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Real time PCR for rapid determination of susceptibility of adenovirus to antiviral drugs

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ABSTRACT

Human adenoviruses (HAdV) are associated with respiratory, ocular and gastrointestinal infections as well as potentially fatal disseminated disease in highly immunocompromised patients. Although there is no specific FDA approved treatment for HAdV infections, some antivirals are used in certain patients. The in vitro antiviral assays for HAdV are not standardized and are usually time consuming. The objective of this study was to evaluate a real time PCR assay for rapid determination of susceptibility of HAdV to antiviral drugs. The nucleoside analogue stavudine (d4T) was used as test drug in A549 cells infected with HAdV5. The antiviral assay measured the reduction of the HAdV DNA levels in culture supernatants by real time PCR using specific primers that amplify a conserved region of the hexon gene. This real time PCR assay demonstrated that stavudine was a selective inhibitor for HAdV5, since the effective concentration 50% (EC_{50}) ranged from 0.08 to 0.12 mM at multiplicity of infection between 0.001 and 1. Furthermore, EC_{50} showed a high correlation with plaque reduction and virus yield inhibition assays (r^2 = 0.9938 and r^2 = 0.9468, respectively). In conclusion, the real time PCR-based antiviral assay is rapid, reproducible and could replace classical and more labor-intensive infectivity assays.

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1. Introduction

Human adenoviruses (HAdV) belong to the *Mastadenovirus* genus in the *Adenoviridae* family. They are divided into seven species from A through G, based on immunological, biological, and biochemical properties. To date, 52 serotypes have been described (Jones et al., 2007) and different genome types can be distinguished within the same serotype.

Primary HAdV infections usually occur in young children and are often mild and self-limited. Approximately 5% of acute respiratory illnesses in children less than 5 years old are caused by HAdV infection. Although HAdV infections were traditionally responsible for respiratory, ocular and gastrointestinal diseases, many other clinical manifestations have been associated with HAdV replication, especially in immunocompromised patients where more severe consequences have been observed. Clinical manifestations depend on the host and the serotype and include pharyngitis, pharyngoconjunctival fever, conjunctivitis, keratoconjunctivitis, bronchiolitis, pneumonia, hemorrhagic cystitis, gastroenteritis, myocarditis and hepatitis. Paediatric patients undergoing allogeneic stem cell transplantation are particularly prone to disseminated HAdV infections with high morbidity and mortality. In 2005, a rare human adenovirus serotype 14 emerged in the USA among civilians and military trainers causing severe respiratory disease (Lewis et al., 2009; Tate et al., 2009).

There is currently no FDA approved antiviral therapy specific for HAdV infections, although, some antivirals have been used for patient care. There are three clinical circumstances in which an effective HAdV chemotherapy would have a considerable impact. The first involves immunocompromised individuals. Given the extent of the immunocompromised population, including individuals infected with human immunodeficiency virus (HIV) as well as transplanted patients, HAdV have been recognized increasingly as a significant viral pathogen in these individuals. In fact, mortality rates between 6 and 70% are reported in paediatric and adult transplant patients (Wasserman et al., 1988; Hale et al., 1999; Venard et al., 2000). The second circumstance is the occurrence of ocular infection resulting in significant patient morbidity, as well as substantial economic losses (Gordon et al., 1991). Furthermore, keratoconjunctivitis, a more serious condition involving the cornea and conjunctiva, may have long-term consequences on visual acuity. The third circumstance is lower respiratory infections in paediatric patients who may develop long-term pulmonary sequel.

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Several drugs such as ganciclovir, vidarabine, ribavirin and cidofovir have been used for HAdV infection with variable results (Arav-Boger et al., 2000; Bordigoni et al., 2001; Hoffman et al., 2001).

The in vitro methods used to determine the anti-HAdV activity are not well standardized. In addition, most of them are time consuming and labor-intensive.

This study focused on the evaluation of a real time PCR assay for rapid determination of antiviral drug susceptibility for HAdV type 5 (HAdV5). The test drug used was the nucleoside analogue stavudine (d4T), an FDA approved drug used for HIV therapy, which reported in vitro activity against some HAdV serotypes (Uchio et al., 2007). Stavudine was an easy drug to obtain; therefore, it was used to validate the assay.

