Application of Real-Time PCR for Determination of Antiviral Drug Susceptibility of Herpes Simplex Virus

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A quantitative real-time PCR (TaqMan) assay was developed for determination of antiviral drug susceptibility of herpes simplex virus (HSV). After short-time culture of the virus, the antiviral drug susceptibility of HSV isolates for acyclovir (ACV) was determined by measuring the reduction of the HSV type 1 (HSV-1) DNA levels in culture supernatants using real-time PCR. The 50% inhibitory concentration was reported as the concentration of antiviral drug that reduced the number of HSV-1 DNA copies by 50%. A total of 15 well-characterized ACV-sensitive or -resistant strains and clinical isolates were used for assay evaluation. The new assay with real-time PCR readout permitted rapid (3 days), objective, and reproducible determination of HSV-1 drug susceptibilities with no need for stringent control of initial multiplicity of infection. Furthermore, the real-time PCR assay results showed good correlation (r = 0.86) with those for the plaque reduction assay. In conclusion, the real-time PCR assay described here is a suitable quantitative method for determination of antiviral susceptibility of HSV-1, amenable for use in the routine diagnostic virology laboratory.

Extensive use of acyclovir and other antiviral drugs for prophylaxis and treatment of herpes simplex virus (HSV) infections exerts a continuous selection pressure on the HSV virus population. HSV antiviral drug resistance occurs frequently especially in immunocompromised patients such as those undergoing bone marrow (6 to 12%) or solid organ transplantation (∼4%) or AIDS patients (∼6%) and can be associated with serious disease (4; B. Lina, D. Thouvenot, C. Danve, F. Morfin, A. Boucher, I. Berlin, and M. Aymard, abstract from European Virology 2000, J. Clin. Virol. 18:47, 2000). The frequency of HSV-resistant infections may increase because of the increasing number of severely immunocompromised patients with chronic or recurrent HSV infections who require prolonged administration of antiviral drugs. In this patient group susceptibility testing is needed to detect drug-resistant HSV strains and to reconsider the antiviral treatment (1; F. Morfin, D. Thouvenot, G. Souillet, M. Michallet, and M. Aymard, abstract from Progress in Clinical Virology V, Acta Microbiol Immunol Hung. 46:429, 1999).

Safirin et al. (24) has shown a good correlation between the failure of HSV suppression by acyclovir (ACV) in vivo and the determination of ACV resistance in vitro. These observations emphasize the clinical relevance of antiviral resistance determination in the laboratory.

Several phenotypic assays have been described, and some of them are used in clinical practice, with the plaque reduction assay (PRA) as the most frequently used drug susceptibility assay. Although this technique is laborious and time-consuming, it still remains the “gold standard” method by which other tests are evaluated (24). The majority of alternative susceptibility assays is based on reduction in cytopathic effect (CPE), which is either microscopically evaluated or colorimetrically detected (7, 13, 15, 19, 23, 29). Assays based on enzyme-linked immunosorbent assay (ELISA) include the sandwich ELISA (33) and the microplate in-situ ELISA (MISE-test) (16, 21, 25). The latter has been shown to correlate well with PRA. Other currently used antiviral susceptibility assays involve the use of DNA hybridization (9, 30, 31), flow cytometric analysis (20) and transgenic HSV inducible reporter cells (32).

With the increasing numbers of immunocompromised individuals, there is a need for the widespread routine availability of antiviral drug susceptibility assays, which would be rapid, reproducible and clinically relevant. Currently used methods, except for the MISE-test, suffer from certain pitfalls, which preclude their routine use. Most of the assays are time-consuming and labor-intensive; some may have subjective endpoints, require special equipment or trained laboratory personnel. Therefore we set out to develop an assay, which would overcome most of the aforementioned restrictions and could be easily implemented in the diagnostic laboratory.

We describe the development and evaluation of a new approach for HSV-1 drug susceptibility determination using quantitative real-time PCR (TaqMan) to measure viral DNA production.

