lítica de JCPyV arquetípic, comparada amb soques reorganitzades, es deu a reorganitzacions específiques de les regions promotores. Aparentment les soques arquetípiques són incapaces de produir els nivells necessaris del mRNA del TAg per activar la replicació (Daniel i col. 1996).

La corba de creixement de BKPyV arquetípic en cèl·lules SVG (figura 3A) mostra una replicació molt ineficient i no es va observar CPE. A la bibliografia es poden trobar descripcions de la interacció de BKPyV amb altres poliomavirus (Caputo i col. 1986, Ryder i col. 1983). Per estudiar la mecànica de transmissió de BKPyV vam estudiar la interacció entre aquest i JCPyV en una línia cel·lular permissiva per aquest últim (figura 3B). Els resultats van mostrar que, encara que BKPyV presenta una replicació molt ineficient, la replicació del DNA de JCPyV sembla més eficient comparada amb la infecció de JCPyV sol en aquesta mateixa línia cel·lular (figura 2) arribant a la fase estacionaria 50 hores abans. Els anàlisis estadístics de la regressió comparant les dues corbes de creixement van demostrar que hi ha diferències estadísticament significatives en la pendent de les rectes de regressió (p-valor de 0,0034 a un 99% de nivell de confiança). Això suggereix que la coinfecció amb BKPyV podria incrementar l'eficiència de replicació de JCPyV en fases primerenques de la infecció. Els resultats obtinguts indiquen que BKPyV podria facilitar la infecció de

JCPyV reduint la fase primerenca, ajudant així a l'establiment de la infecció en l'organisme humà, encara que es necessiten més estudis per confirmar aquesta hipòtesi. Mentre que la població es veu infectada per BKPyV en la primera infància, la infecció per JCPyV requereix més temps per estendre's entre la població i ho fa més tard que BKPyV (Taguchi i col. 1982; Walker i Padgett, 1983). La presència de BKPyV podria doncs facilitar la subsegüent infecció per JCPyV i l'establiment d'una infecció persistent per part de les soques arquetípiques menys infectives presents a l'orina.

Per definir la cinètica de replicació d'una soca reorganitzada de BKPyV, es va realitzar una corba de creixement de la soca Dunlop en cèl·lules HNK-1 (figura 4A). La figura mostra una replicació molt eficient, incrementant 3 logaritmes en 150 hores. Es va observar CPE a les 100 hores post-infecció. SV40 presenta una replicació molt ineficient en aquesta línia cel·lular, no es va observar CPE després de 200 hores post-infecció (figura 4B). Aquest virus presenta un 70% d'homologia amb el genoma de BKPyV (Hirsh i Steiner, 2003). Com s'ha comentat anteriorment, hi ha estudis que descriuen la interacció entre BKPyV i SV40 (Caputo i col. 1986, Ryder i col. 1983). Per explorar la possible interacció entre aquests dos virus en cèl·lules de ronyó es va realitzar una corba de creixement coinfectant BKPyV Dunlop i SV40 777 (figura 4C). SV40 presenta una replicació molt similar a quan infecta en solitari. Encara que es pot observar un petit increment en la eficiència de replicació de BKPyV quan coinfecta amb SV40 respecte a quan infecta en solitari, els anàlisis estadístics conclouen que no hi ha diferències significatives.

En aquest estudi s'ha usat PCR quantitativa per definir les cinètiques d'infecció dels virus descrits quantificant les còpies genòmiques generades. Les cinètiques d'infecció descrites aquí poden representar informació molt útil per estudis dels aspectes clínics i biològics de la infecció de JCPyV. Tot i les limitacions de les condicions experimentals per la multiplicació de JCPyV, els suggereixen que una infecció inicial per BKPyV (que infecta la població en els primers anys de vida) podria afavorir l'establiment d'una infecció permanent èr JCPyV, contribuint a explicar el fet que JCPyV infecta la població més tard que BKPyV encara que s'excreta amb més freqüència per la orina.

Analysis of the kinetics of infection and co-infections of human polyomaviruses JCPyV and BKPyV and adenovirus type 2 in diverse cell lines.

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Abstract

Humans are frequently orally exposed to human adenoviruses (HAdV), human polyomaviruses JV (JCPyV) and BK (BKPyV), which are highly prevalent viruses in urban sewage and contaminated environments, water or food. JCPyV is the causative agent of progressive multifocal leukoencephalopathy (PML), affecting people with specific immunocompromised status. There is a lack of information about the efficient systems and multiplication kinetics of JCPyV, which is required in order to further the study of biological and clinical aspects of the infection. Therefore, one-step growth curves of JCPyV and BKPyV and of co-infections also with HAdV serotype 2 and SV40 were analyzed using qPCR methods. The strains studied were HAdV 2 and JCPyV Mad-4 (rearranged) in A549 and CaCo-2 cells; JCPyV Mad-4, JCPyV archetypal and BKPyV archetypal in SVG cells and BKPyV Dunlop (rearranged) and SV40 777 (rearranged) in HNK-1 cells. The results show that, under the best conditions assayed, HAdV serotype 2 DNA increases by 2.5 logs, reaching a plateau in 48 hours in A549 cells; JCPyV Mad-4 increases by about 2 logs in 120 hours in SVG cells; and BKPyV Dunlop strain, by about 3 logs in 150 hours in HNK-1 cells. HAdV 2 showed no interaction with JCPyV Mad4 infection in a colon cancer cell line as CaCo-2 under the conditions analyzed. Although infection by archetypal BKPyV in SVG cells seemed very inefficient, co-infection between JCPyV Mad-4 and BKPyV archetypal significantly reduced the time required to achieve maximum DNA replication of JCPyV. The transmission of JCPyV in the population is less efficient than BKPyV: although high numbers are excreted, the population tends to seroconvert years later than BKPyV. The results observed are limited by the difficulties in achieving suitable experimental approaches for the culture of human polyomaviruses. However, they tend

to support the hypothesis that initial BKPyV infection may favor the further multiplication of JCPyV and the establishment of JCPyV persistent infections.