2. Materials and methods

2.1. Cell culture and virus

A549 cells were grown in Eagle's minimum essential medium (MEM) (GIBCO, New York, USA) and supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin G and 100 μg/ml streptomycin. For maintenance medium, the serum concentration was reduced to 2.0%.

HAdV5 was propagated in A549 cells using 75 cm^2 cell culture flasks. Virus was harvested from cultures exhibiting >95% cytopathic effect (CPE) by freezing and thawing the cell culture flasks. Supernatant was cleared by centrifugation at $3000 \times g$ for 5 min and stored at −70 °C for further use. Stocks of HAdV were titrated by plaque formation in A549 cells.

2.2. Antiviral drug

Stavudine (d4T: 2′,3′-didehydro-3′-deoxythymidine) was provided generously by Laboratories Microsules, Argentina. It was dissolved in 20 mM aqueous stock solution and stored at −70 °C for further use.

2.3. Cytotoxicity assay

Cell viability was measured by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) method (Sigma–Aldrich, St. Louis, MO, USA). Confluent cultures in 96-well plates were exposed to different concentrations of the drug, in triplicate, using incubation conditions equivalent to those used in the antiviral assays. Then 20 μl of MTT (final concentration 5 mg/ml in PBS) was added to each well. After 2 h incubation at 37 °C, the supernatant was removed and 200 μl of ethanol was added to each well to solubilize the formazan crystals. After vigorous shaking, absorbance was measured in a microplate reader at 595 nm. The cytotoxic concentration 50% (CC_{50}) was calculated as the drug concentration required to reduce cell viability 50% by regression analysis.

2.4. Virus DNA replication kinetics

The kinetics of HAdV5 DNA replication were examined by measuring the increase of HAdV5 DNA in culture supernatants and infected cells over a 3-day time-course, after infection at multiplicity of infection (MOI) of 1 and 0.1.

A549 cells grown in 96-well plates were infected with HAdV5 diluted in maintenance medium to obtain a MOI of 1 and 0.1 PFU/cell. After 1 h at 37 °C, virus was aspirated and replaced by fresh medium. After 3, 24, 48 and 72 h post-infection (p.i.), cell culture supernatants and cells were separately collected and DNA was extracted using the QiaAmp DNA Blood Mini Kit (Qiagen, California, USA). HAdV5 DNA load was measured using real time PCR. The baseline value of HAdV5 DNA concentration was determined after virus adsorption and internalization 3 h p.i.

2.5. Real time PCR for HAdV5

A validated real time PCR for HAdV5 was performed in a Smart Cycler II (Cepheid, California, USA) using Smart Mix beads (Cepheid, California, USA). The primers for real time PCR amplify a highly conserved region of the hexon gene (Echavarria et al., 1998). A specific TaqMan probe for HAdV species C was labeled with FAM and Black hole quencher (Claas et al., 2005). Additional probes to detect species A, B, D, E and F have been developed for a generic real time PCR. The reaction mix (25 μl final volume) consisted of the DNA template, 200 μM of each primer, 200 μM of the probe and Smart Mix bead (reaction buffer, Taq DNA polymerase, dNTPs, $MgCl_2$). A non-template negative control, in which the DNA template was replaced with water, was included in each run. Cycling conditions were 120 s at 95 °C, followed by 45 cycles of 15 s at 95 °C, 30 s at 55 °C, and 30 s at 72 °C. A standard curve was generated using 10-fold dilutions of HAdV5 purified DNA at 10^8 copies per ml. This standard curve was used to convert the cycle threshold values into the absolute number of HAdV DNA copies.

2.6. Real time PCR-based antiviral assay

A549 cells grown in 96-well plates were infected with HAdV5 diluted in maintenance medium to obtain a MOI of 0.0001, 0.1 or 1 PFU/cell. After 1 h at 37 °C, virus was aspirated and replaced by different concentrations of d4T in maintenance medium. After 2, 3 and 7 days of incubation at 37 °C at a MOI of 1, 0.1 and 0.0001, respectively, cell culture supernatants were collected and virus DNA was extracted. HAdV5 DNA load was measured by real time PCR. The effective concentration 50% (EC_{50}) was defined as the concentration of antiviral drug that reduced the number of DNA copies by 50% as compared to the virus control in the absence of drug, and was calculated by regression analysis. All assays were performed twice and each one in triplicate.

