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Rapid determination of antiviral drug susceptibility of herpes simplex virus types 1 and 2 by real-time PCR

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Abstract

An antiviral drug susceptibility assay of herpes simplex virus (HSV) was developed using real-time PCR quantification of intracellular viral DNA load. The number of HSV DNA copies within Vero cells after 24 h infection was strongly correlated with the number of plaques obtained after 72 h infection. Antiviral drug susceptibility of HSV was determined after virus growth for 24 h by measuring the reduction of intracellular HSV DNA in the presence of increasing concentrations of either acyclovir (ACV) or foscarnet (PFA). This assay required neither preliminary titration of infectious stock nor follow-up of cytopathic effect. The 50% inhibitory concentrations (IC_{50}s) obtained with 27 isolates of HSV types 1 and 2 by using this test were significantly correlated with those obtained in parallel with plaque reduction assay taken as the reference method ($r = 0.91$, $p < 0.0001$ and $r = 0.51$, $p = 0.009$ for ACV and PFA, respectively). The threshold real-time PCR IC_{50}s for ACV and PFA resistance did not differ according to HSV type and were determined to be 1.0 and 100 μM, respectively. The real-time PCR susceptibility assay reported here is rapid, reproducible, applicable for HSV-1 as well as HSV-2, and suitable for automation.

Keywords: Herpes simplex virus; Acyclovir; Foscarnet; Resistance; Real-time PCR

1. Introduction

Over the past two decades, acyclovir (ACV) and, to a lesser extent, foscarnet (PFA) have been widely used to treat herpes simplex virus (HSV) infections. As a consequence of the important selection pressure on HSV, the resistance of this virus to antiviral drugs has emerged as a concern for therapy, especially in the case of immunocompromised patients (Danve-Szatanek et al., 2004; Gaudreau et al., 1998; Morfin and Thouvenot, 2003). This in turn has led to the development of HSV susceptibility assays in order to detect resistance in clinical virus isolates with simple rapid reproducible methods. For that purpose, virus growth in cell cultures is quantified in the presence of increasing concentrations of antiviral compounds. The inhibition curve obtained by this way permits to derive the 50% inhibitory concentration (IC_{50}) defined as the antiviral concentration that reduces the rate of virus multiplication by 50%. For a long time, the most conventional read-out of the assay has been based on virus-induced cytopathic effect (CPE) measuring either the count of plaques (plaque reduction assay, PRA) or the staining of residual cell monolayer (dye uptake assay) (Danve et al., 2002). Other approaches included the detection of viral antigen using ELISA, hybridization of HSV DNA, and generation of β-galactosidase activity using an HSV-inducible reporter cell line (Agut et al., 1990; Standring-Cox et al., 1996; Stranska et al., 2004a). The quantitation of HSV type 1 (HSV-1) DNA in the supernatant of cell cultures by means of real-time PCR has permitted the reproducible determination of virus susceptibility to ACV and has shown a good correlation with PRA (Stranska et al., 2002). However, this method displayed some limitations: virus yield in cell culture supernatant was not sufficient to enable early read-out, i.e., at 24 h post-infection (p.i.); the follow-up of CPE until completion in control wells was necessary to assess the time for the collection of samples prior to PCR analysis; the relevance of the assay for HSV type 2 (HSV-2) which is assumed to be less sensitive to ACV than HSV-1 remained to be demonstrated.

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We thus decided to set up a novel PCR-based susceptibility assay with the general objective of more complete automation, earlier read-out and extended use pattern. As presented here, the quantification of HSV DNA in infected cell lysates instead of culture supernatant fulfilled these criteria. The susceptibility assay based on this approach appeared to be fully convenient for both ACV and PFA against a wide panel of HSV-1 and HSV-2 clinical isolates.

2. Materials and methods

2.1. Cells and viruses

Vero cells (African Green Monkey Kidney) were propagated in Minimum Essential Medium (MEM, GIBCO, Cergy, France) supplemented with 3% fetal bovine serum (FBS, GIBCO, Cergy, France), amikacin (20 μg/ml, Bristol Myers Squibb, Rueil-Malmaison, France), vancomycin (20 μg/ml, Lilly, Suresnes, France), glutamin (2 μM, Eurobio, Courtaboeuf, France), non-essential amino acid solution (1%, Sigma, Saint-Quentin Fallavier, France), referred thereafter as the culture medium.