Introduction

Human polyomaviruses, JC (JCPyV) and BK (BKPyV), infect a high number of the world's population. In a study by Knowles et al. (2003) analyzing 2,435 residual sera by hemagglutination inhibition (HI), BKPyV seroprevalence reached 91% at 5-9 years of age, but JCV seroprevalence reached only 50% by age 60-69 years. The initial infection occurs in childhood, but is not apparent (Major et al. 1992). By 10 years of age, 40-60% of the population is JCPyV-seropositive (Taguchi et al. 1982; Walker and Padgett, 1983) and the kidney is considered to be a site of persistent viral infection, with viruria being a common event not only in immunocompromised individuals, but also in healthy ones (Arthur et al. 1989; Chang et al. 2002; Jeong et al. 2004; Kaneko et al. 2005; Shah, 1995).

The JCPyV genome is double-stranded, supercoiled circular DNA that consists of early and late coding regions separated by two non-coding regions at both the 5' and 3' ends. Within the non-coding sequences is the transcriptional control region (TCR), which contains the promoter and enhancer for the early and late proteins; the intergenic region separates the T antigen and the capsid proteins 3' regions.

JCPyV is associated with progressive multifocal leukoencephalopathy (PML), a fatal demyelinating disease. The pathogenicity of the virus is commonly associated with immunocompromised states, but has attracted most attention due to AIDS-linked immunosuppression (Berger et al. 1987). Recently too, PML cases have been identified in multiple sclerosis and Crohn's disease patients under new immunomodulator treatments (Yousry et al. 2006).

Some laboratories have also suggested a relationship between JCPyV and human cancer such as colon cancer (Enam et al. 2002; Hori et al. 2005; Ricciardiello et al. 2001).

Depending on its TCR structure, JCPyV can be differentiated in the archetype and rearranged variants. The archetype strains are excreted in urine of people with and without PML (Shah 1995) and have been found in urban sewage and river water in widely divergent geographical areas (Albinana-Gimenez et al. 2006; Bofill-Mas et al. 2000). The rearranged variants are direct descendants of the archetype and result from deletions and duplications of sequences within the regulatory region (Yogo and Sugimoto, 2001). These rearranged variants are related to pathogenesis; strains Mad-1 and Mad-4 are associated with PML cases (Padgett et al. 1976).

The transmission mechanism of JCPyV is still unknown. Though the presence of the virus in tonsillar tissue suggests that the virus could be transmitted by the respiratory pathway (Monaco et al. 1998) however, some studies fail to detect the virus in the oropharyngeal fluids of infected individuals (Berger et al. 2006). A previous study described the detection of JCPyV in sewage samples and reported that the viral particles are stable in the environment and relatively resistant to acidic pH, which supports the theory of transmission by oral pathway (Bofill-Mas et al. 2003).

Initial BKPyV infection occurs independently of JCPyV during childhood at a median age of 4-5 years (Knowles, 2001; Shah et al. 1973). After primary infection, BKPyV persists in the reno-urinary tract as its main site (Chesters et al. 1983). Reactivation of the virus is associated with immunosuppression states causing polyomavirus-associated nephropathy, especially in kidney transplantation patients (Hirsch and Steigner, 2003).

The BKPyV genome shares 75% overall homology with JCPyV and can be divided into regulatory (TCR) early and late regions (Hirsch and Steigner, 2003). BKPyV TCR may also have archetypal or rearranged structure. BKPyV strains excreted into the urine correspond to the archetype variants (Rubinstein et al. 1987) and have been detected in sewage and river water (Albinana-Gimenez et al. 2006; Bofill-Mas et al. 2000;

McQuaig et al. 2006). The rearranged variants are associated with pathogenesis (Olsen et al. 2006). Its transmission pathway is still unknown, but respiratory transmission has been suggested (Goudsmit et al. 1982).

Simian Virus 40 was first described as a contaminant in polio vaccines, potentially exposing several million people to the virus between 1955 and 1961 (Shah and Nathanson, 1976). Serology studies showed 20% prevalence in vaccinated children and 27% in people working with *rhesus* monkeys, the natural host of the virus (Shah et al. 1971). However, several studies have demonstrated that there is cross-reactivity between BKPyV and SV40 antibodies (Viscidi and Clayman, 2006) and the seroprevalence values for SV40 have not been definitely established.

The SV40 genome shares high homology with both human polyomaviruses, JC and BK: the human polyomaviruses can also be divided into archetypal and rearranged strains depending on the TCR structure (Yogo and Sugimoto, 2001). According to some studies performed in naturally infected *Cynomolgus* monkeys, its transmission is believed to be fecal-oral (Bofill-Mas et al. 2004; Minor et al. 2003).