2.7. Reproducibility of the real time PCR antiviral assay

The reproducibility of the real time PCR antiviral assay was assessed using the supernatants from three wells from drug-treated and -untreated supernatants during the antiviral assay. In order to assess inter-assay variability, EC_{50} values were determined in three different experiments.

2.8. Plaque reduction assay

A549 cells grown in 12-well plates were infected with HAdV5 (MOI 0.0001 PFU/cell). After 1 h at 37 °C, virus was aspirated and replaced by maintenance medium containing 1.4% methylcellulose and the corresponding dose of each drug. Plaques were counted after 7 days of incubation at 37 °C. EC_{50} was calculated as the drug concentration able to reduce virus plaque formation by 50% according to regression analysis. All assays were performed twice and each one in duplicate.

2.9. Virus yield inhibition assay

A549 cells grown in 96-well plates were infected with HAdV5 at a MOI of 0.0001 PFU/cell. After 1 h at 37 °C, virus was aspirated and replaced by different concentrations of d4T in maintenance medium. After 7 days of incubation at 37 °C, cell culture supernatants were collected and the virus yields were determined by

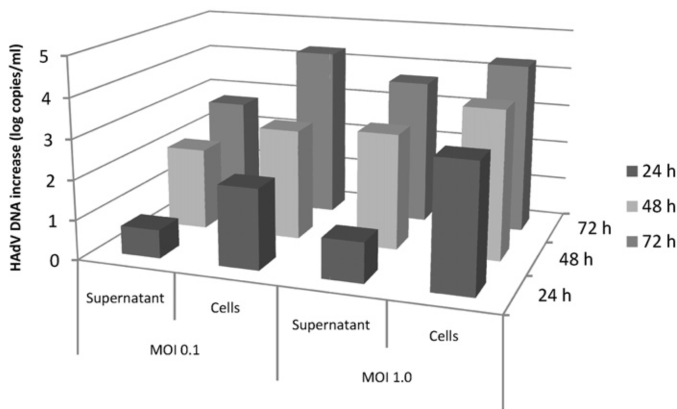

Fig. 1. Kinetics of HAdV DNA replication in A549 cells determined by real time PCR.

plaque formation in A549 cells. EC_{50} was calculated as the drug concentration able to reduce virus plaque formation by 50% according to regression analysis. All assays were performed twice and each one in duplicate.

3. Results

3.1. Kinetics of HAdV5 DNA replication

HAdV5 DNA levels increased over the basal value (3 h p.i.) in both culture supernatants and infected cells between 24 and 72 h p.i., when the maximum values were reached at the two MOIs tested (Fig. 1). Viral DNA load after 24 h of infection was significantly higher than the amount of DNA initially internalized into cells with a log increment ranging from 0.71 to 4.3, according to MOI and kind of sample. The final viral DNA yields depended on the original MOI. The use of culture supernatants was selected for the subsequent antiviral assays, since it was simpler than the use of infected cells and yielded similar results. However, to obtain a rapid readout, the use of supernatant is only useful when high MOI are involved. With low MOI, a rapid readout can be obtained when infected cells are used.

3.2. Real time PCR-based antiviral assay: effect of MOI

The real time PCR assay was further used to evaluate the susceptibility of HAdV5 to d4T by determining the viral DNA copies in supernatants of infected cells treated with the drug at a concentration ranging from 0.05 to 0.5 mM. To analyze the influence of MOI on the antiviral assay, cells were infected at the previously tested MOIs of 1 and 0.1, as well as at a very low MOI of 0.0001. The latter value was chosen for comparative purposes with the classical plaque reduction antiviral assay. For each MOI, the supernatant collection for DNA quantification was performed when cultures exhibited >90% CPE, i.e., at 7, 3 and 2 days after infection for the cultures infected at a MOI of 0.0001, 0.1 and 1, respectively. As shown in Fig. 2, a dose dependent reduction in the HAdV5 DNA copies was observed in the range of the d4T concentrations tested, regardless of the MOI.