MATERIALS AND METHODS

Cells and viruses. Vero cells (African green monkey kidney) were propagated and maintained in Iscove’s modified Dulbecco’s medium (Life Technologies, Breda, The Netherlands) supplemented with 5% fetal bovine serum and gentamicin (10 µg/ml; Life Technologies, Breda, The Netherlands). A stock of HSV-1 strain McIntyre counted by electron microscopy (EM) was obtained from Advanced Biotechnologies, Inc., Columbia, Md. The HSV-1 strain KOS and the KOS-derived ACV-resistant mutants (AraA7, AraA8, AraA13, E891C, PfaA5, and PaaA5) (5, 6, 10, 17) were kindly provided by D. M. Coen (Harvard Medical School, Boston, Mass.). The HSV-1 ACV-sensitive strains McIntyre and R39 were generously provided by A. Linde (Swedish Institute for Infectious Disease Control, Solna, Sweden). Well-characterized ACV-resistant clinical isolates HSV 98.25733-MA/3, HSV 98.15779-VA/2 and HSV 98.14742-PE/1 (Morfin et al., Acta Microbiol Immunol Hung. 46:429, 1999) were a gift of M. Aymard (Université Claude Bernard, Lyon, France). Other HSV-1 clinical isolates were selected from our own collection; two originated from patients after bone marrow transplantation who had recurrent HSV infections not responding to ACV, and one was obtained from a patient with oral HSV infection which resolved over time.
spontaneously. Virus stocks were grown in Vero cells and the infectious titer was determined by plaque assay in Vero cells as previously described by Schaffer et al. (28).

Real-time PCR assay for HSV-1: assay setup. HSV-1-specific PCR primers and a fluorescent probe directed to the HSV-1 glycoprotein G (gG) gene were used for real-time PCR analysis as described by Ryncarz et al. (22). Each 25 μl of PCR mixture contained 7 μl of a 1:100-diluted culture supernatant, 900 nM concentrations of both forward and reverse primer, and 150 nM probe. Amplification was performed using the Applied Biosystems Sequence Detector 7700 under the following conditions: incubation for 2 min at 50°C, and then for 10 min at 95°C, followed by 45 cycles of 15 s at 95°C and 1 min at 60°C. Each PCR run contained two negative controls and a dilution series of HSV-1 DNA (6 × 10^6 to 6 × 10^2 copies/ml) derived from EM-counted virus stock (HSV-1, McIntyre), which was used to generate the standard curve. Each sample was analyzed in duplicate.

Assay optimization. The kinetics of HSV-1 DNA replication was examined by measuring the time course of increase in HSV-1 DNA yield in cell culture supernatants. Vero cells in 24-well culture plates were infected at a multiplicity of infection (MOI) of 0.1, 0.01, and 0.001 PFU/cell of HSV-1 strain McIntyre. The development of CPE was monitored, and the levels of HSV-1 DNA were measured by real-time PCR in supernatant samples collected at 12-h intervals after infection.

Experiments were also performed to evaluate the effect of ACV in the cell culture supernatant on PCR efficiency. Virus infected cell cultures (MOI, 0.01) were incubated with or without a high concentration of ACV (48 μg/ml) for 48 h to resemble the conditions of the assay described here. Subsequently cell culture supernatants were collected and spiked with HSV-1 DNA. These spiked samples were amplified using the real-time PCR assay, either as undiluted supernatant or as a dilution series.

The effect of the MOI on the ACV IC50 was determined in parallel experiments in which cell cultures were infected with a half-log6 incremental range of infectious doses (MOI, 0.001 to 0.5 PFU/cell) of HSV-1 in the presence of serial concentrations of ACV. The levels of HSV-1 DNA were measured by real-time PCR in supernatant samples collected at 24, 48, and 72 h postinfection, and the 50% inhibitory concentrations (IC50s) were determined. The CPE of the virus control was scored at the time of supernatant collection.

Real-time PCR assay for HSV-1 antiviral susceptibility testing: final setup. Virus isolates at an MOI of 0.01 (50 μl) were dispensed in duplicate into wells of the 24-well culture plate containing 450 μl of culture medium with different concentrations of ACV and a suspension of Vero cells (6 × 10^3 cells/ml). Serial twofold dilutions of ACV (Sigma, Zwijndrecht, The Netherlands) ranging from 0.006 to 32 μg/ml were used. Plates were incubated at 37°C for 2 days and monitored for development of CPE. When complete CPE was reached in control wells, 300 μl of cell culture supernatants was collected, cleared by centrifugation (11,000 x g, 1 min, 4°C), and examined in real-time PCR or stored at −70°C until assayed. Reference ACV-susceptible (KOS) and ACV-resistant (AraA r8) strains were included as controls in each PCR assay. The IC50 was used to express virus drug susceptibility and was defined as the concentration of antiviral drug that reduced the number of DNA copies by 50% relative to the no-drug virus control.

