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Rapid determination of antiviral drug susceptibility of human cytomegalovirus by real-time PCR

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ABSTRACT

A quantitative real-time PCR-based assay was developed for determination of cytomegalovirus (HCMV) susceptibility to antiviral drugs. After HCMV isolate-growth for 4 days, antiviral drug susceptibility was determined by measuring the reduction of intracellular HCMV DNA in the presence of increasing concentrations of either ganciclovir, or foscarnet or cidofovir. The 50% inhibitory concentration (IC_{50}) was the drug concentration that reduced the number of HCMV genome copies by 50%. The IC_{50} values were measured for seven HCMV reference strains sensitive or resistant to one or more antiviral drugs. The antiviral susceptibility of 21 HCMV isolates was then tested and the results were consistent with prior determination of their phenotype and/or genotype by plaque reduction assay and sequencing. The real-time PCR susceptibility assay reported here was found to be highly reproducible, simpler to perform than the plaque reduction assay, and amenable to use in the routine diagnostic virology laboratory.

1. Introduction

Cytomegalovirus (HCMV) remains a major opportunistic pathogen in immunocompromised hosts including transplantation recipients and patients with AIDS. Antiviral drugs currently approved for the treatment of disseminated HCMV infections include ganciclovir, its prodrug valganciclovir, foscarnet and cidofovir. Most immunocompromised patients with severe HCMV disease will require long-term, suppressive anti-HCMV therapy and will be at risk for developing HCMV resistance to antiviral drugs (Emery, 2001; Li et al., 2007; Martin et al., 2007). Two gene products are implicated in HCMV resistance: the UL97 kinase that phosphorylates ganciclovir to its monophosphate form and the UL54 DNA polymerase that is the target of the currently available antiviral compounds. Point mutations or deletions in gene *UL97* can lead to resistance to ganciclovir. Mutations in gene *UL54*, depending on their location, can confer resistance to one or more antiviral drugs. Two types of methods are used for assessing the susceptibility of HCMV to antiviral drugs (Gilbert and Boivin, 2005). Phenotypic resistance is measured by the ability of HCMV to grow in the presence of an anti-HCMV drug. Genotypic resistance is defined by the presence of a mutation known to confer a resistant phenotype, and DNA sequencing is the reference method for detecting resistance-related mutations (Gilbert and Boivin, 2005). These tests do not require virus isolation and can be completed within 48 h. Nevertheless, genotypic analysis is limited to already known mutations. Therefore, phenotypic tests remain necessary to identify the role of novel mutations and to evaluate the consequences of mutation combinations.

Methods for phenotypic testing include plaque reduction assay (PRA), in situ enzyme-linked immunosorbent assay, DNA reduction assay, and flow cytometry-based assay (Lee et al., 2005). PRA is considered the gold standard for evaluating the HCMV susceptibility to antiviral drugs (Landry et al., 2000). However, this conventional phenotypic assay is time-consuming, not automated and labor-intensive. The aim of this study was to develop a real-time PCR-based assay to measure the drug concentration inhibiting by 50% (IC_{50}) the viral DNA synthesis and to evaluate its ability to determine whether the HCMV strains were sensitive or resistant to the antiviral drug tested.

2. Materials and methods

2.1. Viruses and cells

The reference strains susceptible to antiviral drugs were AD169, Davis (ATCC, Rockville, MD), Towne and Toledo (generous gifts from

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Dr. S Michelson, Pasteur Institute, Paris). Mutant ADm773 with the L773V mutation in pUL54 selected by propagating AD169 in the presence of foscarnet was used as a reference strain resistant to foscarnet as L773V has already been reported as the single change in a foscarnet-resistant laboratory strain (Mousavi-Jazi et al., 2003). Recombinant viruses Rec545 and Rec495 harbouring either L545S or N495K changes in pUL54 were used as reference strains resistant to ganciclovir and cidofovir, and to foscarnet, respectively (Ducancelle et al., 2006). Twenty-one clinical isolates from patients failing antiviral therapy were studied. The HCMV strains were propagated in human embryonic MRC-5 fibroblasts (BioMérieux, Lyon, France).