The twenty-seven clinical HSV isolates used in the present study included 11 HSV-1 and 16 HSV-2. They were obtained from patients suffering HSV-induced disease with diverse locations (Table 1). The determination of HSV type was performed by means of an immunofluorescence assay (IFA) using specific monoclonal antibodies (Biotest, Buc, France). Five out of these 27 isolates were selected for optimizing the PCR-based susceptibility assay, three being characterized as ACV-sensitive and the other two as ACV-resistant using PRA, as indicated in Table 1. Virus stocks were generated from the supernatant of HSV-infected Vero cell cultures exhibiting 80% CPE. Their infectious titer was determined by plaque assay in Vero cells at 72 h p.i., using serial 10-fold dilutions tested in duplicate, and expressed as the number of plaque-forming units per volume unit (PFU).

According to a general procedure of infection, Vero cells were inoculated with virus stocks appropriately diluted in MEM without FBS. After adsorption at 37 °C for 1 h, viral input was removed, and cells were washed twice with MEM without FBS. They were further incubated with culture medium in the absence or presence of antivirals for a time ranging from 24 to 72 h, depending on the parameter tested (see below).

2.2. Plaque reduction assay

Confluent Vero cell monolayers in 24-well culture plates were infected with 200 μl of serial 10-fold dilutions of virus stocks per well as described above and further incubated with 1 ml of

Table 1
Phenotypic properties and drug susceptibility of the 27 HSV isolates studied

Isolate	Source	Type	ACV IC_{50} (μM)		PFA IC_{50} (μM)	
			PRA[a]	Real-time PCR	PRA[a]	Real-time PCR
0401773	Chin	HSV-1	0.4 (S)	0.1	14.8 (S)	5.1
0406619	Cornea	HSV-1	0.6 (S)	0.2	75.9 (S)	36.3
0204471	Cornea	HSV-1	1.0 (S)	0.2	80.8 (S)	32.3
0306410[b]	BAL[c]	HSV-1	1.0 (S)	0.2	56.1 (S)	24.7
0205540	Cornea	HSV-1	1.1 (S)	0.3	49.5 (S)	47.8
0303135	BAL[c]	HSV-1	1.9 (S)	0.2	29.7 (S)	23.1
0204090	Nose	HSV-1	2.0 (S)	0.2	99.0 (S)	27.1
0302582	Plasma	HSV-1	7.8 (R)	4.3	33.0 (S)	21.1
0400070	Anus	HSV-1	14.5 (R)	2.6	15.2 (S)	7.3
0301098	Neck	HSV-1	>50.0 (R)	10.0	89.1 (S)	19.3
0401446	BAL[c]	HSV-1	>50.0 (R)	10.0	41.2 (S)	20.5
0401737	Genital tract	HSV-2	1.1 (S)	0.4	69.3 (S)	14.5
0303414[b]	Buttock	HSV-2	1.6 (S)	0.1	34.6 (S)	21.4
0206020	Buttock	HSV-2	1.8 (S)	0.2	44.5 (S)	23.1
0205662	Perineum	HSV-2	2.2 (S)	0.2	37.9 (S)	31.0
0304052[b]	Penis	HSV-2	2.3 (S)	0.5	23.1 (S)	7.7
0401436	Buttock	HSV-2	2.9 (S)	0.3	77.5 (S)	29.7
0301020	Vulva	HSV-2	3.0 (S)	0.2	28.4 (S)	16.8
0300650	Buttock	HSV-2	3.0 (S)	0.3	29.7 (S)	17.8
0302061	Scrotum	HSV-2	7.7 (R)	1.0	102.3 (S)	41.2
0303225	Vagina	HSV-2	21.0 (R)	4.0	46.2 (S)	6.3
0406508	Penis	HSV-2	40.0 (R)	2.0	528.0 (R)	115.5
0302477	Vulva	HSV-2	>50.0 (R)	7.8	102.3 (S)	20.8
0402996	Anus	HSV-2	>50.0 (R)	19.0	85.8 (S)	6.6
0304279[b]	Genital tract	HSV-2	>50.0 (R)	20.0	49.5 (S)	9.2
0305883[b]	Vagina	HSV-2	>50.0 (R)	25.0	79.2 (S)	9.9
0203333	Scrotum	HSV-2	>50.0 (R)	>60.0	>660.0 (R)	112.2

[a] Susceptibility as determined by PRA is indicated in parentheses: S, sensitive; R, resistant. Resistance was defined by an $IC_{50} \geq 7.0$ μM for ACV and 330.0 μM for PFA (see text).