From data in Fig. 2, the EC_{50} for d4T was similar at each MOI, confirming that this antiviral assay has a comparable efficiency in the presence of high or low virus inoculums. The CC_{50} for d4T varied according to the incubation time, with values of 4.4 ± 0.5, 3.1 ± 0.4, and 0.69 ± 0.04 mM for 2, 3 and 7 days of drug treatment, respectively. The calculated selectivity index (SI) defined as the ratio between cytotoxic and antiviral activity (CC_{50}/EC_{50}) ranged from 8 to 55 (Table 1). Results were consistent even at more prolonged periods of treatment.

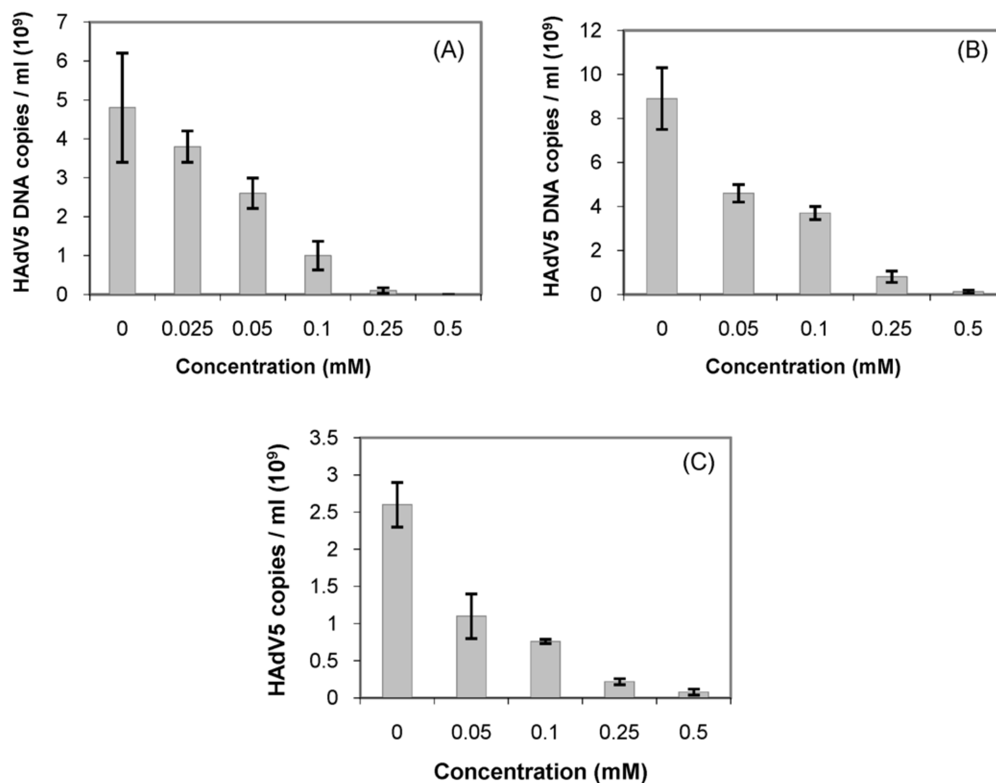

Fig. 2. Inhibition of HAdV5 by d4T at different multiplicities of infection. A549 cells were infected with HAdV5 in the absence or presence of increasing concentrations of d4T. After 2, 3, and 7 days incubation at 37 °C at a MOI of 0.0001 (A), 0.1 (B), and 1 (C), respectively, cell culture supernatants were collected and virus DNA was extracted. HAdV5 DNA load was measured by real time PCR.

Table 1
Antiviral activity of d4T against HAdV5 as determined by real time PCR.

