Herpesvirus infections in immunocompromised patients : treatment, treatment failure and antiviral resistance

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Rapid susceptibility testing for Herpes Simplex Virus type 1 using real-time PCR

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ABSTRACT

Background: Susceptibility testing of herpes simplex virus type 1 (HSV-1) is traditionally performed by a plaque reduction assay (PRA), but this is labor intensive, time consuming and has a manual read out.
Objectives: The goal of this study was to develop an internally controlled real-time PCR-based phenotypical susceptibility test for HSV-1 that is suitable for use in a clinical diagnostic setting.
Study design: A DNA reduction assay (DRA) was developed and validated on a test panel of 26 well-characterized isolates of varying susceptibility to aciclovir or foscarnet, including low-level resistant isolates. The DRA consisted of pre-culture of a clinical sample for 48 hours and subsequent culture in the presence of antivirals for 24 hours. Viral DNA concentration in the culture lysates was measured by an internally controlled quantitative real-time HSV-1 PCR and corrected for cell count and lysis by beta-globin PCR. DRA results were compared to results from PRA and sequence analysis.
Results: DRA results were in accordance with PRA results for both aciclovir and foscarnet susceptibility and appeared to have good discriminative value for low-level resistance due to UL30 gene mutations. Although the direct application of DRA in clinical samples appeared not possible, short pre-culture of 48 hours was sufficient and ensured results within a clinically relevant time frame of 5 days.
Conclusions: DRA is an accurate, rapid and easy to perform phenotypical susceptibility test for HSV-1.

BACKGROUND

Severe and persistent infections with herpes simplex virus type 1 (HSV-1) are common in immunocompromised patients, especially patients receiving chemotherapy and hematopoietic stem cell transplants, and are frequently associated with antiviral resistance.[1] The fastest approach to HSV-1 susceptibility testing is sequence analysis of the UL23 gene of the HSV-1 thymidine kinase that catalyzes a necessary phosphorylation step of aciclovir or of the UL30 gene of the HSV-1 DNA polymerase. Sequencing of these genes may reveal a resistance conferring mutation, but since nucleotide variations are common, mutations of unknown significance are also found frequently.[1-4] In such cases, phenotypical susceptibility testing of HSV-1 is still required which is traditionally performed by a plaque reduction assay (PRA).[5] PRA requires viral titration and prolonged incubation until viral cytopathogenic effect (CPE) is visible and is labor intensive, subjective and time consuming.

Faster phenotypical assays using more sensitive and objective endpoints are preferable. Real-time PCR has previously been applied successfully to measure viral concentrations in HSV-1 phenotypical susceptibility tests.[6;7] Stranska et al[6] measured inhibition of viral DNA replication by antivirals in culture supernatant, which may be less indicative of intracellular viral replication. The protocol described by Thi et al[7] measured viral DNA in cells but used crude cell lysate in an uncontrolled PCR.

OBJECTIVES

The goal of this study was to design, optimize and validate a rapid internally controlled real-time PCR-based phenotypical susceptibility test for HSV-1 for routine use in a clinical diagnostic setting.

STUDY DESIGN

Viral isolates and clinical samples

For DNA reduction assay (DRA) validation using viral isolates (Table 1), susceptible reference strain HSV F (ATCC number VR-733 Manassas, VA, USA), and 25 previously characterized viral isolates from 14 patients that were clinically suspected of having a resistant virus and that had been sent for susceptibility testing to an external laboratory

Table 1. Features of previously characterized HSV-1 isolates used for DNA reduction assay validation.