[b] Used as a prototypic strain for the optimization of assay.

[c] BAL, bronchio-alveolar lavage.

culture medium per well containing different concentrations of either ACV (from 1 to 50 μM) or PFA (from 66 to 660 μM), and 0.6% methylcellulose. Plaques were counted at 72 h p.i. after treatment of cell monolayers with 10% formaldehyde (Sigma, Saint-Quentin Fallavier) and staining with 0.5% crystal violet. The IC_{50} was defined as the antiviral concentration that reduced the number of plaques by 50% compared to the control infected cells in the absence of any antiviral. Isolates were considered resistant at IC_{50}s ≥7 μM and 330 μM for ACV and PFA, respectively. All ACV-resistant isolates tested but one (#0302061) had either single nucleotide substitutions or frameshift mutations of thymidine kinase gene, most of which had already been reported as important determinants of ACV resistance (Chibo et al., 2004, 2002; Morfin et al., 2000; Stranska et al., 2004b).

2.3. Real-time PCR for HSV

HSV-specific primers and a fluorescent probe directed to the DNA polymerase gene (UL30) were used for real-time PCR as described previously (Kessler et al., 2000). Ten microlitre of DNA preparation were mixed with 40 μl of PCR mixture containing 400 nM of each forward and reverse primer, 200 nM of probe and 25 μl of master mix (Applied Biosystems, Courtaboeuf, France) including 2.5 mM $MgCl_2$ and 8% glycerol. The amplification which works equally well for both HSV-1 and HSV-2 was performed using the Applied Biosystems Sequence Detector 7000 (Applied Biosystems, Courtaboeuf, France) under the following conditions: incubation for 2 min at 50 °C, 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 10-fold serially diluted reference DNA obtained from HSV-infected cells in order to generate the standard curve. This reference DNA had previously been quantified in terms of number of HSV DNA copies using the same real-time PCR technique and serial dilutions of a recombinant plasmid containing the 92-bp long target fragment of DNA polymerase (derived from cloning plasmid pCR2.1-TOPO^R, Invitrogen, Cergy, France). This recombinant plasmid was not used during the study itself in order to prevent any carry-over phenomenon. Fluorescence measurements were performed at each cycle, which permitted to determine the cycle threshold (Ct) value for each DNA sample. The detection threshold of the assay was 10 copies per run. Viral load was derived from Ct using the standard curve generated in parallel and expressed as the number of copies (or viral genome equivalents) per volume unit.

2.4. Preparation of DNA from virus stocks and cell cultures

Diverse approaches were used for DNA preparation throughout the study according to the sample studied and the step of assay set-up. The DNA from HSV stocks was initially obtained by using the QIAamp DNA blood Minikit (Qiagen, Courtaboeuf, France) according to the Manufacturer's instructions. Alternatively, 30 μl of stock were added with 270 μl of rapid lysis buffer containing 200 μg/ml of proteinase K, 50 mM of KCl, 2.5 mM of $MgCl_2$, 0.45% Tween 20, 0.45% Nonidet P-40 in Tris–HCl 10 mM pH 8. The lysate was incubated at 56 °C for 90 min, then at 95 °C for 10 min and kept frozen at −20 °C till further analysis. As far as HSV-infected cell cultures are concerned, the supernatant was submitted to ultracentrifugation at 20,000 rpm for 15 min after which the pellet was treated with 30 μl of rapid lysis buffer in the same way as virus stocks. After the supernatant culture has been removed, infected-cell monolayers were similarly treated with 300 μl of rapid lysis buffer.