Human adenovirus (HAdV) infections occur worldwide, but many of them are asymptomatic. They have been classified in the *Mastadenovirus* genus and comprise 51 serotypes distributed in six species. The most common HAdVs (Ad1, 2 and 5) infect 40-60% of children (Brandt et al. 1969). Some serotypes, such as 40 and 41, are unique in being responsible for most cases of Ad-associated gastroenteritis in children (Wold and Horwitz, 2007). Some HAdV serotypes infect the respiratory tract; others infect the gastro-intestinal or urinary tracts. After acute infection, some HAdV types can be shed in stool for months to years (Wadell et al. 1988). HAdVs may be transmitted in various ways, such as the fecal-oral pathway. Inhalation of aerosolized droplets is also an important pathway for HAdV infection (Couch et al. 2006). In previous studies, HAdVs

have been detected in urban sewage, shellfish, river water and even in drinking water (Formiga-Cruz et al. 2003; Pina et al. 1998; Puig et al. 1994; Van Heerden et al. 2005).

The overall aim of this study is to define the multiplication kinetics of human polyomaviruses in diverse cell cultures. In addition, the study explored interactions that might affect the transmission mechanisms of JCPyV.

Humans are frequently orally exposed to HAdV, JCPyV and BKPyV, which are highly prevalent viruses in urban sewage and contaminated environments, water or food. To explore potential facilitation of infection by JCPyV with HAdV in the intestinal track, the replication kinetics of JCPyV in human CaCo-2 cells was analyzed by using qPCR in co-infection experiments with HAdV type 2. Adenoviruses, previously reported to interact with polyomaviruses (Goldman et al. 1981; Heath et al. 1992; Miyamura and Takemoto, 1979), could act as helpers by activating the replication of JCPyV in the intestinal tract as a site of primary infection. Previous studies also described human cytomegalovirus inducing JCPyV DNA replication in human fibroblasts and glioblastoma cells (Heilbronn et al. 1993; Winklhofer et al. 2000). The replication activity of JCPyV and BKPyV in human fetal glial cells, considered the most efficient systems for JCPyV cultivation (Major et al. 1985), is described. BKPyV has been described as efficiently multiplying in human kidney cells (Randhawa and Brennan, 2006) and its interaction with SV40 in these cells has also been analyzed.

Materials and methods.

Cell lines. Human neonatal kidney cells (HNK) (Hybrid diagnostics Inc. USA), a primary culture, were propagated in Dulbecco's minimal essential medium (DMEM) supplemented with 1% glutamine and 10% heat-inactivated FBS. A549 (kindly

provided by Annika Allard) is an epithelial cell line from human lung carcinoma and was propagated in Earl's minimum essential medium (EMEM) supplemented with 1% glutamine, 50 µg of gentamicin per mL and 5% (growth medium) or 2% (maintenance medium) of heat-inactivated FBS. BGM (obtained from the "Coleccion Española de Cultivos Tipo" at a low passage) is an epithelial cell line from African green monkey kidney and was propagated in EMEM supplemented with 1% glutamine, 50 µg of gentamicin per mL and 5% (growth medium) or 2% (maintenance medium) of heat-inactivated FBS. CaCo-2 (obtained from the "Coleccion Española de Cultivos Tipo" at a low passage) is an epithelial cell line from human colorectal cancer propagated in EMEM supplemented with 1% glutamine, 1% non-essential amino acids, 1% sodium pyruvate, 50µg of gentamicin per mL and 20% (growth medium) or 7% (maintenance medium) of heat-inactivated FBS. SVG is a fibroblast cell line from human astroglia transformed with the T-Ag of SV40 (kindly provided by Eugene O. Major) (Major et al. 1985) and was propagated in EMEM supplemented with 1% glutamine, 50 µg of gentamicin per mL and 10% (growth medium) or 2% (maintenance medium) heat-inactivated FBS.

Viruses. The strain of HAdV 2 isolated from a clinical sample was grown in A549 cells. JCPyV Mad-4 rearranged strain (kindly provided by Eugene O. Major) was grown in SVG cells. JCPyV archetypal strain was obtained from the urine of a healthy woman in the 38th week of pregnancy and was grown in SVG cells as described in Bofill-Mas et al. (2000). BKPyV archetypal strain was obtained from a urine sample of a polyomavirus nephropathy kidney transplant patient and was grown in BGM cells. BKV Dunlop strain (kindly provided by Michael Imperiale) was grown in Vero cells. To recover the viruses, the infected cultures were frozen-thawed three times and the

supernatant was ultracentrifuged to concentrate the viral particles. SV40 strain 777 was obtained from a polio vaccine, was pooled and stored at -80ºC.

Culture preparation and infection. One step-growth curves were analyzed in triplicated experiments. Cell cultures in low passage number were trypsinized and the cells were counted. Forty or fifty cell culture tubes were prepared for every multiplication experiment, with $3x10^5$ cells in 2 ml of medium planted in every tube. The cells were incubated at 37°C until a monolayer was formed. The medium from the confluent cell culture tubes was removed and 100µl of viral suspension was added. The infections always took place at multiplicity of infection (MOI) >5. The tubes were incubated at 37°C for 3h for virus adsorption. Three washes of PBS were performed to eliminate the non-adsorbed viral particles and 2 ml of fresh media were added. Three replicates were immediately frozen, corresponding to time 0. The rest were incubated at 37°C and three tubes were collected and frozen every 12 and 24 hours and tested by qPCR for the specific viruses. On collection, the cultures were observed for the presence of cytopathic effects (CPE). Typical CPE from adenoviruses consists in rounded, swollen cells and basophilic intranuclear inclusions. Polyomaviruses' CPE is the result of two linked processes: accumulation of newly formed virions (virus inclusions) and subsequent cell lysis.