patient/ source	isolate	clinical & treatment details	UL23 mutation	UL30 mutation
ATCC	HSV-F	susceptible reference strain	none	none
A	#1	SCT, mucositis, pre-treatment	none	none
	#11	mucositis, ACV iv 13 days	delG180 → fs61	none
	#12	ulcerative esophagitis, ACV iv 13 days & vACV 4 days	delG180 → fs61	none
	#13	ulcerative esophagitis, ACV iv 22 days & vACV 9 days	delG180 → fs61	none
B	#2	SCT, lip lesion, pre-treatment	none	none
	#21	severe facial an days oral lesions, ACV iv 52 days & vACV 32 days	G488A → subR163H	none
	#24	lip lesion, ACV iv 44 days	none	G2171A → subS724N
C	#3	SCT, mucositis, pre-treatment	none	none
	#22	persistent mucositis, ACV iv 13 days	C566T → subA189V	none
D	#4	SCT, mucositis, pre-treatment	none	none
	#14	persistent mucositis, ACV iv 5 days & GCV iv 12 days	insG430 → fs146	none
E	#5	SCT, mucositis, pre-treatment	none	none
	#23	persistent mucositis, vACV 8 days & ACV iv 7 days	insG430 → fs146	none
F	#10	SCT, encephalitis, ACV iv 15 days	T1033C → subS345P	G1684A → subA562T
G	#20	SCT, persistent mucositis, ACV iv 26 days	A314C → subH105P	none
H	#7	SCT, stomatitis, pre-treatment	none	none
	#8	persistent stomatitis, ACV iv 5 days	none	none
	#15	persistent stomatitis, ACV iv 12 days	insG430 → fs146	none
I	#16	SCT, treatment unknown	delG430 → fs146	none
J	#18	SCT, treatment unknown	insC548 → fs185	none
K	#19	SCT, treatment unknown	C310T → stop104	none
L	#25	SCT, treatment unknown	none	C2156T → subA719V
M	#6	chemotherapy, mucositis, vACV 5 days	none	none
	#17	persistent mucositis, vACV 12 days & ACV iv 10 days	delC460 → fs155	none
N	#9	immunocompetent, recurrent genital HSV-1 despite vACV prophylaxis	none	G1947T → subE649D

All samples were isolated from swab samples from mucous membranes or skin, except for sample 10 which was isolated from cerebrospinal fluid. Patient B[10], patients I-L[8] and patients A, C, D and H[9] were described in previous studies.

ACV = aciclovir; d = days; del = deletion; fs = frameshift; GCV = ganciclovir; ins = insertion; SCT = stem cell transplantation; sub = substitution; vACV = valaciclovir.

or that were described in previous studies were used.[8-10] The test panel included pre-treatment viral isolates if available. Aliquots of viral isolates were stored at -80°C. For DRA validation directly on clinical samples (swabs), the clinical samples from which isolates #5 and #17 had been cultured and 7 randomly selected HSV-1 positive clinical samples were used.

UL23 and UL 30 gene sequence analysis

Genotypical resistance analysis was performed by cycle sequencing after PCR amplification on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems). Amplification and sequencing primers and PCR conditions are shown in Table 2. UL23 amplification was performed in 50 μl containing 25 μl HotStart Taq mastermix (Qiagen, Hilden, Germany) and 15 pmol of each primer. For UL30 amplification, nested PCR was necessary for clinical samples, but not for viral isolates. PCR and nested PCR were performed in 50 μl containing 1 μl Advantage®-GC 2 Polymerase mix (Clontech, Westburg, Leusden, The Netherlands), 10 μl Advantage®-GC 2 PCR Buffer, 25 μmol GC-melt, 0.2 mM dNTP mix and 15 pmol of each primer. All cycle sequencing reactions were performed in 20 μl containing 2 μl Big Dye Terminator v1.1 (Applied Biosystems, Carlsbad, CA, USA), 6 μl sequencing buffer and 8 pmol primer. The detection limit of the assay was around 1000 copies/ml for the UL23 gene and around 5000 copies/ml for the UL30 gene. Sequences were compared to pre-treatment isolates if available and to the sequence of HSV F.

