

Two-day detection of infectious enteric and non-enteric adenoviruses by improved ICC-qPCR

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Abstract In order to provide a more suitable response to public health concerns, we improved the detection of infectious human adenoviruses in water by optimising the commonly used integrated cell culture–PCR method. Risk evaluation studies seek for rapid detection of infectious adenoviruses, including the enteric types 40 and 41 that are considered as the second most common agents of gastroenteritis in children next to rotaviruses. The here-employed 293A cell line used for infectious status assessment showed its ability to multiply adenoviruses including type 41. Two modifications were moreover applied to the workflow for viral detection. The first occurred at the nucleic acid extraction step performed directly on all infected cells, while the second was the application of real-time quantitative PCR as detection tool. All adaptations led to a 3-day reduction of the response delay and an improved sensitivity especially for the enteric adenoviral types. The infectious status of laboratory strain types 2 and 41 was demonstrated by a more than 2- \log_{10} increase in genome quantity. These conclusions were confirmed and reinforced by the analysis of water samples applying the improved assay. Naturally occurring infectious adenoviruses were detected in wastewater and river water, within 2 days. Types belonging to the species *human adenoviruses C* and type 31 were observed, but the most frequently identified type was 41 (71 % of identified sequences, $n=34$). This highlights the usefulness of our method for a wide range of

types, and especially for the most prevalent and public health-relevant enteric adenoviruses.

Keywords Human adenoviruses · Type 41 · ICC-qPCR · HEK 293A · Infectivity

Introduction

Adenoviruses are double-stranded DNA viruses that are commonly causing a wide range of respiratory, ophthalmic and gastrointestinal illnesses. Among human adenoviruses (HAdVs), types 40 (HAdV-40) and 41 (HAdV-41), also called enteric adenoviruses, are the two major types with a relevant public health issue. They are second as leading causes of childhood gastroenteritis after rotaviruses (Mena and Gerba 2009). Epidemiological (Dey et al. 2013) and environmental (Bofill-Mas et al. 2010) studies using molecular tools reported that HAdV-41 was clearly the most prevalent enteric strain. Unfortunately, types 40 and 41 cannot be grown easily in most in vitro cell culture systems that support growth of other HAdVs.

HAdVs are currently considered as emerging pathogens and have been widely detected in faecally polluted water all over the world (Jiang 2006). The potential for waterborne transmission of all adenovirus types exists, not just the enteric ones, because all of them are excreted in faeces. Contaminated water could therefore be a source of exposure, either through ingestion, inhalation or by direct contact with the eyes during recreational water activities (Mena and Gerba 2009; Sinclair et al. 2009). Due to their prevalence and their survival characteristics, HAdVs are part of the 12 microorganisms listed on the Candidate Contaminant List (CCL3) established by the US Environmental Protection Agency. A majority of the available data on environmental samples has been gathered with molecular methods only, such as PCR, nested PCR (nPCR) or real-time PCR, that do

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not yield any information about the infectious status of the viral pathogen. Detection of infectious particles in environmental samples using rapid, sensitive and reliable methods is however crucial (Hamza et al. 2011), also with regards to the recent reports on waterborne outbreaks of human enteric viruses, including HAdVs (Maunula et al. 2009).