Nucleic acid extraction. The cell culture tubes were frozen-thawed three times and the nucleic acids were extracted by the QiAamp RNA Mini Kit (Qiagen GmbH, Inc., Hilden, Germany), following manufacturer's instructions.

Quantitative Real-Time PCR (qPCR). Amplification was performed in a 25-µL reaction mixture with the PCR Universal Master Mix (Applied Biosystems). The reaction contained 10 µL of a DNA sample or 10 µL of a quantified plasmid DNA, 1X TaqMan master mix, and the corresponding primers and TaqMan probes at their

corresponding concentrations. HAdV genomes were quantified with 0.9 μM of the primers AdF and AdR and 0.225 μM of the AdP1 probe described by Hernroth et al. (2002). JCPyV genomes were quantified with 0.5 μM of the primers JE3F and JE3R and 0.15 μM of the fluorogenic probe JE3P, described in Pal et al. (2006). BKPyV genomes were quantified with 0.5 μM of the primers BE3F and BE3R and 0.15 μM of the fluorogenic probe BE3P, also described in Pal et al. (2006). Following activation of the uracil N-glycosilase contained in the core mix (2 min at 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 with an Mx3000P sequence detector system (Stratagene). All samples were run in triplicate, and positive and negative controls were included. The amount of DNA was defined as the average of the triplicate data obtained. Each value represented on the plots corresponds with the average values of the three qPCR analyses of the three culture replicates (total of nine values).

Results

Human adenovirus 2 and human polyomavirus JC growth curves.

To study the infection and DNA replication kinetics of HAdV in optimal conditions, we performed a one-step growth curve of HAdV 2 in A549 cell culture, known to be permissive for HAdV 2 infection. Figure 1A shows that HAdV 2 infects efficiently this cell line, showing an exponential growth of 2.5 logs at 48 hours post-infection and increasing 3 logs at 120 hours. Cytopathic effects (CPE) could be observed after 70 hours post-infection. HAdV 2 multiplication infecting CaCo-2 cells is shown in Figure 1A. The virus infects less efficiently as A549, with exponential growth of ~2 logs and reaching a plateau at 200 hours post-infection. CPE was observable after 96 hours post-

infection. Previous studies showed that JCPyV Mad-4 does not cause infection in CaCo-2 cells (Bofill-Mas et al. 2003).

The co-infection of HAdV 2 and JCPyV Mad-4 in CaCo-2 cells is shown in Figure 1B. HAdV 2 shows similar growth as in Figure 1A when infecting alone, growing exponentially ~2 logs and reaching a plateau at 200 hours post-infection. Mad-4 shows no increase in GC number and a tendency to reduce their number in these cells. The JCPyV detected at 250 hours post-infection may correspond to the input viral particles, since the technique used does not allow the determination of whether the viral particles were internalized or remained adsorbed on the cell membranes. These results suggest that HAdV 2 does not act as a helper for the productive infection of JCPyV Mad-4 under the experimental conditions used in these colon cancer cells.

Human polyomavirus JC archetypal and rearranged strain infection in SVG cells.

JCPyV strain Mad-4 were inoculated in SVG cell cultures, known to be permissive for rearranged strains infection. Figure 2 shows that Mad-4 infects SVG efficiently, with a short incubation phase followed by exponential growth for about 2 logs and then reaching a plateau at 120 hours post-infection. CPE was observed after 96 hours post-infection.

The one-step growth curve of JCPyV archetypal strain in SVG cells (also plotted in Figure 2) showed no JCPyV DNA amplification in the culture during the time studied.

Human polyomavirus BK archetypal strain infection in SVG cells.

The one-step growth curve of BKPyV archetypal strain in SVG cells, plotted in Figure 3A, shows very inefficient DNA replication. No CPE was observed during the experiment.

To explore the possible interaction of JCPyV and BKPyV, one-step growth experiments of JCPyV Mad-4 co-infecting with BKPyV archetypal strain (Figure 3B) were

developed. The co-infection shows that, even though BKPyV DNA replication in the entire experiment was very inefficient, JCPyV DNA replication appears to be facilitated (compared with Mad-4 infection in SVG cells, shown in Figure 2), growing over 2 logs in less than 100 hours after the infection, with clear signs of CPE at 96 hours post-infection. Statistical regression analyses comparing the two growth curves showed that there are significant differences in the slope of the regression lines (p-value of 0.0034 at 99% confidence level).

Human polyomavirus BK and SV40 infection in HNK-1 cells.

The infection kinetics of BKPyV strain Dunlop (rearranged) in HNK primary cell culture. Figure 4A shows highly efficient infection kinetics. The DNA replicates exponentially for about 3 logs, reaching stabilization at 150 hours post-infection. CPE was observed after 100 hours of infection. Figure 4B shows the one-step growth curve of SV40 strain 777 in HNK-1 cells. Very inefficient DNA replication was observed for SV40 in the human cell line and no CPE was observed after 200 hours. Finally, to explore the possible interaction of BKPyV and SV40 in these human cells a one-step growth curve co-infecting these two viruses was performed. Figure 4C shows that SV40 has the same kinetics as when it infects alone. However, accelerated BKPyV DNA replication kinetics was observed, reaching the plateau (10^{10} GC/ml) in fewer than 100 hours, compared with the 150 hours needed when BKPyV infects alone. Statistical regression analyses comparing the two growth curves showed no significant differences in the slope of the regression lines (p-value of 0.5402 at 99% confidence level).

Discussion.