Plaque Reduction Assay

The protocol M33-A *Antiviral Susceptibility Testing: Herpes Simplex Virus by Plaque Reduction Assay* of the Clinical and Laboratory Standards Institute[5] using Vero cells was modified by including the use of microcrystalline cellulose (Avicel® RC/CL, FMC BioPolymer, Philadelphia, USA) as overlay.[11] For viral titration, the overlay consisted of 1.5 ml of 0.6% Avicel® RC/CL in sterile water mixed 1:1 with 2x EMEM without phenol red (Gibco) with 4% FCS and 4 mM glutamine. After incubation, cells were fixed with formalin and, after aspiration of the overlay, stained with crystal violet. For PRA, the overlay consisted of twofold serial dilutions of aciclovir (acycloguanosine, Sigma-Aldrich, Schnelldorf, Germany) or foscarnet (sodium phosphonoformate tribasic hexahydrate, Sigma-Aldrich) in 2x EMEM without phenol red with 4% FCS and 4 mM glutamine mixed 1:1 with 0.6% Avicel® RC/CL in sterile water. The aciclovir concentration range was 0,12 to 16 mg/L and for foscarnet the concentration range was 16,7 to 400 mg/L. Isolates having an IC_{50} value $\geq$ 2 mg/L for aciclovir or $\geq$ 100 mg/L for foscarnet were considered resistant.[5]

Table 2. Primers, probes and PCR conditions used for UL23 and UL30 gene sequence analysis and DNA reduction assay.

target	orientation	sequence (5′→3′)	position[a]	amplicon size (bp)	pcr protocol
UL23 gene HSV-1 amplification	sense	TCCACTTCGCATATTAAGGT	-146	1335	15′ 95°C 40x: 1′ 95°C 1′ 50°C 1′ 72°C 1x: 10′ 72°C
	antisense	CTGTCTTTTTATTGCCGTCA	+38		
UL23 gene HSV-1 sequencing	sense	GCTTAACAGCGTCAACAGC	-84		1′ 96°C 25x: 10″ 96°C 5″ 50°C 4′ 60°C
	sense	AGACAATCGCGAACATCTAC	284		
	antisense	CCGATATCTCACCCTGGTC	321		
	sense	ATACGGTGCGGTATCTGC	731		
	antisense	AAAGCTGTCCCCAATCCT	770		
	antisense	TGTCTTTTTATTGCCGTCAT	+37		
UL30 gene HSV-1 amplification	sense	GAGGACGAGCTGGCCTTTCCG	1126	1810	3′ 94°C 40x: 30″ 94°C 30″ 60°C 2′ 68°C 1x: 3′ 68°C
	antisense	AAAACAGCAGGTCGACCAGGGC	2914		
UL30 gene HSV-1 nested amplification	sense	GACCTCCCCGAATCCCA	1252	1652	3′ 94°C 30x: 30″ 94°C 30″ 60°C 2′ 68°C 1x: 3′ 68°C
	antisense	CGGTTGATAAACGCGCAGTTG	2883		
UL30 gene HSV-1 sequencing	sense	GAGGACGAGCTGGCCTTT	1126		1′ 96°C 25x: 10″ 96°C 5″ 50°C 4′ 60°C
	sense	GAGTACTGCATACAGGATTC	1726		
	antisense	TGGCCGTCGTAGATGGTG	1833		
	antisense	TGCTGCACTCCCGTGAAC	2457		
	antisense	AAAACAGCAGGTCGACCAG	3708		
	sense[b]	GACCTCCCCGAATCCCA	1252		
	antisense[b]	CGGTTGATAAACGCGCAGTTG	2883		
Glycoprotein B gene HSV-1 amplification	sense	TCAAGACCACCTCCTCCATC	1484-1503	130	15′ 94°C 50 x: 30″ 94°C 30″ 55°C 30″ 72°C
	antisense	AGGGTCAGCTCGTGATTCTG	1594-1613		
	probe	FAM AACATATCGTTGACATGG BHQ-1	1545-1562		
Glycoprotein B gene PhHV-1 amplification	sense	GGGCGAATCACAGATTGAATC	159-179	89	15′ 94°C 50 x: 30″ 94°C 30″ 55°C 30″ 72°C
	antisense	GCGGTTCCAAACGTACCAA	229-247		
	probe	Cy5 TTTTTATGTGTCCGCCACCATCTGGATC BHQ-2	197-224		
ß hemoglobin gene	sense	AAGTGCTCGGTGCCTTTAGTG	200-220	97	15′ 94°C 50 x: 30″ 94°C 30″ 55°C 30″ 72°C
	antisense	ACGTGCAGCTTGTCACAGTG	222-245		
	probe	YAK TGGCCTGGCTCACCTGGACAACCT BHQ-1	277-296		