Cell culture combined with PCR, also called integrated cell culture-PCR (ICC-PCR), is an approach that has been used to detect infectious viruses more rapidly than conventional cell culture assays. As it does not require the observation of cytopathic effects, ICC-PCR also has the advantage of detecting viable viruses that are fastidious to culture. The use of ICC-PCR has been described for the detection of enteroviruses (Reynolds et al. 1996), astroviruses (Grimm et al. 2004), rotaviruses (Li et al. 2010b), hepatitis A virus (Reynolds et al. 2001) and HAdVs. For these last ones, the association of A549 or BGM cells with nPCR is the most commonly used protocol (Dong et al. 2010; Greening et al. 2002; Lee et al. 2004; Lee et al. 2005; Wyn-Jones et al. 2011; Xagorarakis et al. 2007). After several days of incubation, the infected cells are submitted to successive freeze/thaw cycles for cell lysis and virus release. A small fraction of the supernatant is then used to perform viral DNA purification and qualitative amplification. Using such an approach, a number of studies have demonstrated a wide distribution of infectious HAdVs in water bodies (Lee et al. 2004, Lee et al. 2005; Rigotto et al. 2010; Xagorarakis et al. 2007). Nevertheless, the main drawbacks of this protocol are incubation time of at least 5 days and the lack of enteric HAdVs detection when A549 or BGM cells are employed (Wyn-Jones et al. 2011). An alternative method using 293 cells and based on (real-time) RT-PCR targeting mRNA was also proposed (Ko et al. 2003, 2005). However, mRNA quantification involves a time- and cost-intensive reverse transcription step, and an enzymatic digestion of contaminating DNA, whose efficiency has to be checked. As suggested by Ko et al. (2005), the genes may present differences in rates of expression leading to variation of sensitivity according to the mRNA targeted. Historically, the 293 cell line, also called HEK293 or Graham 293, was proposed to detect HAdVs (Graham et al. 1977). Renewed interest for these cells could recently be observed in the literature for culturing HAdVs, and especially the enteric types (Li et al. 2009; Siqueira-Silva et al. 2009), even though an inherent problem with 293 cells is their poor capacity to adhere on surfaces, resulting in easy dislodgement and tricky maintaining in culture flasks. To overcome this experimental limitation, a sub-clone of 293 cells, named 293A and displaying better adhesion properties, was recently proposed for environmental assessment of HAdV contamination (Jiang et al. 2009; Li et al. 2010a).

The purpose of this study was to propose an improved ICC-quantitative PCR (ICC-qPCR) assay that notably decreases the response delay for infectious status assessment,

through modifications at the DNA extraction and amplification steps. Application of a qPCR approach further allows monitoring the increase in the initial genome copy numbers over time, thus reflecting viral replication upon infection of the host cells. Moreover, we used the 293A cell line, in order to detect all infectious HAdVs including the enteric types. The combination of all improvements was validated on two laboratory strains (HAdV types 2 and 41) before evaluation on contaminated environmental surface water and wastewater samples.

Materials and methods

Cell lines and adenoviruses

Human embryonic kidney cell line 293A (Life Technologies, R705-07) was used in this study between passages 12 and 30. Cell line 293A is a sub-clone of 293 cells with a rather flat morphology which strongly adheres to conventional culture surfaces, without need for flask coating. 293A cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing a high glucose concentration and Glutamax (Life Technologies). This medium was supplemented with 1 % of non-essential amino acids (Life Technologies) and 5 % of heat-inactivated foetal bovine serum (Life Technologies). Human lung carcinoma A549 cell line was obtained from the Health Protection Agency Culture Collections (HPACC, 86012804) and was used in this work between passages 6 and 11. Growing medium was DMEM containing a high glucose concentration, Glutamax (Life Technologies) and 5 % of heat-inactivated foetal bovine serum (Life Technologies).

Human adenovirus type 2 (HAdV-2; HPACC, NCPV#213) and HAdV-41 (American Type Culture Collection, VR-930) were propagated in 293A cells. After 1 week, adenoviruses were extracted by freezing/thawing the flasks three times, followed by centrifugation (175×g for 5 min) to remove cell debris. The viral stock was supplemented with 20 % foetal bovine serum and 30 % sterile glycerol before storage at −80 °C. The infectivity titre of adenovirus was estimated by most-probable-number analysis and expressed as most probable number of cytopathic units (MPNCU). HAdV-2 concentration was determined in microtitre plates by serial log₁₀ dilutions of the viral stock and inoculation of 50 µL of dilution in each of the 96 wells (40 wells/dilution), containing 200 µL of cell suspension (3×10⁴ cells/mL). Plates were incubated at 37 °C with 5 % CO₂ during 11 days before cytopathic effect (CPE) examination. For HAdV-41, two successive passages in cell culture were necessary to determine the viral concentration. For the first passage, 750 µL of each dilution of HAdV-41 stock were inoculated on the six wells of the microtitre plate containing 2 mL of the cell suspension (2×10⁵ cells/mL). After 15 days of culture, the six-well microtitre

plates were frozen at -20°C . Cell lysate was produced for the next passage by thawing the plates at room temperature and using a cell scraper to physically disrupt the cell monolayer (Mahalanabis et al. 2010). For the second passage, 750 μL of the lysates were transferred in a new six-well plate also containing 2 mL of cell suspension (2×10^5 cells/mL) and incubated for another 7 days before CPE examination.