In the human organism, polyomavirus JC only replicates in a limited number of cells, including oligodendrocytes, in which it can be detected by DNA hybridization or by PCR (Azzi et al. 1996). The same cell tropism can be observed *in vitro* and has been attributed to glial-specific transcription factors required for the trans-activation of JCPyV early promoter. However, persistent infections are described in the kidney and viruses are consequently excreted in urine (Eash et al. 2006). HAdV serotypes grow with variable efficiency in a variety of cell lines, such as BGMK, CaCo-2, HeLa, Hep-2, A549 and 293 Human Embryonic Kidney (HEK) (Jiang, 2006). To define the replication kinetics of HAdV 2, we used two cell lines that were permissive to adenovirus infection. The data show that HAdV 2 efficiently multiplies in A549 cells and, to a lesser extent, in CaCo-2 cells. Other studies have described the activation of JCPyV in non-permissive cell cultures by means of a helper virus (Heilbronn et al. 1993; Winklhofer et al. 2000). Interactions of polyomaviruses with adenoviruses have also been described. Miyamura and Takemoto (1979) reported that BKPyV is a helper in the adenovirus infection of CV-1 cells. Helper functions for adenoviruses have also been reported when these co-infect with SV40 (Goldman et al. 1981; Heath et al. 1992). One hypothesis is that HAdVs act as helpers in JCPyV infection of intestinal epithelial cells and so provide clues to the JCPyV primary infection site in the human organism. The data produced, limited by the experimental conditions available, show no interaction between HAdV 2 and JCPyV infection in CaCo-2 cells. JCPyV does not grow in CaCo-2, as reported previously (Bofill-Mas et al. 2003), and showed no multiplication in CaCo-2 in the presence of HAdV 2. The qPCR shows the presence of input JCPyV DNA, but the technique used did not allow to determine whether the virus had been internalized or remained on the cell surface.

The replication kinetics of the JCPyV rearranged strain Mad-4 were defined in the permissive glial cell line SVG. JCPyV replicates in these cells and the genome copies increase by 2 logs at 150 hours post-infection. JCPyV archetypal variants are very difficult to grow in cell cultures. Hara et al. (1998) reported the growth of archetypal JCPyV after infection of Cos-7 cells (these cells contain simian virus 40 (SV40) T-Ag). There is only one description of archetypal JCPyV growth by transfection in human fetal brain (HFB) culture that does not contain T-Ag (O'Neill et al. 2003). In the present study, JCPyV archetypal strain did not show amplification in SVG cells, suggesting that there is no productive infection or it may require more time. Longer incubation time for JCPyV archetypal strain multiplication was described by Bofill-Mas et al. (2003) in SVG cells (human glial cells that contain SV40 T-Ag), showing significant increase in the genome copies after 40 days infection when using an inoculum of urine containing archetypal JCPyV and also BKPyV in lower concentration. These cultures were passed several times in this process and this was not feasible for the assays developed in this study.

Rearranged variants can be grown in human glial cell cultures and some human B cell lines (Atwood et al. 1992). It has been suggested that the restricted lytic behavior of Archetype JCPyV, when compared with rearranged strains, might be due to specific rearrangements in the promoter region. Apparently, archetypal strains are unable to express adequate levels of T-antigen-specific mRNA to efficiently support T-antigen-mediated JCPyV DNA replication (Daniel et al. 1996) under the study's conditions.

BKPyV infects kidney cells *in vivo* and *in vitro*, although infection of other cell types has been observed *in vitro* (Sugimoto et al. 1989; Van der Noorda et al. 1986). BKPyV archetypal variants are known to grow very inefficiently in cell culture (Accott et al.

2006; Marshall et al. 1990; Rinaldo et al. 2005) and rearranged variants multiply more efficiently in kidney cell cultures.

GC numbers of BKPyV archetypal strain increased very little during the infection experiments, which comprised 250 hours post-infection. There have been reports on the interaction between BKPyV and other polyomaviruses, such as SV40 (Caputo et al. 1986; Ryder et al. 1983). As an approach to the potential transmission mechanisms, we explored the interaction of JCPyV and BKPyV in a cell line that was permissive to JCPyV such as SVG. A co-infection one-step growth curve of JCPyV Mad-4 (which infects productively these cells) and BKPyV was analyzed. The data indicate that there was no trans-activation of the BKPyV DNA replication by JCPyV early proteins. However, when Mad-4 alone infects SVG, the exponential replication phase ends at near 150 hours post-infection, while when it co-infects with BKPyV the exponential phase ends 50 hours earlier, reaching the same DNA levels (~ 1.5 x 10^7 GC/ml). This suggests that BKPyV co-infection could increase the replication activity of JCPyV DNA in the early stages of infection. Statistical regression analyses comparing the two growth curves showed that there are significant differences. Although future studies would be needed to evaluate these potential interactions, the results obtained indicate that BKPyV may facilitate JCPyV infection by reducing the early phases, which might help to establish JCPyV infection in the human body. While humans are infected with BKPyV in early infancy, JCPyV infection, even if it is more frequently shed in urine, requires longer for transmission in the population and seems to do it later than BKPyV does (Taguchi et al. 1982; Walker and Padgett, 1983). The presence of BKPyV infection could then facilitate further JCPyV infection and the establishment of persistent infection by the more inefficiently multiplying archetypal strains present in urine.