MOI	Incubation time (days)	EC_{50}[a] (mM)	SI[b]
0.0001	7	0.09 ± 0.01	8
0.1	3	0.12 ± 0.02	26
1	2	0.08 ± 0.02	55

[a] Effective concentration 50%: concentration required to reduce HAdV DNA copies by 50%. Each value is the mean of duplicate assays ± standard deviation.
[b] Selectivity index: ratio CC_{50} (cytotoxic concentration 50%)/EC_{50}. The values of CC_{50} for d4T in A549 cells after 2, 3 and 7 days of drug treatment were 4.4 ± 0.5, 3.1 ± 0.4 and 0.69 ± 0.04 mM, respectively.

Table 2
Comparison of the determination of antiviral activity of d4T against HAdV5 by different methods.

Method	EC_{50}[a] (mM)
Plaque reduction assay	0.22 ± 0.02
Real time PCR	0.09 ± 0.01
Virus yield inhibition assay	0.04 ± 0.03

[a] Effective concentration 50%. Each value is the mean of duplicate assays ± standard deviation.

3.3. Reproducibility of real time PCR antiviral assay

The mean intra-assay coefficient of variation for Ct values was 0.33 (range 0.05–0.60), evidencing, a high level of reproducibility. The mean inter-assay coefficient of variation for Ct values was 0.57 (range 0.35–0.96), indicating a high level of reproducibility.

3.4. Comparison of real time PCR antiviral assay with infectivity methods

The real time PCR antiviral assay was evaluated in parallel with infectivity antiviral methods such as plaque reduction and virus yield inhibition assays. For a more reproducible comparison, the three assays were performed under the same experimental conditions: A549 cells infected at a MOI of 0.0001 for an incubation period of 7 days. The EC50 determined by real time PCR assay correlated well with those values obtained from plaque reduction and virus yield inhibition assays ($r^2 = 0.9938$ and $r^2 = 0.9468$, respectively). The EC50 determined by the real time PCR assay for d4T against HAdV5 was 2.4-fold lower than the EC_{50} from the plaque reduction assay and 2.2 higher than the value from virus yield inhibition (Table 2).

4. Discussion

The study data have shown that the real time PCR assay could be the basis for a rapid and useful readout system for anti-HAdV drug susceptibility determination. The in vitro methods used to determine the activity of antiviral drugs against human HAdV include tests based on evaluation of CPE, plaque formation and virus yield. Though relatively easy and inexpensive, the microscopic examination of CPE may be somewhat subjective. Alternatively, the viability of the infected cells can be measured with a spectrophotometric formazan-based method (Kodama et al., 1996). Direct quantification using immunofluorescence with antibodies directed against a HAdV protein has also been used. However, microscopic quantitation of fluorescent cells is relatively time consuming. More recently, a real time PCR method has been applied to evaluate antiviral drugs, based on the quantitative detection of HAdV DNA in infected cells (Wildner et al., 2003; Naesens et al., 2005; Stock et al., 2006; Uchio et al., 2007). Since the use of cell DNA adds a level of complexity to the assay when compared to the collection of culture supernatant, this study has shown the utility of culture supernatant for the detection of human HAdV DNA.

The antiviral activity of d4T against HAdV studied was similar independently of the MOI used. As seen in Table 1, the incubation time required for the antiviral assay could be reduced from 7 to 2 days when the MOI was increased from 0.0001 to 1, to get a similar level of cytopathic effect. Consequently, this result indicates that screening of new antiviral drugs may be assessed using real time PCR assays in high MOI infections at shorter times in comparison with infectivity antiviral assays. The MOI dependence reported for various drug–virus combinations is very variable and can be affected by diverse parameters such as antiviral assay, type of compound, viral replication cycle in which the drug acts, and others. The lack of dependence of the antiviral potency of d4T with the infecting HAdV inoculum is not an unusual property for several types of antiviral agents (Kruppenbacher et al., 1994; Rice et al., 1997; Stranska et al., 2002; Whitby et al., 2005; Talarico and Damonte, 2007), but represents a clear advantage for those compounds, such as d4T against HAdV, able to block infection even in the presence of high initial virus doses. The final optimal conditions determined for this assay are MOI 1, incubation time of 2 days and the use of culture supernatant for the detection of human HAdV DNA.

