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Rapid susceptibility testing for herpes simplex virus type 1 using real-time PCR

Martha T. van der Beek[a,*], Eric C.J. Claas[a], Caroline S. van der Blij-de Brouwer[a], Florence Morfin[b], Lisette G. Rusman[a], Aloys C.M. Kroes[a], Ann C.T.M. Vossen[a]

[a] Department of Medical Microbiology, Leiden University Medical Center, P.O. Box 9600, 2300 RC Leiden, The Netherlands
[b] Laboratory of Virology, EMR, University Lyon 1, Hospices Civils de Lyon, Lyon, France

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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 h and subsequent culture in the presence of antivirals for 24 h. 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 h 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.

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.

2. 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.

3. Study design

3.1. 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,

Abbreviations: ACV, aciclovir; d, days; del, deletion; DRA, DNA reduction assay; FOS, foscarnet; fs, frameshift; GCV, ganciclovir; HSV-1, herpes simplex virus type 1; ins, insertion; n.d., not determined; PhHV, Phocid Herpes Virus; PRA, plaque reduction assay; R, resistant; S, susceptible; SCT, stem cell transplantation; sub, substitution; vACV, valaciclovir.

* Corresponding author at: Department of Medical Microbiology, Post Zone E4-P, Leiden University Medical Center, P.O. Box 9600, NL-2300 RC Leiden, The Netherlands. Tel.: +31 71 5263931; fax: +31 71 5266761.
E-mail address: m.t.van_der_beek@lumc.nl (M.T. van der Beek).

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

Patient/source	Isolate	Clinical and 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 and vACV 4 days	delG180 → fs61	None
	#13	Ulcerative esophagitis, ACV iv 22 days and vACV 9 days	delG180 → fs61	None
B	#2	SCT, lip lesion, pre-treatment	None	None
	#21	Severe facial and days oral lesions, ACV iv 52 days and 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 and GCV iv 12 days	insG430 → fs146	None
E	#5	SCT, mucositis, pre-treatment	None	None
	#23	Persistent mucositis, vACV 8 days and 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 and 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.

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 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.

3.2. UL23 and UL30 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.

3.3. 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 2× 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 2× 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 0.12–16 mg/L and for foscarnet the concentration range was 16.7–400 mg/L. Isolates having an IC_{50} value ≥2 mg/L for aciclovir or ≥100 mg/L for foscarnet were considered resistant.[5]

3.4. 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