Detection of infectious adenoviruses by ICC-qPCR

For each viral sample (laboratory strain suspensions or water concentrates), a 1-mL volume of viral suspension was inoculated per individual tissue culture flask of 4-day-old 293A or A549 cells, with a growth area of 25 cm^2 . The number of infected flasks depended on the experiments. For experiments using laboratory strain suspensions, nine flasks (one T_0 , two T_1 , two T_2 , two T_3 and two T_5) were needed, while five flasks (one T_0 , two T_2 and two T_3) were used for analyses of water concentrates. A negative control was included in all our experiments, consisting in the inoculation of a cultured cell monolayer with 1 mL of DMEM medium. After inoculation, flasks were incubated for 60 min at 37°C for adsorption of viral particles. Afterwards, the inoculum was removed, and the cells were washed three times with 4 mL of DMEM. Finally, 4 mL of DMEM containing 2 % of foetal bovine serum and supplemented with a 1 % final concentration of an antibiotics–antimycotic solution (10,000 units/mL of penicillin, 10,000 $\mu\text{g}/\text{mL}$ of streptomycin and 25 $\mu\text{g}/\text{mL}$ of amphotericin B; Life Technologies) were added to each flask. One flask was immediately analysed without incubation (T_0), while the other flasks were incubated at 37°C in an atmosphere of 5 % CO_2 for 1 to 5 days, before viral DNA purification and amplification.

Extraction of viral DNA

Two protocols were applied for viral DNA extraction from incubated 293A or A549 cells cultured in 25 cm^2 flasks for predetermined numbers of days at 37°C .

Protocol 1 The flasks were frozen at -20°C and thawed at room temperature three times. Cell suspensions were centrifuged at $175 \times g$ for 5 min, and 140 μL of supernatant (4 mL) were recovered to extract viral nucleic acids using the QIAamp Viral RNA mini kit (Qiagen) to a final volume of 100 μL .

Protocol 2 The entire cell monolayer was disrupted using a scraper. Cells and medium were transferred in a 15-mL centrifuge tube. Cells were then harvested by centrifugation at $150 \times g$ for 5 min (4°C), and the supernatant was discarded. The recovered cell pellet was lysed with 900 μL of lysis buffer (AVL buffer from the QIAamp

Viral RNA mini kit) and viral nucleic acids were extracted using the QIAamp Viral RNA mini kit to a final volume of 100 μL .

Thus, only a fraction of the sample (140 μL out of 4 mL) was examined with protocol 1, whereas all scraped cells were analysed with protocol 2, allowing the investigation of a 30-fold higher sample volume. Given the failure of normality tests or equal variance tests for some groups of data, statistical comparisons of the performance of both protocols were performed through a nonparametric test (Mann–Whitney rank sum test) using SigmaStat software v.2.03.

Real-time PCR assay conditions

Quantification of the adenovirus genome was performed by a generic real-time PCR system, according to the protocol described by Hernroth et al. (2002) using degenerated primers (AdF and AdR) and a TaqMan probe (Ad/ACDEF). This assay is based on the amplification of the HAdV hexon gene and recognises most of HAdVs (Bofill-Mas et al. 2006). Amplifications were performed in a 25- μL reaction mixture containing 10 μL of template DNA and 15 μL of QPCR Master Mix Low ROX (Eurogentec) or TaqMan Universal MasterMix (Life Technologies) containing 0.9 μM of each primer and 0.225 μM of TaqMan probe. Temperature cycles were performed either in an ABI Prism 7500 Fast PCR system or in a StepOnePlus PCR system, according to the involved laboratory. Real-time PCR standard curves were validated and compared between both laboratories, and no significant difference was observed. The standard curve used for this quantitative PCR was generated by using 10-fold dilutions of a linearised plasmid containing the HAdV-41 hexon sequence (Bofill-Mas et al. 2010). The bacterial carrier (*Escherichia coli* JM109 competent cells) of this plasmid (pBR322) was kindly provided by Professor Girones (Department of Microbiology, University of Barcelona).