2.2. Real-time PCR

The assay was based on SYBR Green dye technology. A 201 base-pair conserved region in gene *UL54* was amplified using forward primer HCMVSBpol1 (5′-TACGAGACGGGAGGAAACAC-3′) and reverse primer HCMVSBpol2 (5′-GAAAAGCATAAAGCCCAGCA-3′). Real-time PCR was carried out using QuantiTech SYBR Green PCR master mix (Qiagen, Courtaboeuf, France) and 0.3 μM of each primer in 25 μL final volume with the following amplification conditions: initial denaturation of 15 min at 95 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 52 °C and 30 s at 72 °C. The reaction, data acquisition and analysis were performed by using the Rotor-Gene 3000 analyser (Corbett, Labgene, Archamps, France) and the Rotor-Gene 6.0 software. Quantitative standards were obtained from purified plasmid pAMVpol2 harbouring the entire *UL54* gene of reference strain Towne (Ducancelle et al., 2005).

2.3. Real-time PCR-based susceptibility assay

Human fibroblasts grown as monolayers in 24-well-plates were inoculated with cell-associated virus and fed with medium containing serial twofold dilutions of each antiviral compound. Each drug concentration was tested in quadruplicate. After incubation in the absence and the presence of the drug to test, the cell culture supernatant was discarded and 100 μL of lysis buffer (10 mM Tris–HCl, pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, 0.45% Nonidet P-40, 0.45% Tween 20, 6 μg/mL proteinase K) were added to each well. The cells were incubated at 56 °C for 1 h and 900 μL of distilled water were added to each well. The four diluted lysates per drug concentration were mixed, heated to 95 °C for 5 min, then cooled at room temperature, and stored at −20 °C until use. Seven microlitres of each lysate were tested in duplicate in the real-time PCR assay.

2.4. Kinetics of HCMV DNA replication

The time course of increase in HCMV DNA yield in infected cells was measured.

2.5. DNA sequencing

The entire *UL54* and *UL97* genes were amplified as previously described (Ducancelle et al., 2006). The nucleotide sequences of both DNA strands were determined for each gene and drug-resistance associated mutations were searched for.

3. Results

3.1. Performance of real-time PCR

Specificity and identity of the PCR products were verified by performing melting curve analysis. In addition, no amplification was observed from mock-infected cells. The sensitivity of the assay was 50 genome copies per reaction. The correlation coefficient was at least 0.99.

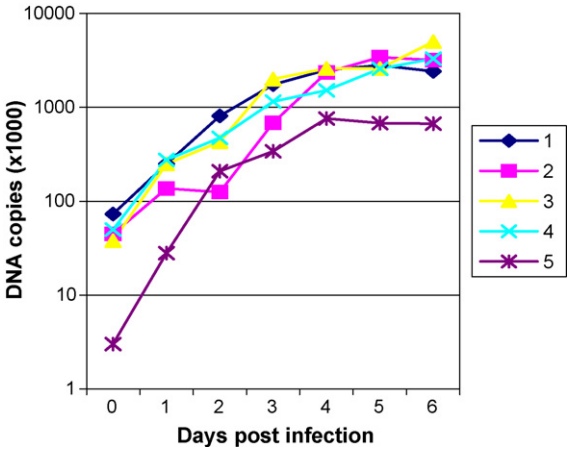

Fig. 1. Kinetics of HCMV DNA replication. Strains Davis (1), Toledo (3), Rec545 (4), AD169 (5), and a clinical isolate (2), were inoculated into MRC-5 cells seeded in 24-well plates. Inoculums were constituted of 5000 cells recovered from HCMV infected tissue culture at 50–70% cytopathic effect. At the time of infection and every day post-infection, the cells were submitted to in situ lysis and HCMV DNA was quantified by real-time PCR assay. The result was expressed as the number of HCMV genome copies per 7 μL of diluted lysate tested in the PCR assay.

3.2. Kinetics of HCMV DNA replication

The DNA levels were measured in infected cells by real-time PCR for strains AD169, Toledo, Davis, Rec545 and a ganciclovir-resistant clinical isolate (Fig. 1). The viral DNA levels increased sharply between 2 days and 4 days post-infection. At 4 days post-infection the DNA levels were at least 30-fold higher than the original amount of viral DNA at the time of infection.