Despite the high correlation with the plaque reduction assay, the EC_{50} values determined by real time PCR were lower than those obtained by plaque reduction. This was also observed by Stock et al. (2006) for adenoviruses and by Stranska et al. (2002) in other viruses. Since both assays measure the effect of drugs on viral replication using different readout parameters, it is easy to understand the differences in absolute EC_{50} between these assays. Furthermore, plaque reduction assays do not consider the effect of antiviral agent on the plaque size. Smaller plaques in drug-treated wells consist of lower numbers of virus-infected cells but are counted in the same way as plaques of normal size in control wells, which leads to underestimation of viral susceptibility. The real time PCR assay, however, measures the true reduction of viral DNA production and may give a more accurate estimation of the drug effect on viral replication. Accordingly, the values of EC_{50} determined by real time PCR were more comparable to those obtained by virus yield inhibition, an assay that determined the reduction of infective viral particles production.

Most drugs reported to have anti-HAdV activity are nucleoside or nucleotide analogues that target the adenovirus DNA polymerase, such as cidofovir [(S)-HPMPC; (S)-1-(3-hydroxy-2-phosphonylmethoxypropyl) cytosine], (S)-HPMPA [(S)-9-(3-hydroxy-2-phosphonylmethoxypropyl) adenine] and 2′-nor-cyclic GMP (Baba et al., 1987; De Clercq, 2003). Among these drugs, cidofovir has been used in clinical studies with variable outcome despite its significant side effects such as nephrotoxicity, myelosuppression, and uveitis, cidofovir is currently used among bone marrow transplant recipients and solid organ recipients. Ribavirin, a purine nucleoside analogue with in vitro activity against RNA and DNA viruses, has also shown anti-HAdV activity in clinical studies, although both successes and failures have been reported. A study evaluating in vitro drug susceptibility found that all HAdV serotypes were susceptible to cidofovir, but only strains from species C (serotypes 1, 2, 5 and 6) were sensitive to ribavirin (Morfin et al., 2005). In contrast, a recent study from the same group showed that ribavirin was active on most isolates from species A, B and D (Morfin et al., 2009). The first study has been performed on reference strains and the second has been performed on clinical isolates. In addition, diverse types of drugs have been reported to have activity against HAdV in cell culture, but have not yet been evaluated in patients. Among these are cyclic D,L-α-peptides (Horne et al., 2005); cycloferon (Zarubaev et al., 2003); lactoferrin (Arnold et al., 2002); medicinal plant drugs (Chiang et al., 2003); nitric oxide (Cao et al., 2003); heterocyclic Schiff bases of aminohydroxyguanidine tosylate (Das et al., 1999); RGD peptidomimetic molecules

(Hippenmeyer et al., 2002) and small interfering RNAs targeting Ad E1A mRNA (Chung et al., 2007).

The present study has demonstrated the anti-HAdV activity of d4T, an HIV reverse transcriptase inhibitor used in the treatment of HIV infection. The effectiveness of d4T for the inhibition of viral replication in HAdV serotypes 3, 4, 8, 19 and 37 has been recently reported (Uchio et al., 2007). Stampeding, a phenylphosphoramidate derivate of stavudine, was also shown to be effective against HAdV5 (Uckun et al., 2004). The study described in this report shows that other viral serotype, HAdV5, is also susceptible to stavudine. However, it must be noted that the value of EC50 for stavudine against HAdV5 determined in this study is 100-fold higher than the reported EC50 for HIV-1 (Paolucci et al., 2004), a difference which must be taken in consideration for any putative in vivo assay of this compound in HAdV infections. In addition, the anti-HAdV5 activity of ritonavir was evaluated, a protease inhibitor of HIV. Inhibition of the HAdV cysteine protease may be another antiviral approach, since this enzyme is indispensable for virus uncoating, maturation and infectivity (Mangel et al., 2003). Unfortunately, HAdV5 was not susceptible to ritonavir inhibition (data not shown).

In conclusion, rapid and objective results generated by this real time PCR-based antiviral assay confirmed the inhibitory activity of d4T against HAdV. This drug and its derivatives merit further examination in both experimental and clinical studies. The established real time PCR-based protocol is sensitive, reproducible and it may also speed up the screening of new antiviral agents against human HAdV, replacing classical and more labor-intensive antiviral assays.

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