2.5. Real-time PCR-based susceptibility assay

Several procedures were tested for assay optimization (see Section 3). The virus stocks were obtained from infected cell cultures exhibiting 80% CPE and 10-fold diluted to the final dilution of 10^{-3}. This diluted stock was used to inoculate Vero cell monolayers in a 24-well culture plate according to the general infection procedure described above. Antivirals were then added at the concentrations of 0.1, 0.5, 1, 10, 30, 60 μM for ACV, and 3.3, 16.5, 66, 165 μM for PFA. At 24 h p.i., supernatant was discarded and cell monolayers were treated with 300 μl of rapid lysis buffer (see above). Ten microlitre of two dilutions of crude lysate (undiluted and 10-fold diluted) were then submitted to real-time PCR for HSV in parallel. The IC_{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.

2.6. Statistical analysis

All analyses were carried out using StatView software (Abacus Concepts, Berkeley). Continuous variables were compared using non-parametric tests as appropriate: Mann–Whitney test for unpaired comparisons, Wilcoxon rank sum test for paired comparisons, and Spearman rank correlation test for correlation studies. Variables exceeding the upper quantification limit were given the highest measurable value. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Relationship between infectivity and viral DNA load applied to virus quantification

The first objective was to demonstrate that the measurement of HSV DNA by means of real-time PCR was a relevant method for virus quantification as compared with plaque formation assay taken as the reference.

In a first step, the number of HSV DNA copies and the number of PFU (infectious titer), were determined in parallel, each in duplicate, for each stock of HSV isolate tested (data not shown). There was only a weak correlation between both parameters ($r = 0.12$, non-significant), for both HSV types taken together. In addition, the number of viral genome copies far exceeded that of PFU, with a ratio ranging from 103 up to 30,763 (median: 1922), which confirmed that viral particles or subparticles carrying DNA but lacking infectivity represented most of the virus yield.

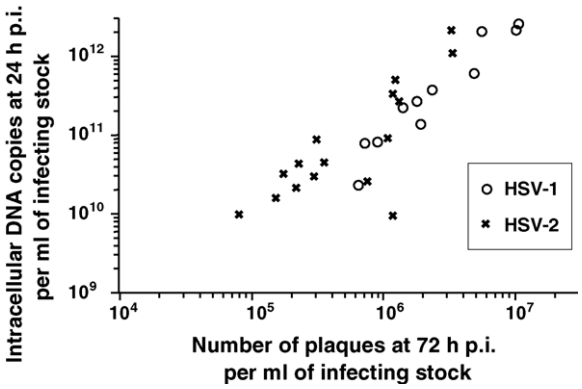

Fig. 1. Comparison between the number of plaques obtained at 72 h p.i. and intracellular HSV DNA load at 24 h p.i. The numbers of plaques and DNA copies obtained when using 1 ml of virus stock for the infection of Vero cells were determined in parallel using serial dilutions of these stocks as described in Section 2.

In a second step, the number of HSV DNA copies present within infected cells at 24 h p.i. was compared with the number of virus-induced plaques obtained at 72 h p.i., using similar viral inputs and cell substrates (Fig. 1). There was a strong correlation between both parameters ($r = 0.88$, $p < 0.0001$). HSV DNA cell load at 24 h p.i. was significantly higher than overall HSV DNA inoculated to cells at the starting of infection, with an apparent amplification factor ranging from 3 up to 1244 (median: 43). As expected, the higher the ratio of HSV DNA to PFU in virus stocks, the lower the amplification factor of DNA at 24 h p.i. ($r = -0.89$, $p < 0.0001$), confirming that intracellular DNA replication resulted from the uptake of infectious particles and cell infection worked as a positive filter for replication-competent viruses. There was a trend for a higher amplification factor in the case of HSV-1 than HSV-2 (median values: 166 and 30 for HSV-1 and HSV-2, respectively) but this difference was not considered significant ($p = 0.08$).