Environmental water samples

Over a 2-month period (March–April 2012), a total of 16 samples were collected from the influent or the effluent of two wastewater treatment plants (WWTP) located in Schiffange (capacity, 90,000 inhabitants equivalent) and Beggen (capacity, 300,000 inhabitants equivalent), Luxembourg. Wastewater samples were concentrated as previously described (Skraber et al. 2011). Briefly, 200 mL of influent or 400 mL of effluent were clarified by centrifugation ($3,000 \times g$ for 15 min). The supernatant was ultracentrifuged at $100,000 \times g$ for 90 min, and the resulting pellet was resuspended in 10 mL of growth medium (DMEM containing 5 % of foetal bovine serum). To remove bacteria from the concentrate, the suspension was passed

through a combination of pre-filter (5 μm , Sartorius Minisart) and membranes (0.8 and 0.2 μm , PALL, Acrodisc PF). Samples were stored at $-80\text{ }^\circ\text{C}$ prior to cell infection.

Ten river water samples were collected in the Meurthe River (Nancy), in February, March, October and November 2012, at two different sampling sites (S1 and S2). A glass wool method was used for virus concentration, as previously described (Wyn-Jones et al. 2011). Briefly, river samples (10 L) were acidified with 1 M HCl (pH 3.5) and 10 g of sterile glass wool was used for the filtration column. The resulting concentrate containing viruses was dissolved to a final volume of 10-mL 5 % DMEM. As performed for wastewater samples, the suspension was passed through a combination of pre-filter (5 μm , Sartorius Minisart) and membranes (0.8 and 0.45 μm , Sartorius Minisart) before freezing at $-80\text{ }^\circ\text{C}$.

The environmental sample analysis was performed using only the most sensitive ICC-qPCR method.

Sequencing analysis of water samples

Before sequencing, DNA template of real-time PCR-positive samples (wastewater and river samples) was amplified by a nPCR assay described by Allard et al. (2001). The amplicons obtained were purified using ExoSAP-IT (Affimetrix) and subsequently directly sequenced on both strands using a Big Dye Terminator v 3.1 Cycle Sequencing kit (Life Technologies) and a capillary automatic sequencer (ABI Prism 3130 genetic analyser), following the manufacturer's instructions. Conditions for the 25-cycle sequencing amplification were: denaturing at $96\text{ }^\circ\text{C}$ for 10 s, annealing at $50\text{ }^\circ\text{C}$ for 5 s and extension at $60\text{ }^\circ\text{C}$ for 4 min. The primers hex1deg and hex2deg or the nested primers nehex3deg and nehex4deg described by Allard et al. (2001) were used for sequencing at a concentration of 0.125 μM . The obtained sequences were then compared with the GenBank database using the basic BLAST program of the NCBI (National Centre for Biotechnology Information, <http://www.ncbi.nlm.nih.gov/BLAST/>).

Results

Viral stock suspension assay by CPE and real-time PCR

The titre of the HAdV-2 stock was determined to be 3×10^9 MPNCU/mL. For HAdV-41, the stock titre was estimated at 7×10^5 MPNCU/mL after one passage and 1×10^7 MPNCU/mL after a second passage.

The standard curve of the generic TaqMan assay showed a reliable detection ranging from 10^1 to 10^6 genome copies/reaction. The detection limit was thus estimated at

10 genome copies/reaction. From the standard curve obtained with the plasmid, the amount of genome was determined to be similar for both viral stocks (HAdV-2 and HAdV-41) and reached 1×10^{10} genome copies/mL.

Permissibility of A549 cells to HAdVs

Cell line A549 was infected with 100 MPNCU per flask of HAdV-2 or HAdV-41 in order to compare its sensitivity to different adenovirus types. After an incubation period of 5 days, cell monolayers were submitted to DNA extraction using protocol 2. The real-time PCR results allowed quantifying $4.99 \pm 0.88 \log_{10}$ genome copies of HAdV-2 per reaction, while no amplification was observed from cells infected with HAdV-41.