To define the replication kinetics of BKPyV rearranged strain Dunlop, one-step growth curves in HNK-1 cells were studied. The virus had efficient DNA replication in these cells, increasing by 3 logs in 150 hours. This virus shares 70% genome homology with SV40 (Hirsh and Steiner, 2003). As mentioned above, there are reports on the interaction of BKPyV with SV40: BKPyV T-Ag can bind the promoter region of SV40 and *vice versa* (Caputo et al. 1986; Ryder et al. 1983). Some studies reported the presence of both viruses in urine and polyomavirus-associated nephropathy patients (Li et al. 2002; Vanchiere et al. 2005). SV40 rearranged strain 777 grows very inefficiently in human kidney cells (HNK-1), not only when infecting alone but also when co-infecting with BKPyV. Although BKPyV is a little more efficient when co-infecting with SV40, increasing by 3 logs in 100 hours, statistical regression analyses comparing the two growth curves showed that there are no significant differences. When Low et al. (2004) co-infected these two viruses in human kidney tubular epithelial cells, their analysis of growth by immunoblotting resulted in similar kinetics as those reported in the present study and SV40 did not multiply, supporting the results found here.

In this study, quantitative real-time PCR was used to define the infection kinetics of the viruses studied and quantification is based on the viral genome copies generated. The multiplication kinetics described could represent useful information for studies of biological and clinical aspects of JCPyV infection. Despite the limitations of the experimental conditions for the multiplication of JCPyV, the results suggest that the initial infection by BKPyV (which takes place during the early childhood) could facilitate the establishment of a persistent infection by JCPyV, hence contributing to explain the fact that JCPyV infection in the population takes place later thn BKPyV even if it is more frequently excreted in urine.

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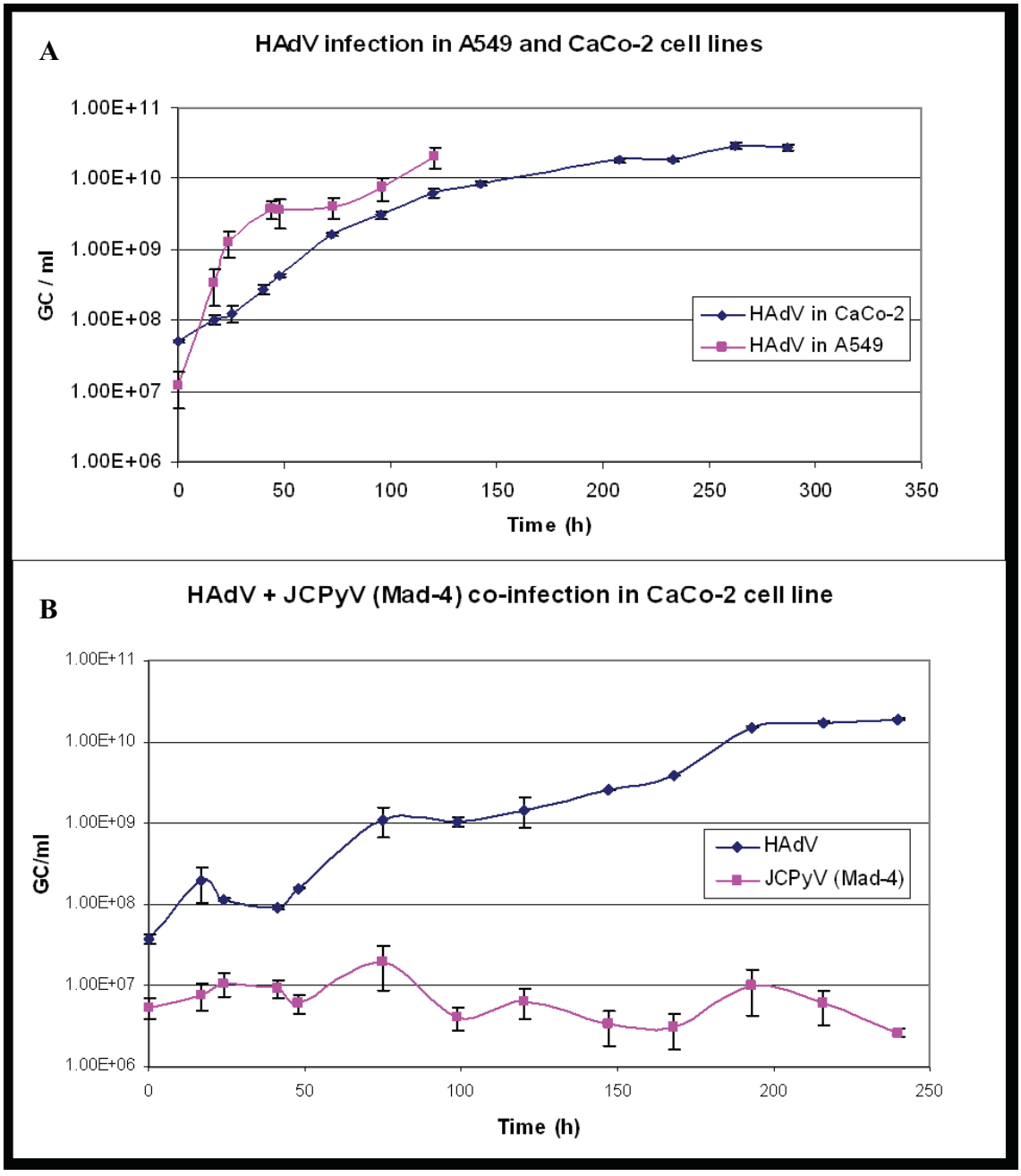

Fig.1. A) HAdV infection in A549 and CaCo-2 cell lines. B) HAdV and JCPyV Mad-4 co-infection in CaCo-2 cell line.