PRA. The PRA for drug susceptibility determination was performed as previously described by Erlich et al. (8) with minor modifications, using neutral red staining for plaque detection. Briefly, confluent Vero cell monolayers in 24-well culture plates were inoculated with 40 to 60 PFU of virus. After incubation at 37°C for 1 h, the viral inoculum was replaced with culture medium containing various concentrations of ACV and 0.5% agar. The same ACV concentrations were used as in the real-time PCR assay. Each drug concentration was tested in quadruplicate. The plates were incubated at 37°C for 2 to 3 days until plaques were observed in the control wells without the drug. Subsequently, the monolayers were stained overnight using a second overlay medium containing 0.08% neutral red in 0.8% agar. The same reference control strains were used in each PRA as were used in the in real-time PCR assay. The IC50 was defined as the ACV concentration that reduced the number of plaques by 50% compared to the untreated control wells. Isolates were considered resistant to ACV at IC50 of ≥2 μg/ml.

Statistical analysis. Results of real-time PCR assay and PRA were analyzed and compared using Wilcoxon’s signed rank test and Spearman’s correlation coefficient.

RESULTS

Our goal was to develop an easy-to-perform assay for HSV antiviral susceptibility testing, suitable for implementation into the modern routine diagnostic laboratory. To determine and optimize its characteristics, several parameters of the assay were studied in detail, such as the viral replication kinetics, the effect of ACV in culture supernatant on PCR efficiency, and the effect of MOI and incubation time on drug susceptibility values. Once the optimal format of the assay was set, the test was validated on a panel of well-characterized HSV-1 strains and clinical isolates.

The sensitivity of the real-time TaqMan PCR to detect HSV DNA was evaluated by using serial dilution of HSV-1 DNA extracted from EM-counted virus stock (McIntyre). The quantification was linear over the range of concentrations examined, from at least 10^3 to 1,000 DNA copies per ml.

Kinetics of HSV-1 DNA replication. Complete CPE was observed at 36, 48, and 60 h after infection for the cultures infected at an MOI of 0.1, 0.01, and 0.001 PFU/cell, respectively. This corresponded to an HSV-1 DNA yield in the culture supernatant of approximately 9 log10 copies/ml. Culture wells showing less than 30% CPE were found to have DNA levels at the detection limit of the real-time PCR assay (Fig. 1).

Effect of ACV in culture supernatant on performance of real-time PCR. Using a viral culture supernatant directly in a PCR analysis may introduce inhibitory substances in the PCR. Particularly the presence of ACV, which is a DNA polymerase inhibitor, in culture supernatants could inhibit the PCR (18). The presence of ACV at 48 μg/ml in the cell culture supernatants decreased the yield of amplified products approximately sixfold. The inhibitory effect of ACV was completely overcome by diluting the cell culture supernatant 100-fold in water prior to PCR, which was routinely done in later experiments.

Effect of MOI and incubation time on IC50. The IC50 of ACV in the TaqMan PCR assay at different MOIs and incubation times varied between 0.16 and 0.64 μg/ml (Table 1). Only a slight increase in IC50s at higher MOIs was observed at each time point. At 24, 48, and 72 h of incubation, respectively, a maximum 1.8-, 3.2-, and 2.1-fold difference in IC50 was observed among the cultures infected with a large range of MOIs from 0.001 to 0.5 PFU/cell. For each MOI an increase in IC50 with incubation time was observed (Table 1). The IC50 de-
tected at 48 and 72 h were on average 2- and 2.4-fold higher than those determined at 24 h after infection. The IC$_{50}$ results were comparable at each MOI regardless of incubation time as long as the susceptibility was determined when the CPE in the virus control was between 50 and <100%. The same findings were obtained for drug-resistant viruses (data not shown). Based on these results, an incubation time of 48 h and an MOI of 0.01 PFU/cell were subsequently selected for routine use.

**Antiviral susceptibility testing using real-time PCR assay.** The real-time PCR-based HSV-1 drug susceptibility assay was evaluated in parallel to PRA in a pilot study of nine well-characterized ACV-sensitive and resistant laboratory strains and six clinical isolates (Table 2). ACV IC$_{50}$ values determined by real-time PCR assay correlated well with those from PRA ($r = 0.99; P < 0.0001$) (Table 2). In addition, the observed fold differences in drug susceptibility between the reference strain KOS and each of the tested strains were highly comparable between the two assays ($r = 0.99; P < 0.0001$) (Fig. 2). The absolute IC$_{50}$ values determined by the real-time PCR assay were significantly lower ($P < 0.0001$) than those from PRA for all ACV-sensitive and resistant laboratory strains and clinical isolates (Table 2). The average difference in IC$_{50}$ values was 7.7-fold (range, 4.1- to 15.0-fold) between the two assays.

