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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.

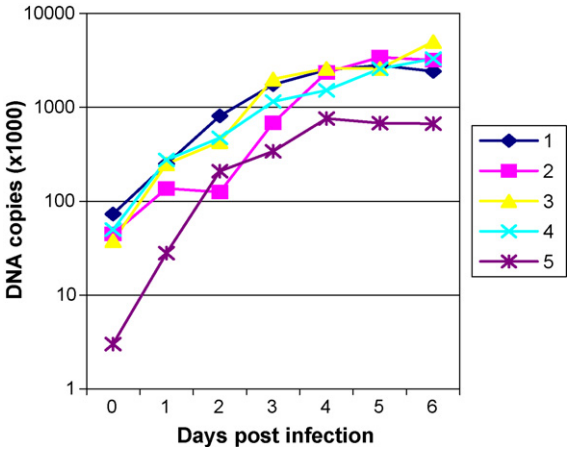


**Fig. 1.** Kinetics of HCMV DNA replication. Strains Davis (1), Toledo (3), Rec545 (4), AD169 (5), and a clinical isolate (2), were inoculated into MRC-5 cells seeded in 24-well plates. Inoculums were constituted of 5000 cells recovered from HCMV infected tissue culture at 50–70% cytopathic effect. 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: $1.31 \pm 0.44$ 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: $40.5 \pm 10.39$ μM) with controls between 3.6 and 7.2 $\log_{10}$. Cidofovir $IC_{50}$ values were between 0.37 and 0.70 μM (mean: $0.47 \pm 0.10$ 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