[a] from startcodon in gene in sense orientation, [b] in case of nested amplification

Real-time PCR based phenotypical susceptibility test

DRA was performed on supernatants from either HSV-1 culture isolates (Table 1) or directly on positive clinical samples inoculated onto A549 cells and incubated for 48 hours. Supernatants from pre-cultured clinical samples were diluted 1:100 in EMEM regardless of viral titers whereas stored viral isolates were diluted to obtain viral loads between 10^4 and 10^7 copies/ml. A confluent monolayer of Vero cells was cultured in 24 wells plates. Subsequently 300 μl of the diluted sample was added to each well after removal of culture medium. After one hour of incubation at 37°C in a humidified atmosphere of 5% CO_2 the inoculum was removed and replaced by 1 ml of aciclovir or foscarnet in EMEM with 5% FCS. The concentration range of the serial dilutions (6 dilutions per isolate) of aciclovir was 0.12 to 16 mg/L and for foscarnet 8.35 to 100 mg/L. All samples were assayed in quadruplicate and incubated for 24 hours.

Then, after removal of the culture medium, 200 μl of AL Lysis buffer (QIAamp® DNA Mini and Bloodkit, Qiagen) spiked with Phocid Herpes Virus (PhHV, kindly provided by Dr. M Schutten, Erasmus Medical Center, Rotterdam, The Netherlands) was added to the monolayer and incubated for 15 minutes at room temperature. Lysate was harvested and all wells were rinsed with 200 μl PBS to include any remaining viral DNA which was then added to the lysate. After addition of 20 μl Proteinase K (Qiagen), samples were incubated 10 minutes at 56°C and 10 minutes at 95°C. Samples were mixed with 200 μl ethanol 100% and transferred to a Qiamp® mini spin column (Qiagen) and centrifuged for 1 minute at 6000 x g. Washing steps were omitted to limit hands on time. DNA was eluted in 200 μl AE buffer (Qiagen) by centrifugation for 1 minute at 6000 x g. A real-time PCR for beta-globin and a multiplex real-time PCR for PhHV and HSV-1 were performed on a CFX96 real-time detection system (Bio-Rad, Veenendaal, The Netherlands). Primers, probes and PCR conditions are shown in Table 2. PCRs were carried out in 50 μl volume containing 25 μl HotStart Taq mastermix (Qiagen) and 4 mM $MgCl_2$ (5 mM for beta-globin).

For quantitation, a standard of HSV-1 (cultured field isolate or ATCC KOS strain VR-1493D) was calibrated using a quantitated DNA control of the HSV-1 MacIntyre strain (Advanced Biotechnologies Inc., Columbia, MD). PhHV served as an internal control for DNA extraction and PCR inhibition[12] and those wells that were outliers in the PhHV PCR (more than 3 CT-values different from assay average) were considered inhibited and were excluded from the analysis. The beta-globin PCR was performed as a control for cell concentration and cell lysis for which the same exclusion criteria were used. The concentration of antiviral agents to inhibit viral DNA replication by 50% (IC_{50}) was calculated after plotting the viral concentration versus the concentration of antiviral agent. Provisional resistance breakpoints were adapted from Thi at al.[7] and set at ≥0.23 mg/L for aciclovir and at ≥ 13 mg/L for foscarnet.

RESULTS

Viral isolates

PRA and sequence analysis of the UL23 and UL30 genes were repeated on aliquots of the viral isolates to be used for DRA. PRA results for aciclovir were concordant with genotypic analysis in 25 isolates including 11 with no mutations in either the UL23 or UL30 genes, 13 with a mutation in UL23, and 1 with a mutation in UL30. One isolate (#25) had a UL30 mutation but was susceptible to aciclovir by PRA but with an elevated IC50 of 1.03 mg/L (Table 3). PRA results for foscarnet were concordant in 23 isolates, including 10 with no mutations and 13 with a mutation in UL23, failed in one isolate (#2) and were discordant with genotypical analysis in the two isolates with UL30 mutations (#24 and #25, Table 3). Both isolates #24 and #25 showed diminished susceptibility to foscarnet although not meeting the proposed resistance criteria according to CLSI.