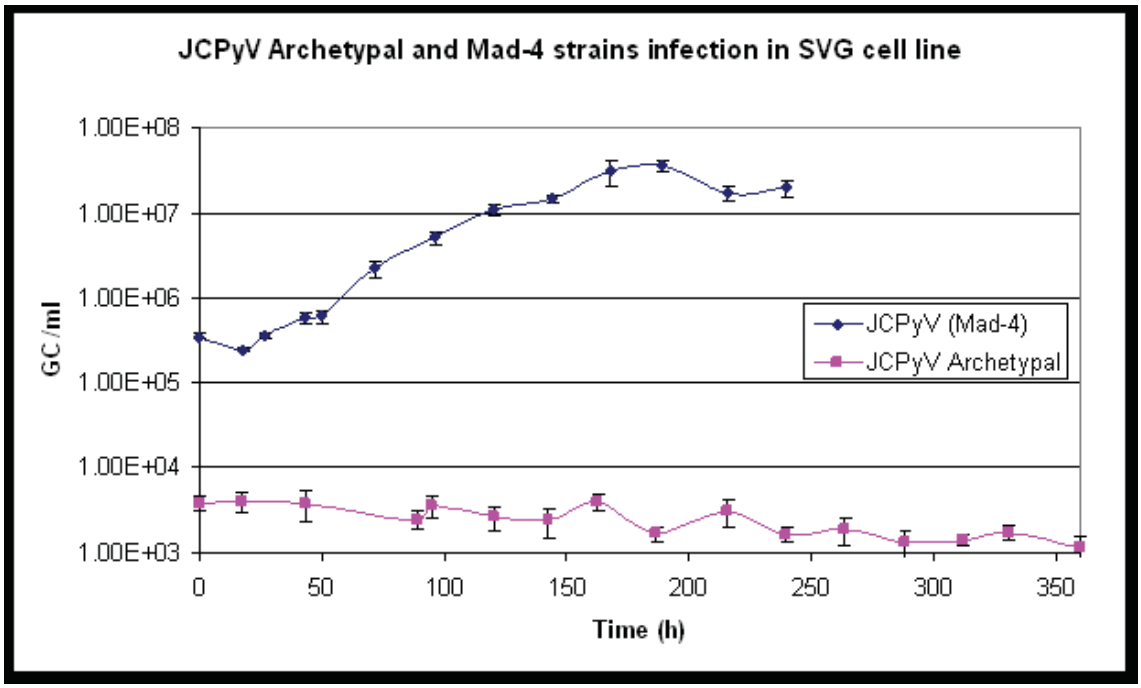

Fig.2. Kinetics of multiplication of JCPyV archetypal and rearranged strain infection in SVG cell line in independent experiments.