3.3. Optimisation of the test

The IC_{50}s of ganciclovir, foscarnet and cidofovir were measured using 5000 cells per well as inoculum in 10 independent experiments. Although inoculums were fixed, variation in the control DNA levels at 4 days post-infection was noticeable. Fig. 2 shows the results according to the number of genome copies measured in the control wells at 4 days post-infection. Ganciclovir IC_{50} values remained between 1 and 2.1 μM (mean: 1.31 ± 0.44 μM) whereas the number of copies varied from 4.5 to 7 $\log_{10}$. Foscarnet IC_{50} values ranged between 30 and 65 μM (mean: 40.5 ± 10.39 μM) with controls between 3.6 and 7.2 $\log_{10}$. Cidofovir IC_{50} values were between 0.37 and 0.70 μM (mean: 0.47 ± 0.10 μM) with controls between 5.7 and 7 $\log_{10}$s The final format of the susceptibility assay included the following steps: isolation of the virus and recovering the infected cell suspension at 50–70% cytopathic effect, infection of MRC-5 cells with 5000 cells per well, quantification of CMV DNA using real-time PCR after a 4-day incubation, plotting the figures onto a graph, calculation of the antiviral drug concentration required to reduce the viral DNA copy number by 50% (IC_{50}) as compared to controls from the inhibition curve obtained.

3.4. Reproducibility

The reproducibility of the cycle threshold (Ct) values used for IC_{50} calculation was assessed on two aliquots of the same lysates collected from control and drug-treated wells, during the susceptibility testing of three isolates. The mean intra-assay coefficient

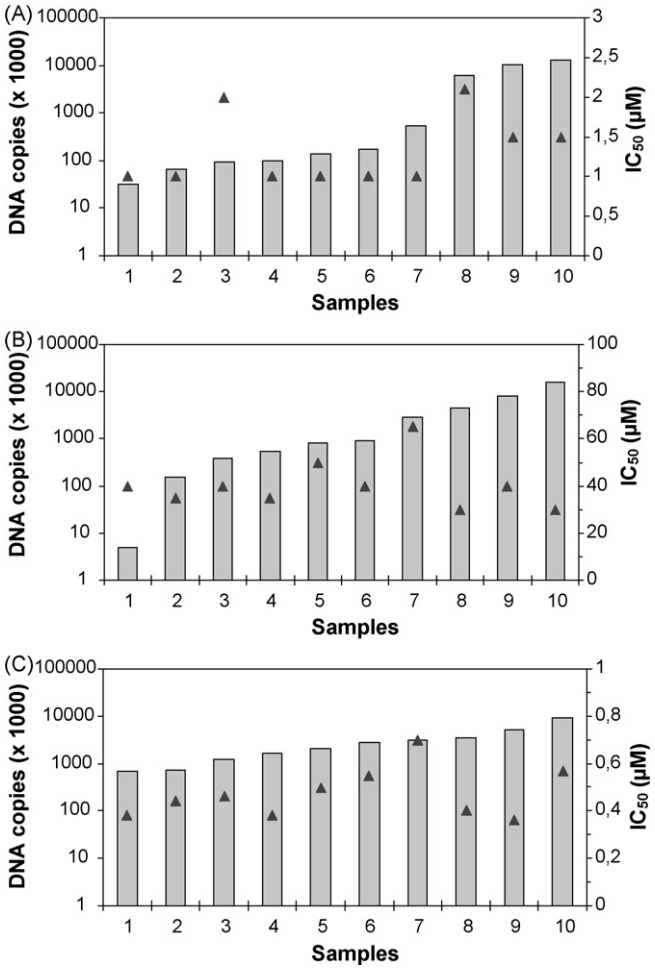

Fig. 2. Antiviral drug concentrations inhibiting by 50% the DNA synthesis of strain AD169 according to the genome copy number measured in the virus controls at 4 days post-infection. IC_{50}: 50% inhibitory concentrations. Black triangles: IC_{50} values. Columns: HCMV DNA copies in control wells at 4 days post-infection. (A) Antiviral activity of ganciclovir. IC_{50} values ranged from 1 to 2.1 μM (mean: 1.31 μM; standard deviation: 0.44). (B) Antiviral activity of foscarnet. IC_{50} values ranged from 30 to 65 μM (mean 40.5 μM; standard deviation: 10.39). (C) Antiviral activity of cidofovir. IC_{50} values ranged from 0.38 to 0.70 μM (mean 0.47 μM; standard deviation: 0.10).

of variation calculated from replicate Ct values was 1.08 ± 0.84%, indicating a high level of reproducibility. To assess the inter experimental variability, the IC_{50} values of ganciclovir, foscarnet and cidofovir for reference strain AD169 were found in at least 10 independent experiments at 1.2 ± 0.5, 50 ± 8.66 and 0.47 ± 0.10 μM, respectively.