DRA was validated for aciclovir on all isolates and for foscarnet in UL30 mutants and in a subset of UL23 mutants. DRA failed in one isolate (#9). DRA results (Table 3) for aciclovir were in concordance with genotyping of all 25 isolates using the provisional breakpoint of 0.23 mg/L, but not in concordance with PRA for 1 isolate (#25). By DRA, IC50 values of all 15 UL23 and UL30 mutants, including isolate #25, were at least at least 2.8-fold the tentative breakpoint and 6.4-fold the IC50 value of the susceptible reference isolate. DRA results (Table 3) for foscarnet were concordant with genotypical results in the 8 tested isolates, but were not concordant with PRA in two isolates (#24 and #24) . These UL30 mutants had IC50-values that were at least 1.7 fold the tentative breakpoint and at least 2.5 fold the IC50 value of the susceptible reference isolate.

Clinical samples

DRA was applied to 9 clinical samples (Table 4) and demonstrated aciclovir resistance in two UL23 mutants (Table 4) and failed in one of the clinical samples that had a relatively low viral load after 48 hours of pre-culture (7.9×10^4 copies/ml).

DISCUSSION

This study optimized and validated a protocol for phenotypical susceptibility testing of HSV-1. A fast and easily applicable protocol was developed that compared very well to results obtained by genotypical tests and by PRA. Although only tentative breakpoints

Table 3. UL23 and UL30 sequence analysis, plaque reduction assay and DNA reduction assay results on HSV-1 isolates.

isolate	resistance-associated mutation	PRA			DRA		
		IC50 ACV mg/L	**IC50 FOS** mg/L	**conclusion**	**IC50 ACV** mg/L	**IC50 FOS** mg/L	**conclusion**
HSV-F	none	0.08	21.9	ACV S FOS S	0.10	8.75	ACV S FOS S
#1	none	0.10	21.3	ACV S FOS S	0.08	n.d.	ACV S FOS n.d.
#2	none	0.45	failed	ACV S FOS n.d.	0.06	n.d.	ACV S FOS n.d.
#3	none	0.16	18.2	ACV S FOS S	0.12	n.d.	ACV S FOS n.d.
#4	none	0.09	26.0	ACV S FOS S	0.07	n.d.	ACV S FOS n.d.
#5	none	0.15	19.4	ACV S FOS S	0.07	n.d.	ACV S FOS n.d.
#6	none	0.23	16.7	ACV S FOS S	0.12	n.d.	ACV S FOS n.d.
#7	none	0.28	11.4	ACV S FOS S	0.08	n.d.	ACV S FOS n.d.
#8	none	0.28	17.8	ACV S FOS S	0.18	n.d.	ACV S FOS n.d.
#9	none	0.06	10.5	ACV S FOS S	failed	failed	failed
#10	none	0.11	35.7	ACV S FOS S	0.08	12.81	ACV S FOS S
#11	UL23	**16.21**	11.3	ACV **R** FOS S	**2.65**	n.d.	ACV **R** FOS n.d.
#12	UL23	**15.16**	16.3	ACV **R** FOS S	**3.67**	n.d.	ACV **R** FOS n.d.
#13	UL23	**11.20**	10.1	ACV **R** FOS S	**0.76**	n.d.	ACV **R** FOS n.d.
#14	UL23	**11.82**	12.5	ACV **R** FOS S	**6.17**	n.d.	ACV **R** FOS n.d.
#15	UL23	**15.56**	9.1	ACV **R** FOS S	**2.40**	n.d.	ACV **R** FOS n.d.
#16	UL23	**13.92**	25.0	ACV **R** FOS S	**3.18**	5.98	ACV **R** FOS S
#17	UL23	**16.70**	17.4	ACV **R** FOS S	**3.06**	n.d.	ACV **R** FOS n.d.
#18	UL23	**37.95**	25.0	ACV **R** FOS S	**4.62**	4.81	ACV **R** FOS S
#19	UL23	**6.88**	25.0	ACV **R** FOS S	**2.42**	9.11	ACV **R** FOS S
#20	UL23	**10.55**	15.5	ACV **R** FOS S	**1.63**	n.d.	ACV **R** FOS n.d.
#21	UL23	**6.15**	21.6	ACV **R** FOS S	**4.69**	n.d.	ACV **R** FOS n.d.
#22	UL23	**6.33**	5.8	ACV **R** FOS S	**0.67**	n.d.	ACV **R** FOS n.d.
#23	UL23	**14.25**	11.7	ACV **R** FOS S	**0.95**	5.63	ACV **R** FOS S
#24	UL30	**5.81**	88.7	ACV **R** FOS S	**0.64**	**22.13**	ACV **R** FOS **R**
#25	UL30	1.03	43.4	ACV S FOS S	**0.99**	**29.73**	ACV **R** FOS **R**

