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Protocols

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

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A B S T R A C T

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

1. Introduction

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

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

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

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

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

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

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

2. Materials and methods

2.1. Cell lines and viral stocks

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

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

2.2. River water samples

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

2.3. Sewage samples

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

2.4. Antibodies

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

2.5. Immunofluorescence assay (IFA)

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

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

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

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

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

2.6. Plaque assay

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

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

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

2.8. Quantitative PCR (qPCR)

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

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

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

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

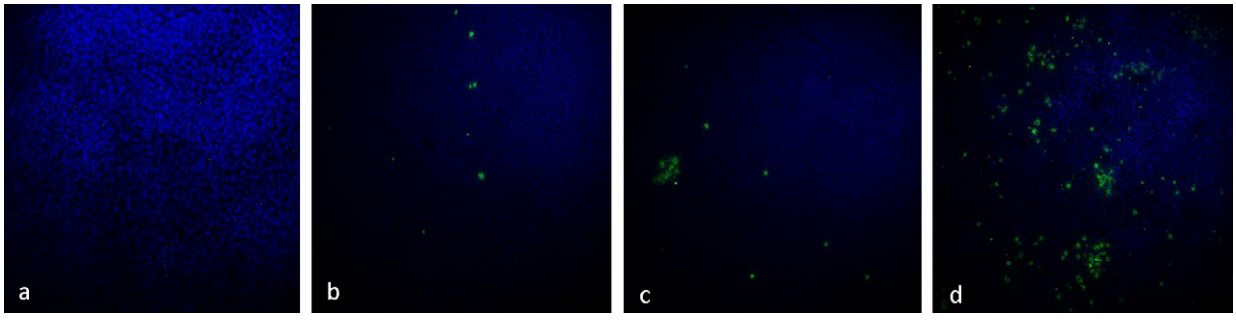

Fig. 1. Infected SVG-A cells with JCPyV strain Mad-4 by IFA. (a) Negative control (b) 4th day of infection, individual infected cells are detected, (c) 8th day of infection, individual infected cells and foci are detected, (d) 11th day of infection, the cells infected from the virus spiked are not distinguished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

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

3. Results

3.1. Immunofluorescence assays

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

4. Discussion

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

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

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

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

behavior of JCPyV in environmental conditions and inactivation treatments.

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

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

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

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

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