3.2. Optimization and reproducibility of real-time PCR susceptibility assay

The strength of the correlation between intracellular viral DNA load and infectivity prompted us to set-up a simplified rapid assay based on real-time PCR that could substitute for the conventional PRA. The format of culture remained unchanged, Vero cells being seeded in 24-well microplates. To obtain results more quickly, it was tested whether the titration of virus stocks prior to susceptibility assay could be avoided. Preliminary assays were performed using diluted stocks ranging from 1:10,000 to 1:100. ACV IC_{50} could be determined in all cases and a decrease of IC_{50} was consistently observed in parallel to the decrease of multiplicity of infection (MOI) as previously reported (Stranska et al., 2002). However, the variation of IC_{50} was modest (being less than two-fold) within this range of stock dilutions: as examples, the ranges of IC_{50} for the dilutions from 1:10,000 to 1:100 were 0.11-0.20, 12.0–21.5, 17.0–28.5 μM in the case of the isolates #3414 and ACV, #4279 and ACV, #3414 and PFA, respectively. Infection with the single intermediary 1:1000 dilution of virus stocks was ultimately chosen to permit the reproducible determination of IC_{50} without the need for previous titration of these stocks.

The quantification of HSV DNA in supernatant at 24 h p.i. was not retained for two reasons: (i) DNA load was too low, in particular with virus stocks exhibiting a low infectivity (data not shown), which confirmed previous results (Stranska et al., 2002); (ii) residual input DNA might interfere with the quantification of virus yield. In contrast, the evaluation of HSV DNA cell content was readily possible from 24 h p.i., as shown above. When a later read-out (at 48 or 72 h p.i.) was used, IC_{50} increased but again the overall variation was lower than two-fold (data not shown).

Lastly, after the supernatant had been discarded, the recovery of infected cell DNA was carried out in situ within 24-well microplates with rapid lysis buffer (see Section 2). This simple method proved to be as efficient for the preparation of DNA as more complex procedures including the collection of cells using trypsin treatment before lysis and phenol–chloroform extraction of DNA. The use of the crude lysate at 1:10 dilution permitted to circumvent the possible presence of PCR inhibitors.

As a result of this optimization process, the final format of the susceptibility assay included the following steps (see Section 2): isolation of the virus and constitution of the stock at 80% CPE, infection of Vero cells for 24 h p.i., quantification of HSV DNA on undiluted and 10-fold diluted crude cell lysate using real-time PCR, plotting the figures onto a graph, calculation of IC_{50} from the inhibition curve obtained. This assay was repeatedly applied to the five prototypic isolates depicted in Table 1, exposed to either ACV or PFA. The mean intra-assay and inter-assay standard deviations of Ct used for IC_{50} computing were 0.12 and 0.07 cycles, respectively, while the mean inter-assay coefficient of variation of IC_{50} was 21%. These values demonstrated a good reproducibility of assay results.

3.3. Application of the susceptibility assay to the panel of HSV isolates

Twenty-seven HSV isolates were tested both for their susceptibility to ACV and PFA using PRA and real-time PCR assays in parallel (Table 1; Figs. 2 and 3). No significant difference of

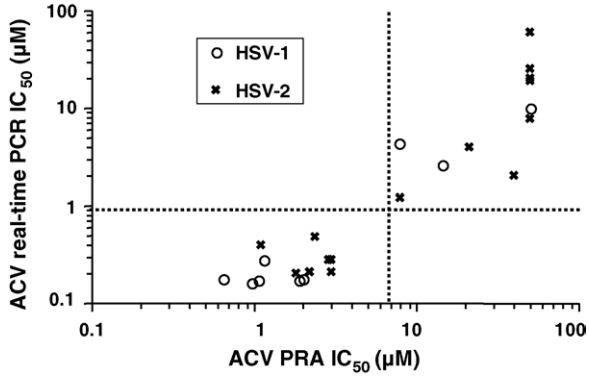

Fig. 2. Comparison between the acyclovir inhibitory concentrations 50% (ACV IC_{50}s) obtained by using plaque reduction assay (PRA) and real-time PCR. Dotted lines correspond to resistance IC_{50} thresholds (see text).

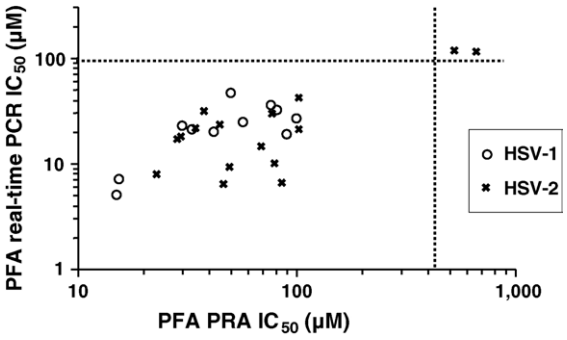

Fig. 3. Comparison between the foscarnet inhibitory concentrations 50% (PFA IC_{50}s) obtained by using plaque reduction assay (PRA) and real-time PCR. Dotted lines correspond to resistance IC_{50} thresholds (see text).