IC50 values greater than or equal to resistance breakpoint (see text) in bold. ACV = aciclovir; DRA = DNA reduction assay; FOS = foscarnet; n.d. = not determined; PRA = plaque reduction assay; R = resistant; S = susceptible.

exist for susceptibility testing of HSV-1 using a real-time-PCR based approach, classification of the DRA results according to the tentative breakpoints adopted from Thi et al.[7] resulted in very high concordance. DRA failed in only one isolate.

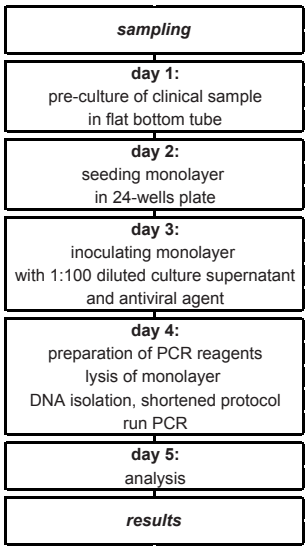
DRA provided results within five days after sampling (Figure 1). No visible viral CPE is required when viral replication is measured by sensitive methods such as real-time

Table 4. UL23 sequence analysis and DNA reduction assay results performed directly on HSV-1 positive clinical samples.

patient/ source	**clinical & treatment details**	**viral load (^{10} log copies/ml)**				**resistance associated UL23 gene mutations**		**IC50 ACV in DRA (mg/L)**
		in sample	*after pre-culture*	*at input in DRA*	*at read-out of DRA*	*in sample*	*after pre-culture*	*after pre-culture*
E**	SCT, mucositis, pretreatment	7.4	8.4	4.9	7.2	none	none	0.07
M*	chemotherapy, persistent mucositis, vACV 12 days & ACV iv 10 days	5.3	7.3	5.4	6.6	delC460 → fs155	delC460 → fs155	**3.06**
O	SCT, HSV mouth, no antivirals	4.1	5.9	3.9	5.9	none	none	0.06
P	chemotherapy, HSV lip, no antivirals	5.3	7.1	5.1	6.1	none	none	0.16
Q	infant, eczema herpeticum, treatment unknown	5.4	4.9	2.9	3.9	none	none	Failed***
R	SCT, HSV mouth, no antivirals	6.4	7.1	5.1	7.5	none	none	0.06
S	unknown	6.9	6.5	4.5	6.1	none	none	0.06
T	SCT, HSV mouth, ACV iv 17 days	6.9	6.6	4.6	6.5	insG430 → fs146	insG430 → fs146	**5.74**
U	infant, eczema herpeticum, treatment unknown	8.3	6.2	4.2	6.6	none	none	0.06

IC50 values greater than or equal to resistance breakpoint (see text) in bold. * see Table 1, isolate #17, ** see Table 1 isolate #5, *** IC50 value 0.06 mg/L at 1:10 dilution; ACV = aciclovir; d = days; del = deletion; DRA = DNA reduction assay; fs = frameshift; ins = insertion; SCT = stem cell transplantation; vACV = valaciclovir.