Comparison of both extraction protocols using 293A cells

Both extraction procedures were compared using 293A cells infected with HAdV types 2 or 41 at a concentration of 10 MPNCU/flask. Infected cells were analysed daily during 3 days of incubation. Higher genome quantities were systematically detected using protocol 2, whatever the studied virus (Fig. 1). As the volume of sample tested differed between both methods, an extrapolation of the quantities present in the entire sample was performed with the results obtained with protocol 1 (calculation of equivalents for 4,000 μL with the results obtained for 140 μL ; Fig. 1). The deviation observed between both protocols could be directly related to the higher volume of sample analysed for HAdV-2, since no significant difference was observed between the quantities of genome detected with protocol 2 and those estimated for the whole sample using protocol 1 (Mann–Whitney U tests, $P=0.150$, $P=0.534$, $P=0.844$ for 1, 2 and 3 days of incubation, respectively). It was instead not the same for HAdV-41 since a significantly different viral load was calculated between both approaches (Mann–Whitney U tests, $P<0.001$ for each incubation time).

Detection limits

In order to investigate the minimum incubation time required to detect low concentrations of infectious HAdVs, confluent 293A cells in 25-cm² flasks were infected with varying amounts of HAdV-2 (10, 1 and 0.1 MPNCU) or HAdV-41 (10, 1, 0.1 and 0.01 MPNCU), followed by analyses with both extraction methods up to 5 days post-infection (Table 1). We observed that 293A cells inoculated with 1 MPNCU of HAdV-2 exhibited a positive result at 2 days post-infection using both protocols, with higher DNA quantities detected with protocol 2. Cells infected with the same concentration of HAdV-41 were declared positive the third day post-infection with protocol 1; whereas, a

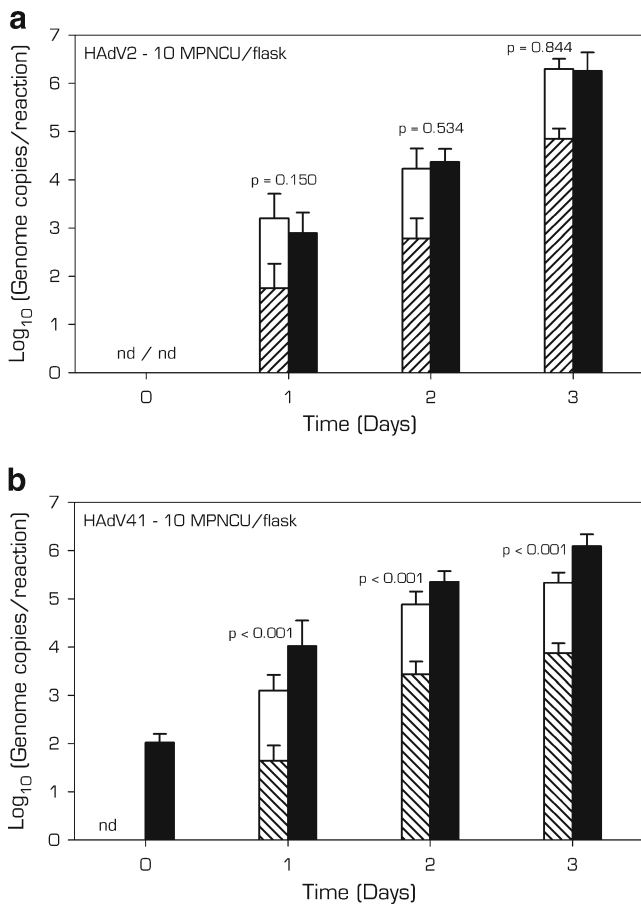


Fig. 1 Comparison of adenoviral DNA concentrations detected in cells infected by HAdV-2 (**a**) and HAdV-41 (**b**) throughout a 3-day incubation period, using protocol 1 (hatched bars) and protocol 2 (black bars). Each bar represents the average of five replicates. The white bars represent an estimation of the adenoviral DNA concentration if the whole sample had been analysed using protocol 1. *P* values corresponding to the comparison of protocol 1 (estimated values) with protocol 2 using the Mann–Whitney *U* test are given for each incubation day. *nd* not detected or below the detection limit