IC_{50} was evidenced according to HSV type whatever the method or drug used. The IC_{50}s determined by real-time PCR assay were significantly correlated with PRA IC_{50}s in the case of both drugs ($r = 0.91$, $p < 0.0001$, and $r = 0.51$, $p = 0.009$ for ACV and PFA, respectively). The real-time PCR IC_{50}s were significantly lower than PRA ones both for ACV and PFA ($p < 0.0001$ in both cases). The mean ratio of PRA IC_{50} to real-time PCR IC_{50} was 7.0 (range: 0.8–20.0) and 3.5 (range: 1.0–13.0) in the case of ACV and PFA, respectively. However, taking into account real-time PCR IC_{50}s did not alter the classification into sensitive and resistant isolates established by means of PRA (Table 1). These values effectively permitted to distinguish sensitive from resistant isolates for both drugs. The median real-time PCR IC_{50}s of sensitive and resistant isolates were 0.2 μM (range: 0.1–0.5) and 8.9 μM (1.0 to >60), respectively, for ACV (Fig. 2); these values were 23.1 μM (5.1–47.8) and 113.8 μM (112.2 and 115.5 for the two isolates tested), respectively, for PFA (Fig. 3). These values correspond to the values obtained for the PRA IC_{50}s for resistance (7 and 330 μM for ACV and PFA, respectively). Therefore the threshold real-time PCR IC_{50}s for resistance might be set provisionally at 1.0 and 100 μM for ACV and PFA, respectively.

4. Discussion

We report a novel HSV susceptibility assay based on the early measurement of intracellular virus DNA by means of real-time PCR. This assay works equally well for both HSV types and was validated for ACV as well as PFA. Due to the high amount of intracellular HSV DNA at 24 h p.i. and the wide dynamic range of real-time PCR, the read-out was possible after 1-day culture without the need for virus stock titration or CPE follow-up. These properties provide a good basis for automation of the assay, once the virus has been isolated by conventional procedures, and should accelerate the transition from the collection of clinical sample to the adaptation of HSV therapy. Since the real-time PCR procedure corresponds to a method also used for HSV detection in human specimens (Kessler et al., 2000), implementation of this susceptibility assay in clinical virology laboratories is easy and does not require any additional equipment.

The use of cell DNA after rapid cell lysis undoubtedly adds a level of complexity to the assay when compared to the collection of culture supernatant. However, this permits an earlier read-out and the use of diluted crude lysate without any need for DNA purification is rather simple. In addition, at least in our hands, the correlation of intracellular HSV DNA load with virus infectivity is stronger than that of culture supernatant DNA. Thus, to some extent, intracellular HSV DNA appears to be more relevant than culture supernatant DNA in regard of the process of DNA replication and its inhibition by antiviral drugs. Lastly, this procedure can be extended to other herpesviruses such as varicella-zoster virus and human cytomegalovirus which need easier susceptibility assays but for which the release from culture cells is poor and spread out over time. Also a real-time PCR susceptibility assay was successfully developed for human herpesvirus 6 (HHV-6) but the persistence of DNA in culture medium might jeopardize the calculation of IC_{50} in case of late read-out (Bonnafous et al., 2005; Mace et al., 2003).