Figure 1



Time frame (workings days) of susceptibility testing by DNA reduction assay versus plaque reduction assay.

PCR and hence incubation time can be reduced compared to PRA. Pilot experiments using both susceptible and resistant isolates showed comparable IC50 values over broad ranges of viral inoculum, given sufficient viral input (10.000 copies/ml), hence allowing a fixed dilution of pre-cultured clinical samples without the need for viral titration. Hands on time could be reduced by the use of a simplified DNA isolation procedure. DRA was applicable to all clinical samples with a viral load of at least 10.000 copies/ml. In the final format of quadruplicate testing the coefficient of variation of the log-transformed viral concentrations was 5% on average. On average 0.3 wells were excluded per 24-wells assay because of PCR inhibition and 1.2 wells because of insufficient beta-globin concentration.

As shown in previous studies,[1-3] mutations of unknown significance are commonly found in HSV-1 clinical isolates and a pre-treatment sample for comparison of mutations is often not available. Three isolates contained UL30 mutation Asp-672→Asn which has recently been described both as a natural polymorphism[2] and as an aciclovir and foscarnet resistance-associated mutation.[13] Phenotypical susceptibility tests showed aciclovir and foscarnet susceptibility in an isolate with no other resistance-associated mutations making Asp-672→Asn unlikely as a resistance-associated mutation. This nicely demonstrates the usefulness of phenotypical susceptibility testing.

Low level resistance to aciclovir and foscarnet due to mutations in the UL30 gene is possibly more accurately detected by DRA than by PRA. Both our PRA and PRA performed initially (by others) showed a level of resistance around or below the specified breakpoint, as previously described for UL30 mutations A719V and S724N.[8;10;14;15] This may be due to the specific UL30 gene mutations or because low-level resistance or intermediate susceptibility may not be accurately defined by the CLSI breakpoints.[5] Nevertheless, DRA consistently showed increased IC50 values above the tentative breakpoints in these isolates. Because knowledge on the clinical significance of such mutations is lacking, it would be interesting to study how infections with such isolates should be treated.

Some limitations apply to the DRA. Firstly, without a short pre-culture, no viral replication was observed in DRA, but our protocol was suitable for clinical samples after a fixed short term pre-culture. This may be due to the low inoculum present in clinical samples, although pre-culture did not increase viral loads very much. It can also be related to the short duration of incubation of the DRA, although prolonging the assay to 48 hours did not solve the problem. Possibly, adaptation of virus to cell culture is necessary for a successful DRA. To study the possible selection pressure during pre-culture of a clinical sample, nine clinical samples were cultured for 48 hours. The nucleotide sequence of the entire UL23 gene was compared between the clinical samples and the viral isolates after 48 hours of pre-culture (Table 4) and no differences were found.

Secondly, the detection limit of DRA precluded its use for weakly positive samples. This limitation may be partly overcome by adaptation of the dilution in samples with a lower viral load, which was applied successfully in one of our clinical samples in which a 1:10 diluted culture supernatant was used instead of 1:100 (Table 4). Finally, the assay was validated for HSV-1 only.

In clinical diagnostics, a genotypical approach has the advantages of speed and technical ease. A two-step approach may therefore be practical in this setting, starting with UL23 and UL30 gene sequencing of, preferably, a pre- and on-treatment sample and subsequent phenotypical confirmation of resistance if mutations of unclear significance are encountered. A multicenter trial evaluating assay applicability and reproducibility and the clinical outcome in relation to the tentative breakpoints, should confirm the role of DRA in phenotypical susceptibility testing of HSV-1.

In conclusion, DRA is an accurate, rapid and easy to perform phenotypical susceptibility test for HSV-1.

AUTHOR CONTRIBUTIONS

MB, EC, AK and AV conceived and designed the experiments. MB, LR and CB performed the experiments. MB, EC, FM and AV analyzed the data. MB wrote the paper. All authors read and approved the final manuscript.

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