positive signal was already observed the day of infection using protocol 2. The lowest concentration detected with protocol 1 was 1 MPNCU/flask for both HAdV-2 and HAdV-41; whereas, protocol 2 permitted the detection of 0.1 MPNCU/flask for HAdV-2 and HAdV-41 after 5 and 1 day(s) post-infection, respectively. Interestingly, it could be noticed that using protocol 2 one replicate out of two was positive after infection with 0.1 MPNCU of HAdV-2 and 0.01 MPNCU of HAdV-41 at 2 and 3 days post-infection, respectively.

Environmental samples

The modified protocol (protocol 2) was performed to detect infectious adenoviruses in wastewater and river water

Table 1 Sensitivity of both ICC-qPCR protocols (P1/P2) for detection of infectious HAdV-2 and HAdV-41 ($n=2$)

	MPNCU/flask	Incubation time (days)				
		T_0 P1/P2	T_1 P1/P2	T_2 P1/P2	T_3 P1/P2	T_5 P1/P2
HAdV-2	10	-/-	+/+	+/+	+/+	+/+
	1	-/-	-/-	+/+	+/+	+/+
	0.1	-/-	-/-	-/-	-/-	-/+
	0	-/-	-/-	-/-	-/-	-/-
HAdV-41	10	+/+	+/+	+/+	+/+	+/+
	1	-/+	-/+	-/+	+/+	+/+
	0.1	-/-	-/+	-/+	-/+	-/+
	0.01	nd/-	nd/-	nd/-	nd/-	nd/-
	0	-/-	-/-	-/-	-/-	-/-

A sample was declared positive if both flasks presented a positive signal by qPCR

“-” negative ICC-qPCR results, “+” positive ICC-qPCR results, *nd* not determined

samples. After 2 days of incubation (T_2), the percentage of positive samples for WWTP influents, WWTP effluents and river water was 100.0 (eight of eight), 75.0 (six of eight) and 90.0 % (nine of ten), respectively. For all positive water samples, the copy numbers of adenovirus detected in infected cells increased by more than 2- \log_{10} between T_0 and T_2 , and remained stable between T_2 and T_3 (Table 2). Twenty-five cell culture samples from wastewater (11 T_0 and 14 T_3) and 15 cell culture samples from river water (6 T_0 and 9 T_3) were further analysed by sequence-based typing of the HAdVs present. Thirty-four amplicons were obtained by nPCR. The most frequently detected HAdV type was 41 (70.6 %). Types belonging to the species *HAdV C* (HAdV 1/2/5/6; 17.6 %) and type 31 (11.8 %) were observed with lower frequency.

Discussion

The combination of the 293A cell line with a quantitative PCR tool for HAdV infectivity assessment has to date never been reported. So far, literature data show a consensus with the use of A549 or BGM cell lines for HAdV detection in aquatic environments by ICC-PCR, albeit sparse studies proposed the employment of 293 cells (Ko et al. 2005). In a Europe-wide surveillance performed in recreational waters, the HAdV type 41 was the most frequently observed by direct nPCR, whereas its presence could not be evidenced in a parallel ICC-nPCR assay based on A549 cells due to a lack of host cell compatibility (Wyn-Jones et al. 2011). Using the same cell line, our results corroborated the latter

Table 2 Detection of infectious adenoviruses in wastewater and river samples using the improved ICC-qPCR protocol