Despite the high correlation with PRA, real-time PCR IC_{50}s were consistently lower than those obtained with plaque formation infectivity assay. This was also observed by Stranska et al. (2002), although the target genes for real-time PCR and the format of the assay were different. The same result was basically found in the case of HHV-6 when comparing real-time PCR and antigen expression (Mace et al., 2003). Overall this confirms that real-time PCR may represent the most sensitive approach to measure the effects of antivirals targeted at viral DNA polymerase activity, as also illustrated with other herpesviruses (Sergerie and Boivin, 2003). Otherwise, real-time PCR IC_{50}s of HSV tend to increase with MOI and with the time of read-out, as yet reported by others (Stranska et al., 2002). However, this variability of IC_{50}s was intrinsically modest and did not affect the classification of isolates in terms of sensitivity or resistance to antivirals. Moreover, there is no evidence from literature that PRA, considered the reference method in the field, is less sensitive to this type of fluctuations than real-time PCR assays. Of note, the cutoff value of 0.3 μg/ml of ACV proposed as a discriminative concentration for sensitive and resistant isolates in a previously reported real-time PCR assay (Stranska et al., 2002) is close to the value of 1.0 μM provisionally defined from our results. This similarity makes us confident that, despite marked differences in the concept of the assays, real-time PCR is a valuable tool for the read-out of HSV susceptibility tests.

As a whole, the resistance of HSV to antivirals remains a limited phenomenon (Danve-Szatanek et al., 2004). It is much more frequent in immunocompromised patients than immunocompetent ones and has, in the clinical context, an important impact on the control of severe HSV infections (Boivin et al., 1993; Safrin et al., 1990; Schmit and Boivin, 1999; Snoeck, 2000). The perspective of developing a direct genetic test for resistance combined with the PCR-based detection of HSV in clinical samples remains a relevant objective (Andrei et al., 2001). However, the ACV-resistant HSV-2 isolate #0302061, which has no mutation in the thymidine kinase gene, points to the fact (Stranska et al., 2004b), that the genetic profile of HSV cannot predict the phenotypic resistance to antivirals in all situations. This isolate is on the borderline of ACV resistance with IC_{50}s of 7.7 and 1.0 μM for the PRA and real-time PCR assay, respectively (Table 1). In further experiments using real-time PCR assay, this isolate did not consistently show IC_{50} values above the resis-

tance threshold, provisionally defined as 1.0 μM. This result was expected, since the mean inter-assay coefficient of variation of IC_{50} was found to be 21%. Alternatively, a mutation of DNA polymerase gene may confer a low level ACV resistance, an hypothesis which deserves further investigations. Whatever the final classification of the #0302061 isolate may be in terms of resistance, this apparent discrepancy between genetic analysis and susceptibility assay justifies to continue studies based on phenotypic approaches. There is no doubt that the extended use of real-time PCR susceptibility assays may contribute to a better detection of HSV resistance, a better understanding of molecular mechanisms underlying this phenomenon, and, eventually, better therapeutic options for immunocompromised patients.

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References

Agut, H., Aubin, J.T., Ingrand, D., Blanc, S., Clayton, A.L., Chantler, S.M., Huraux, J.M., 1990. Simplified test for detecting the resistance of herpes simplex virus to acyclovir. J. Med. Virol. 31, 209–214.

Andrei, G., Fiten, P., De Clercq, E., Snoeck, R., Opdenakker, G., 2001. Evaluating phenotype and genotype of drug-resistant strains in herpesviruses. Mol. Biotechnol. 18, 155–167.

Boivin, G., Erice, A., Crane, D.D., Dunn, D.L., Balfour Jr., H.H., 1993. Acyclovir susceptibilities of herpes simplex virus strains isolated from solid organ transplant recipients after acyclovir or ganciclovir prophylaxis. Antimicrob. Agents Chemother. 37, 357–359.

Bonnafous, P., Gautheret-Dejean, A., Boutolleau, D., Caiola, D., Agut, H., 2005. Persistence of DNA in cell cultures may jeopardize the analysis of human herpesvirus 6 dynamics by means of real-time PCR. J. Virol. Methods 125, 95–98.

Chibo, D., Druce, J., Sasadeusz, J., Birch, C., 2004. Molecular analysis of clinical isolates of acyclovir resistant herpes simplex virus. Antiviral Res. 61, 83–91.

Chibo, D., Mijch, A., Doherty, R., Birch, C., 2002. Novel mutations in the thymidine kinase and DNA polymerase genes of acyclovir and foscarnet resistant herpes simplex viruses infecting an immunocompromised patient. J. Clin. Virol. 25, 165–170.