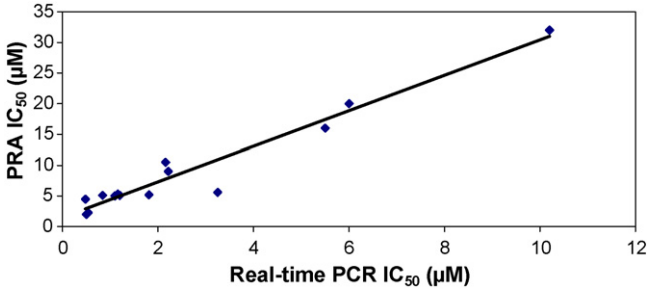

Fig. 3. Comparison between the ganciclovir inhibitory concentrations 50% (IC_{50}s) obtained by using plaque reduction assay (PRA) and real-time PCR assay. Regression line for IC_{50}s determined by PRA and real-time PCR assay of sensitive and resistant strains.

3.5. Antiviral susceptibility testing using real-time PCR assay

The 50% susceptibility index (SI_{50}) is defined as the fold difference in IC_{50} values between AD169 and the studied isolate tested in the same experiment. The antiviral drug IC_{50} values for the reference strains are shown in Table 1. The real-time PCR-based HCMV susceptibility assay was evaluated in parallel to PRA in a pilot study in 15 characterised laboratory and clinical strains (12 ganciclovir-sensitive and three resistant). The results are shown in Fig. 3. Ganciclovir IC_{50}s from real-time PCR assay correlated well with those from PRA ($r = 0.97$) and ranged from 0.5 to 2.22 μM for the ganciclovir-sensitive isolates and were 5 and 6 μM for the resistant isolates tested with the UL97 mutations M460V and A494V, respectively. Ganciclovir-IC_{50} values ranged from 0.5 to 3.5 μM (mean: 1.8 ± 1.12 μM) with corresponding SI_{50} values between 0.4 and 2.3 for eight isolates without resistance-associated mutations. Ganciclovir-IC_{50} values were 5.5 μM for one strain with the M460V mutation in UL97, 6, 20 and 40 μM, respectively, for isolates with the L595S mutation in UL97, and 80 μM for one isolate with the L595S and A494P mutations in UL97 combined with the P522S mutation in UL54. The foscarnet IC_{50} values ranged from 25 to 105 μM (mean: 67.28 ± 27.29 μM) for seven clinical isolates without UL54 resistance-associated mutations, whereas those for two clinical strains with the N495K in UL54 mutation were 190 and 600 μM, respectively. The cidofovir IC_{50} values ranged from 0.5 to 1.48 μM (mean: 1.18 ± 0.36 μM) for five clinical isolates without any resistance-associated mutation.

4. Discussion

Phenotypic antiviral drug susceptibility assays use different read-out parameters. As the reduction of viral DNA production is the basic mechanism underlying the antiviral effect of the drugs currently available for the treatment of HCMV infections, an assay

Table 1
Antiviral drug phenotypic testing of cytomegalovirus reference strains by real-time PCR-based assay.

Strain	Ganciclovir		Foscarnet		Cidofovir	
	IC_{50} (μM)	SI_{50} range	IC_{50} (μM)	SI_{50} range	IC_{50} (μM)	SI_{50} range
AD169	1.2 ± 0.5	–	50 ± 8.66	–	0.48 ± 0.10	–
Davis	1.16 ± 0.28	0.5–1.0	45 ± 5	0.72–1.25	0.33 ± 0.11	0.4–0.8
Toledo	1.81 ± 0.27	1.0–1.3	53.3 ± 23.6	0.64–1.6	0.31 ± 0.10	0.39–0.52
Towne	3.25 ± 0.35	1.5–2.3	118.3 ± 10.4	2.1–2.6	0.41 ± 0.15	0.4–1.07
Rec495	1.1 ± 0.28	0.9–1.13	**230 ± 70.7**	**4.5–5.6**	0.46 ± 0.12	1.0–1.12
Rec545	**9.35 ± 1.2**	**7.08–8.5**	Nd		**5.7 ± 0.56**	**13–13.5**
ADm773	Nd		**183.7 ± 37.1**	**3.5–4.21**	Nd	