Site	Water type	Volume of water analysed (L)	T_0 number of positive samples ^a	Log_{10} gc/react (average \pm SD)	Types or species	T_2 number of positive samples	Log_{10} gc/react (average \pm SD)	T_3 number of positive samples	Log_{10} gc/react (average \pm SD)	Types or species
Schiffflange	Influent	0.2	3/4	2.49 \pm 0.43	41, <i>HAdV C</i>	4/4	4.91 \pm 1.38	4/4	5.87 \pm 0.40	41, <i>HAdV C</i>
	Effluent	0.4	0/4	–	–	2/4	3.12 \pm 1.05	2/4	3.14 \pm 0.88	31
Beggen	Influent	0.2	4/4	2.53 \pm 0.42	41	4/4	5.12 \pm 0.47	4/4	5.77 \pm 0.30	41
	Effluent	0.4	4/4	2.34 \pm 0.24	41	4/4	5.53 \pm 0.33	4/4	5.99 \pm 0.28	41
Meurthe	S1	10	3/5	1.82 \pm 0.08	41, <i>HAdV C</i>	4/5	4.70 \pm 0.41	4/5	4.95 \pm 1.07	41, 31
	S2	10	3/5	2.06 \pm 0.38	<i>HAdV C</i>	5/5	4.88 \pm 0.22	5/5	5.40 \pm 0.27	41

The major types or species were identified by sequencing analysis

gc/react genome copies per qPCR reaction

^aBased on qPCR results

since no HAdV-41 DNA was detectable after 5 days, even in employing the most sensitive extraction protocol (P2, cell scrapers). The improved ICC-qPCR protocol proposed in the present study was based on an initial culture step using the 293A cell line. Our results of the infectivity assay using a most probable number method confirmed the ability of 293A cells to support the growth of HAdV-41, as previously described (Jiang et al. 2009).

In addition to a change in the choice of cell line, we also introduced here two technical modifications to improve the detection sensitivity of the fastidious enteric HAdVs. First, the collection of all the cells from a flask using a scraper was preferred to freeze/thaw cycles. Alternatively, the use of trypsin or TRYzol would also allow analysing the whole sample (Gerrity et al. 2008; Ko et al. 2005; Li et al. 2010b). Using a scraper, the cells are harvested in about 5 min; whereas, the three freeze/thaw cycles require half a day. Beyond the time saved during the test, the collection of the entire cell monolayer before DNA extraction permitted analysing a 30-fold higher quantity of sample than that commonly tested after freeze/thaw cycles. Larger quantities of genome were systematically detected with our modified ICC-qPCR protocol, demonstrating a better detection sensitivity. For HAdV-2, the difference between both approaches could be directly related to the increase in sample volume with the modified assay. For HAdV-41 however, the deviation between both protocols was significantly more pronounced. In accordance with our results, it appears that applying freeze/thaw cycles is not the most effective way to harvest HAdV-41 genomes from infected cells. A longer multiplication cycle of HAdV-41 could be a possible explanation. Siqueira-Silva et al. (2009) described that the HAdV-41 multiplication cycle had a slower progression than the one of HAdV-2 on 293 cells. Cells infected with HAdV-2 were lysed at 72-h post-infection, while more than 132 h were needed to observe the spreading of HAdV-41 to

neighbouring cells. Moreover, HAdV-41 was described to have a non-lytic cycle, but the release process of newly formed viral particles remains unknown (Siqueira-Silva et al. 2009). Protocol 2, which relates to DNA content release based on chemical treatment of the cells using guanidine salts under highly denaturing conditions (lysis buffer of extraction kit), seems to allow a better recovery efficiency for HAdV-41 than freeze/thaw cycles.

The second methodological improvement was the choice of the molecular biology tool combined to cell culture. The infectious status of viruses can be evidenced by a change of magnitude in viral genome quantities during cell infection. The quantitative aspect of the real-time assay provides thus a simple, reliable method to monitor the inoculated cells for the increase in viral genome over time, contrary to the qualitative nPCR. In the work described here, nPCR was only employed prior to DNA sequencing of environmental water samples for type identification. Along with the quantitative aspect, our results showed a better sensitivity of qPCR than nPCR. Eleven samples of wastewater out of sixteen were tested positive by real-time PCR the day of infection (T_0), but only six of these eleven remained positive by nPCR (data not shown). This difference of sensitivity was less evident after 3 days of incubation, when samples contained superior amounts of viral DNA. Also, we notably chose a qPCR approach because it is more time- and cost-effective than the RT-real-time PCR previously proposed (Ko et al. 2005), even though mRNA detection can be directly related to the presence of infectious viruses. Our experiments performed with low numbers of infectious adenoviruses showed that the modified protocol allowed detecting 1 MPNCU/flask after 2 days of infection for HAdV-2 and 0.1 MPNCU/flask after 1 day of infection for HAdV-41. It appears hazardous to directly compare these two detection limits because an underestimation of the infectious titre within our HAdV-41 stock was still probable,