Danve-Szatanek, C., Aymard, M., Thouvenot, D., Morfin, F., Agius, G., Bertin, I., Billaudel, S., Chanzy, B., Coste-Burel, M., Finkielsztejn, L., Fleury, H., Hadou, T., Henquell, C., Lafeuille, H., Lafon, M.E., Le Faou, A., Legrand, M.C., Maille, L., Mengelle, C., Morand, P., Morinet, F., Nicand, E., Omar, S., Picard, B., Pozzetto, B., Puel, J., Raoult, D., Scieux, C., Segondy, M., Seigneurin, J.M., Teyssou, R., Zandotti, C., 2004. Surveillance network for herpes simplex virus resistance to antiviral drugs: 3-year follow-up. J. Clin. Microbiol. 42, 242–249.

Danve, C., Morfin, F., Thouvenot, D., Aymard, M., 2002. A screening dye-uptake assay to evaluate in vitro susceptibility of herpes simplex virus isolates to acyclovir. J. Virol. Methods 105, 207–217.

Gaudreau, A., Hill, E., Balfour Jr., H.H., Erice, A., Boivin, G., 1998. Phenotypic and genotypic characterization of acyclovir-resistant herpes simplex viruses from immunocompromised patients. J. Infect. Dis. 178, 297–303.

Kessler, H.H., Muhlbauer, G., Rinner, B., Stelzl, E., Berger, A., Dorr, H.W., Santner, B., Marth, E., Rabenau, H., 2000. Detection of herpes simplex virus DNA by real-time PCR. J. Clin. Microbiol. 38, 2638–2642.

Mace, M., Manichanh, C., Bonnafous, P., Precigout, S., Boutolleau, D., Gautheret-Dejean, A., Agut, H., 2003. Real-time PCR as a versatile tool for investigating the susceptibility of human herpesvirus 6 to antiviral agents. Antimicrob. Agents Chemother. 47, 3021–3024.

Morfin, F., Souillet, G., Bilger, K., Ooka, T., Aymard, M., Thouvenot, D., 2000. Genetic characterization of thymidine kinase from acyclovir-resistant and -susceptible herpes simplex virus type 1 isolated from bone marrow transplant recipients. J. Infect. Dis. 182, 290–293.

Morfin, F., Thouvenot, D., 2003. Herpes simplex virus resistance to antiviral drugs. J. Clin. Virol. 26, 29–37.

Safrin, S., Assaykeen, T., Follansbee, S., Mills, J., 1990. Foscarnet therapy for acyclovir-resistant mucocutaneous herpes simplex virus infection in 26 AIDS patients: preliminary data. J. Infect. Dis. 161, 1078–1084.

Schmit, I., Boivin, G., 1999. Characterization of the DNA polymerase and thymidine kinase genes of herpes simplex virus isolates from AIDS patients in whom acyclovir and foscarnet therapy sequentially failed. J. Infect. Dis. 180, 487–490.

Sergerie, Y., Boivin, G., 2003. Evaluation of susceptibility of human herpesvirus 8 to antiviral drugs by quantitative real-time PCR. J. Clin. Microbiol. 41, 3897–3900.

Snoeck, R., 2000. Antiviral therapy of herpes simplex. Int. J. Antimicrob. Agents 16, 157–159.

Standring-Cox, R., Bacon, T.H., Howard, B.A., 1996. Comparison of a DNA probe assay with the plaque reduction assay for measuring the sensitivity of herpes simplex virus and varicella-zoster virus to penciclovir and acyclovir. J. Virol. Methods 56, 3–11.

Stranska, R., van Loon, A.M., Polman, M., Schuurman, R., 2002. Application of real-time PCR for determination of antiviral drug susceptibility of herpes simplex virus. Antimicrob. Agents Chemother. 46, 2943–2947.

Stranska, R., Schuurman, R., Scholl, D.R., Jollick, J.A., Shaw, C.J., Loef, C., Polman, M., van Loon, A.M., 2004a. ELVIRA HSV, a yield reduction assay for rapid herpes simplex virus susceptibility testing. Antimicrob. Agents Chemother. 48, 2331–2333.

Stranska, R., van Loon, A.M., Polman, M., Beersma, M.F., Bredius, R.G., Lankester, A.C., Meijer, E., Schuurman, R., 2004b. Genotypic and phenotypic characterization of acyclovir-resistant herpes simplex viruses isolated from haematopoietic stem cell transplant recipients. Antiviral Ther. 9, 565–575.