**Reproducibility.** The reproducibility of the cycle threshold (Ct) values used for IC$_{50}$ calculation was assessed on two aliquots of the same supernatant samples collected from drug treated and drug control wells during ACV susceptibility testing of three random isolates. The mean intra-assay coefficient of variation calculated from replicate Ct values was 0.71% (range, 0.07 to 1.8%), indicating a high level of reproducibility.

To assess the interexperimental variability, the IC$_{50}$ of the HSV-1 strain KOS was determined in seven repeated experiments. This resulted in a mean IC$_{50}$ of 0.15 µg/ml (range, 0.10 to 0.25 µg/ml; standard deviation = 0.06 µg/ml).

**DISCUSSION.** The real-time PCR assay described here could be the basis for a useful novel readout system for antiviral drug susceptibility determination. The assay developed and evaluated for HSV-1 may be generally applicable to other viruses.

The assay measures inhibition of HSV-1 DNA production by quantification of viral DNA using the TaqMan technology, whereas in classical PRA the reduction of numbers of virus induced plaques is used to determine the antiviral effect of the drug. Thus, both assays measure the effect of viral replication, though using different read-out parameters. The determination of these different parameters may explain the differences in absolute IC$_{50}$ values between the two assays. Moreover, PRA does not...
not take into account the effect of antiviral agent on the plaque size. In PRA the antiviral effect of the drug is often manifested as a decrease in plaque size without complete prevention of plaque formation (2). Smaller plagues in drug-treated wells consist of lower numbers of virus-infected cells but are counted equally to plagues of normal size in control wells, which leads to overestimation of viral susceptibility. The real-time PCR assay, however, measures the true reduction of viral DNA production, which is the basic mechanism underlying the antiviral effect of the drug. As such, the real-time PCR assay may give more accurate estimation of the effect of the drug on viral replication.

In the real-time PCR-based HSV-1 drug susceptibility assay, the effect of the MOI on the ACV susceptibility was limited, which was demonstrated by only small differences in IC50 among the cultures infected with a large range of MOIs (500-fold difference). The effect of the MOI was small as long as the virus had not infected all the cells. An incubation time of 48 h was routinely used in our assay. However, considering the reported differences in growth rates of clinical isolates, it cannot be excluded that longer incubation times will be needed for particular isolates to reach sufficient amount of CPE (50%) required for reproducible real-time PCR analysis. Therefore, rather than harvesting the virus at a fixed reading time, we would recommend monitoring CPE and subsequent susceptibility testing at CPE levels ranging from 50% to <100%.

The real-time PCR assay was evaluated by testing 15 HSV-1 strains for ACV susceptibility and by comparing the results with those from the conventional PRA. The test showed good correlation with PRA on IC50s and also the fold differences in susceptibility between the reference sensitive and tested strains highly correlated.

Based on the range of IC50s obtained for sensitive HSV strains and clinical isolates, a cutoff value of 0.3 μg/ml for ACV was considered as a discriminative concentration for sensitive and resistant strains in the real-time PCR assay. For a better and more accurate determination of the in vitro resistance threshold of this assay, a larger number of clinical isolates needs to be analyzed. Nevertheless, as the threshold values defining sensitive and resistant virus can differ depending on the assay utilized (26), the fold-differences in drug susceptibility compared to a reference strain may provide more relevant information for comparing results generated with different drug susceptibility assays (27).

The real-time PCR assay described here allows the rapid determination of the ACV susceptibility of HSV strains. The test was only mildly affected by variation in the MOI, while quite accurate titration of the clinical isolate is required for PRA. The real-time PCR assay has an objective readout and a good reproducibility, furthermore it is more rapid and easier to perform than the PRA. Full susceptibility testing results from the real-time PCR assay were obtained within 3 days, in contrast to the usual 4 to 6 days required for PRA. This is a considerable improvement and in combination with the technology already available in many routine diagnostic laboratories may render it a useful test for the clinical virology laboratory.

At present, real-time PCR-based assays are increasingly implemented into diagnostic clinical virology because of their high sensitivity, high throughput, and ease of use format (3, 11, 12, 14). The real-time PCR assay described here uses the same PCR components and is performed under the same standard amplification conditions that are routinely used for detection of HSV in clinical specimens. Thus, the assay fits in well with methods already available in the clinical virology laboratory and as such it could be easily implemented in many clinical laboratories. In house availability of antiviral susceptibility testing would enable physicians to obtain results on drug susceptibility in a clinically useful time frame and may help explaining therapeutic failure in patients not responding adequately to treatment.

In conclusion, we demonstrated the real-time PCR assay as a suitable method for the determination of antiviral drug susceptibility for HSV-1. Application of the assay for clinical practice needs to be further evaluated.

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REFERENCES