even after a second passage. The difference of infectivity titre between HAdV-2 and HAdV-41 was certainly related to a less conspicuous CPE with the HAdV-41. In order to balance sensitivity and detection time, the promising results obtained here with laboratory strains suggest that a 2-day incubation time may be a sufficient assay time for water samples, contrary to a 5-day period recommended when using the common ICC-nPCR assay with the A549 cells. This faster response can be explained by the better permissibility of the 293A cell line to HAdVs than A549 cells (Jiang et al. 2009), resulting thus in a quick and effective viral multiplication.

Three kinds of water samples naturally contaminated with different levels of faecal material were analysed using the improved protocol, following a viral concentration step. In all samples, it appeared that the viral DNA concentration measured after 2 days was always at least 100-fold higher than that initially present. An incubation of 2 days is thus optimal to give evidence for the occurrence of infectious adenoviral particles. As a consequence, the volume of the viral concentrate can be reduced in accordance with the concentration method used, resulting in an increase of the concentration factor; and the likelihood of detecting viruses will be greater. With our protocol, the equivalent of 1 L of environmental water is inoculated per flask. Using these experimental conditions, the incidence of infectious HAdVs was 100.0 ($n=8$), 75.0 ($n=8$) and 90.0 % ($n=10$) of samples from WWTP influents, effluents and river water, respectively. Not surprisingly, these data were quite higher than those described in previous studies. The recent Europe-wide surveillance study has shown that 20.0 % ($n=226$) of freshwater samples contained infectious adenoviruses (Wyn-Jones et al. 2011). In two Korean studies, the authors reported the presence of infectious adenoviruses in 43.5 ($n=69$), 42.0 ($n=50$) and 32.5 % ($n=40$) of surface water, tap water and river water samples, respectively (Lee et al. 2004, 2005). Similarly, infectious particles were detected in 36.0 % ($n=42$) of samples from a water treatment plant (Dong et al. 2010). This discrepancy might be easily explained by the accessibility to all adenovirus types thanks to the modified protocol. In our water samples, sequencing of the hexon gene retrieved types 41 was the most predominant type. Type 31 as well as types belonging to the *HAdV C* species were also found. Noteworthy, the presence of a mixture of diverse DNA sequences was observed in several samples, revealing larger type diversity. These results are consistent with previous studies based on A549 or BGM cells (Lee et al. 2004; Wyn-Jones et al. 2011), except accurate detection of infectious enteric adenoviruses. These latter authors reported that the most frequently observed types belonged to the species *HAdVA* (types 31 and 12), *HAdV B* (types 3 and 7) and *HAdV C* (types 1, 2, 5 and 6). As proven by these results, our improved ICC-qPCR protocol expands

the range of targeted types, and specifically the enteric ones. To confirm these findings and obtain more details on the spectrum of adenoviruses detectable by our ICC-qPCR assay, a more discriminative taxonomic identification (e.g. sequencing after cloning of PCR products) would add further health-related value, as the selected sequencing approach reveals the most frequent sequences only.

To conclude, the proposed combination of modifications improves the ICC-PCR method in terms of sensitivity, range of types detected, incubation and handling times, without any additional steps. Benefits of the methodological improvements were especially measurable for the enteric type 41. The usefulness of our ICC-qPCR is, for the moment, restricted to presence/absence information. The next step will be to optimise this assay to permit the quantification of infectious adenoviruses occurring in water samples. The ICC-qPCR could be, for instance, associated either to the MPN assay (Dong et al. 2010) or to a growth curve approach as recently described for the poliovirus (Lambertini et al. 2010). In any event, the work described here should be an important contribution to efforts in monitoring all infectious adenoviruses in the environment.

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