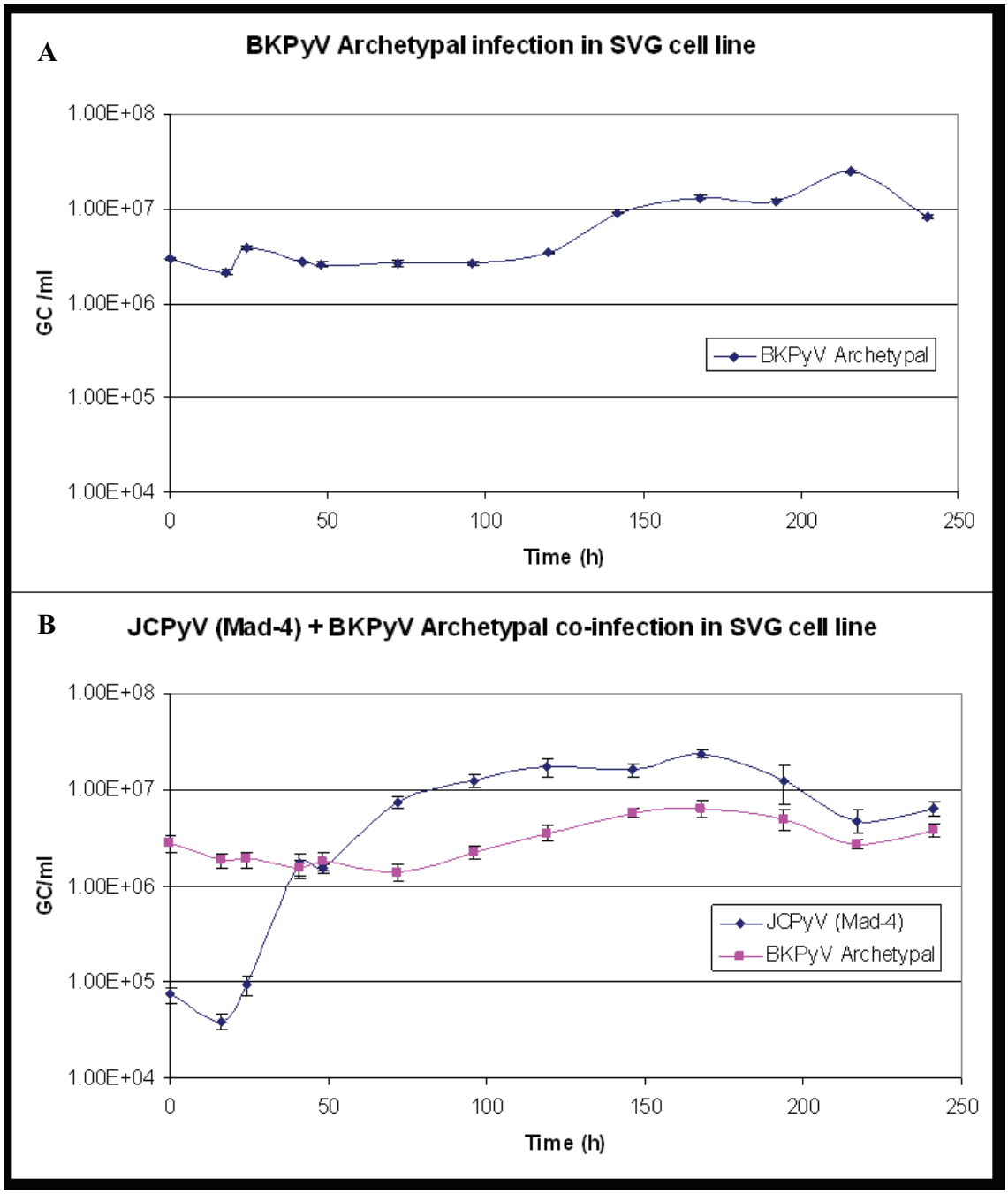

Fig.3. A) BKPyV archetypal strain infection in SVG cells. B) JCPyV Mad-4 and BKPyV archetypal strain co-infection in SVG cell line.

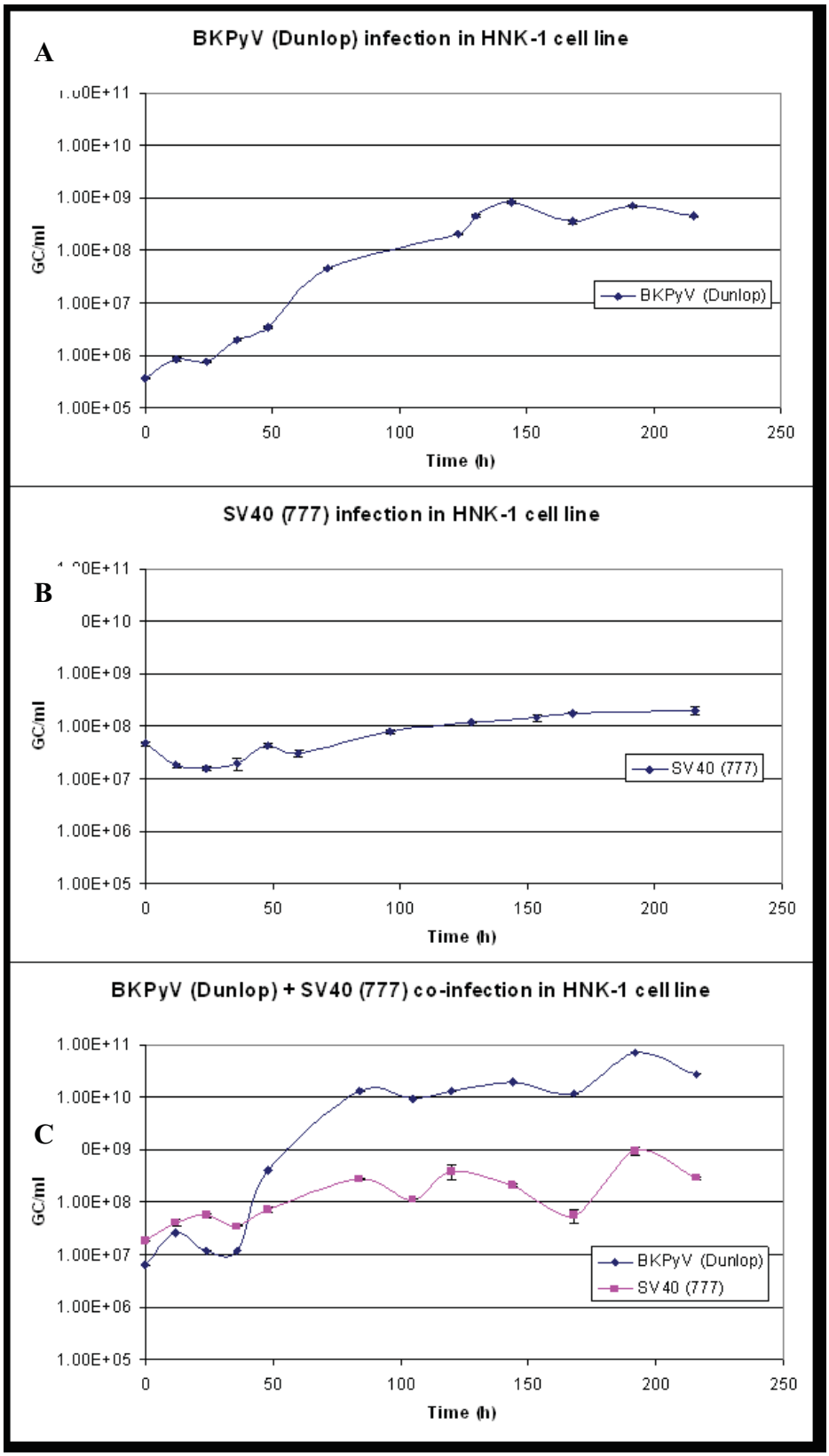

Fig. 4. A) BKPyV rearranged strain Dunlop infection in HNK-1 cell line. B) SV40 rearranged strain 777 infection in HNK-1 cells. C) BKPyV Dunlop and SV40 777 co-infection in HNK-1 cells.