IC_{50}: concentration of drug inhibiting by 50% the viral DNA synthesis. SI_{50}: fold difference in IC_{50}s between AD169 and each of the other strains tested in the same experiment. Nd: not done. In bold, IC_{50} and SI_{50} values indicating resistance to the antiviral drug tested. Mean values and standard deviations were calculated from at least three replicate experiments for sensitive reference strains and from two experiments for the resistant ones.

measuring viral DNA synthesis in the presence and the absence of the drug should be efficient. Real-time PCR has proven to be accurate for determination of antiviral drug susceptibility of herpes viruses including herpes simplex virus, human herpes virus 6 and human herpes virus 8 (Stránská et al., 2002; Sergerie and Boivin, 2003; Isegawa et al., 2007; Thi et al., 2006). The real-time PCR assay described here is applicable to HCMV drug susceptibility determination.

To routinely perform the real-time PCR assay in the clinical virology laboratories, the cost of the PCR reagents must remain moderate and the DNA preparation be rapid and cheap. The assay was based on SYBR Green fluorescent dye technology. SYBR Green by binding to double-strand DNA is a generic indicator of amplification. The specificity of the reaction is verified by producing a melting profile representing the specific product. SYBR Green-based detection is the most economical format for detection and quantification of PCR products. DNA preparation is a crucial step in the PCR process. Cell lysis is a simple, rapid and very cheap method for DNA isolation with no amplification-inhibiting effect. Performing lysis in situ avoided a possible loss of cells occurring with handling while removing cells from the plates, and thus a bias in quantification.

The standardisation of inoculums is an important concern for PRA as IC_{50} values depend on the multiplicity of infection (Prix et al., 1998; Landry et al., 2000). As low-passage clinical HCMV isolates are highly cell-associated, the testing of cell-free virus is inadequate and the use of cell-associated virus circumvents this problem. However, standardisation of the inoculums is difficult because, on the one hand, the preparation of infected cell suspensions requires the visual evaluation of the cytopathic effect and, on the other hand, infected cells contain different amounts of viruses, depending on the strain studied and the conditions of cell culture. Although the cell suspensions were adjusted at 5000 infected cells per well, variations in DNA loads could be noticeable. Importantly, IC_{50} values from real-time PCR based assay were not affected by these variations. A possible drawback to the use of cell-associated virus could be a significant background caused by the input-virus infected cells. Due to the high amount of intracellular DNA after a 4-day culture, this possible effect was not perceptible.

The ganciclovir-IC_{50} values obtained with real-time PCR assay were compared with those from PRA. Despite the high correlation with PRA, they were found consistently lower, as already reported in herpes simplex virus drug susceptibility studies (Thi et al., 2006; Stránská et al., 2002). PRA takes into account only the effect of the drug on the plaque number, whereas the effect of the drug is also manifested as a decrease in plaque size without complete inhibition of plaque formation. Therefore, IC_{50} values are over estimated by PRA.

The ganciclovir-IC_{50} values obtained for the resistant isolates (over 5 μM) were clearly higher than those for the sensitive strains (below or equal to 3.5 μM). A threshold value of 5 μM could be provisionally considered as a discriminative concentration for sensitive and resistant strains in the real-time PCR assay. However, this threshold needs to be confirmed by testing a larger number of isolates. The fold differences in drug susceptibility compared with a reference strain also helped to determine HCMV strains as sensitive (below 3) or resistant (above 3.5). The feasibility of real-time PCR assay for assessing HCMV susceptibility to foscarnet and cidofovir was demonstrated with reference strains and a panel of clinical isolates. For an accurate definition of in vitro resistance threshold of this assay, a large number of clinical isolates needs to be analysed.

The real-time PCR assay can overcome the shortcomings of PRA. The test is not affected by variation in virus loads. It has an objective readout and a high reproducibility. Furthermore, as the measure of viral load is automated, the test is easier to perform than PRA. This assay may well be a useful alternative to PRA in the clinical virology laboratory.

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